

Microphysiological Tumor-on-Chip Models
Recapitulating Tumor-Microenvironment Interaction
Applicable for Cell Therapy Testing

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Table of Contents

Table of Contents	I
Abstract	IV
Zusammenfassung	V
Abbreviations.....	VI
List of Figures	X
List of Publications.....	XI
1. Background & State of the Art	1
1.1 Tumor Microenvironment	1
1.2 Adoptive Cell Therapy: a Promising Approach?	3
1.3 Challenges of Current Cancer Models	6
1.4 Tumor-on-chip to Investigate Tumor-Immune-Microenvironment.....	7
2. Objective of the Thesis	11
3. Results & Discussion I.....	13
3.1 Model and Setup Establishment.....	14
3.2 Demonstration of CAR-T cell Efficacy Assessment on Chip	16
3.3 Demonstration of CRS-related Safety Assessment on Chip	18
3.4 Dasatinib-mediated Pharmacologic Intervention of CAR-T Cells on Chip..	19
3.5 Impact of Endothelial Cell Barrier Function and Sourcing Strategy	21
3.6 Impact of Macrophages' Integration	22
4. Results & Discussion II.....	29
4.1 Model and Setup Establishment.....	30
4.2 BC and WAT Bi-Directional Crosstalk on Chip	32
4.3 Impact of WAT on Chemotherapeutic Efficacy	33
5. Conclusion and Outlook	37
6. Additional Methods	43
6.1 Monocyte Isolation and Differentiation into Macrophages	43
6.2 Maturation and Polarization of Macrophages	44
6.3 Flow Cytometry Analysis of Differentiated Macrophages	44
6.4 Integration and Culture of Macrophages in the Tumor-on-chip	45
6.5 Cytokine Quantification Assay	46
6.6 Image Analysis	46
References	49
Acknowledgements	69
Declaration	71
Appendices.....	73

Abstract

The tumor microenvironment (TME) plays a crucial role in tumor growth and treatment response. Traditional animal models fail to accurately replicate the human TME, carcinogenesis, immune responses, metabolism, pathophysiology, and adverse events, limiting their usefulness in studying cancer development and therapies, especially those involving the immune system. To address these limitations, this thesis aimed to develop microphysiological tumor-on-chip models that closely mimic human TME and immune responses, enabling detailed studies of cancer-TME interactions and immunotherapy responses. The first part of the work focused on the development and utilization of a tumor-on-chip model for evaluating the efficacy and safety of chimeric antigen receptor (CAR)-T cell immunotherapy in a solid TME. This model examined CAR-T cell responses in relation to target antigen levels, tumor size, and TME components, such as endothelial barriers and macrophages. Excessive antitumor response by CAR-T cells was captured through the monitoring of inflammatory cytokine release kinetics, which allowed the testing of acute safety intervention strategy on the chip. This model offers a rapid and adaptable platform for comparing different CAR-T cell products and exploring strategies to overcome TME barriers. The second part of the work focused on establishing a breast cancer-on-chip model to recreate the breast TME for studying interactions between breast tumors and white adipose tissue (WAT). This platform uniquely integrated breast cancer spheroids or patient-derived organoids central to the surrounding WAT-associated cells in a perfusable microfluidic chamber, allowing long-term co-culture that lasted for up to two weeks. Using this model, it was demonstrated that the presence of breast cancer increased lipolytic activity in adipocytes and that WAT increased lipid accumulation and promoted growth of breast cancer cells. Notably, WAT protected breast cancer cells from the chemotherapeutic agent daunorubicin, highlighting the TME's critical role in modulating cancer treatment efficacy. Overall, this thesis established tumor-on-chip models to study tumor-TME interactions and cancer immunotherapies, laying the foundation for mechanistic research and clinical translation of therapies designed to overcome TME barriers.

Zusammenfassung

Die Tumormikroumgebung (TME) spielt eine entscheidende Rolle bei Krebsentwicklung und -behandlung. Traditionelle Tiermodelle können die menschliche TME, die Immunantworten, den Stoffwechsel, die Pathophysiologie und Nebenwirkungen nicht genau nachbilden, was ihre Nützlichkeit bei der Untersuchung der Krebsentwicklung und -therapien, insbesondere solcher, die das Immunsystem nutzen, einschränkt. Ziel dieser Arbeit ist, mikrophysiologische Tumor-on-Chip-Modelle zu entwickeln, die menschliche TME und die Immunantworten besser nachbilden und dadurch detaillierte Studien zu Wechselwirkungen zwischen Krebs, TME, und Immuntherapien ermöglichen. Der erste Teil dieser Arbeit konzentrierte sich auf die Entwicklung eines Tumor-on-Chip-Modells zur Evaluierung der Wirksamkeit und Sicherheit der chimären Antigenrezeptor (CAR)-T-Zell-Immuntherapie in einer soliden TME. Dieses Modell untersuchte die CAR-T-Zell-Reaktionen in Bezug auf Zielantigen, Tumorgroße und TME-Komponenten wie Endothelbarrieren und Makrophagen. Übermäßige Antitumorreaktionen von CAR-T-Zellen wurden durch das Monitoring der Freisetzungskinetik von entzündungsfördernden Zytokinen erfasst, was die Testung akuter Sicherheitsintervention auf dem Chip ermöglichte. Dieses Modell bietet eine schnelle und anpassbare Plattform zum Vergleich verschiedener CAR-T-Zell-Produkte und zur Erforschung von Strategien zur Überwindung von TME-Barrieren. Der zweite Teil der Arbeit befasste sich mit der Etablierung eines Brustkrebs-on-Chip-Modells zur Nachbildung der Brust-TME, um Wechselwirkungen zwischen Brusttumoren und weißem Fettgewebe (WAT) zu untersuchen. Diese Plattform integrierte Brustkrebssphäroide oder patientenabgeleitete Organoide in der Mitte der umliegenden WAT-assoziierten Zellen in einer perfundierbaren mikrofluidischen Kammer, was eine Kokultur für bis zu zwei Wochen ermöglichte. Darüber hinaus wurde es untersucht, ob der Brustkrebs die lipolytische Aktivität in Adipozyten und WAT die Lipideinlagerung und das Wachstum von Brustkrebszellen beeinflusste. Resultate zeigen, dass WAT die Brustkrebszellen vor dem chemotherapeutischen Wirkstoff Daunorubicin schützen konnte. Insgesamt etablierte diese Arbeit neuartige Tumor-on-Chip-Modelle zur Untersuchung von Wechselwirkungen zwischen Tumor und TME sowie Krebsimmuntherapien und schafft damit die Grundlage für mechanistische Forschungen und die klinische Umsetzung von Therapien, die darauf abzielen, TME-Barrieren zu überwinden.

Abbreviations

3D	Three-dimensional
4-1BB	Tumor necrosis factor receptor superfamily member 9
BC	Breast cancer
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CCR2b	C-C chemokine receptor type 2b
CCR4	C-C chemokine receptor type 4
CD11b	Cluster of differentiation molecule 11B (also: Integrin alpha M)
CD14	Cluster of Differentiation 14
CD163	Cluster of Differentiation 163 (also: Scavenger receptor cysteine-rich type 1 protein M130)
CD19	Cluster of Differentiation 19
CD206	Cluster of Differentiation 206 (also: Macrophage mannose receptor 1)
CD25	Cluster of Differentiation 25 (also: Interleukin-2 receptor alpha chain)
CD28	Cluster of Differentiation 28
CD31	Cluster of Differentiation 31 (also: Platelet endothelial cell adhesion molecule [PECAM-1])
CD36	Cluster of Differentiation 36
CD3-ζ	T cell surface glycoprotein CD3 zeta chain (also: Cluster of Differentiation 247)
CD4	Cluster of Differentiation 4
CD45	Cluster of Differentiation 45 (also: Protein tyrosine phosphatase, receptor type C)
CD68	Cluster of Differentiation 68
CD69	Cluster of Differentiation 69
CD8	Cluster of Differentiation 8
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CMAC	7-amino-4-Chloromethylcoumarin
CO ₂	Carbon dioxide

CPT-11	Irinotecan, a chemotherapy drug
CR	Cytokine release
CRS	Cytokine release syndrome
CSF-1R	Colony stimulating factor 1 receptor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DMEM/F-12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ET-1	Endothelin-1
FAP	Fibroblast activation protein
FCS	Fetal calf serum
FDA	Food and Drug Administration
FSC-A	Forward Scatter Area (used in flow cytometry)
FSC-H	Forward Scatter Height (used in flow cytometry)
GFP	Green Fluorescent Protein
GM-SCF	Granulocyte-macrophage colony-stimulating factor
HER2	Human Epidermal Growth Factor Receptor 2
HiPSC	Human induced pluripotent stem cell
HLA	Human leukocyte antigen
HLA-DR	A major histocompatibility complex class II cell surface receptor
ICAM-1	Intercellular Adhesion Molecule 1
IFN- γ	Interferon gamma
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
irAOP	Immune-related Adverse Outcome Pathway
KE	Key event
LAG-3	Lymphocyte Activation Gene-3
LPS	Lipopolysaccharide

M0	Non-polarized macrophage
M1	Pro-inflammatory macrophage
M2	Anti-inflammatory macrophage
M-CSF	Macrophage Colony-Stimulating Factor
MiE	Molecular initiating event
mvEC	Microvascular endothelial cell
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS ⁻	Dulbecco's phosphate-buffered saline without calcium chloride and without magnesium chloride
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDMS	Polydimethylsiloxane
PDO	Patient-derived organoid
RGD	Arginine-Glycine-Aspartic Acid (a peptide sequence)
rH	Relative humidity
ROR1	Receptor tyrosine kinase-like Orphan Receptor 1
RPMI-1640	Roswell Park Memorial Institute medium 1640
RT	Room temperature
SSC-A	Side Scatter Area (used in flow cytometry)
SVF	Stromal vascular fraction
TCR	T cell receptor
TGF- β	Transforming Growth Factor Beta
TGN1412	Anti-CD28 monoclonal antibody
Th1	T helper cell type 1
TIL	Tumor-infiltrating lymphocytes
TIM-3	T cell Immunoglobulin and Mucin-domain containing-3
TME	Tumor microenvironment
TNF- α	Tumor Necrosis Factor alpha
Treg	Regulatory T cell
VCAM-1	Vascular Cell Adhesion Molecule 1
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
WAT	White adipose tissue
VIII	

ZAP70

Zeta-chain-associated protein kinase 70

List of Figures

Figure 1: TME characteristics and mechanisms that support tumor growth.	3
Figure 2: CAR-T cell therapy and its clinical challenges.	6
Figure 3: Tumor-on-chip as a physiologically relevant human model for cancer research.....	9
Figure 4: Tumor-on-chip establishment: chip design, tissue generation, and readout methods.....	15
Figure 5: Macrophages' integration into the tumor-on-chip, characterization, and impact towards CAR-T cell response.	24
Figure 6: Breast cancer-on-chip establishment: chip design, tissue generation, and readout methods.....	31

List of Publications

a. Accepted publications

= authors contributed equally

2021

- [1] **T. I. Maulana**[#], E. Kromidas[#], L. Wallstabe, M. Cipriano, M. Alb, C. Zaupa, M. Hudecek, B. Fogal, P. Loskill. “Immunocompetent Cancer-on-Chip models to assess immuno-oncology therapy”, *Adv. Drug. Deliv. Rev.* 173, 281–305 (2021), <https://doi.org/10.1016/j.addr.2021.03.015>
- [2] A. Apostolou, R. A. Panchakshari, A. Banerjee, D. V. Manatakis, M. D. Paraskevopoulou, R. Luc, G. Abu-Ali, A. Dimitriou, C. Lucchesi, G. Kulkarni, **T. I. Maulana**, M. Kasendra, J. S. Kerns, B. Bleck, L. Ewart, E. S. Manolakos, G. A. Hamilton, C. Giallourakis, K. Karalis. “A Novel Microphysiological Colon Platform to Decipher Mechanisms Driving Human Intestinal Permeability”, *Cell. Mol. Gastroenterol. Hepatol.* 12(5), 1719-1741 (2021), <https://doi.org/10.1016/j.jcmgh.2021.07.004>
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- [7] **T. I. Maulana**, N. Wevers, T. Kristoforus, M. Chandler, J. Joore, S. Kustermann, R. Villenaeve, P. Loskill, P. Vulto, K. Bircsak. “Opportunities in using MPS for toxicity testing of new drug modalities.” *Annu. Rev. Pharmacol. Toxicol.* (in press) (2024).
- [8] **T. I. Maulana**, C. Teufel, M. Cipriano, L. Lazarevski, F. E. van den Hil, V. Orlova, A. Koch, M. Hudecek, #M. Alb, #P. Loskill. “Breast cancer-on-chip for patient-specific efficacy and safety testing of CAR-T cells”, *Cell Stem Cell* (2024), <https://doi.org/10.1016/j.stem.2024.04.018>

b. Manuscripts ready for submission

- [9] **T. I. Maulana**, L. Lazarevski, M. Cipriano, L. Christ, J. Rogal, A. Koch, P. Loskill. “Breast cancer-on-chip integrating white adipose tissue microenvironment to study tumor-white adipose tissue crosstalk” (2024)

1. Background & State of the Art

1.1 Tumor Microenvironment

Cancers account for 1 in 6 deaths globally, with 10 million fatalities in 2019 alone¹. The incidence and mortality rates are rising worldwide and are projected to continue for at least the next two decades due to an aging population and various risk factors, including suboptimal diets, sedentary lifestyles, air pollution, tobacco use, and alcohol consumption^{2–5}. Consequently, it has become increasingly crucial to deepen our understanding of the disease to enhance prevention measures and treatment options. In recent decades, our understanding of cancer has significantly shifted. Rather than viewing it merely as a genetic disease, we now perceive cancer as a complex disease involving intricate relationships between cancerous and non-cancerous cells within an abnormally vascularized extracellular matrix (ECM), shaping a highly structured ecosystem known as the tumor microenvironment (TME) (**Figure 1A**). The TME dramatically influences immune response and evasion⁶, tumor growth⁷, invasiveness⁸, metastasis⁹, and response to therapy^{10,11}. This environment is occupied by many non-cancerous cell types, including cancer-associated fibroblasts, diverse immune cells, endothelial cells (ECs), pericytes, and other tissue-specific cells. In this microenvironment, cancer cells are the major orchestrators that actively shift the ecosystem from tumor-suppressing toward tumor-supportive as the tumor progresses. For example, as early-stage tumors grow, their increasing need for nutrients and oxygen leads to the release of pro-angiogenic signaling factors, which trigger the development of vascular networks in the tumors¹². This process, known as the “angiogenic switch”, is a critical stage that transforms tumors into more malignant forms¹³. Compared to normal tissues, however, the resulting vascular network in tumors is significantly different. Due to the imbalanced expression of angiogenic factors and inhibitors, as well as the rapid disruption of homeostasis by the tumor cells, tumor vasculature becomes highly disorganized and lacks a hierarchical vessel structure¹⁴. This poses a barrier to infiltrating immune cells and impedes drug delivery, hindering the control of tumor growth^{15,16}.

In addition to the tumor stage, the state of TME also highly depends on the organ and tissue in which the tumor grows¹⁷. For instance, white adipose tissue (WAT) is a predominant component of the microenvironment of breast cancer, where an intricate

interplay between cancer cells and WAT-associated cells influences tumor behavior and treatment responses. WAT primarily consists of mature adipocytes, comprising 90% of its volume and 50% of its cellular content¹⁸. It additionally contains the stromal vascular fraction (SVF), which encompasses mesenchymal stem cells, adipocyte and vascular progenitors, endothelial cells, fibroblasts, and diverse immune cell types¹⁹. As a metabolically active tissue, adipocytes support tumor growth and influence treatment response through various mechanisms. These include providing energy in the form of fatty acids^{20–28}, producing growth-supporting adipokines²⁹, hormones^{30,31}, and exosomes^{32,33}, metabolizing chemotherapeutic drugs³⁴, and shaping the inflammatory microenvironment^{35,36}. When glucose is scarce, cancer cells can actively induce lipolysis in adipocytes and metabolically shift from glycolysis towards oxidative phosphorylation, which allows them to utilize these adipocyte-derived fatty acids for sustaining their growth³⁷ (**Figure 1B**). This metabolic adaptation is seen across various cancer types found in tissues abundant with WAT, such as prostate and ovarian cancer^{21,23}, indicating that the TME is distinct and highly specific to each tissue.

Anti-tumor immunity is also constantly evolving throughout tumor development^{38,39}. Cancer cells are initially recognized and eliminated, for example by T cells, due to their expression of non-self-antigens. The presence of infiltrating T cells correlates with improved patient survival, with higher densities and activation signatures within tumors associated with better outcomes^{6,40,41}. However, at a certain point, tumor breaks the immune control: the balance shifts from immune attack to immune evasion^{39,42}. The timing and mechanisms of immune evasion are likely to vary based on tissue context and can happen through a multitude of mechanisms¹⁷. For instance, the high metabolic needs of the tumor force the tumor-infiltrating immune cells to adapt to various types of metabolic stress. Elevated glucose metabolism and lactate release by the tumors, along with amino acid scarcity, hypoxia, and increased lipid abundance in the TME, suppress T cell effector functions while simultaneously promoting the suppressive functions of Tregs, M2-like (anti-inflammatory) macrophages, and myeloid-derived suppressor cells⁴³ (**Figure 1C**). Increased density of ECM in tumor stroma also limits T cell infiltration and causes exhaustion of effector T cells^{44,45}.

However, within every challenge lies opportunity. Here, therapeutic strategies targeting the TME are gaining traction, with approaches aimed at not only reprogramming the immune landscape but also normalizing aberrant vasculature, breaking ECM barriers, and disrupting the supportive interactions between stromal cells and cancer cells⁴⁶.

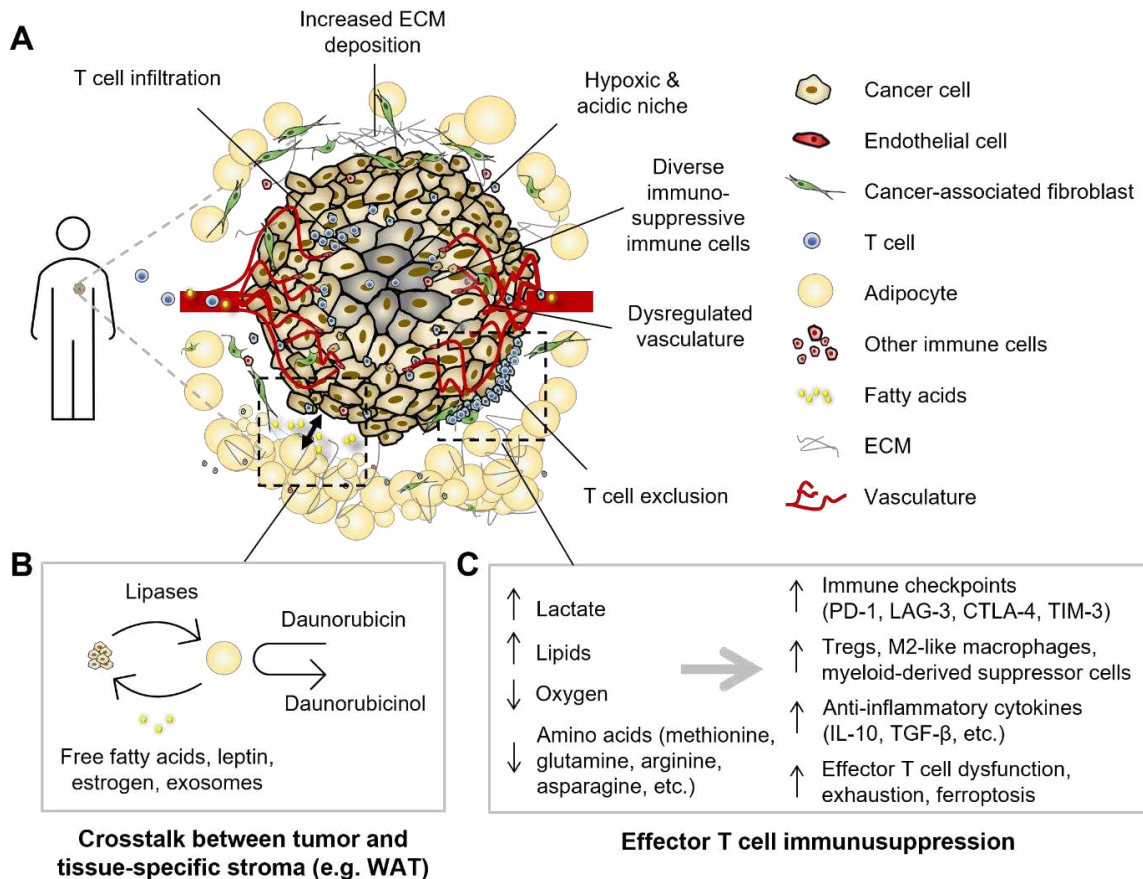


Figure 1: TME characteristics and mechanisms that support tumor growth.

(A) The TME is characterized by the presence of diverse immunosuppressive immune cells such as Tregs, M2-like macrophages, and myeloid-derived suppressor cells, hypoxic and acidic environment, dysregulated vasculature, and different densities of effector T cells that depend on tumor immunogenicity and immunosuppressive state of the TME. **(B)** The developing tumors interact with tissue-specific stromal cells, such as breast cancer cells that interact with adipocytes in the breast TME. Cancer cells can secrete lipases, which increase the lipolytic activity of adipocytes, providing fuel for cancer growth. Adipocytes can also supply tumors with adipokines, hormones, exosomes, as well as catabolizing a chemotherapeutic drug, such as daunorubicin into daunorubicinol. The figure is adapted from a reference⁴⁷. **(C)** Tumor metabolism increases lactate and lipid amount and decreases oxygen and amino acid availability in the TME, directly and indirectly causing effector T cell immunosuppression through increased recruitment, activity, immune checkpoint upregulation, and proliferation of immunosuppressive cells, and increased amount of anti-inflammatory cytokines⁴³.

1.2 Adoptive Cell Therapy: a Promising Approach?

Since the late 19th century, many discoveries have revealed that the immune system plays a crucial role in regulating malignantly transformed cells, resulting in the emergence of a promising additional treatment option for cancers called cancer immunotherapy⁴⁸. This approach leverages the immune system, predominantly relying

on T cells, to target and eradicate tumors. Cancer immunotherapy is an umbrella term for many treatment modalities, including adoptive cell therapy, immune checkpoint inhibition, T cell bispecific antibody therapy, oncolytic viruses, cytokine therapy, and cancer vaccines^{49–52}. These immunotherapeutic strategies have transformed cancer treatment landscape and prolonged the survival of patients facing aggressive malignancies.

One of the fastest growing approaches is adoptive cell therapy (ACT)^{53,54}. In this therapy, cancer-reactive immune cells such as T cells are isolated from the body of a patient or healthy donor, which are then enhanced (e.g. genetically modified, purified) and expanded ex vivo, and reinfused back into the patient to eliminate the tumors (**Figure 2A**). This treatment approach uses either endogenous tumor-infiltrating lymphocytes (TILs) or genetically engineered T cells. In the case of the latter, T cells are genetically modified to express either highly specific T cell receptors (TCRs) or chimeric antigen receptors (CARs). To date, CAR technology has been successfully incorporated not only into T cells but also into NK cells and macrophages^{55,56}. CAR-modified cells, unlike TCR-T cells, have the ability to recognize tumor-associated antigens without the need for major histocompatibility complex (MHC) expression on cancer cells. This is particularly advantageous since cancer cells often downregulate MHC to evade immune surveillance^{53,57}.

Despite the initial success stories of CD19-targeting CAR-T cell therapy against liquid tumors, that has led to the FDA approval for the first cancer cell therapy for cancer treatment⁵⁸, a significant question mark remains on its potential application in solid tumors due to various clinical challenges (**Figure 2B**). An example is the challenge of identifying an optimal target due to the antigen heterogeneity in solid tumors⁵⁹. Other challenges are associated with the immunosuppressive nature of the solid TME, which is quite different from liquid tumors^{60–63}. In solid tumors, CAR-T cells have to function within an environment that has limited nutrients, low oxygen levels, dense extracellular matrix, abundant immunosuppressive cytokines, high interstitial fluid pressure, upregulated immune checkpoints, and plenty of suppressive regulatory myeloid cells⁶⁰. This led to the development of new CAR designs equipped with additional functions to specifically overcome these challenges^{64,65}.

One key cell type in the TME is the macrophage, which can negatively influence CAR-T cell treatment response through various mechanisms, including the expression of inhibitory ligands such as PD-L1 and the secretion of immunosuppressive factors such

as IL-10, leading to T cell exhaustion or anergy⁴³. However, in certain cases, macrophages can also enhance CAR-T cell therapy efficacy. Here, macrophages exhibit an M1-like pro-inflammatory phenotype in response to IFN- γ secreted by the CAR-T cells during tumor killing⁶⁶ and can consequently promote the recruitment and activation of additional immune effector cells, such as natural killer (NK) cells and dendritic cells, thereby amplifying the overall antitumor response⁶⁷. Furthermore, they can contribute to the clearance of apoptotic tumor cells following CAR-T cell-mediated cytotoxicity, which prevents secondary necrosis and the release of immunosuppressive signals⁶⁸. However, in extreme cases, macrophages, along with monocytes and endothelial cells, can become overactivated and amplify the CAR-T cell-mediated pro-inflammatory response, such that it induces severe side effects such as cytokine release syndrome (CRS)⁶⁹.

CRS is a systemic inflammatory reaction triggered by the induction of powerful immune effector responses, marked by a significant surge in proinflammatory cytokines within the peripheral blood⁶⁹. It is one of the most common adverse events linked to advanced therapy medicinal products, including T cell bispecific antibodies and CAR-T cell therapy for cancers^{70–74}. During CAR-T cell therapy, its development is commonly linked with various additional complications, such as hemodynamic instability (capillary leakage, tachycardia, hypotension) and neurotoxicity (cerebral edema, seizures, aphasia, delirium), potentially resulting in organ failures and patient's death, if not optimally treated^{75,76}. Predicting its occurrence is extremely challenging⁷⁷, and the underlying mechanism of this patient variability remains unclear⁷⁸. Understandably, the pathophysiology of CRS is complex, involving T cells, tumor cells, endothelial cells, and endogenous immune cells such as monocytes and macrophages both at the local tumor site and in the bloodstream⁶⁹. One way to describe this systemic complexity is through a conceptual framework known as the immune-related adverse outcome pathway (irAOP)^{79,80}. The irAOP breaks down the toxicity mechanism into a molecular initiating event (MiE) and a series of subsequent key events at the molecular, cellular, organ, and organism levels that eventually lead to the adverse outcome. For CAR-T cell-associated CRS, the MiE involves the CAR-T cell recognizing the target antigen on tumor cells, followed by CAR-T cell activation and proliferation (key event [KE] 1), increased proinflammatory mediators by bystander immune cells (KE 2), subsequent activation of monocytes, macrophages, dendritic cells, and endothelial cells (KE 3), leading to systemic inflammation (KE 4)^{79,81} (extensively described in **Publication [6]**).

Through this deconvolution, appropriate model systems can be identified to study certain KEs and KE relationships, thereby providing opportunities to better understand the toxicity mechanism, potentially determine relevant toxicity biomarkers, and ultimately improve the toxicity profile of CAR-T cell therapies.

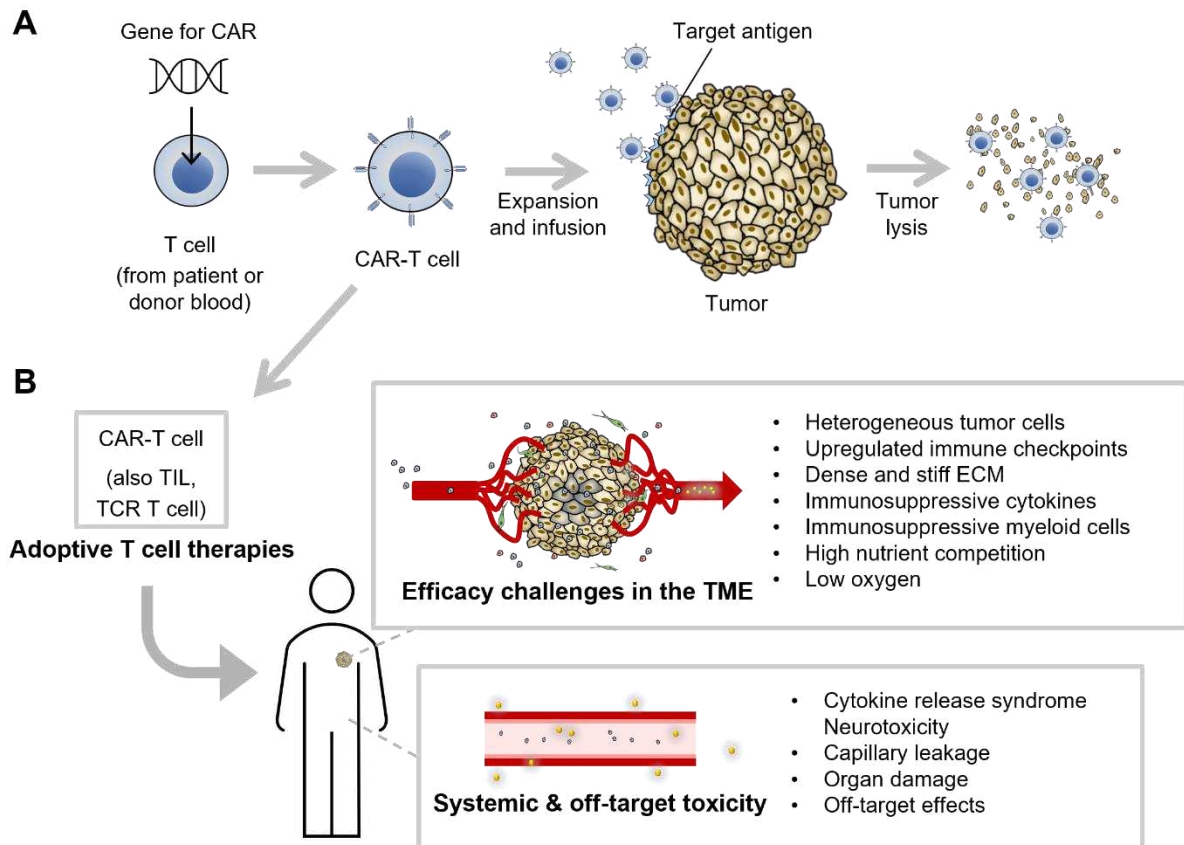


Figure 2: CAR-T cell therapy and its clinical challenges.

(A) In CAR-T cell therapy, T cells are first isolated from the patient's or donor's blood, where they are genetically modified to express CAR targeting specific tumor antigen. CAR-T cells are then expanded until reaching a sufficient number of cells and (re-)infused back into the patient's body. The CAR-T cells recognize the target antigen on the tumor cells and eradicate the tumor. **(B)** Adoptive T cell therapies, specifically for solid tumors, face clinical challenges related to both efficacy and safety.

1.3 Challenges of Current Cancer Models

One major hurdle causing limited translation of cancer immunotherapy, including CAR-T cell therapy, is the absence of accurate nonclinical models that recapitulate human immune responses, carcinogenesis, and therapy response⁸². Today, animal models remain the gold standard^{83–86}. Artificially inducing carcinogenesis in animals does not mirror the age- and lifestyle-related causes of humans. Moreover, these models often fail to replicate antitumor immunity in response to tumor progression due to the

species-specific differences in key immunological processes⁸⁷. Different species have different immune cell subsets that are crucial for controlling tumor growth. For example, certain key cell types, such as subsets of $\gamma\delta$ T cells, are entirely absent in mice⁸⁸. Importantly, discrepancies in the immune cell subsets, relative composition, and receptor expression between animals and humans can be a matter of life and death for patients, as they have failed to predict fatal toxicities. For instance, in the clinical trial of a T cell engaging monoclonal antibody therapy TGN1412, non-human primate models failed to predict severe CRS in the first-in-human trial because macaques lacked CD28 expression on CD4⁺ effector memory T cells, which are crucial for T cell activation and cytokine release^{89,90}. Indeed, the incapacibilities of most animal models to capture immune-mediated toxicities in the human context have been highlighted by a growing body of literature^{53,74,91–98}.

In terms of understanding the TME, causal relationships and individual TME contributions are difficult to identify in animal models due to their complicated interdependencies⁹². Moreover, the majority of existing in vitro models for testing cancer immunotherapy lack the inclusion of relevant cell types, a physiologically relevant cell ratio, organotypic tumor microarchitecture, and the interaction between different tissues implicated in the disease progression^{77,79,82,99}. The current nonclinical models poorly predict clinical outcomes, where numerous safety and efficacy issues are increasingly apparent^{53,61,74,91–95,100}. For all these reasons, alternative model systems that faithfully recapitulate human disease by including patient cancer cells, human immune cells, and other relevant TME factors are crucial for better understanding the role of each TME component and improving therapy prediction, thereby providing the potential to improve immunotherapy success in patients.

1.4 Tumor-on-chip to Investigate Tumor-Immune-Microenvironment

An optimal in vitro cancer model should consist of three-dimensional cancer tissue, along with a TME that incorporates aspects such as vascularization, tumor-associated stromal cells, and immune components. In recent years, organ-on-chip technology has become a promising platform to achieve this, demonstrated by its ability to mimic the key functional units of organs in “healthy” and “diseased” states within a microfluidic device^{101,102} (demonstrated in **Publication [2], [3], [4], [5]**). The opportunity to create complex vascularized tissues within a precisely controlled microenvironment has been made possible through advances in microfluidics, microfabrication, biomaterials, and

tissue engineering research¹⁰³. This microtissue environment, which can be analyzed in situ using a variety of methods, allows for the study of communication between individual cells, tissue components, or entire tissues¹⁰⁴. Leveraging these capabilities, organ-on-chip can integrate tumor cellular materials to mimic specific tumors and their TME, referred to as tumor-on-chip (**Figure 3**)^{82,105}.

Microfluidic cell culture systems have been extensively applied for cancer research. These systems are used to generate defined gradients of soluble factors for migration studies, study the behavior of circulating tumor cells, detect cancer cells in suspension, and pair individual immune cells with cancer cells to study direct cell-cell interactions^{106–112}. More recently, more complex tumor-on-chip systems integrating immune components have been introduced to model specific TME and simultaneously recapitulate human immune response (comprehensively reviewed in **Publication [1]**). These systems increasingly integrate patient-derived materials to improve the translatability of results to patients, including the use of tumor biopsies and organoids¹¹³.

A notable feature of the tumor-on-chip models is their ability to incorporate cell types relevant to specific TME and to replicate tissue-specific cellular organization at various tumor development stages, which can be tailored to meet specific experimental requirements and research questions¹⁰⁵. For understanding the TME, this modular approach facilitates the study of the individual roles of TME factors in a physiologically relevant environment. In these systems, a defined three-dimensional spatial organization also enables precise control of the ratio between cellular types, their three-dimensional arrangements, cytokine gradients, oxygen distributions, as well as other biophysical forces. This allows for the investigation of factors impacting tumor growth, immune-cancer cell interaction, and mechanism of action of cancer therapies⁸².

While offering great potential, new drug modalities to treat cancers, such as CAR-T cell therapy, antibody-drug conjugates, and multi-specific antibodies, still pose challenges by triggering complex and often unpredictable adverse effects. Adverse effects in cell therapies can arise not only from direct interactions between cells and drugs but also from a complex cascade of interactions among various specialized cell types and the drug within a specific microenvironment. This can be addressed with tumor-on-chip technology that possesses important features that enable complex toxicological processes to be modeled in vitro in a controllable and fully human setting

(comprehensively reviewed in **Publication [7]**). Tumor-on-chips can be valuable in dissecting the role of specific cell type(s) in initiating/perpetuating toxicity and recapitulating certain KEs within irAOP networks that describe certain toxicity mechanisms, for example in CRS during CAR-T cell therapy. In addition, connecting multiple organ-on-chips is useful to recreate systemic interactions between multiple tissues, tumors, and organs to study adverse, off-target effects of drugs¹¹⁴.

By combining these aspects, the research community may now have complex human-relevant model systems capable of providing answers to questions that animal models could not^{115,116}. To showcase this capability, this thesis presents the development of two fit-for-purpose tumor-on-chip models and their application in assessing CAR-T cell therapy for solid tumors, as well as investigating the impact of breast TME in promoting tumor development and treatment response.

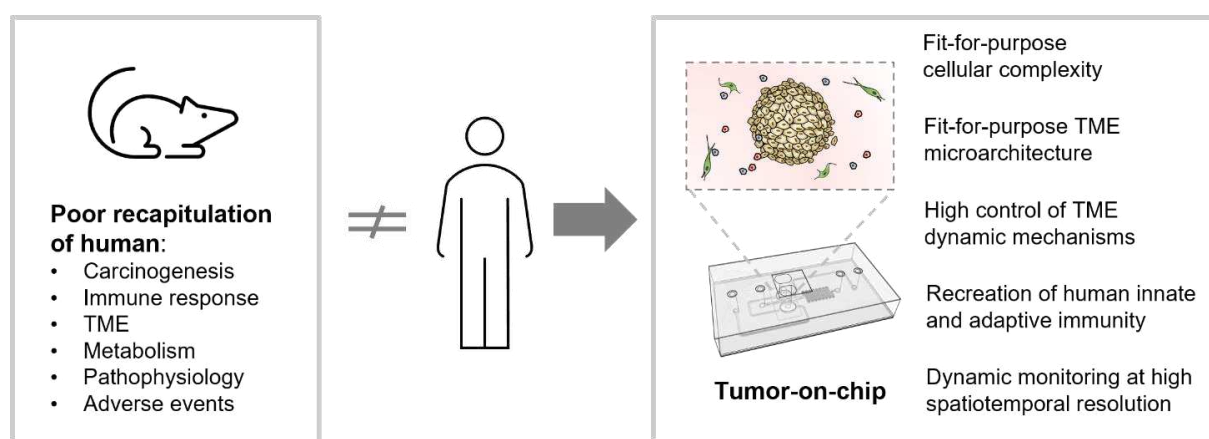


Figure 3: Tumor-on-chip as a physiologically relevant human model for cancer research.

Tumor-on-chip models can address the limitations of the gold-standard mouse models in cancer research, which often fail to recapitulate human carcinogenesis, immune response, TME, metabolism, pathophysiology, and adverse events. Tumor-on-chip features include the ability to fit-for-purposely adjust cellular complexity, TME microarchitecture, and dynamic mechanisms to mimic specific tumor development stages and key events during an adverse event. Tumor-on-chip can also recreate human innate and adaptive immune responses and integrate dynamic monitoring methods at high spatiotemporal resolution, allowing the study of the dynamic antitumor immune response. Figure is adapted from **Publication [1]** and **[7]**.

2. Objective of the Thesis

The overarching goal of the thesis is to develop human tumor microphysiological systems (tumor-on-chips) that are fit-for-purposely designed to recapitulate specific stages of tumor development and treatment, addressing the current need for physiologically relevant human models for cancer research. To comprehensively demonstrate the technological capabilities of such models, two objectives were pursued:

1. The establishment of a tumor-on-chip model for CAR-T cell therapy assessment. The model should recapitulate the initial events occurring upon CAR-T cell administration in solid tumor patients, including CAR-T cell infusion through the bloodstream, extravasation towards the tumor, targeted eradication of tumor aggregates/organoids in the TME, and cytokine release kinetics for at least one week. Upon model establishment, this model should be used to assess:
 - a. CAR-T cell efficacy,
 - b. CAR-T cell safety with regards to early KE that could initiate CRS,
 - c. Acute safety intervention strategy using pharmacologic off-switch, and
 - d. The impact of the endothelial cells and macrophages on efficacy and safety.
2. The establishment of a tumor-on-chip (breast cancer-on-chip) model for studying the crosstalk between breast tumor and WAT. The model should recreate the microarchitecture of a developing breast tumor in mammary ducts/lobules surrounded by WAT-associated cells and remain functional for at least two weeks to allow kinetic measurement of tumor-WAT interactions. Upon model establishment, this model should be used to assess:
 - a. The impact of tumor on adipocyte lipolytic activity,
 - b. The impact of WAT on lipid accumulation and growth of the tumor, and
 - c. The impact of WAT on the efficacy of chemotherapeutic daunorubicin.

3. Results & Discussion I

Tumor-on-chip for CAR-T cell assessment targeting solid tumor malignancies

Content is based on the following appendix:

Publication [8]:

T. I. Maulana, C. Teufel, M. Cipriano, L. Lazarevski, F. E. van den Hil, V. Orlova, A. Koch, M. Hudecek, #M. Alb, #P. Loskill. "Breast cancer-on-chip for patient-specific efficacy and safety testing of CAR-T cells", *Cell Stem Cell* (2024), <https://doi.org/10.1016/j.stem.2024.04.018>

3.1 Model and Setup Establishment

Based on the criteria described above, several features were integrated throughout the course of the model development (**Figure 4A**). First, pre-aggregation of tumor cells was essential to resemble dense solid tumor tissue that CAR-T cells need to penetrate and was conducted off-chip before chip loading. Target cells (ROR1[Receptor Tyrosine Kinase Like Orphan Receptor 1]-overexpressing MDA-MB-231 triple-negative breast cancer cell line, ROR1-negative fibroblast spheroids, and ROR1-high/low PDOs) were aggregated until they reached ~120 μm in size before chip loading (see attached **Publication [8] Methods and Figures S1A and S1B**). Tumor aggregates and PDOs were kept below 250 μm during cell injection to avoid the presence of a necrotic core at the beginning of the experiment^{117,118}. This size restriction also facilitated their potential to grow to at least 10 times their initial size within the microfluidic tumor chamber. A dextran-based, chemically inert, soft (~200 Pa) hydrogel was used in the experiments to facilitate the migration of CAR-T and permit tumor growth. This stiffness falls within the range of normal breast tissue¹¹⁹ and was selected to minimize the effects exerted by the ECM components during chip culture, thereby isolating the interaction between (CAR-)T cell and tumor aggregates. Higher ECM stiffness (>500 Pa) would reduce T cell proliferation, infiltration, CD8+ to CD4+ T cell ratio, and downregulated cytotoxic activity markers^{120,121}. Although not the primary focus of the study, the chip allows for the integration of various ECMs and stiffness levels to investigate their impact on CAR-T cell efficacy in a solid tumor context.

The chip fabrication and model generation (i.e. cell seeding and chip culture) methods are described in detail in the attached **Publication [8]**. The microfluidic channels' arrangement was designed to allow for constant (CAR-)T cell perfusion through the vasculature-like channel that was previously endothelialized (see attached **Publication [8] Figures 1C and S3A**) without disturbing the loaded tumor aggregates/PDOs. This arrangement also allows the subsequent (CAR-)T cell extravasation and migration into the underlying tumor chambers while simultaneously enabling high-resolution imaging by sealing the bottom side of the chip with a glass coverslip (**Figure 4B**, see also **Publication [8] Figures 1A and B**).

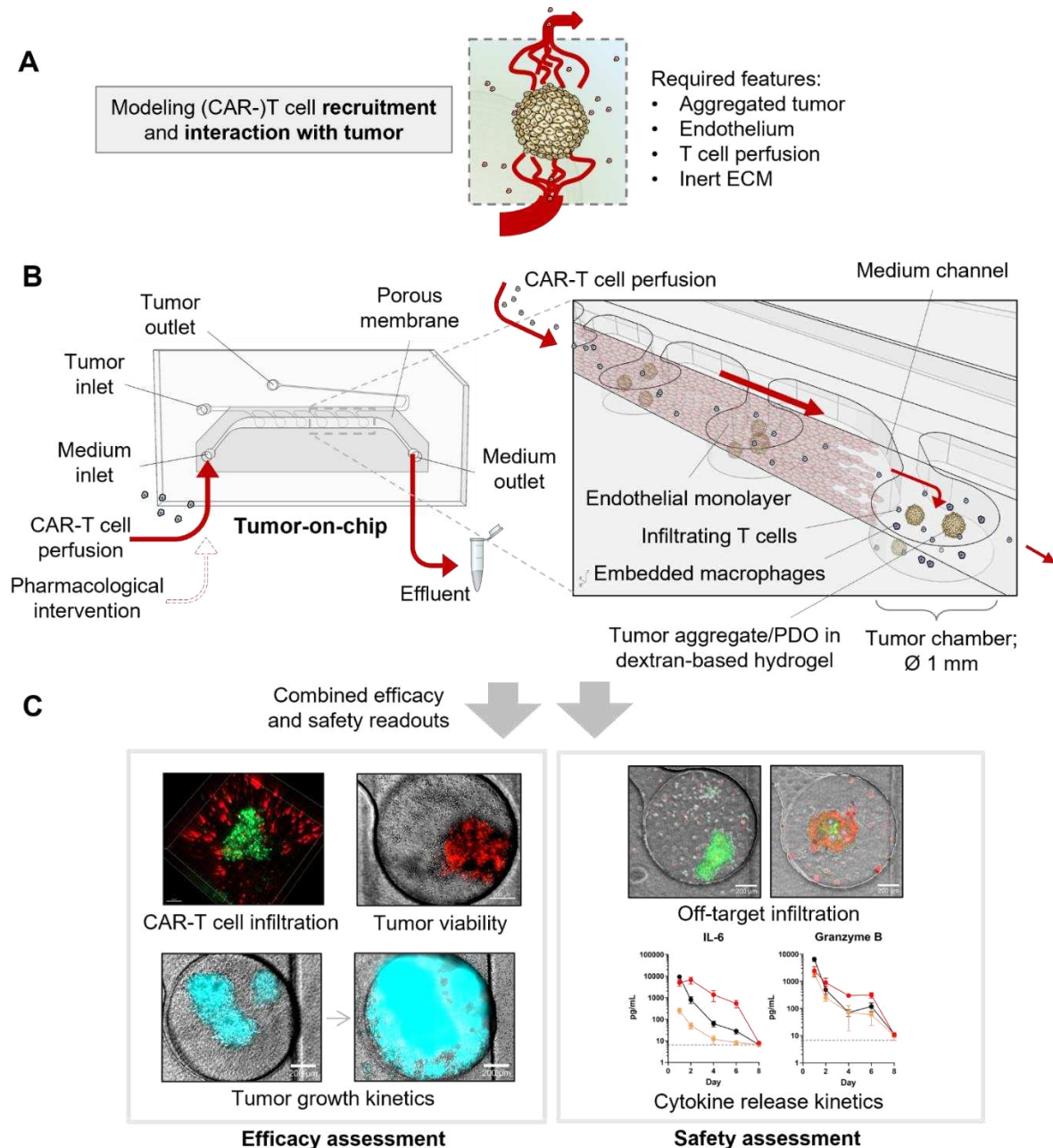


Figure 4: Tumor-on-chip establishment: chip design, tissue generation, and readout methods.

(A) Model features that are required to model CAR-T cell recruitment in the early phase of the therapy and its subsequent interaction with the solid tumor. **(B)** Schematic representation of the tumor-on-chip model. Tumor aggregates or PDOs were embedded in a dextran hydrogel (together with macrophages) and cultured in the cylindrical tumor chambers of the chip. The tumor chambers are situated beneath a medium channel, which is lined with an endothelial monolayer. CAR-T cells were linearly perfused through the medium channel, allowing them to migrate into the tumor chambers. **(C)** The experimental setup includes readouts to assess the efficacy and safety of CAR-T cell therapy, such as target-specific CAR-T cell infiltration, tumor growth kinetics and viability, and cytokine release kinetics. Images adapted from figures in the attached **Publication [8]**.

To demonstrate the feasibility of assessing the efficacy and safety of CAR-T cells in solid tumors, ROR1-targeting CAR-T cells were used. Following tumor-on-chip model generation, the chips were perfused with either ROR1-targeting CAR-T cells, untransduced T cells (negative control), or CD19-targeting CAR-T cells (negative control) from the same healthy donor. The timeline of the experiment was designed to simulate the first week of CAR-T cell therapy in patients after undergoing lymphodepletion (see attached **Publication [8] Figure 1D**). As described in the thesis objective, one of the aims is to develop a model that can simulate the initial KEs of CAR-T cell inflammatory response at the TME that could potentially develop into CRS at the organism level. Hence, CAR-T cell concentrations that reflect the range of peripheral T cell counts observed in patients experiencing CRS following a CD19-CAR-T cell therapy were chosen for the experiments (100,000, 500,000, and 1,000,000 (CAR-)T cells/mL) (see attached **Publication [8] Figure S1E**)^{122,123}. In both the acellular chip and tumor-on-chip systems, 70–80% T cell viability was sustained by the selected perfusion process and parameters (see attached **Publication [8] Figure 1E**). The applicability to integrate clinically relevant endpoints for assessing the safety and efficacy of CAR-T cell therapy for multiple days was iteratively optimized throughout the model development. In the clinic, measurements of T cell infiltration and tumor growth are typically done to monitor treatment efficacy^{124–126}. Moreover, measuring the kinetics of inflammatory cytokines (IL-2, IL-6, IL-10, TNF- α , IFN- γ , granzyme B) in patient's sera is also a clinical routine to monitor the risk of CRS development, which would require safety measures⁶⁹. To this end, fluorescence imaging-based on- and off-target CAR-T cell infiltration, tumor viability, tumor growth kinetic, as well as “real-time” kinetic measurement of the released inflammatory cytokines were implemented as readouts in the tumor-on-chip experiments (**Figure 4C**). Such combined safety and efficacy readouts for CAR-T cell therapy assessment are so far only achievable using animal models. By offering this capability, the tumor-on-chip advances current models in this aspect while still ensuring human relevance and fully controllable, yet complex, TME components.

3.2 Demonstration of CAR-T cell Efficacy Assessment on Chip

The tumor-on-chip was first utilized to assess CAR-T cell efficacy in a typical solid tumor condition. The feasibility was demonstrated using CAR-T cells targeting ROR1 antigen, which are expressed in a subset of B cell tumors and many epithelial tumors,

including breast^{127–129}. The expression levels differ between patients and tumors^{128,130}. In this study, ROR1⁺ and GFP-expressing MDA-MB-231 tumor aggregates, PDOs with varying ROR1 expression levels, and ROR1⁻ fibroblast spheroids were used as the target cells (see attached **Publication [8] Figure 2H**). PDOs, that were derived from four female patients with metastatic breast cancer that had varying ROR1 expression levels, were integrated to test the model's ability to evaluate patient-specific effects upon CAR-T cell therapy, given their ability to recapitulate tumor cell heterogeneity¹³¹ (see attached **Publication [8] Figures 5A, S5A, and Table S1**).

The results showed that the chip successfully captured differences in T cell infiltration into the tumor, tumor growth, viability, and cytokine response that was target-specific, meaning it depended on ROR1-CAR integration of the modified T cells and ROR1 expression on the target cells throughout the tumor-on-chip culture (see attached **Publication [8] Figures 2, 3, 5, S2, S4, S5**). In patients, the extent of (non-engineered) T cell infiltration into tumors varies widely and can be classified as either immunologically hot (high T cell infiltration) or cold (low T cell infiltration)¹³². The presence of immunologically hot tumors is reported to predict the subset of cancer patients who are responsive to cancer immunotherapy^{6,133}. In the tumor-on-chip, the untransduced T cell control condition reflected this T cell exclusion phenomenon and was only observable at a later timepoint (day 8). This was not observed in ROR1-CAR-T cell condition, where they infiltrated into ROR1⁺ MDA-MB-231 aggregates and ROR1-high PDOs in high density and persisted over 8 days.

This observation, however, seems to depend on the initial concentration of perfused ROR1-CAR-T cells. At a lower CAR-T cell dose, i.e., 100,000 cells/mL, CAR-T cells tend to initially migrate toward single tumor cells and smaller tumor aggregates <50 μ m instead of the larger ones on day 1, which resulted in no tumor growth control over time (see attached **Publication [8] Figure 2F**). This observation better aligns with the current clinical challenges of CAR-T cell therapy in solid tumors compared to the high-dose setting. Indeed, the environment surrounding and within larger tumors is less oxygenated, and due to metabolic competition, has less accessible amino acid nutrients (such as tryptophan, arginine, methionine, asparagine, and cysteine), which are critical for the metabolism, function, and differentiation of CAR-T cells^{43,134,135}. Additionally, larger tumors are more likely to develop immune evasion mechanisms, such as upregulating inhibitory molecules like PD-L1 and downregulating the target antigen, a phenomenon known as antigen escape^{136,137}. Future studies using tumor-

on-chip models could explore different CAR designs and combinations to overcome the hostile TME in solid tumors. These strategies might include engineering chemokine receptors (e.g. CSF-1R, CCR4, CCR2b), equipping CAR-T cells with additional functions to break the stromal barriers (e.g. targeting fibroblast activation protein [FAP] or secreting heparinase), performing PD-1 gene knockout, and enabling CAR-T cells to self-secrete anti-PD-1 and cytokines^{64,65}. These approaches can be monitored in the tumor-on-chip system with high spatiotemporal resolution over potentially weeks, which is currently unachievable with traditional in vitro methods. In terms of potential clinical application, the capability to generate the PDOs directly from patient tumor samples and establish the tumor-on-chip model within a week, as opposed to several months with murine models, offers the potential for assisting clinical decision-making within a shorter timeframe. Such testing using PDOs has been successfully utilized to inform clinical decision-making for combinatorial therapies of chemotherapeutics^{138–145} and immune checkpoint therapies^{146–148} with impressive predictivity of therapeutic outcomes. More recently, PDOs have also been used to assess CAR-T cell treatment efficacy^{149–151}. Despite providing valuable insights into treatment efficacy and CAR-T cell mode of action, these models still lack the TME components crucial in determining CAR-T cell recruitment and persistence.

3.3 Demonstration of CRS-related Safety Assessment on Chip

In solid tumors, efficacious CAR-T cells are often accompanied by high inflammatory cytokine release originating from the tumor site, resulting in recruitment of circulating monocytes and amplification of inflammatory response that eventually initiates systemic CRS^{69,152–154}. By measuring the cytokine concentration in the tumor-on-chip effluents, it was evident that the cytokine response (IL-2, IL-6, IL-10, TNF- α , IFN- γ , granzyme B) was target- and CAR-type-dependent and originated from tumor-CAR-T cell interaction. Throughout the observation period, cytokine concentrations in the ROR1-CAR-T cell condition consistently surpassed those in the control T cell condition (see attached **Publication [8] Figures 3 and S4**) and mimicked the kinetics observed in patients⁶⁹. A positive correlation between target antigen density and cytokine response was seen in tumor-on-chips containing PDOs with varying ROR1 expression levels treated with ROR1-CAR-T cells (see attached **Publication [8] Figures 5 and S5**).

From all experiments conducted, the data revealed that the peak cytokine concentrations in the ROR1-CAR-T cell condition (in combination with ROR1⁺ MDA-MB-231 tumor aggregates) consistently occurred on either day 1 or 2 for all measured cytokines. Cytokine levels typically decreased afterwards, although the mechanism is not yet clear. Future studies should look at whether this was due to activation-induced T cell death following tumor cell eradication or T cell exhaustion. Indeed, this decrease in cytokine levels could be attributed to successful MDA-MB-231 tumor lysis by the CAR-T cells, given that the tumors stopped growing in this condition, as observed in previous results regarding treatment efficacy. Additionally, replacing the non-recruited CAR-T cells with a cell culture medium in the perfusate from day 1 onwards might limit cytokine secretion to only the recruited CAR-T cells in the following days. Further aspects are discussed in detail in **Publication [8]** in the *Discussion* section.

While possessing many potential applications in future studies, the developed tumor-on-chip and the experimental setup have already effectively reproduced clinically relevant cytokine levels and kinetics, mimicking the initial surge in inflammatory cytokine secretion in the TME induced by target antigen recognition of CAR-T cells, which could potentially trigger CRS. These initial key molecular and cellular events that trigger CRS mediated by CAR-T cells can be described using the irAOP⁷⁹, as introduced in section 1.2 and extensively reviewed in **Publication [6]**. Within this irAOP context, the results from the tumor-on-chip show that the model is capable of recapitulating the MiE, followed by KE 1 and 2. This finding can be valuable in further research to understand the role of these KEs in contributing to the adverse outcome. This irAOP approach provides a framework not only for understanding the toxicity mechanisms and KE relationships but also for investigating the most optimal safety intervention stage to prevent CRS without compromising CAR-T cell efficacy. The next section demonstrates the applicability of the tumor-on-chip to test CRS-related safety intervention strategies via pharmacologic control using dasatinib.

3.4 Dasatinib-mediated Pharmacologic Intervention of CAR-T Cells on Chip

Having successfully replicated clinical cytokine release kinetics using the CAR-T cell-treated tumor-on-chips, the next step was to investigate whether these kinetics could be managed pharmacologically on chip to model therapeutic intervention and prevention of CRS onset. Predicting CRS is not an easy feat⁷⁷, and modeling the

release kinetics of key CRS mediators requires a tailored human model comprising relevant cell types within a perfused microenvironment. Upon CRS onset, a pharmacological intervention capable of temporarily halting CAR-T cell activity could potentially prevent adverse reactions in patients⁶⁴. Dasatinib, an FDA (Food and Drug Administration)-approved tyrosine kinase inhibitor, was chosen for its ability to reversibly inhibit the phosphorylation of CD3 ζ and ζ -chain of T cell receptor-associated protein kinase 70 (ZAP70) in CAR constructs containing either CD28_CD3 ζ or 4-1BB_CD3 ζ activation modules¹⁵⁵. In preliminary experiments using cell culture plates, it was initially confirmed that dasatinib (50 nM) did not compromise CAR-T cell viability and effectively inhibited the upregulation of early (CD69) and late (CD25) activation markers after 24 h of interaction with MDA-MB-231 cells (see attached **Publication [8] Figures 4A and 4B**). Subsequently, three different treatment regimens were tested to quantify timepoint-dependent effects of dasatinib: either throughout the CAR-T cell treatment period on chip (day 0–8; cytokine release (CR) prevention regimen), before the peak of cytokine release (day 0–1; CR delay regimen), or after (day 1–8; CR regulation regimen) (see attached **Publication [8] Figure 4C**). Within the context of irAOP, the CR prevention and delay regimen impacts CAR-T cell function during the MiE by delaying CAR binding to the target antigen. Meanwhile, the CR regulation regimen interferes during KEs 1 and 2⁷⁹.

Administering dasatinib after the peak of cytokine release (day 1–8; CR regulation) led to a rapid decrease in TNF- α and IFN- γ levels compared to those in the control CAR-T cell condition (see attached **Publication [8] Figures 4D and 4E**), thereby mimicking acute management of CRS mediators' release. This treatment regimen also facilitated CAR-T cell infiltration into the tumor within the initial 24 h (prior to dasatinib treatment), but subsequent excessive infiltration was mitigated, as indicated by the lower fluorescence intensity (~2-fold) relative to that in the untreated CAR-T cell condition (see attached **Publication [8] Figure 4F**). Moreover, the tumor GFP signal intensity on day 8 was comparable to the control CAR-T cell condition without dasatinib, indicating that the baseline antitumor cytotoxicity was maintained (see attached **Publication [8] Figure 4G**). Other strategies, namely CR prevention and delay, resulted in the failure of CAR-T cell-mediated tumor growth control despite successfully suppressing cytokine release (see attached **Publication [8] Figures 4D and 4G**, and further described in detail in the *Results* section). This, therefore, demonstrates the applicability of the model to test safety intervention strategies of CAR-T cells and

identify regimens to achieve the balance of safety and efficacy. In clinical settings, the standard treatment for patients experiencing CRS typically involves administering multiple doses of anti-IL-6 receptor antibodies, glucocorticoids, or vasopressors in severe cases¹⁵⁶. Other proposed safety intervention strategies include the integration of suicide gene systems that enable the elimination of CAR T cells by inducing apoptotic pathways¹⁵⁷, knockout of relevant cytokine genes, and equipping CAR-T cells with the ability to secrete factors that neutralize relevant cytokines⁶⁴. Future experiments using the tumor-on-chip model could test these strategies while also monitoring their effect on treatment efficacy.

3.5 Impact of Endothelial Cell Barrier Function and Sourcing Strategy

In all tumor-on-chip experiments, control T cell and CAR-T cell recruitments were measured in the presence of a microvascular endothelial cell (mvEC) barrier. The barrier was characterized by the expression of CD31 and vascular endothelial (VE)-cadherin as well as physiologically relevant permeability for macromolecules (see attached **Publication [8] Figures 1C and S3A**). Interestingly, when endothelial cells were absent from the system, a greater number of CAR-T cells in the tumor chambers and a stronger inhibition of tumor growth were observed in both control T and CAR-T cell conditions, suggesting an inhibitory effect of the endothelium (see attached **Publication [8] Figures S3B-D**). Indeed, tumor vasculature is known to be a significant obstacle for T cell trafficking and has long been considered one of the therapeutic targets in cancer immunotherapy^{158–161}. To extravasate, T cells rely on the expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by endothelial cells, which can be downregulated by tumor cells through the secretion of tumor-derived angiogenic factors, such as vascular endothelial growth factor-A (VEGF) and endothelin-1 (ET-1)^{162–164}. Future studies using tumor-on-chip or similar models could explore the mechanism by which the tumors alter endothelial cell phenotype, as well as methods to overcome this inhibitory influence of the tumor vasculature, thereby improving CAR-T cell homing into the tumor. In addition, it is important to note that conventional well plate culture settings can underestimate these inhibitory effects of the TME, as evidenced by the significantly higher infiltration of T and CAR-T cells and lower GFP intensity values of tumor aggregates on day 1 compared to those in the chip setup (see attached **Publication [8] Figures S2D-E**).

In the experiments with PDOs, the model was refined by using isogenic human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) derived from the same (CAR-)T cell donors to avoid potential background alloreaction. When T cells were co-cultured with mvECs from multiple donors (allogeneic setting), the cytokine secretion data showed higher fold changes in TNF- α , IFN- γ , and granzyme B levels compared to when T cells were cultured with isogenic hiPSC-ECs. Unsurprisingly, the fold change in cytokine levels in the isogenic setting was comparable to the condition without endothelial cells (see attached **Publication [8]** *Figure S3E*). HiPSC-ECs were confirmed to express CD31 before their integration into the chip. Despite facilitating human leukocyte antigen (HLA)-matched autologous systems, hiPSCs-ECs expressed a certain level of ROR1, which led to the induction of IFN- γ and granzyme B secretion in preliminary plate assays (see attached **Publication [8]** *Figure S3G and S3H*). As an oncofetal antigen, ROR1 is also a known marker of stem cell derivatives¹⁶⁵. Despite this background effect, patient-specific reactions arising from relative ROR1 expression by the PDOs were evident in the tumor-on-chip systems. This may be irrelevant for various types of CARs, and future advances in hiPSC-derived EC maturation protocol should allow the mitigation of this effect for ROR1-CAR-T cells. Nevertheless, this underscores the need for careful consideration, thorough characterization, and assurance of maturation when using non-malignant hiPSC-based cells in cell-mediated toxicity assays targeting tumor-associated antigens, specifically those that are also expressed during the embryonic stage, categorized as oncofetal and carcinoembryonic antigens^{166,167}.

3.6 Impact of Macrophages' Integration

The interplay between macrophages and CAR-T cells is complex and context-dependent. While macrophages within the TME often exert immunosuppressive effects that promote tumor progression and limit the efficacy of CAR-T cell therapy¹⁶⁸, they can also facilitate antitumor immune responses under certain conditions⁶⁸. Understanding the mechanisms underlying macrophage-tumor and macrophage-CAR-T cell interactions is essential for developing strategies to harness the therapeutic potential of macrophages and optimize CAR-T cell-based immunotherapy for cancer, or ensuring CAR-T cell safety by controlling macrophages-mediated proinflammatory response that can induce CRS. Accordingly, macrophages were introduced in the tumor-on-chip model.

To avoid potential alloreaction in the CAR-T cell-treated tumor-on-chip experiments, macrophages were differentiated from the monocytes-fraction of peripheral blood mononuclear cells (PBMCs) derived from the same donor as the (CAR-)T cells using a published protocol¹⁶⁹. Prior to macrophages' integration into the chip, their surface markers after the differentiation from monocytes at their baseline state (M0), pro-inflammatory (M1-like), and anti-inflammatory (M2-like) phenotypes were characterized using flow cytometry (**Figure 5A**).

From two healthy donors tested, the percentage of macrophages was 80-90% of the cells after the differentiation from monocytes, defined by the gated population of CD45⁺ CD14⁺ CD68⁺ CD11b⁺ cells. These macrophages were able to polarize towards M1-like phenotype upon IFN- γ and LPS stimulation for 24 h, marked by the upregulation of CD80, CD86, and HLA-DR. In addition, IL-4, IL-10, and IL-13 were able to induce M2-like phenotype expression of the macrophages, as shown by elevated expressions of CD163 and CD206 (**Figure 5B**).

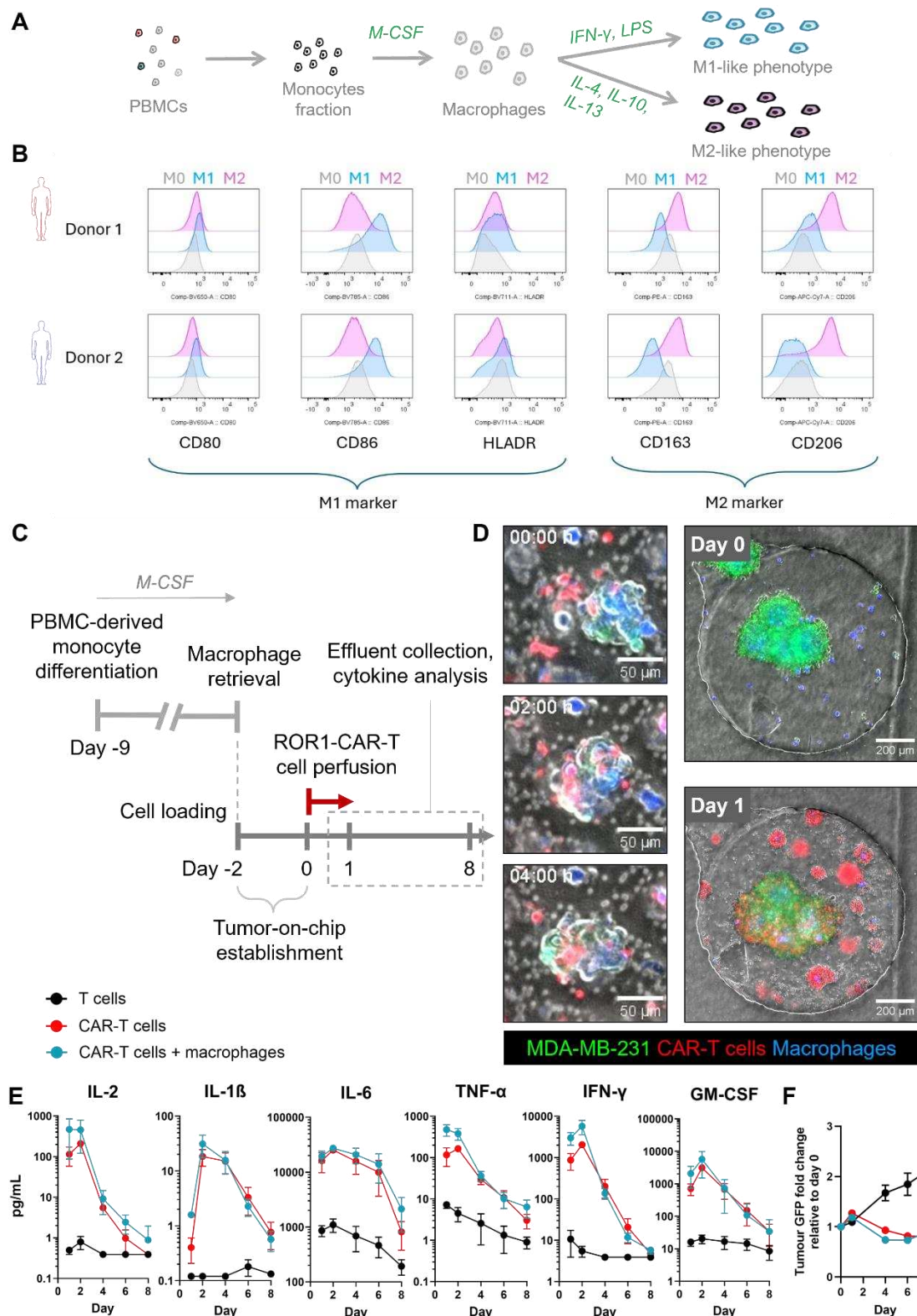


Figure 5: Macrophages' integration into the tumor-on-chip, characterization, and impact towards CAR-T cell response.

(A) Primary macrophages were differentiated from PBMC-derived monocytes, autologously sourced from the same donor as the CAR-T cells used in the experiment. The differentiated macrophages were tested with regards to their polarization ability into M1- and M2-like phenotypes. (B) Flow cytometry analysis of macrophages derived from two healthy donors,

showing the ability of M0 macrophages to polarize into M1-like (CD80, CD86, and HLA-DR) and M2-like (CD163 and CD206) phenotypes after polarization either with IFN- γ and LPS (for M1-phenotype induction) or IL-4, IL-10, and IL-13 (for M2-phenotype induction). **(C)** The timeline of the experiment of CAR-T cell efficacy and safety assessment using tumor-on-chips integrating macrophages. Primary microvascular endothelial cells were expanded and MDA-MB-231 tumor aggregates were generated up to 3 days before chip seeding (see attached **Publication [8], Methods**). After the differentiation for 7 days, macrophages were collected and mixed with the tumor aggregates in a dextran-based hydrogel, injected into the tumor-on-chip, followed by the injection of endothelial cells. Cells were cultured in the chip with perfusion for 2 days. On day 0, either control T cells or ROR1-CAR-T cells were perfused through the chip for 20 h, followed by medium perfusion. Chips were imaged, and effluents were collected every one or two days until day 8. **(D)** CAR-T cell (red) infiltration into the MDA-MB-231 tumor aggregates (green) in the presence of macrophages within the first 4 h (left panel) or on day 0 and day 1 after CAR-T cell perfusion (right panel). **(E)** Release kinetics of cytokines IL-2, IL-1 β , IL-6, TNF- α , IFN- γ , and GM-CSF from days 1 to 8 after control T cell or CAR-T cell (with or without macrophages in the tumor-on-chip) perfusion from days 0 to 1. Samples were collected every 1–2 days; $n = 4$ chips. Data are presented as the mean $\pm$ SEM. **(F)** Quantification of the fold change in the mean GFP intensity of MDA-MB-231 tumor aggregates in each tumor chamber on days 1-8 after the perfusion of (control) T cells or ROR1-CAR-T cells (with or without macrophages in the tumor-on-chip) relative to day 0; $n = 24$ tumor chambers from 4 chips. Data are presented as the mean $\pm$ SEM.

Once the differentiation protocol was successfully validated, the differentiation timeline was fit to the timepoint of tumor-on-chip loading (day -2 before ROR1-CAR-T cell perfusion) (**Figure 5C**). Macrophages were embedded in the dextran-based hydrogel and loaded into the chip together with MDA-MB-231 tumor aggregates. During the first 4 h of ROR1-CAR-T cell perfusion at 500,000 cells/mL, CAR-T cells were able to infiltrate the tumor aggregate despite the macrophages' presence (**Figure 5D, left panel**). On day 1, aggregates of CAR-T cells were observed surrounding the macrophages. CAR-T cells were also able to infiltrate the larger tumor aggregate (**Figure 5D, right panel**). These observations indicate a possible tendency of proinflammatory macrophage phenotype within this TME, which can be induced by IFN- γ secretion by T helper cells Type 1 (Th1)¹⁷⁰. Indeed, on days 1 and 2 after CAR-T cell perfusion, higher proinflammatory cytokine levels (IL-2, IL-1 β , IL-6, TNF- α , IFN- γ , and GM-CSF) were detected in the effluents of tumor-on-chips containing macrophages (up to 4-fold) compared to without (**Figure 5E**). Tumor shrinkage was observed in CAR-T cell treatment conditions both in tumor-on-chip with and without macrophages, supporting the observation of a rather proinflammatory influence and therefore tumor-suppressing role of the macrophages (**Figure 5F**).

These results may be caused by the ratio between tumor cells, macrophages, and recruited CAR-T cells that shift the balance towards proinflammatory TME during CAR-T cell response. In this experiment, the macrophages:tumor cell ratio was 1:2. In solid tumors, however, macrophages can occupy more than half of the tumor mass (roughly 1:1 ratio)^{168,171}. The number of macrophages in the tumor-on-chip may therefore be too low to achieve an immunosuppressive effect. Second, macrophages were integrated into the chip as dispersed single cells surrounding the tumor aggregates, which may limit their contact-dependent inhibitory influence via e.g. PD1-PD-L1 axis exerted by the tumor cells and expose the macrophages to higher nutrient and oxygen availability¹⁷². In solid tumors, macrophages are located within the tumor bulk and are often exposed to hypoxic and nutrient-deprived environments, which polarize them toward an anti-inflammatory tumor-promoting phenotype^{173,174}. Future experiments should explore different macrophage integration methods, for instance, by mixed tumor-macrophage aggregation before chip injection, to study its influence on macrophage phenotype and simultaneously better mimic solid TME. Moreover, as highlighted in previous sections, the tumor-on-chip was treated with high-dose CAR-T cells to simulate the initiation of CRS and to specifically examine the role of macrophages in this context. The amplification of cytokine response was successfully mimicked in this setting. To put this into the CRS-irAOP perspective, this setting extended previous results by mimicking KEs 2 and 3 during CRS development, namely increased pro-inflammatory mediators released by CAR-T cells followed by innate immune cells, specifically macrophages⁷⁹. The tumor-on-chip system may therefore be used to study direct intervention on macrophage function as a safety measure for CRS.

4. Results & Discussion II

Breast cancer-on-chip for studying tumor- white adipose tissue interactions

The contents of this chapter are based on the appended manuscript:

Manuscript [9]:

T. I. Maulana, L. Lazarevski, M. Cipriano, L. Christ, J. Rogal, A. Koch, P. Loskill.

“Breast cancer-on-chip integrating white adipose tissue microenvironment to study tumor-white adipose tissue crosstalk”, (2024)

4.1 Model and Setup Establishment

Based on the criteria described in the aim of the study, several features were integrated throughout the course of the model development (**Figure 6A**). Similar to the tumor-on-chip described in the previous section, pre-aggregation of tumor cells (BC spheroid or PDO) was conducted, in this case, to resemble ductal carcinoma in situ filling an entire mammary duct or lobule. To mimic its development within the TME, the breast cancer-on-chip was designed to allow the placement of the pre-aggregated tumors surrounded by mature adipocytes along with other WAT-associated cellular components (SVF and tissue-resident immune cells) in two separated microfluidic chambers (**Figure 6B**). These tissue chamber compartments are located above a constantly perfused medium channel separated by a semipermeable membrane. BC spheroids (BT474 [HER2+ cell line], HCC70 [triple-negative cell line]) or PDOs were formed until they reached 150-250 μm in size before chip loading (see attached **Manuscript [9] Methods and Figures 1 and S1**). The size was kept below 250 μm during cell injection to avoid the presence of a necrotic core at the beginning of the experiment. A key feature of this breast cancer-on-chip platform is the barrier-free lateral separation between tumor and WAT area within the tissue chamber, allowing the monitoring of tumor growth in response to WAT's presence or other experimental perturbations. WAT-associated cells were injected through the fluidic tissue channel, whereas the BC spheroids/PDOs were loaded from the open-top tumor chambers to ensure their placement at the central region. To encapsulate WAT cellular components in the chip, collagen-I (5 mg/mL) hydrogel was used, since it is the predominant ECM component in breast adipose TME and becomes increasingly abundant and stiffer as the primary tumor progresses¹⁷⁵. The stiffness value (~ 700 Pa) falls within the range of dense breast tissue during cancer progression¹⁷⁶ and simultaneously allows a stable anchorage of WAT cellular components during the model generation process (e.g. PDMS pin removal, BC injection) (see attached **Manuscript [9] Methods and Figure 1**). To encapsulate BC spheroids or PDOs in the chamber's tumor region, a softer hydrogel (~ 200 Pa) composed of denatured collagens and hyaluronic acids (HyStem®-C) was used, as hyaluronic acids accumulate specifically at the peri-tumor stroma during BC development^{177–179}.

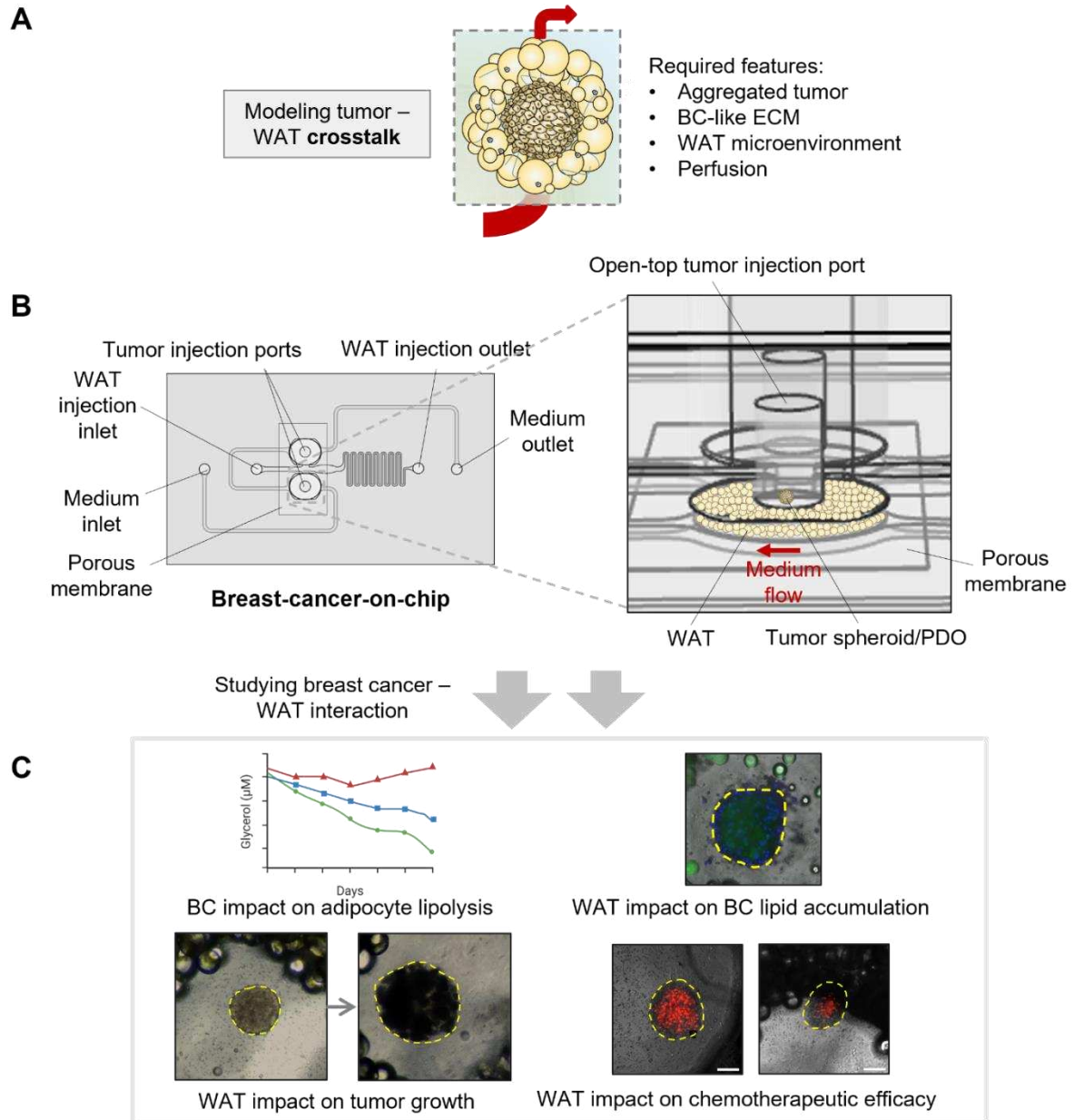


Figure 6: Breast cancer-on-chip establishment: chip design, tissue generation, and readout methods.

(A) Model features that are required to study BC-WAT interaction. **(B)** Schematic representation of the breast cancer-on-chip model. WAT-associated cells were injected through the WAT injection inlet to occupy the WAT chamber area. BC spheroids or PDOs were injected through the open-top tumor injection ports and cultured in the middle of the previously injected WAT-associated cells. The tumor chambers are located above a medium channel, where perfusion of medium was applied from day 1 to 14. **(C)** The experimental setup includes readouts to study BC-WAT interaction, such as glycerol release (lipolytic activity of adipocytes), BC lipid accumulation, and BC growth. Imaging-based viability assay using propidium-iodide staining was conducted to assess the impact of WAT on chemotherapeutic efficacy. Images adapted from figures in the attached **Manuscript [9]**.

The chip fabrication and model generation (i.e. cell seeding and chip culture) methods are described in detail in the attached **Manuscript [9]**. The developed culture method allowed for the co-culture of BC spheroids or PDOs with surrounding WAT, including tissue-resident macrophages with various phenotypes, in a perfused microenvironment for up to two weeks while preserving tissue structure (see attached **Manuscript [9] Figure 2**). Although various breast cancer-on-chip models exist for studying cancer-microenvironment interactions, none have previously incorporated the immunocompetent WAT microenvironment.

4.2 BC and WAT Bi-Directional Crosstalk on Chip

The chip design and experimental setup were designed to allow the investigation of bidirectional impacts between cancer and WAT (**Figure 6C**). Cancer cells can obtain nutrients in the form of fatty acids from adipocytes to fuel their growth via the activation of lipolytic pathway²⁸. During lipolysis, a glycerol molecule previously broken down from a triacylglyceride molecule in adipocytes is released¹⁸⁰. In the breast cancer-on-chip systems, glycerol levels in the effluent of chips containing WAT, BT474, and HCC70 BC spheroids were lower than baseline (WAT only) in a high glucose (17.5 mM) environment and gradually decreased over time. Conversely, in a low glucose (5 mM) environment, glycerol release increased when BC spheroids were present (see attached **Manuscript [9] Figure 3**). This suggests that BC spheroids may induce adipocyte lipolysis to potentially support their growth when glucose is scarce in the TME, which is aligned with the growing number of studies reporting the ability of cancer cells to induce adipocyte lipolysis^{23,25,181–183}. This metabolic adaptation from glycolysis to fatty acid uptake and oxidation in glucose-deprived conditions supports cancer survival¹⁸⁴, which shows the ability of malignant cells to benefit from alternative available resources in TME. Future experiments should investigate the exact mechanism of this lipolysis induction (discussed in **Manuscript [9] Results & Discussion** section).

When looking at the accumulation of lipids in the BC spheroids after co-culture with WAT-associated cells, a higher lipid-derived fluorescence signal (BODIPY 493/503) from the BC spheroids was detected compared to when WAT was not present (see attached **Manuscript [9] Figures 4A, 4C, and S3**). This indicates that BC spheroids may obtain the excessive fatty acids available in the adipose TME, as shown by increasing glycerol concentrations produced in this condition described above. In

addition, the presence of WAT induced faster BC spheroid growth (see attached **Manuscript [9]** *Figure 4B*). This effect was exacerbated by glucose, implying a synergistic effect of glucose and fatty acids in supporting tumor growth. Future experiments need to confirm whether this lipid accumulation in the BC spheroids occurs through exogenous uptake of free fatty acids instead of de novo-produced ones. Furthermore, it remains to be investigated whether tumor growth was supported through increased oxidative phosphorylation in combination with glycolysis, as we observed synergistic effects of glucose and fatty acids. Although many in vitro models have successfully produced insights into the bidirectional crosstalk between cancer cells and adipocytes with regards to fatty acid transport mechanism, these models do not yet enable long-term co-culture of tumor and WAT for multiple weeks to monitor tumor growth in a three-dimensional, organotypic, and perfused TME (described further in **Manuscript [9]**). This breast cancer-on-chip addresses this gap by providing a novel platform to culture cancer and WAT in a perfused microenvironment for up to two weeks with retained tissue structure.

4.3 Impact of WAT on Chemotherapeutic Efficacy

Adipocytes can promote cancer progression by metabolizing chemotherapeutic drugs, rendering them less effective in the TME⁴⁷. This was demonstrated using the breast cancer-on-chip model, where the presence of WAT retained HCC70 spheroids' viability upon 4-day-treatment with 1 μ M daunorubicin. These findings are consistent with a previous study showing a chemoprotective effect of adipocytes against daunorubicin³⁴, although the exact mechanism in the breast cancer-on-chip system remains to be investigated. Adipocytes can protect cancer through direct daunorubicin sequestration and metabolism, which extends to other chemotherapeutic agents like doxorubicin and mitoxantrone³⁴. In addition to drug sequestration, lipids can also promote drug accumulation¹⁸⁵. In leukemia, another mechanism in which adipose tissue protects malignant cells from chemotherapy has been reported. In this context, gonadal adipose tissue serves as a supporting TME for a subpopulation of leukemic stem cells, elevating their CD36 fatty acid transporter-dependent fatty acid metabolism. This increased fatty acid oxidation rendered them more resistant to various chemotherapeutic drugs, including cytarabine, doxorubicin, etoposide, CPT-11, and dasatinib¹⁸⁶. Acquired chemoresistance through increased fatty acid metabolism has

indeed been extensively reported and recently reviewed¹⁸⁷, also in the context of breast cancers, and may be further studied in the breast cancer-on-chip system.

5. Conclusion and Outlook

This thesis presents the successful development and utilization of two distinct tumor-on-chip models tailored to address specific applications. The first model is designed to assess the efficacy and safety of CAR-T cell therapy in the context of solid tumors. The second model aims to recreate the breast cancer TME in vitro for studying the crosstalk between tumors and WAT. Each of these purposes required a bottom-up integration of unique features, including the selection of microfluidic chip materials, channel design, cellular components, cellular scaffolds, tissue microarchitecture, dynamic mechanisms during culture, and readout methods. Developing these models was a highly iterative process requiring careful monitoring of all these interconnected aspects and evaluation against multiple criteria to achieve the specific study's objectives described in section 2. This thesis only focuses on the latest versions of both tumor-on-chip models and the results achieved with those models.

Tumor-on-chip for CAR-T cell assessment targeting solid tumor malignancies: This tumor-on-chip specifically modeled the initial events occurring in the TME upon CAR-T cell infusion in patients and the effects that manifest over the following week. This consisted of CAR-T cell flow through the vasculature, extravasation toward the tumor, tumor infiltration, and cytokine release. By monitoring these events, the efficacy and safety profile of specific T cell treatment and/or its administration strategy were evaluated. Four major findings are attributed to these assessments:

- 1.) CAR-T cells infiltrated tumor and hampered its growth on chip in target-specific manner. The extent of CAR-T cell recruitment, tumor infiltration, and tumor growth inhibition depended on the CAR expression on T cells as well as the abundance of target antigen expression on (patient-specific) tumor cells. This response was highly influenced by the cellular components and tumor microarchitecture. This antitumor response was overestimated in conventional in vitro settings that lack an endothelial barrier, ECM, and controlled CAR-T cell perfusion.
- 2.) High CAR-T cell infiltration on chip was only achieved when high-dose CAR-T cells treatment was mimicked. CAR-T cells effectively infiltrated the larger tumor aggregates (>50 μm) only when a mid-to-high CAR-T cell concentration range

(relative to the CAR-T cell count measured in the peripheral blood of patients with CRS) was applied.

- 3.) Excessive CAR-T cell inflammatory response could be controlled using dasatinib without compromising treatment efficacy. The model can be used to identify a potentially optimal acute safety intervention strategy to bring pro-inflammatory cytokine levels down while keeping the baseline antitumor efficacy of the CAR-T cells. This proof-of-principle assessment can be applied to other intervention strategies to manage adverse events caused by CAR-T cell therapy.
- 4.) The presence of endothelial cells and macrophages in the TME altered CAR-T cell recruitment and inflammatory response. The model showed the crucial role that endothelial cells and macrophages in the TME play in dampening or amplifying the recruitment and pro-inflammatory cytokine response during CAR-T cell treatment. With regards to macrophages, this effect may depend on their state and relative abundance to tumor and CAR-T cells during CAR-T cell treatment.

While there are a variety of aspects in which the model could be further developed, this tumor-on-chip already presents huge potential for various future applications. For example, it could be used to compare the efficacy and safety profiles of different CAR-T cell products targeting the same antigen but vary in their CAR designs, gene transfer methods (e.g. viral vs. non-viral vectors), CAR-T cell expansion methods, starting T cell phenotypes, or donors. Due to the modular concept of the model, challenges of TME typically found in solid tumors can systematically be recreated, including the dense tumor stroma by incorporating higher ECM stiffness along with the abundant presence of cancer-associated fibroblasts and immunosuppressive myeloid cells. Systematically assessing the impact of each barrier within the TME and comparing various T cell engineering and administration strategies to overcome these barriers and increase persistence could be extremely beneficial for advancing the field of adoptive T cell therapies beyond just CAR-T cells. Another major advantage of the tumor-on-chip model is the rapid platform establishment and results analysis compared to animal experiments, offering opportunities for direct clinical application to predict treatment efficacy and safety.

Breast cancer-on-chip for studying tumor-white adipose tissue interactions: This tumor-on-chip specifically modeled the early development of primary BC within a

microenvironment rich in adipose tissue. It integrated BC spheroids or PDOs and WAT-associated cellular components (mature adipocytes, stromovascular cells, and tissue-resident macrophages), which enabled long-term co-culture to monitor BC-WAT interactions. Throughout the culture period, the influence of BC on adipocyte lipolytic activity, as well as the effects of WAT on BC lipid accumulation, growth, and drug response, were evaluated. Four major demonstrations were performed:

- 1.) Breast cancer-on-chip enabled controlled compartmentalization of BC spheroids/PDOs and WAT that mimicked breast adipose TME and allowed long-term co-culture. The chip design and cell seeding method allowed the compartmentalization of BC spheroids or PDOs central to the surrounding WAT, thereby mimicking the microarchitecture of breast adipose TME and simultaneously allowing the monitoring of BC growth. In addition, stacking another microfluidic channel below this generated tissue enabled the constant supply of nutrients and removal of metabolic waste products via medium perfusion. The tissue maintained its structure and cells remained viable upon two weeks of culture.
- 2.) BC's presence induced higher lipolytic activity of adipocytes. Throughout the co-culture period of WAT-containing chips, both the presence of BCs and a glucose-deprived condition resulted in higher glycerol concentrations in the effluent of chips. This indicates that BC cells may induce adipocyte lipolysis due to limited glucose availability as a way to obtain an alternative nutrient source.
- 3.) WAT's presence increased lipid accumulation and growth of BCs. In WAT microenvironment, BC spheroids accumulated more lipids and grew faster compared to their single-culture on-chip. This effect is exacerbated by higher glucose availability, indicating a synergistic effect of glucose and fatty acids in supporting BC growth.
- 4.) WAT protected BC from chemotherapeutic daunorubicin. Upon 4 days of treatment with daunorubicin, BC viability remained stable when WAT was present in their surroundings. In contrast, daunorubicin potently induced BC cell death when cultured alone. This implies a protective effect of WAT from daunorubicin and potentially other chemotherapeutic agents.

The tailored breast cancer-on-chip model represents a significant advancement in human-based models for studying interactions between BC and WAT. By closely

mimicking the human breast TME and incorporating relevant WAT-associated cell types, this model overcomes the limitations of previous in vitro systems and animal models (described in more detail in **Manuscript [9]**).

Designed to facilitate research beyond BC, this model can be adapted to other tumor types where tumor-WAT interactions are crucial in cancer progression and therapy. This versatility holds great potential for exploring novel therapeutic strategies that target metabolic pathways and tumor-WAT interactions. Such approaches could ultimately lead to more effective treatments for breast cancer and potentially other cancers where WAT plays a central role.

6. Additional Methods

Most of the methods used for the research experiments in this thesis are thoroughly detailed in the attached publications and manuscripts. The following chapter describes the additional methods applied for the macrophages' integration into the tumor-on-chip model in section 3.6. The remaining methods using this particular tumor-on-chip were described in the attached **Publication [8]**.

6.1 Monocyte Isolation and Differentiation into Macrophages

Isolation of monocytes and their subsequent differentiation into macrophages was performed according to a published protocol¹⁶⁹. Briefly, 15 mL of whole blood from each donor was mixed with 15 mL of PBS⁻ (1:1) at room temperature (RT). Peripheral blood mononuclear cells (PBMCs) were separated via density gradient centrifugation using SepMate™ according to the manufacturer's instructions. For each donor, 15 mL Histopaque 1077 (10771; Sigma Aldrich) was added into the central hole of a 50 mL SepMate™ tube, followed by gently adding 30 mL of the previously diluted whole blood. The tubes were then centrifuged for 12 min at 1200 x g at RT. PBMCs attaching to the walls of the tube were carefully detached, and the PBMC-containing supernatants were transferred into fresh 50 mL falcon tubes. To wash the PBMCs, the tube was filled up to 50 mL with PBS⁻ containing 2 mM of EDTA (15575020; Invitrogen) and 10% of BSA (referred to as isolation buffer hereafter), followed by centrifugation for 8 min at 300 x g. The washing step is repeated twice. Lastly, the cell pellet was resuspended in 20 mL of isolation buffer for cell counting. Cryopreserved PBMCs from the same (CAR-)T cell donor were alternatively used for chip experiments. In this case, the thawed cell pellet was first resuspended and incubated in 100 µg/mL DNase (diluted in isolation buffer) for 15 min at RT, followed by straining the cells through a 40 µm cell strainer.

Monocytes were isolated using Pan Monocyte Isolation Kit (130-096-537; Miltenyi Biotec) according to the manufacturer's instructions. Monocytes were seeded at a concentration of 500,000 cells/mL in a 24-well plate (250,000 cells/cm²) in a macrophage differentiation medium: RPMI-1640 (61870036, Thermo Fisher Scientific) supplemented with 10% (v/v) FCS (SH30066.03, HyClone™ FetalClone™ II Serum; Cytiva), 100 U/mL penicillin-streptomycin (15140122; Thermo Fisher Scientific), and

50 ng/mL M-CSF (300-25; PeproTech). Cells were incubated for 2 days at 37 °C, 5% CO₂ and 95% rH. Medium was refreshed on day 2 and incubated for another 3 days.

6.2 Maturation and Polarization of Macrophages

For the differentiation into M0 macrophages, the medium was refreshed once more on day 5 of culture and cultured for another 2 days. To induce macrophage polarization into M1-like phenotype, the culture medium was replaced on day 5 of culture with RPMI-1640 (61870036, Thermo Fisher Scientific) supplemented with 10% (v/v) FCS (SH30066.03, HyClone™ FetalClone™ II Serum; Cytiva), 100 U/mL penicillin-streptomycin (15140122; Thermo Fisher Scientific), 50 ng/mL M-CSF (300-25; PeproTech), 50 ng/mL LPS (00-4976-93; Invitrogen), and 100 ng/mL IFN-γ (78141.1; STEMCELL Technologies). To induce macrophage polarization into M2-like phenotype, the culture medium was replaced on day 5 of culture with RPMI-1640 (61870036, Thermo Fisher Scientific) supplemented with 10% (v/v) FCS (SH30066.03, HyClone™ FetalClone™ II Serum; Cytiva), 100 U/mL penicillin-streptomycin (15140122; Thermo Fisher Scientific), 50 ng/mL M-CSF (300-25; PeproTech), 40 ng/mL IL-4 (130-093-921, Miltenyi Biotec), 10 ng/mL IL-10 (200-10; PeproTech), and 20 ng/mL IL-13 (200-13; PeproTech).

6.3 Flow Cytometry Analysis of Differentiated Macrophages

For the analysis of the extracellular markers' expression on the differentiated and polarized macrophages, the following antibodies were used in the indicated final dilution: anti-human CD80 BV650 [1:100] (305227; BioLegend), anti-human CD206 APC/Fire750 [1:100] (321134; BioLegend), anti-human CD163 PE [1:500] (333606; BioLegend), anti-human CD45 AF700 [1:200] (304024; BioLegend), anti-human CD11b BV421 [1:300] (101235; BioLegend), anti-human CD14 AF488 [1:300] (367130; BioLegend), anti-human CD86 BV785 [1:100] (395442; BioLegend), anti-human HLA-DR BV711 [1:200] (307644; BioLegend), and anti-human CD68 PE/Cy7 [1:200] (333816; BioLegend). 96-well V-bottom cell culture plate was used for staining and flow cytometry reading. For staining of extracellular markers, cells in each well were first washed with the staining buffer: 3% FCS with 2 mM EDTA (15575020; Invitrogen) in PBS⁻, followed by incubation in a blocking solution containing Human BD Fc Block (564220; BD Biosciences) diluted 1:25 (v/v) in PBS⁻ for 15 min at 4 °C. Afterwards, cells were incubated for 30 min at 4 °C in the extracellular antibody-mixture

solution. Zombie aqua dye (423102; BioLegend) was added 1:200 in the antibody-mixture solution to exclude dead cells during analysis. Cells were then washed twice with the staining buffer and fixed with ROTIHistofix 4% (P087.6; Carl Roth GmbH) for 10 min at RT. Next, the cells were washed twice at 300 x g for 3 min in a permeabilization buffer using 0.5% Saponin and 0.01% sodium azide (NaN_3) in 3% FCS-PBS⁻ solution to permeabilize cells for the staining of intracellular markers. Cells were then incubated in the intracellular antibody-mixture solution for 30 min at 4 °C in the dark, followed by washing twice by centrifuging at 300 x g for 3 min in a permeabilization buffer. The samples were resuspended in the staining buffer and analyzed directly using flow cytometry (BD LSR II or BD LSRFortessa™, BD Biosciences) or stored at 4 °C for maximum overnight. In every staining procedure, autofluorescence and isotype controls were included. For the analysis, FSC-A and SSC-A properties were used to identify cells, and cell doublets were excluded based on FSC-H vs. FSC-A. Dead cells were excluded from the analysis. The population of macrophages was defined by the gated population of CD45⁺ CD14⁺ CD68⁺ CD11b⁺ cells. M1-like macrophages were defined by the gated population of CD80, CD86, and HLA-DR. M2-like macrophages were defined by the gated population of CD163 and CD206.

6.4 Integration and Culture of Macrophages in the Tumor-on-chip

Chip fabrication, MDA-MB-231 tumor aggregate generation, and chip priming methods are described in detail in **Publication [8] Methods**. To retrieve the MDA-MB-231 aggregates from the plate, cell culture medium was first aspirated from the wells before adding 500 µL of 2 mg/mL cold dispase II solution, followed by pipetting up and down for mechanical dissociation of the gel. This is followed by incubation for 10 min at 37 °C, 5% CO₂ and 95% rH. Afterwards, each well was resuspended with 1 mL of cell culture medium. The retrieved aggregates were subsequently centrifuged at 500 x g for 5 min.

In the meantime, macrophages were retrieved after day 7 of differentiation from PBMC-derived monocytes. Here, each cell culture well was gently washed with PBS⁻, followed by incubation with 250 µL Accutase (P10-21500; Pan-Biotech) for 10 min at 37 °C. Afterwards, each well was resuspended with 750 µL of RPMI 1640 medium to retrieve the cells, followed by centrifugation at 300 x g for 5 min. Cell pellet was then resuspended in 10 µM of CellTracker™ CMAC dye solution (C2110; Invitrogen) in

phenol red-free DMEM/F-12 medium and incubated for 20 min at 37 °C, 5% CO₂ and 95% rH. Phenol red-free DMEM/F-12 medium was added up to 10 mL and subsequently centrifuged at 300 x g for 5 min to wash the cells. In experimental conditions containing macrophages, cell pellet containing 600,000 macrophages was resuspended in dextran hydrogel (3-D Life Dextran-CD Hydrogel SG; Cellendes GmbH) supplemented with RGD peptide (09-P-001; Cellendes GmbH) with a final concentration of 0.5 mmol/L. This solution was then used to resuspend the previously centrifuged tumor aggregate pellet from four AggreWell™ plate wells, with a total cell number of roughly 1,200,000 cells, yielding a macrophage:tumor cell ratio of 1:2. The total volume of macrophage-tumor hydrogel solution is 160 µL. 10 µl is used to load one chip. The subsequent endothelial cell seeding, (CAR-)T cell perfusion, and chip culture methods are described in detail in the Methods section of **Publication [8]**.

6.5 Cytokine Quantification Assay

Cytokines from undiluted chip effluent samples were analyzed by fluorescent bead-based multiplex sandwich immunoassays (Custom ProcartaPlex Multiplex Panel; PPX-08, Invitrogen) containing capture beads targeting IL-1β, IL-2, IL-6, TNF-α, IFN-γ, and GM-CSF, using ProcartaPlex™ Flat Bottom 96-well plate (EPX-88182-000, Invitrogen) and read by FLEXMAP 3D according to manufacturer's protocol.

6.6 Image Analysis

To perform timepoint live imaging of (CAR-)T cell infiltration, an epifluorescence microscope (Axio Observer 7, Carl Zeiss) was used, with the focal plane set to focus on the aggregate. Throughout all live cell imaging acquisitions of the chips, the microscope chamber temperature was maintained at 37 °C. The analysis of GFP fluorescence intensity of the MDA-MB-231 tumor aggregates was conducted using ImageJ-Fiji software¹⁸⁸. Here, the mean gray value of tumor aggregates in each chamber was measured and compared with their initial mean gray value on day 0. Tumor aggregates with a diameter smaller than 50 µm were deemed too small and thus excluded from the analysis.

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Declaration

„Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel: *„Microphysiological Tumor-on-Chip Models Recapitulating Tumor-Microenvironment Interaction Applicable for Cell Therapy Testing“* selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen (alternativ: Zitate) als solche gekennzeichnet habe. Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen (Beschluss des Senats vom 25.5.2000) beachtet wurden. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe. Mir ist bekannt, dass die falsche Abgabe einer Versicherung an Eides statt mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft wird.

Stuttgart, 21.06.2024

Tengku Ibrahim Maulana

Appendices

Publication [1]

Immunocompetent cancer-on-chip models to assess immune-oncology therapy

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Abstract:

The advances in cancer immunotherapy come with several obstacles, limiting its widespread use and benefits so far only to a small subset of patients. One of the underlying challenges remains to be the lack of representative nonclinical models that translate to human immunity and are able to predict clinical efficacy and safety outcomes. In recent years, immunocompetent Cancer-on-Chip models emerge as an alternative human-based platform that enables the integration and manipulation of complex tumor microenvironment. In this review, we discuss novel opportunities offered by Cancer-on-Chip models to advance (mechanistic) immuno-oncology research, ranging from design flexibility to multimodal analysis approaches. We then exemplify their (potential) applications for the research and development of adoptive cell therapy, immune checkpoint therapy, cytokine therapy, oncolytic virus, and cancer vaccines.



Immunocompetent cancer-on-chip models to assess immuno-oncology therapy [☆]



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ABSTRACT

The advances in cancer immunotherapy come with several obstacles, limiting its widespread use and benefits so far only to a small subset of patients. One of the underlying challenges remains to be the lack of representative nonclinical models that translate to human immunity and are able to predict clinical efficacy and safety outcomes. In recent years, immunocompetent Cancer-on-Chip models emerge as an alternative human-based platform that enables the integration and manipulation of complex tumor microenvironment. In this review, we discuss novel opportunities offered by Cancer-on-Chip models to advance (mechanistic) immuno-oncology research, ranging from design flexibility to multimodal analysis approaches. We then exemplify their (potential) applications for the research and development of adoptive cell therapy, immune checkpoint therapy, cytokine therapy, oncolytic virus, and cancer vaccines.

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Contents

1. Introduction	282
2. New opportunities provided by immunocompetent Cancer-on-Chip models	283
2.1. Fit-for-purpose tailoring of models for specific questions	283
2.2. Modeling and manipulating the tumor microenvironment	285
2.2.1. Cellular components	286
2.2.2. Local oxygen content	286

Abbreviations: ACT, adoptive cell therapy; ADCC, antibody-dependent cellular cytotoxicity; APC, antigen-presenting cells; BITEs, bispecific T cell engagers; CAF, cancer-associated fibroblast; CAR, chimeric antigen receptor; CRS, cytokine release syndrome; CTLA-4, cytotoxic T-lymphocyte-associated Protein 4; ECM, extracellular matrix; DC, dendritic cell; iPSC, induced pluripotent stem cell; irAE, immune related adverse events; HER2, human epidermal growth factor receptor 2; ICI, immune checkpoint inhibitor; iCoC, immunocompetent Cancer-on-Chip; NK cells, natural killer cells; OoC, Organ-on-Chip; OV, oncolytic virus; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; PMN, polymorphonuclear neutrophil; TAM, tumor associated macrophage; TIL, tumor-infiltrating lymphocyte; TME, tumor microenvironment; TCR, T cell receptor; Treg, regulatory T cell.

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2.2.3.	Chemokine gradient	286
2.2.4.	Cell scaffolds and other biophysical forces	287
2.2.5.	Vasculature and perfusion	287
2.3.	Optical accessibility and high-content analysis	288
2.4.	Short platform development time	289
3.	Applications of iCoCs to test immunotherapy approaches	289
3.1.	Adoptive cell therapy	289
3.1.1.	iCoC approaches to study TME effects on ACTs	290
3.1.2.	Modeling ACT-mediated toxicities	291
3.2.	Immune checkpoint inhibitors	291
3.2.1.	Identification of responders in patient-specific iCoCs	291
3.2.2.	iCoCs as high(er)-throughput screening tools	292
3.2.3.	Overcoming resistance by defining synergistic drug combinations in iCoCs	292
3.2.4.	Dissecting effects of the immunosuppressive TME on ICI therapies	293
3.2.5.	An unmet need: Researching immune-related adverse events of ICIs	296
3.3.	Cytokine therapy	296
3.4.	Oncolytic viruses	298
3.5.	Cancer vaccines	298
4.	Outlook	298
	Funding	299
	Declaration of Competing Interest	299
	Acknowledgments	299
	Appendix A. Supplementary material	299
	References	299

1. Introduction

Cancer is one of the most frequent causes of death worldwide. Conventionally, therapeutic strategies based on radiation, chemotherapy, and surgical removal are applied. In the last decades, immunotherapy has established itself as an additional treatment option. Cancer immunotherapy aims to eliminate cancer cells by unleashing the immune system, in particular T cells. It encompasses a broad range of modalities, such as adoptive cell therapy, immune checkpoint inhibition, cytokine therapy, oncolytic viruses, and cancer vaccines [1–4]. The first immune system-related cancer treatment dates back to the end of the 19th century [5]. Since then, numerous discoveries have elucidated the central role of the immune system in the control of malignantly transformed cells. Nowadays, immunotherapeutic approaches have led to a breakthrough in cancer treatment by prolonging the survival of patients suffering from rapidly progressing malignancies.

Despite the explosion of interest in cancer immunotherapy in the past years, the failure rate in oncology drug development is still 2-fold higher than for non-oncology drugs; spanning throughout the entire process from phase I studies to approval. In fact, the likelihood of approval is currently only 5% [6]. A major issue is that the translation from preclinical animal models often fails due to poor recapitulation of the human carcinogenesis and human response to cancer therapy [7], including tumor resistance and immune escape through defects in (neo)antigen presentation, upregulation of additional inhibitory checkpoints and immunosuppressive features of the tumor microenvironment [8–12]. The induction of artificial carcinogenic processes in animals does not parallel the multifactorial and life-style related tumor etiology in humans. Furthermore, in humans, cancer is often associated with aging which is not reflected in most mouse models, thereby additionally hampering the translatability of preclinical studies to the situation in patients [13]. As most tumor mouse models are still relying on inoculation of *in vitro* grown tumor cell lines, these models also fail to recapitulate e.g. the interplay of the malignant cells with immune cells and other cells within the tumor microenvironment such as fibroblasts [14]. Similarly, the development of immune cells in response to tumor growth are not always recapitulated in these models, such as e.g. tissue-resident memory T cells, which have been shown to play an important role in controlling tumor

growth and express many of the immune checkpoint receptors targeted by immunotherapy [15]. The key immunological processes that are essential to allow cancer progression or to effectively mimic the therapeutic responses to immuno-oncology therapies differ significantly between species [16]. Many immune cell subsets important to control tumor growth differ in development, regulation, and signaling pathways, and some important cell types, such as certain subsets of $\gamma\delta$ T cells are not represented in mice at all [17]. The differences in the composition of immune cell subsets and expression of receptors between mice and humans can, however, be of utmost importance for the prediction of possible toxicities, e.g. differences in CD28 expression caused serious side effects in the first-in-human trial of TGN1412 which were not foreseen during preclinical development [17–19]. Last, the safety of many immune therapies is assessed in healthy animals lacking tumors, and as such, tumor-induced changes to the targeted immune cells are not represented. Despite this, more than 10% (more than 1 million) of all animals used in research and testing in the EU were used for human cancer studies in 2017. In the case of applied/translational science, cancer research accounted for the most used animals overall; In basic science, cancer research holds on to the 3rd “place” [20]. The limited predictive ability of the current preclinical models is depicted in the clinical results, where various issues related to safety and efficacy are becoming more apparent [21–29]. Hence, alternative model systems that are able to recapitulate the human disease and feature both patient cancer cells and human immune cells are of utmost importance to increase the success rates in immunotherapy discovery and development.

To this end, advanced human-based *in vitro* models have been developed to allow targeted mechanistic research, gain early information about on-target off-tumor and off-target toxicities, and increase confidence in the mechanisms of action. Ideally, an *in vitro* cancer model should comprise three-dimensional (3D) cancer tissue integrated within a tumor microenvironment (TME) featuring vascularization and immune components [30]. The latter is an essential aspect of both carcinogenesis and cancer therapy: Tumor growth often depends on the silencing of the immune response [31]. The vasculature is also crucial as it provides nutrient and oxygen supply. It can dynamically adapt to stimuli from the tumor and is a key element of immune cell interaction [32].

In the last decade, Organ-on-Chip (OoC)-technology has emerged as a promising platform for the generation of microphysiological tissue models that mimic the key functional units of organs. By integrating 3D co-cultures of relevant cell types with vasculature-like perfusion, OoCs allow to study the communication between individual cells, tissue components, or entire tissues [33–35]. By combining microfabrication and biomaterials with tissue engineering and cell biology, the research community now has complex human-relevant model systems at its disposal that are able to provide answers to questions that animal models were not [36,37]. Cancer research has been a major application area for microfluidic cell culture systems; for instance, to generate defined gradients and investigate tumor cell migration, to study the behavior of circulating tumor cells, or for cancer cell detection [38–40]. More recently, Cancer-on-Chip systems with higher degrees of complexity have been introduced to model tumor heterogeneity and tumor microenvironments, comprehensively covered by several recent overview articles [41–43]. In this review, we first take a closer look at the state of the art of Cancer-on-Chip models that integrate immune components and discuss the advances they bring to (mechanistic) immuno-oncology research. Afterwards, we highlight examples of how these human-based immunocompetent Cancer-on-Chip (iCoC) models can be applied to assess the efficacy and safety of various immunotherapy modalities.

2. New opportunities provided by immunocompetent Cancer-on-Chip models

Traditionally, microfluidics technology has been utilized broadly for (immuno-)oncology research, for instance, to isolate circulating tumor cells, immobilize single cells, and pair individual immune-cancer cells to study direct cell-cell interactions [44–47]. The convergence of microfluidics, microfabrication, biomaterials, and tissue engineering research has provided the opportunity to create complex vascularized and perfused structures in a tightly controlled microenvironment accessible by a wide range of analysis methods. Immunocompetence is added to Cancer-on-Chip models by integrating human immune cells capable of generating an immune response into models that feature human cancer cells presenting target antigen and carefully tailored TME relevant to a specific human tumor type. First-generation iCoC models focused mostly on recapitulating and studying 2D cell migration in response to cytokine/chemokine gradients. More recent models aim at mimicking the complex 3D cancer microenvironment including the extracellular matrix (ECM), dynamic vasculature-like perfusion, for instance with self-assembled microvessels. Moreover, novel iCoCs shift from cancer cell lines to tumor biopsies, spheroids, or organoids and are combined with high-content imaging to gain intricate insights in immune-cancer interaction dynamics. In the following sections, we give an overview of the concepts and potentials of iCoC models specifically for immuno-oncology research. We highlight how they can provide useful mechanistic insights about immune-cancer interaction by leveraging aspects, such as their adjustable degree of complexity through a fit-for-purpose approach, the opportunity to tune tumor microenvironmental parameters, the applicability of high-content imaging and analysis, as well as the accelerated platform development time relative to e.g. animal models.

2.1. Fit-for-purpose tailoring of models for specific questions

OoC technology provides a platform of engineered compartments, channels, and barriers for the co-culture of different cell types and tissues in a fit-for-purpose manner [48,49]. The ability

to design customized cellular microenvironments enables researchers to determine flexibly the desired cellular organization and relative position between different cell populations according to the experimental requirements and research questions. In the human body, immune cells are highly mobile and actively migrate from one tissue to another. They move under flow conditions inside the vasculature, extravasate, infiltrate the TME, and interact with cancer cells, all of which can be readily recapitulated inside iCoCs through guided spatial compartmentalization. This compartmentalization also serves further purposes, such as the generation of concentration gradients, temporal control of cell-cell interaction, creation of multilayered 3D-structures, immobilization of tissue fragments, as well as monitoring the behavior (viability, morphology, motility, etc.) of cancer and immune cells.

In simple microfluidic systems, monocultures of cancer or immune cells were exposed to gradients of chemoattractants or conditioned media to study migratory behavior. Gradients were thereby generated by mixing continuous laminar flows based on the pressure difference between in- and outlets [50–52] or by adding soluble factors directly into the micro-chamber with a microinjector for short-term gradients [53]. Via real-time imaging, cell migration without directional constraints [50,51,53] or with directional movement guided through channels [52] was observed. Cultivation of more than one cell type was implemented in various ways: Most simply, two different cell types were mixed and cultivated in the same culture chamber for stromal support [54] or to observe direct interactions of tumor cells with immune cells [55]. Businaro et al. designed a microfluidic device with two main culture chambers connected with a central compartment via microchannels [56]. This platform allows chemical and physical contact between tumor cells and immune cells and was used for migration studies (Fig. 1A). A slightly modified version of this chip was used to characterize migration of immune cells in competition conditions: Dendritic cells (DC) in the central compartment were exposed to the secretome of treated cancer cells (RI) and either matrix or untreated (NT) cancer cells that were embedded in the right and left chamber, respectively (Fig. 1B, supplementary video) [57]. To mimic varying physical confinement and complex pathways that cells encounter *in vivo*, Um et al. oriented channels of 5 μm or 50 μm width in a maze [58]. Bait cells (normal or cancerous cells) were placed at the outlets of the maze and the path pattern of dendritic cells was monitored (Fig. 1C). This platform sheds light on the physical and chemical microenvironment and their impact on the trajectories of immune cells.

Fluidic manipulation in iCoC models allows both temporal and directional control over the media flow, which has been used to temporally control paracrine signaling between different cell types. For example, Hsu et al. used pneumatic microvalves to control the interaction between cancer cells, immune cells, and fibroblasts that are cultured in separate chambers [59]. The media was directed into the different compartments to pretreat/activate cells in a temporally controlled manner. In another platform, developed by Wu et al., microvalves were leveraged to pattern a well-defined antibody barcode in a channel between two adjacent cell culture chambers [60]; thereby, intercellular communication could be assessed via surface-enhanced Raman scattering on-chip. Guo et al. employed precise fluidic flow control to assess the invasive behavior of tumor cells [61]. In one chamber, tumor cells and macrophages were cultivated in close proximity on both sides of a membrane, which induced epithelial-to-mesenchymal transition (EMT) in tumor cells and tumor-associated macrophage (TAM)-like phenotype in macrophages. Conditioned media of the co-culture was directed to a distant tumor cell culture chamber to activate cancer cells. Subsequent redirection of the medium via microvalves lead to a gradient in a hydrogel-loaded migration chamber and promoted cancer cell invasion. Rather than manipulating the

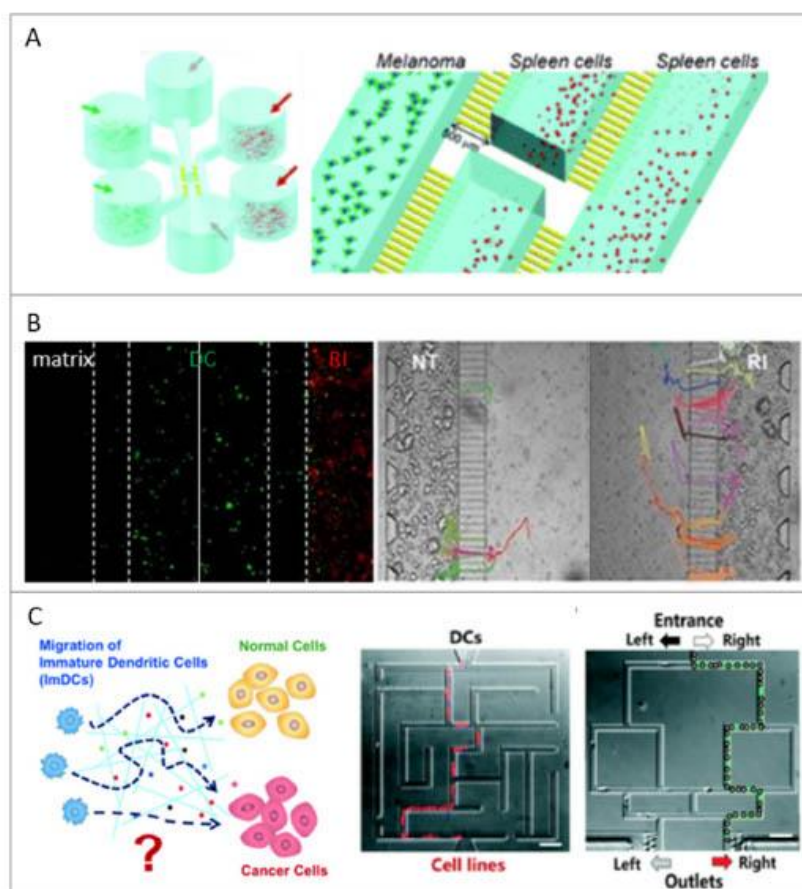


Fig. 1. Different examples of fit-for-purpose iCoC Designs: A) In a co-culture iCoC model, two main culture chambers and a central compartment are connected via micro-channels and allow chemical and physical contact between tumor cells and immune cells. The secretion of chemokines by melanoma cells induces migration of spleen cells through micro-channels that mimic body venules (Republished with permission of Royal Society of Chemistry, from Ref. [56]). B) In a similar iCoC as in A), dendritic cells (DC) in the central channel were cultivated in no competition conditions in between treated cancer cells (RI) and a channel filled with matrix as shown in the fluorescent image (DC = green, RI = red). In competition conditions, the matrix was replaced by untreated cancer cells (NT) and the migratory pattern of DCs was analyzed as depicted in the bright field image by colored lines. DC preferentially migrated towards treated cancer cells rather than an empty matrix or untreated cancer cells. Reproduced from [57], CC BY 4.0. C) Channels with varying sizes oriented in a maze allow the investigation of directional choices of immune cells towards bait cells in a physically confined environment (Republished with permission of Royal Society of Chemistry, from Ref. [58]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluidics, Yu et al. temporally controlled the paracrine signaling between immune and cancer cells using a system with reconfigurable stacks of cultured tissues. With this system, each tissue compartment can be cultured separately and interfaced with the other compartments at arbitrary time points. This enabled the generation of organotypic differentiated tumor-associated macrophages in a tumor-specific and patient-specific manner [62].

More recent iCoC models integrate ECM scaffolds to provide a 3D environment for different cell populations (Fig. 2A). These scaffolds enable the creation of a more physiologically relevant biophysical/chemical landscape within the TME. The integration of the TME components is crucial as it is known to heavily attenuate tumor immunosurveillance, influence treatment responses, and mediate resistance, hence have been aimed as potential targets

in immuno-oncology [63–66]. A number of studies demonstrated that 3D microfluidic cancer model incorporating multiple cell populations exhibited a substantially different response to immunotherapeutic drugs compared to 2D models [67–71]. In fact, drug effects are often overestimated in 2D models [72–74]. However, an underestimation of the effects of immunomodulatory agents targeting immune checkpoints in 2D immune-cancer models was observed when compared with an iCoC model that integrates multiple aspects of the TME. This was caused by the upregulation of immune exhaustion markers in the iCoC model, but not in the 2D plate control [71]. This suggests that understanding the contribution of the tumor microenvironment is crucial, and using a relevant model for drug testing forms the basis of designing effective cancer immunotherapy [66].

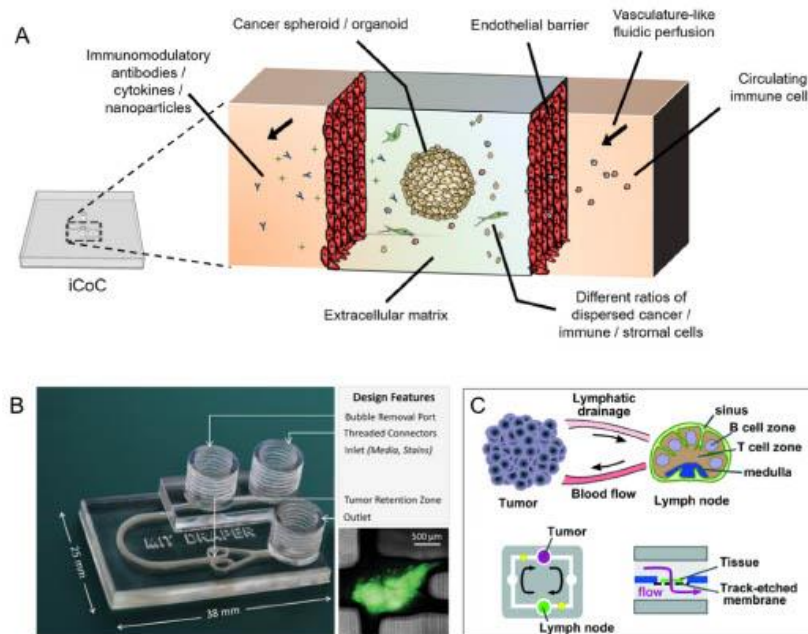


Fig. 2. Compartmentalization of 3D tissues in iCoC models. A) Various components can be integrated into the iCoC model to create a complex 3D tumor microenvironment, shown here in an exemplary iCoC with vertical tissue-tissue interfaces. B) Trapping patient-derived tumor fragments and applying perfusion through the construct have enabled multiple-days culture of the tumor and personalized immune checkpoint therapy assessment (Reproduced from Ref. [83]. Copyright © 2019 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.). C) Applying continuous circulating flow between *ex vivo* tissues in iCoC platform has emulated distant bidirectional communication between tumor and lymph node tissue that are placed in different chambers (Republished with permission of the Royal Society of Chemistry from Ref. [84]).

One of the most common architectures of 3D iCoC models includes a vertical tissue-tissue interface (Fig. 2A). One advantage of such an interface is the easy visualization of the dynamic cellular interaction and migration in the lateral dimension, as the resolution and optical accessibility in x-y-direction is generally higher than in z-direction. Several techniques have been developed to create this interface, including sequential hydrogel photopatterning [68,75], micro-molding [76], as well as capillary pinning e.g. by using a series of regularly spaced pillars [69,70,77–80]. These approaches have been used to analyze the migration dynamics of natural killer (NK) cells, genetically engineered T cells, dendritic cells, neutrophils, and monocytes into the tumor mass [57,68,69,76,80]. Moreover, such interfaces also allow lateral fluid diffusion and have been utilized to assess the diffusion of immunomodulatory nanoparticles across the endothelial barrier into the tumor mass [81,82].

Besides establishing tissue barriers for dispersed cell populations and cellular monolayer, spatial confinement also enables the integration of spheroids, organoids, and tumor fragments. Patient-derived tumor fragments have been used for *ex vivo* profiling of immune checkpoint blockade in a microfluidic system, showing early promise in its use for evaluating combination therapies (Fig. 2B) [83,85,86]. Additional to short-distance cell-cell or tissue-tissue interactions, compartmentalization also allows the modeling of distant inter-tissue crosstalk between *ex vivo* tumor and lymphoid tissue slices; enabling e.g. the study of tumor-mediated lymph node immunosuppression (Fig. 2C) [84].

Design features of microfluidic iCoC can readily be modified according to the application or to answer specific immuno-

oncological research questions. Simpler iCoC models can be used, for example, for studies that require higher throughput. On the other hand, more comprehensive systems that integrate tumor spheroids/organoids, mobile immune cells, vasculature, and stromal components with multiple microenvironmental factors can be utilized to investigate the mechanisms of action of immunotherapies and immune-cancer interaction in a more complex biological setting.

2.2. Modeling and manipulating the tumor microenvironment

Understanding the TME is crucial, as it currently presents major barriers to effective cellular immunotherapy, especially in solid tumors [87]. For engineered T and NK cells, for example, they first need to infiltrate the dense stromal elements of solid tumors and overcome hostile microenvironment characterized by i) altered metabolic conditions such as hypoxia, oxidative stress, and acidic pH, ii) the presence of immunosuppressive cytokines, as well as iii) the recruitment of immunosuppressive myeloid cells, regulatory T cells (Tregs), and TAMs [88–92]. Immunosuppressive innate immune cells play a major role in shaping the tumor microenvironment as they release immunosuppressive cytokines (IL-10, TGF- β) and express receptors (PD-L1) that negatively affect the adaptive immune response/cellular immunotherapy [14,93]. Additionally, the dense cancer cell-cell junctions in a tumor can limit therapeutic antibodies penetration [76]. Therefore, modeling these factors to better assess the efficacy of immunotherapy is of great interest to drug developers [94,95].

The use of animal models to study the TME is particularly challenging because the various immunosuppressive aspects are interdependent and not readily controllable, thereby making it difficult to interpret their individual contribution [14,25]. One additional drawback is the partial HLA mismatch in mouse models using tumor cell lines of human origin and e.g. CAR-T cells from a third-party donor [96]. Mouse models using patient-derived tumors (PDX models) as well as patient-derived stem cells for engraftment of a functional immune system may overcome such drawbacks but are highly dependent on the appropriate cells for engraftment of the immune system and the TME might only reflect partially the TME in patients [93,96]. Additionally, special considerations need to be taken in all models to avoid xeno-graft-versus-host-disease against mouse tissues [97]. iCoC models present the potential to make experimental modifications of cellular and biochemical composition. This allows to dissect individual roles of immunosuppressive factors and increase physiological relevance at the same time. In these systems, a defined 3D spatial organization also enables tight control of cytokine gradients [98], oxygen level [68,70,75], as well as other biophysical forces [99], which allows the investigation of how these factors might alter immune/cancer cell behavior and interaction. Testing and validating tumor microenvironmental factors in iCoC models can be done via computational approaches, modeling e.g. oxygen and chemokine transport, interstitial fluid flow, or shear stress [68,75,99,100].

2.2.1. Cellular components

Along with their relative positions, the ratio of the different cell types can be easily modified to mimic physiological conditions. By adjusting the population density of immune and cancer cells, it is possible to analyze different states of early cancer-immune interaction, such as early infiltration (immune cells are more excluded at the tumor periphery and in relatively small number compared to cancer cells) and post-infiltration phase (higher immune cell/cancer cell ratio) [101]. This could serve as a powerful solid tumor model to evaluate the efficacy of engineered immune cells and other immunomodulatory drugs in a temporally specific fashion. In addition, it allows the investigation of cancer treatment's mode of actions when multiple cell types are incorporated. For instance, by increasing the proportion of immune cells in co-culture with cancer cells and cancer-associated fibroblasts (CAFs) in their iCoC model, Nguyen et al. recapitulated one of trastuzumab's mode of actions: it increases the antibody-dependent cellular cytotoxicity (ADCC) against the cancer cells. Increasing the ratio of immune cells (PBMCs) to cancer cells (human epidermal growth factor receptor 2 (HER2)+ BT474 cell line) from 5:1 to 25:1 allowed them to reveal that trastuzumab-induced ADCC may require high immune cell density that infiltrates the TME from the vasculature, as the number of apoptotic cancer cells was ~10-fold higher in 25:1 immune cell/cancer cell ratio compared to 5:1 [79]. Moreover, increasing effector to target (E:T) ratio of chimeric antigen receptor (CAR)-T cells and ovarian cancer cells (SKOV3) from 1:1 to above 10:1 allowed Ando et al. to reveal a significant increase of CAR-T cell-mediated cytotoxicity using 2D multiwell plate, but not in their 3D iCoC model [68]. Differences in cytotoxic activity between 2D system and 3D microfluidic model were also observed when comparing the killing ratio of HeLa cells by NK-92 cells and HepG2 target cells by T cell receptor (TCR)-T cells [69,70,78]. This exemplifies how different factors within the 3D TME might reduce engineered T cells cytotoxic ability even in a higher E:T ratio [68]. For another T cell-based immunotherapy, such as bi-specific T cell engagers (BiTEs), the ratio of Tregs within the peripheral blood can determine the outcome of the therapy [102]. Potentially, varying different E:T ratios in iCoC models can be used for BiTEs dose prediction and safety evaluation.

Besides bottom-up approaches based on single cells, the integration of organoids and spheroids provides a great synergistic potential [103–105]. For iCoC platforms, in particular, cancer organoids can add *in vivo*-like cancer cell heterogeneity. This heterogeneity and the loss of antigenicity and immunogenicity by a subset of cancer cell population is a crucial aspect of immune escape mechanisms [106,107]. In fact, heterogeneity of cancer antigen expression remains a challenge in designing an effective CAR-T/NK cell construct [108]. The advantage of integrated iCoC systems over conventional cancer organoid models is the surrounding TME [109].

One consideration for cellular components in immunocompetent iCoC platforms is the potential for HLA-mismatch between immune cells and co-cultured tissue cells, which might complicate interpretation of results both for safety and for efficacy investigations. Similar limitations apply to other preclinical models, as discussed above, and could be overcome through the utilization of HLA-matched or induced pluripotent stem cells (iPSCs) derived tissues as well as careful testing to understand where these limitations apply.

2.2.2. Local oxygen content

A widely-studied characteristic of the TME is the oxygen level. Hypoxic conditions often occur in solid tumors where oxygen delivery is no longer sufficient to sustain ATP production by the cancer cells via oxidative phosphorylation. This is regarded as one of the main obstacles in immunotherapy due to its negative effect on anti-tumor immune response [110–113]. Using microfluidics, oxygen gradients/levels in solid tumors can be modeled in various ways: by physical barriers that selectively block oxygen diffusion (Fig. 3A) [68], by using tumor spheroid to naturally establish a hypoxic core within its own structure (Fig. 3B) [75,76], or by placing the entire system inside a hypoxic chamber (Fig. 3C) [70]. Other Cancer-on-Chip models utilize flanking channels filled with oxygen supplying and scavenging medium (e.g. sodium sulfite) respectively to create a precise gradient across the tissue compartment [114], e.g. to reproduce the HIF-1 α -mediated tumor growth and migratory behavior in lower (<5%) oxygen level [115].

The microscale characteristic of iCoCs offers a more accurate regulation of local oxygen levels than traditional *in vitro* models in hypoxia cancer research. Hypoxia chambers, for instance, do not allow control of pericellular oxygen conditions [116]. Using microfluidics, a defined oxygen level in a certain location of the tumor mass can be tailored to investigate immune and cancer response. Understanding the molecular mechanisms of hypoxia-regulated tumor immunity could provide a therapeutic value of targeting hypoxia-induced immunosuppressive cells and other molecular mechanisms. This could potentially serve as a combination therapy along with other cellular immunotherapies; thereby improve the overall efficacy of current treatment approaches.

2.2.3. Chemokine gradient

In vivo, various soluble factors from tumor cells, intratumor stromal cells, and intratumor leukocytes can stimulate the recruitment of tumor-infiltrating T cells, natural killer cells, and dendritic cells into the tumor site [117,118]. This process, however, is extremely complex and can be both tumor-promoting and -suppressive [119]. Precisely controlled levels and gradients of chemokines can be generated similarly to oxygen gradients by leveraging microfluidic approaches [120], for instance, using source/sink approaches [121].

To this end, microfluidic chips have been used in several immune-cancer interaction studies to perform chemokine-guided migration assays; looking at, for example, how macrophages induce cancer cells' migration [52,61,122] and how pre-activated NK cells migrate towards cancer cells [55]. Gradient control in

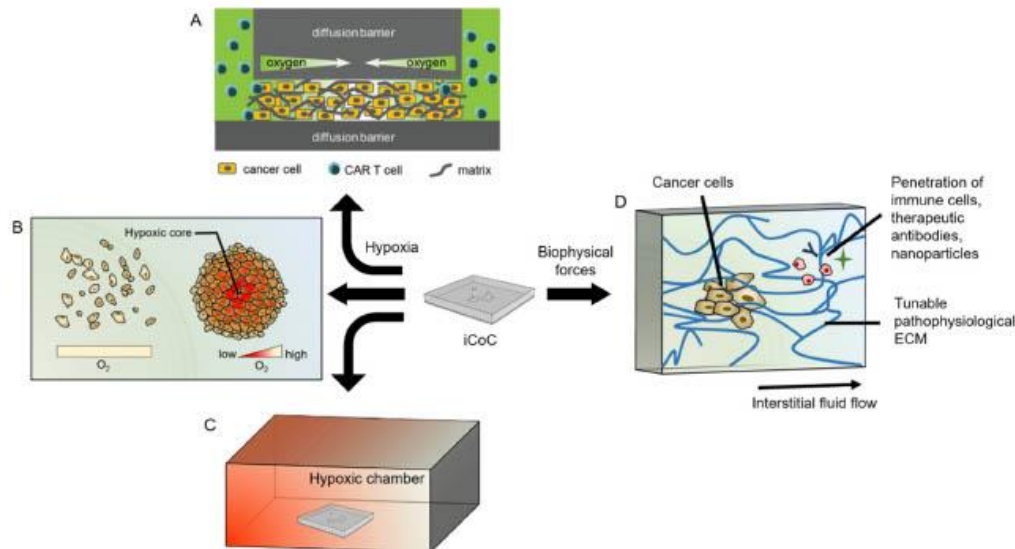


Fig. 3. Different approaches to modulate oxygen and biophysical forces in iCoC models. A) Selectively blocking oxygen diffusion by applying gas-impermeable chip material and varying cancer cell density within the solid tumor mass allowed Ando et al. to create an oxygen gradient and investigated its effect on CAR-T cell cytotoxicity (Reproduced from Ref [68]. Copyright © 2019 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.). B) By creating cancer spheroids, hypoxic cores that are naturally generated can be recapitulated. C) Another approach is to place the whole iCoC platform into a hypoxic chamber, which allowed the adjustment of oxygen concentrations. D) Tuning biophysical microenvironments allow the investigation of how ECM stiffness, porosity, as well as interstitial fluid flow affect immune cell infiltration and therapeutic antibodies and nanoparticles penetration towards the cancer cells.

3D scaffold has often been employed for studying endothelial sprouting in response to VEGF gradient [115,123,124], directed migration/sprouting of cancer cells/spheroids [125], chemotherapeutic screening of different anticancer drugs [126], as well as investigating chemokine-guided immune cell migration into 3D TME [99,121,127].

2.2.4. Cell scaffolds and other biophysical forces

In iCoC systems, scaffold modulation allows researchers to investigate how stiffness, porosity, and composition of the ECM affect the migratory behavior of immune cells into the tumor site, viability of cancer cells, as well as cancer cell growth [68,75]. *In vivo*, the ECM presents a physical barrier that hinders therapeutic antibody penetration as well as engineered immune cell migration into the TME [88,128–131]. In the case of adoptive cell therapy, this challenge has already led to the development of CAR-T cells that target cancer-associated fibroblasts [132] and the ECM itself [133]. iCoCs featuring pathophysiological ECM conditions provide a platform to test these approaches and to optimize the transport efficiency of therapeutic antibodies and other immunomodulatory drugs (Fig. 3D) [76,81].

ECM of varying stiffness can be patterned *in situ* to mimic different types and stages of solid tumors. Ando et al., for instance, utilized a UV-crosslinkable gelatin methacryloyl (GelMA) hydrogel to generate a tailored ECM with stiffness (~6 kPa) and porosity resembling multiple cancer matrices. This model was used to evaluate CAR-T cell cytotoxicity against the cancer cells embedded in the hydrogel (Fig. 3A) [68]. A further correlation between ECM and immune cell's cytotoxicity was revealed by Park et al.: Using a microfluidic hydrogel patterning array, collagen-I concentration (2–4 mg/mL) was varied to investigate its impact on NK cells (NK-92) migration and cytotoxicity towards cancer cells (HeLa).

Their preliminary 3D cytotoxicity assay revealed that although denser ECM indeed impedes the migration of NK cells, it enhances the effectiveness of contact-mediated killing [78].

In addition to tumor-secreted cytokines, Lee et al. mimicked interstitial fluid pressure; a further important aspect within the TME [99]. This aspect is a critical tumor-associated biophysical factor that promotes tumor growth and can be integrated into the iCoC model by adding fluidic perfusion through the TME (Fig. 3D) [134]. In this work, the authors investigated the individual role of interstitial fluid pressure and tumor-secreted factors in promoting macrophage migration into the tumor site (macrophage density in the TME correlates with increased metastasis [135]). They demonstrated that interstitial pressure and tumor-secreted cytokines can act independently in increasing migration directedness and speed. Interestingly, however, no additive effect was observed under the simultaneous presence of both factors. They revealed that antibody blockade against IL-8 and/or CCL2 inhibited macrophage migration, but could be restored by interstitial flow, indicating the presence of a cytokine-independent pathway.

2.2.5. Vasculature and perfusion

The vasculature is a crucial component of the TME: it affects immune-cancer interaction in various ways, such as the active suppression of the recruitment, adhesion, and activity of T cells, the support of immunoevasion, as well as the build-up of cancer cells' resistance to immunotherapies [32]. Despite its significance, the vasculature is inherently lacking in traditional *in vitro* models in immuno-oncology research. In this regard, microfluidic cancer models provide a direct advantage by integrating vasculature-like perfusion and channels. Various groups have integrated endothelial cells into their iCoC models to specifically study immune-cancer-endothelial cells crosstalk investigating angiogenesis

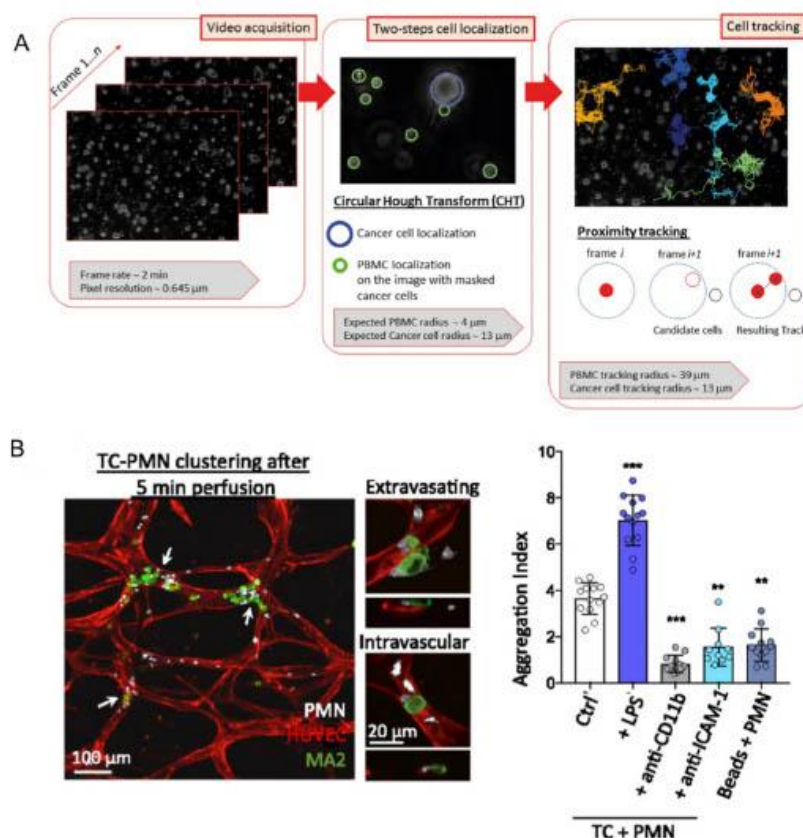


Fig. 4. Examples of high-content imaging performed on iCoC models. A) In an iCoC platform that includes multiple cell types (PBMCs, BT474 cancer cells, and Hs578T cancer-associated fibroblasts), advanced live imaging and automated image analysis was utilized to enable high-throughput tracking at the single-cell level and massive quantifications of cell-cell interaction. This allowed the assessment of real-time drug response and dissect the roles of each individual cell type [79]. B) High-content time-lapse imaging was used to monitor and quantify aggregate formation and motility of neutrophils (PMNs) and A375-MA2 tumor cells (TCs) inside self-assembled microvasculature after different pretreatment conditions. This revealed the role of neutrophils in supporting cancer cells' extravasation (Republished with permission from the National Academy of Sciences from Ref. [77]).

[136], cancer cells intravasation [137], and immune/cancer cells extravasation [75,77,80,82,138]. Others have established endothelial barriers to mimic *in vivo*-like nutrient/immunomodulatory drug delivery to the surrounding tissue [76,81]. Such endothelial barriers can be formed in microfluidic channel via different approaches, such as the creation of planar monolayers, vascular lumen, or self-assembled microvessels [139]. As human endothelial cells can express both MHC class I and II [140], and have the ability to present antigens [141], it is important to consider the interpretation of results that HLA mismatched endothelial cells might result in T cell activation independent of tested immunotherapies. However, endothelial cells can be derived from the same donors, e.g. from blood outgrowth endothelial cells [142].

In addition to the cellular component of the vasculature, the biophysical aspect of perfusion further increases physiological relevance and accessible endpoints: It enables i) constant nutrient delivery and removal of metabolic waste products from the TME [143], ii) the study of immune cells dynamics under vascular intraluminal flow (e.g. adhesion and extravasation) as well as the

migratory behavior inside the TME under interstitial flow [77,99], and iii) the integration into multi-organ-chips to study off-target toxicity and pharmacokinetics of immunotherapeutic drugs [144]. Furthermore, perfusion enables convective transport of nutrients through thick tissue and has therefore enabled multiple-days culture of thick tumor fragments for analyzing its response to circulating tumor-infiltrating lymphocytes (TILs) and immune checkpoint inhibitors for personalized testing [83,145].

2.3. Optical accessibility and high-content analysis

Due to their transparency and microscale, iCoC platforms provide superior optical accessibility allowing high-resolution real-time imaging to study spatiotemporal dynamics. Compared to *in vivo* intravital imaging, imaging of microfluidic devices requires less specialized equipment and expertise [146]. Compared to traditional 3D *in vitro* models, the height of the tissues and their distance to the microscope objective are typically significantly smaller. Primary cancer organoids, for instance, are grown in a

millimeters-thick 3D matrix, where they are located at different focal planes. Co-culturing cancer organoids with immune cells and monitoring key parameters for cell-mediated cytotoxicity such as effector cell recruitment, motility, and interaction with cancer cells and therefore extracting quantitative data from such live imaging experiments are challenging.

Live-cell imaging in iCoC platforms has been used for quantifying immune/cancer cell migration speed and directedness in many studies [57,99,138] as well as to uncover specific interaction behaviors between heterotypic cell-cell populations [77,79]. Nguyen et al., for example, conducted parallelized single-cell tracking and quantification of cell-cell interactions on chip to assess the immunosuppressive effect of CAFs [79]. They quantified the growth, motility, mitosis, and apoptosis of individual cancer cells (HER2+ BT474 cell line) when co-cultured with immune cells (PBMC), CAFs (Hs578T), with or without trastuzumab treatment (Fig. 4A). Employing high-resolution live-cell imaging in self-assembled microvessels, Chen et al. revealed the dynamic roles of inflamed neutrophils in exerting proextravasation effects of cancer cells (A375-MA2 melanoma) through the formation of intraluminal clusters of neutrophils and cancer cells. By quantifying aggregate formation and the motility of cluster-associated/free neutrophils over time, they revealed that the cluster formation is dependent on physical trapping and adhesive interactions between neutrophils and endothelial cells (HUVECs) (Fig. 4B) [77]. These visualizations can provide useful insights not only for studying immune-cancer interaction, but also for assessing cellular therapies and other immunomodulatory drugs in real-time, which would be more challenging to perform *in vivo*.

Additional to endpoint analysis via live-cell imaging, immune-cancer interactions in iCoCs can be monitored using a variety of *in situ* as well as terminal assays: Besides conventional cell/tissue culture assays such as qRT-PCR, RNA-Seq, or immunohistochemistry, the perfusion provides the opportunity to dynamically sample the effluent (perfused media) and analyze, for instance, the kinetics of secreted factors such as released cytokines and the state of circulating tumor/immune cells with a high temporal resolution [77,84,147].

2.4. Short platform development time

For personalized screening applications, the establishment of iCoC platforms can be much faster than patient-derived xenograft (PDX) murine models, which can take up to 6 months or longer [148]. Once established, iCoC models also have a much better potential for parallelized, quantitative clinical analysis than animal studies [96,146]. Early development phase can be completely independent of the final cell source, for example by using cell lines in proof of concept studies before integrating patient-derived cancer, immune, and stromal cells. The platform can be developed via iterative design optimization assisted by a computational modeling approach and exploiting different microfabrication methods.

3. Applications of iCoCs to test immunotherapy approaches

As elaborated on in the previous chapter, iCoC models provide the opportunity to model immune-cancer interactions in entirely new ways and can be utilized to unravel mechanisms of cell-mediated cytotoxicity. In this chapter, we now discuss how these novel iCoCs can be translated and applied to advance the development of novel immunotherapies. We address challenges the different immunotherapy modalities are currently facing and exemplify how iCoC models can provide nonclinical insights to address those challenges.

The advances in cancer immunotherapy come with several roadblocks limiting their widespread use and their benefits to so far only a small subset of patients. The lack of representative preclinical models that translate to human immunity, thereby, remains one of the greatest challenges [13,26]. Current nonclinical model systems are mostly unable to provide predictive information on the efficacy and safety of immunotherapy. The efficacy is clinically hampered by the immunosuppressive TME [26,149,150], which conventional model systems do not recapitulate. This is due to the dense nature of tumor extracellular matrix, high interstitial fluid pressure, abundant immunosuppressive cytokines, presence of suppressive regulatory myeloid cells, upregulated immune checkpoints, hypoxia, and nutrient starvation. Additionally, the inherent heterogeneity in antigen expression within solid tumors poses challenges to identify an optimal target [21,23]. In terms of safety evaluation, a growing body of literature has pointed out the inadequateness of most animal models to capture immune-mediated toxicities [22–25,27–29,151–153]. It is currently difficult to model on-target, off-tumor toxicities, and systemic inflammatory response syndrome using animal models. This is partly due to a lacking homology of tumor-associated antigen proteins in humans and animals [23]. Advances in humanized murine models enabled the reproduction of cytokine release syndrome (CRS) and neurotoxicity after CAR-T treatment [154–156]. The underlying mechanisms, however, could not be completely identified due to complicated interdependencies of many factors [25]. In general, many of the mechanisms of immune-mediated toxicities in humans are still unknown and human-based alternative models are much-needed.

In this chapter, we review application examples and discuss how iCoC models can help to elucidate the underlying mode of actions in adoptive cell therapy, immune checkpoint therapy, cytokine therapy, oncolytic virus therapy, and cancer vaccines. We believe that innovative models are needed to assist in the nonclinical-assessment of immunomodulatory therapy safety and efficacy. Such models could not only improve the development of safer and more efficacious therapies, but also help identify responders and better predict therapeutic window and combinations.

3.1. Adoptive cell therapy

Through progress in genetic engineering and *ex vivo* T cell manufacturing processes, adoptive cell therapy (ACT) has risen to be one of the fastest-growing immuno-oncology fields in the past decades [22,157]. This therapy encompasses broad approaches to isolate cancer-reactive immune cells from the body, enhance and expand them *ex vivo*, and reinfuse them back into the patient. ACT utilizes endogenous tumor-infiltrating lymphocytes or genetically engineered T cells that express either highly specific TCRs or CARs. Genetic modification to generate CAR expression has been successfully implemented not only on T cells, but also on NK cells and macrophages [158,159]. In contrast to TCR-T cells, CAR-modified cells can detect epitopes of tumor-associated antigens independent of the presence of the major histocompatibility complex (MHC) molecules on the cancer cell's surface, which they often lose to evade the immune system [22,160]. Growing interest in ACT is marked by remarkable results from clinical trials using anti-CD19 CAR-T cells against B cell malignancies. These results led to the FDA approval of the first cell therapy for cancer treatment, and subsequently its integration into standard cancer care [21,161]. In fact, compared with other immuno-oncology therapies, ACT represents the largest growth of development in 2017–2019, with nearly 1200 new cell therapy products that are currently in preclinical and clinical evaluation [162,163].

One of the major question marks for ACT, specifically CAR-T cell therapy, is whether it will expand beyond B cell malignancies to

other cancer types, including solid tumors [161]. So far, the success of CAR-T cell therapy against solid tumors remains limited due to various challenges associated with the TME [88]. Moreover, ACT can be accompanied by severe side effects such as CRS, which is an overreaction of the immune system that can be lethal if not treated [164]. Besides CRS, there are a number of further complications associated with ACT, such as neurotoxicity (cerebral edema, seizures, delirium, aphasia) and hemodynamic instability (capillary leak syndrome, hypotension, tachycardia), which can lead to widespread organ dysfunction [22,165]. Below, we discuss how iCoC platforms have been utilized to elucidate the underlying complex mechanisms of both the immunosuppressive TME and ACT-mediated adverse events.

3.1.1. iCoC approaches to study TME effects on ACTs

Various aspects of the TME are suggested to be the major obstacle to CAR-T therapy: The TME presents both physical and biochemical barriers to the infiltrating T cells and might consequently induce their exhaustion [21,88]. Attempts to gain further insights into these mechanisms in 3D microenvironment have been done using 3D *in vitro* models such as spheroids, organoids, and scaffold-based constructs [166–170]. Wallstabe et al., for instance, tried to assess the efficacy of CAR-T cells against human lung and mamma carcinoma by measuring their infiltration, proliferation, cytolytic activity, and cytokine secretion in a dynamic flow setting using decellularized porcine jejunum matrix [170].

As next-generation *in vitro* models, microfabrication and microfluidics-based iCoCs now enable to precisely manipulate the spatial location of the cells, vascular barriers, oxygen/chemokine transport, and biophysical forces on physiological length scales. Ando et al., for instance, investigated the effect of hypoxia on CAR-T cell infiltration into a solid tumor utilizing a microphysiological tumor model featuring defined oxygen gradients and comprising HER2+ ovarian cancer cells (SKOV3) embedded in a GelMA hydrogel [68]. By combining computational modeling of oxygen transport with fluorescent oxygen sensor-based characterization of the gradient, they were able to precisely tune oxygen levels in the tumor mass in the microfluidic channel. Their findings showed that lower oxygen levels (6–17%) actually enhance the cytotoxic activity of HER2-targeting CAR-T cells in a spatially-modulated manner. Despite the evidence of increased therapeutic resistance, heterogeneity, and progression of tumor under a hypoxic microenvironment, certain hypoxic levels may actually support higher cytotoxic effector function of T cells [112], which was revealed in the iCoC model. This result also suggests that clinical observations of T cell suppression in lower oxygenated TME might not be a direct effect of oxygen level, but rather due to metabolic alterations driven by hypoxia [113,171]. Their iCoC model also recapitulated further clinical observation, namely that effector T cells enter the periphery of the hypoxic tumor mass but are excluded from the hypoxic core [172,173]. Despite this shallow penetration, the treatment still increases cancer cell death in the hypoxic core in the iCoC. Interestingly, this hypoxic core is absent of Granzyme B, a serine protease secreted by cytotoxic T cells during cellular immune response that induces cancer cell death. Granzyme B has been proposed as a potential early biomarker of efficacious cellular immunotherapy to identify responders, as it indicates the presence of actively engaged cytotoxic T cells [174,175]. The absence of Granzyme B in the core region might suggest that the noncontact mediated CAR-T cell cytotoxicity may not be dependent on Granzyme B but rather due to metabolic competition between cancer and CAR-T cells.

Another approach to model oxygen gradients was demonstrated by Aung et al., who recapitulated the hypoxic core of solid tumors by using spheroids of breast cancer cell lines (MCF-7 and

MDA-MB-231) in their iCoC model [75]. By co-culturing the hypoxic-core cancer spheroids with monocytes (THP-1) encapsulated within a GelMA hydrogel, they showed that monocytes promoted T cell (TALL-104) infiltration through the endothelial barrier (HUVECs) into the tumor site by secreting CCL4, CCL5, CCL11, CCL20, and CXCL8. Their platform also provided mechanistic insights about the regulation of T cell infiltration by monocytes: their findings showed that the chemokine release might be associated with hypoxia as their concentrations were not as high as when dispersed cancer cells (no oxygen gradient within the tumor mass) were employed.

Modeling the immunosuppressive TME is not only beneficial to compare cytotoxic ability of genetically engineered versus untreated T cells, but also of the same engineered T cells that underwent different preparation methods. By exposure to different microenvironmental conditions, for instance, the efficacy of TCR-T cells prepared via retroviral transduction and via mRNA electroporation was compared [70]. Thereto, TCR-T cells were injected into a microfluidic channel where they encountered and penetrated a three-dimensional solid tumor mass in the adjacent channel (human HepG2 liver carcinoma cells embedded in collagen type I hydrogel). Interestingly, they demonstrated that mRNA-TCR-T cells were overall less cytotoxic than retroviral-transduced TCR-T cells, which is not in agreement with the results from conventional 2D assays. This suggests that the cytotoxic ability of specific TCR-T cell variants might be influenced by T cell motility, which is not fully recapitulated in a 2D environment. The exact mechanisms are, however, still under investigation. Moreover, this microfluidic model was able to recapitulate a reduced cell killing of dispersed cancer cells by TCR-T cells by exposing the entire microfluidic chip to a low oxygen level (2%). The authors also showed that the addition of pro-inflammatory cytokines (IFN γ and TNF α) into the culture condition increases TCR-T cell cytotoxic ability against cancer cell aggregates, particularly in higher oxygen level (20%).

Using a similar model, Lee et al. investigated the immunosuppressive role of monocytes towards hepatitis B virus (HBV)-specific TCR-T cells with regards to programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) signaling contribution [69]. Here, they showed that the proportion of PD-L1+ monocytes is significantly higher compared to those observed on cancer cells. This shows that the monocytes (not the cancer cells) might have a fundamental impact on impeding the TCR-T cells cytotoxic activity. Indeed, multiple myeloid cells express inhibitory molecules and therefore have been proposed to serve as therapeutic targets [176,177]. The iCoC model also confirmed this potential of TCR-T cells inhibition by monocytes through PD1/PD-L1 signaling; no difference of TCR-T cell cytotoxicity was observed in standard 2D cytotoxicity assays. Remarkably, however, such inhibition by monocytes was only seen on retrovirally transduced TCR-T cells, while mRNA electroporated TCR-T cells remained unaffected. Similar to the study described above, this implies that differences in engineering methods, culture, and expansion processes of TCR-T cells might render them more sensitive toward PD-1 inhibition, and that oversimplified models might not capture the implication of such differences. Potentially, such screening can be implemented in early drug development phases to evaluate ACT preparation methodologies, as well as in clinical setting to identify the most efficacious TCR-T cell types for personalized therapy.

Taken together, iCoC models were able to provide new insights about the roles of different immunosuppressive factors in the TME on ACT by precisely adjusting the relevant proportion of specific cell population, ECM, and oxygen level. The platforms can potentially be used to investigate further biophysical and biochemical cues listed in chapter 2. Moreover, such models have also been employed to evaluate drug candidates and recreate scenarios that

are often encountered in clinical setting, such as pretreatment with immunosuppressive agents and co-treatment with immune checkpoint inhibitor [68,70].

3.1.2. Modeling ACT-mediated toxicities

The Organ-on-Chip technology presents a great potential to address ACT-mediated toxicities, as it is able to recapitulate immune responses in different organs [178–181]. In addition, linking multiple organ chips allows the recreation of systemic interaction between multiple tissues and organs to predict unexpected adverse drug and inflammatory effects (Fig. 5) [182]. For example, the significant increase of inflammatory cytokine release (such as IFN γ and IL-2) by CAR-T cells upon their activation has been modeled *in vitro* [170]. This can potentially be recreated using OoC technology and coupled to other OoC platforms, such as the vasculature, to assess the effect of inflammatory cytokines to the endothelial activation, capillary leakage, and its subsequent effects (e.g. neurotoxicity) in a distant organ, similar to previous studies on neurovascular units [183,184]. A recent study reported the use of OoCs to study on-target off-tumor toxicities of BiTEs in the lung and the intestine [185]. The same concept can be applied to study on-target off-tumor toxicities of ACTs as these have been reported to occur in the liver, colon, and lung [186–188].

Additionally, the use of iCoCs is not limited to the assessment of endothelial cells activation and permeability, but also for investigating the crosstalk between engineered T cells and innate immune cells (e.g. macrophages, dendritic cells) and how they might contribute to adverse events leading to CRS. Owing to their ability to model immune response and simultaneously combine multiple organ systems, iCoC models present enormous potential to provide new insights into the pathogenesis of CRS and other ACT-mediated toxicities in a controllable and fully human setting.

3.2. Immune checkpoint inhibitors

Numerous immune cells, e.g. T cells, bear distinct “recognizing” receptors for receiving a primary, antigen-specific signal for initiation of a response to extracellular stimuli [189]. However, the direction and magnitude of the response are decided by the interplay of co-stimulating and co-inhibitory signals that are mediated by immune checkpoints: For initiation of the immune response, co-stimulating signals are required. Co-inhibitory signals serve as a negative feedback loop and play an important role in discontinua-

tion of immune response and prevention of autoimmune reactions. The expression of immune checkpoints is differentially and tightly regulated through every stage and location of immune cell activation. For instance, co-stimulating CD28 is constitutively expressed on T cells, while one of the major negative regulators cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) is rapidly transported to the cell surface upon T cell activation, downregulating the immune response through competitive inhibition. Hence, inhibition of the checkpoint CTLA-4 leads to an enhanced activation and priming of antigen-specific T cells, acting primarily at the level of T cell activation and proliferation in the draining lymph nodes [189]. To the contrary, PD-1 is highly expressed on T cells with exhausted phenotypes as a result of constant exposure to antigen stimuli, as present in the TME. PD-1 interacts with its ligand PD-L1, which is expressed on many cancer cells and tumor-infiltrating immune cells and may contribute to immune escape. Inhibition of the PD-1/PD-L1 axis can remove suppression and restore preexisting T cell effector function, primarily at the site of the tumor [190]. While ICIs targeting these two axes are now standard treatments in oncology, several additional checkpoints expressed on exhausted T cells and associated with tumor-mediated immune modulation are currently of interest and might benefit from iCoC systems to improve understanding of efficacy, appropriate patient population, and most suitable combination therapies. This next generation of checkpoint inhibitors includes both receptors modulating T cells, such as e.g. LAG-3, TIM-3, VISTA (PD-H1), GITR, OX-40, IDO, ICOS, and TIGIT, but also Killer inhibitory receptors expressed by NK cells [191–193], and receptors modulating tumor-associated macrophage activity [194], with about 800 targets currently being investigated in clinical trials [162].

The treatment modality of immune checkpoint inhibitors (ICI) aims at restoring immune function in a dysregulated cancer immune environment by blocking key inhibitory regulators of the immune system. Since the approval of the CTLA-4 inhibitor ipilumab by the FDA for the treatment of metastatic melanoma in 2011 [195,196], the interest in ICI has grown enormously due to its substantial clinical benefit and durable response in multiple tumor types. The number of preclinical and clinical trials evaluating T cell-targeted modulators has increased to more than 4000 in 2020 [162]. To date, seven ICIs were approved by the FDA, including the aforementioned CTLA-4 inhibitor and three inhibitors each targeting PD-1 and PD-L1 [197]. Despite the promising long-term response with approved therapies in multiple carcinomas, some tumor types are completely refractory, and a significant number of patients who initially respond to the treatment eventually acquire resistance. Several resistance mechanisms have been identified, such as defects in antigen presentation, upregulation of additional inhibitory checkpoints, neoantigen depletion, and the immunosuppressive features of TME (reviewed by [8–11]). Thus, the lack of detailed understanding of the immunosuppressive TME and the numerous additional resistance mechanisms that are still unknown pose critical challenges in ICI therapy [198]. In addition, recent failures of ICI therapies in combination trials [199] despite promising pre-clinical data highlight the need for better pre-clinical models. Together with challenges in fully understanding the patient-specific risk for immune-related adverse events in patients treated with ICIs, the need for more physiologically relevant *in vitro* models is clearly highlighted.

3.2.1. Identification of responders in patient-specific iCoCs

The dynamic variation of immunogenicity in patients and cancer types makes it difficult to identify prognostic and predictive indicators for treatment success, contributing to a low response rate to ICI [11,200]. Many cancer patients with the targeted tumor type that fit all defined inclusion criteria for the use of an ICI emerge as non-responders [201]. The discovery by Chowell et al.

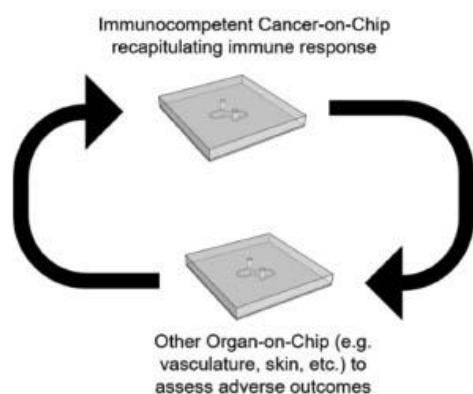


Fig. 5. Multi-organ-Chip approach to recapitulate systemic toxicities. Linking iCoC models with other OoC models may enable the recapitulation of immune-related adverse events in distant organs.

that a greater diversity of HLA molecules results in a larger repertoire of presented neoantigens and influences survival to ICIs underlines the importance of including the patient's genotype [202]. To capture the genetical phenotype, primary cells or iPSCs can be integrated in *in vitro* models. While stem cell derived sources are considered a solution when primary tissues from donors cannot be accessed, the immature phenotype, as well as the time and resources needed, represent drawbacks [203]. iCoCs reflecting the patient-specific genetic background pose a promising (screening) tool to identify responders to specific ICIs and are able to provide a balanced and personalized safety and efficacy profile before treatment. Moreover, they can be applied to establish biomarkers that guide the use of ICIs in the most appropriate patients.

Beckwith et al. and Moore et al. developed iCoC models that integrate tumor with native features of the TME such as the heterogeneity of the tumor and its microenvironment, containing resident and potentially exhausted TILs [83,145]. Beckwith et al. captured fragments of human non-small-cell lung carcinoma (NSCLC) biopsies in a retention zone in a microfluidic device (Fig. 2B). Constant perfusion not only delivered nutrients and removed metabolic waste via convective transport into the thick tissue fragments, but also mimicked high intratumoral hydrostatic pressure and enabled dynamic PD-1 inhibitor exposure. In tumor fragments of one of the two NSCLC patients, PD-1 inhibitors decreased tumor cell viability over the time course of 72 h. Whether these results correlate with the clinical benefits of PD-1 inhibitor treatment for (exclusively) one of the patients is unknown, but cross-correlation of *ex vivo* and *in vivo* results are aimed for and can be anticipated in the future. Decreased cancer cell viability was co-localized with tumor resident lymphocytes, supporting a hypothesized involvement of tissue-resident lymphocytes in viability decline. In a proof-of-concept study, Moore et al. captured 12 fragments from murine MC38 colorectal carcinoma biopsies in parallel in a platform termed EVIDENT (*ex vivo* immuno-oncology dynamic environment for tumor biopsies). In order to assess the killing efficiency of TILs, TILs were isolated from the same tumor biopsies, perfused through the system, and allowed to infiltrate into the tumor fragments. The authors claim that pre-treatment of TILs with PD-1 inhibitor resulted in increased tumor cell death in proximity to infiltrated TILs in the murine model. Similar effects were observed in human NSCLC patients' specimen with autologous TILs. In addition to these proof-of-concept experiments, images of tumor viability and TIL infiltration were analyzed and quantified automatically based on machine learning, laying the foundation for a scalable platform to evaluate a patient's response to ICI.

While these two biopsy fragment-based *ex vivo* models are rather quick to set-up, *ex vivo* platforms do not allow the control of individual parameters similar to bottom-up approaches as described in Section 2.2. Al-Samadi et al. chose a bottom-up approach for their iCoC by embedding HSC-3 cancer cells in myogel, a human tumor-derived ECM [204]. Thereby, they could show that the migratory behavior of PBMCs from healthy donors was dependent on cancer cell density in the hydrogel, potentially reflecting gradients of chemokines. Using an advanced version of the model integrating patient-derived cancer cells and autologous serum, interpatient variability in migratory behavior of PBMCs upon PD-L1 inhibitor treatment was observed. To the contrary, migration of immune cells towards cancer cells in both patients was increased upon inhibition of IDO 1, an immune checkpoint protein that evokes T cell apoptosis and promotion of Treg differentiation and is known to be upregulated in HNSCC patients. By using the patients derived cancer and immune cells with autologous serum in a human tumor-derived matrix, they provide a fully human TME to the cancer cells; this is an important step towards

precision medicine. Investigating new immune checkpoints as targets for ICI poses an important opportunity in the fight against resistance. Since inflamed "hot" tumors are associated with favorable response to ICI therapy [205], induction of immune infiltration with IDO 1 inhibitor opens the door to sensitize tumors to PD-L1 treatment, and testing synergistic effects of IDO 1 and PD-L1 inhibitors in a follow-up study would be intriguing.

3.2.2. iCoCs as high(er)-throughput screening tools

Compared to 2017, the number of T cell-targeted as well as other immunomodulators in the development pipeline increased by more than 220% in 2020 [162], underlining the high demand for high-throughput screening tools. Sarkar et al. applied a droplet-based screening platform as a high throughput microfluidic cytotoxicity assay, in which a multiple myeloma cell line and donor-derived NK cells or an NK cell line were encapsulated [206]. They observed a significantly increased target cell death upon treatment with α PD-L1 as well as lenalidomide, an immunomodulator that has demonstrated activation of T and NK cells and anti-cancer effects. However, basic interactive characteristics such as contact or death times were not affected by the treatments. With the gained information, a computational drug screening approach was implemented to determine the efficiency of NK-mediated immunotherapies against multiple myeloma. The droplet-based platform paves the way for patient-specific cytotoxicity assays in immunotherapy screenings, but has its limitations with regards to the microenvironment and long-term cultivation. In an advanced droplet-based screening platform, immunogenic diffuse large B cell lymphoma (DLBCL) spheroids were generated, cultivated, and assessed in a single device [207]. Tumor-stromal-immune spheroids comprised a mixture of cells similar to the TME in Non-Hodgkin lymphoma and were embedded in a composite hydrogel. Subsequently, these spheroids were trapped in a docking array and continuously perfused with a flow rate similar to the velocity of blood in the tumor. In these co-culture spheroids, lenalidomide exhibited direct anti-proliferative effects on both DLBCL and stromal cells while enhancing the proliferation of activated immune cells. Via secretome profiling, dynamic changes of several pro- and anti-inflammatory cytokines upon lenalidomide treatment were detected. Compared to the perfused 3D spheroids, stromal and tumor cell proliferation and death were notably higher when cultivated in monolayers in microplates. In this study, the immunomodulatory compound lenalidomide was applied. However other immunotherapies such as ICIs may also be tested using this platform. This platform succeeded in recapitulating many parameters of the TME while keeping higher-throughput modalities. In the future, the system could be advanced to a patient-specific screening tool to validate immunotherapies.

3.2.3. Overcoming resistance by defining synergistic drug combinations in iCoCs

A pressing challenge in immunotherapy is to increase the population that would benefit from immunomodulating agents [201]. Underlying resistance mechanisms can be rooted in every step of the immune-cancer interaction and enable therapeutic interventions at distinct targets and stages of the immune response. Different immunotherapeutic strategies can be combined with each other and with chemotherapy, targeted therapy, and radiotherapy to achieve therapeutic effects that are superior to the additive effect of each of the components [208]. Hence, there is a great need to further elucidate mechanisms of resistance and determine effective and tolerable combination therapies, including dosing and scheduling thereof [9]. iCoCs can recapitulate several steps of the cancer-immune interaction, facilitating the simultaneous testing of therapeutic strategies that intervene at different stages of the immune response in the same model.

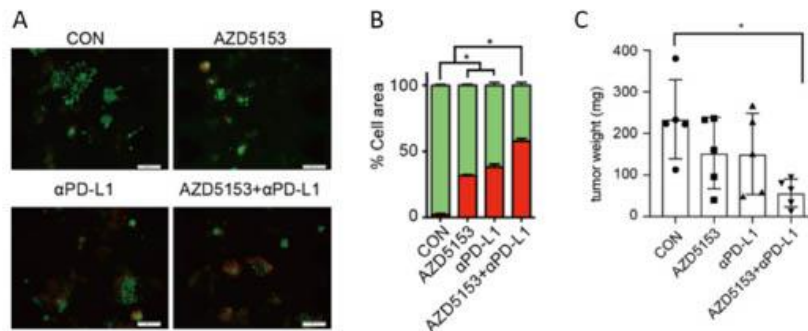


Fig. 6. Comparing *in vitro* response of PDOTS in iCoCs to combinational therapy with *in vivo* data of a mouse model. A) PDOTS from ascites of an untreated ovarian cancer patient were treated with a single exposure to AZD5153 or αPD-L1, and combinational strategy demonstrates synergistic effects of both agents. B) Corresponding live/dead quantification of A). C) Mice were treated with the same conditions as in (A) and cytotoxic and synergistic effects of AZD5153 and αPD-L1 with PDOTS in iCoCs were confirmed in mouse models (Reproduced from [211], CC BY).

A common combination tested in immunotherapy is the combination of a CTLA-4 inhibitor, leading to enhanced priming and activation, and inhibition of the PD-/PD-L1 axis, acting on the effector phase in the TME [209]. This combination was studied by Jenkins et al. and Aref et al. [85,86] on murine- and patient-derived organotypic tumor spheroids (MDOTS/PDOTS). MDOTS of several cell sources with contrasting sensitivities were created and *in vitro* efficacy of PD-1 inhibition in terms of cell death was compared to *in vivo* data in terms of mouse survival. Furthermore, PDOTS from patients with the shortest progression-free survival elaborated immunosuppressive cytokines/chemokines in the microfluidic device, of which several are components of the recently reported innate PD-1 resistance gene expression signature. The concept of dual therapy was applied to PDOTS derived from melanoma, thyroid, and pancreatic adenocarcinoma, determining the upregulation of the cytokines CCL19/CXCL13 as a readout. Upregulation of CCL19/CXCL13 is a measure of early immune activation and an important feature of the immune response to PD-1 blockade, as shown in biopsy samples. Dual treated PDOTS did not show an increased upregulation of the cytokines compared to PD-1 alone, and was minimal upon CTLA-4 treatment. In a follow-up study, Aref et al. [86] used the same platform and confirmed that αCTLA-4 is not contributing to immune-mediated killing with PD-1 inhibition by measuring direct cytotoxic effects. However, cellular changes were mapped via RNA-sequencing, revealing an expansion of CD8⁺ T cells and M0 macrophages in PDOTS treated with PD-1 + CTLA-4 inhibitor. Neither upregulation of cytokines nor cytotoxicity upon ICI treatment could be replicated in multi-well plates, where cells were grown in 2D. Furthermore, Jenkins et al. combined inhibition of PD-L1 and TBK1/IKKε, two immune signaling kinases that have been shown to restrain T cell activation. In an attempt to render the immunosuppressive TME more permissive for T cell function, TBK1/IKKε were inhibited with Cmpd1 in MDOTS and mice. In combination with PD-L1 inhibitor, *ex vivo* killing of MDOTS was enhanced upon TBK1/IKKε inhibition with Cmpd1, and survival of syngeneic mice was prolonged compared with either treatment alone.

Li et al. exposed PDOTS of high-grade serous ovarian cancer as well as mice to a combination of a PD-L1 inhibitor and the BRD4 inhibitor AZD5153, known to reprogram tumor-associated macrophages from M2 to M1-like macrophages *in vitro* [210]. Tumor weight of mice receiving the combination therapy was more reduced compared to a single administration of the molecules and synergistic effects were replicated in the microfluidic system,

showing extended cell death with the combination compared to the single agents (Fig. 6).

Combinations of immune checkpoint inhibitors and ACTs are also promising and have already been tested in iCoCs: Ando et al. modeled the heterogeneous oxygen landscape within a tunable ECM for the evaluation of CAR-T cell cytotoxicity and infiltration in solid tumors [68] (Section 3.1). The authors claim an overall upregulation of PD-L1 under hypoxic conditions (oxygen levels of 0–17%) and an increased surface expression, making PD-L1 a promising target to render the hostile microenvironment more responsive to CAR-T cell therapy. Hence, in an attempt to rescue low CAR-T induced cytotoxicity, combination treatment of PD-L1 inhibition and CAR-T cells was tested but did not yield any significant therapeutic benefits. Lee et al. increased the architectural complexity of the TME by adding cellular components [69]. Monocytes were reported to impede natural T cell functions via PD-L1/PD-1 pathway in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Therefore, the authors investigated the immunosuppressive potential of monocytes towards HBV-specific TCR-T cells. Suppression of HBV-specific TCR-T cell cytotoxicity by monocytes depended on the production method of TCR-T cells and could be rescued by blocking the PD-L1/PD-1 signaling pathway. These mechanistic insights provide the potential to optimize the currently described therapeutic strategies.

3.2.4. Dissecting effects of the immunosuppressive TME on ICI therapies

Immune cell extravasation, migration, and effector functions are tightly regulated and potentially hindered by immunosuppressive factors present in some solid tumors, making the TME one of the major causes of failure of ICI therapies. In the TME, there are many factors that influence each other, and dissecting the role of each parameter provides valuable mechanistic insight in this interplay. In the following studies, the immunosuppressive TME was modeled to dissect the roles of immunosuppressive components.

In the study of Cui et al., a glioblastoma model with TAMs as stromal cells and T cells as immune components was utilized to dissect the heterogeneity of the immunosuppressive TME and optimize PD-1 therapy [212]. Glioblastoma (GBM) is a genetically heterogeneous disease with a highly immunosuppressive TME, marked by a high abundance of TAMs and a low number of CD8⁺ T cells. The microfluidic model contains human brain microvascular endothelial cells in an outer ring, and TAM as well as patient-derived GBM cells of three distinct GBM subtypes in an adjacent

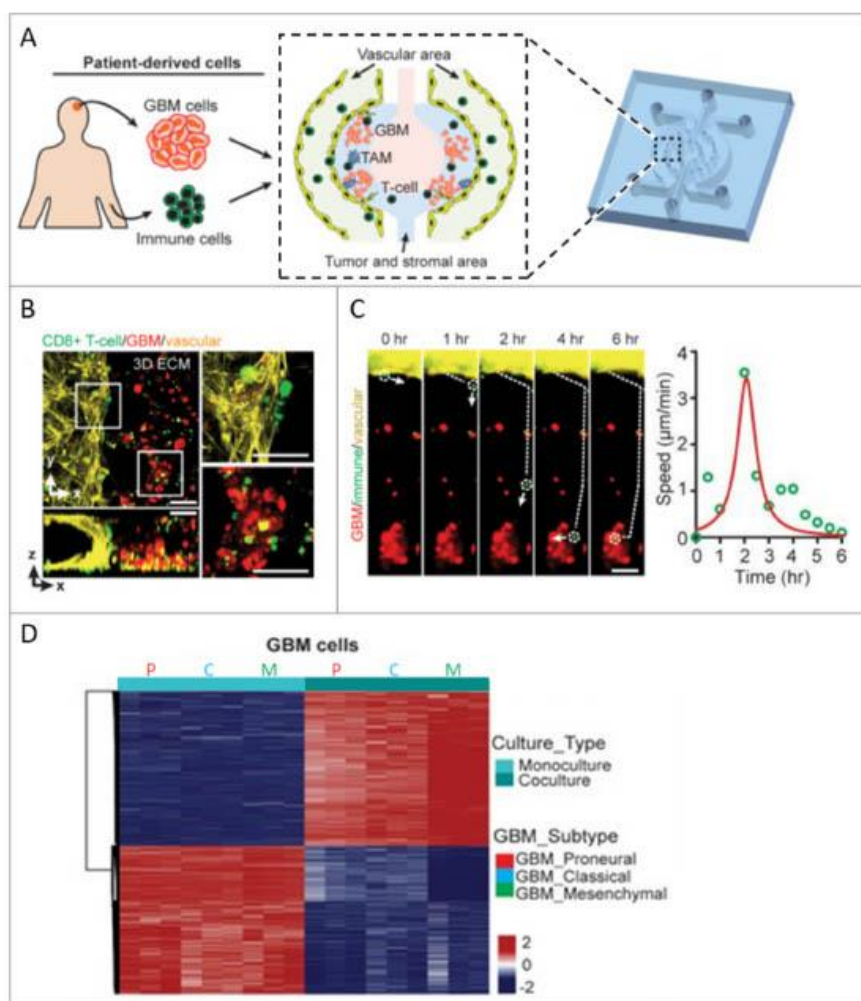


Fig. 7. Glioblastoma-on-Chip for dissecting the effects of TAMs. A) Schematic illustrating the iCoC with patient-resected glioblastoma (GBM) cells and human primary immune cells. The peripheral vascular region (yellow) is perfused and delivers T-cells. The middle area contains tumor cells and TAMs in a hydrogel mimicking ECM (blue) and surrounds a central region for medium supply (pink). B) Confocal immunofluorescence image showing endothelial lining of the microvessel (yellow) and GBM cells (red) with T cells (green) in close proximity to both. Scale bar is 50 μm . C) Time-lapsed images showing extravasation, infiltration, and interaction of a single T cell with GBM tumor (colors as in B). Scale bar is 50 μm . Quantification of migration speed indicates maximum speed after extravasation and before contacting GBM cells. D) GBM cells from patients of the proneural (P), classical (C), and mesenchymal (M) subtype were cultivated in the iCoC for 3 days. Up to 10,000 probes were differentially methylated when cultivated in coculture with macrophages. (This section is an excerpt of the original, since the annotation was modified. Reproduced from [212], CC BY). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compartment (Fig. 7A). Allogeneic IL-2 activated CD8+ T cells were perfused through the endothelial lined microvessels to investigate dynamic extravasation, migration, interaction with TAMs and GBM, and cytotoxic activity (Fig. 7B and C). In their model, the therapeutically least responsive subtype of GBM showed the highest number of immunosuppressive features, consistent with clinical data. Specifically, compared to the classical and proneural subtypes, the mesenchymal subtype (MES) showed the lowest number of extravasated T cells and the highest number of M2-

like TAMs. The presence of TAMs not only reduced T cell extravasation which suggests inhibitory effects of TAMs, but changed the methylation of GBM cells of all subtypes (Fig. 7D). Migration trajectories and speed showed reduced motility of T cells in MES models. Accordingly, expression of T cell markers for activation and cytotoxicity was lowest and PD-1 expression highest in MES models compared to the other subtypes. These immunosuppressive features could be attributed to the presence of MES GBM cancer cells, which also exhibited the highest PD-L1 levels, as their

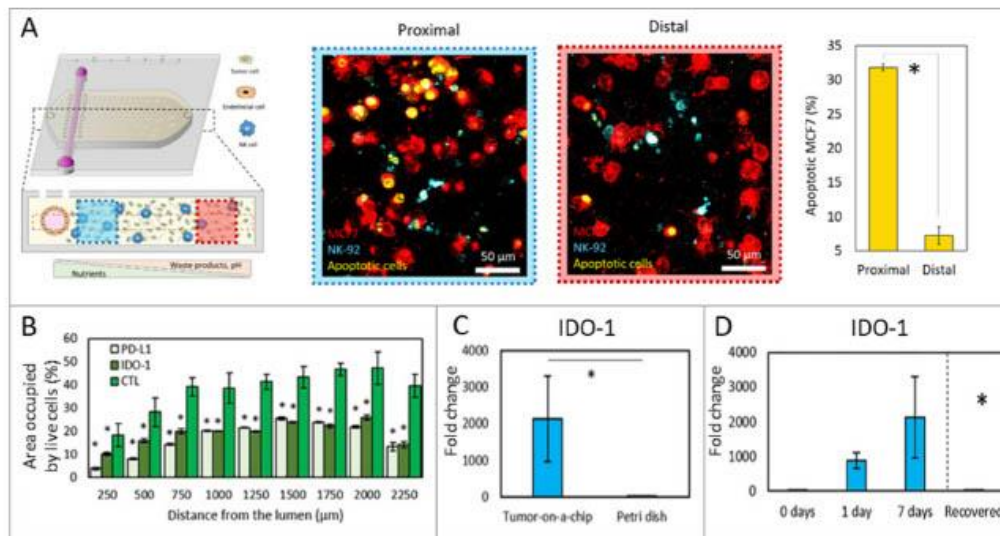


Fig. 8. MCF-7 induced suppressive environment induces exhausted phenotype in NK cells. A) Schematic of the iCoC and confocal images showing NK-92 cells (cyan) and MCF-7 cells (red) proximal to the lumen (blue box) or in greater distance (red box). NK cell cytotoxicity was reduced in the distal region as reflected by the number of apoptotic cells (yellow), quantifications are shown in the bar graph. B) Cytotoxic capacity of NK-92 was restored with the α PD-L1 antibody or IDO-1 inhibitor. C) Bar graphs depicting the change in gene expression of IDO-1 in an iCoC versus a petri dish. D) Bar graphs showing IDO-1 upregulation in NK-92 during 7 days of co-culture with MCF-7 in the iCoC. Upon 1 week of recovery in a monoculture in a traditional flask, IDO-1 returned to the basal expression. (This section is an excerpt of the original, since the annotation was modified. Reproduced from [71], CC BY-NC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

absence reversed these effects. MES GBM highly secreted M2-promoting CSF-1 and further cytokine profiling revealed a milieu that favors TAM polarization towards M2, which is reflected in the profound number of M2-like TAMs in the MES models. The presence of TAMs not only impacted CD8⁺ T cell infiltration, but also changed the epigenetic signature in GBM cells in co-culture and vice versa.

Other than dissecting the influence of the cellular components of the GBM model, Cui et al. also optimized PD-1 therapy by simultaneous applying the CSF-1R inhibitor BLZ945. BLZ945 suppressed M2 phenotype without affecting PD-L1 expression on TAM or GBM, suggesting an insufficient reversion of immunosuppression in monotherapy. However, CSF-1R inhibition significantly enhanced CD8⁺ T cell extravasation. Inhibition of PD-1 enhanced CD8⁺ activation, and dual blockade combined both effects, resulting in reversed TNF α and TGF β profiles and augmented cytotoxicity, in particular in the MES subtype.

This study demonstrates how a complex model of the immunosuppressive TME can profoundly increase our understanding of immune-cancer interaction by dissecting the influence of its single components. Correlation with clinical data confirms the relevance and indicates translatability to patients. Testing of adjuvant therapy that simultaneously targets M2-polarization via CSF-1 signaling and PD-1 proves to be a promising strategy, in particular in the MES subtype that is associated with a highly immunosuppressive TME and poor prognosis.

Nguyen et al. investigated the effects of CAFs and the targeted therapeutic trastuzumab on the immune response [79]. While the mechanism of action of trastuzumab differs from ICIs, both therapies apply a monoclonal antibody and the investigation of cellular components of the TME is just as relevant for ICI therapy as for all immunotherapies. Trastuzumab targets HER2 on breast

cancer cells, but does not only act via direct inhibition of proliferation and induction of apoptosis, but also indirectly by inducing immune response mediated through ADCC. The central compartment of the microfluidic platform was lined by a monolayer of endothelial cells and flanked by two chambers that contained HER2⁺ breast cancer cells with PBMCs in collagen hydrogels. Exclusively one of the chambers also comprised CAFs in order to be able to compare conditions within the same chip. The platform mirrored the *in vivo* microarchitecture and proportion of cell types and enabled modulation of the experimental setup to dissect the effects of specific cellular components or a therapeutic antibody. Cytostatic and cytotoxic effects of trastuzumab as well as increased frequency of interactions of immune cells with cancer cells was observed via live-cell imaging and quantified with automated image analysis (Section 2.3). Trastuzumab promoted long-term cancer-immune interactions (>50 min) with correlated stimulation of cancer apoptosis, but failed with cancer cells that were not HER2⁺. These results suggest a recapitulation of anti-tumoral ADCC as a consequence of trastuzumab binding the HER2 receptor on-chip. In terms of stromal effects, this study revealed that trastuzumab-induced ADCC is dependent on the local density of infiltrating immune cells. Furthermore, immune-suppressive CAFs antagonized increased frequency of contacts between cancer and immune cells and slightly inhibited trastuzumab-induced ADCC. In the absence of trastuzumab, augmentation of the velocity of cancer cell migration was detected, suggesting pro-invasive effects of CAFs. This iCoC could be further used for basic studies focusing on the TME and the investigation of tumor response to treatments such as ICI.

A further iCoC from Ayuso et al. created a suppressive environment that compromised NK cell cytotoxic capacity [71]. In the microfluidic device, a HUVEC-lined lumen was surrounded by

MCF-7 tumor cell-containing collagen with NK-92 either dispersed within the hydrogel or added via perfusion through the lumen (Fig. 8A). A gradient of nutrients, pH, waste, etc. was generated by the metabolic activity of MCF-7 and led to environmental stress on NK-92 cells. As a result, the cytotoxic activity of NK-92 in greater distance from the lumen was reduced (Fig. 8A). Furthermore, exhaustion genes such as IDO-1, PD-1, and CTLA4 were upregulated and activation/prosurvival markers such as GZMB and BCL1 downregulated. The exhausted phenotype could not be replicated in a 3D culture in a traditional dish, suggesting that chronic activation through co-culture plays a minor role in immune exhaustion compared to the environmental stress generated by MCF-7 in the iCoC (Fig. 8C). α PD-L1 antibody atezolizumab and IDO-1 inhibitor epacadostat restored cytotoxic activity in the tumor-chip particularly in close proximity to the lumen (Fig. 8B), with little efficacy in traditional well plates. Recovery of NK-92 from the microfluidic platform and cultivating exposed NK-92 in a traditional flask for a week showed that multiple genes returned to basal level (IDO-1, Fig. 8D), while others showed only partial (GZMB) or no (IL-1) recovery compared to naïve NK-92 cells. This platform provides a tool to investigate epigenetic and molecular alterations that lead to long-lasting immunosuppressive effects of environmental stress on NK cell biology.

3.2.5. An unmet need: Researching immune-related adverse events of ICIs

Although ICIs are well tolerated, undesired immune responses appear as a result of unleashing the immune system by inhibiting the physiological brake of immune activation and disrupting the balance between tolerance and immunity. The mechanism of immune-related adverse events (irAE) differs between ICIs: PD-1/PD-L1 inhibition, for instance, occurs in peripheral tissue on T cells that are already engaged in an ongoing effector T cell response. Hence, irAE seem less likely than for CTLA-4 inhibition, which regulates early T cell activation and might take place in all lymph nodes where an immune response is initiated [213]. Effects on non-tumor-specific T cells that target self-antigens on healthy tissue result in immune-mediated inflammation of almost any organ or tissue with varying incidences and severities [214–216]. Up to 70% of patients treated with a PD-1/PD-L1 antibody experience severe irAEs, frequently affecting the skin, the digestive tract, and the liver [217–221]. More rare, but significant due to associated mortality, are reported events of ICI-associated myocarditis [222,223]. Toxicities of ICI combination therapy are associated with a markedly greater risk of severe adverse events such as diarrhea/colitis, transaminitis, and fatigue [218]. In severe cases, additional immunosuppressive therapy or discontinuation of immunotherapy may be required [224]. However, currently no robust testing, diagnostic and management approaches for ICI toxicity are established and represent an unmet need [225]. To our knowledge, no iCoCs were yet applied for toxicity studies despite the relevance of irAE in ICI therapy. As in the previously discussed efficacy studies, predictive biomarkers for irAE are absent and patient-specific *in vitro* models may provide the opportunity to take irAE into consideration for treatment-related decisions [226]. For instance, to identify the population of certain cancer types where the benefit of combination therapy outweighs risk [227]. Furthermore, combining iCoCs with organs such as skin, intestine, liver, or heart might provide a valuable tool to investigate off-target effects (Fig. 5).

3.3. Cytokine therapy

Cytokines can act during every phase of cancer-immune interactions by, for instance, improving antigen priming, enhancing the recruitment of effector immune cells into the TME, or increas-

ing their cytotoxicity [190]. Cytokines impact tumor growth directly via anti-proliferative or pro-apoptotic activity or indirectly by stimulating cytotoxicity of the effector cells [4]. Since the potent anti-tumor activities of pro-inflammatory cytokines were discovered, interferon-alpha (IFN- α) and interleukin-2 (ILN-2) were approved by the FDA for several malignant diseases. Moreover, inhibition of immunosuppressive cytokines as well as novel targets are currently explored. In general, however, cytokine therapy is limited by the short half-life of most cytokines, a narrow therapeutic window with only modest anti-tumor efficacy, and a less favorable safety profile compared to ICI. As with the previously discussed modalities, synergistic combinations (e. g. with ICI or CAR-T) to increase efficacy are a potential option. Furthermore, several strategies are being tested to enhance local concentration, such as intratumoral gene therapy vectors that encode cytokines. In this section, we review studies of cytokines in iCoCs, which encompass target discovery, combinational therapy, and targeted chemokine delivery.

To identify and validate cytokines as potential therapeutic targets, Xie et al. [51] and Wu et al. [60] exposed iCoCs integrating monolayers of cancer cells and adhering immune cells to immunosuppressive cytokines. Xie et al. studied the inhibitory effect on cell migration by monitoring the migratory responses of metastatic breast cancer cells and neutrophils in the microfluidic platform [51]. Cancer or immune cells were aligned at docking structures before exposure to a stable gradient of activin A, a cytokine of the transforming growth factor beta 1 (TGFB-1) superfamily that is overexpressed in breast cancer cells. Cell migration analysis at the single-cell level provided information on migration distance and chemotaxis, including transendothelial migration. Using this rather simple iCoC, the authors successfully characterized the effect of activin A on neutrophil-cancer migratory interactions and identified activin A as a potential therapeutic target.

Wu et al. employed a more complex optofluidic drug-screening platform that enables simultaneous cancer secretion profiling, analysis of cancer-immune system communication and drug screening on a single device [60]. The immunosuppressive secretion of HeLa cells was quantified on-chip by a surface-enhanced Raman scattering (SERS)-assisted multiplex immunoassay with high sensitivity. Conditioned culture medium was perfused from the culture chamber into the antibody barcode, which was patterned with TGFB-1, prostaglandin E2 (PGE2) and interleukin 10 (IL-10) antibodies and captured corresponding secreted proteins. After profiling of the cancer secretion, the medium was redirected into the extracellular environment of T cells and their proliferation monitored. Thereby an inhibitory effect on immune cell proliferation was observed which could be partially recovered by addition of neutralizing antibodies or artemunate, a drug that inhibits the secretion of these cytokines. This confirmed anti-proliferative effects of TGFB-1, PGE2 and IL-10 induced by HeLa secretions. This integrated SERS-microfluidic platform nicely highlighted how target identification as well as discovery and validation of novel drugs could be accelerated in preclinical studies.

The first two steps in the immune-cancer response are the release of cancer cell antigens and the subsequent antigen uptake and presentation by DC initiating an antigen-specific response. These two steps are targeted in the iCoC of Parlato et al., who enhanced immunogenic cell death of cancer cells with a histone deacetylase inhibitor (HDACi) and activated DCs with IFN- α [57]. Their iCoC consists of a central chamber containing IFN- α activated DCs and two flanking compartments with colorectal cancer cells embedded in a hydrogel. The central chamber and flanking compartments are connected via micro-channels featuring dimensions and geometry similar to the blood vessel and tissue interstitial areas. The microarchitecture, porosity, and stiffness of the hydrogel were adapted to recapitulate physical and biochemical cues *in vivo*.

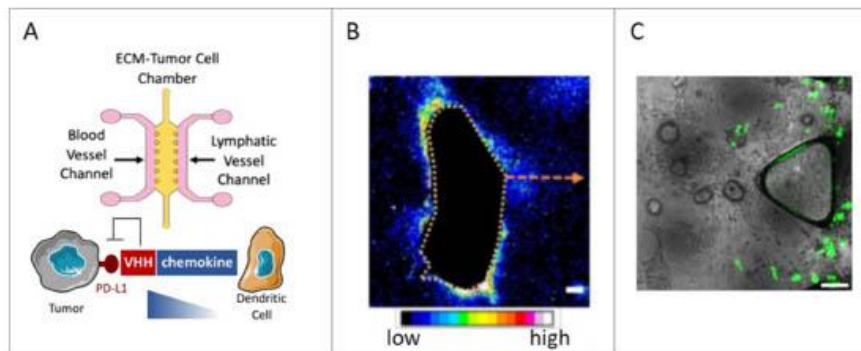


Fig. 9. Applying iCoC to evaluate the dynamics of chemokine-conjugated antibodies and their impact on immune cells. A) The microfluidic device contains an ECM–tumor cell chamber positioned between two microfluidic channels containing media, each lined with HUVECs or human lymphatic dermal microvascular endothelial cells. B) CCL21(T)–B3 effectively targets the tumor cells and creates a steep concentration gradient in close proximity of the tumor spheroid (outlined with dashed line). Scale bar is 10 μm . C) CCL21(T)–B3 increases infiltration BMDC (green) and directs the movement towards tumor cells. Scale bar is 50 μm . Reprinted with permission from [229]. Copyright 2019 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Monitoring of the infiltration of IFN- α treated DCs through the micro-channels into the cancer compartment revealed an enhanced migration towards HDACi treated cancer cells. This was largely guided by an upregulation of the chemokine CCL12 and the respective receptor CXCR4 on DCs. Migration velocity reversely correlated with longer interaction times, suggesting pausing of DCs for endocytosis. Subsequent to antigen uptake, there is evidence that antigen-loaded DC may make a direction choice and choose to leave the tumor environment, indicating trafficking towards lymph nodes. This model shows how two different anti-tumor agents can have complimentary anti-cancer effects and enable mechanistic analysis of immune-cancer interaction.

The type, density, and location of tumor-infiltrating immune cells are critical determinants of patient outcome [228]. Enhancing the homing of effector cells with chemokines may improve immune response and efficacy of immunotherapies such as CAR-T therapy or ICI. In the following iCoC studies, the immunosuppressive TME was modeled to investigate dynamics, specificity, and effects of targeted chemokines and immune cells on cancer.

Fang et al. conjugated the chemokine CCL21 to a PD-L1 blocking single-domain antibody fragment to exploit its therapeutic effects as well as its specific targeting properties; thereby the bispecific molecule should be localized in close proximity to the tumor (Fig. 9A and B) [229]. In the iCoC model, B16 melanoma cells were embedded in a collagen hydrogel, flanked by two channels lined with human umbilical vein endothelial cells (HUVECs) and human lymphatic microvascular endothelial cells (Lym-HMVEC) that mimic blood and lymph vessels respectively (Fig. 9A). Utilizing this system, the specific binding of the fusion protein to PD-L1 expressing melanoma cells could be verified, eliciting a concentration gradient in the tumor spheroids vicinity (Fig. 9B). Indeed, in the presence of the bispecific molecule, bone-marrow-derived dendritic cells (BMDCs) extravasated through the lymphatic channel and migrated through the hydrogel towards the tumor spheroid with increased speed and persistence (Fig. 9C). In this study, a therapeutic molecule that could enhance immune cell infiltration as well as a model that elucidates drug distribution and its influence on cell migration within the TME was developed.

Ayuso et al. applied an iCoC to elucidate the dynamics of NK cells and a chemokine-conjugated antibody, as well as subsequent cytotoxic effects and NK-mediated ADCC [76]. The microfluidic platform incorporated an MCF-7 breast cancer spheroid with a

hypoxic core in a collagen hydrogel, mimicking a solid tumor model that may obstruct drug penetration and cell migration. Two lumens lined with endothelial cells crossed the hydrogel and could be perfused with media, NK cells, macromolecules, or combinations thereof. Perfusion with an α -EpCAM antibody revealed its ability to cross the endothelial barrier and passively diffuse through the collagen hydrogel. However, cell-cell junctions of the cancer spheroid posed an impermeable barrier and hindered antibody penetration into the spheroid. Antibodies on the tumor cells' surface were endocytosed into intracellular lipids, which was not observed in spheroids of healthy breast cell lines MCF-10A and suggests an immune escape mechanism by the MCF-7 tumor cells. In contrary to the antibody, NK cells exhibited intense chemotactic migration towards the spheroid with increased directionality in proximity to the tumor. In a cell density-dependent manner, NK cells penetrated the spheroid by squeezing through the cell-cell junctions and killed tumor cells in the center of the spheroid, despite hypoxia. In the presence of an IL-2 conjugated α -EpCAM antibody, NK cell-mediated cytotoxicity was significantly increased and located mainly at the spheroid periphery, presumably due to the limited penetration of the targeted cytokine into the tumor. This study sheds light on endothelial permeability, tumor penetration, and antibody clearance by tumor cells and identifies several potential targets for immunotherapy. In this model, compounds targeting the tumor shedding mechanism could be tested. Further research with this model may elucidate how NK cells move through the tumor cell-cell junctions to generate NK cells with higher penetration capabilities as well as identification of new therapeutic targets that prevent NK cell exhaustion in long-term studies.

In cytokine therapy, iCoCs were applied for target discovery, showing the potential of an integrated platform and the testing of a synergistic combination, gaining insights in migration characteristics and antigen uptake by DCs. In more complex models, dynamics and effects of a targeted cytokine and immune cells were investigated and evidence of replicating a tumor evasion mechanism was demonstrated. Such a complex iCoC represents a valuable tool that could further contribute to examine other immunotherapeutics, including but not limited to the investigation of antibody dynamics within a TME for ICI therapy or T cell engagers. Moreover, many additional synergistic combinations could be tested as well as alternative delivering strategies, e.g. intratumoral

gene therapy, which would greatly benefit from the 3D TME context.

3.4. Oncolytic viruses

Oncolytic viruses (OV) encompass immunotherapy and cytotoxic drug properties. They preferentially target tumor cells inside which they replicate to lead to their destruction along with the release of progeny virions. The new viral particles can then attack additional tumor cells while sparing healthy cells. Such virus-driven attack awakes the immune response against the infected cells including innate and adaptive immunity leading to a specific antitumor immune response [230]. Over the years, numerous clinical trials have proven the good safety record of OV derived from different virus families. In 2015, Tamoligene Laherpapvec (T-vec), an HSV-1 derived virus expressing GM-SCF, was approved by the USA, European Union, and Australia for advanced recurrent melanoma [231]. Nevertheless, clinical efficacy of OV as a single agent is extremely limited compared to what has been anticipated by preclinical assessments. Several obstacles encountered by the OV are underestimated and not fully understood. Among them are 1) their tumor delivery in a perfused environment, comprising immune components that can sequester and clear the virus 2) their spreading inside a tumor mass with high interstitial pressure, rich in ECM and stromal cells 3) the interplay with the antiviral immune response [232]. As the virus tropism varies greatly between species, human iCoC models appear as privileged tools to address these questions. For example, evaluation of oncolytic Vesicular Stomatitis Virus (VSV) properties in an OoC comprised of vessel-like channels and 3D multicellular tumor spheroids, showed a more restricted tropism and replication than previously established in 2D culture [233]. Combined with real-time monitoring, this system successfully demonstrated the propagation of oncolytic VSV from a primary-infected tumor spheroid to a secondary one mimicking *in vivo* targeting of metastasis site by OV [234]. The model, however, was lacking immune cells, which are crucial to fully capture OV mode of action. Indeed, interplays between OV, immune system, and tumor are numerous and complex with the immune response being an essential part of OV activity while limiting OV spread [235]. iCoCs will be valuable assets to decipher such dynamics. By mimicking an immunocompetent tumor microenvironment with the ability to control its key components (Section 2.2), iCoCs should enable to break down the impact of different immune and stromal cells either on antitumor immunity or antiviral immunity and ultimately on the tumor destruction. Such mechanistic insights will allow the identification of cells that need to be boosted or impeded to optimize OV efficiency. These functions may then be implemented with the expression of therapeutic genes by OV [236]. Furthermore, OV offer a privileged approach to inflame tumor and potentialize the effect of other immunotherapies like ICI or ACT [237]. Due to iCoC's ability to emulate human TME with its physical barriers, intercellular crosstalk, and realistic cell motion in and out of the tumor, assessment of combination therapies with multiple variables (doses, timing of injection) holds the promise to more accurately predict clinical outcome. Moreover, it will avoid the need of surrogate viruses or agents with the suitable species-specificity for *in vivo* model. Lastly, iCoCs open the door for personalized medicine with the addition of immune and tumor components from each patient to assess their own susceptibility to different OV families and payloads.

3.5. Cancer vaccines

Cancer vaccines aim to induce *de novo* or boost antitumor immunity in "cold" tumors and in "hot" tumors, respectively. Different types of cancer vaccines have been developed including

molecular vaccines (DNA-, RNA-, or peptide-based)-, virus vector vaccines, and cellular vaccines [238]. Molecular- and viral-based vaccine components can be delivered to the patient via direct intranodal injection, passively through the lymphatic route, as well as active targeting of antigen-presenting cells (APCs) specific receptors [239]. Upon activation by the vaccine, the APCs can then mature, present tumor antigen, undergo trafficking to the tumor-draining lymph nodes, and ultimately prime effector T cells. Consequently, this should promote the proliferation of activated T cells and their migration to the tumor site to eventually kill cancer cells that display the associated target-antigen [239]. Cellular vaccines, on the other hand, rely on the *ex vivo* modification of patient-derived APCs to express tumor antigens, which are then reinfused back to the patient. In fact, the only cancer vaccine approved by the FDA so far, Provenge (Sipuleucel-T), uses this approach by activating autologous dendritic cells *ex vivo* and reinfusing them to prime the endogenous T cells and elicit an antitumor response [240]. Despite the enthusiasm that it brought to the field since its FDA approval a decade ago, broad adoption of cancer vaccines remains limited so far due to different sets of challenges. Clinical translation of therapeutic efficacy has often been poor [241,242], which mostly stem from the low immunogenicity of the vaccine and the negative regulatory mechanisms orchestrated by the tumor that induce immune tolerance [243]. Here, the use of nonclinical models that can translate to human immunity will be important to better predict clinical response upon vaccination and thereby optimize immunization strategy. A number of different approaches to model lymphoid tissues including microfluidic-based models have been introduced in recent years [244–247]. Linking iCoC models to these platforms is of high interest and can be used, for example, to evaluate effector T cells cytotoxicity after cancer vaccine treatment and assess the overall efficacy of combination therapy (e.g. with ICI or targeted adjuvants).

To improve the therapeutic efficacy and the delivery of cancer vaccines, other approaches have been explored such as the use of multiple immunogenic antigens, and the integration of nanoparticles to facilitate vaccine transport to the lymph node [248,249]. The nanocarrier design, such as the particle size, composition, shape, chemistry, stiffness, etc., is influenced by the choice of antigen to be delivered [239]. As previously reported, OoC models can be used to evaluate the transport mechanism of nanoparticles through 3D tissue and barriers [250]. Connecting lymph-node chip to iCoC models could therefore also allow the assessment of nanoparticles' delivery and efficacy, thereby potentially accelerate nanocarrier design development. Taken together, iCoC models have the potential to boost cancer vaccine development by providing a human platform for nonclinical basic research and personalized screening in the complex TME.

4. Outlook

In 2016, Boussommier-Calleja et al. proposed the need to employ microfluidic systems to model the complex tumor microenvironment for studies on immune-cancer interactions due to their tremendous potential [146]. Now, iCoC models have been increasingly utilized to characterize mechanisms of actions of different types of immunotherapies and will potentially become a valuable asset in immuno-oncology research. The iCoC platforms highlighted here show that leveraging recent advances in micro-fabrication, microfluidics, biomaterials, and tissue engineering, complex immunocompetent cancer models that are human-relevant can be generated. The potential application areas of these systems are manifold, ranging from i) basic research of immune-cancer interaction, ii) efficacy and safety studies of different immunotherapies, to iii) personalized immunotherapy screening.

To reach the ambitious goal of broad application among biomedical research communities and drug developers, however, several challenges remain to be solved; many of which are related to the challenges OoC technology in general is facing [203,251]. For instance, iCoC models need to increase their throughput, manufacturability, and scalability in order to incorporate them in different stages of the immunotherapy development pipeline such as biomarker identification, target validation, lead selection, and lead optimization [203]. Owing to the bottom-up approach of the technology, it could in principle be possible to use the simpler iCoC models for higher throughput screenings for target identification, whereas the more complex iCoC models could be used for non-clinical efficacy and toxicity studies.

In the future, further research should target at incorporating multiple immune cell types, such as other myeloid-derived suppressor cells and Tregs, due to their critical immunosuppressive role within the TME [252,253]. Additionally, tissue/organ-specific resident cell types could be included to elucidate their possible role in modulating immune-cancer interaction within that specific organ. For example, in fat-rich tissues such as breast and prostate, cancer cells grow either nearby or in direct contact with adipocytes, which, in obese condition, can promote cancer progression by triggering chronic inflammatory responses. This is characterized by an increase of macrophages infiltration, chronic dendritic cell activation, and eventually T cell exhaustion [254,255]. This aspect could be addressed by direct integration of cancer tissue in immunocompetent "host" organ-on-chip models such as lung, liver, or adipose [256–258]. Linking iCoCs with other OoC-models has the potential to recapitulate systemic toxicities or other adverse effects of immunotherapy, such as CRS.

In terms of clinical translation, close interaction with biomarker development could be mutually beneficial: iCoCs could be used to discover novel biomarkers; established biomarkers could be utilized to correlate iCoC data to clinical data. Future works should also capitalize on the high-content characteristic of iCoC models by applying AI-based data analysis, e.g. for image interpretation. Other computational modeling approaches, such as modeling transport phenomenon in microfluidic devices [68,75] and molecular biocomputation for modeling signaling pathways [99], could be more widely integrated to support experimental data. Lastly, we believe that the utilization of iCoC models can provide entirely new and human-relevant insights for immunotherapy research, add an additional immune-competent nonclinical model for safety and efficacy testing of immune-based therapeutics, promote patient-specific medicine, and ultimately improve patient outcomes.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cecile Zaupe is employee of Transgene S. A. Michael Hudecek is an inventor on granted patents and patent applications

relating to CAR-T cell therapy. Birgit Fogal is an employee of Boehringer Ingelheim Pharmaceuticals, Inc.

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Appendix A. Supplementary material

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Publication [2]**A Novel Microphysiological Colon Platform to Decipher Mechanisms Driving Human Intestinal Permeability**

A. Apostolou, R. A. Panchakshari, A. Banerjee, D. V. Manatakis, M. D. Paraskevopoulou, R. Luc, G. Abu-Ali, A. Dimitriou, C. Lucchesi, G. Kulkarni, **T. I. Maulana**, M. Kasendra, J. S. Kerns, B. Bleck, L. Ewart, E. S. Manolakos, G. A. Hamilton, C. Giallourakis, K. Karalis
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Abstract/Summary:

Colon Intestine-Chip (Emulate, Inc, Boston, MA), seeded with human colon crypt-derived epithelial and primary microvascular endothelial cells, was built to investigate leaky gut in human beings. This ex vivo platform recapitulated the effects of proinflammatory cytokines in the intestinal epithelial barrier and identified novel mechanisms of action.

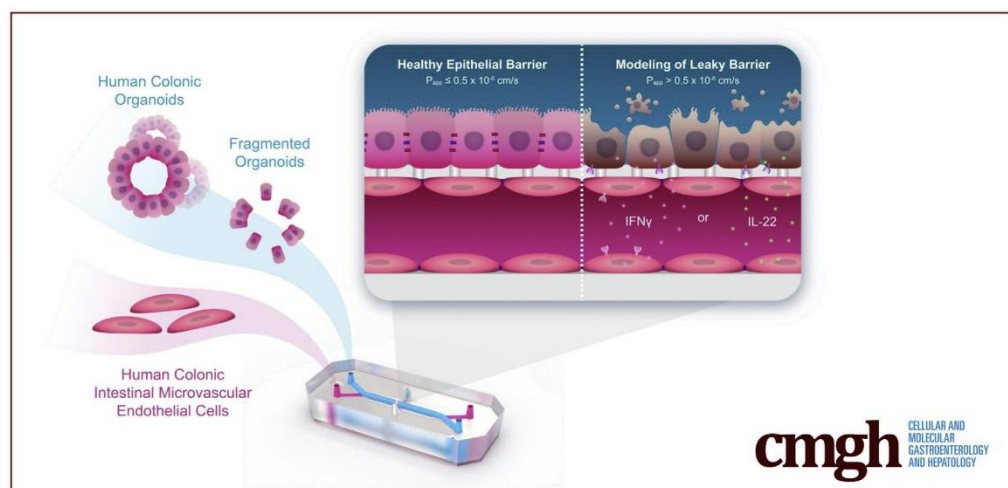
ORIGINAL RESEARCH

A Novel Microphysiological Colon Platform to Decipher Mechanisms Driving Human Intestinal Permeability



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SUMMARY

Colon Intestine-Chip (Emulate, Inc, Boston, MA), seeded with human colon crypt-derived epithelial and primary microvascular endothelial cells, was built to investigate leaky gut in human beings. This *ex vivo* platform recapitulated the effects of proinflammatory cytokines in the intestinal epithelial barrier and identified novel mechanisms of action.

BACKGROUND & AIMS: The limited availability of organoid systems that mimic the molecular signatures and architecture of human intestinal epithelium has been an impediment to allowing them to be harnessed for the development of therapeutics as well as physiological insights. We developed a microphysiological Organ-on-Chip (Emulate, Inc, Boston, MA)

platform designed to mimic properties of human intestinal epithelium leading to insights into barrier integrity.

METHODS: We combined the human biopsy-derived leucine-rich repeat-containing G-protein-coupled receptor 5-positive organoids and Organ-on-Chip technologies to establish a micro-engineered human Colon Intestine-Chip (Emulate, Inc, Boston, MA). We characterized the proximity of the model to human tissue and organoids maintained in suspension by RNA sequencing analysis, and their differentiation to intestinal epithelial cells on the Colon Intestine-Chip under variable conditions. Furthermore, organoids from different donors were evaluated to understand variability in the system. Our system was applied to understanding the epithelial barrier and characterizing mechanisms driving the cytokine-induced barrier disruption.

RESULTS: Our data highlight the importance of the endothelium and the *in vivo* tissue-relevant dynamic microenvironment

in the Colon Intestine-Chip in the establishment of a tight monolayer of differentiated, polarized, organoid-derived intestinal epithelial cells. We confirmed the effect of interferon- γ on the colonic barrier and identified reorganization of apical junctional complexes, and induction of apoptosis in the intestinal epithelial cells as mediating mechanisms. We show that in the human Colon Intestine-Chip exposure to interleukin 22 induces disruption of the barrier, unlike its described protective role in experimental colitis in mice.

CONCLUSIONS: We developed a human Colon Intestine-Chip platform and showed its value in the characterization of the mechanism of action of interleukin 22 in the human epithelial barrier. This system can be used to elucidate, in a time- and challenge-dependent manner, the mechanism driving the development of leaky gut in human beings and to identify associated biomarkers. (*Cell Mol Gastroenterol Hepatol* 2021; 12:1719–1741; <https://doi.org/10.1016/j.jcmgh.2021.07.004>)

Keywords: Organoids; Organ-on-Chip; Leaky Gut; Interleukin 22.

The gastrointestinal barrier is the most extensive epithelial barrier of the body that is colonized by commensal bacteria, segregated from the host's immune system to maintain homeostasis.¹ The characteristics of the intestinal barrier are attributed mainly to the epithelial cell interconnections via specialized tight junctions (TJs). Supporting elements include locally secreted factors, the mucin layer, and microbiome-derived humoral factors. Disruption of the barrier was first identified in infections and inflammatory bowel disease (IBD)²; with the list continuously expanding to include additional diseases, drugs, and toxic factors.^{3,4} Intestinal crypt stem cells renew the intestinal epithelial cells (IECs) during the lifespan, thus maintaining barrier function and enabling the repair of the injured barrier. Dysregulation of epithelial regeneration is critical for the progression of chronic gastrointestinal diseases.

The significance of cytokines in disruption, repair, or accelerated damage of the intestinal barrier has been shown both *in vivo* and in cell-based disease models. Mechanisms used by proinflammatory cytokines to disrupt the barrier include induction of epithelial cell apoptosis and degeneration of the TJs. Interferon- γ (IFN γ) is the prototype cytokine used, either alone or in combination with tumor necrosis factor α (TNF α) or interleukin (IL)1 β , to induce barrier disruption in several intestinal disease models. Specifically, prolonged exposure of IECs to IFN γ results in activation of apoptotic mechanisms,⁵ increased expression of Dickkopf-related protein 1 (DKK1) and inhibition of epithelial proliferation,⁶ or phosphorylation of Myosin-Light Chain (MLC) via activation of the Myosin-Light Chain Kinase (MLCK), which drives internalization and, thus, disruption of TJs.^{5,7}

A major advancement in our ability to understand the regulation of intestinal epithelial homeostasis has been the discovery of organoid technology.^{8,9} This development enables the use of patient-derived samples to capture the spectrum of individual responses¹⁰ and significantly advances our understanding of IEC biology. Recently, a culture

system was developed in Transwells that enables expansion of organoids to multicellular polarized monolayer epithelia and was applied to model intestinal infection.¹¹ This system bypasses the main caveat of the organoids, which is the lack of accessibility to the apical side of IECs, the equivalent of the luminal tissue side. A missing element of the earlier-described systems that is addressed by the Organ-on-Chip technology is the potential to incorporate conditions that emulate the intestinal microenvironment.^{12,13} Specifically for the gut, significant features include stretching to emulate the peristalsis-like motion of the tissue, exposure to shear stress, and interactions of the epithelium and endothelium or other cells via tissue-relevant extracellular matrix proteins. The Duodenum Intestine-Chip (Emulate, Inc, Boston, MA) supports the synchronous culture of different, tissue-relevant cell types of the intestinal lumen and, thus, enables functionality, such as the activity of CYP3A4, much closer to that measured *in vivo* compared with other systems.¹⁴

Similarly, in this work, we leveraged the Organ-on-Chip technology^{14–16} and the proprietary Human Emulation System (Emulate, Inc, Boston, MA) to develop a human Colon Intestine-Chip (Emulate, Inc, Boston, MA) platform including organoid-derived IECs and primary microvascular intestinal endothelial cells. First, we confirmed *in vivo* relevant cytoarchitecture and tissue-relative abundance of the key colon epithelial cell subtypes. Next, we showed the reproducibility of the system among 3 donors including the establishment of a tight epithelial barrier. The latter provided the basis for modeling the well-established IFN γ -elicited barrier disruption and assessed the effects of IL22 on epithelial integrity. We show that IL22, upon engaging its receptors expressed in the Colon Intestine-Chip, acts as a barrier-insulting cytokine, in line with recent publications.^{17–19} These findings considered together indicate that the Colon Intestine-Chip renders a promising human platform for studying intestinal epithelial barrier development and homeostasis. Considering the use of epithelial modulatory effectors such as IL22 in clinical trials for indications such as IBD (<https://clinicaltrials.gov/ct2/show/NCT03558152>),²⁰ it also will be of interest to further understand the relationship of model systems, such as ours, to clinical read outs.

In summary, we present the development of a new biopsy-derived Colon Intestine-Chip as a human organoid-

[§]Authors share co-senior authorship.

Abbreviations used in this paper: cHIMEC, colonic human intestinal microvascular endothelial cell; DGE, differential gene expression; DKK1, Dickkopf-related protein 1; ECM, extracellular matrix; EdU, 5-ethynyl-2'-deoxyuridine; GO, gene ontology; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN γ , interferon- γ ; IL, interleukin 6; IL22BP, interleukin 22 binding protein; NHS, N-hydroxysuccinimide; P_{app}, apparent permeability; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA sequencing; STAT, signal transducer and activator of transcription; TJ, tight junction; TNF α , tumor necrosis factor α ; Wnt, wingless-related integration site; ZO-1, zonula occludens-1.

 Most current article

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based platform to study cell–cell interactions. The Colon Intestine-Chip is a significant development for targeting unmet needs such as leaky barrier implicated in the pathogenesis of gastrointestinal diseases, toxicity by frequently used drugs, and aging.

Results

Development of a Colon Intestine-Chip Platform

As previously described, juxtaposed culture of intestinal epithelial and endothelial cells in micro-engineered Organ-Chips results in the formation of a 3-dimensional mucosal layer.¹³ Here, we applied this technology to develop a Colon Intestine-Chip based on human organoids isolated from colonic biopsy specimens, along with colon-specific microvascular endothelial cells (Figure 1A). The top and bottom surface of the membrane, precoated uniformly with extracellular matrix (ECM) proteins, as depicted by fluorescent staining with N-hydroxysuccinimide (NHS)-ester dye (Figure 1B), was seeded with enzymatically fragmented organoids and endothelial cells, respectively (Figure 1C). Culture conditions included fluid flow, supported by the Zoë Culture Module (Emulate, Inc) (see the Materials and Methods section for more detail), at 60 μ L/h initiated on day 1, followed by the removal of the stemness supporting factors, Y-27632 and CHIR99021, on day 2 of the culture (Figure 1D). Periodic stretch initiated on day 3 at 2% intensity and after 24 hours of pre-acclimation was switched to 10% until the completion of the study. The key point for switching to 10% stretching is when a confluent epithelial monolayer is established (Figures 2A and 3A), and the apparent permeability to 3 kilodaltons dextran becomes stabilized at less than 0.5×10^{-6} cm/s²¹ (Figures 2B and 3B). Twenty-four hours after exposure to 10% stretching (day 5), we characterized the formation of TJs in the expanded colonic epithelial monolayer by immunostaining for zonula occludens-1 (ZO-1) and E-cadherin (Figures 2C and 3C). Next, we evaluated the cell polarization by immunostaining on the same day of culture for ion channels such as the apically expressed SLC26A3,²² and the basolaterally expressed Na⁺/K⁺ adenosine triphosphatase transporter²³ (Figures 2D and 3D). These results show the establishment of a tight, polarized, human colonic epithelial monolayer by day 5 of the culture of colonic organoids in the Intestine-Chip. We show here the apparent epithelial permeability of chips maintained in culture for 8 days (Figure 2B), with reproducible and robust data across different donors. To date, we have maintained the culture for 12 days.

Role of the Endothelium in the Establishment of the Epithelial Barrier

In the Colon Intestine-Chip, we observed that co-culture with endothelial cells significantly enhanced the epithelial barrier integrity in a nonadditive manner, as indicated by the apparent permeability to 3 kilodaltons dextran (day 4, endothelium alone: 7.38×10^{-6} cm/s vs minus endothelium: 3.01×10^{-6} cm/s vs plus endothelium: 0.89×10^{-6} cm/s) (Figures 2B and 3B). In line with this observation, a mature TJ network was established as early as day 5 and maintained up to day 8 of culture (Figures 2C, 3C, and 4A). On the

contrary, in the absence of endothelium, the TJs were poorly defined, and ZO-1 and E-cadherin staining showed proteins localized in the cytoplasm, rather than on the cell membrane, as expected in tight epithelial barriers (Figures 2C and 4A). These data suggest that the endothelium supports the establishment and the maturation/differentiation of IECs and highlights the advantages of dynamic co-culture systems. Furthermore, immunostaining against the basolateral and apical transporters, Na⁺/K⁺ adenosine triphosphatase and SLC26A3, respectively, showed the compromised epithelial cell polarity in the absence of endothelium (Figures 2D and 4B). On the other hand, stretching did not have a clear impact on the polarization of IECs (Figure 3D). The enhanced expression of the TJ-associated proteins, *Cdh1* and *Tjp1*, and the ion transporters, *Atp1a1* and *Slc26a3*, also was confirmed at the transcriptome level throughout the culture (Figure 4C and D). Parallel scanning electron microscopy analysis showed the formation of a mature brush border, with densely packed microvilli, only in IECs co-cultured with endothelium (Figures 2E and F, 3E and F, and 4E and F).

To comprehensively characterize the co-culture effects on the colonoids and endothelial cells at a transcriptome level, we performed RNA sequencing (RNA-seq) analysis of colonoids cultured in suspension and colonoid-derived IECs on-Chip, in the presence and absence of endothelium and/or stretch. Principal component analysis used on the highly variable genes across libraries showed distinct gene expression profiles between the different on-Chip culture conditions and colonoids in suspension. Our data highlight the effect of endothelium on the divergence of the transcriptome of colonic IECs cultured on-Chip as opposed to colonoids in suspension (Figure 5A and B). We performed functional analysis on the 573 Differentially Expressed Genes and 881 Differentially Expressed Genes, identified of the 58,342 genes annotated in the genome between IECs cultured with or without endothelium in the presence of stretch, on days 5 and 8 of culture, respectively (Figure 5C and D). Significantly enriched terms related to cell development and morphogenesis, cytoskeletal remodeling, and mechanosensory behavior were identified over the course of the culture (Figure 5E and F). Linear differential gene expression analysis confirmed the role of the endothelium as a differentiation driving factor at various time points of experimentation (Figure 5G–I). These findings are consistent with the aforementioned experimental readouts because the increased expression of *Alpi* transcripts in colonocytes cultured juxtaposed to the endothelium (Figure 6D), complementing the scanning electron microscopy analysis (Figure 2E and F). At the transcriptome level, the application of stretch enhanced terms related to ion, lipoprotein, and water transportation, highlighting the importance of tissue-relevant mechanical forces for the functional maturation of the organoid-derived epithelial cells (Figure 5J–M).

Multilineage Differentiation of Colonoids in the Colon Intestine-Chip

Because the use of organoids in research is rapidly expanding, several protocols have been developed for

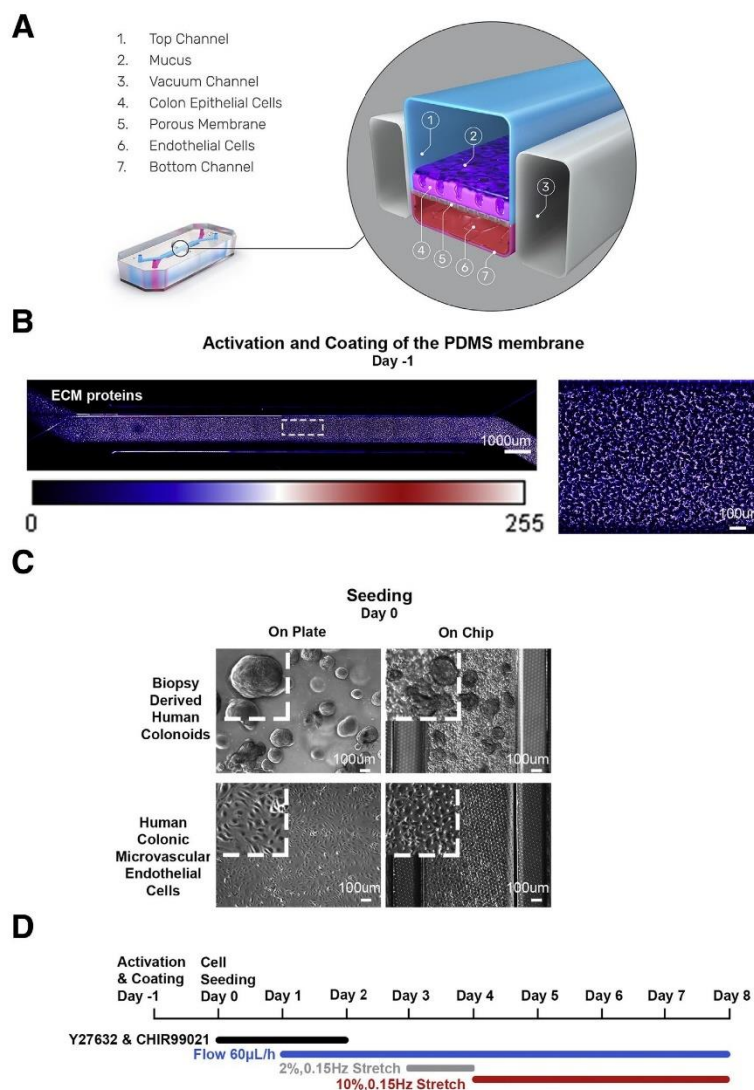
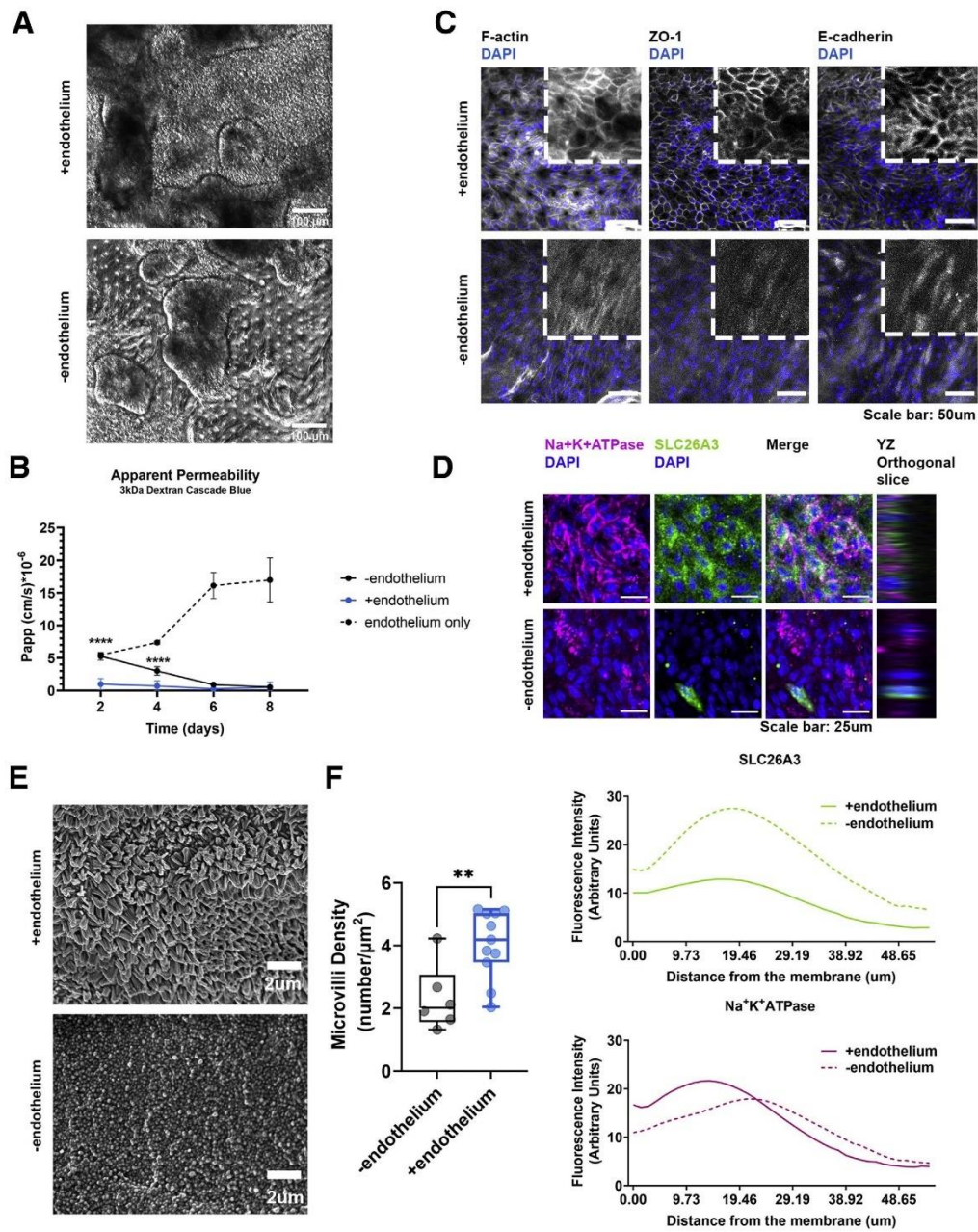


Figure 1. Establishment of the Colon Intestine-Chip. (A) Cross-section of the Colon Intestine-Chip showing the epithelial channel (1), mucus layer (2), vacuum chambers (3), colonic IECs (4), PDMS membrane (5), cHMECs (6), and endothelial channel (7). (B) NHS-ester dye staining against the ECM proteins of the apical channel and the respective Look Up Table color bar, representing the values of an 8-bit image pixel intensity. (C) Representative contrast-phase microscopy images of each cell type morphology, on a plate culture (left) and in the Colon Intestine-Chip (right), on day 0 of the culture. (D) Experimental timeline of the 8-day fluidic culture.

differentiation of the intestinal organoid-derived cells toward mature cells of absorptive and secretory lineage. These methods are based either on engraftment of the organoids in animals,²⁴ or in supporting signaling pathways required to define cell fate in the crypt-villus axis. In the latter, activation of wingless-related integration site (Wnt) and inhibition of p38 pathways²⁵ are the main options, often coupled with inhibition of the Notch pathway.²⁶ In the current study, the stemness factors, Wnt-3a/epithelial growth factor/noggin/R-spondin 1, were maintained in

the culture medium over the length of the culture. Immunofluorescence analysis on day 8 of culture shows physiological differentiation of the colonoid-derived IECs, toward absorptive enterocytes, goblet cells, and enteroendocrine cells (Figure 6A). This finding was verified by quantitative polymerase chain reaction (qPCR) and RNA-seq analysis for markers specific for the absorptive (*Alpi*) and secretory (*Muc2*, *ChgA*) subsets (Figure 6B and C). The presence of endothelium promoted the acquisition of a mature epithelial phenotype (Figure 6D). As can be deduced, the multilineage



differentiation occurred at the expense of the proliferating cells. Reduced expression of the cycling intestinal stem cell marker, *Lgr5*, over the course of the culture (Figure 6B), and drastic elimination of 5-ethynyl-2'-deoxyuridine (EdU)-positive cells by day 5 of culture (Figure 6E), are in further support of the gradual depletion of progenitor cells in the organoid-derived monolayer of IECs. Importantly, the Colon Intestine-Chips recapitulated the expected donor-to-donor variability in the differentiation process, as shown by the varying representation of the absorptive and secretory lineage among 3 different healthy individuals. Notably, donors presented with higher proliferative capacity had lower differentiation toward the absorptive phenotype, and vice versa (Figure 6A and E).

Barrier Disruption Model in the Colon Intestine-Chip

The intestinal barrier maintains mucosal homeostasis via compartmentalization of the resident commensal bacteria from the host immune cells. Disruption of the epithelial barrier results in activation of immune cells and cytokine release, propagating the epithelial injury. IFN γ , a type II IFN produced in the intestinal lamina propria, has been implicated in the pathogenesis of IBD.²⁷ Early studies showed the ability of IFN γ to compromise the intestinal epithelial barrier.⁷ Here, to simulate the *in vivo* action of IFN γ , we exposed the basolateral side of the epithelium to different concentrations of fluidically administered IFN γ in the endothelial channel (bottom channel) of the Colon Intestine-Chip. Two days after stimulation, we observed morphologic changes in the IECs (Figure 7A, red arrows), together with an associated concentration-dependent increase in the apparent permeability (P_{app}) to 3 kilodaltons of dextran (Figure 7B). The basolateral challenge of the IECs with IFN γ increased caspase 3 cleavage and activity (Figure 7C), while prolonged exposure to IFN γ resulted in decreased abundance of IECs (Figure 7D). Fluorescent staining for F-actin showed compromised cytoarchitecture, depicted by poorly defined cell borders and the appearance of enlarged, squamous-shaped cells (Figure 7E) and displaced localization of the TJ proteins ZO-1 and claudin 4 (red arrows).

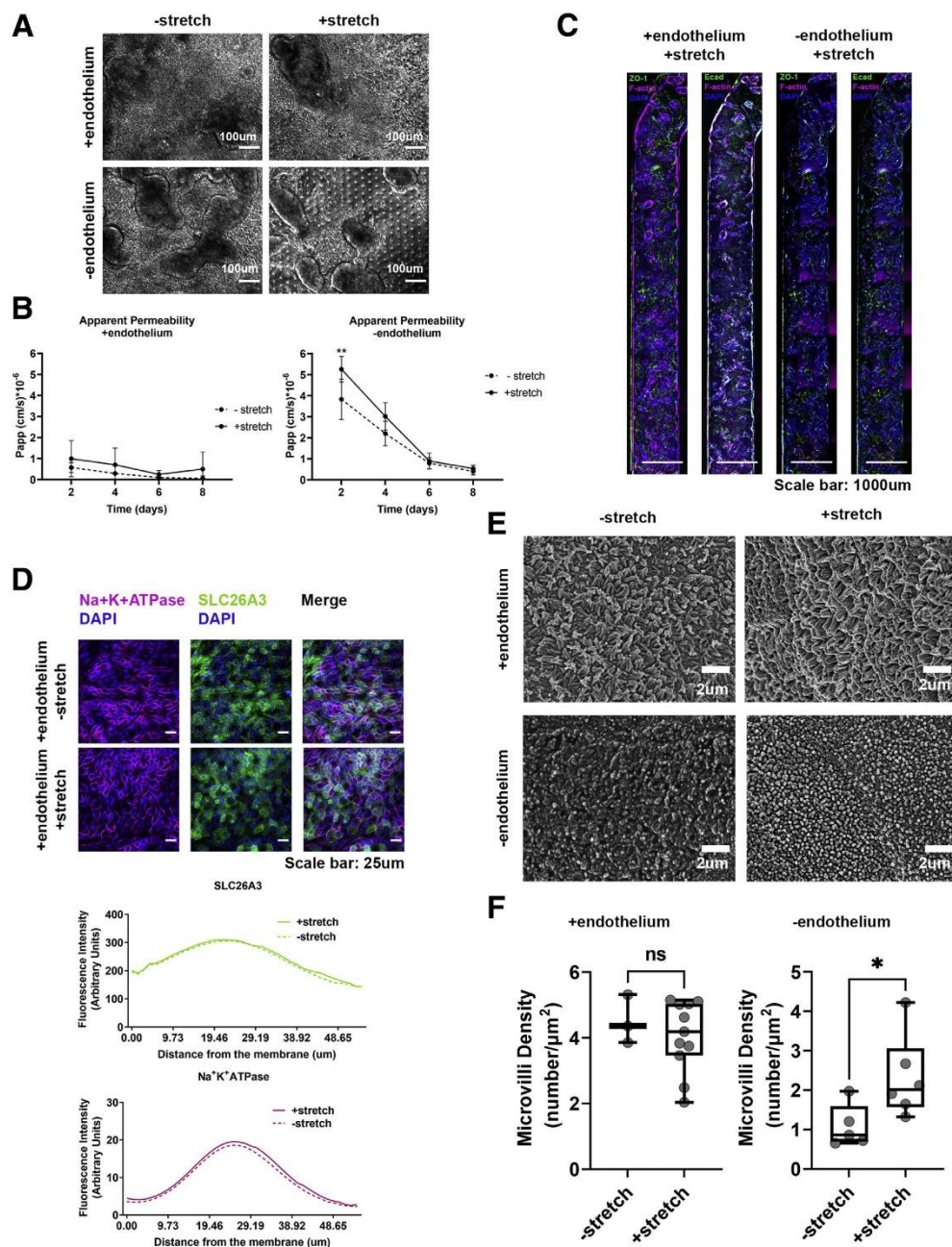
Expression of E-cadherin and occludin was reduced in TJs, in contrast to an increase in the cytoplasmic expression (red arrows). This finding is reminiscent of findings after cotreatment of T84 cells with IFN γ and tumor necrosis factor α , in which reduced affiliation of occludin, junctional adhesion molecule 1, and E-cadherin with membrane lipid rafts triggered redistribution of TJs and weakened cell-cell adhesion.²⁸ Furthermore, upon 24 hours of stimulation with IFN γ , we detected increased levels of IL6 and vascular cell adhesion molecule-1 in the effluent of the vascular channel (Figure 7F). Interestingly, intercellular cell adhesion molecule-1²⁹ and serum amyloid A³⁰ were detectable in the effluent of the top channel (Figure 7G), as reported for the sera of patients with inflammation.²⁹

IL22 Acts as a Barrier-Disruptive Agent for the Mature Colonic Epithelial Monolayer in the Colon Intestine-Chip

Because IL22, a cytokine implicated in IBD, has been shown to protect but also to worsen colitis in different mouse models,^{31,32} we sought to assess its effects in the Colon Chip, a human platform. The binding of IL22 to its receptor at the basolateral side of IECs³³ triggers the phosphorylation of signal transducer and activator of transcription (STAT)3.³⁴ We first confirmed the expression of the 2 IL22-receptor subunits, *Il22ra1* and *Il10rb*, in RNA-seq from organoid-derived IECs (Figure 8A). Interestingly, the expression of both *Il22ra1* and *Il10rb* was higher in the organoid-derived IECs in the chip compared with organoids in suspension culture, and was enhanced further in the presence of endothelium. These data signify the supportive effects of endothelium and the potentially incomplete understanding of human IL22 biology in the absence of a tissue-relevant microenvironment as in suspension cultures.¹⁹

Next, we stimulated the IECs in the Colon Intestine-Chip through the vascular channel with either 0.1, 0.3, or 1 nmol/L of IL22 in the presence or absence of IL22BP, for 1 hour, to determine the optimal concentration, resulting in activation of STAT3 (Figure 8B). The activation of STAT3 was maximal in the acute phase of treatment (1 h),

Figure 2. (See previous page). The presence of endothelium enhances the establishment of the epithelial barrier. (A) Representative phase-contrast images of the colonic epithelial cells on day 5 of the Colon Intestine-Chip culture in the presence or absence of endothelial cells. **(B)** The P_{app} to 3 kilodaltons dextran Cascade blue, of endothelial or epithelial cells, where stretch (10% strain, 0.15 Hz) was applied in the presence or absence of endothelium, over the course of an 8-day culture. Data shown correspond to 1 representative of 3 independent experiments. $n = 3$ –11 chips/condition, means $\pm$ 95% CI, 2-way analysis of variance, Tukey post hoc test. **(C)** Representative confocal fluorescent images showing the establishment of strong epithelial TJs on day 5 of culture in the presence of endothelium. TJs were stained with anti-ZO-1 and anti-E-cadherin and cytoskeleton with phalloidin (gray). Cell nuclei are shown in blue. **(D)** Representative confocal immunofluorescence images against the basolateral transporter Na⁺K⁺ adenosine triphosphatase (ATPase) (magenta) and the apical transporter SLC26A3 (green), indicating the establishment of a polarized epithelial monolayer on day 5 of culture only during the co-culture of colonocytes with endothelium. Cell nuclei are shown in blue. The plots indicate the mean fluorescent intensity distribution for each channel along the basal-apical axis of the epithelial cells. The quantification was performed across 3 different fields of view in 3 individual chips for each condition. **(E)** Representative scanning electron microscopy (SEM) images of the colonic epithelial cells on day 5 of the Colon Intestine-Chip culture, indicating maturation of the epithelial brush border in the presence of endothelial cells. **(F)** Box plots showing the density of microvilli per μm^2 , according to quantification of SEM images on day 5 of culture. The presence of endothelium significantly increases the density of microvilli per μm^2 . The quantification was performed across 6–11 different fields of view for each condition. Each individual point represents a single field of view. Unpaired *t* test. ***P* < .01 and *****P* < .0001. DAPI, 4',6-diamidino-2-phenylindole.



whereas it still was evident up to 72 hours after stimulation (Figure 8C), indicating the sustained sensitivity and responsiveness of the system for potential secondary readouts. Unlike the downstream effects of IL22, treatment with IFN γ did not result in significant activation of STAT3, highlighting the specificity of the event (Figure 8D).

IL12 and IL23 regulate IL22 and are central to the pathogenesis of IBD. The exact role and directionality of the IL22 effects in intestinal epithelial homeostasis are not clearly defined. Current reports include anti-inflammatory effects, induction of homeostatic factors or IEC proliferation,³⁵ as well as a reduction in epithelial integrity in mouse models or the Caco2 cells.^{17,18} Interestingly, a recent study showed induction of endoplasmic reticulum stress response in healthy and IBD colonoid-derived IECs.¹⁹ We next assessed the effects of IL22 on the epithelial permeability of the Colon Intestine-Chip, as described earlier, with the reproducible effects of IFN γ . As shown, challenge with 1 nmol/L IL22 for 48 hours resulted in compromised morphology of the epithelial monolayer (Figure 8E), with poorly defined cell borders and an increased number of floating cells in the effluent medium. We also detected increased P_{app} of the colonic epithelium to 3 kilodaltons of dextran, abrogated when co-treated with 30 nmol/L IL22BP (Figure 8F). Moreover, cleavage of caspase 3 was increased by IL22 (Figure 8G), likely affecting apoptosis in a similar way to IFN γ (Figure 8H). Transcriptomic analysis and downstream functional characterization of the differentially regulated genes support these data (Figure 8I and J). Functional enrichment of gene ontology (GO) terms indicated significant enrichment of biological processes associated with apoptosis and inflammatory responses (Figure 8K). Importantly, our data strongly suggest that IL22 targets human colonic epithelial cells to disrupt the barrier function, acting as an injurious factor.^{36,37} Notably, the IL22-driven effects shown with the Colon Intestine-Chip refer to an epithelium–endothelium system at a healthy state, in which healthy subjects have been stratified as a cell source

and cells have not been exposed previously to other inflammatory insults.

Discussion

Gastrointestinal diseases affect all ages and account for a significant part of acute and chronic diseases across the world. More than 11% of the US population is estimated to suffer from a digestive disorder, often compromising the quality of life or is associated with decreased life expectancy.³⁸ Despite advancements in population screening and improved diagnostic methods,³⁸ the treatment for a number of these diseases remain an unmet need.

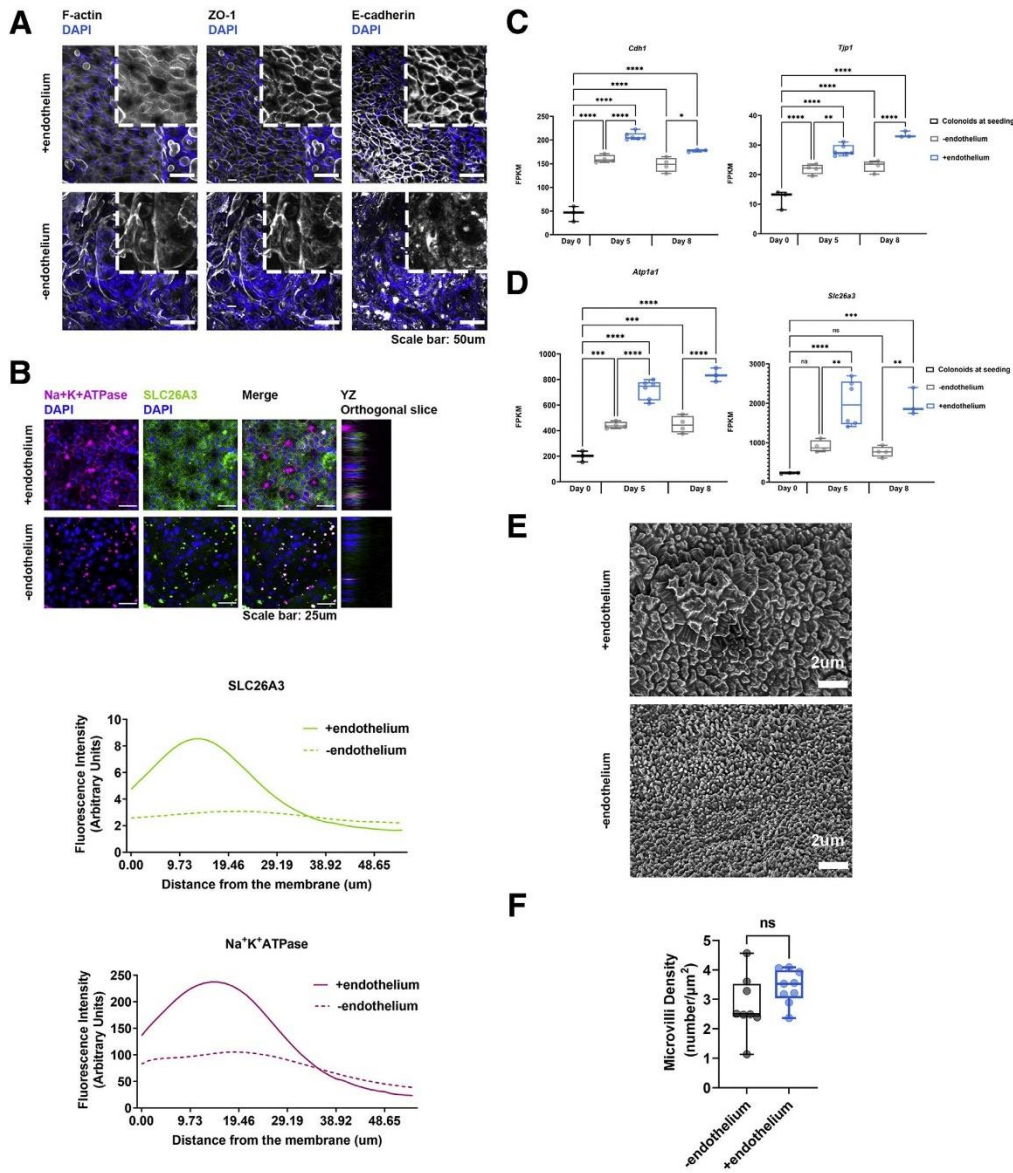
Intestinal epithelial homeostasis is intimately linked to the development of a tight epithelial barrier, owing to the specialized TJs connecting adjacent polarized IECs and associated supporting factors, such as the mucin layer. The significance of the barrier function is shown by the devastating consequences from its disruption, including diseases such as IBD. Our understanding of gastrointestinal physiology and pathogenesis has increased dramatically lately, but the translation of these findings to efficient therapeutics and associated biomarkers still lags behind. A significant reason for the latter is the limited availability of human cell systems that recapitulate cell interactions and functionality of the human tissue. The recent development of organoids, as patient-specific cell sources with unrestricted potential for propagation, has revolutionized our capabilities for experimentation with human cells. A major advantage of organoids is the potential to uncover a spectrum of disease phenotypes by direct comparisons between patient samples. A caveat of the system is that it cannot maintain the tissue microenvironment and cell–cell interactions critical for the identification of potential disease mechanisms for targeting.³⁹

Reproducible establishment of organoids from the small and the large intestine²⁵ is owing to the unique ability of the gut for lifelong renewal from the villous crypts. An important restriction of the organoids is that they do not allow for

Figure 3. (See previous page). The presence of stretch does not significantly contribute in the establishment of a tight epithelial barrier. (A) Representative phase-contrast images of the colonic epithelial on day 5 of the Colon Intestine-Chip culture in the presence or not of endothelium and/or stretch. (B) The P_{app} of the colonic epithelial cells to 3 kilodaltons of dextran Cascade blue, over the course of an 8-day culture, in the presence or absence of stretch (10% strain, 0.15 Hz) and/or endothelium. Stretch does not contribute significantly to the establishment of the epithelial barrier. Data shown correspond to 1 representative of 3 independent experiments. $n = 3$ –11 chips/condition, means $\pm$ 95% CI, 2-way analysis of variance Tukey post hoc test. (C) Representative widefield fluorescent tile images showing the uniform expression of the TJ proteins, ZO-1 (green) and anti-E-cadherin (magenta), along the Colon Intestine-Chip (day 5) when endothelium is present. Cell nuclei are shown in blue. (D) Representative confocal immunofluorescence images indicating the basolateral localization of the transporter Na⁺K⁺ adenosine triphosphatase (ATPase) (magenta) and the apical localization of the transporter SLC26A3 (green) on day 5 of culture. Cell nuclei are shown in blue. The application of stretch does not affect the establishment of the polarity of the epithelial cells. The plots indicate the mean fluorescent intensity distribution for each channel, SLC26A3 (green) or Na⁺K⁺ATPase (magenta), along the basal–apical axis of the epithelial cells. The quantification was performed across 3 different fields of view in 3 individual chips for each condition. (E) Representative scanning electron microscopy (SEM) images of the colonic epithelial on day 5 of the Colon Intestine-Chip culture in the presence or not of endothelium and/or stretch, show the significant contribution of stretch on the maturation of the epithelial brush border only in the absence of endothelial cells. (F) Box plots showing the density of microvilli per μm^2 , according to quantification of SEM images on day 5 of culture, in the presence (top graph) and absence (bottom graph) of endothelium and/or stretch. The quantification was performed across 3–11 different fields of view for each condition. Each individual point represents a single field of view. Unpaired t test. * $P < .05$ and ** $P < .01$. DAPI, 4',6-diamidino-2-phenylindole.

direct access to the apical side of the cells, which face inward of the enclosed cystic cell structure.²⁵ This becomes a bottleneck in particular when mechanisms that drive epithelial barrier homeostasis are investigated because they

are linked to the strictly polarized expression of key proteins in the IECs. To address this need, methods for expansion of 3D organoids into monolayered epithelial structures were established in the Transwell culture



systems.^{40,41} These developments enabled studies with infectious agents, and co-culture of different cell populations, although in a nondynamic microenvironment. In the current study, we leveraged the developments in microphysiological systems and organoid technology to develop a human Colon platform for preclinical studies that incorporates critical aspects of the tissue microenvironment. We previously reported in a micro-engineered Duodenum Intestine-Chip the method for establishing a juxtaposed culture of human organoid-derived IECs and primary endothelium on tissue relevant-derived ECM.⁴² The microenvironment of the Colon Intestine-Chip includes shear stress and stretching to recapitulate aspects of the intestinal peristaltic motion. Although none of the stemness supporting factors is removed during the culture, unlike other protocols described previously^{25,43} we achieved a gradual differentiation of the organoid-derived IECs for the 8 days of culture. Furthermore, the presence of endothelium from the beginning of the culture was found to support the earlier establishment of a tight epithelial barrier, a consistent finding in all donors tested and aligned with former studies using a duodenum-based Intestine-Chip.¹³ As described earlier, up-regulation of key signaling pathways related to epithelial differentiation, metabolism, and ion transportation unveil the physiological relevance of the transcriptomic phenotype of the Colon Intestine-Chip. The robustness and reproducibility of the protocol and the demonstrated interindividual variability in the pace of differentiation of colonoids to epithelial cell subtypes were highly suggestive of the potential of the Colon Intestine-Chip as a platform for human studies. For further confirmation of the latter, we proceeded with an assessment of the functional response of the Colon Intestine-Chip to a well-established barrier disruption cytokine, IFN γ .⁷ As has been shown by studies with cell lines and *in vivo* experimental models, IFN γ modulates the integrity of both the intestinal epithelial⁷ and endothelial⁴⁴ barriers. Disruption of the epithelial barrier, specifically, is driven by apoptosis²⁸ and cytoskeletal remodeling, via up-

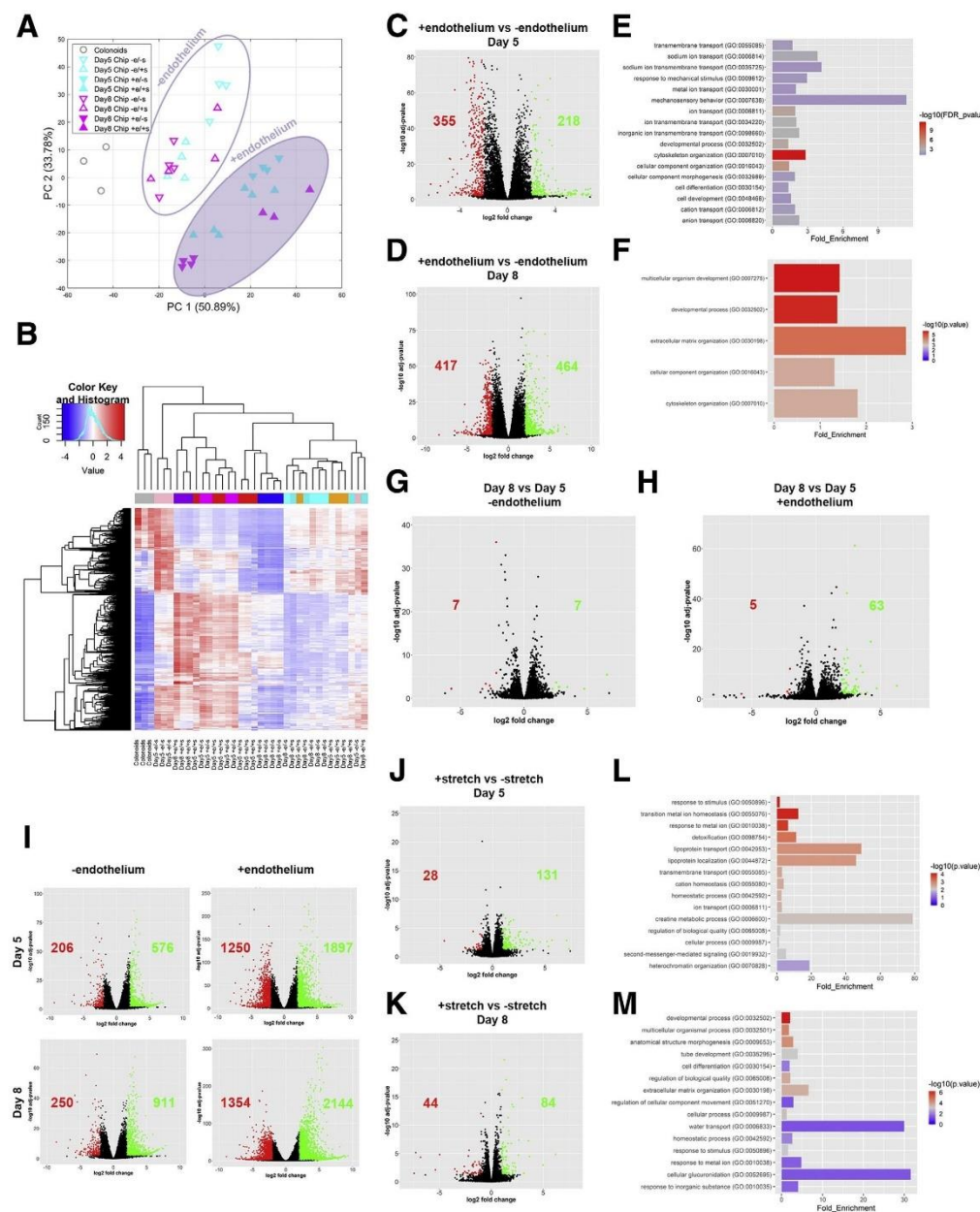
regulation of MLCK.⁵ We found that upon exposure to IFN γ , the Colon Intestine-Chip showed loss of the epithelial barrier integrity in a concentration- and time-dependent manner reproduced in all donors used in this study. Activation of apoptosis, as indicated by the cleavage of caspase 3, and erosion of the epithelial TJ were detected as early as 48 hours after stimulation in comparison with previously described models using T84 and Caco2 monolayers.^{7,28,45} These results indicate the response and sensitivity of the Colon Intestine-Chip platform to a well-established, disease-relevant, barrier-disruption bioassay and provide reassurance for its potential to complement and further advance existing models.

Next, we applied the Colon Intestine-Chip to elucidate the effect of IL22, a member of the IL10 family of cytokines secreted in human beings primarily by subpopulations of CD4⁺ T cells, including T_H22, T_H17, T_H1, and $\gamma\delta$ T cells, as well as innate lymphoid cell 3, natural killer cells, and dendritic cells.^{31,46–48} Increased levels of IL22 have been detected in the intestinal mucosa of IBD and the serum of Crohn's disease patients.⁴⁹ Although several reports advocated for a protective role of IL22 in the inflammatory state because it promotes cell survival and proliferation as well as secretion of mucins, chemokines, and antimicrobial peptides of the S100 and Regenerative Genes families,^{50–52} the overall effects of IL22 in the epithelial barrier homeostasis, as per animal and human studies, remain unclear.^{53,54} Our findings in the Colon Intestine-Chip showed a concentration-dependent increase of epithelial permeability in response to IL22, consistent with studies in Transwells with Caco2 cells,¹⁸ although they do not support its regenerative effect on the crypt stem cells, as described in murine experimental models.³⁷ This observation could be linked to the low representation of proliferating cells at the time of IL22 administration in our platform and suggest a differential effect of the cytokine on differentiated enterocytes. IL22 acts via activation of STAT3 and STAT1. It has been hypothesized that the cross-talk between type I IFNs and

Figure 4. (See previous page). Endothelium supports the establishment of a tight epithelial barrier over the course of the culture. (A) Representative confocal fluorescent images showing the establishment of strong epithelial TJs on day 8 of the culture in the presence of endothelium. TJs were stained with anti-ZO-1 and anti-E-cadherin and cytoskeleton with phalloidin (gray). Cell nuclei are shown in blue. (B) Representative confocal immunofluorescence images, against the basolateral transporter Na⁺K⁺ adenosine triphosphatase (ATPase) (magenta) and the apical transporter SLC26A3 (green), indicating the establishment of a polarized epithelial monolayer only during the co-culture of colonocytes with endothelium, on day 8 of culture. Cell nuclei are shown in blue. The plots indicate the mean fluorescent intensity distribution for each channel along the basal-apical axis of the epithelial cells. The quantification was performed across 3 different fields of view in 3 individual chips for each condition. (C) The gene expression of the 2 epithelial junction proteins, *Cdh1* and *Tjp1*, was confirmed in epithelial cells on days 5 and 8 of culture of the Colon Intestine-Chip. The on-chip culture enhances the expression of both proteins. The graph represents fragments per kilobase of transcript per million mapped reads (FPKM) values generated from bulk RNA-seq analysis of the epithelial cells and plotted on a box plot, where each individual point represents a single chip. n = 3–6 chips/condition, means $\pm$ 95% CI, 1-way analysis of variance, Tukey post hoc test. (D) The gene expression of the 2 ion transporters, *Atp1a1* and *Slc26a3*, was confirmed in epithelial cells on days 5 and 8 of culture of the Colon Intestine-Chip. The on-chip culture enhances the expression of both transporters. The graph represents FPKM values generated from bulk RNA-seq analysis of the epithelial cells and plotted on a box plot, where each individual point represents a single chip. n = 3–6 chips/condition, means $\pm$ 95% CI, 1-way analysis of variance, Tukey post hoc test. (E) Representative scanning electron microscopy (SEM) images of the colonic epithelial on day 8 of the Colon Intestine-Chip culture, where stretch (10% strain, 0.15 Hz) is applied in the presence or not of endothelium. Endothelium does not significantly contribute on the establishment of a dense microvilli network at a later stage of the culture. (F) Box plots showing the density of microvilli per μm^2 , according to quantification of SEM images on day 8 of culture. The quantification was performed across 8–9 different fields of view for each condition. Each individual point represents a single field of view. Unpaired *t* test. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. DAPI, 4',6-diamidino-2-phenylindole.

the members of the IL10 cytokine family, IL10 and IL22, may alter the downstream pathway landscape by enhancing a STAT1-mediated proinflammatory response.^{55,56}

Moreover, recent reports have indicated an IL22-elicited inhibition of Wnt and Notch pathways in small intestinal organoids, limiting the renewal of the leucine-rich repeat-



containing G-protein-coupled receptor 5-positive stem cell pool.³⁵ These data collectively suggest a biological action of IL22 dependent on the specific characteristics of the experimental model used and further support the detrimental effect of IL22 during a time window during which few self-renewing stem cells still are present. Independent of its short-term effects, prolonged exposure to IL22 has been associated with chronic diseases such as intestinal inflammation and cancer.^{57,58} Additional complexity is added by secretion of IL22BP, which binds the cytokine with higher affinity than the transmembrane receptor⁵⁹ and modulates the threshold of its action on intestinal epithelium. Even if accumulating evidence suggests a pathophysiological role of IL22BP in experimental colitis, the different cell source and levels of expression among rodents and human beings still provide room to elucidate the role of IL22BP in the progression of the human disease.⁶⁰ Blockade of the IL22 effect on STAT3 activation and loss of the epithelial barrier integrity by co-treatment of the Colon Intestine-Chip with IL22BP shows the specificity of the effect. Moreover, it suggests that this platform can be used to decipher species-specific discrepancies in the mechanism of action of multifaceted cytokines. Investigation of the potential compensatory actions of IL22 in the inflamed epithelium is beyond the scope of this study.

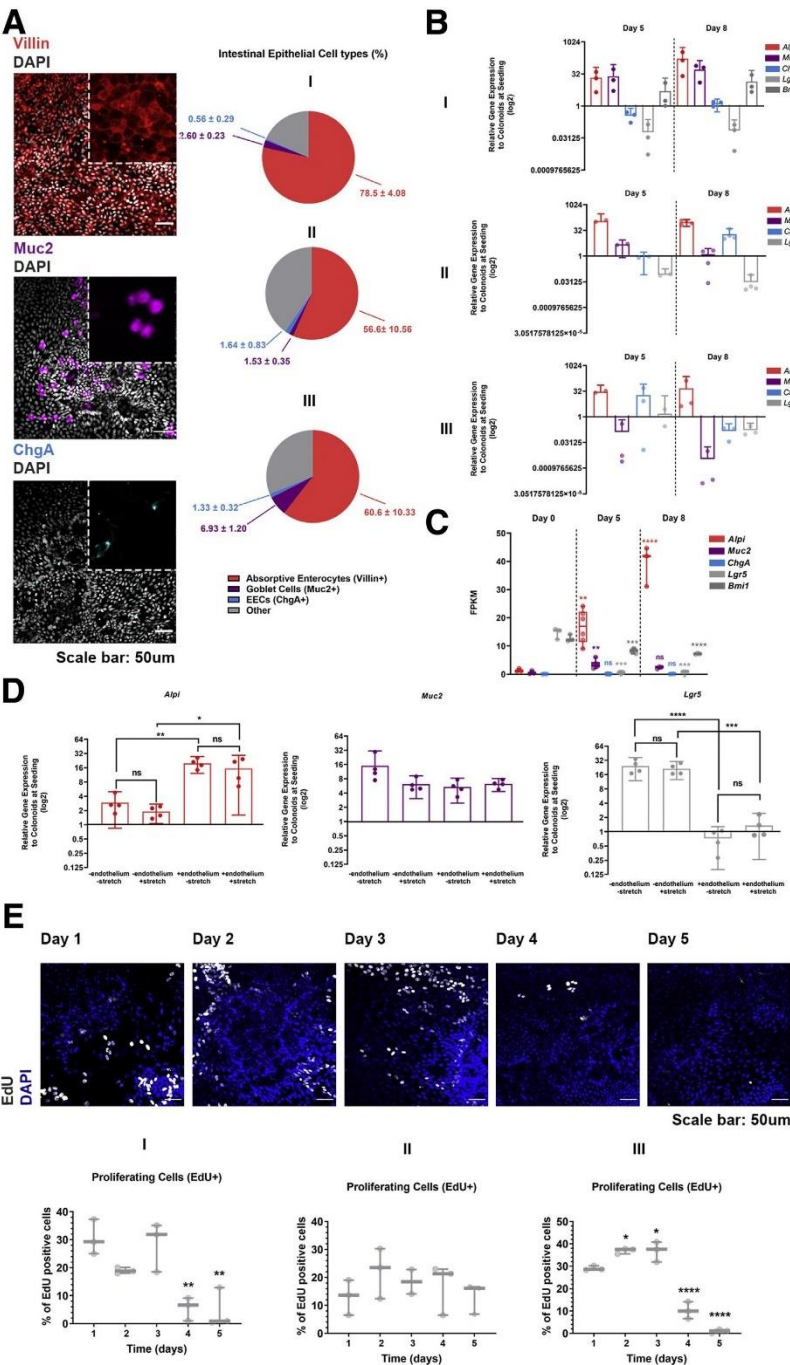
In summary, we report here the development of a Colon Intestine-Chip, a microphysiological system that provides the microenvironment to enable differentiation of human organoids to an epithelial monolayer of polarized cells forming a tight barrier. We show that this human platform can be used to study potential barrier-disruptive factors in a reproducible and specific manner. The implications of these findings for understanding the regulation of barrier function are very important given the list of conditions causing leaky barrier and the association of the latter with degenerative and other diseases. It is not lost on us that this system ultimately can be used to study biology beyond barrier function including the functionality of novel proteins in different cell types represented in the system via genetic manipulation systems such as clustered regularly interspaced short palindromic repeats (CRISPR).

Materials and Methods

Cell Culture

Human biopsy-derived colonoids, from 2 male donors and 1 female donor, isolated according to experimental protocols approved by Johns Hopkins University Institutional Review Board (NA.00038329), were provided by Professor Mark Donowitz's research group.

Figure 5. (See previous page). On-Chip culture enhances biological processes related to the maturation of the intestinal epithelium. (A) Principal component analysis shows that the presence of endothelium clearly separates the epithelial transcriptome of the Chips cultured with and without endothelium. (B) Heatmap representation of the 1000 most variant genes. Samples cultured in the presence of endothelium are clustered separately from all other conditions. (C) The volcano plot resulting from the differential gene expression analysis between the epithelial cells on day 5 of culture, where stretch (10% strain, 0.15 Hz) is applied in the presence and absence of endothelium. The number of the up-regulated and down-regulated differentially expressed genes in the presence of endothelium is shown in green and red, respectively. (D) The volcano plot resulting from the differential gene expression analysis between the epithelial cells on day 8 of culture, where stretch (10% strain, 0.15 Hz) is applied in the presence and absence of endothelium. The number of the up-regulated and down-regulated genes in the presence of endothelium is shown as green and red, respectively. (E) List of biological processes identified by the GO enrichment analysis using the differentially expressed genes between the epithelial cells on day 5 of culture, where stretch (10% strain, 0.15 Hz) is applied in the presence and absence of endothelium. The results indicate the significant enrichment of biological processes related to the morphogenesis and function of the epithelial barrier. (F) List of biological processes identified by the GO enrichment analysis, using the differentially expressed genes between the epithelial cells on day 8 of culture, where stretch (10% strain, 0.15 Hz) is applied in the presence and absence of endothelium. The results indicate significant enrichment of biological processes related to the morphogenesis of the epithelial barrier. (G) The volcano plot resulting from the differential gene expression analysis between the epithelial cells cultured in the absence of endothelium and the presence of stretch (10% strain, 0.15 Hz), on days 5 and 8 of culture. The number of up-regulated and down-regulated genes at the end of the culture is shown in green and red, respectively. (H) The volcano plot resulting from the differential gene expression analysis between the epithelial cells cultured in the presence of endothelium and stretch (10% strain, 0.15 Hz), on days 5 and 8 of culture. The number of the up-regulated and down-regulated genes at the end of the culture is shown in green and red, respectively. (I) The volcano plots resulting from the differential gene expression analysis between the epithelial cells cultured on chip on days 5 and 8, with the presence of stretch (10% strain, 0.15 Hz) and the presence or absence of endothelium and colonoids. The number of the up-regulated and down-regulated differentially expressed genes in chips is shown in green and red, respectively. (J) The volcano plot of the differential gene expression analysis between the epithelial cells on day 5 of culture with endothelium, in the presence and absence of stretch (10% strain, 0.15 Hz). The number of the up-regulated and down-regulated differentially expressed genes in the presence of stretch is shown in green and red, respectively. (K) The volcano plot resulting from the differential gene expression analysis between the epithelial cells on day 8 of culture with endothelium, in the presence and absence of stretch (10% strain, 0.15 Hz). The number of the up-regulated and down-regulated differentially expressed genes in the presence of stretch is shown in green and red, respectively. (L) List of biological processes identified by the GO enrichment analysis, using the differentially expressed genes between the epithelial cells on day 5 of culture with endothelium, in the presence and absence of stretch (10% strain, 0.15 Hz). The results indicate the significant enrichment of biological processes related to the homeostasis and function of the epithelial barrier. (M) List of biological processes identified by the GO enrichment analysis, using the differentially expressed genes between the epithelial cells on day 8 of culture with endothelium, in the presence and absence of stretch (10% strain, 0.15 Hz). The results indicate the significant enrichment of biological processes related to the morphogenesis, function, and homeostasis of the epithelial barrier. n = 3–6 chips/condition.



Colonoids were cultured in Matrigel (356231; Corning, Corning, NY) and in the presence of the commercially available medium IntestiCult Organoid Growth Medium (human) (06010; STEMCELL Technologies, Vancouver, Canada), supplemented with the antibiotic primocin (ant-pm-2; Invivogen, San Diego, CA) in a concentration of 1:500 (vol/vol). Passaging was performed every 7 days, colonoids were recovered from the Matrigel using Cell Recovery Solution (cat. no. 354253; Corning), and fragmented for 2 minutes at 37°C in a digestion solution containing TrypLE (12604013; Thermo Fisher Scientific, Waltham, MA), diluted with phosphate-buffered saline (PBS) 1× 1:1 (vol/vol), and supplemented with 10 μ mol/L Y-27632. Both 10 μ mol/L Y-27632 (Y0503; Sigma-Aldrich, St. Louis, MO) and 5 μ mol/L CHIR99021 (04-0004-10; Stemgent, Cambridge, MA) were present in the culture medium for 72 hours after passaging.

Colonial human intestinal microvascular endothelial cells (cHIMECs) were obtained from Cell Biologics (H-6203; Chicago, IL) and Cell Systems (ACBRI 666; Kirkland, WA). cHIMECs were cultured in gelatin-coated (6950; Cell Biologics) flasks in endothelial cell growth medium MV2 (C-22121; Promo Cell, Heidelberg, Germany), which contained 5 ng/mL recombinant human epidermal growth factor, 10 ng/mL recombinant human basic fibroblast growth factor, 20 ng/mL R3 insulin-like growth factor, 0.5 ng/mL recombinant human vascular endothelial growth factor, 1 μ g/mL ascorbic acid, 0.2 μ g/mL hydrocortisone, 5% fetal bovine serum (F4135; Sigma-Aldrich), and 1:1000 (vol/vol) primocin.

Cells were cultured in a humidified environment at 37°C in 5% CO₂. For the experiment, human colonoids of passage 30 or earlier, and cHIMECs of passage 7 or below, were used.

Establishment of the Colon Intestine-Chip

The Colon Intestine-Chip (10231-2; Emulate, Inc) consists of 2 parallel channels separated by a porous polydimethylsiloxane (PDMS) membrane (diameter, 7 μ m; spacing, 40 μ m; thickness, 50 μ m), and 2 lateral channels where vacuum is applied. The dimensions of the top channel, where colonic epithelial cells reside, are 1000 × 1000 μ m (width × height), its volume is 28.041 μ L, and its cell culture surface is 28 mm². The endothelial cells were introduced in the bottom channel, the dimensions of which are 1000 × 200 μ m (width × height), its volume is 5.584 μ L, and its cell culture surface is 24.5 mm². The interface of the 2 cell types extended in an area of 17.1 mm². The timeline of the establishment of the Colon Intestine-Chips was as follows (Figure 1).

Day -1. The PDMS membrane was activated using Emulate Reagent 1 (ER1) and Emulate Reagent 2 (ER2) reagents (10465; Emulate). A solution of 0.5 mg/mL ER1 in ER2 was prepared and 50 μ L was introduced in each channel. Chips were incubated under UV light for 10 minutes, followed by 3 minutes of incubation at room temperature and another 10 minutes of UV treatment. Subsequently, both channels were washed with ER2, then PBS 1×, and coated overnight at 37°C, with 200 μ g/mL collagen IV (C5533; Sigma-Aldrich) and 100 μ g/mL Matrigel (Corning), and 200 μ g/mL collagen IV (Sigma-Aldrich) and 30 μ g/mL fibronectin (356008; Corning), in the top and bottom channels, respectively.

Day 0. To be seeded on the Chips, colonoids were recovered from Matrigel using Cell Recovery Solution (Corning), and fragmented by incubation at 37°C for 2 minutes in a digestion solution containing TrypLE (Thermo Fisher Scientific), diluted with PBS 1× 1:1 (vol/

Figure 6. (See previous page). (See previous page) On-Chip culture supports the multilineage differentiation of the colonic epithelial cells. (A) Representative confocal fluorescent images showing the presence of absorptive enterocytes (anti-villin, red), Goblet cells (anti-mucin 2, magenta), and enteroendocrine cells (anti-ChgA, cyan) on day 8 of culture. Cell nuclei are shown in gray. The pie charts plot the percentage of the respective cell types for each of the 3 different donors. The total number of cells is evaluated based on 4',6-diamidino-2-phenylindole (DAPI) staining. The quantification was performed across 5 different fields of view in 3 individual chips for each cell type and donor. Means $\pm$ SD. (B) Identification of each epithelial cell type by qPCR for the *Alpi*, *Muc2*, *ChgA*, *Lgr5*, and *Bmi1* genes. The relative gene expression values of each gene at the respective time points (day 5 and day 8) are normalized to colonoids at seeding. 18S has been acquired as the housekeeping gene. The expression of indicative for the mature enterocytes and quiescent stem cells genes increase over the course of the culture whereas the expression of the cycling stem cells driving gene *Lgr5* decreases. A donor-dependent expression was observed for the mature enterocytes and cycling stem cells driving genes. Each individual spot represents a single chip. n = 2–4 chips/day, means $\pm$ 95% CI. (C) The gene expression of *Alpi*, *Muc2*, *ChgA*, and *Lgr5* on days 0, 5, and 8 of culture of donor 1. The expression of indicative for the mature absorptive enterocytes and Goblet cell genes increases over the course of the culture, whereas the expression of the cycling stem cells driving gene *Lgr5* and of the quiescent stem cell gene *Bmi1* decreases. Fragments per kilobase of transcript per million mapped reads (FPKM) values generated from bulk RNA-seq analysis of the epithelial cells are plotted on a box plot, where each individual point represents a single chip n = 3–6 chips/condition, 1-way analysis of variance, Tukey post hoc test. (D) Identification of each epithelial cell type by qPCR for *Alpi*, *Muc2*, *ChgA*, and *Lgr5* on day 5 of culture in the presence of stretch and/or endothelium. Endothelium significantly enhances the gene expression of the absorptive enterocyte marker *Alpi* and decreases the expression of the cycling stem cells driving gene *Lgr5*. Each individual spot represents a single chip. n = 4 chips/condition, means $\pm$ 95% CI, 1-way analysis of variance, Tukey post hoc test. n = 2–4 chips/day for each donor, means $\pm$ 95% CI. (E) Confocal fluorescent images of 3 different donors indicating the abundance of proliferating cells (EdU, gray) over the course of the first 5 days of culture. Cell nuclei are shown in blue. The total number of cells is evaluated based on DAPI staining and the percentage of the EdU-positive cells is shown on a box plot, where each individual dot represents a single chip. The quantification was performed across 3 different fields of view in 3 individual chips for each day of the culture and donor. The abundance of the proliferating cells was decreased significantly on days 4 and 5 of the culture. One-way analysis of variance, Tukey post hoc test. *P < .05, **P < .01, ***P < .001, and ****P < .0001.

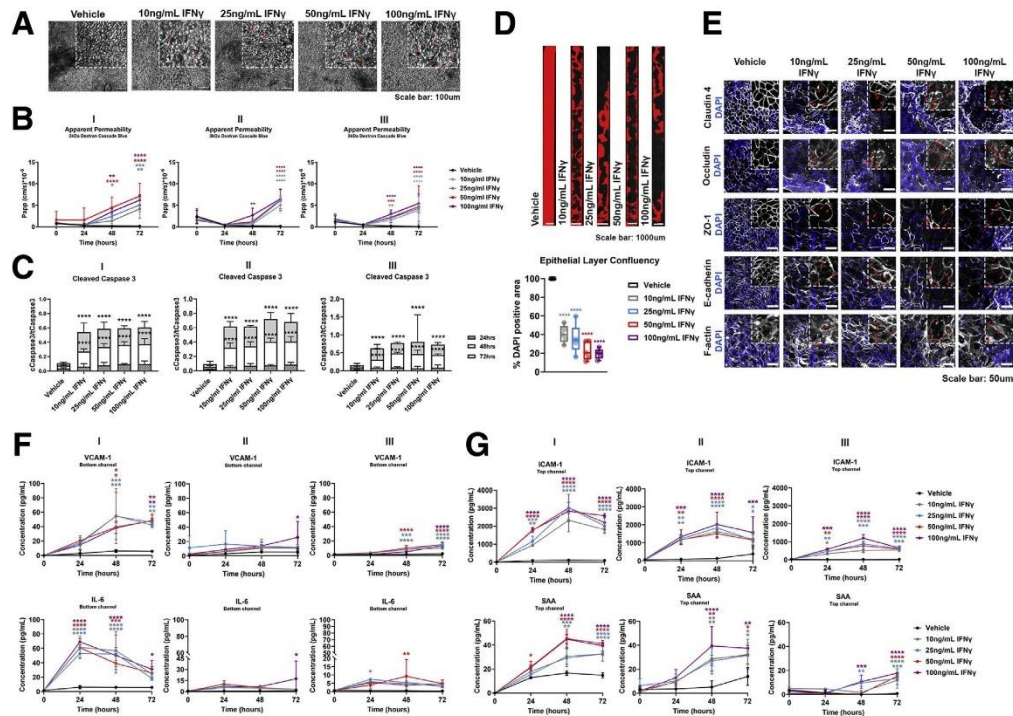
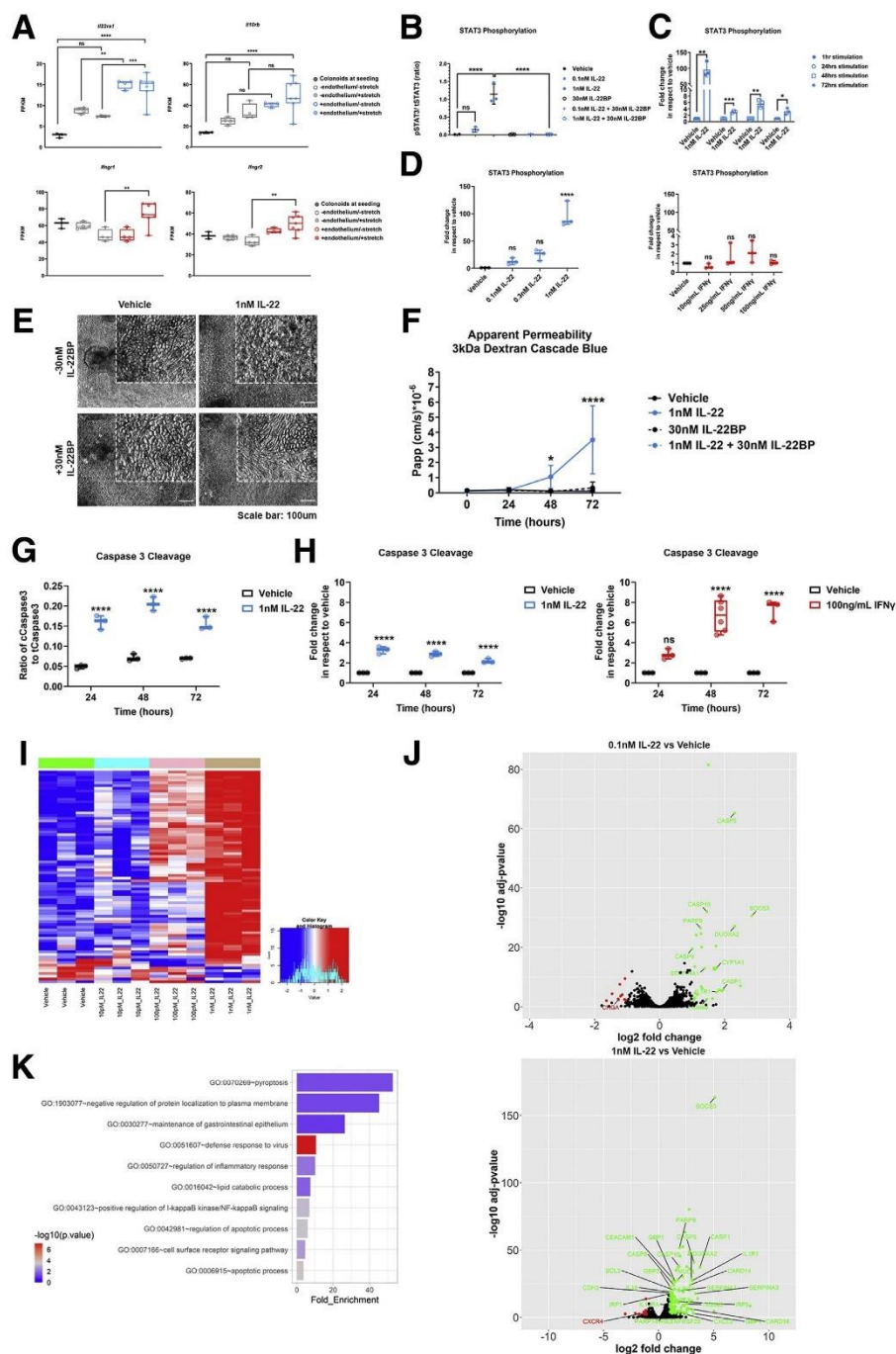


Figure 7. The established epithelial barrier responds in a time- and concentration-dependent manner to IFN γ , a barrier-disruptive agent. (A) Representative phase-contrast images of the colonic epithelial monolayer 48 hours after treatment with IFN γ . Gradual degeneration of the morphology of the epithelial cells was observed as the concentration of IFN γ increased, indicating a transition from columnar to the squamous-shaped epithelium (red arrows). (B) The P_{app} of epithelial cells to 3 kilodaltons dextran Cascade blue, over the course of 72 hours of basolateral stimulation with IFN γ , indicates a dose-, donor-, and time-dependent barrier disruption. Data shown correspond to 1 representative of 3 independent experiments. $n = 3$ –12 chips/condition, means $\pm$ 95% CI, 2-way analysis of variance, Tukey post hoc test. (C) Quantification of the cleaved over total caspase 3 in epithelial cells shows a significant and concentration-dependent activation of apoptosis 48 and 72 hours after stimulation across 3 different donors. $n = 3$ chips/condition means $\pm$ 95% CI, 2-way analysis of variance, Tukey post hoc test. (D) Representative tile fluorescent images highlighting the area of the Colon Intestine-Chip covered with epithelial cells, determined by positive 4',6-diamidino-2-phenylindole (DAPI) staining (red), 48 hours after stimulation with different concentrations of IFN γ . The epithelial layer confluency is expressed as a percentage of the DAPI-positive area over the total area of the chip, depicted as a box plot where each individual point represents a single chip. A significant and concentration-dependent decrease of the epithelial layer confluency was observed 48 hours after treatment with IFN γ . $n = 4$ –5 chips/condition, means $\pm$ 95% CI, 1-way analysis of variance, Tukey post hoc test. (E) Representative confocal immunofluorescence images indicating the dose-dependent degeneration of the epithelial TJs, as shown by the redistribution of claudin 4, ZO-1, and F-actin, and internalization of occludin and E-cadherin (red arrows). Cell nuclei are shown in blue. (F) Time-course quantification of cytokines and vascular injury-related serum proteins in the Colon Intestine-Chip shows a concentration-, donor-, and time-dependent secretion of IL6 and vascular cell adhesion molecule-1 (VCAM-1) in the bottom channel, upon treatment with IFN γ . $n = 3$ chips/condition, means $\pm$ SD, 2-way analysis of variance, Tukey post hoc test. (G) Time-course quantification of intercellular cell adhesion molecule-1 (ICAM-1) and serum amyloid A (SAA) in the top channel, upon treatment with IFN γ . $n = 3$ chips/condition, means $\pm$ SD, 2-way analysis of variance, Tukey post hoc test. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

vol) and supplemented with 10 μ mol/L Y-27632. They were resuspended in IntestiCult medium, supplemented with 10 μ M Y-27632 (Sigma-Aldrich) and 5 μ M CHIR99021 (Stemgent), and introduced in the apical

channel of the Colon Intestine-Chip in a seeding density of 7×10^6 cells/mL. To assess the cell density, part of the seeding cell suspension was incubated in ACCUTASE (07920; STEMCELL Technologies) for 30 minutes and



fragmented to a single-cell level. Both 10 $\mu\text{mol/L}$ Y-27632 and 5 $\mu\text{mol/L}$ CHIR99021 were maintained in the culture medium for 72 hours thereafter.

When investigating the interaction between epithelial and endothelial cells, cHIMECs were seeded on the basal side of the PDMS membrane 1 hour before the seeding of epithelial cells in the top channel. Briefly, 20 μL of the suspension of endothelial cells, in a seeding density of 8×10^6 cells/mL, were introduced in the bottom channel, and Colon Intestine-Chips were incubated inverted at 37°C, in a humidified environment. The bottom channel was washed and thereafter immersed in endothelial cell growth medium MV2 medium.

Day 1. Both channels were washed with the corresponding media to remove the nonadherent cells. Colon Intestine-Chips were attached to devices called Pod Portable Modules (10153; Emulate, Inc) and connected in the automated cell culture system Zoë Culture Module (Emulate, Inc). Each Zoë is connected to an Orb Hub module that supplies the gas, vacuum, and power. The flow rate was set at 60 $\mu\text{L/h}$ in both channels.

Day 2. Medium without Y-27632 and CHIR99021 was replenished in both channels.

Day 3. Colon Intestine-Chips were acclimated to mechanical forces using 2% strain and 0.15-Hz frequency.

Day 4. Stretching was increased to 10% strain and 0.15-Hz frequency, and culture medium was replenished. Both

channels were perfused at 1000 $\mu\text{L/h}$ for 5 minutes to flush out any detached cells.

Days 5–8. Culture media was refreshed, and detached cells were removed by flushing (1000 $\mu\text{L/h}$, 5 min) every other day of the culture.

Assessment of Apparent Permeability

The epithelial barrier integrity was assessed based on the apparent permeability of 3 kilodaltons of dextran Cascade Blue molecule (100 $\mu\text{g/mL}$, D7132; Thermo Fisher), applied in the medium of the top channel of the Colon Intestine-Chip. The diffusion of the dye to the bottom channel was monitored based on a standard curve and the absorbance of the bottom channel effluents at 375–420 nm. For the calculation of the P_{app} , the following mathematical formula was used:

$$P_{\text{app}} = \frac{V_{\text{rec}} \cdot dC_{\text{rec}}}{A \cdot dt \cdot C_{\text{don}, t=0}}$$

Where P_{app} is the apparent permeability (cm/s), V_{rec} is the volume of the retrieved effluent from the bottom channel (mL), dC_{rec} is the difference of the concentration of the dye in the effluent and input medium of the bottom channel (mg/mL), A is the surface area on which the diffusion of the dye occurs (cm^2), dt is the time period on which the P_{app} is assessed (s), and $C_{\text{don}, t=0}$ is the concentration of the dye in the dosing epithelial medium (mg/mL).

Figure 8. (See previous page). IL22 induces disruption of the epithelial barrier in the Colon Intestine-Chip, an effect rescued by the presence of its soluble receptor IL22BP. (A) The gene expression of the 2 subunits of the transmembrane IL22 receptor, *IL22ra1* and *IL10rb*, and IFN γ receptor, *Ifngr1* and *Ifngr2*, was confirmed in epithelial cells on day 5 of culture of the Colon Intestine-Chip. The on-chip culture enhances the expression of both receptors. Fragments per kilobase of transcript per million mapped reads (FPKM) values generated from bulk RNA-seq analysis of the epithelial cells are plotted on a box plot, where each individual point represents a single chip. $n = 3$ –6 chips/condition, 1-way analysis of variance, Tukey post hoc test. (B) Quantification of the phosphorylated over the total STAT3 in epithelial cells of the Colon Intestine-Chip, shows a concentration-dependent activation of STAT3 1 hour after stimulation with 0.1 or 1 nmol/L IL22. In the presence of IL22BP the activation of STAT3 is negated. $n = 3$ chips/condition means $\pm$ SD, 1-way analysis of variance, Tukey post hoc test. (C) Quantification of the phosphorylated over the total STAT3 in epithelial cells of the Colon Intestine-Chip, expressed as fold increase over the vehicle, shows a sustained activation of STAT3 over the course of 72 hours of stimulation with 1 nmol/L IL22. $n = 3$ chips/condition means $\pm$ SD, unpaired t test performed for each time point. (D) Quantification of the phosphorylated over the total STAT3 in epithelial cells of the Colon Intestine-Chip, expressed as fold increase over the vehicle, shows a cytokine-specific activation of STAT3 upon stimulation with various concentrations of IL22 and IFN γ . Each individual point in the box plot represents a single chip. $n = 3$ chips/condition, 1-way analysis of variance, Tukey post hoc test. (E) Representative phase-contrast images of the colonic epithelial monolayer 48 hours after treatment with 1 nmol/L IL22, shows a compromised morphology of the epithelial cells. The epithelial cell morphology is rescued in the presence of 30 nmol/L IL22BP. (F) The P_{app} of epithelial cells to 3 kilodaltons dextran Cascade blue, over the course of 72 hours of stimulation with 1 nmol/L IL22 and/or 30 nmol/L IL22BP. IL22 triggers loss of the epithelial barrier integrity, which is rescued upon co-treatment with IL22BP. $n = 3$ –6 chips/condition, means $\pm$ 95% CI, 2-way analysis of variance, Tukey post hoc test. (G) Quantification of the cleaved over the total caspase 3 in epithelial cells of the Colon Intestine-Chip, indicates a significant and sustained increase in the levels of activated caspase 3 over the course of 72 hours of stimulation with 1 nmol/L IL22. Each individual point in the box plot represents a single chip. $n = 3$ chips/condition, 2-way analysis of variance, Tukey post hoc test. (H) Quantification of the cleaved over the total caspase 3 in epithelial cells of the Colon Intestine-Chip, expressed as fold increase over the vehicle, shows a cytokine- and time-dependent activation of apoptosis over the course of 72 hours of stimulation with either 1 nmol/L IL22 or 100 ng/mL IFN γ . Each individual point in the box plot represents a single chip. $n = 3$ –6 chips/condition, 2-way analysis of variance, Tukey post hoc test. (I) Heatmap of the 1000 most variant genes across samples cultured in the presence of different conditions (ie, vehicle and 3 different concentrations of IL22). $n = 3$ chips/condition. (J) The volcano plot resulting from the differential gene expression analysis between the epithelial cells on day 5 of culture under baseline conditions and upon stimulation with 0.1 or 1 nmol/L IL22. Genes up-regulated and down-regulated in the presence of IL22 are shown in green and red, respectively. (K) List of biological processes identified by the Gene Ontology enrichment analysis, using the differentially expressed genes between the epithelial cells on day 5 of culture under baseline conditions and upon stimulation with 1 nmol/L IL22. The results indicate significant enrichment of biological processes related to the inflammatory response and apoptotic pathway in the presence of IL22. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

Table 1. List of Antibodies Used in Immunofluorescence

Target epitope	Host species	Vendor	Catalog number	Dilution ratio
Mucin 2	ms	Santa Cruz Biotechnology	sc- 515032	1:300
Villin	rb	Abcam	ab130751	1:100
Chromogranin A	rb	Abcam	ab15160	1:100
ZO-1	ms	Thermo Fischer	339194	1:100
E-cadherin	ms	Abcam	ab1416	1:100
Occludin	ms	Thermo Fischer	33-1500	1:100
Claudin 4	rb	Thermo Fischer	36-4800	1:100
Na ⁺ K ⁺ adenosine triphosphatase	ms	Abcam	ab7671	1:100
SLC26A3	rb	Abcam	ab244452	1:100

ms, mouse; rb, rabbit.

Cytokine Stimulation

IL22 (782-IL-010; R&D, Minneapolis, MN), in the presence or absence of 30 nmol/L IL22BP (8498-BP-025; R&D) and IFN γ (300-02; Peprotech, Rocky Hill, NJ) was introduced in the culture medium of the endothelial channel at various concentrations. The cytokine-supplemented medium was refreshed every 24 hours and the appropriate vehicle controls were used in each stimulation experiment.

Immunofluorescence

At the indicated time points Colon Intestine-Chips were fixed at room temperature for 15 minutes in 4% paraformaldehyde solution, or at -20°C for 20 minutes in ice-cold methanol according to the target epitope. Briefly, chips were washed in both channels with PBS 1 $\times$, immersed in permeabilization solution (10% normal donkey serum, 0.1% Triton X-100 in PBS 1 $\times$) for 15–30 minutes at room temperature followed by blocking solution (10% NDS in PBS 1X) for 1 hour, and incubated with primary antibody (Table 1), overnight at 4°C. Subsequently, chips were stained with the appropriate combination of secondary Alexa Fluor antibodies (Abcam, Cambridge, MA) and counterstained with 50 μ g/mL 4',6-diamidino-2-phenylindole (D1306; Invitrogen, Carlsbad, CA). The antibody solutions were prepared using 5% normal donkey serum in PBS 1 $\times$ in the dilution ratios mentioned in Table 1. In the case of morphologic analysis of the epithelial cytoarchitecture, F-actin was stained with phalloidin, conjugated with the appropriate Alexa Fluorophores (Abcam), added in the secondary antibody solution. To assess epithelial cell proliferative capacity,

epithelial cells in the chips were treated with 20 μ mol/L, harvested 24 hours later, and EdU reactivity was assessed as per the manufacturer's instructions (C10337; Thermo Fisher).

Assessment of ECM Coating

ECM proteins were stained using an Alexa Fluor 488-conjugated NHS ester (A20100; Thermo Fisher). After membrane activation and coating, each channel was pre-treated with borate buffer (pH 8.5) at room temperature for 5 minutes and stained with NHS ester dye solution in borate buffer (1:500) at room temperature for 25 minutes in the dark. Both channels were rinsed gently with PBS 1 $\times$ before and images were acquired.

Imaging and Imaging Quantification

Phase-contrast images were acquired using the Zeiss (Peabody, MA) AXIOvert.A1 brightfield microscope (objective: Zeiss LD A-Plan 10 $\times$ /0.25 Ph1) and the Zeiss Axiocam 503 mono camera. Fluorescent z-stack images and fluorescent tile images of the Colon Intestine-Chip were acquired using the inverted laser-scanning confocal microscope Zeiss LSM 880 (objective: Zeiss LD Plan-Neofluar 20 $\times$ /0.40 Korr M27), and the epifluorescence inverted microscope Olympus (Waltham, MA) IX83 (objective: Olympus CPlanFL N 10 $\times$ /0.30 RC1) and the Hamamatsu (Boston, MA) ORCA Flash 4.0 camera, respectively. Image processing and quantification were performed using Fiji (version 2.0, NIH, Bethesda MD), as per standard methods.

Table 2. List of Human TaqMan Gene Expression Assays Used in qRT-PCR

Gene ID	Gene name	TaqMan assay ID
18S	18S ribosomal RNA	Hs99999901_s1
Alpi	Alkaline phosphatase intestinal	Hs00357579_g1
Muc2	Mucin 2	Hs00894025_m1
Chga	Chromogranin A	Hs00900375_m1
Lgr5	Leucine-rich-repeat-containing G-protein-coupled receptor 5	Hs00969422_m1
Bmi1	Polycomb complex protein BMI-1	Hs00409825_g1

Table 3. List of Epithelial Cell Samples Isolated at Different Conditions and Time Points of the Colon Intestine-Chip Culture, and Analyzed Using RNA-Seq

Time point	Experimental group	Replicates, n
Day 0	Colonoids	3
Day 5	-endothelium/-stretch	4
	-endothelium/+stretch	4
	+endothelium/-stretch	4
	+endothelium/+stretch	6
Day 8	-endothelium/-stretch	4
	-endothelium/+stretch	4
	+endothelium/-stretch	4
	+endothelium/+stretch	3

Scanning Electron Microscopy

At the indicated time points, the Colon Intestine-Chips were fixed at room temperature for 2 hours in 2.5% glutaraldehyde solution and washed 3 times with 0.1 mol/L sodium cacodylate buffer. Concomitantly, the Chip was trimmed using a razor so that the lateral and top portions of PDMS were removed and the top channel was shown. Afterward, the samples were fixed with 1% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer for 1 hour at room temperature and dehydrated in graded ethanol. The Chip samples were dried using the chemical drying agent hexamethyldisilazane, sputter-coated with platinum, and images were acquired using the Hitachi (Westford, MA) S-4700 Field Emission Scanning Electron Microscope. The morphologic analysis of the microvilli was performed according to a previously described method.⁶¹

Assessment of STAT3 Phosphorylation, Caspase 3 Cleavage, and Cytokine Secretion

At the indicated time points after stimulation with IL22 or IFN γ , the Colon Intestine-Chip was disconnected from pods, endothelial cells were removed enzymatically, and, subsequently, epithelial cells were lysed with a protein lysis buffer (R60TX-3; Meso Scale Discovery, Rockville, MD) containing protease (78425; Thermo Fisher) and phosphatase inhibitors (P0044 and P5726; Sigma-Aldrich). Protein extraction was performed according to standard protocols and the quantification of the total amount of protein/sample was performed by Bradford assay (1856210; Thermo Fisher). Total and phosphorylated STAT3 and total and cleaved caspase 3 were detected using Meso Scale Discovery

technology (totalSTAT3-K150SND, phosphorylatedSTAT3-K150DIA, cleaved/total-caspase 3-K15140D).

To assess the secretion of proinflammatory cytokines and acute inflammatory response proteins, effluents were collected from both channels of the Colon Intestine-Chip at the indicated time points after stimulation with IFN γ . IL6, intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and serum amyloid A were detected using the Meso Scale Discovery technology (K15198D and K15049G; Meso Scale Discovery).

RNA Isolation, Reverse Transcription, and qPCR

Each channel of the Colon Intestine-Chip was lysed using TRI Reagent (T9424; Sigma-Aldrich) and RNA was isolated using the Direct-zol RNA Purification Kit (R2060; Zymo Research, Irvine, CA). Genomic DNA was removed using the TURBO DNA-free Kit (AM1907; Thermo Fisher), and reverse transcription to complementary DNA was performed with the SuperScript IV Synthesis System (18091050; Thermo Fisher). TaqMan Fast Advanced Master Mix (4444963; Applied Biosystems, Foster City, CA) and TaqMan Gene Expression Assays (Table 2) were used for qPCR. The qPCR was detected on a QuantStudio 3 PCR System (Thermo Fisher Scientific, Waltham, MA). The raw data were transformed to linear data by calculating the $2^{-\Delta\Delta Ct}$ values.

RNA-seq Analysis

Total RNA was extracted using TRI Reagent (T9424; Sigma-Aldrich). Bulk RNA sequencing was performed in colonoids, in suspension culture, and colonoid-derived epithelial cells cultured in the presence and absence of endothelium and/or stretch on days 5 and 8 of culture, using a minimum of 3 technical replicates per experimental group (Tables 3 and 4).

The bulk RNA sequencing platform used was the Illumina (San Diego, CA) TruSeq paired-end sequencing with a maximum read length of 2×100 bp. The sequencing depth was at approximately 48 million paired-end reads/sample. The sequence reads were trimmed using Trimmomatic v.0.36 to remove poor-quality adapter sequences and nucleotides. Using the Spliced Transcripts Alignment to a Reference aligner v.2.5.2b, we mapped the trimmed reads to the Homo sapiens reference genome GRCh38 (available on ENSEMBL) and generated the corresponding BAM and feature count files. Next, using the v.1.5.2 subread package, we calculated the gene hit counts. It is worth mentioning that only unique reads that fell within exon regions were counted. Genes with very low expressions across samples were filtered out and the remainder was used to form our data set, which was used for the differential gene expression (DGE) analysis. We performed DGE analysis using the DESeq2 R package by Bioconductor. For all but 2 DGE analyses, we applied the following widely accepted strict thresholds: $P < .01$ and $|\log_2 \text{foldchange}| > 2$. A more relaxed version of these thresholds (ie, $P < .05$ and $|\log_2 \text{foldchange}| > 1$) was used to identify the differentially

Table 4. List of Epithelial Cell Samples Treated With Various Concentrations of IL22, and Analyzed Using RNA-Seq

Time point	Experimental group	Replicates, n
Day 5	Vehicle	3
	0.01 nmol/L IL22	3
	0.1 nmol/L IL22	3
	1 nmol/L IL22	3

expressed genes in the presence and absence of stretch (Figure 5J and K).

All RNA-seq data described in this article are accessible through the Gene Expression Omnibus database (accession number: GSE179130).

Functional Enrichment Analysis

The genes identified from the DGE analysis were used to perform GO enrichment analysis with the GO biological processes. The GO term enrichment analysis was performed using the GO knowledgebase (Gene Ontology Resource available at: <http://geneontology.org>).

Statistical Analysis

Results are expressed as boxplots, depicting the minimum, first quartile, median, third quartile, and maximum values, or means $\pm$ 95% CI and means $\pm$ SD, as appropriate. The statistical analysis was performed using GraphPad Prism, version 8.3.0 (GraphPad Software, Inc), by applying a 2-tailed, unpaired *t* test, 1-way or 2-way analysis of variance followed by the Tukey post hoc multiple comparison tests, according to the experimental design. Any *P* value less than .05 was considered statically significant.

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Conflicts of interest

These authors disclose the following: Athanasia Apostolou, Dimitris V. Manatakis, Raymond Luc, Alexandra Dimitriou, Carolina Lucchesi, Gauri Kulkarni, Lorna Ewart, Magdalena Kasendra, Jordan S. Kerns, Geraldine A. Hamilton, and Katia Karalis are current or former employees of Emulate, Inc, and may hold equity interest in Emulate, Inc; and Rohit A. Panchakshari, Antara Banerjee, Maria D. Paraskevopoulou, Galeb Abu-Ali, Bertram Bleck, and Cosmas Giallourakis are current or former employees of Takeda Pharmaceutical Company, Ltd, and may hold equity interest in Takeda Pharmaceutical Company, Ltd. The remaining authors disclose no conflicts.

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Publication [3]**Modeling alcohol-associated liver disease
in a human Liver-Chip**

J. C. Nawroth, D. B. Petropolis, D. V. Manatakis, **T. I. Maulana**, G. Burchett, K. Schlünder, A. Witt, A. Shukla, K. Kodella, J. Ronxhi, G. Kulkarni, G. Hamilton, E. Seki, S. Lu, K. Karalis

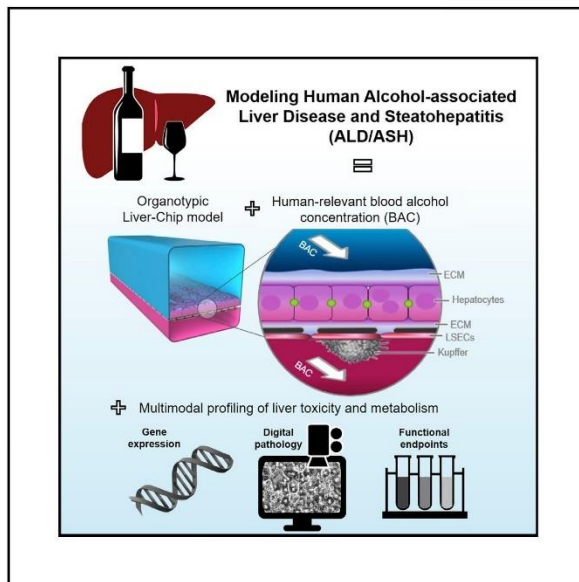
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Abstract/Summary:

Alcohol-associated liver disease (ALD) is a global health issue and leads to progressive liver injury, comorbidities, and increased mortality. Human-relevant preclinical models of ALD are urgently needed. Here, we leverage a triculture human Liver-Chip with biomimetic hepatic sinusoids and bile canaliculi to model ALD employing human-relevant blood alcohol concentrations (BACs) and multimodal profiling of clinically relevant endpoints. Our Liver-Chip recapitulates established ALD markers in response to 48 h of exposure to ethanol, including lipid accumulation and oxidative stress, in a concentration-dependent manner and supports the study of secondary insults, such as high blood endotoxin levels. We show that remodeling of the bile canalicular network can provide an in vitro quantitative readout of alcoholic liver toxicity. In summary, we report the development of a human ALD Liver-Chip as a powerful platform for modeling alcohol-induced liver injury with the potential for direct translation to clinical research and evaluation of patient-specific responses.

Modeling alcohol-associated liver disease in a human Liver-Chip

Graphical abstract



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In brief

Nawroth et al. develop an advanced human Liver-Chip model of alcohol-associated liver disease (ALD) with direct translation to clinical research. The chip recapitulates early critical events of ALD, such as lipid accumulation and oxidative stress, mimics disease worsening due to leaky gut syndrome, and models recovery following alcohol abstinence.

Highlights

- The Liver-Chip models early critical events of ALD
- Key readouts include markers of steatosis, cholestasis, and oxidative stress
- The chip recapitulates second-hit injury through circulating endotoxins (leaky gut)
- The chip also models injury recovery following abstinence from alcohol



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Report

Modeling alcohol-associated liver disease in a human Liver-Chip

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SUMMARY

Alcohol-associated liver disease (ALD) is a global health issue and leads to progressive liver injury, comorbidities, and increased mortality. Human-relevant preclinical models of ALD are urgently needed. Here, we leverage a triculture human Liver-Chip with biomimetic hepatic sinusoids and bile canaliculi to model ALD employing human-relevant blood alcohol concentrations (BACs) and multimodal profiling of clinically relevant endpoints. Our Liver-Chip recapitulates established ALD markers in response to 48 h of exposure to ethanol, including lipid accumulation and oxidative stress, in a concentration-dependent manner and supports the study of secondary insults, such as high blood endotoxin levels. We show that remodeling of the bile canicular network can provide an *in vitro* quantitative readout of alcoholic liver toxicity. In summary, we report the development of a human ALD Liver-Chip as a powerful platform for modeling alcohol-induced liver injury with the potential for direct translation to clinical research and evaluation of patient-specific responses.

INTRODUCTION

Fatty liver disease (FLD), or hepatic steatosis, is a growing global health problem that affects up to 30% of the general population in Western countries (Asrani et al., 2019; Bedogni et al., 2014; Wong et al., 2019). The disease is classified based on the causative trigger as either alcohol-associated liver disease (ALD) or diet-induced non-alcoholic fatty liver disease (NAFLD) (Bedogni et al., 2014), which share several genetic susceptibility markers (Hotta et al., 2010; Meroni et al., 2018) and progress from simple hepatic steatosis to steatohepatitis and liver fibrosis and finally to irreversible cirrhosis and hepatocellular carcinoma (Chacko and Reinus, 2016; Magdaleno et al., 2017; Orman et al., 2013; Rehm et al., 2009; Scaglioni et al., 2011; Toshikuni et al., 2014). In Western countries, approximately half of all deaths from liver cirrhosis are due to ALD resulting from alcohol abuse (Magdaleno et al., 2017; Rehm et al., 2009; Singal et al., 2018). ALD is also a co-factor in the progression of chronic viral hepatitis, NAFLD, iron overload, and other liver diseases (Crabb et al., 2020).

Currently, diagnosis of ALD relies on a combination of history and clinical and laboratory findings. Because alcohol intake is the established cause of clinical alcohol-associated steatohepatitis (ASH), patients presenting with hepatic steatosis and a his-

tory of alcohol intake above 20 g/d for women and 30 g/d for men are commonly diagnosed with ALD/ASH (Bedogni et al., 2014). To improve diagnostics and develop effective treatments, it is important to develop faithful models of the human liver responses to disease-relevant challenges. However, the great majority of the experimental studies on FLD have employed animal models that display aspects of the liver disease phenotype but do not capture the spectrum of the metabolic, inflammatory, and fibrotic responses found in human patients (Bertola, 2018; Santhekadur et al., 2018). Similarly, *in vitro* models such as hepatocyte sandwich culture and liver spheroids may recapitulate several of the features of liver diseases but are missing the dynamics of the tissue microenvironment and cell-cell interactions. Common models have been unable to recapitulate systemic effects induced by alcohol in humans, such as compromised intestinal epithelial barrier function and increased gut permeability ("leaky gut"), a response subject to significant species-dependent variability (Bishehsari et al., 2017). Leaky gut and the ensuing inflammatory response due to the escape of bacterial endotoxins to the portal and systemic circulation are thought to directly contribute to the development of ASH (Bishehsari et al., 2017).

We hypothesized that a microphysiological system (MPS) of the human liver (Liver-Chip) using clinically relevant blood-



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alcohol concentrations (BACs) could recapitulate alcohol-induced and endotoxin-mediated hepatic tissue injury and hence provide a platform for studying ALD/ASH. Ethanol-induced alterations in hepatic cytochrome P450 CYP2E1 and their role in the mechanisms driving liver damage (Seitz and Stickel, 2006) can be studied in human MPS, whereas in commonly used cell lines (e.g., HepG2, HuH7 cells) or the standard sandwich primary hepatocyte culture, the expression of these enzymes is not maintained beyond the early time of the culture (Jang et al., 2019).

Here, we advanced our previously published Liver-Chip for drug toxicity screening (Jang et al., 2019) with additional capabilities to recapitulate early, critical events in human ALD using alcohol at concentrations as those found in the blood of patients. We demonstrate the ability of the ALD Liver-Chip to model recovery of early injury through alcohol abstinence, as well as the worsening of the phenotype in a two-hit model of co-exposure to alcohol and bacterial endotoxin. We optimized the extracellular matrix (ECM) scaffolding to achieve robust formation of a biomimetic BC network, and we propose that ethanol-induced disruptions of the BC network are a sensitive and early marker of toxicity.

Together, we demonstrate that our ALD Liver-Chip recapitulates early events in human-relevant alcohol-induced steatosis and remodeling that may be missed by other *in vitro* systems.

RESULTS

Development of the Liver-Chip for modeling ALD/ASH

To model human ALD, we continuously perfused an advanced version of our recently reported Liver-Chip, an organotypic MPS using primary human cells (Jang et al., 2019), with ethanol doses within clinically relevant BACs, followed by multimodal phenotyping and functional analysis. The Liver-Chip is made of polydimethylsiloxane (PDMS) and contains an upper channel and a lower channel separated by a porous membrane to allow for cell-cell interactions. The tri-culture configuration used in this study includes primary hepatocytes cultured in the upper channel and primary liver sinusoidal endothelial cells (LSECs) and Kupffer cells cultured in the lower channel (Figure 1A). The co-culture of primary hepatocytes with LSECs is essential for sustained hepatocyte physiological functions over time, and Kupffer cells modulate important inflammatory responses (Bale et al., 2015; Jang et al., 2019). We further aimed to recreate the extensive bile canaliculi (BC) network that is established between neighboring hepatocytes in the liver. In mice, changes to the BC network structure are an early and sensitive indicator of liver drug toxicity (Meyer et al., 2017). BC network structure is also altered in human patients with NAFLD (Segovia-Miranda et al., 2019). We reasoned that alcohol-induced toxicity would generate similar remodeling suitable for digital pathology. However, in contrast to rodent hepatocytes, human hepatocytes do not readily form biomimetic bile canaliculi networks *in vitro* but usually exhibit disconnected BC pockets (Abe et al., 2009; Deharde et al., 2016; Nakakariya et al., 2012; Reif et al., 2015; Swift et al., 2010). Inspired by the recent finding that hepatocyte polarization and BC lumen formation are driven by symmetric mechanical anchoring to the matrix (Li et al., 2016), we probed

whether optimizing ECM scaffold composition, thickness, and homogeneity in the Liver-Chip could improve BC network integrity (see STAR Methods and Figure S1). We tested five different scaffolding designs consisting of Matrigel and collagen-I-based gels ("ECM-A" to "ECM-D" in Figure S1G) and used a digital pathology approach to quantify the MRP2-stained BC network branching density, overall area fraction (porosity), and branching radius, which describe BC network integrity (Meyer et al., 2017) (Figure S1H). We chose for the Liver-Chip the matrix protocols "ECM-C" and "ECM-D," which reproducibly elicited the best BC metrics throughout the chip channel (Figure 1C).

Hepatic intracellular lipid accumulation, or steatosis, is the key histological finding of both diet- and alcohol-induced FLD (Scaglioni et al., 2011; Toshikuni et al., 2014). To confirm that the Liver-Chip is sufficiently sensitive to ethanol exposure for modeling ALD-like hepatocyte injury, we measured lipid accumulation upon short-term (48-h-long) treatment with ethanol. During the treatment, hepatocyte morphology and albumin levels were monitored routinely, which we had found to be a more sensitive method than lactate dehydrogenase activity (LDH) assays for detecting early liver toxicity effects. No evidence of significant liver toxicity effects was found. As a positive control for induction of steatosis, we exposed the chips to fatty acids, a reproducible experimental method for induction of the steatotic phenotype (Breher-Esch et al., 2018). Ethanol concentrations ranging from 0.08% to 0.16% were chosen to mimic the BAC of human patients after alcohol consumption (Elgammal et al., 2015). A BAC of 0.08% is the upper limit for legal driving in the United States and United Kingdom. Based on flow and material properties of chip and perfusion system, the expected loss of ethanol from the medium during the treatment duration was minimal (see STAR Methods). To increase the sensitivity of the Liver-Chip to ethanol in a reproducible manner, we adjusted the glucose, insulin, and cortisol concentrations of the cell culture medium toward more physiological ranges (see STAR Methods). Steatosis was induced following treatment with either ethanol (at BACs ranging from 0.08% to 0.16%) or oleic acid (1 μ g/mL) for 48 h as visualized by AdipoRed staining (Figure 2A, i). The average lipid droplet size increased with rising ethanol concentrations (Figure 2A, ii), indicating the sensitivity of the Liver-Chip to operate within a range of clinically relevant BACs, as required for disease modeling (Bala et al., 2016; Takahashi and Fukusato, 2014). Triglyceride (TG) storage did not change significantly, likely due to the short treatment window.

Next, we assessed whether the lipid accumulation observed in the Liver-Chip in response to ethanol treatment was associated with signs of metabolic dysregulation, although this usually requires more prolonged exposure to the challenge (Tsukamoto, 2015). The 48-h exposure to human-relevant BACs resulted in a significant increase in cholesterol levels in the effluent (Figure 2B), and to a lesser degree, in the cell lysates (Figure S2A). We confirmed this finding with hepatocytes from two different donors, which showed that despite the donor-to-donor variability in baseline cholesterol release (Figure S3A), there was no significant difference in response to ethanol (Figure S3C). Glycogen storage, measured in cell lysates, showed a slight, but not significant, increase upon exposure to 0.08% ethanol (Figure 2C), while no changes were detected in glucose release

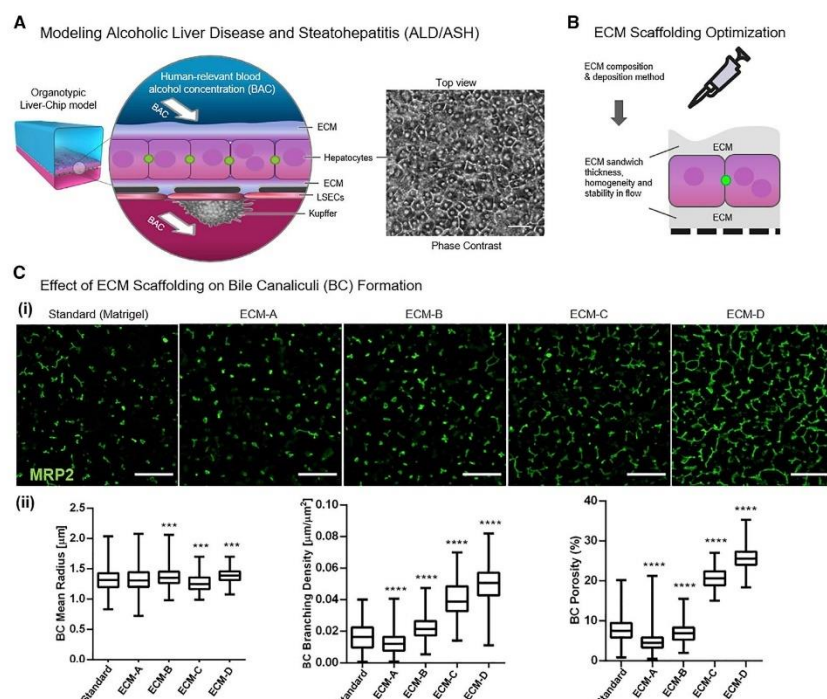


Figure 1. Development of the ALD Liver-Chip

(A) Approach for modeling human ALD/ASH by exposing the organotypic Liver-Chip to human-relevant blood-alcohol concentrations (BACs). Scale bar, 50 μm . (B) Different ECM composition and deposition methods were tested in the Liver-Chip to improve ECM scaffold thickness, homogeneity, and stability under flow. (C) Optimization of BC network integrity in the Liver-Chip. Scale bar, 100 μm . (i) Representative images of BC networks (MRP2, green) of the triculture Liver-Chip as a function of ECM conditions. (ii) Effects of different ECM conditions on the radius, branching density, and area fraction of BC networks. Biomimetic hepatobiliary architecture (condition ECM-C and ECM-D) is characterized by higher branching density, higher porosity, and more narrowly distributed mean radius compared to control (standard). Data are from one experiment with $n = 3$ chips per condition. Data represent median $\pm$ (minimum and maximum). *** $p < 0.001$; **** $p < 0.0001$ versus control (branching density and porosity, Kruskal-Wallis and Dunnett's multiple comparisons test; radius, Kolmogorov-Smirnov (KS) test and Bonferroni correction for multiple comparisons test).

(Figure S2B), except with toxic ethanol concentrations above 0.32% (data not shown). Similarly, albumin release was not affected by this short-term exposure to ethanol (Figure 2D), in line with clinical data in patients where such changes develop over time and reflect severe deterioration of liver function (Dyson et al., 2014; Petersen et al., 2017). Exposure to ethanol had a minimal effect on the number of hepatocytes nuclei counted per field of view but increased polyploidy, thought of as a marker of tissue stress (Zhang et al., 2018) (Figure 2E). High-fat treatment did not have this effect (Figure S2C).

To assess for changes in gene expression underpinning the above phenotypic treatment responses, we performed RNA sequencing (RNA-seq) analysis and identified the differentially expressed genes (DEGs) and the associated stratification, based on the magnitude of differences between ethanol-exposed and

control chips (Figure S4A). Ethanol-exposed chips (at concentrations of either 0.08% or 0.16%) had 123 DEGs (red dots, adjusted p value < 0.05 and $|\log_2$ fold change > 1), of which 87 were upregulated and 36 were downregulated (Figure S4B). Pathway analysis of the DEGs using the Kyoto Encyclopedia of Genes and Genomes (KEGG) bioinformatics database revealed changes in the expression of genes implicated in functions consistent with the readouts discussed above (Figure S4C). Relevant to lipid metabolism, we found upregulation of three genes related to cholesterol transport, *NR0B2* (Smalling et al., 2013), *APOA4* (Kang et al., 2017), and *APOA1* (Castera et al., 2019); and another seven genes related to cholesterol biosynthesis, *MVK*, *MVD*, and *HMGCS1* (Iravani et al., 2018), *FDFT1* (Stättermayer et al., 2014), *HMGCR* (Min et al., 2012), *FDPS* (Tommasi and Besaratinia, 2019), and *CYP51A1* (Kakehashi et

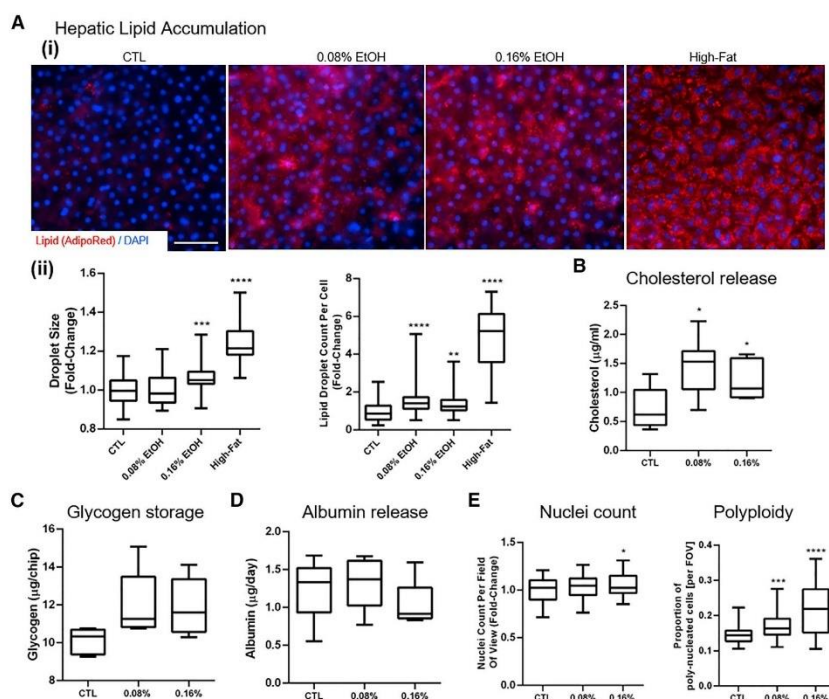


Figure 2. Assessment of liver toxicity, metabolic changes, and polyploidy in the ALD/ASH Liver-Chip
(A) Hepatic lipid accumulation. (i) Lipid droplet accumulation in hepatocytes visualized using AdipoRed staining after administration of fat (oleic acid 1 μg/mL; positive control) or ethanol (0.08% and 0.16%) for 48 h. Scale bar, 50 μm. (ii) Number of lipid droplets per cell and lipid droplet size (projected area). (B–D) Quantitative analysis of hepatic functional markers in the Liver-Chip after 48 h of exposure to physiologically relevant BACs. Fluorometric assessment using ELISA of cholesterol levels in the effluent (B), glycogen storage in cell lysate (C), and albumin release (D). (E) Nuclei count per field of view and proportion of hepatocytes with multiple nuclei per cell (polyploidy). Data represent median ± (minimum and maximum). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 versus control (Kruskal-Wallis and Dunnett's multiple comparisons test).

al., 2017); along with downregulation of *AKR1D1* (Nikolaou et al., 2019), involved in cholesterol catabolism (Figure 3A). Glycolysis/gluconeogenesis, steroid hormone biosynthesis, and insulin resistance/type II diabetes were also among the key pathways affected by ethanol treatment (Figure 3B). Further, ethanol induced significant changes in the expression of members of the alcohol dehydrogenase (ADH) gene family, including *ADH1C* (Aljomah et al., 2015), *ADH1B* (Li et al., 2018), and *ADH1A*, all involved in alcohol metabolism (Li et al., 2018). Notably, alcohol induced the expression of *CYP2E1*, critical for ethanol oxidation and the associated induction of oxidative stress (Figure 3C), a major pathogenic mechanism of ALD (Caro and Cederbaum, 2004). Further, exposure to ethanol affected the expression of clinically relevant genes involved in bile acid production and processing, including the major BC transporters *ABCB4* (*MDR3*) (Farrell et al., 2012), *ABCB1* (*MDR1*) (Pirola and Sookoian, 2018), *ABCC3* (*MRP-3*) (Atilano-Roque et al., 2016), *SLC10A1* (*NTCP*) (Brunt, 2017), *ABCB11*

(*BSEP*) and *ABCC2* (*MRP2*) (Arabet al., 2017), and *ADCY8* (Liang et al., 2018); along with genes associated with bile acid processing, such as *SLC27A5*, involved in fatty acid elongation (Benedict and Zhang, 2017); *CYP7A1* (Chiang, 2018; Liu et al., 2017), which regulates the overall bile acid production rate; and *CYP27A1* (Chiang, 2017), associated with the bile acids synthesis alternative pathway (Figure 3D). Our data show that the ALD Liver-Chip recapitulated the impact of ethanol treatment on genes involved in the metabolism of alanine, aspartate, and glutamate. In line with previous data showing the link between FLD and DNA damage in hepatocytes (Seitz and Stickel, 2006), our analysis revealed upregulation of *POLE* and *POLD2* (Kuttippurathu et al., 2016), both involved in DNA replication and repair, as well as *RAD51* and *FANCB*, which are expressed at DNA damage sites and implicated in homologous recombination (Nomura et al., 2007; Owada et al., 2018) (Figure 3E). Further, we saw upregulation of *E2F1* (Denechaud et al., 2016) and *CCNB1* (Gentric et al., 2015) and downregulation of *KIF14* (Yang et al., 2013) and

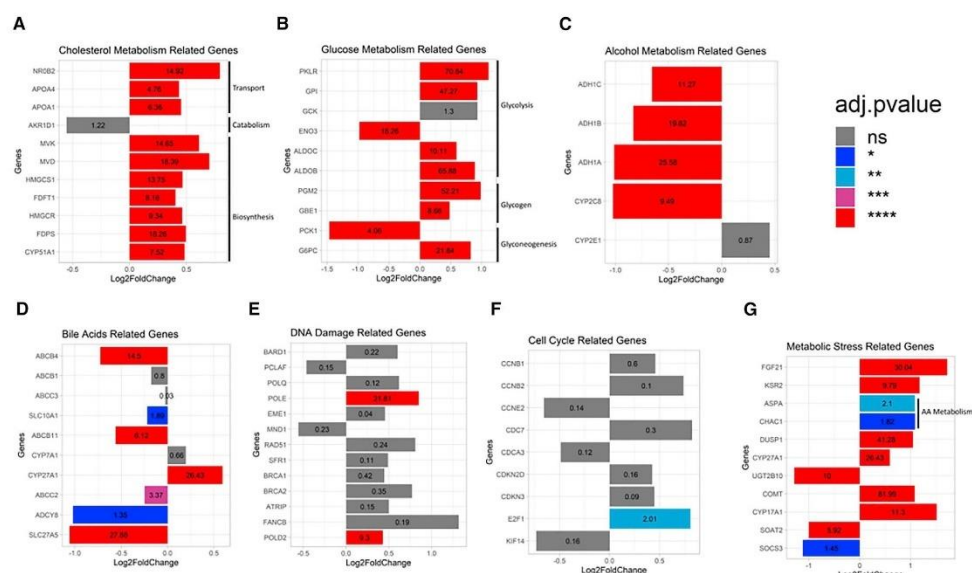


Figure 3. Gene expression profiling of the Liver-Chip induced by physiologically relevant ethanol concentrations
(A–G) Differential gene expression analysis in hepatocytes from ethanol-treated (exposed for 48 h at either 0.08% or 0.16%; see STAR Methods) and control Liver-Chip revealed significant differences in the expression of genes related to alcohol metabolism (A), cholesterol metabolism (B), glucose metabolism (C), bile acid production and maintenance (i.e., cholestasis) (D), DNA damage (E), cell-cycle regulation (F), and oxidative and metabolic stress (G). The adjusted p value is listed within each bar, with statistical significance indicated by bar color and * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Data are from one experiment with two to five chips per condition.

CCNE2 (Natarajan et al., 2017), all participating in cell-cycle regulation (Figure 3F). As several of the DNA-damage-associated genes identified in our data have not yet been implicated in the pathogenesis of FLD, this suggests the potential of the ALD Liver-Chip platform for identification of unknown mechanisms involved in alcohol-induced damage. Lastly, ethanol exposure led to the dysregulation of several markers of oxidative stress in the Liver-Chip, such as genes of the metallothionein family (*MT1*) (Ruttkay-Nedecky et al., 2013; Si and Lang, 2018; Waller-Evans et al., 2013) (Figures S4C and 3G) and altered the expression of *DUSP1* (Ye et al., 2019) (Figure 3G). While some of the gene expression changes shown in Figure 3 are nonsignificant or present with $|\log_2 \text{fold change}| < 1$, they are listed for context, as they are part of known pathways involved in ALD.

Modeling the two-hit hypothesis for ALD/ASH development

Having established that the Liver-Chip responds to ethanol, we next explored the possibility of modeling the two-hit hypothesis, which states that simple steatosis induced by alcohol requires a second insult for progression to ASH (Tsukamoto et al., 2009). Ethanol consumption compromises the intestinal barrier function, resulting in increased permeability to intestinal endotoxins, such as bacterial lipopolysaccharide (LPS), from the gut to the

liver via the portal vein (Bishehsari et al., 2017). The endotoxins are thought to act as the critical second hit in the progression of ALD to inflammation (ASH) (Szabo and Bala, 2010). Therefore, we tested whether co-exposure of the Liver-Chip to ethanol and LPS would worsen steatosis and the oxidative stress in the ALD Liver-Chip. We found that the size of hepatic lipid droplets was increased after 48 h of exposure to 0.08% ethanol + LPS (Figure 4A). We also found a dose-dependent increase in mitochondrial reactive oxygen species (ROS) events in response to ethanol, which was further exacerbated upon co-exposure to ethanol and LPS. Treatment with LPS alone had no effect, highlighting the specificity of the response (Figure 4B). These results are in line with our RNA-seq data showing the effects of alcohol on genes related to DNA damage and the cell cycle and are consistent with reported disease mechanisms. Induction of CYP450 by ethanol and free fatty acids and the consequential oxidative stress rampage have been implicated in the increased DNA damage in hepatocytes and the associated progression to hepatocellular carcinoma (HCC) (Seitz and Stickel, 2006).

Inflammatory cytokines are significantly elevated in patients with alcoholism with advanced liver disease (Urbaschek et al., 2001) and have been proposed as therapeutic targets, as inhibition of tumor necrosis factor α (TNF- α) action was protective against alcohol-induced liver injury in mouse models (Hines

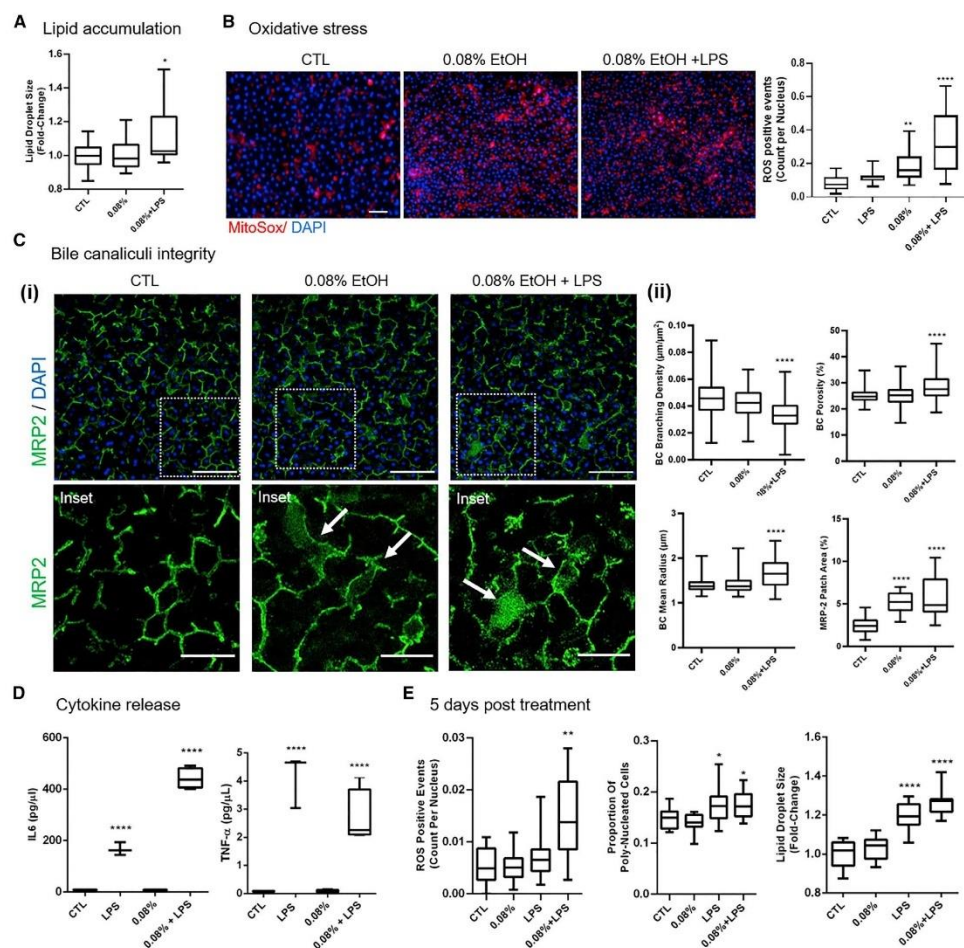


Figure 4. Modeling the two-hit hypothesis for ALD/ASH and alcohol abstinence in the ALD Liver-Chip

Data were collected after 48 h of exposure to 0.08% ethanol or ethanol + LPS.

(A) Quantification of mean lipid droplet size in the hepatocytes. Scale bar, 100 µm.

(B) Representative images of MitoSox staining in the hepatocytes (left) and quantification of ROS events (right).

(C) BC network changes in responses to treatment. (i) Representative images of changes in MRP2 BC staining (green) showing large MRP2-positive patches in the treatment conditions (arrows). Main scale bar, 100 µm; inset scale bar, 50 µm. (ii) Quantification of the changes in BC radius, branching density, and porosity, as well as the relative area of MRP2 patches.

(D) Release of IL-6 and TNF-α as measured by multiplexed immunoassays. Data are from two (48 h ethanol) or one (48 h high-fat diet; 5 days of recovery) independent experiments; minimally n = 2 chips per condition, five to eight images per chip where applicable. Data represent median ± (minimum and maximum). *p < 0.05; **p < 0.01; ****p < 0.0001 versus control (Kruskal-Wallis and Dunnett's multiple comparisons test).

(E) Quantification of mean lipid droplet size and frequency of oxidative stress events and polyplody in hepatocytes after 48 h of exposure of the Liver-Chip to either ethanol or ethanol + LPS followed by 5 days of recovery without exposure to ethanol.

and Wheeler, 2004). Therefore, we assessed the secretion of cytokines in response to ethanol and LPS, as our Liver-Chip model contains Kupffer cells, which are a major source of proinflammatory cytokines (Jang et al., 2019). Whereas treatment with LPS robustly increased the release of the proinflammatory cytokines interleukin-6 (IL-6) and TNF- α , co-treatment with 0.08% ethanol + LPS resulted in further increase in the production of IL-6 (Figure 4D), similar to findings from clinical and experimental studies demonstrating its role in disease progression (Bird et al., 1990; Remmler et al., 2018; Urbaschek et al., 2001). The cytokine rise is most likely linked to oxidative stress (Figure 4B) and the associated cell injury or other mechanisms as previously described (Schmidt-Arras and Rose-John, 2016).

Patients with ALD frequently manifest clinical and/or histologic evidence of cholestasis (Tung and Carithers, 1999), defined as decrease in bile secretion or obstruction of bile flow through the intra- or extrahepatic bile transport network. Currently, the mechanisms underlying the alcohol-induced cholestasis remain poorly understood. Our RNA-seq data revealed altered expression of BC-related genes in response to ethanol treatment, indicative of potential damage to BC integrity, a known finding in cholestatic livers (Jansen et al., 2017). We have recently shown the ability of our human Liver-Chip to recapitulate *in vivo* findings on drug-induced destruction of BC transporters (Jang et al., 2019). In the current study, the Liver-Chip was optimized to develop biomimetic BC networks, allowing us to directly measure the presence of cholestasis-associated changes, such as BC dilation (Chung et al., 2002; Fickert et al., 2002; Popper, 1981). For this, we quantitatively measured the MRP2-stained BC network to assess alcohol-induced cholestatic structural changes at the level of the canaliculi (Figure 4C, i). In the Liver-Chips exposed to 0.08% ethanol, we identified large patches of MRP2 expression (Figure 4C, ii), which could either indicate cholestatic protrusions of the BC network (Jansen et al., 2017) or abnormal MRP2 internalization, as described in human cholestatic livers (Chai et al., 2015). These MRP2 patches were significantly expanded in those with 0.08% ethanol + LPS together, consistent with the more severe hepatic injury (Figure 4C, ii). Further, in the latter the average BC radius increased throughout the BC network, whereas the branching density was decreased. BC dilation, leading to reduced biliary clearance (Meyer et al., 2017), together with the loss of connectivity indicate a worsening of hepatic damage, in line with the two-hit hypothesis for the pathogenesis of ASH/NASH.

Modeling of hepatocyte recovery following abstinence from alcohol with the Liver-Chip

Clinical data indicate the potential of the steatotic liver to repair the associated hepatocyte injury following timely abstinence from alcohol or with diet modifications (Menon et al., 2001). Thus, we assessed the sensitivity of the “alcoholic” Liver-Chip to respond to withdrawal from ethanol or ethanol + LPS. Treatment of the Liver-Chip for 48 h with ethanol or ethanol + LPS as above was followed by a 5-day-long treatment-free “recovery” period. By the end of the recovery period, oxidative stress, ploidy, and lipid droplet size normalized in ethanol-only-treated chips, whereas this was not the case for

the ethanol + LPS condition (Figure 4E). Although it is possible that a longer recovery period might lead to recovery of all treatment groups, these data suggest that the ALD Liver-Chip shows an insult-dependent recovery, which parallels the lack of repair of hepatic injury in patients with more advanced alcoholic disease and indicates the potential of the Liver-Chip to model recovery from ethanol-induced damage in a clinically relevant way.

DISCUSSION

Here, we leveraged a human Liver-Chip to enable the preclinical modeling of progressive alcohol-induced liver injury, a major health concern that is on the rise globally. We chose the Liver-Chip as it supports a complex co-culture system containing the main cell types found in the liver. Overwhelming evidence from the literature indicates that non-parenchymal cells are heavily involved in driving ALD (Cohen and Nagy, 2011). This implies that responses of monocultures to ethanol are unlikely to reflect the response of the multi-cell models, which is also driving the growing interest in using precision cut liver slices with multi-cell-type architecture for studying ethanol toxicity (Klassen et al., 2008). Applying a short-term experimental design to probe the system’s sensitivity, we showed that the ALD Liver-Chip model recapitulates critical disease signs such as intracellular accumulation of lipids, development of oxidative stress, and cholesterol synthesis dysregulation upon exposure to alcohol for 48 h (Lieber, 2004; Menon et al., 2001). Our study also suggested that changes to the biomimetic BC network are a sensitive marker of liver injury, a finding corroborated by altered gene expression of BC transporters. This rapid and multimodal response of the chip to ethanol could facilitate fast go/no-go decisions in drug development studies.

The ALD Liver-Chip recapitulated known effects of LPS on hepatocytes, demonstrating the model’s sensitivity to the two-hit injury described in ASH (Tsukamoto et al., 2009). In extension, our results suggest that chronic alcohol intake coupled with systemic inflammation worsens the liver damage and may significantly compromise recovery in ALD/ASH, similar to the responses of patients in advanced disease stages (Zhou and Zhong, 2017). In animal experiments, administration of antibiotics to reduce endotoxemia or inactivation of Kupffer cells with gadolinium chloride helped prevent liver injury (Thurman, 1998). The ALD Liver-Chip model could be useful as a platform to determine human relevancy of proposed mechanisms for new therapeutic approaches and the role of LPS in ASH progression, support patient-specific responses using donor-specific cells, and probe the progression of alcoholic FLD to fibrosis and cirrhosis (Lieber, 2004). Here, it is worth mentioning that the optimized ECM scaffold (Figures 1B and S1) supports the embedding and co-culture of hepatic stellate cells to generate a quad-culture Liver-Chip (data not shown), as needed for modeling fibrosis and progression of alcohol-induced steatosis.

In summary, the ALD Liver-Chip provides a promising platform toward modeling human ALD and study clinically relevant metabolic events, including ethanol metabolism, lipogenesis, biliary function, and oxidative stress.



Limitations of study

The present article was submitted for consideration in September 2019, and reviewers' comments were received in October 2020. While we resolved the majority of the reviewers' concerns, we were unable to conduct additional studies due to the consequences of the coronavirus disease 2019 (COVID-19) pandemic in our operations, leading to three main limitations of the present study. First, a future full characterization of tight junctions, cell-cell contacts, and bile acid distribution would be very desirable to better understand the mechanisms and context of the altered BC architecture. Second, while our previous study strongly suggested that tricultures containing hepatocytes, LSECs, and Kupffer cells are essential for modeling multiple mechanisms of action (Jang et al., 2019), it would be informative to study the effect of ethanol on various mono- and co-culture configurations in order to further elucidate the role of the different cell types in ALD. Lastly, the 48-h window was likely too short to see significant changes in TG storage and other metabolic responses, and it limited the changes in gene expression detected by RNA-seq. Further, the recovered lysate from the Liver-Chip was not sufficient to conduct western blots and investigate the associated protein-level changes. In closing, our data on the ALD Liver-Chip's responses to ethanol is robust and clinically relevant. Nonetheless, the platform's value to the community would benefit from additional studies on hepatocyte remodeling, the role of individual cell types, longer-term exposure to ethanol, and changes in protein expression.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
 - Lead contact
 - Materials availability
 - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
 - Liver-Chip culture
 - Optimization of Liver-Chip ECM for BC network formation
 - Ethanol and high-fat treatment
 - Robustness of ethanol dosing
 - Immunofluorescence staining and imaging
 - Live cell staining and imaging
 - RNA extraction and sequencing
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
 - Image analysis
 - Cell lysing
 - Albumin quantification assay
 - Cholesterol quantification assay
 - Glycogen quantification assay
 - Glucose quantification assay
 - RNA-seq/pathway analysis
 - Cytokine quantification assay
 - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2021.109393>.

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AUTHOR CONTRIBUTIONS

D.B.P., K.C.K., and J.C.N. developed the overall strategy of the experimental studies. D.B.P. and J.C.N. supervised the studies. D.B.P., G.B., T.I.M., K.S., A.W., K.K., J.R., G.K., and J.C.N. performed the experiments and analyzed data. J.C.N., A.S., and T.I.M. developed the quantitative imaging methods. D.V.M. conducted the bioinformatics analyses studies. D.B.P., J.C.N., and D.V.M. wrote the manuscript. K.C.K. reviewed all data, discussed it with G.H., and edited and reviewed the manuscript. E.S. and S.L. reviewed the manuscript and provided critical input for discussion and interpretation.

DECLARATION OF INTERESTS

All authors except S.L. and E.S. are current or former employees or interns of Emulate and may hold patents related to this work or equity interests in Emulate.

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Cell Reports Report



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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
MRP2	Abcam	ab172630
Chemicals, peptides, and recombinant proteins		
AdipoRed	Lonza	PT-7009
TMRM	Thermo Fisher	T668
MitoSox	Thermo Fisher	M36008
Cholyl-l-lysyl-fluorescein (CLF)	Corning	451041
Critical commercial assays		
PureLink RNA Mini Kit lysis buffer	Thermo Fischer	12183018
Human Albumin SimpleStep ELISA® Kit	Abcam	ab179887
Amplex Red Cholesterol Assay Kit	Thermo Fischer	A12216
Glycogen Assay Kit	Abcam	ab65620
Glucose Assay Kit	Abcam	ab65333
Deposited data		
RNA-sequencing data	Illumina HiSeq	GEO: GSE175396 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175396
Experimental models: cell lines		
Cryopreserved primary human hepatocytes from 1 healthy donor	Triangle Research Labs (Lonza)	QHum15063
Cryopreserved primary human hepatocytes from 1 healthy donor	GIBCO (Thermo Fisher)	HU8305
Software and algorithms		
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
ImageJ-Fiji	https://imagej.nih.gov/ij/	Schindelin et al., 2012
CellProfiler	https://cellprofiler.org/	McQuin et al., 2018
ICY	http://icy.bioimageanalysis.org/	de Chaumont et al., 2012
RStudio Team (2021). Integrated Development Environment for R. "DESeq2" R package (Bioconductor)	https://www.rstudio.com/	Love et al., 2014
Trimmomatic v.0.36	The USADEL LAB http://www.usadellab.org/cms/	Bolger et al., 2014
STAR (Spliced Transcripts Alignment to a Reference) v.2.5.2b	https://github.com/alexdobin/STAR	Dobin et al., 2013
Other		
Emulate Organ-Chip System (Zoe, Orb and Chip-S1)	Emulate Inc.	https://www.emulatebio.com/our-technology

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Janna Nawroth (jnawroth@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The RNA-seq data generated during this study are available at the NCBI Gene Expression Omnibus (GEO) database repository (GEO: GSE175396).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cryopreserved primary human hepatocytes from two healthy donors, Donors G and Q, were purchased from Triangle Research Labs (Lonza) and GIBCO (Thermo Fisher Scientific). Cells were cultured under standard human cell culture conditions (37°C, 5% CO₂). Donor Q: Lot QHum15063, male, 6 years of age, ethnicity unknown; donor G: Lot HU8305, male, 53 years of age, Caucasian.

METHOD DETAILS

Liver-Chip culture

Liver-Chip culture was established according to our previously published protocol (Jang et al., 2019). Briefly, primary human hepatocytes (3.5 million cells/mL) from two healthy donors, Donors G and Q, were cultured on one side of a porous membrane (pore size ~7 microns) in the Emulate dual-channel microfluidic chip (Kasendra et al., 2018). Extracellular matrix sandwiching and composition in the Liver-Chip was optimized to support BC integrity (Figures 1B, 1C, and S1; STAR Methods sections below). On the other side of the membrane (the lower chamber of the chip) human primary LSECs (3 million cells/mL) and Kupffer cells (0.5 million cells/mL) were seeded to mimic the hepatic sinusoid architecture (Figure 1A). The two cell compartments were perfused independently, and flow rates were optimized to enable optimal survival and maturation of the different cell types (Jang et al., 2019). The seeded hepatocytes were overlaid with ECM as described in section below and maintained in William's E Medium (WEM) containing Glutamax (GIBCO), ITS+ (Corning), dexamethasone (1 μM, Sigma-Aldrich), ascorbic acid (0.05 mg/mL, Sigma-Aldrich), fetal bovine serum (Sigma-Aldrich), and penicillin/streptomycin (Sigma-Aldrich), and incubated at 37°C, 5% CO₂. The vascular channel of the Liver-Chip was maintained with human endothelial media (Emulate, Inc.). Two days after seeding, the Liver-Chips were connected to the Human Emulation System (Emulate, Inc.) and both chip channels were perfused at 30 μL/h to provide a continuous supply of fresh medium for the duration of the experiments. Note that the hepatocytes were protected from the shear stress generated by this flow through the optimized gel coating, as discussed in the section on ECM optimization. Meanwhile, at 30 μL/h, the endothelial and Kupffer cells in the bottom channel experience a shear stress of ca. 0.008 dyn/cm², which is lower than the average normal fluid shear level of 4 dyn/cm² reported for microvasculature (Topper and Gimbrone, 1999). Hence, no shear-mediated damage of either parenchymal or non-parenchymal cells is expected.

In order to increase the sensitivity required for stimulus-dependent metabolic readouts, the traditional hepatocyte cell culture media was modified at day 5 in culture to a lower, more physiological relevant, concentration glucose of 1 g/L (~5.5 mmol/L) DMEM (GIBCO, Cat#11054001) (Wannamethee et al., 1999), and supplemented with non-essential amino acids solution (NEAA, 1:200 dilution), glutaMAX 1:100 dilution (Thermo Fisher), ascorbic acid (0.05 mg/mL, Sigma-Aldrich), dexamethasone (50nM, Sigma-Aldrich) and penicillin/streptomycin (Sigma-Aldrich). Moreover, ITS+ premix (Corning 1:100 dilution) was replaced by ITS (GIBCO, 1:500 dilution) in order to reduce the insulin concentration from 10 to 2 μg/mL, a more physiological relevant concentration (Wannamethee et al., 1999), and the FBS supplement was removed.

Optimization of Liver-Chip ECM for BC network formation

We had observed that the chip ports were frequently site of highly branched bile canalicular networks compared to cholestatic pockets in the chip channels (Figure S1A). BC formation and integrity has recently been shown to be strongly dependent on the presence of a stable extracellular matrix that affords the symmetric mechanical anchoring needed for hepatocyte polarization and elongated BC lumen formation (Figure S1B) (Li et al., 2016). Indeed, ECM gel thickness was consistently greater in the ports than in the channels (Figure S1C), where shear stress was found to significantly remodel and remove ECM gels depending on their composition. Matrigel-based scaffolds commonly used for human hepatocyte sandwich culture fared worse than collagen-I based gels which exhibited greater initial thickness and in-flow stability; however, gels of both compositions displayed great inhomogeneity of gel thickness across and within chips (Figures S1C and S1D). Based on these preliminary findings, we tested whether improved deposition of collagen-I based gels to create thick, flow-stable, and homogeneous ECM sandwich scaffolds throughout the hepatic channel could improve BC network formation. Consequently, iteratively modified the original ECM coating protocol (Jang et al., 2019) and optimized the planar deposition of various collagen-I based gels to achieve homogeneous and robust thickness in the chip through pressured injection of liquid into the channels filled with gelled ECM (Figure S1E). We also developed a luminal scaffold design based on viscous fingering (Figure S1F). Five different combinations of ECM composition and deposition strategy were tested for their effect on BC network formation (Figure S1G), where "Standard" denotes the original protocol (Jang et al., 2019). Briefly, for "Standard" ECM condition, top and bottom channels were coated by incubating with 100 μg/mL rat tail collagen-I (Corning) and 25 μg/mL fibronectin (GIBCO) overnight at 37°C. Hepatocytes were seeded as described above. After seeding, Matrigel® prepolymer solution was pre-



pared on ice and injected into the top channel, which was then incubated at 37°C overnight. The following day, the top channel was gently flushed with warm medium. In conditions ECM-A, -B, -C, and -D, the hepatocytes are sandwiched between two 3D-ECM gel of different compositions, including Collagen-I (FibriCol®), Fibronectin (GIBCO), Collagen-IV (Sigma), and the collagen cross-linking agent, microbial transglutaminase (MTG) (Modernist Pantry LLC). The underlying gel was prepared by injecting the gel prepolymer solution after surface activation and incubated overnight at 37°C. The next day, the gel was then flushed twice with 100 μ L of warm medium at 187.5 μ L/s flow (Eppendorf Xplorer automatic pipet), generating a 3D-ECM on the membrane with a thickness of 30–50 μ m (data not shown). Afterward, hepatocytes were seeded as described above. One day after the hepatocyte seeding, the same method was used to prepare the overlaying gel in conditions ECM-A, -B, and -C. In condition ECM-D, the overlay was prepared via viscous fingering method (Bischof et al., 2012). Briefly, 5 mg/mL of bovine collagen-I (FibriCol®) was injected into the top channel and a pipette tip filled with 200 μ L of warm medium was immediately injected into the channel inlet to apply hydrostatic pressure. This will cause an interface instability between medium and prepolymerized collagen-I, enabling the medium to flow through the middle part of the prepolymerized collagen-I, thereby creating a lumen (Figure S1F). The chips were then immediately moved into humidified incubator to promote gelation of the lumen formed thick collagen-I ECM on top of the hepatocyte monolayer. ECM-C was used in the experiments in this study.

To assess and confirm ECM scaffold formation during the optimization procedure, we stained the gels with a fluorescent dye: Briefly, 1 mg/mL of N-hydroxysuccinimide (NHS) ester dye (Atto 488 NHS Ester, Sigma Aldrich) was mixed with 50 mM borate buffer (pH 9) in 1:500 ratio. Directly after ECM formation, the prepared staining solution was injected into the top channel and the chips were incubated for 25 minutes at room temperature in the dark. The top channel was then rinsed three times with PBS prior to fluorescence imaging.

To assess the effect of each ECM protocol on BC network formation, we quantified the MRP-2 stained BC network topology (see method sections on IF staining and Quantification). Optimal conditions thus identified were ECM-C and ECM-D, and these protocols were then used for ALD Liver-Chip studies.

Ethanol and high-fat treatment

To model FLD, cell culture medium was supplemented at day 7 in culture either with ethanol (0.08% or 0.16%, i.e., 80 mg/ml to 160 mg/ml, or 17.4 mM to 34 mM), with or without lipopolysaccharides (LPS, 1 μ g/ml), or fatty acid (oleic acid, 1 μ g/ml). The Liver-Chip was maintained for 48h in treatment medium and either assayed or allowed to recover for 5 days in basal medium. During treatment, liver toxicity was routinely monitored by albumin quantification and morphology assessment, which we had found to be a more sensitive method for detecting early liver toxicity effects than LDH assays.

Robustness of ethanol dosing

Minimal loss of ethanol from the medium is expected due to the material properties of the system. Though the inlet reservoirs allow ethanol and water vapor to off-gas, dissolving ethanol in water drastically decreases ethanol's vapor pressure, which means that at the low ethanol/water ratios used in this study ethanol's vapor pressure is much lower than that of water (Liu et al., 2008). Therefore, the usual slow rate of water evaporation in the reservoir is still faster than that of ethanol, such that the concentration of ethanol would not be significantly impacted. Further, the primary reservoir and conduit material that contacts the fluid is COC and is known not to allow significant permeation of gas (Ochs et al., 2014).

The diffusion coefficient of ethanol through PDMS, the most porous material in our microfluidic system, is on the order of $D = 5 \times 10^{-8}$ cm²/s (Lan et al., 2016). Assuming steady-state flux, we can apply Fick's First law along the length of the Chip to compute the diffusive flow $= DA \frac{dC}{dx}$, with wall thickness $dx = 600$ μ m and internal channel surface area $A = 0.28$ cm². This model predicts a 2.8% loss of the initially dosed concentration C of ethanol; e.g., the 0.08% ethanol dose in the chip would be reduced to 0.077%.

During the initial onset of flow, absorption of ethanol into PDMS also contributes to total loss. However, the partition coefficient of ethanol in PDMS is on the order of 2.98 (Qiu et al., 2019) which is reasonably low. We expect an effective system equilibrium to be achieved after less than 20h during the 48h study. This initial loss to the PDMS at early time points would also primarily impact cellular exposure concentrations at the outlet of the chip channel rather than the inlet since the loss occurs along the length of the chip.

Taken together, we conclude that the ethanol dosing protocol is robust, as changes to the ethanol concentrations due to loss are expected to occur only briefly and locally over the course of the treatment.

Immunofluorescence staining and imaging

Cells on the Liver-Chips were washed 3 times in 1X PBS then fixed with 4% paraformaldehyde for 20 minutes at room temperature (RT). Chips were then washed 3 times with cold 1X PBS and blocked using 1% bovine serum albumin (BSA) in 1X PBS for 30 minutes to 2h at RT. Cells were permeabilized using a 1X PBS solution containing 1% saponin and 10% serum matching the species of the secondary antibody for 30min at RT. Cells were then washed 3 times in 1X PBS and blocked again in a 1X PBS solution containing 1% BSA for 2h to overnight at 4°C. All incubations with primary antibodies against MRP2 (1:50, Abcam) were carried out in this blocking buffer overnight at 4°C. This was followed by a two-hour incubation with secondary antibodies (Cell Signaling, Danvers, MA, USA) and DAPI in the blocking buffer at RT. Images were acquired with either an Olympus fluorescence microscope (IX83) or a Zeiss

confocal microscope (LSM880). The fields of view were chosen randomly but fell always within the same three locations (near inlet, center, and near outlet) as to span the length of the chip channel. Two images were taken per region, providing six images per chip sample for analysis.

Live cell staining and imaging

Liver-Chips were stained in the upper channel with AdipoRed (1:40 dilution in PBS, Lonza) to visualize lipid droplet accumulation, Tetramethylrhodamine, methyl ester, perchlorate (TMRM) (0.1 μ M in hepatocyte medium, Thermo Fisher) to visualize active mitochondria, and MitoSox® (5 μ M in hepatocyte medium, Thermo Fisher) to visualize cellular oxidative stress, and cholyl-L-lysyl-fluorescein (CLF, Corning) to visualize bile canaliculi. Each staining solution was prepared and added to the upper channel, incubated for 15–30 minutes at 37°C, and washed three times before imaging. NucBlue (Thermo Fisher) staining was used to identify cell nuclei during live imaging. The stained chips were imaged using either an Olympus fluorescence microscope (IX83) or Zeiss confocal microscope (LSM880) and were de-blurred with Olympus cellSens software.

RNA extraction and sequencing

The cells from both top and bottom channel were separately lysed from the Liver-Chip according to the Protocol for Emulate Organ-Chips: Cell Lysis for RNA Isolation (EP161 v1.0). In brief, we used PureLink RNA Mini Kit lysis buffer (Thermo Fischer #12183018) to directly lyse the cells while still adhering to the chip, collected the lysate, and immediately frozen in dry ice. RNA sequencing was performed by GENEWIZ.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis

Analyses of lipid droplet accumulation, ROS events, and nuclei were conducted using the software tool ICY (de Chaumont et al., 2012), ImageJ-Fiji (Schindelin et al., 2012), CellProfiler (McQuinn et al., 2018), and MATLAB (MATLAB, MathWorks Inc., Natick, MA). For ROS measurements, the histogram of the fluorescent images was adjusted to remove the background signal, followed by quantification of ROS events in the region of interest (ROI) based on minimum and maximum size and fluorescent intensity using the batch processing tool in ICY. ImageJ-Fiji was used to preprocess the AdipoRed images for analysis of lipid droplet accumulation. Here, the AdipoRed channel was median filtered, corrected for illumination, and then filtered with a Laplacian filter to emphasize droplet edges and remove larger background structures. The DAPI channel was filtered with an adaptive contrast enhancement algorithm (CLAHE) and then thresholded, followed by multiple dilation and erosion steps to yield a binary image in which nuclei in very close proximity, which likely belong to a single poly-nucleated cells, are merged. These preprocessed images were further processed in CellProfiler where a pipeline first automatically segmented the fields of view into estimated cell boundaries using the nuclei as reference points, followed by thresholding and detection of lipid droplets in the AdipoRed channel within each estimated cell boundary. Using CellProfiler modules as well as MATLAB scripts, we then computed mean droplet size (i.e., the projected area of the droplet in μm^2) and the number of droplets per cell. Values of treatment groups were normalized to the median values of the associated control group in order to express fold-change values and thereby mitigate baseline variability due to donor-to-donor and cell batch variability. Furthermore, we computed the proportion of poly-nucleated cells by binning the detected nuclei according to their perimeter, which revealed two distinct populations, i.e., single, well separated nuclei, indicating mononucleated cells, and closely neighboring nuclei fused during thresholding, indicating poly-nucleated cells.

For measuring bile canicular network properties, MRP-2 stained chips were imaged at 40x using a confocal point-scanning microscope (Zeiss LSM880, Airyscan). Fields of views were randomly chosen along the entire length of the channel to catch heterogeneity caused by ECM deposition or erosion in flow. In each field of view, a z stack was recorded and combined using maximal intensity projection in order to fully capture the bile canicular network. In subsequent image analysis, each field of view was first segmented into 16 sub-windows, and analyzed for three quantitative metrics: Porosity, branching density, and average radius (Figure S1H). The area fraction taken up by the BC network, which is a measure of porosity, was determined using an ImageJ-Fiji macro that filters and thresholds the signal, followed by the ImageJ particle analysis which detects the area occupied by BC elements (Area BC). BC porosity was then computed as the ratio of Area BC to the total area of the field of view. Average radius and branching density were determined by applying the ImageJ-Fiji plugin “ridge detection” (Steger, 1998) to detect and measure the radius and length of all BC segments in each window. Then, we computed the average radius measured in each window as well as the branching density, defined as the summed length of the BC branches in the sub-window divided by the area of the sub-window.

Cell lysing

Cells in the Liver-Chip were lysed according to the protocol for Emulate Organ-Chips (Cell Lysis for Protein Extraction (EP135 v1.0)). In brief, we used Tris lysis buffer (MSD, #R60TX-3) to directly lyse the cells while still adhering to the chip, collected the lysate, and performed downstream assays.



Albumin quantification assay

Albumin secretion was quantified in Liver-Chip effluent collected from the top channel using the Human Albumin SimpleStep ELISA® Kit (Abcam, #ab179887) according to the manufacturer's protocol.

Cholesterol quantification assay

Cholesterol was quantified in Liver-Chip effluent according to manufacturer's protocol for fluorometric detection (Amplex Red Cholesterol Assay Kit, Thermo Fisher). Medium in the top channel was changed to standard hepatocyte medium without FBS prior to the experiment. The following sample quantification was used to determine amount of cholesterol in the hepatocytes channel effluent: Net effluent cholesterol = [cholesterol from effluent] $\mu\text{g/ml}$ MINUS [Cholesterol from dosing medium] $\mu\text{g/ml}$. The same quantification method was used to determine the cholesterol concentration of the hepatocytes cell lysate as described above.

Glycogen quantification assay

Hepatocytes in the top channel of the Liver-Chip were lysed as described above, then diluted at a range of 1:500 to 1:1000. Glycogen levels were determined using a standard assay according to manufacturer's instructions for fluorometric detection (Abcam, #ab65620).

Glucose quantification assay

Glucose was quantified in Liver-Chip effluent collected from the top channel. Sample concentration were adjusted be within a 50 mg/dl to 200 mg/dl range. Glucose was quantified using a standard kit according to manufacturer's instruction (Abcam, # ab65333).

RNA-seq/pathway analysis

The RNAseq dataset consisted of 2 vehicle samples, 3 samples treated with 0.08% alcohol, and 2 samples treated with 0.16% alcohol. Since differential gene expression analysis between the 0.08% and 0.16% groups yielded no significant differentially expressed genes, we pooled the samples of the two ethanol treatment groups together and constructed a single larger "ethanol-treated" group (consisting of 5 samples) which we compared to the vehicle group.

To remove poor quality adaptor sequences and nucleotides, the sequence reads were trimmed using Trimmomatic v.0.36. Next, using the STAR (Spliced Transcripts Alignment to a Reference) aligner v.2.5.2b, we mapped the trimmed reads to the *Homo sapiens* reference genome GRCh38 (available on ENSEMBL). Using the generated BAM files and the feature Counts from the v.1.5.2 subread package, we calculated the unique gene hit counts. Of note, only unique reads that fell within exon regions were counted. We prepared a strand-specific library; therefore, the reads were strand-specifically counted. Using the gene hit counts table, we filtered out genes with very low expression across the samples. The remainder were used for differential gene expression (DGE) analysis. For the DGE analysis, we used the "DESeq2" R package (Love et al., 2014) (Bioconductor), which normalizes the gene expression data and RNA composition using the "median-of-ratios" method (Anders and Huber, 2010; Love et al., 2014). In order to select the differentially expressed genes, we applied the following thresholds: adjusted p value < 0.05 and $|\log_2\text{FoldChange}| > 1$. Of the 57,500 genes annotated in the genome, 123 were found to have significant differential expression between the vehicle (n = 2) and the ethanol exposed chips (n = 5). More specifically, 87 (36) genes were found to be significantly upregulated (downregulated) in the ethanol exposed chips (0.08% and 0.16%). These 123 differentially expressed genes were used for the KEGG pathway analysis.

Cytokine quantification assay

Liver-Chip bottom channel (containing Kupffer cells) effluent cytokine levels were measured using the U-PLEX® Biomarker Group 1 Human Assays (MSD® Cat No. K15067L) according to the manufacturer's instructions.

Statistical analysis

As indicated in the figure legends, one-way ANOVA, Sidak's and Dunnett's multiple comparisons tests were used for comparing the mean values of parametric data, and the Mann-Whitney U test or Kruskal-Wallis tests followed by Dunnett's multiple comparisons test was used for comparing the median value of nonparametric data. As noted in figures, the KS-test was occasionally used to test for differences in distributions. All statistical analyses were performed using Prism v6, 7 or 8 (GraphPad). All data was collected from at least 2 independent experiments with at least 3 chips per condition and imaged at 5-10 fields of view per chip (where applicable), unless stated otherwise in the figure legends.

Publication [4]**Development of a bi-layered cryogenic electrospun polylactic acid scaffold to study calcific aortic valve disease in a 3D co-culture model**

K. Stadelmann, A. Weghofer, M. Urbanczyk, **T. I. Maulana**, P. Loskill, P. D. Jones, K. Schenke-Layland

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Abstract/Summary:

Calcified aortic valve disease (CAVD) is the most prevalent valve disease in the elderly. Targeted pharmacological therapies are limited since the underlying mechanisms of CAVD are not well understood. Appropriate 3D in vitro models could potentially improve our knowledge of the disease. Here, we developed a 3D in vitro aortic heart valve model that resembles the morphology of the valvular extracellular matrix and mimics the mechanical and physiological behavior of the native aortic valve fibrosa and spongiosa. We employed cryogenic electrospinning to engineer a bi-layered cryogenic electrospun scaffold (BCES) with defined morphologies that allowed valvular endothelial cell (VEC) adherence and valvular interstitial cell (VIC) ingrowth into the scaffold. Using a self-designed cell culture insert allowed us to establish the valvular co-culture simultaneously by seeding VICs on one side and VECs on the other side of the electrospun scaffold. Proof-of-principle calcification studies were successfully performed using an established osteogenic culture protocol and the here designed 3D in vitro aortic heart valve model.



Full length article

Development of a bi-layered cryogenic electrospun polylactic acid scaffold to study calcific aortic valve disease in a 3D co-culture model



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ABSTRACT

Calcified aortic valve disease (CAVD) is the most prevalent valve disease in the elderly. Targeted pharmacological therapies are limited since the underlying mechanisms of CAVD are not well understood. Appropriate 3D *in vitro* models could potentially improve our knowledge of the disease. Here, we developed a 3D *in vitro* aortic heart valve model that resembles the morphology of the valvular extracellular matrix and mimics the mechanical and physiological behavior of the native aortic valve fibrosa and spongiosa. We employed cryogenic electrospinning to engineer a bi-layered cryogenic electrospun scaffold (BCES) with defined morphologies that allowed valvular endothelial cell (VEC) adherence and valvular interstitial cell (VIC) ingrowth into the scaffold. Using a self-designed cell culture insert allowed us to establish the valvular co-culture simultaneously by seeding VICs on one side and VECs on the other side of the electrospun scaffold. Proof-of-principle calcification studies were successfully performed using an established osteogenic culture protocol and the here designed 3D *in vitro* aortic heart valve model.

Statement of Significance

Three-dimensional (3D) electrospun scaffolds are widely used for soft tissue engineering since they mimic the morphology of the native extracellular matrix. Several studies have shown that cells behave more naturally on 3D materials than on the commonly used stiff two-dimensional (2D) cell culture substrates, which have no biological properties. As appropriate 3D models for the study of aortic valve diseases are limited, we developed a novel bi-layered 3D *in vitro* test system by using the versatile technique of cryogenic electrospinning in combination with the influence of different solvents to mimic the morphology, mechanical, and cellular distribution of a native aortic heart valve leaflet. This 3D *in vitro* model can be used to study valve biology and heart valve-impacting diseases such as calcification to elucidate therapeutic targets.

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1. Introduction

The aortic valve consists of three leaflets, and each of them is composed of three different layers: the fibrosa, the spongiosa, and the ventricularis [1]. The fibrosa layer faces the blood outflow side of the valve and predominantly contains collagen fibers, which

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represents the load-bearing component [2]. The intermediate spongy layer is mainly composed of proteoglycans and glycosaminoglycans, which are capable of binding a high amount of water to absorb occurring forces during the compression of the valve [2]. The ventricularis layer faces the blood inflow side of the valve and is rich in an elastic fiber network that provides elasticity [3,4]. This architecture guarantees to withstand compressive loads while providing a high degree of elasticity as the aortic valve is exposed to the highest pressure gradient in the body [2,5]. To date, we know of at least two distinct cell phenotypes that are resident in the aortic valve leaflet: valvular endothelial cells (VECs), which line the outer surface of the fibrosa and ventricularis, and valvular interstitial cells (VICs), which are localized throughout the extracellular matrix (ECM) of all three layers and mainly organize ECM production and remodeling [5,6].

Environmental changes such as chemical, morphological and/or mechanical changes in the ECM of the aortic heart valves can lead to a disrupted valvular homeostasis of VICs and VECs, which can lead to calcified aortic valve disease (CAVD). CAVD is considered to be an active and progressive disease that causes chest tightness and pain [7–9]. Age plays a major role in the development of the disease, resulting in an increased risk of CAVD starting at the age of 65 [3,10]. The pathology of CAVD begins on the fibrosa side of the aortic valve, and with disease progression calcific nodules are formed, leading to stenosis and obstruction of blood flow, which can ultimately lead to death [5]. To date, the pathobiological mechanisms of CAVD are not fully understood and effective pharmacological therapies are lacking [11]. An improved understanding of the processes that lead to or advance CAVD would potentially allow the development of better therapies or early intervention.

For example, appropriate (preferably human-based) *in vitro* models could help to further decipher CAVD. However, the currently used routine *in vitro* models are based on two-dimensional (2D) platforms, utilizing tissue culture polystyrene (TCPS) or glass substrates. These 2D surfaces are non-physiologically stiff, which is particularly critical for CAVD studies as VICs exhibit an activated phenotype on stiff substrates in more than 90% of the cells, which is the first sign of disease progression, whereas in a healthy aortic valve leaflet less than 5% of VICs exhibit the activated phenotype [12]. Consequently, the results from those studies are associated with great limitations introduced by non-physiological substrates. Wang et al. [13] demonstrated in their study the upregulation of the phosphoinositid-3-kinase pathway when VICs were cultured on stiff substrates, resulting in a pathogenic myofibroblast phenotype. In contrast, when VICs were cultured on soft hydrogels, the quiescent phenotype was maintained [14,15]. To date, soft suitable three-dimensional (3D) aortic valve *in vitro* models are limited, especially those that can be transferred to perfused and dynamic systems.

3D electrospun substrates that can mimic native valve leaflets are promising scaffold candidates for 3D *in vitro* aortic heart valve models as they resemble the fibrillar histoarchitecture of the valvular ECM, mimic mechanical valve properties, and they can be transferred into dynamic culture systems [16,17]. Electrospun scaffolds can be produced by an advanced method called cryogenic electrospinning [18]. During this process, electrospun fibers are collected on a low-temperature drum (-30°C) while ice crystals, formed by water vapor in the surrounding atmosphere, build up a removable void template of ice, which leaves behind a loosely packed fiber structure after the lyophilization of ice crystals [19].

In this study, we developed a 3D bi-layered cryogenic electrospun scaffold (BCES) that is composed of polylactic acid (PLA), which is a widely used polymer for tissue engineering and clinical applications due to its biodegradability, biocompatibility, and advantageous mechanical properties [20]. We investigated valvular cell response to BCES and whether it is possible to chemically-

induce calcification in the 3D aortic heart valve co-culture (AHVCC) model under the same conditions as in 2D models.

2. Materials and methods

2.1. Electrospinning of BCES

The conductivity of solvents and 15% (w/v) PLA solutions (dissolving process, see SI section 1.1, properties of solvents see SI Table 1) was measured with a conductivity meter (GMH 3431-LTG, Greisinger, Regenstauf, Germany). The rheological properties of the PLA solutions were measured using a MCR 302 rheometer (Anton Paar, Ostfildern, Germany). For more information, see SI section 1.1.

15% (w/v) PLA solutions were conventionally electrospun on a flat collector, and cryogenically electrospun on a low-temperature drum in a temperature- and humidity-controlled electrospinning apparatus (EC-CLI, IME Technologies, Eindhoven, Netherlands). For detailed process parameters, see SI section 1.1 and SI Table 2. A gas shield (EM-GSM, IME Technologies, Eindhoven, Netherlands) of trichloromethane (TCM)-saturated air was used during the cryogenic electrospinning process.

2.2. Characterization of BCES

The mechanical properties of BCES were analyzed by uniaxial tensile testing in an adaption of the DIN EN ISO 527-1,-2:2012-06 with a ZwickRoell Z020 (Ulm, Germany). For detailed information, see SI section 1.2.

The water uptake capacity of untreated and treated (disinfected and biofunctionalized (see next Section 2.3)) BCES was analyzed by swelling ratio experiments. Triplicates of BCES punches with a diameter of 10 mm were immersed in 2 ml ddH₂O at RT for 1, 4, 8, and 16 h, as previously described [21].

The contact angle of both sides of untreated and treated BCES was measured using an OCA 40 (DataPhysics Instruments GmbH, Filderstadt, Germany), as previously described [21].

The morphology of BCES was analyzed with scanning electron microscopy (SEM) using an Auriga® 40 (Zeiss, Oberkochen, Germany). BCES were sputtered with platinum/gold (SCD040, Balzers Union) for 20 s. Argon was used for evacuation and flooding. For detailed information on processing and fiber analysis, see SI section 1.2.

2.3. Biofunctionalization of BCES, TCPS, and glass substrates

BCES and glass substrates were disinfected with a wash of 70% ethanol (EtOH) for 20 min and two more washes for 20 min with phosphate-buffered saline (PBS⁻). Then, BCES, glass, and TCPS were biofunctionalized with a 10 µg/ml bovine plasma fibronectin (FN) solution (F1141, Sigma Aldrich, USA) by protein adsorption during an incubation time of 2 h at 37°C, 5% CO₂. Excess protein was removed by washing with PBS⁻ for 5 min.

In addition, BCES, glass, and TCPS were biofunctionalized by coating with rat collagen type I (8 µg/mL, C3867-1VL, Sigma Aldrich, St. Louis, Missouri, US) in PBS⁻ and mouse laminin (12 g/mL, 3400-010-02, R&D Systems) in DMEM without BSA. The coated samples were incubated at 37°C for 4 h. Fibrin coating was prepared by adsorbing bovine plasma (1 mg/mL, 341573-1GM, MERCK Millipore, Darmstadt, Germany) to BCES, glass, and TCPS overnight at 4°C. Followed by a bovine thrombin reaction (25 U/mL, 605157-1KU, MERCK Millipore, Darmstadt, Germany) for 4 h at 37°C, followed by rinsing three times 15 min with PBS-T (PBS with 0.1% Tween-20).

2.4. VIC and VEC isolation and cell culture on BCES

Porcine aortic heart valves from 6-month-old pigs were purchased from a local slaughterhouse (Emil Färber, Balingen, Germany). VICs and VECs were isolated from porcine aortic heart valve leaflets by dispase (Neutral Protease, LS02104, Worthington, USA) and collagenase (Collagenase NB8 broad range, S1745601, SERVA, USA) as previously described by Butcher et al. [22]. Immunofluorescence (IF) staining was used for cell characterization, which indicates no cross-contamination between the distinct valvular cell types of VICs and VECs and cell-material interaction (SI Fig. 1), see SI section 1.3.

For monoculture experiments, BCES punches with a diameter of 10 mm were inserted into 24-well cell crowns (CellCrown™ 12NX, Scaffoldex) and transferred into 24-well cell culture dishes. The punches were then biofunctionalized with different ECM proteins: fibronectin, laminin, collagen type I and fibrin. For VIC monoculture, a total amount of 3×10^4 cells (passage (P) 2–3) per cm^2 were seeded onto the infiltration layer (IL) side of the BCES and subsequently centrifuged (Universal 320, Hettich Zentrifugen, Tuttingen, Germany) at 1000 rpm for 10 min. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) at 37°C and 5% CO_2 for 3 and 6 days. The medium was changed every 2–3 days. As VEC monoculture did not infiltrate into the scaffold, a total number of 2×10^4 cells (P2–3) per cm^2 were seeded on the non-infiltration layer (n-IL) side of FN-biofunctionalized BCES punches. VECs were cultured for 3 and 6 days in endothelial cell growth medium (EM) at 37°C and 5% CO_2 . The medium was changed every 2–3 days.

For the co-culture experiments, both sides of the 10 mm diameter BCES punches were used. Firstly, VICs were seeded on the FN-biofunctionalized IL side as described above and cultured for 4 h in a self-designed cell insert (SI Fig. 2). Then, the self-designed cell insert was inverted and VECs were seeded onto the FN-functionalized n-IL side with EM as described previously. The next day, EM was removed and co-culture medium (DMEM/EM 1:1 ratio (v/v)) was added.

2.5. Analyses of cell-seeded BCES

The morphology of cell seeded BCES was analyzed by SEM with an Auriga® 40 (Zeiss, Oberkochen, Germany). For detailed information on sample processing, see SI section 1.4. VICs infiltration into the BCES was analyzed by measuring z-stacks with a laser scanning microscope (LSM880, Carl Zeiss AG, Oberkochen, Germany), see SI section 1.4 for a detailed processing protocol. The number and position of infiltrated VICs in the z-direction of the IL were analyzed by using the spot detection function of the Imaris® software (64 9.5.1, Oxford Instruments bitplane).

Cell viability was assessed by live/dead staining as previously described [23]. Samples were imaged using a fluorescence microscope (Axio Observer, Carl Zeiss AG, Oberkochen, Germany). VIC metabolic activity on BCES was determined via 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS, CellTiter 96Aqueous One Solution Cell Proliferation Assay, G358A, Promega, Madison, WI, US), which was adapted from the manufacturer's protocol.

Live cell imaging in different medium ratios was assessed by 3×10^3 VICs/well and 1.5×10^3 VECs/well, which were seeded in monoculture in 96 well plates (TC 96 Well, 3595, Corning costar, Germany). The tested medium ratios were DMEM/EM (v/v); 100/0, 90/10, 70/30, 50/50, 30/70, 10/90, and 0/100. The cellular proliferation rate was determined by live cell imaging using the IncuCyte®S3 Live-Cell Analysis System (Essen BioScience, Hertfordshire, United Kingdom). The total cell number and cell viability was analyzed with a NucleoCounter®NC-200™ (ChemoMetec, Allerød, Denmark) based on the manufacturer's protocol. Cell confluence

of 3 replicate wells was determined every hour for the duration of the experiment. The medium was changed every 3 days.

Cell phenotype, cell-cell contact and cell-material interactions were assessed by IF staining as previously described [24]. All images were acquired using a fluorescence microscope (Axio Observer, Carl Zeiss AG, Oberkochen, Germany). Antibodies are listed in the SI section 1.4. Fluorescence images were analyzed with the open-source software Fiji [25] as previously described [24]. For routine histological analysis with hematoxylin-eosin (HE), samples were fixed with 4% PFA (see SI section 1.4), embedded in paraffin, cut in 7 μm thick slices and staining was performed as previously described [26].

2.6. Calcification assay

VICs cultured on FN-biofunctionalized BCES, TCPS (24-well plate) and glass were exposed to an osteogenic medium (OM) consisting of DMEM with 10% FBS, 1% penicillin/streptomycin (P/S, 15070-063) and supplemented with 10 nmol/L dexamethasone (101-B1-001), 50 $\mu\text{mol}/\text{ml}$ ascorbic acid (A4403) and 10 mmol/L beta-glycerolphosphate (G9422) all from Merck (Darmstadt, Germany) [27]. VICs were used in P2-3 and the OM was changed every 1–2 days. DMEM supplemented with 10% FBS and 1% P/S was used as control medium (CM). Alizarin red staining was performed as previously reported [28]. Samples were analyzed using an Axiovert 200M with the adapted camera Axiocam MRC5 (Carl Zeiss, Oberkochen, Germany).

2.7. qPCR using RT2 profiler PCR array

RNA extraction of VICs was performed using TRI-Reagent (Sigma Aldrich) for 10 min and sequentially incubating and centrifuging the lysate with chloroform (Sigma-Aldrich), isopropanol (Carl Roth), and 75% EtOH (Applchem). RNA was resuspended in nuclease-free water, incubated for 10 min at 55°C, and quantified using a NanoQuant Plate and the Infinite 200 Pro (both TECAN). RNA was reverse transcribed to cDNA using the RT2 First Strand Kit (330404, Qiagen). Custom RT2 profiler plates (330171, Qiagen) containing the primers from Qiagen; GAPDH (PASS-000Z), COL1A1 (XM_021067153), ACTA2 (NM_001164650), BMP2 (NM_001195399), RUNX2 (XM_013977989.1), SPP1 (NM_214023) were used to measure the gene expression levels. All genes were normalized to GAPDH expression. qPCR measurements were performed using the Bio-Rad CFX96 system data according to the manufacturer's instructions. Data analysis and quality control was performed using the Qiagen data analysis center online tool.

2.8. Statistical methods

Statistical analyses were performed using GraphPad Prism (Windows, version 9.0.0, San Diego, CA, USA). All results shown in this study are mean $\pm$ standard deviations for $n \geq 3$ specimens. Data sets were tested for normal distribution with the Kolmogorov-Smirnov test, and Grubb's test was performed to identify outliers. Material analyses were assessed with one-way ANOVA/Tukey's multiple comparisons test. Semiquantitative fluorescence intensity and MTS assays were analyzed with a two-tailed *t*-test (normal distribution) and with the Mann-Whitney test (non-normal distribution). Calcification studies were analyzed with two-way ANOVA/Tukey's multiple comparisons test. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Solvent properties allow tailoring of distinct layer morphologies

Conventional electrospinning resulted in a scaffold with ECM morphologies comparable to the native leaflet (SI Fig. 3). A PLA-binary solvent system of trichloromethane (TCM)/ *N,N*-dimethylformamide (DMF) was used to fabricate a microfiber layer referred to as the “infiltration layer” (IL) within this study, which was designed to facilitate VIC ingrowth. The nanofibrous layer, the “non-infiltration layer” (n-IL), was spun using a PLA-single solvent system of 1,1,1,3,3,3-hexafluoro-propan-2-ol (HFP) to facilitate VEC adherence. Scaffolds, which were prepared by using the conventional electrospinning method revealed large pores in IL and small pores in the n-IL (SI Fig. 3). Since the same polymer was used for both polymer solutions and the process parameters were kept constant, the different morphologies between the distinct layers were attributed to the solvents [29]. The conductivity of the pure binary solvent system TCM/DMF showed a 12-fold lower conductivity (0.8 $\mu\text{S}/\text{cm}$) compared with the single solvent system utilizing HFP (10.2 $\mu\text{S}/\text{cm}$) ($p < 0.0001$). The same amount of PLA was dissolved in either the binary solvent system or the single solvent system, significantly decreasing the conductivity of the resulting polymer solutions (binary solvent: 0.8 $\mu\text{S}/\text{cm}$ versus binary solvent in combination with PLA 0.3 $\mu\text{S}/\text{cm}$, conductivity reduced by 62%, $p = 0.015$ and the single solvent: 10.2 $\mu\text{S}/\text{cm}$ versus single solvent in combination with PLA: 7.2 $\mu\text{S}/\text{cm}$, conductivity reduced by 29%, $p = 0.0001$). The viscosity measurement of the distinct polymer solutions (SI Fig. 4, SI Table 3) indicated a shear-thinning behavior for the PLA-binary solvent (16546 mPa·s at 1s^{-1} and 441 mPa·s at 10s^{-1}), and a nearly ideal viscous behavior for the PLA-single solvent (391 mPa·s at 1s^{-1} and 429 mPa·s at 10s^{-1}).

Although different solvents lead to distinct scaffold morphologies with a large pore diameter and with a small pore diameter, these scaffolds were based on a dense fibrillar layer-by-layer morphology that is not ideal for cell infiltration. In contrast, cryogenic electrospinning allowed the generation of a loosely-packed fibrillar layer-by-layer architecture. Fig. 1 shows the process of cryogenic electrospinning with the low-temperature drum, covered by an ice crystal layer and the electrospun BCES (Fig. 1A,B). The n-IL was directly spun onto the IL to generate a bi-layered scaffold. The cross-section of BCES is illustrated in Fig. 1C showing the IL in blue and n-IL in green.

SEM analysis of the cryogenic electrospun IL and n-IL (Fig. 1D–I) revealed distinct fiber morphologies within the bead-free bi-layered scaffold. Since the parameters were kept constant for the cryogenic electrospinning process for both polymer solutions, it can be hypothesized that the solvents and the low evaporation rate are responsible for the distinct morphologies. In detail, due to the void template of ice, the IL exhibited randomly oriented microfibers, which were loosely packed (Fig. 1D). The morphology of n-IL showed a dense nanofibrous membrane covering a self-assembled honeycomb structure (Fig. 1E). For both scaffold morphologies a homogeneous fiber distribution was obtained, whereby the mean microfiber diameter of the IL was 5.3 times larger than the mean nanofiber diameter of the n-IL ($1.6 \pm 0.6 \mu\text{m}$ versus $0.3 \pm 0.1 \mu\text{m}$, $p < 0.0001$ Fig. 1F and G). The interconnected porous structure of the IL was 8.5 times larger than the mean pore diameter of the n-IL ($23.9 \pm 12.1 \mu\text{m}$ versus $2.8 \pm 1.0 \mu\text{m}$, $p = 0.0012$, Fig. 1H and I). Interestingly, when the polymer solution of the n-IL was spun with the conventional electrospinning setup, the self-assembled honeycomb morphology was not obtained (SI Fig. 3). This result indicates a high impact of humidity, the presence of ice crystals, and the low solvent evaporation rate on the generation of a nanofibrillar architecture.

3.2. FN-biofunctionalization increases water storage capacity of BCES

Before cell seeding experiments were conducted, BCESs were disinfected with 70% EtOH, which was followed by a FN-biofunctionalization. The EtOH disinfection did not alter the morphology of the scaffolds (Fig. 2A–D). Untreated and the disinfected BCES showed comparable hydrophobic properties (IL untreated $136.7^\circ \pm 1.3^\circ$ versus disinfected $127.9^\circ \pm 1.5^\circ$ IL, $p = 0.267$; and n-IL untreated $136.3^\circ \pm 1.0^\circ$ versus disinfected $132.0^\circ \pm 0.3^\circ$, $p = 0.487$, Table 1). However, the disinfection significantly increased the water uptake capacity by 64% (untreated $515 \pm 63\%$ versus disinfected $1470 \pm 613\%$, $p = 0.0015$; Table 1, SI Fig. 5A). In addition, the Young's modulus increased significantly after the EtOH disinfection (untreated $16.5 \pm 3.0 \text{ MPa}$ versus disinfected $25.2 \pm 3.3 \text{ MPa}$, $p = 0.0025$; Table 1). Interestingly, the IL alone showed a significantly lower tensile strength and Young's modulus compared with BCES (Table 1, SI Table 4, SI Fig. 5B,C), indicating the n-IL as the load-bearing layer similar to the fibrosa of the aortic leaflet.

FN-biofunctionalization did not alter the BCES pore and fiber diameter (Fig. 2E–H). An increased wettability indicates the expected effect of FN-biofunctionalization, as the hydrophobic IL and n-IL became hydrophilic. Furthermore, FN did not create a film on the bi-layered scaffold, which would close the porous structure, FN rather covered each single fiber (Fig. 2G,H) allowing the pores to be maintained and to absorb a large amount of water, which increased almost 6-fold compared with the untreated BCES (untreated $515 \pm 63\%$ versus FN-biofunctionalized $2948 \pm 527\%$, $p < 0.0001$; Table 1, SI Fig. 5A). A high water uptake was confirmed by the absorption of a water droplet in less than 5 seconds into the BCES, which did not allow the measurement of the contact angle. Our data showing that the BCES absorbs and stores a large amount of water is being highly encouraging as it corresponds to the properties of the native valve spongiosa layer [17].

3.3. BCES morphology is stable after exposure to culture medium and does not delaminate

The exposure of the FN-biofunctionalized BCES to DMEM for 7 days showed stable pore diameter (FN-biofunctionalized $23.0 \pm 11.5 \mu\text{m}$ versus immersed $21.5 \pm 10.8 \mu\text{m}$, $p = 0.357$; Fig. 2E and I). However, the fiber architecture of the n-IL honeycomb walls became more flat when compared with the samples prior to DMEM immersion (Fig. 2J versus Fig. 2F). The Young's modulus of the immersed BCES increased significantly when compared with the untreated BCES (immersed: $41 \pm 9 \text{ MPa}$ versus untreated: $17 \pm 3 \text{ MPa}$, $p < 0.0001$, Table 1).

The tensile strength and Young's modulus of the aortic heart valve differs among species [30–33], as shown in Table 2. The mechanical parameters of BCES before the disinfection and FN-functionalization were in the range of human aortic valves, and in the range of porcine aortic valves and several tissue-engineered heart valve (TEHV) constructs after disinfection and FN-functionalization of BCES (Table 2).

One of the most crucial considerations in manufacturing a multilayer structure is the interface between the individual layers. The adhesion between the two distinct layers, IL and n-IL, of the BCESs was investigated for untreated, disinfected, and FN-biofunctionalized samples after exposure to DMEM for 7 days. No delamination was observed and the strong adhesion between the layers was shown by a single breaking point during the mechanical tensile testing, which is shown in Fig. 3.

3.4. VIC and VEC interact with IL and n-IL of the BCES

The IL is designed to provide high water uptake and to enable infiltration of cells into the bi-layered scaffold. A centrifuga-

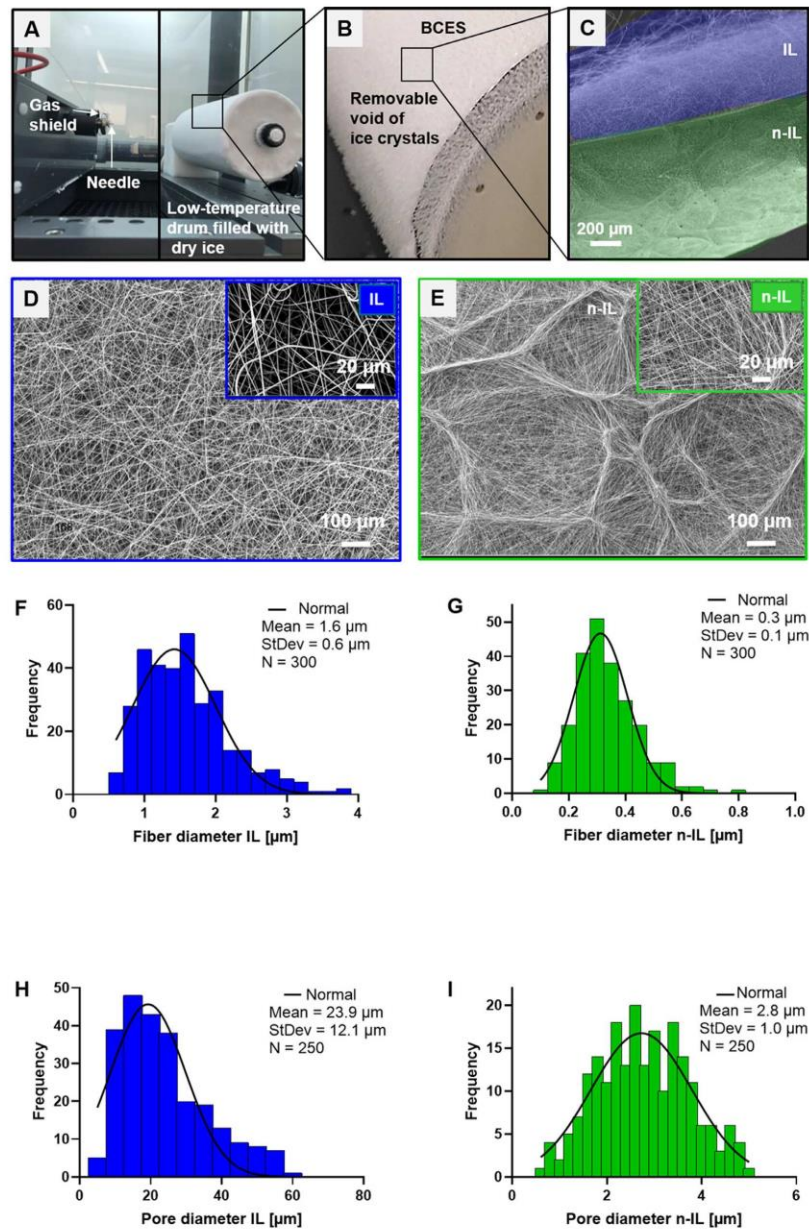


Fig. 1. Distinct layer morphologies show large pores on the IL and small pores on the n-IL of BCES. A) Setup of the cryogenic electrospinning process. B) Rotating low-temperature drum with covered ice crystals and electrospun bi-layered scaffold. C) SEM images of the cross-section of the bi-layered scaffold after lyophilization. D) SEM image of IL shows randomly-oriented and loosely-packed fibers. E) SEM image of the n-IL indicates a honeycomb structure, with a self-closing membrane. F) Fiber size distribution of the IL shows a mean fiber size in the micrometer scale. G) The n-IL shows a mean fiber diameter in the nanometer scale. H) The pore size distribution of the IL shows a mean pore diameter larger than 10 µm. I) The pores size distribution for the n-IL shows a mean pore size diameter less than 10 µm.

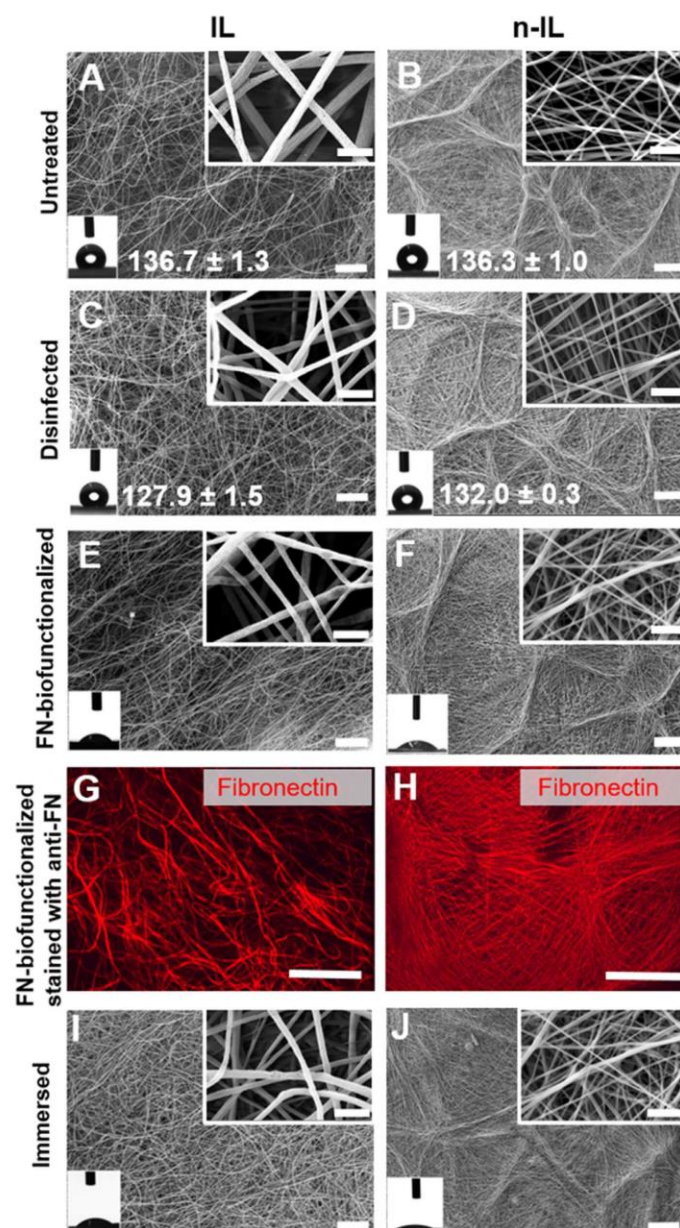


Fig. 2. Effect of treatments on the BCES and immersion effect on the fiber as well as on the pore morphology. A–F) SEM images of A) untreated IL, B) untreated n-IL, C) disinfected IL, D) disinfected n-IL, E) FN-biofunctionalized IL, and F) FN-biofunctionalized n-IL. Immunofluorescence staining (anti-FN antibody (red)) of G) FN-biofunctionalized IL and H) FN-biofunctionalized n-IL. SEM images of I) culture-medium exposed (immersed) IL and J) immersed n-IL. Insets at the bottom left of the images illustrate the contact angle measurements. Non-biofunctionalized scaffolds (A–D) are hydrophobic, and scaffolds after the FN-biofunctionalization (E–J) are hydrophilic. Scale bars equal 100 μm / in the insets 5 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Physical properties of BCES before and after treatments, as well as after immersion in DMEM.

Treatments	Young's modulus	Tensile strength	Water absorption	Contact angle	
	BCES	BCES		IL	n-IL
	MPa	MPa	%	°	°
Untreated	17 ± 3	2.1 ± 0.6	515 ± 63	136.7 ± 1.3	136.3 ± 1.0
Disinfected	25 ± 3 **	2.4 ± 0.5	1470 ± 613**	127.9 ± 1.5	132.0 ± 0.3
FN-biofunctionalized	–	–	2948 ± 527****	†	†
Immersed	41 ± 9 ****	1.8 ± 0.3	–	†	†

Values are mean ± SD. Young's modulus, tensile strength, and water absorption each n=3. Contact angle each n=4. One-way ANOVA *p<0.05 **p<0.01, ***p<0.001, ****p<0.0001 versus untreated. Water absorption after 16 h. – not measured as treatment has no significant influence [44] or the maximum was already achieved in the previous treatment, † not measurable because of the rapid water absorption into BCES.

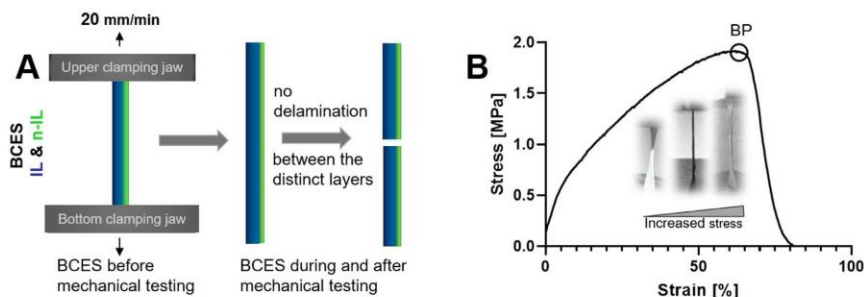


Fig. 3. Strong adhesion between IL and n-IL prevents delamination of the BCES under mechanical stress. A) The IL and n-IL of BCES show no delamination after tensile testing. B) The stress-strain curve of BCES indicates a single breaking point (BP) demonstrating a strong adhesion between the two layers.

Table 2
Comparable data of the mechanical properties of native human and porcine valves, TEHV, and BCES.

Native valve	Young's modulus [MPa]	Tensile strength [MPa]	Reference
human	15	2.6	[31]
human	15.3 ± 3.8	1.7 ± 0.3	[32]
porcine	28.0	–	[32]
porcine	42.3 ± 4.9	1.6 ± 0.3	[33]
TEHV	Young's modulus [MPa]	Tensile strength [MPa]	Reference
PLLA	28.0	0.2	[36]
PCL	46.6	2.5	[36]
PLA	64.1 ± 6.5	2.0 ± 0.2	[21]
BCES	Young's modulus [MPa]	Tensile strength [MPa]	
Untreated	16.5 ± 3.0	2.1 ± 0.7	–
Disinfected	25.2 ± 3.3	2.4 ± 0.5	–
Immersed	40.7 ± 8.7	1.8 ± 0.3	–

tion step immediately after the seeding of VICs improved the infiltration. We analyzed whether the centrifugation step had an impact on VIC viability. Live/dead staining (Fig. 4A) and an MTS assay (Fig. 4B) showed comparable results between the control and centrifuged cells and confirmed the healthy state of centrifuged VICs (control 100 ± 24 versus centrifuged 92 ± 16, $p = 0.440$, Fig. 4B). The reduction of MTS to colored formazan by VICs demonstrated a homogeneous distribution of cells without an accumulation of cells on the surface or outer edge of IL for the control and centrifuged samples after 1 day of culture (SI Fig. 6).

The ingrowth depth of VICs into the biofunctionalized BCES was analyzed after 3 and 6 days of culture. F-actin staining showed a spread cell morphology of VICs and served as the first indicator for the well-being of the cells in the z-direction (Fig. 4C). Anti-FN staining and DAPI was utilized to assess VIC distribution throughout the IL (Fig. 4C). Using the DAPI signal, we determined the distribution of VICs in z-direction (Fig. 4D). After 3 days of culture, the

maximum depth of VIC ingrowth in the z-direction was observed at 70 µm and at 100 µm after 6 days of culture (Fig. 4E). Based on the significantly increased number of cell nuclei counted from day 3 to day 6 (536 ± 102 versus 1242 ± 227 , $p = 0.0044$, shown in Fig. 4E), we hypothesize that after the infiltration of VICs into the IL of the BCES, both VIC proliferation and VIC migration occurs in x- and y-direction as well as in deeper sections of the z-direction. Overall, VICs showed good cell adherence at the IL surface and homogenous migration and proliferation within the z-direction. Furthermore, the DAPI signal of the cross-section of VECs seeded on n-IL of the BCES (Fig. 4F) showed no infiltration of VECs into the bi-layered scaffold (Fig. 4G).

F-actin staining of the VIC monoculture indicated an elongated fibroblast-like morphology (Fig. 5A–B). SEM images confirmed the typical spindle-like shape and showed homogeneously adhered cells on the IL, which were able to spread over the large porous surface (Fig. 5C). After 6 days of culture, a confluent VIC layer was formed (Fig. 5D). Also, VICs infiltrated into the underlying fiber sections (Fig. 5E, green arrow). Moreover, the formation of filopodia indicated cell-cell contacts and VIC-IL interaction (Fig. 5E, red arrows). H&E staining showed a consistent ingrowth of VICs into deeper sections of the microfibrillar IL (Fig. 5G–H).

After culture of VECs on BCES for 3 and 6 days, the VEC phenotype was confirmed by antibody staining for PECAM1 (Fig. 5I–J). Typical cobblestone-like VEC growth patterns were identified especially after 6 days of culture (Fig. 5J). SEM images confirmed VEC adherence and spread morphology after 3 days of culture (Fig. 5K), and a confluent cell layer was seen after 6 days of culture (Fig. 5L). Filopodia formation was observed along the electrospun fibers, which confirmed VEC-n-IL interactions (Fig. 5M, red arrow), and between the cells VEC-VEC contacts were seen (Fig. 5N, red arrow). H&E staining confirmed the adherence of the cells on the nanofibrous n-IL surface and that the cells did not grow into deeper regions of the bi-layered scaffold (Fig. 5O–P).

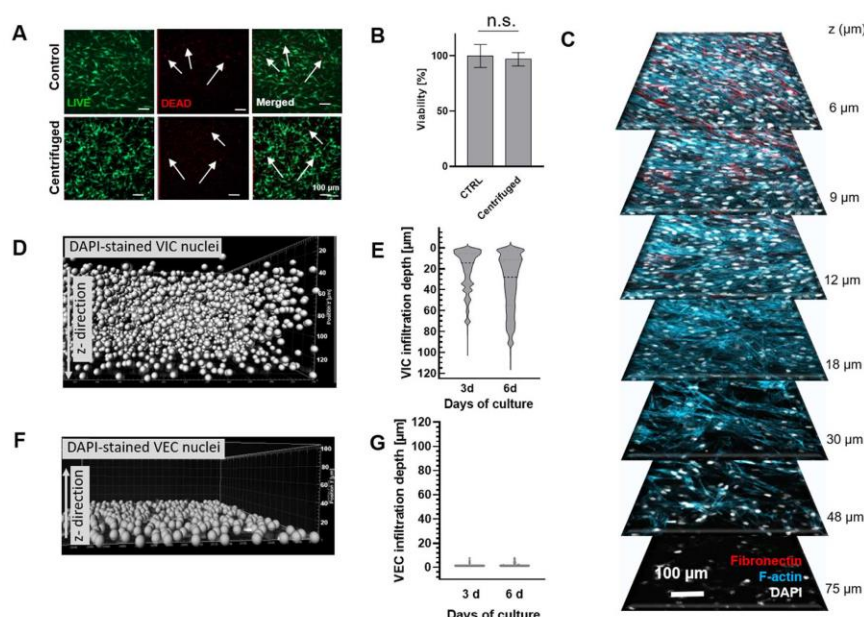


Fig. 4. The loosely packed and interconnected porous IL enables VIC infiltration and migration through the layer. A) Live/dead staining of control and centrifuged VICs cultured on BCES after 1 day of seeding, white arrows indicate dead cells, $n=3$. B) Viability of control and centrifuged VICs was comparable, two-tailed t-test, mean $\pm$ SD, $n=3$, $p<0.05$, n.s. = not significant. C) z-stack of infiltrated VICs in the BCES of a representative sample measured after 6 d of culture, showing fibronectin (red), F-actin (blue) DAPI (white). D) Cross-section of infiltrated VICs into IL after 6 days of culture, shown by the detection of the DAPI signal. Ingrowth depth and distribution of VICs were determined by counting the DAPI signal using the spot function algorithm of Imaris®. E) VICs reached a maximum ingrowth depth of 70 μ m after 3 days and 100 μ m after 6 days of culture, $n=4$. F) Cross-section of VECs seeded on n-IL after 6 days of culture. G) VECs adhered to the n-IL and did not infiltrate into the BCES, $n=4$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Overall, both valvular cell types, VICs and VECs, homogeneously adhered and proliferated on the respective side of BCES within 6 days (Fig. 5).

3.5. VIC and VEC co-culture on BCESs mimics the native valvular cell distribution

A 3- and 6-day co-culture of VICs and VECs was realized by utilizing a self-designed cell insert (SI Fig. 2) that allowed cell seeding on both BCES sides. The co-culture on BCES is referred to as the aortic heart valve co-culture (AHVCC) model and the co-culture medium was adapted for both cell types to ensure growth and cell viability (SI Fig. 7). F-actin expression of VICs confirmed a typical elongated fibroblast-like morphology (Fig. 6A-B). PECAM1 expression confirmed the VEC phenotype and typical cobblestone morphology (Fig. 6C-D). Representative SEM images showed cell adherence and cell spreading of VICs on the IL (Fig. 6E) and VECs on the n-IL (Fig. 6G) after 3 days of culture. Both cell types proliferated well and formed a confluent cell layer on the BCES at 6 days of culture (Fig. 6F,H). Moreover, H&E and IF staining of cross-sections showed an overall physiological histoarchitecture of the AHVCC (Fig. 6I-L). However, the embedding and cutting process of the cell-seeded BCES samples must be further improved as the temperature treatment during the paraffin embedding lead to a shrinking of the scaffold, and the cutting process of the samples lead to the separation of single sections, as it can be observed especially in Fig. 6I.

In addition to the static co-culture performed in the cell insert, we also assessed feasibility of the AHVCC model in a perfused mi-

crofluidic chip (SI Fig. 8). In this system, VECs seeded on the n-IL of the BCES were continuously perfused in the upper channel of the microfluidic chip with a laminar flow. In contrast, VICs seeded on the IL side of the BCES inserted in the lower channel were indirectly supplied with medium and essential nutrients by the upper flow.

To further investigate the valvular cell response to BCES in the AHVCC model, we compared the protein expression of cell-cell contact, cell-BCES interaction, and cell phenotype-specific markers between the monoculture and the co-culture of VIC and VEC. The adhesion markers vinculin (Fig. 7A1-B3) and FAK (Fig. 7C1-D3), as well as the cell-cell contact marker β -catenin (Fig. 7E1-F3) were comparably expressed in both VIC and VEC monocultures as well as in the AHVCC model. VICs expressed the phenotype-typical marker α -SMA (Fig. 7G1-G3) and vimentin (Fig. 7I1-I3) in the monoculture and the AHVCC model. The expression of the endothelial cell-specific marker PECAM1 (Fig. 7H1-H3) confirmed the phenotype of VECs, although the protein expression was significantly reduced in the 3D model (monoculture 1.6 ± 0.2 versus AHVCC model 1.0 ± 0.2 , $p = 0.035$, Fig. 7J3). Vimentin expression of VECs was significantly increased in the 3D model compared to VEC monoculture (monoculture 0.6 ± 0.1 versus AHVCC model 2.2 ± 0.3 , $p = 0.011$, Fig. 7J1-J3). While VICs showed a comparable proliferation profile in the monoculture and the AHVCC model (Fig. 7K1-K3), the cell number in the VEC monoculture was significantly increased compared to the 3D model (monoculture 31.6 ± 7.6 versus AHVCC model 22.4 ± 3.4 , $p = 0.045$, Fig. 7L1-L3).

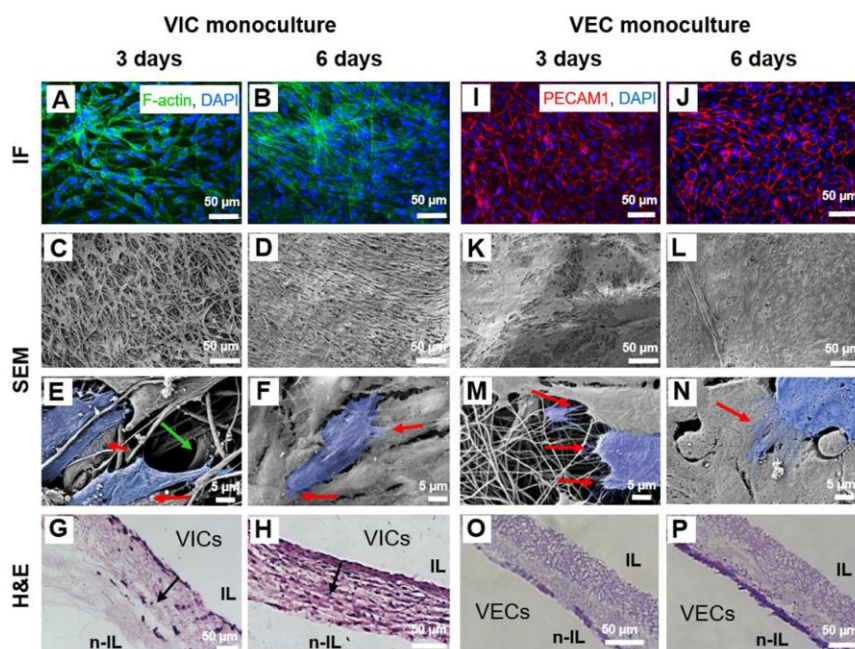


Fig. 5. The biofunctionalized layers of the BCES fulfill their function to enable infiltration of VICs and adherence of VECs. A–B) F-actin expression of VICs indicates an elongated fibroblast-like morphology. C–F) SEM images show a homogeneous adherence on the FN-biofunctionalized IL side of the BCES. E) The infiltration of VICs into the IL was observed (see green arrow). The formed filopodia (red arrows) (E, F) of the VICs indicate VIC–VIC contact and VIC–IL interactions. G–H) Histological analysis shows the migration of VICs into the microfibrillar IL. I–J) VECs express PECAM1 and show a typical cobblestone cell morphology. K–N) SEM images show a homogeneous adherence of VECs to the n-IL of BCES and the formation of filopodia (see red arrows (M, N)) indicating VEC–VEC contact and VEC–n-IL interaction. O–P) Histological staining displays VEC adherence on the nanofibrous n-IL, whereas VEC migration into the IL after 3 and 6 days of culture was not observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Bi-layered AHVCC model is appropriate to study calcification processes under more physiological conditions in 3D

We demonstrated that BCES did not delaminate in VIC and VEC co-culture, even after a culture period of 4 weeks, making it suitable for long-term culture (SI Fig. 9). It has previously been shown that the activation of VICs towards a myofibroblast phenotype can be initiated with an osteogenic medium, which includes dexamethasone, ascorbic acid and β -glycerophosphate (DAG) [34]. We tested the osteogenic potential of the OM in our VIC monoculture on stiff 2D substrates as it was described in the literature [35]. VICs were cultured on stiff FN-biofunctionalized glass (Fig. 8A,D) and TCPS (Fig. 8B) substrates. The functionality of OM in the 2D model was demonstrated by the typical calcific nodule formation and alizarin red staining (Fig. 8A,B). The formation of nodules in the VIC monoculture was also observed on the FN-biofunctionalized 3D BCES (Fig. 8C and E). VICs cultured on BCES that was biofunctionalized with other ECM proteins such as collagen type I, fibrin and laminin also showed the typical nodule formation and positive alizarin red staining (SI Fig. 10). qPCR analyzing gene expression patterns of VICs from these 3D BCES cultures in OM confirmed the upregulation of run-related transcription factor 2 (RUNX2) (SI Fig. 11A–C). VICs on ECM protein-biofunctionalized TCPS in OM also showed an increased expression of RUNX2 and osteopontin (SPP1) (SI Fig. 11D–F). Interestingly, in the FN-biofunctionalized 3D BCES cultures in OM, the nodules had a diameter of around 10 μ m compared with those on the stiff 2D substrates with a diameter of

around 200 μ m (2D glass $203 \pm 49 \mu$ m and 2D TCPS $197 \pm 30 \mu$ m versus 3D BCES $9 \pm 7 \mu$ m, $p < 0.0001$).

We also compared VIC α -SMA expression in the 3D BCES cultures and on the 2D glass, which is known to enhance the activation of VICs by the high E-modulus [36]. α -SMA expression of VICs cultured on glass was significantly increased compared to VICs seeded on BCES in CM and OM after 3 days of culture (CM: glass 23.2 ± 6.3 versus BCES 7.2 ± 1.8 , $p < 0.0001$; and OM: glass 25.5 ± 3.1 versus BCES 8.2 ± 3.1 , $p < 0.0001$; Fig. 8F,G).

To verify whether we could induce the complex osteogenic process in our AHVCC model, we cultured the 3D co-culture construct for 4 weeks in CM or OM (Fig. 8H and I). The 3D model showed nodule formation under exposure to OM in VICs but less in CM, and less in VECs (Fig. 8H, red-colored), similar to what had previously been reported [37]. Statistical analyses confirmed a significant increase in spherical nodules in OM-exposed VICs in the AHVCC model (VICs+CM 0.3 ± 0.1 versus VICs+OM 2.0 ± 0.4 , $p = 0.0006$, Fig. 8I).

4. Discussion

In this study, we combined the beneficial properties of microfibers and nanofibers to design a BCES suitable for an AHVCC model that mimics the function of the fibrosa and spongiosa of a native aortic valve in order to allow simultaneous VIC and VEC co-culture studies. To the best of our knowledge, we present, for the first time, an innovative and simplified process to create a bi-layered 3D scaffold with the technique of cryogenic electrospinning

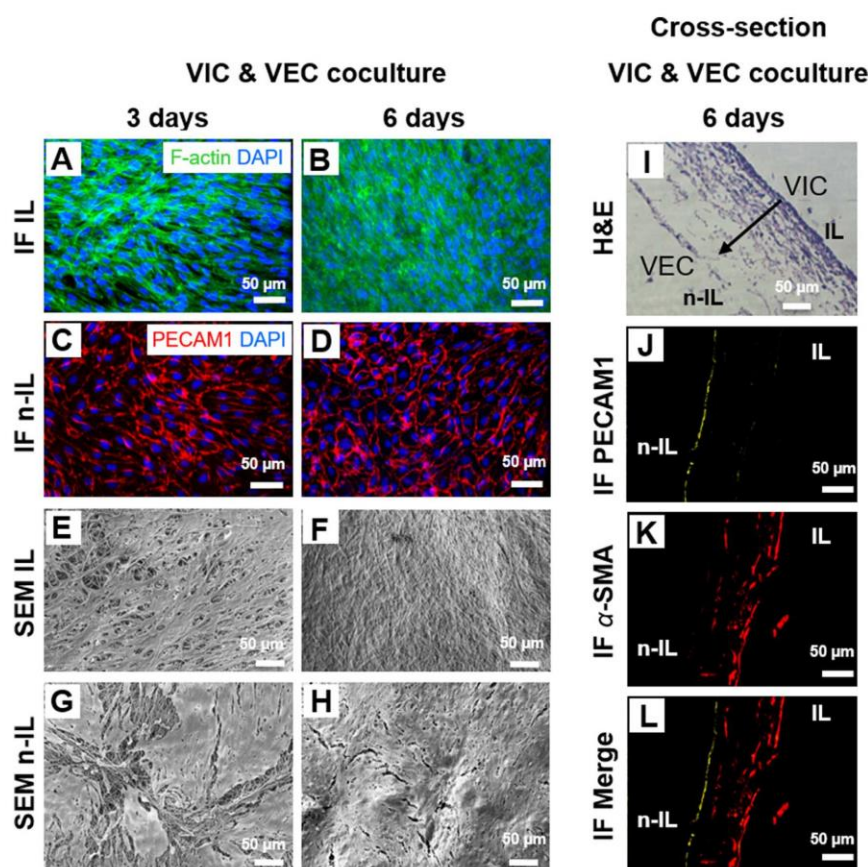


Fig. 6. AHVCC model of VICs and VECs co-cultured on BCES. A–B) F-actin expression of VICs reveals an elongated fibroblast-like morphology. C–D) PECAM1 antibody staining reveals the typical cobblestone morphology of VECs. E–F) SEM images show the successful adherence of VICs on IL. G–H) SEM images highlight VEC adherence on the n-IL. I) H&E staining of the AHVCC cross-section shows the infiltration of VICs into the IL and adherence of VEC on the n-IL. J–L) IF staining of cross-sections of the AHVCC model: J) PECAM1, K) α -SMA, and L) merge of PECAM1 and α -SMA.

by using one kind of PLA to produce distinct fiber and pore morphologies without changing the process parameters.

For this purpose, an understanding of the solvents commonly used in electrospinning was essential. TCM and HFP are known to dissolve PLA based on their Hansen solubility parameters [38,39]. The combination of TCM with the co-solvent DMF stabilizes the electrospinning process by increasing the number of charge carriers, which reduces the surface tension and leads to a lower rate of bead formation and smoothing of the electrospun fibers [38]. The selected PLA concentration ensures the production of bead-free fibrillar scaffolds for both polymer solutions [40].

In our study, the microfiber formation of the IL can be attributed to the low charge carrier concentration of the polymer solution and its low dielectric constant, which causes weak electrostatic repulsion. The surface tension is therefore high and does not elongate the fiber jet, which leads to an increased fiber diameter. Also, the viscoelastic behavior of the polymer solution reduced the mobility and thus the rapid fiber formation. Both, the high viscosity and the low conductivity of the polymer solution increased

the time of flight of the polymer fiber, which led to a higher solvent evaporation and to fibers in the scale of micrometers, which were randomly distributed on the collector [41]. The IL morphology seen in our study is similar to previously reported cryogenic electrospun scaffolds in the literature [19,42–44].

HFP is known to generate fibers in the nanometer range [45], which can be enhanced by the supplementation of salt. The added salts produce a high surface charge density leading to electrostatic repulsion on the jet surface and reduction of the surface tension, which results in an elongated jet during the electrospinning process and fibers in the scale of nanometers [46]. Also, the low viscosity of the PLA solution enables quick fiber formation [41]. In our hands, the low viscosity and the more conductive polymer solution reduced the time of flight and the fibers attached to the collector in a moist state. In combination with the high humidity of 52 % during the electrospinning process and the ice crystals layering on the drum, the nanofibers stayed moist and enabled the formation of the self-assembled morphology, which is referred to as honeycomb structure in the literature [47]. The pro-

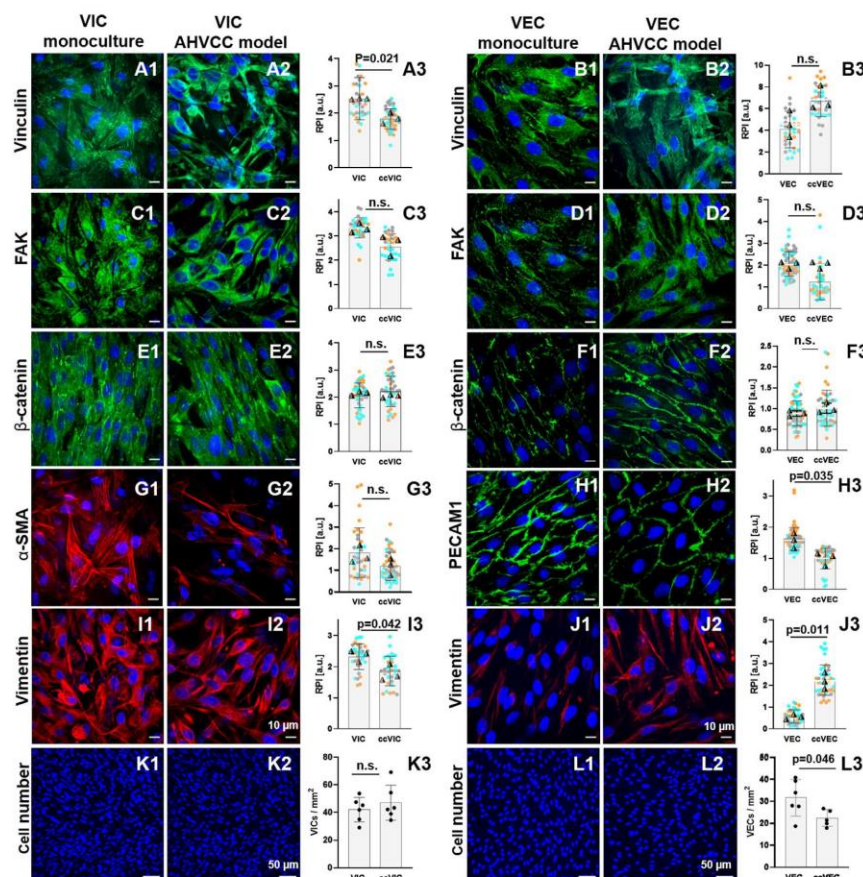


Fig. 7. VIC and VEC response to BCES in monoculture and in the AHVCC model analyzed by the fluorescence intensities of protein expression. Cell-cell contact (A1–B3, E1–F3), cell-material interaction (A1–D3), and cell phenotype-specific markers (G1–J3) are shown. A significant decrease of PECAM1 (H1–H3) and a significant increase of vimentin expression (J1–J3) was observed in the AHVCC culture when compared with VEC monocultures, $n = 3$, scale bar 10 μm . (K1–L3) VIC and VEC cell numbers assessed by DAPI via relative pixel intensity (RPI), and plotted as mean values of $n=6$, scale bar 50 μm . Two-tailed t-test, means $\pm$ SD, p -values < 0.05 are significant, n.s. = not significant.

cess of self-assembled morphology has already been studied and described [47–49]. The honeycomb walls grow upwards as newly arriving positively charged fibers are electrostatically attracted to the negatively charged fibrous walls [38,39]. After reaching a critical value of height to diameter ratio, the self-assembled honeycomb structures are covered with a nanofibrous membrane by a self-closing process that hinders cell infiltration. The longer the spinning time, the less apparent is the honeycomb structure [49].

We showed that the two distinct layers of the BCES did not delaminate, even after 4 weeks of co-culture (SI Fig. 9). To accomplish the stable bi-layer, we used a single polymer type for both polymer solutions to circumvent the influence of the molar mass. Furthermore, the nanofibers of the n-IL resulted in a higher density of fiber per unit area, which increased the van der Waals attraction and led to a stronger adhesion between both distinct electrospun layers [40]. The cryogenic electrospinning process itself may also have supported the strong adhesion between both layers. At low temperatures and a high humidity, the vapor pressure of the polymer solution and the residual solvent on the nanofibers de-

celerated fiber solidification, which enhances the time to build up adhesion forces among the distinct fibers. Also, the residual solvent may have partially dissolved the surface of the first fibers of IL, which would increase the interfacial adhesion between the IL and n-IL during the solidification process [41]. Furthermore, we observed that FN, the intensively studied component of the ECM [50], coated the single fibers and reduced the hydrophobicity of PLA [21,51], which significantly supported the water uptake of the BCES and provided a biological binding domain for the valvular cells via the RGD motif [50]. We demonstrated that the mechanical properties of BCES were similar to the native valve [33] by taking advantage of the nano- and microstructures.

In our study, we aimed to design a 3D in vitro model for future use as disease model, particularly for calcification studies, and therefore one main focus was on the fibrosa since it is the starting point of calcification. We designed the n-IL with nanofibers, which increased the density of the fiber network and generated a high mechanical performance. Thus, the n-IL represents the load-bearing layer of the BCES, similar to the fibrosa, which compen-

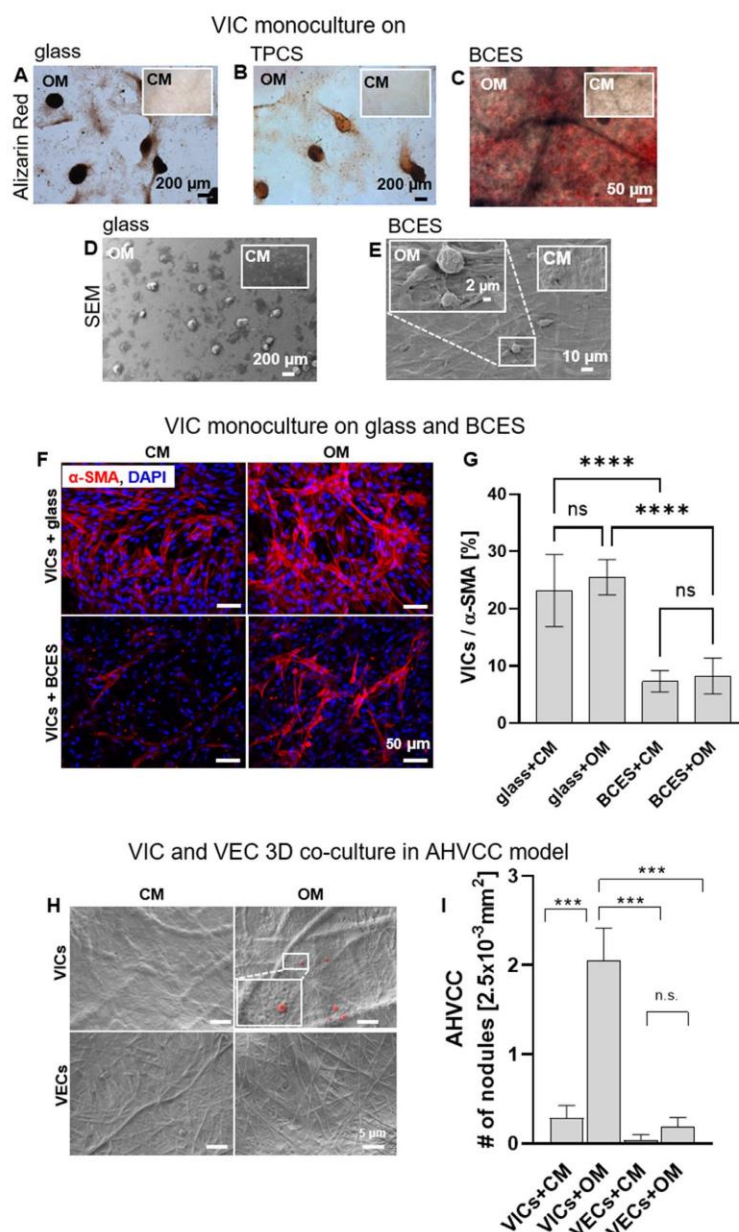


Fig. 8. Comparison of the 2D monoculture and 3D co-culture calcification model. VICs cultured for 4 weeks in OM on FN-biofunctionalized A) glass, B) TPCS and C) BCES (inserts show VICs in CM) stained with Alizarin Red and SEM images of D) glass and E) BCES. The formation of nodules and positive Alizarin Red staining indicates activated VICs. F) VICs on 2D glass and 3D BCES, stained with α -SMA (red) and DAPI (blue). G) Statistical analysis, each group n=3. H) SEM images of VIC and VEC in the AHVCC model cultured for 4 weeks in CM and OM. Nodules (red-colored) were observed under osteogenic culture conditions. I) Statistical analysis, each group n=3 Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005, n.s. = not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sates for the weak mechanical properties of the IL. The design of the IL aimed to support the infiltration and migration of VICs to generate a cell reservoir and store a large amount of water to mimic the cell distribution and function of the native spongiosa [53]. Simonet et al. [54] reported the ingrowth of human vena saphena cells into a loosely packed 700 μm cryogenic electrospun scaffold, which agrees with our findings of cell infiltration into the IL.

Simultaneous cell culture on both sides of a scaffold has not been feasible until now and was achieved on both sides of the BCES by utilizing a self-designed cell culture insert. Previous applications included the seeding of one cell type in a well plate and the seeding of the other cell types in a cell insert that is clamped into the well. However, the problem with this is that both cell types are not experiencing the same substrate morphology, which could have a major impact on the study. The AHVCC model presented here overcomes such co-culture limitations and resembles a more native cellular distribution similar to the one in an aortic valve leaflet. We observed that VECs formed a single endothelial layer, and the endothelium was demonstrated by the expression of PECAM1, which is an essential component of the EC junctions [55]. In contrast, VICs were detected within the entire IL, and after 4 weeks of culture, VICs were also found in the first fibrillar layers of the n-IL (SI Fig. 9). Both vimentin and α -SMA antibody staining confirmed the presence of VICs. The positive cell response to BCES was shown by the expression of vinculin, FAK, and β -catenin. VECs adhered on the n-IL and assembled to a monolayer without cell infiltration into the scaffold, reflecting the behavior of healthy, “physiologically normal” VECs. A limitation of this study is that diseased VECs were not assessed for their ability to infiltrate the n-IL. However, Leong et al. [52] showed in a previous study that fibroblasts can invade the first sections of an n-IL. Since the n-IL of the BCES in this study had a low thickness of less than 50 μm , comparable to the study by Leong et al., and diseased VECs also develop the disposition to infiltrate into tissue just like (myo)fibroblasts, we hypothesize that they should tend to invade the n-IL after they adapt the diseased state, which needs to be addressed in a follow-up study.

VICs are the predominant cell type in the aortic heart valve leaflet and are arranged in sub-populations. A healthy aortic heart valve contains predominantly quiescent VICs [5]. The phenotype differentiation into activated VICs can initiate the onset of disease and could be triggered by the environment. One such trigger is a high Young's modulus of the substrate on which the cells are seeded. The high stiffness alters the cellular function and ends up in contraction-mediated nodule formation [36,13]. Glass, with a Young's modulus of 60 GPa [56], and TCPS, with a Young's modulus of 1 GPa [57], increased α -SMA expression, which is a key marker for VIC activation [14,15,34,58–60]. Quinlan et al. [61] investigated the relationship between material stiffness and phenotype response of porcine aortic VICs and showed a significant effect of material stiffness on VIC activation and cell morphology changes [58]. Studies by Wyss et al. [62] using collagen matrices and Wang et al. [63] using PD-PEG hydrogels confirmed the findings that porcine aortic VICs are activated by an increased substrate stiffness. These studies are consistent with our findings that the substrate environment has a strong impact on VIC activation. We demonstrated that VICs on the BCES maintained the more quiescent VIC phenotype, avoiding material-induced pathological changes when compared with the culture on 2D stiff glass (Fig. 8D), which is routinely used in cell culture for *in vitro* experiments. Therefore, the BCES and co-culture AHVCC model allowed the generation of more realistic *in vitro* environments to study physiological and pathophysiological processes.

Although we observed the formation of calcific nodules in the AHVCC model, these nodules differed in size and number when

compared to the stiff 2D models. The nodules in the 3D AHVCC model were more spherical in shape, similar to what had been reported by Bertazzo et al. as early signs of calcification in a native aortic heart valve [64,65]. One could speculate that additional molecules may be needed when compared to what has been used classically in the OM of the 2D cultures, and which was also used in this study for the 3D AHVCC model, in order to robustly induce calcification in a multi-cellular 3D *in vitro*-engineered tissue. Such molecular cues may come from cytokines such as TGF- β_1 and TNF- α [66]. Wang et al. [13] reported an increased calcification potential in a soft 3D *in vitro* model made of PEG hydrogel through the induction of TGF- β_1 , which is a key mediator for the transition of VICs. The supplementation with TGF- β_1 to OM in our study showed differences in the gene expression profile of VICs, which was more dominant in the 3D BCES samples compared with the TCPS samples. This leads to the hypothesis that 3D BCES, which mimics the mechanical properties of a native heart valve leaflet much closer than TCPS, may be better suited as a platform material for an advanced aortic heart valve *in vitro* test system. Another molecule to increase calcification is BMP-2, which activates Smad and Wnt/ β -catenin signaling [27,67], that could be of interest as culture supplement in future AHVCC models. Further reasons for the reduced calcification seen in our AHVCC model may be due to the co-culture with VECs, which could inhibit calcification based on the secretion of basic fibroblast growth factor 2 (FGF2), nitric oxide (NO), or prostaglandin [42,43,68], which reduce VIC activation in OM by decreasing the expression of stress fibers, as identified by α -SMA, and stabilize matrix synthesis and degradation in 3D [60,37,42,43]. Richards et al. [68] demonstrated the inhibition of the early phase of valve calcification by the inhibitor NO, which also has protective effects on the early calcification events by inhibiting Rho-associated kinase activity via the cyclic guanosine monophosphate pathway [60,68]. It was shown that VECs significantly decreased the formation of nodules in hydrogels when compared with VICs cultured on hydrogels alone [60]. This highlights the importance of the intercellular crosstalk between VICs and VECs, and emphasizes the need to better understand these processes through more realistic (co-culture and 3D) models. Also, the isolated cells themselves can have a major impact on the osteogenic induction. Chen et al. [69] reported a passage-dependent calcification potential of porcine aortic VICs. Osteoprogenitor frequency had decreased to 14.8% at passage two and to 5.8% at passage three. Goto et al. [27] confirm the passage-dependent calcification potential in human aortic VICs. In this study, porcine valvular VICs were used at passages 2–3 for the long-term calcification experiments (monoculture at passages 2–3; co-culture at passage 2). Furthermore, the here presented 3D *in vitro* model facilitated long-term co-culture experiments of VECs and VICs while mimicking the mechanical properties of native aortic valve leaflets, and it provided the ease of adaption through a variety of potential ECM protein functionalization possibilities. Most importantly, fibrillar 3D scaffolds, such as the BCES shown in this study, allow the integration into perfused or dynamic culture systems such as microfluidic on-chip systems and bioreactors in order to investigate disease progression performed under more physiological conditions, which will be necessary for subsequent CAVD studies.

5. Conclusion

Using one material with different solvents and constant cryogenic electrospinning parameters, we generated a multi-fibrillar histoarchitecture that resembles the native fiber structure and mechanical properties of the aortic heart valve fibrosa and spongiosa. The BCES promoted cellular adhesion and infiltration and supported the maintenance of a more physiological, quiescent VIC phenotype, which is crucial when aiming to develop a valvular test

system for studying aortic heart valve physiological or pathological processes, such as valve calcification. First experiments were promising as calcification was inducible in VICs co-cultured with VECs on the 3D BCES in our AHVCC model. Moreover, we identified that mechanical stimulation potentially impacts the calcification behavior, which makes the BCES a promising platform material to study calcification on a soft substrate that can be integrated into perfused and dynamic *in vitro* systems. The future combination of the here presented AHVCC model with biophysical signals in a dynamic system (e.g. microfluidic on-chip systems) will potentially allow to uncover novel molecules and mechanisms that are involved in the aortic heart valve leaflet calcification process, enhancing our knowledge about the disease, and may contribute to develop valve specific diagnostic tools and effective medical therapies for the prevention and treatment in the future.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2021.11.030.

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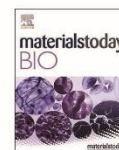
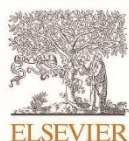
Publication [5]**Breathing on chip: Dynamic flow and stretch accelerate mucociliary maturation of airway epithelium in vitro**

J. C. Nawroth, D. Roth, A. van Schadewijk, A. Ravi, **T. I. Maulana**, C. N. Senger, S. van Riet, D. K. Ninaber, A. M. de Waal, D. Kraft, P. S. Hiemstra, A. L. Ryan, A. M. van der Does. "Breathing on chip: Dynamic flow and stretch accelerate mucociliary maturation of airway epithelium in vitro",

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Abstract:

Human lung function is intricately linked to blood flow and breathing cycles, but it remains unknown how these dynamic cues shape human airway epithelial biology. Here we report a state-of-the-art protocol for studying the effects of dynamic medium and airflow as well as stretch on human primary airway epithelial cell differentiation and maturation, including mucociliary clearance, using an organ-on-chip device. Perfused epithelial cell cultures displayed accelerated maturation and polarization of mucociliary clearance, and changes in specific cell-types when compared to traditional (static) culture methods. Additional application of airflow and stretch to the airway chip resulted in an increase in polarization of mucociliary clearance towards the applied flow, reduced baseline secretion of interleukin-8 and other inflammatory proteins, and reduced gene expression of matrix metalloproteinase (MMP) 9, fibronectin, and other extracellular matrix factors. These results indicate that breathing-like mechanical stimuli are important modulators of airway epithelial cell differentiation and maturation and that their fine-tuned application could generate models of specific epithelial pathologies, including mucociliary (dys)function.



Breathing on chip: Dynamic flow and stretch accelerate mucociliary maturation of airway epithelium *in vitro*

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ABSTRACT

Human lung function is intricately linked to blood flow and breathing cycles, but it remains unknown how these dynamic cues shape human airway epithelial biology. Here we report a state-of-the-art protocol for studying the effects of dynamic medium and airflow as well as stretch on human primary airway epithelial cell differentiation and maturation, including mucociliary clearance, using an organ-on-chip device. Perfused epithelial cell cultures displayed accelerated maturation and polarization of mucociliary clearance, and changes in specific cell-types when compared to traditional (static) culture methods. Additional application of airflow and stretch to the airway chip resulted in an increase in polarization of mucociliary clearance towards the applied flow, reduced baseline secretion of interleukin-8 and other inflammatory proteins, and reduced gene expression of matrix metalloproteinase (MMP) 9, fibronectin, and other extracellular matrix factors. These results indicate that breathing-like mechanical stimuli are important modulators of airway epithelial cell differentiation and maturation and that their fine-tuned application could generate models of specific epithelial pathologies, including mucociliary (dys)function.

1. Introduction

The mucosal surface of the airways continuously removes contaminants from inhaled air by mucociliary clearance, which provides an essential defense mechanism against infection and/or damage of the lungs. This function relies on an ensemble of specialized epithelial cells that use motile cilia to transport secreted mucus, along with trapped airborne matter, out of the airways [1]. Although standard *in vitro* cultures of airway epithelial tissues feature all major cell types after three

weeks of differentiation or earlier, a recent detailed examination of the mechanics of ciliary beat in such cultures showed that full maturity of ciliary beat and associated full maturity of mucociliary clearance may require more than 60 days of differentiation [2]. Moreover, these static culture conditions do not provide experimental control over epithelial differentiation towards the cellular composition characteristic of different airway branching generations. The cellular composition instead emerges based on the use of cell culture medium, donor, and culture protocol [3,4]. These limitations complicate translational value of

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(human) airway epithelial cell models, particularly when studying how infectious agents breach mature mucociliary clearance, or why some disease processes preferentially affect the large or the small airways [5]. It is currently unknown what mechanisms contribute to the tight regulation of mucociliary clearance during development and to homeostasis

in the adult lungs.

In addition, breathing-related mechanical stresses, including stretch and shear stresses act on lung epithelial tissues throughout development and adult life *in vivo* [6], differ in magnitude in large versus small airways [7,8], and can improve airway epithelial barrier function *in vitro* [9].

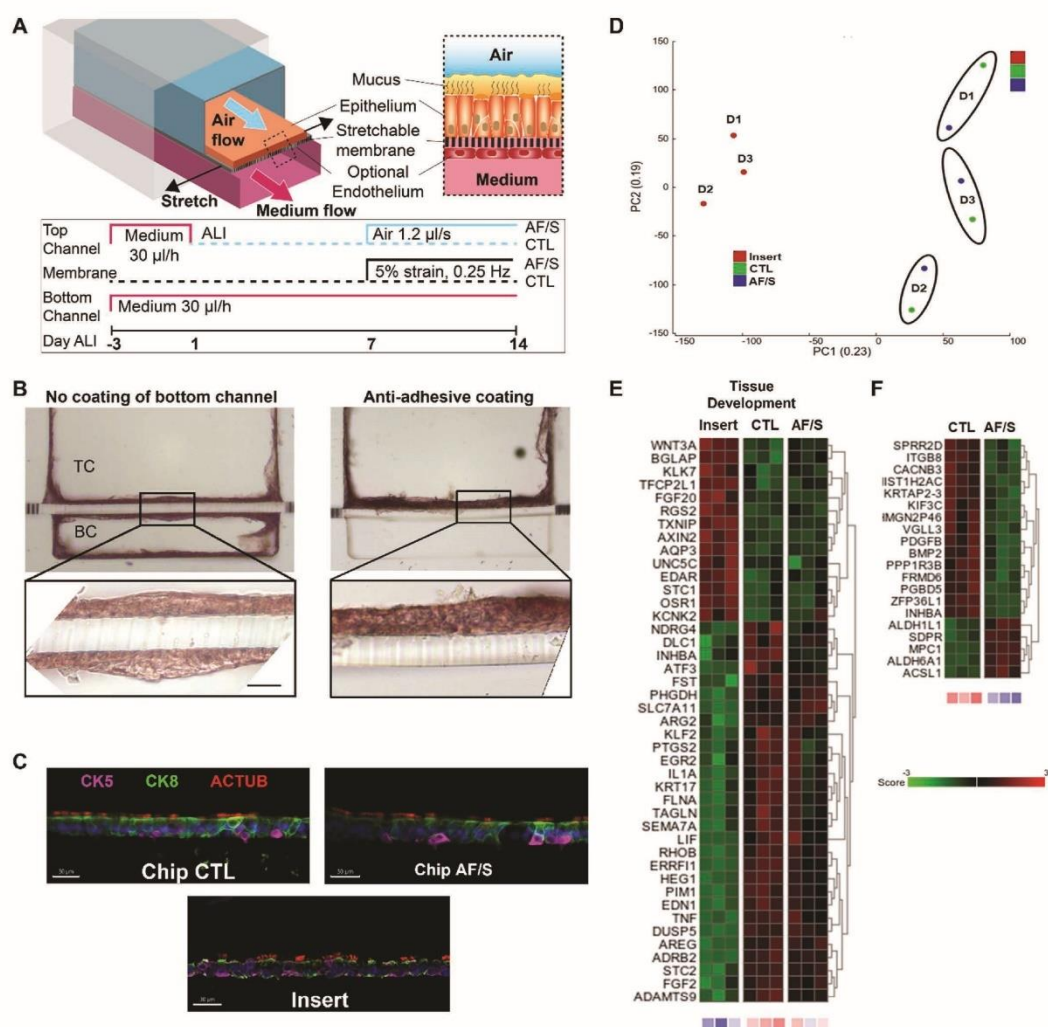


Fig. 1. Dynamic chip culture skews cellular composition of the airway epithelium and accelerates maturation of mucociliary clearance in hPBECs. **A.** Airway basal epithelial cells are cultured in the top channel on a porous stretchable membrane. Endothelial cells can optionally be grown in the bottom vascular channel. Medium flow, air flow and membrane stretch are applied as indicated in the timeline. **B.** Histology cross-sections (H&E stain) of a fully invaded chip with hPBECs growing in both channels on the left, and a Pluronic-treated chip with no invasion of the bottom channel on the right. TC, top channel, BC, bottom channel. Zoom: cells line both sides of the membrane. Scalebar: 50 μ m. **C.** Representative image of chip cross-section of a control (CTL) chip culture, an airflow and stretch-exposed (AF/S) chip culture and an insert culture with stainings for cytokeratin 5 (CK5, basal cells), -8 (CK8), and acetylated alpha-tubulin (ACTUB, cilia). Scalebar: 30 μ m. **D.** PCA on all transcriptomes showing distinct clusters of airway epithelial cells from donors (N = 3) in CTL chips, AF/S chips, or cell culture inserts coated with collagen IV. **E.** Heat map displaying the Z score of DEGs related to the tissue development pathway from Table 2, expression was compared between insert, CTL chip and AF/S chip cultures with N = 3 donors that are paired (one donor per column). The Z scores for individual genes are represented in green and red, while the average Z scores (underneath the heat maps) of all genes in the gene set are represented in blue and red. **F.** Heat map displaying the Z score of all 21 DEGs between AF/S and CTL chip cultures. N = 3 donors that are paired between AF/S and CTL (one donor per column). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Normal breathing creates a diverse mechanical landscape along the respiratory tree. Computational models suggest that breathing-associated cyclic tensile strain tends to increase as a function of branching generation, whereas the shear stress of breathing-associated airflow reduces [7, 8]. *In vitro* alveolar epithelial tissue responds to these physiological cyclic strains by accelerated differentiation of specific cell types [10]. A pivotal study presenting the first alveolar Lung-on-Chip model in 2010 showed benefits for disease modeling in dynamically perfused and cyclically strained alveolar epithelial tissue cultures using cell lines [11]. However, due to a lack of human airway models in which such breathing forces can be mimicked, it remains unclear whether physiological breathing-related forces may also positively impact airway epithelial function. Traditionally used insert culture models are completely static, and the first generation of Airway-on-Chip models, despite other advancements such as perfusion, typically included rigid membranes [12,13], preventing the use of stretch, and lacked dynamic air flow. Here, we hypothesized that normal breathing-associated airflow shear and strain can modulate mucociliary function and development of human primary bronchial epithelial cells (hPBEC).

To test our hypothesis, we developed a robust protocol for differentiation of hPBEC at air-liquid interface (ALI) on a stretchable, porous membrane in an airway-on-chip device. Using this advanced cell culture system, we assessed mucociliary clearance function and differentiation of airway epithelium in response to a dynamic microfluidic environment with application of stretch and airflow shear stress levels that were set to mimic the small airway environment during normal tidal breathing.

2. Results

2.1. Optimization of airway epithelial cell culture on chip

To test our hypothesis, we used the microfluidic Chip-S1® (Emulate Inc.), which features two adjacent, perfusable microchannels separated by a flexible and highly porous poly (dimethylsiloxane) (PDMS) membrane. hPBEC cultured on the apical side of the membrane can be exposed to air while interfacing with the (vascular) bottom compartment that is perfused with medium (Fig. 1A top and Supplementary Fig. 1A). The PDMS membrane is suited for dynamically stretching the epithelial tissue but was found to require extensive optimization of surface functionalization and cell culture protocol for successful hPBEC differentiation. Previously developed protocols for airway chips using cell-culture optimized, but rigid, membranes [12,13], failed to reliably support cellular adhesion on the PDMS substrate in this chip (data not shown). After testing a variety of options, which consisted mainly of various mixtures of collagen I, collagen IV, fibronectin and/or BSA or these compounds alone, we found that coating the activated membrane with high concentrations of collagen IV (300 µg/mL), followed by seeding hPBEC at high density (~300,000 cells/cm²), led to robust cell adhesion and confluent monolayer formation in the top channel. We subjected the hPBEC to dynamic medium perfusion in both top and bottom channel during the submerged culture phase and next switched to air-liquid interface (ALI) in the top channel to initiate differentiation, while medium perfusion continued in the bottom channel (Fig. 1A bottom). The relatively large pore size of the membrane (7 µm) (Supplementary Fig. 1B) stimulated spontaneous and progressive transmembrane migration of hPBEC in the majority of donors, as verified with our newly developed method of chip sectioning (Fig. 1B left and Supplementary Fig. 1C and D). Since bottom channel invasion limited visibility and frequently led to degeneration and delamination of tissue in the top channel, we evaluated a variety of strategies to prevent epithelial cell migration. Chemical cell migration inhibitors and changes to the cell culture medium (Supplementary Table 1) proved to be ineffective or detrimental to differentiation (data not shown), gel barriers were effective but unreliable (Supplementary Fig. 1E and 7), and endothelial cells used as a barrier [14] would require co-culture with endothelial cells which was undesired for this study as it confounds the comparison to

insert culture. We eventually identified a robust solution by functionalizing the bottom channel with the anti-fouling agent Pluronic F-127 [15]. Using this biocompatible and removable anti-adhesion coating, bottom channel invasion was significantly reduced for the cultures from all evaluated donors (Fig. 1B right and Supplementary Fig. 1F), enabling robust hPBEC differentiation as well as optional co-culture with primary endothelial cells at a time point of choice (Supplementary Fig. 2 and Supplementary Videos 1 and 2) and as needed for applications involving vascular and immune cell responses [13].

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.mtbio.2023.100713>

2.2. Comparison between static insert and dynamic chip cultures

Next, to assess the effects of the microfluidic and flexible environment on airway epithelial development, we compared traditional, fully static cell culture inserts with the medium-perfused chip model (Table 1). We evaluated hallmarks of airway epithelial development: pseudotratification and presence of various epithelial cell types [16] at day 14 of ALI [17]. Both chip and insert cultures exhibited a classical pseudotratified layer with basal cells located close to the membrane and luminal cells with visible ciliation (Fig. 1C). We hypothesized that breathing-related mechanical cues may modulate airway epithelial development. To test this hypothesis, we added airflow and stretch to the chip culture and compared epithelial culture development between these airflow/stretch (AF/S) chips, control (CTL) chips with medium perfusion but static ALI, and fully static insert cultures (Table 1). The membrane of AF/S chips was actuated linearly and perpendicular to the channel at a rate of 0.25 Hz to achieve maximally 5% cyclic stretch in the center of the chip. This strain is the estimated physiological stretch in the small airways during tidal breathing at rest [7]. Airflow in AF/S chips was set to generate airflow shear stress of ca. 0.1 mPa on the cells, which is comparable to small airway conditions during tidal breathing [18].

We applied AF/S between day 7 and 14 of ALI (Fig. 1A bottom) to allow for barrier formation before the application of mechanical forces and to provide sufficient time for key cellular markers to change in expression during the course of the experiment [17]. We verified that lactate dehydrogenase (LDH) levels were similar between both chip groups, indicating comparable levels of cell viability (Supplementary Fig. 3A). To compare effects between the various culture conditions on tissue organization and MCC function in hPBECs, we performed an unbiased bulk RNA-sequencing (RNAseq) analysis on RNA isolated from hPBEC in static cultures (inserts) and dynamic cultures (CTL and AF/S chips) from three different donors collected at day 14 of ALI. Principal component analysis (PCA) revealed a pronounced difference between the transcriptional profiles of inserts and chip cultures, and a smaller difference between donors (Fig. 1D). We identified 182 differentially expressed genes (DEGs; q-value <0.05 and >1.5 fold change) between CTL chips and inserts (Supplementary Table 2) and performed pathway analyses (Table 2 with the Top 10 pathways depicted, heatmaps in Fig. 1E and Supplementary Fig. 4). Interestingly, one of the pathways with which the DEGs were associated was tissue development (Fig. 1E). Applying AF/S to the chip cultures shifted the transcriptional profile further. We identified 20 DEGs between AF/S chips and CTL chips

Table 1
Overview of airway epithelial culture conditions.

Culture condition	Cell culture inserts	Medium-perfused chip (CTL-Chip)	Medium-perfused chip with airflow and stretch (AF/S-Chip)
Properties	• ALI • Fully static	• ALI • Static air • 30 µl/h medium flow	• ALI • 1.2 µl/s air flow • 30 µl/h medium flow • 5% membrane stretch at 0.25 Hz

Table 2
Top 10 Pathways associated with DEGs between CTL chip and insert cultures.

Pathway description ^a [# genes]	^b DEGs	ID	p value	q value
Response to oxygen containing compound ^b [1653]	43	GO:1901700	2.76 e ⁻²²	2.87 e ⁻¹⁸
Signaling receptor regulator activity ^b [543]	27	GO:0030545	4.15 e ⁻²¹	2.16 e ⁻¹⁷
Tissue development ^b [1916]	44	GO:0009888	1.03 e ⁻²⁰	3.56 e ⁻¹⁷
Regulation of cell population proliferation ^b [1745]	41	GO:0042127	1.23 e ⁻¹⁹	3.12 e ⁻¹⁶
Molecular function regulator ^b [1953]	43	GO:0098772	1.5 e ⁻¹⁹	3.12 e ⁻¹⁶
Locomotion ^b [1921]	42	GO:0040011	5.59 e ⁻¹⁹	9.7 e ⁻¹⁶
Signaling receptor binding ^b [1550]	38	GO:0005102	8.29 e ⁻¹⁹	1.23 e ⁻¹⁵
Regulation of multicellular organismal development ^b [1394]	36	GO:2000026	1.53 e ⁻¹⁸	1.99 e ⁻¹⁵
Regulation of cell death ^b [1643]	38	GO:0010941	5.73 e ⁻¹⁸	6.62 e ⁻¹⁵
Cell migration ^b [1556]	37	GO:0016477	6.92 e ⁻¹⁸	7.19 e ⁻¹⁵

^a Gene Ontology Biological Process.

^b Gene Ontology Molecular Function.

(Fig. 1F, Supplementary Table 3). Further analysis did not reveal a specific pathway related to these genes; however, several genes related to TGF- β /BMP signaling (i.e., *BMP2*, *INHBA* and *PDGFB*) were reduced in expression in AF/S compared to CTL (Fig. 1F).

2.3. Perfused airway epithelial tissue cultures show accelerated maturation of mucociliary clearance

Further exploring the potential changes in epithelial development, we turned to a central functional marker of mature airway epithelia: mucociliary clearance (MCC). High-speed videomicroscopy revealed a similar surface density and beat frequency of motile cilia in chip cultures compared to static inserts (Fig. 2A and B, and Supplementary Video 3). We next directly assessed MCC function by tracking the displacement of fluorescent microbeads by the ciliated epithelium. Despite the similar beating frequency, we observed markedly better MCC in chip cultures compared to insert cultures. We found that in chip cultures, a significantly higher proportion of the total surface area was cleared by MCC (MCC surface coverage), leading to twice the tissue-averaged MCC speed (Fig. 2C–E, Supplementary Video 4). It was previously shown that insert cultures lack efficient ciliary beat patterns and MCC at day 14 of ALI culture, and that this markedly improves at around day 30 of ALI [2]. We measured MCC in cell culture inserts at day 14 and day 35 and confirmed this improvement (Fig. 2F–G, Supplementary Video 4), ruling out any underlying issues with the insert cultures and instead indicating that MCC function was accelerated on chip. Together, these observations suggest that culturing hPBEC on a flexible membrane combined with dynamic flow provides critical environmental cues that promote functional maturation of multiciliated airway epithelium *in vitro*.

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.mtbio.2023.100713>

We next assessed ciliary beat activity and mucociliary clearance function after AF/S application. We found no significant difference in the density of motile cilia, CBF, average MCC speed, or MCC coverage (Supplementary Fig. 3C to E) between the two chip conditions. We noted that MCC in all chips tended to be aligned along the length of the channel and point towards either end of the chip. Interestingly, while MCC in CTL chips only showed a weak bias towards the inlet, MCC in AF/S chips was commonly directed towards the inlet, i.e., against the direction of the applied air and medium perfusion, as seen in the angle histograms of the fluorescent microbead trajectories for each condition (Fig. 2H). We quantified the polarization of MCC towards either chip inlet or outlet by using a normalized index of direction, the polar order parameter (PO). The PO analysis confirmed unidirectional MCC towards the inlet in AF/S chips, but not in CTL chips, when compared to the null hypothesis of random or perpendicular flow direction (Fig. 2I). Together, our observations suggest that physiological levels of airflow and stretch may have profound effects on *in vitro* airway epithelial ciliary maturation.

2.4. Dynamic airflow and cyclic strain changes *VANGL1* expression related to multiciliated cell maturation

To probe the mechanisms behind the accelerated MCC maturation and polarization in dynamic chip culture conditions (Figs. 1 and 2), we analyzed the expression of genes related to cilia function, e.g. dyneins, in the RNAseq data. Compared to static inserts, chip cultures from two out of three donors showed increased expression levels in this gene set (Genes in Fig. 3A are grouped based on literature [19]). Follow-up qPCR analysis of a selection of these genes using a larger number of donors revealed however no significant changes in the dynein-related genes *DNAH11* and *RSPH4a* (Fig. 3B). We next assessed expression of planar cell polarity (PCP) genes and found that expression of the PCP gene *VANGL1*, which plays an important role in multiciliated cell maturation [2,20], was significantly increased in CTL chips compared to inserts (Fig. 3C). We further investigated *VANGL1* on protein level. IF staining at day 14 ALI showed that localization of *VANGL1* in both chip conditions was asymmetric and appeared in a crescent shape along boundaries of ciliated cells, whereas expression was diffuse with few crescent shapes in insert cultures of same age (Fig. 3D and E) but became comparable to chips at day 35 ALI (Fig. 3F–G). Asymmetric, crescent-like PCP localization to cell boundaries is a key indicator of functional maturation of ciliary beat in airway epithelia [2,20]. Together, these results support that the enhanced MCC functionality on chip might be promoted by accelerated maturation of multiciliated cells.

2.5. Airway epithelial cell composition in static and dynamic conditions

To investigate if the increased mucociliary clearance was accompanied by changes in epithelial cell-type numbers, we assessed cellular composition via gene expression of cell-type marker genes and by immunohistochemistry. Compared to insert cultures, cell cultures in the chip exhibited significantly higher gene expression levels of basal cell markers (*TP63* and *KRT5*) and of keratin 8 (*KRT8*) (Fig. 4A). *KRT8* is a marker gene that is used for describing a population of cells that is variously listed as non-basal [21], luminal [21,22], (suprabasal) early progenitor [23] or basal luminal precursor [24] cells. Due to this lack of consensus in literature, we will address them as *KRT8*⁺ cells. Expression of differentiated luminal cell markers for secretory club cell (*SCGB1A1*), goblet cell (*MUC5AC*) and ciliated cells (*FOXJ1*) was not different from insert cultures (Fig. 4A). IF-staining of cell type-specific proteins confirmed similar levels of differentiated luminal cell in chip compared to insert cultures (Fig. 4B and C).

Compared to CTL chips, AF/S chips expressed significantly lower levels of genetic markers for basal cells (*TP63* and trend for *KRT5* ($p = 0.055$)) and for *KRT8*⁺ cells. Expression of *SCGB1A1*, a marker of club cells, was significantly increased (Fig. 4D). The difference in gene expression may capture an early stage in club cell development as the tissue-level density of immunofluorescent (IF) labeled club cell protein

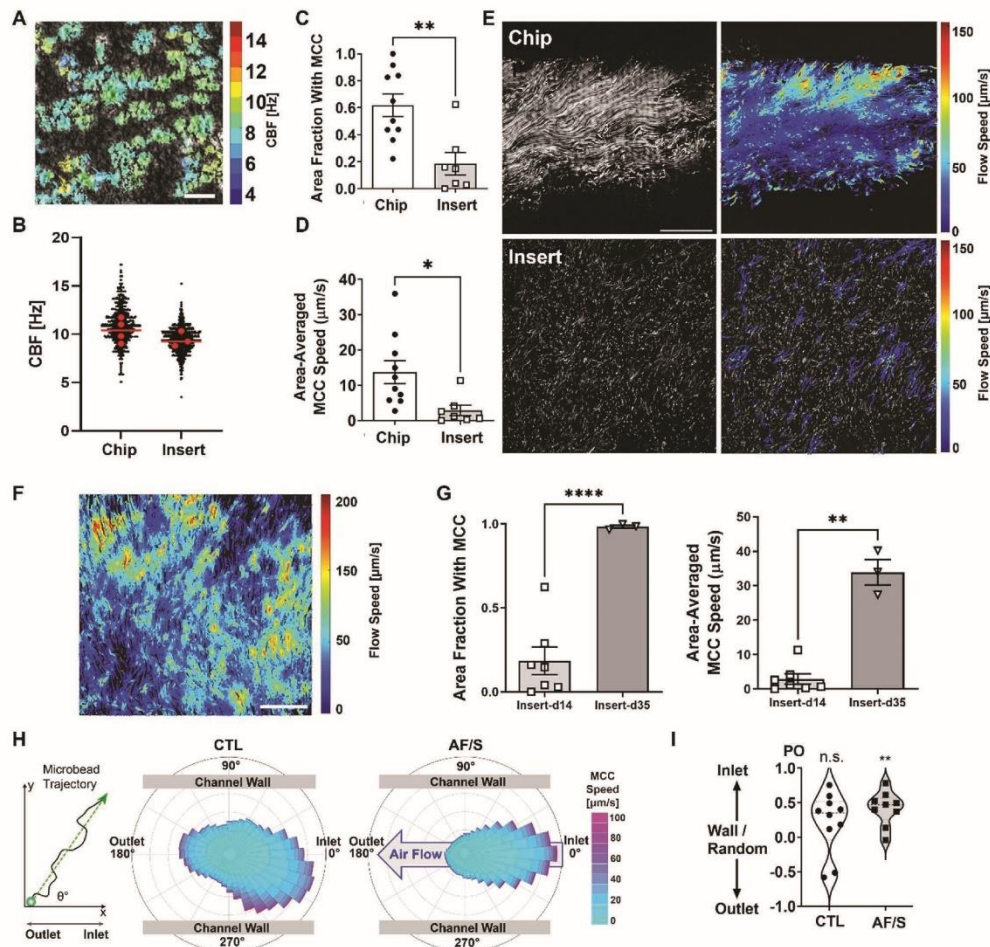


Fig. 2. Exposure to airflow and stretch accelerates mucociliary clearance in hPBEC.

A. Example of CBF heatmap (top; scalebar: 100 μm) and B. quantitative analysis comparing chip and insert (day 14 ALI) cultures. Data from N = 2 donors, 2–4 chips or inserts per donor. Each black dot represents approximately 1 ciliated cell (chips: 2481 measurements; inserts: 2976 measurements). Red dots indicate means of individual inserts or chips and line indicates their median. C. Quantification of area fraction covered by MCC and D. area-averaged MCC speed (right). Depicted are mean $\pm$ SEM of one chip or insert (day 14 ALI) per donor (chips N = 9 donors; inserts: N = 7 donors). *, $p < 0.05$; **, $p < 0.01$; two-tailed Welch's t -test. E. Example of fluorescent bead trajectories (left) and corresponding instantaneous flow speeds (right) in chip (top) and insert (bottom). Scale bar: 50 μm . F. Example of fluorescent bead flow speeds in insert at day 35 ALI. Scale bar: 200 μm . G. Quantification of area fraction covered by MCC (left) and area-averaged MCC speed (right) in inserts at day 14 ALI compared to day 35 ALI. Depicted are mean $\pm$ SEM of 7 inserts at day 14 ALI from N = 7 donors (one insert per donor) and 3 inserts from N = 3 donors (one insert per donor) ** $p < 0.01$, **** $p < 0.0001$; two-tailed Welch's t -test. H. From the overall transport direction of each fluorescent microbead (schematic on left), angle histograms of MCC direction for each condition are created, indicating the flow alignment relative to channel, relative to medium perfusion in bottom channel (from inlet to outlet), and relative to air flow in the AF/S top channel (large arrow, from inlet to outlet). CTL: 140,760 trajectories; AF/S: 119,696 trajectories. Data from N = 9 (CTL) and N = 8 (AF/S) donors, 1 chip per donor. I. Polar order (PO) parameter obtained from the bead trajectories in (e). The PO ranges from -1 to 1, with -1 indicating perfect polarization towards the outlet and 1 towards the inlet, and 0 is random flow or towards the wall. Each data point is PO from 1 chip per donor with N = 9 (CTL) and N = 8 (AF/S) donors. **, $p < 0.01$ as assessed by 1-sample Wilcoxon signed rank test against null hypothesis (random or wall-bound flow with PO = 0). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was similar between groups (Fig. 4E and Supplementary Fig. 3B).

Together, these results indicate that the luminal cellular composition of the epithelium was not dramatically changed in chip cultures compared to inserts, despite a significant improvement in mucociliary functioning.

2.6. Dynamic airflow and cyclic strain reduce expression of inflammation and extracellular matrix-related genes and protein levels in airway epithelial cultures

To assess if other processes were affected by dynamic chip culture

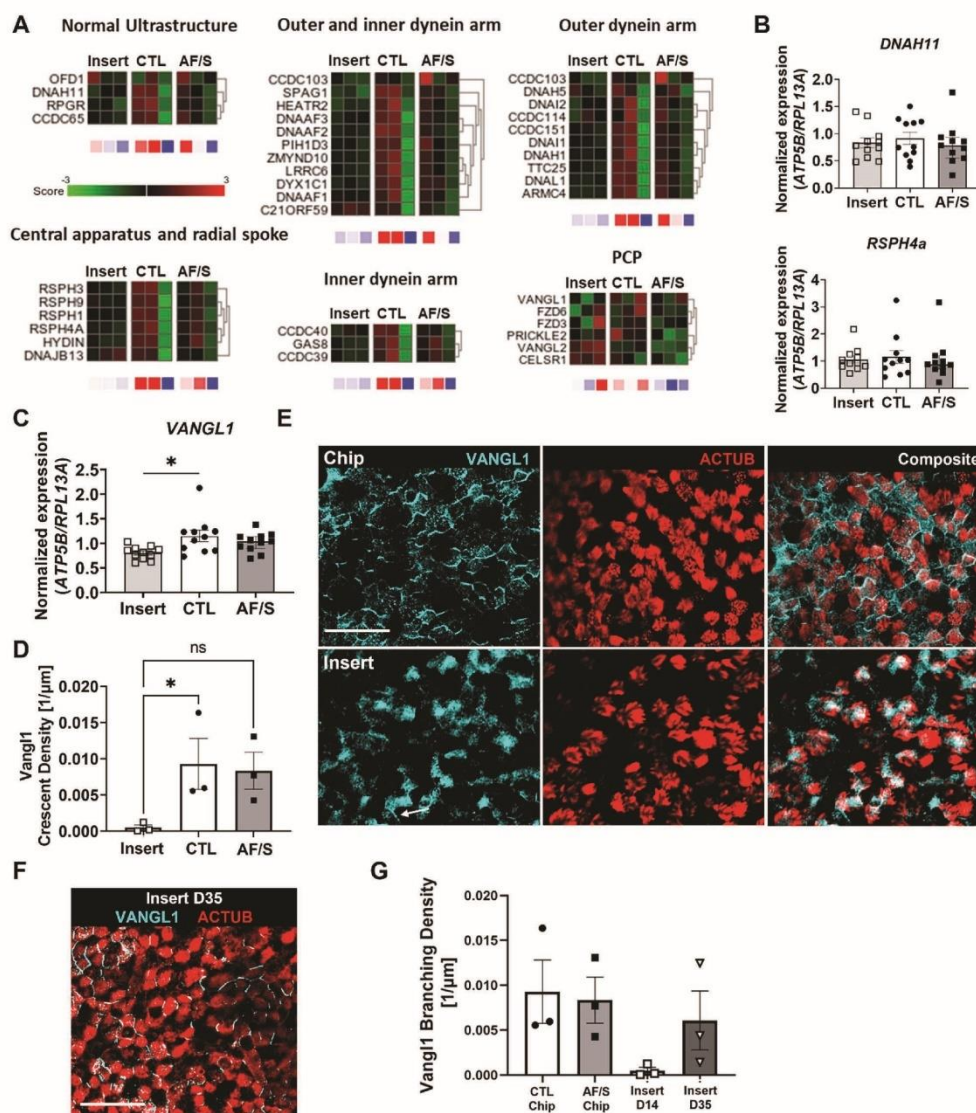


Fig. 3. Culture on chip promotes VANG1 expression

A. Heat maps displaying the Z score of PCP genes between insert, CTL chip and AF/S chip cultures with N = 3 donors that are paired between insert, CTL chip and AF/S chip cultures (one donor per column). B. Gene expression at day 14 ALI of *DNAH11* and *RSPH4a* for cultures on inserts (light gray bars; open squares), chips (open bars; black circles) or chips exposed to AF/S (dark gray; open circles); N = 8–11 donors, one insert or chip per donor. Data are depicted as mean with SEM. C. Gene expression at day 14 ALI of *VANG1* for cultures on inserts (light gray bars; open squares), chips (open bars; black circles) or chips exposed to AF/S (dark gray; open circles); N = 8–11 donors, one insert or chip per donor. Data are depicted as mean with SEM. D. Quantification of VANG1 crescent density in CTL and AF/S chips and inserts at day 14 ALI. N = 3 donors; each point is mean crescent density from multiple FOVs in 1 chip per donor. *, $p < 0.05$ as assessed by one-way paired ANOVA with Dunnett's multiple comparison test. E. Representative IF staining of VANG1 at day 14 ALI. Distinct VANG1 crescents line most ciliated cells in chips of either condition (shown here: CTL). In contrast, static inserts of the same donor exhibit diffuse VANG1 expression in most cells and very few such crescents (arrows). Scalebar 25 μm. F. Representative staining of VANG1 at day 35 ALI in same donor as in Fig. 3 at day 14 ALI. Scale bar: 50 μm. G. Quantification of VANG1 crescent density in chips at day 14 ALI compared to inserts at day 14 ALI and day 35 ALI. N = 3 donors, one chip or insert each; each point is mean value from 1 insert or chip; columns and error bars represent mean ± SEM.

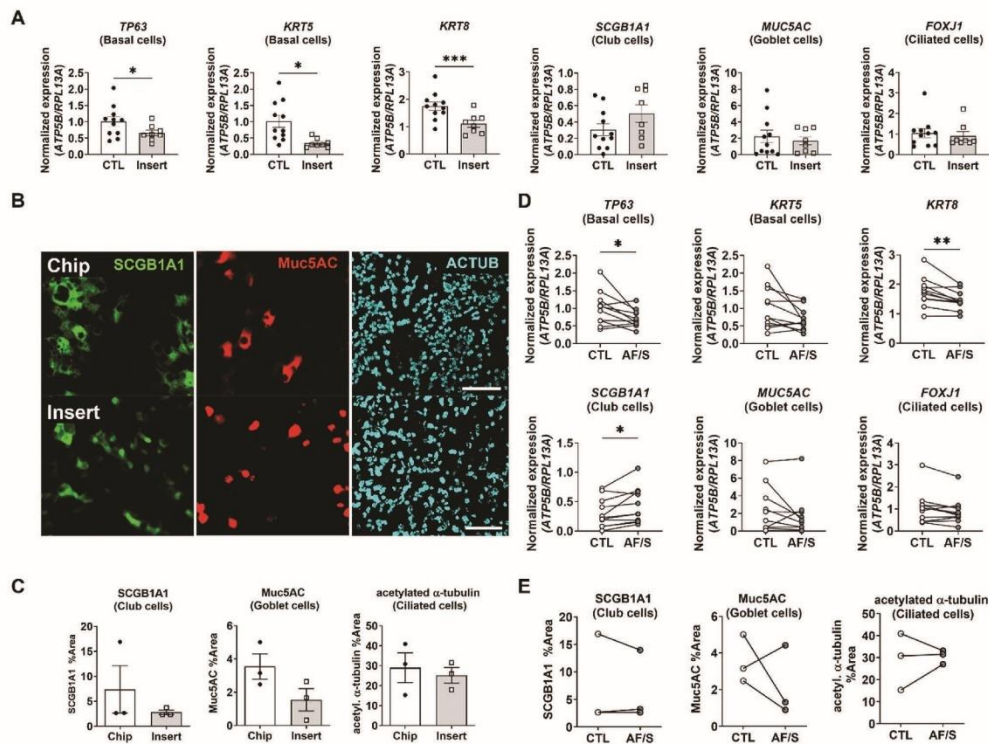


Fig. 4. Effects of dynamic chip culture on epithelial cell differentiation

A. Gene expression at day 14 ALI of *TP63* (basal cells), *KRT5* (basal cells), *KRT8* (differentiated non-basal cells), *SCGB1A1* (club cells), *MUC5AC* (goblet cells) and *FOXJ1* (ciliated cells). Open circles: CTL chips; gray circles: AF/S-exposed chips; N = 8 donors, one chip per donor. Data are depicted as mean with SEM. *, $p < 0.05$; **, $p < 0.01$ as assessed by a two-tailed paired *t*-test. **B.** Representative IF staining of club (*SCGB1A1*), goblet (*Muc5AC*), and ciliated cells (acetylated alpha-tubulin, *ACTUB*) in chip and insert. Scalebar: 100 μ m. **C.** Quantification of the relative surface area labeled with IF markers for each cell type. N = 3 donors; each point is mean relative surface area labeled in multiple FOVs in 1 chip per donor. N.S. as assessed by Wilcoxon matched-pairs signed rank test. **D.** Gene expression of *TP63* (basal cells), *KRT5* (basal cells), *KRT8*, *SCGB1A1* (club cells), *MUC5AC* (goblet cells) and *FOXJ1* (ciliated cells) in collagen IV (COLIV)-coated chips and inserts at day 14 ALI. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B* and *RPL13A*; N = 8–11 donors, one chip or insert per donor. Data are depicted as mean $\pm$ SEM. *, $p < 0.05$; ***, $p < 0.001$, a paired two-tailed paired *t*-test. **E.** Quantification of the cell culture surface area fraction positive for IF markers of each cell type. Depicted are mean $\pm$ SEM from N = 3 donors with 3–6 chips or inserts each. No statistical difference was detected using one-tailed *t*-test.

compared to static insert culture, we assessed markers of inflammation and related to extracellular matrix (ECM) production.

Baseline levels of IL-8, a typical inflammatory chemokine, were significantly lower in chip cultures at five and seven days after exposure to airflow and stretch (Fig. 5A). Expression of another pro-inflammatory marker, *PTGS2*, was also significantly lower in these cultures (Fig. 5B), as were protein levels of several cytokines (G-CSF, IL-9 and MIP-1 β) as assessed by a multiplex assay, confirming an overall lower baseline level of inflammatory mediator production by the epithelial cultures exposed to airflow and stretch (Supplementary Fig. 5A).

To explore effects on ECM production, we assessed expression of collagens, glycoproteins (selection), serpins, MMPs, and Lysyl oxidase homologs (LOXLs)-related genes in our RNAseq data set (Fig. 5C). No overall change in expression in any of these groups was observed; however, expression of specific components, including *MMP1*, *MMP9*, *COL1A1* (collagen type-1) and *FNI* (Fibronectin), was downregulated in AF/S compared to CTL (supplementary Fig. 5B). qPCR analysis of *MMP9* and *FNI* for N = 8 donors supported a significant downregulation of both these genes in AF/S chips compared to CTL chips (Fig. 5D). Together,

these results suggest that physiologically relevant mechanical cues may influence inflammatory signaling and ECM-remodeling in airway epithelial tissues.

3. Discussion

Primary human airway epithelial cells differentiated in organ chips exhibited a notably stronger mucociliary clearance compared to static insert cultures of same age. MCC function depends on several factors, including mucus rheology, ciliated cell density, ciliary beat frequency, and ciliary beat waveform, such as cilia alignment, stroke trajectories, and temporal coordination [2,25–27]. Our data demonstrate that the density of ciliated cells and their beat frequencies were similar in all conditions, suggesting that these factors were not majorly contributing to the pronounced differences in MCC.

Instead, we posit that the chip tissues experienced accelerated maturation of ciliary beat properties, such as force generation and stroke pattern, as supported by the increased expression of PCP genes and localization of PCP proteins in chips compared to inserts. PCP protein

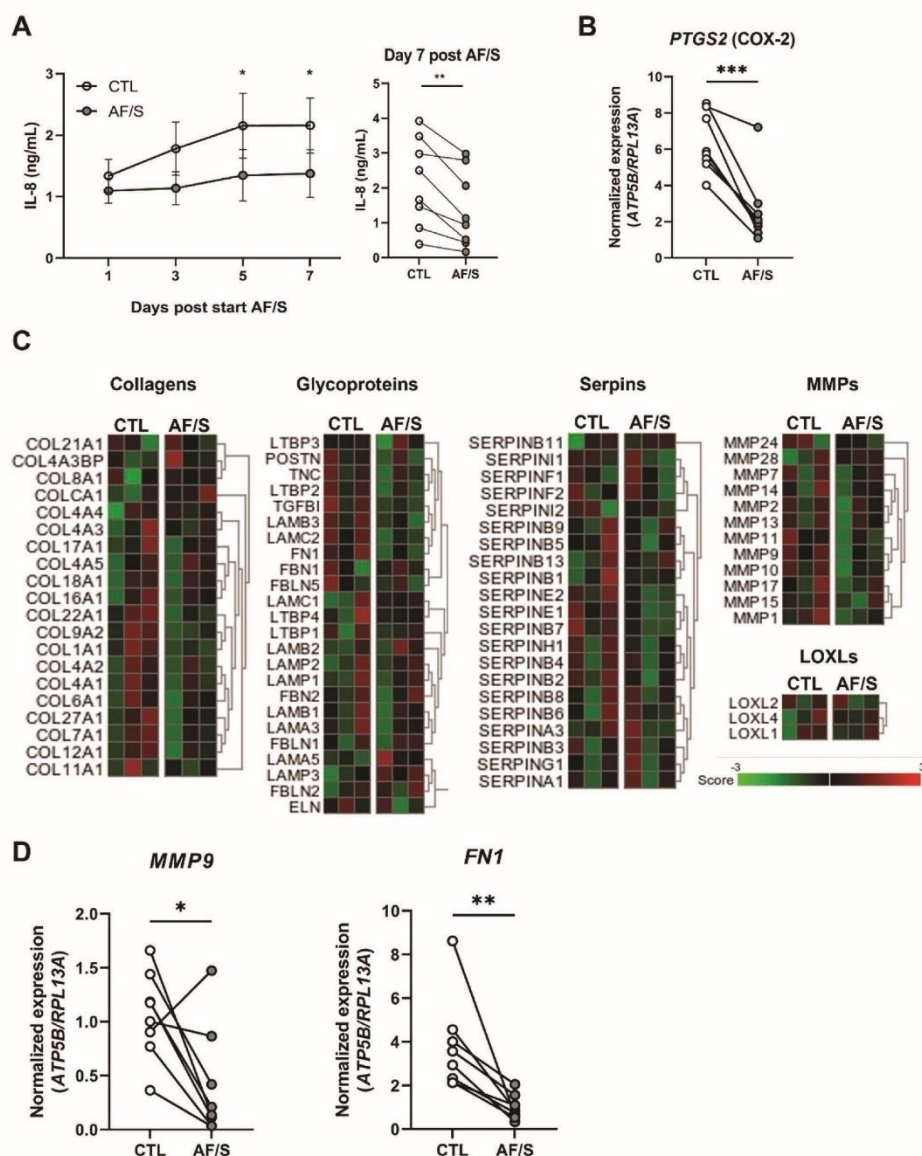


Fig. 5. Exposure to airflow and stretch reduces baseline pro-inflammatory and ECM-related gene expression. **A.** Left: IL-8 protein levels (ELISA) in the basal channel medium collected 24 h after each medium change at the indicated times. Filled circles are CTL chips, open circles are AF/S chips; N = 8 donors with 2 chips per donor, except for 2 donors with 1 AF/S chip each. Right: paired IL-8 levels at day 14 post ALI and 7 days of application of AF/S. *, $p < 0.05$ as assessed by Mixed-effects analysis combined with a Sidak's multiple comparisons test. **, $p < 0.01$ as assessed by paired t-test. **B.** Gene expression of *PTGS2* at day 14 ALI. Open circles: CTL chips, gray circles: AF/S-exposed chips; N = 8 different donors, one chip/donor. Data are depicted as mean $\pm$ SEM. **, $p < 0.01$ as assessed by paired t-test. **C.** Heat map of Z-scores for ECM-related gene expression (RNAseq), i.e., collagens, glycoproteins, matrix metalloproteinases (MMPs), SERPINS and LOXLs, in AF/S and CTL chips at day 14 of ALI. RNA was derived from N = 3 donors that are paired between AF/S and CTL (one column per donor), 1 chip/donor. The Z scores for individual genes are represented in green and red. **D.** Gene expression assessed by qPCR analysis of *MMP9* and *FN1* at day 14 of ALI. Open circles: CTL chips, gray circles: AF/S chips; *, $p < 0.05$; **, $p < 0.01$ as assessed by a paired t-test, N = 8 donors, one chip per donor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

localization is one of the hallmarks of multiciliated cell maturation and determines beat polarity [20]. The comparatively slower maturation of MCC in static inserts matches the timelines previously reported for such cultures, and these studies also showed that, while ciliary beat was detectable after 1 or 2 weeks at ALI, ciliary beat properties took many more weeks to mature until efficient mucus propulsion was observed [2]. Another study showed that beat frequency was regulated by an internal timing mechanism and not a geometric switch point (i.e. a waveform-dependent mechanism), and force was independent of frequency [28], confirming that beat frequency is neither an indicator of applied force nor the presence of a mature waveform.

Another effect we observed on chip was that MCC tended to oppose the direction of the applied perfusion in the bottom channel, an effect that was amplified when we applied additional airflow in the same direction in the top channel, indicating an impact on multiciliated cell polarity and organization in the total culture [29]. By contrast, a recent study showed that human induced pluripotent stem cell (hiPSC) derived airway epithelium generated MCC in the same direction as imposed flow [30]. Hence, different mechanisms may be implicated in determining ciliary beat polarity of primary epithelial cultures versus hiPSC-derived airway epithelial cultures, which may be related to the notion that hiPSC-derived cultures are sometimes considered to be relatively immature. It is tempting to speculate that this effect of airflow on directionality of MCC helps in directed movement of mucus towards the oral cavity, where it can be removed by swallowing or expectoration. This may occur despite the fact that airflow is bidirectional, since flow-rates are higher during inspiration than during expiration. It may furthermore help to explain impaired MCC in e.g. patients with bronchiectasis, a condition with abnormal widening of the airways resulting in reduced airflow.

Importantly, our new chip culture protocol requires less time to achieve physiologically relevant MCC function compared to insert cultures. A variety of factors differ between chip and insert cultures that may have contributed to the accelerated maturation of PCP expression and mucociliary clearance on chip. The chips include a stretchable PDMS membrane with lower stiffness compared to the rigid PET membrane of the inserts. Substrate stiffness influences many cellular responses [31] but the airway epithelium remains understudied in this context and effects on mucociliary clearance have so far not been reported to our knowledge. In addition, a higher turnover of the medium could have provided the cell cultures with exposure to more nutrients (and less waste products) compared to insert models. Finally, increased evaporation under airflow may have increased mucus viscosity which could influence ciliary alignment [25].

Besides effects on ciliary maturation, these factors may also influence epithelial differentiation.

Rat tracheal epithelial cells cultured on four different platforms exhibited significant differences in cellular composition that mimicked either the proximal or distal airways, probably promoted by many factors that varied between platforms, including substrate stiffness and chemical composition, 2D and 3D substrate geometries, and differentiation duration [32]. In our chip cultures, we observed significant changes in gene expression related to the basal cell compartment compared to insert cultures but no major impact on gene expression related to the luminal cell population was observed. Perhaps the time frame of 7 days was too short to measure effects on changes in cellular composition. In future studies of such kind, it will also be important to investigate the role of individual factors in shaping differentiation, maturation, and epithelial functioning, such as mucociliary clearance, since we show significant functional differences despite finding only moderate changes in (basal) cell composition. The methods we developed will facilitate the modeling of different lung tissue compartments *in vitro* and enable future studies into the mechanisms by which mucociliary clearance is impaired in disease or upon damage, and whether this can be reversed by application of targeted mechanical cues or related pathways.

In this study we used human primary cells from multiple human donors (Supplementary Table 4) and studied differentiation in the presence of mechanical stimulation in order to capture robust, longer-term responses rather than acute insults or cell-line-specific behavior. We did not co-culture endothelial cells in the vascular compartment of the chip in this study as this would confound the comparison with inserts without endothelial co-culture and potentially mask the effects of biomechanical cues on airway epithelial biology. It would be interesting to study in the future how the changes in epithelial cellular composition and/or function affect the mesenchymal and the vascular compartment. Although we did not explore the isolated effects of dynamic flow and stretch on airway epithelial biology, the ability to deliver these cues and achieve measurable effects on mucociliary functioning using a commercially available chip model combined with a robust protocol for primary airway culture opens new avenues for investigating ciliogenesis, airway disease and epithelial regeneration in the presence of mechanical stresses and establish beneficial versus adverse effects. Our method could also be applicable to the differentiation of alveolar or airway cells derived from induced pluripotent stems cells [30,33] as well as multi-tissue airway models and enhance their structural and functional maturation *in vitro*.

4. Conflict of interest disclosure

J.N. is a former employee of Emulate, Inc. A.D. was supported by a Global Marie Curie fellowship (No. 748569) that included a 1-yr visit (2018–2019) at Emulate Inc. to work on their Lung-Chips. D.R. and T.M. performed their internship at Emulate Inc in 2018 and 2019, respectively. Materials from Emulate Inc. related to this work were therefore provided by Emulate Inc. The remaining authors have no conflicts of interest relevant to this publication.

5. Methods

5.1. Primary human bronchial epithelial cell sourcing and expansion

Here and in following steps, we report the procedures optimized in parallel in laboratories at LUMC, USC, and Emulate Inc. And specify differences in material and protocol choices as applicable. For donor characteristics see Supplementary Table 4.

5.1.1. Leiden

Primary human bronchial epithelial cells (PBEC) were isolated from tumor-free resected bronchus rings obtained from lung cancer patients undergoing a resection surgery at the Leiden University Medical Center (LUMC, Leiden, the Netherlands). Patients from which this lung tissue was derived were enrolled in the biobank via a no-objection system for coded anonymous further use of such tissue (www.coreon.org). Within this framework, individual written informed consent is not needed. Since 01-09-2022, patients are enrolled in the biobank using active informed consent in accordance with local regulations from the LUMC biobank with approval by the institutional medical ethical committee (B20.042/Ab/ab and B20.042/Kb/kb). hPBEC were thawed in a T75 flask coated with 30 µg/mL bovine type-1 collagen (PureCol®, Advanced BioMatrix), 10 µg/mL fibronectin (Promocell, PromoKine, Bio-connect) and 10 µg/mL BSA (ThermoFisher Scientific) in BEpiCM-b basal medium (ScienCell Research Laboratories, Sanbio), supplemented with 100 U/mL penicillin (Lonza), 100 µg/mL streptomycin (Lonza) and bronchial epithelial cell growth supplement (ScienCell). When reaching ~90% confluency, cells were trypsinized in 0.03% (w/v) trypsin (ThermoFisher Scientific), 0.01% (w/v) EDTA (BDH, Poole, UK), 0.1% glucose (BDH) in phosphate buffered saline (PBS) and prepared for seeding.

5.1.2. USC

Fresh hPBEC were isolated from lung explant tissue from rejected donor transplant from subjects with no prior evidence of chronic lung

disease with IRB approval from the University of Southern California (IRB# USC HS-18-00162) using established protocols [34]. Next, hPBEC were thawed in T75 flasks or 100 mm cell culture dishes coated with PureCol (Advanced Biomatrix, Carlsbad, CA, USA) using complete BEpiCM-b basal medium as described above. When reaching ~90% confluency, cells were dissociated with Accutase (Sigma Aldrich, St. Louis, MO, USA) and prepared for seeding.

5.1.3. Emulate Inc

Hydrogel experiments were performed with hPBECs from a commercial supplier (Lifeline Cell Technologies, Frederick, MD, USA) that were cultured similar to the methods stated for LUMC/USC.

5.2. Chip functionalization and cell culture

5.2.1. Chip membrane activation

To hydrophilize the PDMS membrane, top and bottom channels of the Chip-S1® (Emulate, Inc., Boston, MA, USA) were filled with ER-1 solution (1 mg/mL in ER-2; Emulate Inc) followed by 10 min of UV-light exposure using a 36-Watt UV-chamber (NailStar Professional, Model NS-01-US). Next, channels were washed twice with ER-2 (Emulate Inc.). This ER-1/ER-2 procedure was repeated once more. After the second ER-2 wash, channels were washed and filled with cold PBS.

5.2.2. Chip membrane coating

The chip top channel was emptied and subsequently completely filled with 300 µg/mL human collagen IV (Sigma) solution in PBS (approximately 35 µl). Chips were placed in petri dishes containing a small, open container of PBS to promote humidity and kept overnight in the incubator at 37 °C/5%CO₂. For optimization experiments, also other coating components and their mixtures were used: 0.1% BSA (Gibco), 30 µg/mL bovine type-1 collagen (PureCol®, Advanced BioMatrix), or human fibronectin (Sigma). We found that BSA and collagen IV coating both resulted in reliable cell adhesion; however, collagen IV coated chips exhibited significantly better viability post seeding and significantly higher ciliation compared to BSA coated chips at day 14 ALI (Supplementary Fig. 6).

5.2.3. Epithelial cell seeding and submerged culture on chip

The coating solution in the top channel was removed and replaced with so-called B/D complete medium: a 1:1 mixture of BEpiCM-b and DMEM medium (STEMCELL Technologies), supplemented with Bronchial Epithelial Cell Growth Supplement (ScienCell), and additional 50 nM EC-23 (Tocris); 25 mM HEPES (Cayman Chemical), 100 U/mL penicillin and 100 µg/mL streptomycin (ScienCell). B/D complete was pre-filtered using a 70 µm cell strainer (BD Biosciences) or a 0.22 µm vacuum filter unit (Steriflip™; Millipore, Sigma) to remove any suspended particles that could block flow in the Chip-S1®. Epithelial cells were seeded in B/D complete in the top channel at 3×10^6 cells/mL (ca. 90 K cells per chip, i.e., ca. 320 K cells/cm²) and left to adhere in the incubator for ~6h. At this point, the Pluronic treatment described below was applied to the bottom channel in the final chip protocol. Next, chip channels were washed gently with pre-warmed B/D complete. The chips were connected to the pre-warmed media-filled fluidic manifolds, “Pod®” (Emulate Inc.). After obtaining fluid connection between chips and Pods, these units were placed in the micro perfusion instrument, “Zoë®” (Emulate Inc.). After finishing the initial regulate cycle program (which pressurizes the medium to increase gas solubility and remove nucleating air bubbles while the system calibrates), the chips were continuously perfused with a flow rate of 30 µl/h in both top and bottom channel. Approximately 24h after start of the first regulate cycle, a so-called via wash was performed, dislodging any bubbles in the Pod's reservoirs fluid vials, followed by a second regulate cycle. This was important to minimize the blockage of flow by air bubbles. When following this protocol, no degassing of medium was needed. Approximately 3–5 days after seeding (donor dependent), air-liquid interface

(ALI) was established as described in the next paragraph.

5.2.4. Epithelial differentiation at air-liquid interface (ALI) in the airway chip

ALI was established by removing the medium from the top channel and having only medium flowing through the bottom channel. To equalize (hydrostatic) pressure at the membrane level, reservoirs in the Pod connecting the inlet and outlet of the top channel were sealed with 1 mL B/D complete medium per reservoir. Nonetheless, occasional flooding of the top channel would still occur, requiring daily monitoring. Submerged top channels were emptied manually using a 1000 µl pipette. Epithelial cultures were also monitored daily for flow issues and cellular invasion and morphology. If flow in the bottom channel became impaired, chips were disconnected, channels were rinsed with medium, potential air bubbles were dislodged, and chips were re-connected after establishing liquid-liquid interface between chip and Pod. If this happened repeatedly in the same sample, the Pod was replaced. Every 48h, medium outlet reservoirs were emptied, and inlet reservoirs were filled with fresh medium.

5.2.5. Application of airflow and stretch

At day 7 of ALI, the flow rate of air in the top channel in AF/S chips was set to ca. 1.2 µl/s of airflow. Additionally, the membrane strain rate was set to 5% at a frequency of 0.25 Hz.

5.3. Invasion scoring

We assigned the following scores to the degree of cell invasion into the bottom channel, as assessed by phase contrast microscopy: 0, no invasion; 1, individual cells in the bottom channel; 2, confluent cell layers in the bottom channel; 3, confluent tissue with multiple layers and/or fibroblast-like cell morphology in the bottom channel.

5.4. Prevention of bottom channel invasion

5.4.1. Chemical treatments

Several strategies were tested to assess inhibition of PBEC migration to the bottom channel which are summarized in Supplementary Table 1. Three strategies were included: cell culture media variations, addition of different dose of EC-23 to the currently used medium or addition of dexamethasone at variable concentrations to the cell culture medium. Alternatively, several inhibitors or agonists of signaling pathways were added to the cell culture medium currently used. Neither signaling inhibitors of migration nor altered media composition significantly reduced invasion while preserving differentiation capacity (data not shown).

5.4.2. Hydrogel mechanical barrier

The concurrent seeding of epithelial and endothelial cells onto either side of the membrane can be used as a mechanical barrier to cell migration [35], however, this strategy prevents the study of epithelial cells in isolation and comparison to standard culture conditions. Instead, we tested the use of a mechanical gel barrier. Different compositions of ECM gels in the top channel were tested, including bovine type-1 collagen (FibriCol®, Advanced BioMatrix), Matrigel® (Corning, Corning, NY, USA), Fibronectin (Gibco), collagen IV (Sigma), and the collagen cross-linking agent, microbial transglutaminase (MTG) (Modernist Pantry LLC, Eliot, ME, USA). All ECM mixtures were prepared in PBS-solution and contained the following: collagen I = 0.5 mg/mL collagen I; collagen I + MTG = 0.5 mg/mL collagen I + 4 mg/mL MTG; collagen I + Fibronectin = 0.5 mg/mL collagen I + 200 µg/mL Fibronectin; collagen I + collagen IV = 0.5 mg/mL collagen I + 200 µg/mL collagen IV; collagen I + Matrigel® = 400 µg/mL collagen IV + 200 µg/mL Matrigel®. The gel was prepared by injecting the gel prepolymer solution after membrane surface activation and incubated overnight at 37 °C. The next day, the gel was then flushed twice with 100 µl of warm medium at 187.5 µl/s

flow (Eppendorf Xplorer automatic pipet; Eppendorf). This generated a 3D-ECM layer on the membrane with a thickness of 20–85 μm , as optimized previously [36] (Supplementary Fig. 7A and B). To assess and confirm ECM scaffold formation of different ECM compositions, we stained the gels with a fluorescent dye. Briefly, 1 mg/mL of N-hydroxysuccinimide (NHS) ester dye (Atto 488 NHS Ester, Sigma) was mixed with 50 mM borate buffer (pH 9) in 1:500 ratio. Directly after ECM formation, the prepared staining solution was injected into the top channel and the chips were incubated for 25 min at room temperature in the dark. The top channel was then rinsed three times with PBS prior to fluorescence imaging. Using ImageJ [37], the ECM-covered area was measured as a percentage to the total channel area via automated thresholding. The entire straight length of the channel was evaluated in all conditions ($N = 3$ chips each). To measure the ECM thickness, z-stack images with 4 μm step size were acquired in two defined regions (left and right part) of the top channel. Using ImageJ, the stacks were 3D-projected and rotated to obtain the 90°-side view of the channel, followed by visualizing the stained ECM with different thicknesses via automated thresholding. Average ECM thickness was measured by calculating the threshold area divided by the length of the image. Basal cells seeded onto the different gel types attached well, and significant reduction in basal channel invasion was observed compared to chips without hydrogels (Supplementary Fig. 1E). At day 14 of ALI a well-differentiated epithelium containing club, ciliated, goblet and basal cells had formed (Supplementary Fig. 7C). However, given substantial variability between ECM lots used for hydrogel preparation and other factors influencing spreading, gelling, and final geometry in the microfluidic channel, it was technically challenging to reliably deposit gels without any holes that allowed for transmigration.

5.4.3. Pluronic anti-adhesion surface treatment

Approximately 6 h after hPBEC seeding into the top channel, the bottom channels of all chips were coated with the surfactant Pluronic F-127 (Sigma) to prevent cell adhesion. For this, a minimum of 0.02% w/v Pluronic F-127 was freshly prepared in complete B/D medium and given time to dissolve at room temperature rather than at 37 °C, as solubility of Pluronic decreases with temperature [38]. Next, the solution was sterile filtered using a 0.22 μm filter. The bottom channel of the chips was emptied and filled with the Pluronic solution, followed by 1 h incubation at 37 °C. After the incubation, warm medium was used to gently rinse both channels before proceeding with connecting the chips to the perfusion instrument Zoë as described above. Our preliminary data suggest that the bottom channel functionalization can be safely renewed at later timepoints by adding up to 0.2% Pluronic to the perfusion medium for 24–48 h, which prevents bottom channel invasion even in highly migratory donors.

5.5. Endothelial-epithelial co-culture on chip

Primary Human Lung Microvascular Endothelial Cells (HLMVEC) were purchased from Cell Applications (San Diego, CA, USA) and expanded in Microvascular Endothelial Cell Medium (Cell Applications). Chips were seeded with endothelial cells on day 11 or 12 of ALI. To prepare for endothelial cell seeding, the chips with epithelial tissue in the top channel were disconnected from the Pods. Since Pluronic is thermoreversible and thereby more soluble in medium at lower temperatures [38], we washed the basal chip channels at day 12 of ALI with ice-cold PBS to remove the Pluronic coating. For this, the top channel was filled with warm B/D complete medium, and the bottom channel was incubated three times for 30 s, then rinsed, with ice-cold PBS. The bottom channel was filled with 100 $\mu\text{g/mL}$ human fibronectin (Sigma) in B/D complete medium. The chips were flipped upside down, leveled and incubated for 1 h at 37 °C. Just before endothelial cell seeding, cells were washed with PBS and dissociated using Accutase (Sigma), then resuspended at 7.5×10^6 cells/mL in endothelial cell medium. The fibronectin solution was removed from the bottom channel and ca. 8 μl of the cell

suspension was added, resulting in a seeding density of ca. 200,000 cells/ cm^2 . The chip was again flipped upside down and left undisturbed in the incubator for 2 h. After 2 h, the endothelial cell medium was carefully exchanged in the bottom channel. The chip was reconnected to the Pod and brought to ALI using standard procedures. The bottom channel was perfused with a mix of 50% endothelial cell medium and 50% B/D complete medium at standard flow rates (30 $\mu\text{l/h}$).

5.6. Airway cell culture inserts

5.6.1. Leiden

To match the seeding conditions on the chips, hPBEC were seeded at high density (150,000 cells/well) on ECM-coated 12 mm clear polyester cell culture inserts with 0.4 μm pore size (Corning Costar, Cambridge, USA, cat.#: 3460). Inserts were coated with 300 $\mu\text{g/mL}$ human collagen IV (Sigma) solution in PBS. We also tested a coating mixture consisting of 30 $\mu\text{g/mL}$ Purecol (Advanced BioMatrix), 10 $\mu\text{g/mL}$ fibronectin (Promocell) and 10 $\mu\text{g/mL}$ BSA (ThermoFisher Scientific) in PBS [39,40] but no significant differences in any of our endpoints compared to the collagen IV-coated inserts (Supplementary Fig. 8), hence we discontinued this alternative protocol.

Seeded cells were cultured submerged in B/D complete with EC-23 (50 nM). When cells were confluent, medium on the top side was removed to initiate differentiation at air-liquid interface. Medium was changed 3 times a week and the top side was washed with warm PBS.

5.6.2. USC

Clear polyester 6.5 mm cell culture inserts with 0.4 μm pore size (Corning Costar, Cambridge, USA) were pre-coated with 300 $\mu\text{g/mL}$ human collagen IV (Sigma) solution in PBS. hPBEC were seeded at high density (70,000 cells/well) and cultured as above.

5.7. RNA isolation and bulk RNA sequencing

At day 14 of ALI, cells in the airway chip were lysed by disconnecting the chip from the Pod, lodging an empty filter tip at the outlet of the chip, and pipetting 100 μl lysis buffer (Promega) with another filter tip into the inlet of the chip. This tip was then lodged into the inlet, such that the two tips inserted at either channel end were capturing the overflowing liquid. When visual inspection indicated lysis of the cells, the lysis solution was collected in a tube. Another 100 μl was used to rinse the channel once more, to obtain in total 200 μl of lysis solution from one chip. The lysis solution was stored at –20 °C until RNA extraction. Total RNA was robotically extracted using the Maxwell tissue RNA extraction kit (Promega) and quantified using the Nanodrop ND-1000 UV–Vis Spectrophotometer (Nanodrop technologies, Wilmington, DE, USA). Next, RNA was sent to GenomeScan (Leiden, the Netherlands) for Bulk RNA sequencing, or converted to cDNA for qPCR analysis. RNA sequencing was performed with the cDNA fragment libraries by the Illumina NovaSeq6000 sequencer using 150 bp pair-end sequencing settings.

5.8. RNA sequencing analysis

5.8.1. Data processing

Data processing was performed by GenomeScan. For this, the NEB-Next Ultra II Directional RNA Library Prep Kit for Illumina was used to process the samples. The sample preparation was performed according to the protocol “NEBNext Ultra II Directional RNA Library Prep Kit for Illumina” (NEB #E7760 S/L). Briefly, mRNA was isolated from total RNA using the oligo-dT magnetic beads. After fragmentation of the mRNA, a cDNA synthesis was performed. This was used for ligation with the sequencing adapters and PCR amplification of the resulting product. The quality and yield after sample preparation were measured with the Fragment Analyzer. The size of the resulting products was consistent with the expected size distribution (a broad peak between 300 and 500 bp). Clustering and DNA sequencing using the NovaSeq6000 was performed

according to manufacturer's protocols. A concentration of 1.1 nM of DNA was used. Sequence reads were trimmed to remove possible adapter sequences using cutadapt v1.10. Presumed adapter sequences were removed from the read when the bases matched a sequence in the adapter sequence set (TruSeq adapters). For each sample, the trimmed reads were mapped to the human GRCh37.75 reference sequence (Homo.sapiens.GRCh37.75.dna.primary_assembly.fa). The reads were mapped to the reference sequence using a short-read aligner based on Burrows-Wheeler Transform (Tophat v2.0.14) with default settings. Based on the mapped locations in the alignment file the frequency of how often a read was mapped on a transcript was determined with HTSeq v0.11.0. The hit counts were summarized and reported using the gene_id feature in the annotation file. Only unique reads that fall within exon regions were counted. To enable comparison of gene/transcript expression across all samples outside of the context of differential expression analysis, RPKM/FPKM (reads/fragments per kilobase of exon per million reads mapped), TPM (transcripts per million), and CPM values were calculated with Cufflinks v2.2.1.

5.8.2. Differential expressed genes

The read counts were used in the DESeq2 v2.1.14 package, a statistical package within the R platform R v3.3.0. The differentially expressed genes were identified based on read counts of genes >10, with a log2 fold change of >1.5 and q value of <0.05. Since groups had a small sample size and over-dispersion, an expression curve model based on a negative binomial distribution and local regression was used to estimate the relationship between the mean and variance of each gene. For heat maps, log2 expression of read counts are normalized with Z scores calculated by: $(X \text{ (value of the individual sample)} - \mu \text{ (average of the row)})/\sigma$ (standard deviation of the row).

5.8.3. Gene set analysis

The gene sets of differentially expressed genes were analyzed in GSEA (Geneset Enrichment analysis) which also shows the related pathways. The significantly up- and downregulated genes (False Discovery Rate (FDR) < 0.05) and related pathways between CTL and AF/S chips and between CTL chip and COLIV insert are provided in Table 2 and Supplementary Tables 2 and 3

5.9. cDNA and qPCR

For cDNA conversion, 500 ng of total RNA was reverse transcribed using oligo dT primers (Qiagen Benelux B.V., Venlo, The Netherlands) and M-MLV Polymerase (Thermo Fisher Scientific) at 42 °C. All quantitative PCR (qPCR) reactions were performed in triplicate on a CFX-384 Real-Time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands), using primers shown in Supplementary Table 5 and IQ SYBRGreen supermix (Bio-Rad). The relative standard curve method was used to calculate arbitrary gene expression using CFX-manager software (Bio-Rad). Two reference genes were included to calculate the normalized gene expression.

5.10. CXCL8 ELISA

The protein encoded by CXCL8 (interleukin-8 or IL-8), was measured with use of the Recombinant Human IL-8/CXCL8 Protein ELISA kit of R&D (Abingdon, UK) according to manufacturer's instructions and analyzed with use of 4-parameter logistic curve fitting in Prism 8 (version 8.1.1).

5.11. Multiplex protein measurements

Cytokine and growth factor secretion was measured with the Bio-Plex Pro Human Cytokine Screening Panel (Bio-Rad #M500KCAF0Y), a 27-plex assay. Effluent culture medium was collected from the chips (collected in the Pod's effluent reservoir) from cultures with 8 different

donors. These samples were diluted 1:4 in assay buffer provided with the kit. Manufacturer's instructions were followed to perform this assay, with minor adjustments to the protocol; one third of the proposed number of magnetic beads and detection antibodies was used. Plate readout was performed on a Luminex 100/200 System (Luminex, 's-Hertogenbosch, The Netherlands).

5.12. IF staining and histology

At 14 day of ALI, cells in the Chip were fixed using 4% paraformaldehyde (PFA) solution. The PFA solution was pipetted into both channels and incubated for 20 min at RT, washed with PBS and stored in PBS at 4 °C until staining. Chips were subsequently removed from the carrier and cells were blocked and permeabilized using 0.5% (v/v) Triton-X 100 in PBS with 5% BSA for 60 min at RT. Chips were cut into 2 pieces using a razor blade and channels were filled with primary antibodies (Supplementary Table 6) diluted in the Triton/BSA buffer and incubated for 1 h at RT. Samples were rinsed three times 5 min with PBS. Next, channels were filled with secondary antibodies diluted in Triton/BSA buffer which was left incubating for 1 h at RT, followed by a triple 5 min wash with PBS. 4',6-Diamidino-2-phenylindole (DAPI, Sigma) was used to stain cell nuclei. Channels were next filled with prolong gold anti-fade (Thermo Scientific) and stored in the dark at 4 °C until imaging. Chips were imaged on Bello coverslips, 25 × 75 mm (Electron Microscopy Sciences, Hatfield, PA, USA) with 0.13–0.17 mm thickness using a Leica DMi8 microscope equipped with an Andor Dragonfly 200 spinning disk confocal using a 10× objective (NA 0.30×), 20× water objective (NA 0.50) or 40× water objective (NA 0.80).

5.13. Cross-sectional imaging of thin chip slices

A new sectioning technique was developed to obtain cross-sectional slices of the chip for histology and immunofluorescence staining. Chips were fixed as described as above. The PDMS surrounding the chip channel was cut away as much as possible using a razor blade. Next, the chip channel was mounted on the specimen tube using glue (Pattex instant glue) of a semi-automated vibrating microtome (Compressotome® VF-300-OZ, CMA, Kista, Sweden) and placed in the tube holder. Next, 150 µm thick slices were cut and collected in PBS-filled wells of a 12-well plate. Slices were stained in the well for classical H&E or immunofluorescence as described above. Slices were imaged on bello coverslips as described above for the complete chip.

5.14. IF image processing and quantifications

5.14.1. Cell type quantification

Raw images were split into individual channels, median filtered, cropped and CLAHE-filtered [41] to remove illumination artifacts, and auto-thresholded to retrieve the area fraction positive for each cell type marker. Semi-automated batch processing was conducted using ImageJ-Fiji [42].

5.14.2. VANGL1 crescent quantification

Raw images were split into individual channels and cropped as needed to remove illumination artifacts. We used a Matlab script to denoise the images with Gaussian, median, and Wiener filters and detected the VANGL1 crescents with Hessian based multiscale filtering (Supplementary Fig. 9). The binarized crescents were skeletonized to yield lines of single pixel width and compute their total length. VANGL1 crescent density (D_C) was defined as $D_C = \sum L_i / A$, where A is the area of the field of view in μm^2 , L_i is the length of VANGL1 crescent i in μm , and the sum ranges over all $i = 1 \dots N$ crescents detected in the field of view. For each donor, final crescent density was reported as the average value from all fields of view.

5.15. Measurements of CBF and MCC

5.15.1. Ciliary beat frequency measurement

CBF was measured using high-speed video microscopy followed by signal processing of the recordings as described previously in detail [12]. Briefly, to optimize optical imaging quality, inserts were washed for 10 min with PBS and the returned to ALI whereas Lung-Chips kept submerged in PBS or culture medium during imaging. Samples were imaged using a Leica DMI8 microscope equipped with phase-contrast objectives and a PCO Edge 4.2 high-speed camera with camera link interface that was operated from a PC desktop computer using the ImageJ-Fiji [42] with Micro-Manager [43]. Per sample, 6 to 8 fields of view ($166 \times 166 \mu\text{m}$, with ca. 3 pixel/ μm spatial resolution) were recorded under Koehler illumination at a temporal resolution of 200 frames per second. Using a custom algorithm in Matlab (MathWorks), regions containing ciliary beat activity were detected automatically by computing the standard deviation of each pixel's intensity over time, which reveals motion, and thresholding the resulting image using Otsu's method to segment area with detected motion. Ciliary beat density was computed by dividing the number of pixels with detected motion by the total number of pixels in the image. In regions with ciliary beat, the dominant beat frequency was identified from the smoothed pixel intensity over time using Fast Fourier Transform (FFT). The ciliary beat frequencies and ciliary beat densities measured in all movies per sample were averaged for further statistical analysis.

5.15.2. Mucociliary clearance measurement

MCC was measured by adding fluorescent tracer particles to the top of samples, recording the movement of the tracers over time using video microscopy, and tracking the displacement of the particles in the resulting videos using specialized software to derive transport velocities. Briefly, the samples were washed and prepared as above before adding $30 \mu\text{l}$ of a 1:200 dilution of $1\text{-}\mu\text{m}$ sized fluorescent polystyrene microspheres (ThermoFisher Scientific) in PBS. Samples were imaged using a Leica DMI8 microscope equipped with a fluorescence light source and appropriate filters. Per sample, 3–6 fields of view (ca. $1200 \times 1200 \mu\text{m}$ each) were recorded for 10–20 s at a temporal resolution of 30 frames per second. Using a batch-processing script in ImageJ-Fiji with the Trackmate plugin [44], we tracked the bead displacement per time step to derive their trajectories and flow speed. To identify and filter out potential background flow, we used a supervised machine learning approach in Matlab to classify trajectories as either background or true signal. Briefly, we imaged bead movements in cell-free chips and inserts as well as in strong MCC flow. To generate the ground truth data, we labeled ca. 40,000 trajectories correctly as either true signal (MCC) or background flow. To characterize the trajectories, we measured the following metrics: mean speed, median speed, standard deviation of speed, path length, Euclidian distance covered, the ratio of Euclidian distance covered to path length (directness), mean flow angle, number of nodes, and maximal acceleration. Using the Matlab classification learner app, we trained a decision tree model with 5-fold cross-validation to identify true and background trajectories. We found that Euclidian distance, median speed, and directness below a threshold level were sufficient indicators to classify trajectories as background flow with an accuracy of more than 95%. This simple classification strategy was then used to automatically filter out the background flow in all data sets (Supplementary Fig. 10A). Matlab was used to further analyze the flow as described below.

5.15.3. MCC polar order parameter

The main direction of each bead trajectory was determined by the measuring the angle Θ_i formed between the channel axis and the line between start point and end point of each bead trajectory (Fig. 2D), where i ranges from 1 to the number of trajectories per sample. For each sample, an average unit vector $\hat{u}$ was determined from all measured

angles: $\hat{u} = \langle [\sin(\Theta_i), \cos(\Theta_i)] \rangle$, where the outer brackets indicate averaging. The PO was computed by dotting the average unit vector with the unit vector $\hat{n}$ pointing towards the channel inlet: $PO = \hat{u} \cdot \hat{n}$. The PO hence ranges from -1 to 1 , with -1 and 1 indicating perfect alignment of the mean flow vector towards the channel outlet or inlet, respectively. Intermediate values indicate partial alignment towards one the channel ends, i.e., negative values towards outlet and positive values towards inlet. A PO near 0 indicates isotropic flow or alignment of the mean flow vector towards the walls. Trajectory data from all movies per sample were pooled for the analysis. Therefore, alternating, perpendicular, and random flow without any bias would all be expected to result in an average PO of zero.

5.15.4. MCC coverage and speed

To measure MCC coverage and area-averaged speed, the trajectories were converted into a Eulerian velocity vector field $U(x, y) = u(x, y)\hat{i} + v(x, y)\hat{j}$ by averaging the flow velocity component in x direction (u) and in y direction (v) measured at each coordinate point over time (since multiple beads may pass over the same location) (Supplementary Fig. 10B left and center). The Matlab “alphaShape” function was used to generate coherent areas from these point measurements and estimate MCC coverage C_{MCC} , i.e., the fraction of surface area with measurable MCC: $C_{MCC} = \frac{A_{MCC}}{A_{total}}$, where A_{MCC} is the area with measurable MCC and A_{total} is the total tissue surface area visible in the field of view (Supplementary Fig. 10C right). Area-averaged speed $\bar{M}$ was computed by weighting the average flow speed in active regions by their relative surface fraction: $\bar{M} = C_{MCC} \langle |U| \rangle$, where $|U|$ is the scalar field of flow magnitudes and the brackets indicate averaging. Flow data from all movies per sample were pooled for statistical analysis.

5.16. Statistical methods

Statistical analysis was conducted using GraphPad Prism version 8 and 9 (GraphPad Software Inc). Differences were considered significant at $p < 0.05$. Statistical significance, statistical methods, and the number of donors and samples used are detailed in the figures and figure legends. Where number of donors is reported, this always refers to the number of different donors.

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Credit author statement

Janna C. Nawroth: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. Doris Roth: Conceptualization, Methodology, Investigation, Writing – review & editing, Visualization, Funding acquisition. Annemarie van Schadewijk: Methodology, Investigation, Visualization. Abilash Ravi: Formal analysis, Writing – review & editing, Visualization. Tengku Ibrahim Maulana: Methodology, Investigation, Writing – review & editing, Visualization. Christiana N. Senger: Methodology, Investigation. Sander van Riet: Formal analysis, Investigation, Writing – review & editing, Visualization. Dennis K. Ninaber:

Investigation. Amy M. de Waal: Investigation. Dorothea Kraft: Investigation. Pieter S. Hiemstra: Resources, Writing – review & editing, Supervision, Funding acquisition. Amy L. Ryan: Resources, Writing – review & editing, Supervision, Funding acquisition. Anne M. van der Does: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Anne M. van der Does reports financial support was provided by European Union. Janna C. Nawroth reports financial support and equipment, drugs, or supplies were provided by Emulate Inc. Doris Roth reports equipment, drugs, or supplies was provided by Emulate Inc. Anne M. van der Does reports equipment, drugs, or supplies was provided by Emulate Inc. Tengku Ibrahim Maulana reports equipment, drugs, or supplies was provided by Emulate Inc. Janna C. Nawroth reports financial support was provided by European Union. Janna C. Nawroth has patent #US20220106547A1 pending to Emulate Inc. J.N. is a former employee of Emulate, Inc. A.D. was supported by a Global Marie Curie fellowship (No. 748569) that included a 1-yr visit (2018–2019) at Emulate Inc. to work on their Lung-Chips. D.R. and T.M. performed their internship at Emulate Inc in 2018 and 2019, respectively. Materials from Emulate Inc. Related to this work were therefore provided by Emulate Inc. The remaining authors have no conflicts of interest relevant to this publication.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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Publication [6]**Novel strategies to assess cytokine release mediated by chimeric antigen receptor T-cells based on the adverse outcome pathway concept**

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Abstract:

The success of cellular immunotherapies such as chimeric antigen receptor (CAR) T cell therapy has led to their implementation as a revolutionary treatment option for cancer patients. However, the safe translation of such novel immunotherapies, from non-clinical assessment to first-in-human studies is still hampered by the lack of suitable in vitro and in vivo models recapitulating the complexity of the human immune system. Additionally, using cells derived from human healthy volunteers in such test systems may lead to confounding results, as they will not reflect the altered state of the patient's immune system. This could potentially lead to an underestimated risk of life-threatening conditions, such as cytokine release syndrome (CRS) following CAR T cell therapy. The IMI2/EU project imSAVAR (immune safety avatar: non-clinical mimicking of the immune system effects of immunomodulatory therapies) aims at creating a platform for novel tools and models for early non-clinical prediction of possible adverse events of immunomodulatory therapies. This platform shall in the future guide early non-clinical safety assessment of novel immune therapeutics thereby also reducing the costs of their development. Therefore, we review current challenges and hurdles of non-clinical in vitro and in vivo models ranging from organ-on-chip up to advanced biomarker screening.

The attached file below is a proof version of the accepted article.

Novel strategies to assess cytokine release mediated by chimeric antigen receptor T cells based on the adverse outcome pathway concept

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Abstract

The success of cellular immunotherapies such as chimeric antigen receptor (CAR) T cell therapy has led to their implementation as a revolutionary treatment option for cancer patients. However, the safe translation of such novel immunotherapies, from non-clinical assessment to first-in-human studies is still hampered by the lack of suitable *in vitro* and *in vivo* models recapitulating the complexity of the human immune system. Additionally, using cells derived from human healthy volunteers in such test systems may lead to confounding results, as they will not reflect the altered state of the patient's immune system. This could potentially lead to an underestimated risk of life-threatening conditions, such as cytokine release syndrome (CRS) following CAR T cell therapy. The IMI2/EU project in SAVAR (immune safety avatar: non-clinical mimicking of the immune system effects of immunomodulatory therapies) aims at creating a platform for novel tools and models for early non-clinical prediction of possible adverse events of immunomodulatory therapies. This platform shall in the future guide early non-clinical safety assessment of novel immune therapeutics thereby also reducing the costs of their development. Therefore, we review current challenges and hurdles of non-clinical *in vitro* and *in vivo* models ranging from organ-on-chip up to advanced biomarker screening.

Keywords:

CAR T cells – Cytokine Release Syndrome – Non-clinical Safety Assessment – immune-related adverse outcome pathway – organ-on-a-chip – biomarkers

Abbreviations

ANG1/2, angiopoietin-1/2; AO, adverse outcome; AOP, adverse outcome pathway; APC, antigen-presenting cell; aPPT, activated partial thromboplastin time; BCMA, B-cell maturation antigen; BOEC, blood outgrowth endothelial cells; CAR, chimeric antigen receptor; CRP, c-reactive protein; CRS, cytokine release syndrome; DIC, disseminated intravascular coagulation; EC, endothelial cell; e.g., exempli gratia; ELISA, enzyme-linked immunosorbent assay; et al., et alii; GM-CSF, granulocyte macrophage colony stimulating factor; HLA, human leukocyte antigen; HSPC, hematopoietic stem and progenitor cells; HUVEC, Human umbilical vein endothelial cells; ICANS, immune effector cell-associated neurotoxicity syndrome; i.d., id est; IFN, interferon; IL, interleukin; IND, investigational new drug; irAOP, immune-related adverse outcome pathway; KE, key event; KER, key event relationship; LPS, lipopolysaccharide; mAb, monoclonal antibody; MCP1, monocyte chemoattractant protein 1; MHC, major histocompatibility complex; MIE, molecular initiating event; MIP-1, macrophage inflammatory protein; MoA, mode of action; NO, nitric oxide; OoC, organ-on-chip; PBMC, peripheral blood mononuclear cell; PMA, Phorbol 12-myristate 13-acetate; scFv, single-chain fragment variable; scRNA-seq, single-cell RNA sequencing; SEB, staphylococcal enterotoxin B; TH, T helper cell; TNF, tumor necrosis factor; Treg, regulatory T cell; VEGF, Vascular Endothelial Growth Factor; VWF, von Willebrand factor

1 Introduction

The approval of engineered CD19-specific chimeric antigen receptor (CAR) T cells for the treatment of relapsed/refractory B cell malignancies has revolutionized the field of cancer immunotherapies ¹⁻⁹.

CAR T cells combine the specificity of an antigen-binding domain of monoclonal antibodies (mAb) with the cytotoxic and memory function of T cells. The intracellular domain allows the activation of T cells and incorporates stimulatory and costimulatory domains such as CD28 or 4-1BB ¹⁰. The binding process is independent of the major histocompatibility complex (MHC) so that target cell recognition is unaffected by some of the major mechanisms by which tumors avoid MHC-restricted T cell recognition, such as downregulation of human leukocyte antigen (HLA) class I molecules and defective antigen processing ^{11,12} as illustrated in Figure 1.

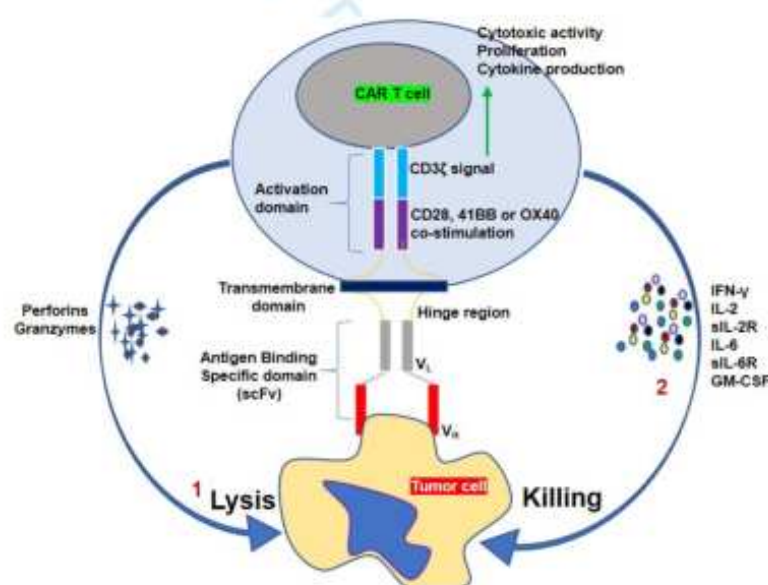


Figure 1 Mode of action of CAR T cells. Binding of the specific antigen on target cells activates antigen-specific CAR T cells. Activated CD8⁺ CAR T cells produce large amount of granzymes and perforins to exert their cytolytic function, which leads to tumor cell lysis (1). CD4⁺ as well as CD8⁺ CAR T cells proliferate and release cytokines (2) upon binding of antigen to the CAR, subsequently leading to the death of tumor cells. CAR, chimeric antigen receptor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; scFv, single-chain fragment variable; sIL-R, soluble interleukin receptor.

Second-generation CAR T cells, like the commercially available axicabtagene ciloleucel (Yescarta®) and tisagenlecleucel (Kymriah®), are equipped with the CD3ζ chain in combination with a CD28 or 4-1BB costimulatory domain that allow for prolonged persistence *in vivo* and improved cytokine production ^{7,13-15}.

Despite the impressive response rates and long-lasting remissions, short-term serious side effects such as cytokine release syndrome (CRS) and/or immune effector cell-associated neurotoxicity syndrome (ICANS) occur with high incidence and varying degrees of severity, ranging from mild to fatal ^{3,4,7,16-18}.

The term "CRS" was first coined in the '90s after the clinical introduction of the anti-CD3 monoclonal antibody OKT3 ¹⁹ and reported as a systemic reaction with a massive elevation of tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ). CRS is currently described as a systemic inflammatory response that results from the activation or engagement of endogenous or infused T cells and/or other immune effector cells with progressive symptoms that must include fever at the onset, and may include hypotension, hypoxia and organ dysfunction ²⁰.

CAR T cell-associated CRS is thought to result from the activation of myeloid cells by highly activated T cells, and although anti-interleukin (IL)-6 receptor antibodies (e.g., tocilizumab ²¹) can ameliorate CRS, they do not prevent or treat CRS. A plethora of other cytokines such as IL-1, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF) and monocyte chemoattractant protein 1 (MCP1) are involved in CAR T cell-mediated CRS (see Figures 2 and 3) ²²⁻²⁵. However, it has been difficult to investigate the complex mechanisms underlying the CAR T cell-mediated CRS because most non-clinical models use human tumors in immunodeficient mice that poorly recapitulate a patient's immune system ²⁶⁻²⁸.

Such a discrepancy between non-clinical testing and clinical observations is a major hurdle for the safe implementation of novel immune therapeutics. More predictive non-clinical test systems are needed to get a better insight in possible safety risks of immunomodulatory drugs.

In this review, we present the IM12/EU project imSAVAR (**immune Safety Avatar**: nonclinical mimicking of the immune system effects of immunomodulatory therapies) that aims at creating an accessible platform for novel models, tools and resources for the early non-clinical prediction of adverse events in immunomodulatory therapeutics.

2 The irAOP concept as exemplified using the pathophysiology of CRS in CAR T cell therapy as a use case

Within imSAVAR, we ~~follow the strategy to~~ define immune-related adverse outcome pathways (irAOPs) to guide the development of novel test systems ~~for immunomodulatory therapeutics~~ ²⁹. Adverse Outcome Pathways (AOPs) are a conceptual framework to describe the current knowledge of biological effects triggered by a molecular initiating event (MIE) leading to an adverse outcome (AO) ³⁰. In detail, AOPs describe the link between a MIE and a series of key events (KEs), i.e., measurable and ~~critical essential~~ biological events, at different response levels (e.g., molecular,

cellular, tissue, organ, organism) and their respective key event relationship (KER) leading to a specific AO ²⁹⁻³³.

The AOP framework emerged in the context of regulatory toxicology to guide the effective paradigm shift towards mechanism- and evidence-based chemical risk assessment ³¹. Rapidly, due to this practical and stressor agnostic approach, the framework gained interest from other fields. AOPs are acknowledged as a useful tool for the design of testing strategies to support safety assessment of nanomaterials and radiation-induced AO ^{34,35} as well as to systematically organize diverse and fast-evolving knowledge on the COVID-19 pathogenesis ^{36,37}.

In the context of immunomodulatory therapeutics, we interpret propose to further expand the scope of the framework and to exploit irAOPs as the to link a certain MIE interacting with the immune system and a series of KEs at different organizational-biological levels of the immune system leading to an adverse immune response such as excessive inflammation, to CRS as the AO. Structuring the KEs along an AOP can provide an instrumental scaffold to anchor relevant test systems. Such an irAOP collects the weight-of-evidence for the MIE, KE, and KER, which can also guide the development of test systems to assess the (proposed) KEs of a given AO ³⁸. In addition, we assigned Hence, we aim to assign suitable *in vitro* and *in vivo* test systems as well as potential and/or already existing biomarkers for the MoA and the KEs involved. Furthermore, the continuous refinement of such an irAOP will also provide a better insight on the existing knowledge gaps in a the given irAOP (Figure 2).

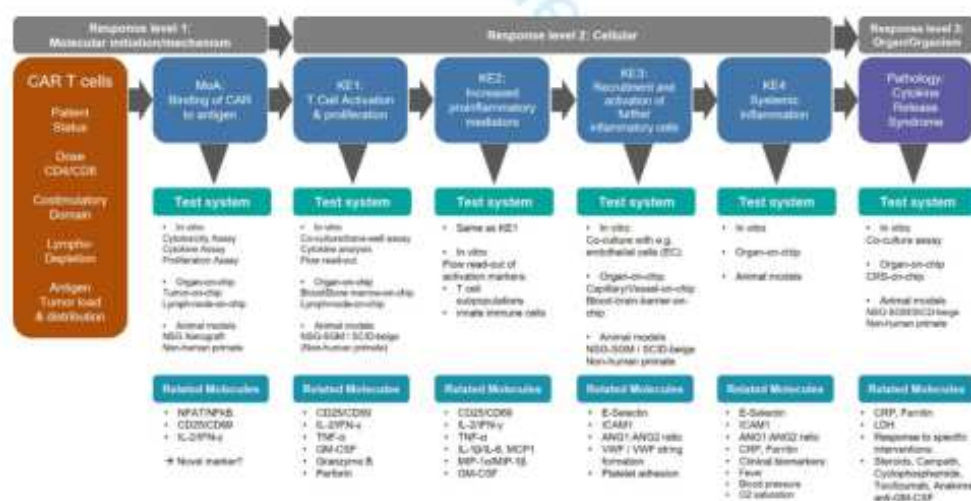


Figure 2 Illustration of the irAOP for CRS mediated by CAR T cells. The binding of CAR T cells to their targets (antigen-expressing cells) represents response level 1 (molecular initiation). The proposed key events 1 to 4 represent the cellular response level and may include interactions between different cell types (KEs 2 and 3). This cellular response to the initial event (binding of CAR T cells to their target) finally leads to the

response on organ and/or organism level (cytokine release syndrome). Possible test systems and a selection of related molecules for the mode of action (MoA; binding to antigen-expressing cells) and the proposed key events are listed. ANG, Angiopoietin; CAR, chimeric antigen receptor; CRP, c-reactive protein; EC, endothelial cell; GM-CSF, granulocyte-macrophage colony stimulating factor; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; IL, interleukin; KE, key event; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein 1; MIP-1, Macrophage Inflammatory Protein-1; TNF, tumor necrosis factor; VWF, von Willebrand factor.

In addition, in contrast to chemical inducing toxicity, the use of an AOP in this context is intended to evaluate the potential of CAR-T cell therapies in balancing the efficacy and safety of the treatment. In that context, the quantitative and time dimension along with other biomarkers might be essential to assess the trade-off between protective and adverse inflammation. Such an irAOP might represent a great opportunity, albeit a challenging one, to clarify within the AOP framework a physiological normal inflammatory response versus a pathological excessive inflammation, relevant also to many chemical stressors ³⁹.

Table 1 summarizes the Mode of Action (MoA) and KEs of the irAOP for CAR T cell induced CRS, which are illustrated in more detail in Figure 3. The binding of the specific antigen on target cells activates antigen-specific CAR T cells enabling them to exert their cytotoxic and cytolytic function by e.g., release of IFN- γ and Granzyme B (MoA and KE 1). Furthermore, CD4⁺ as well as CD8⁺ CAR T cells proliferate and release cytokines (e.g., IL-2) upon binding of antigen to the CAR (MoA and KE 1). Here, the design of the CAR itself, e.g., choice of costimulatory domain, the transmembrane domain, as well as the nature of the single-chain fragment variable (scFv) binding to the respective antigen can have a great impact on the functionality and potency of CAR T cells ^{12,40-43}. The extracellular spacer domain of the CAR can also have an effect on CAR specificity ^{41,42,44,45}. Majzner et al. recently reported that replacement of the CD8 hinge-transmembrane (H/T) region of a 4-1BB ζ CAR with a CD28-H/T lowers the threshold for CAR reactivity despite identical signaling molecules ⁴³. This further highlights, that different CAR designs also impact on the functionality of CAR T cells in tumors with heterogeneous antigen expression and hence also the risk of adverse events like CRS. Furthermore, the intracellular domains of second- and third-generation CARs additionally affect the persistence of CAR T cells *in vivo*, which might also affect the safety of the CAR T cells ⁴⁶⁻⁴⁹. Recently, new strategies in CAR design to avoid tumor escape and toxicities like Dual CARs, On-Switch CARs, Universal CARs or CARs with inducible Caspase-9 (safety switch) were described, all of which can affect the safety of next generation CAR T cell therapy ^{50,51}. In addition, the cellular composition of the infused CAR T cell product affects both the efficacy and the safety of this immunotherapy ^{52,53}.

In addition, as most antigens targeted by CAR T cells are not strictly tumor-specific e.g., CD19, which is also expressed by many non-malignant B cells, this may lead to an on-target, off-tumor effect triggering a massive cytokine release by CAR T cells. This in turn may lead to the recruitment of additional (innate) immune cells and trigger the release of cytokines and other pro-inflammatory mediators by these cells (KEs 1 and 2). In particular, monocytes and macrophages are described

as the main source for e.g., IL-1, IL-6 and MCP1, which are all attributed to play key roles in the pathophysiology of CRS ^{54,55}. Insights from recently available mouse models also elucidated the role of cytokines (IL-1, IL-6) and nitric oxide (NO) produced by monocytes and/or macrophages as contributing factors to the development of CRS (KE 3) ^{56,57}. Recent findings from Liu et al. suggest that granzyme B released from CAR T cells lead to tumor cell pyroptosis, a form of inflammatory cell death, via cleavage of Gasdermin E in tumor cells. The pyroptosis of tumor cells trigger IL-6 and IL-1 β production in macrophages to induce CRS. Therefore, tumor cell death, in addition to cytokines produced from CAR T cells, could be a factor triggering CRS ⁵⁸. The activation of endothelial cells, which may also produce IL-6 ⁵⁹, contributes to a large extent to the clinical presentation of CRS, namely, capillary leak and hypotension (KE 3 and 4). Clinical parameters of endothelial cell activation include von Willebrand factor (VWF) and angiopoietin-2 (ANG2) which were both recently associated with the severity of CRS in patients treated with CAR T cells ⁶⁰.

Table 1: Mode of Action of CAR T cells and key events leading to CAR T cell-mediated CRS

	Description	Strength of Evidence Experimental support	Related molecules
Molecular Initiating Event (MIE) / Mode of Action (MoA)	Binding of CAR to (tumor) antigen on antigen-expressing cell	<u>Strong:</u> Efficacy of novel CAR T cell products always tested against antigen-expressing cells and cells that do not express the target antigen ⁶¹ . Long-lasting remissions were observed in patients treated with CD19 CAR T cells ⁶² .	IL-2/IFN- γ ; CD89/CD25; NFAT/NF κ B
Key Event (KE) 1	(CAR) T cell activation and proliferation	<u>Strong for CAR T cells:</u> Cytolytic as well as proliferative capacity upon antigen encounter tested <i>in vitro</i> for each CAR T cell product ⁴⁵ . Ability to lyse tumors <i>in vivo</i> tested in animal models as a prerequisite for clinical trials ⁴¹ . Monitoring of CAR T cell expansion during clinical trials ⁶⁰ . Long-lasting remissions in patients treated with CD19 CAR T cells ⁶³ .	IL-2/IFN- γ ; CD89/CD25; Perforin, Granzyme B, TNF- α , GM-CSF;
KE 2	Increased pro-inflammatory	<u>Strong for CAR T cells:</u>	IL-2/IFN- γ or Multiplex

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	mediators released by (CAR) T cells and bystander (innate) immune cells	The ability to release IFN-γ and IL-2 upon antigen encounter tested <i>in vitro</i> for each CAR T cell product ⁴¹. Cytokine levels (especially IL-6) in patients' sera monitored during clinical application of CAR T cells ^{80,82}. <u>Intermediate to Strong for bystander (innate) immune cells:</u> Monitoring of IL-6, CRP and ferritin levels in patient sera ^{80,82}. Role of monocyte- and macrophage-derived IL-1 and IL-6 as mediators of CRS elucidated in recent mouse models of CRS ^{56,57}. Recent findings (<i>in vitro</i> co-culture experiments, NSG xenograft <i>in vivo</i> model) suggest that GM-CSF inhibition ameliorates CRS without affecting CAR T cell functionality ^{22,23}.	Cytokine Panel incl. Perforin, Granzyme B (for CAR T cells); IL-1 β , IL-6, NO, IL-8 (for bystander immune cells); clinical parameters like CRP, ferritin, MCP1
KE 3	Activation of other cell types such as endothelial cells, macrophages, dendritic cells, platelets as well as amplification of cytokine release by CAR T cells, bystander immune cells, endothelial cells	<u>Intermediate to Strong:</u> Role of monocyte- and macrophage-derived IL-1 and IL-6 as mediators of CRS elucidated in recent mouse models of CRS ^{56,57}. Monitoring of ANG2:ANG1 ratio in patients as well as serum levels of IL-6, CRP and ferritin and DIC parameters such as fibrinogen, VWF, prothrombin time, aPTT ^{80,82,83}.	IL-2/IFN- γ or Multiplex Cytokine Panel incl. Perforin, Granzyme B (for CAR T cells); IL-1, IL-6, NO, IL-8 (for bystander immune cells); clinical parameters like VWF, ANG2, IL-6, CRP, ferritin, MCP1, DIC parameters like fibrinogen, prothrombin time, aPTT

KE 4	Systemic inflammation	<u>Strong:</u> Increased serum levels of CRP, ferritin and other pro-inflammatory mediators in patients upon CAR T cell infusion ⁶² .	Multiplex Cytokine Panel; clinical parameters like VWF, ANG2, IL-6, CRP, ferritin, MCP1, DIC parameters (see above)
Adverse Outcome	Cytokine Release Syndrome	<u>Strong:</u> CRS is a common adverse event upon CAR T cell infusion ^{3,4,7,16,17} .	Same as for KE4

ANG, angiotensin; aPPT, activated partial thromboplastin time; CRP, c-reactive protein; CRS, cytokine release syndrome; DIC, disseminated intravascular coagulation; IL, interleukin; IFN, interferon; MCP-1, monocyte chemoattractant protein 1; NO, nitric oxide; VWF, von Willebrand factor

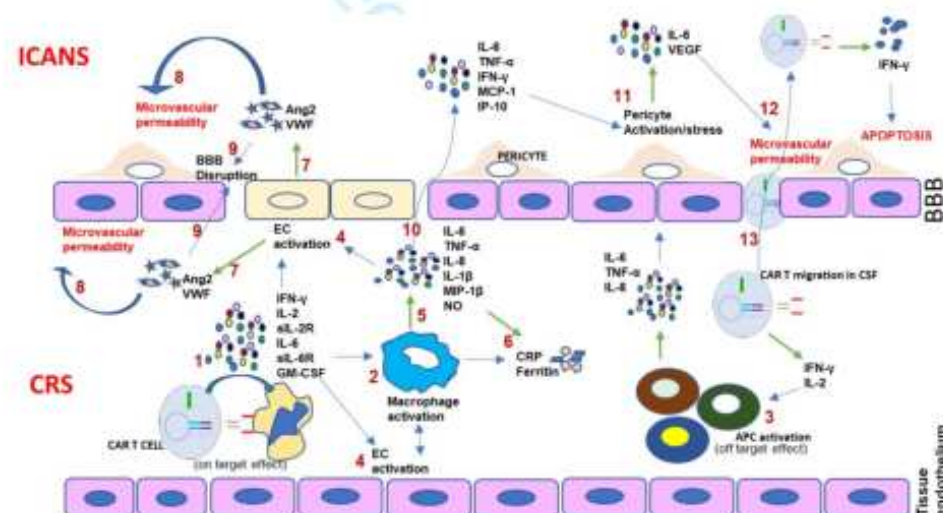


Figure 3 Proposed pathomechanism of CRS and ICANS. Antigen-specific activated CAR T cells (on-target effect) produce inflammatory cytokines (1) resulting in activation of macrophages (2), APC, e.g., dendritic cells, (off-target effect) (3) in the blood circulation and activation of EC (4). Activated macrophages secrete large amounts of pro-inflammatory cytokines (5) contributing to further activation of EC and subsequently produce CRP and ferritin (6). Stimulated EC release VWF and ANG2 (7) associated with CRS and ICANS, resulting in enhanced microvascular permeability (8) and BBB disruption (9). Breach in the BBB allows influx of cytokines into the CNS from blood (10) where these cytokines further activate pericytes (11) which leads to additional IL-6 and VEGF secretion from pericytes. Cytokine storm in the CNS subsequently leads to further enhanced BBB damage and vascular permeability (12) allowing CAR T cell infusion in the brain (13). In the CNS, local cytokines and IFN- γ produced by CAR T cell contribute to apoptosis and ultimate neurotoxicity. ANG2, angiotensin-2; APC, antigen-presenting cells; BBB, blood-brain barrier; CNS, central nervous system; CRP, c-reactive protein; CRS, Cytokine Release Syndrome; CSF, cerebrospinal fluid; EC, endothelial cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICANS, immune effector cell-associated neurotoxicity syndrome; IL, interleukin; IFN, interferon; MIP-1, macrophage inflammatory protein 1.

profile; NO, nitric oxide; sIL-R, soluble interleukin receptor; TNF, tumor necrosis factor; VEGF, Vascular Endothelial Growth Factor; VWF, von Willebrand factor.

In the following sections, we will discuss the currently available *in vitro* and *in vivo* test systems as well as biomarkers for cytokine release and the gaps and limitations of these test systems especially for non-clinical safety assessment of novel CAR T cell products.

3 *In vitro* test systems to assess release of cytokines by CAR T cells (MIE/MoA, KE1-KE3)

Currently available *in vitro* test systems for cytokine release only partly reflect the complexity of the human immune system and/or the situation at the tumor site, as not all relevant cell types are included or at least not in their physiological ratios.

In addition, almost all non-clinical assays "ignore" the influence of lymphodepletion on CAR-T efficacy and safety as well as the influence of the tumor burden (only standardized effector to target ratios) and, importantly, most non-clinical assays are only conducted with cells from healthy donors.

For *in vitro* efficacy testing, CAR T cells are normally co-incubated with antigen-expressing cells or cells that lack antigen expression as a negative control. Here, the cytolytic as well as the proliferative capacity upon antigen encounter can be calculated using standardized effector to target (E:T) ratios which, however, might not reflect the situation *in vivo*. Cytokine release upon antigen encounter has been observed in such co-incubation assays although it is very challenging to draw conclusions regarding the safety of the respective CAR T cell product from these simplistic models.

The addition of e.g., monocytes or endothelial cells in such co-incubation assays can help to get an insight into the interactions of CAR T cells with target and bystander immune cells and thus provide a potentially enhanced model for identifying potential safety issues (hazard assessment) and for investigating associated mechanisms. Ideally, all cell types included in such models would be obtained from the same donor to avoid confounding results due to HLA-mismatch. Such a fully autologous assay was recently described by the Mitchell group. They developed an autologous endothelial cell / peripheral blood mononuclear cell *in vitro* assay that can detect cytokine responses e.g., to mAb⁶⁴. Here, they prepared autologous endothelial cells from peripheral blood progenitors, termed blood outgrowth endothelial cells (BOEC). The BOEC assay is now commercially available at one of imSAVAR's partner sites, LabCorp Drug Development Inc, and is already part of our test concept for the different MoAs covered by imSAVAR, including CAR T cells, giving us the unique opportunity to study e.g., cytokine release by CD19 CAR T cells and the effect on endothelial cells and other immune cells in a fully autologous *in vitro* test system. Here, plans to further improve the BOEC assay to a 3D model are currently under development and will be implemented into imSAVAR's workflow towards improved non-clinical safety assessment. Recently, the Mitchell group described an advanced version of this BOEC assay ("vessel in a dish" bioassay), where also

smooth muscle cells were prepared from progenitors present in peripheral blood⁶⁵. Another group recently demonstrated that antigen-presenting cells (APCs) secrete IL-6 when co-cultured with CD19-specific CAR T cells and tumor cells expressing CD19. This observation was dependent on the co-incubation of APCs with CAR T cells and their respective target cells but not dependent on cell-cell contact as results could be reproduced in a trans-well assay format, thereby confirming the role of cytokines for the activation/engagement of APCs in the CRS pathophysiology⁶⁶. Another approach was published by Norelli et al.⁶⁶, where they used T cells from newborn hematopoietic stem and progenitor cells (HSPC)-humanized SGM3 mice to generate CAR T cells and co-cultured them with either CD33⁺CD44v6⁺ THP-1 leukemic cells or CD19⁺CD44v6⁺B⁺V173 leukemic cells (see also section 5).

Additionally, whole-blood assays were described in recent years as a low-cost alternative to evaluate cytokine release by mAb like TGN1412 and other immunomodulatory drugs⁶⁷⁻⁷¹. Here, minimally diluted whole blood is co-incubated with the respective drug of interest. Then, after e.g., 24 h plasma is collected and cytokines are analyzed. Such an assay is currently not used for studying CAR T cell mediated cytokine release but could be a low-cost method for monitoring cytokines in patient sera.

In general, standardization and harmonization of cytokine release assays is still an open issue. The simplest method to analyze cytokine release in supernatants of such co-culture assays would be a solid-phase enzyme-linked immunosorbent assay (ELISA) to analyze e.g., IFN- γ release. For multiplex cytokine analysis, two main formats are available: bead-based, liquid-phase assays e.g., BDTM cytometric bead array and plate-based, solid-phase assays e.g., the electrochemiluminescence (ECL)-assay from Meso Scale Discovery (MSD)⁷²⁻⁷⁴.

Assays to measure cytokine release are standardized and validated and are also used in the clinic to measure cytokine levels in human plasma or sera⁷⁵. In the experimental *in vitro* setting, where e.g., CAR T cells are co-incubated with target cells, such a standardization is missing: E:T ratios may vary, as well as negative and positive controls, culture conditions, incubation times which also vary depending on the MoA used (e.g., CAR T cells vs. bispecific T cell engagers)⁷¹.

Furthermore, controls are normally included to validate the assay performance and not as a benchmark for a potential safety risk. In addition, it is still difficult to use cytokine levels measured *in vitro* as a guide for the safety of a given immune therapeutic for clinical application⁷¹. Therefore, the imSAVAR consortium aims at establishing a "feedback loop" between biomarker development (based on clinical observations) and analyses in 2D/3D *in vitro* models or animal models, which could help to better define cytokine levels that may be harmful. Here, we collect and analyze samples from patients undergoing CAR T cell therapy with FDA/EMA approved CAR T cell

products. Here, we will also characterize all immune cell subsets prior and after infusion of the cell product at specific time points.

It should however be highlighted, that most test systems are still only conducted with cells from healthy donors and permanent (immortalized/tumor) rather than autologous cell lines (primary tumors, patient-derived tumor organoids)^{71,76}. But, as patients receiving immunotherapies are normally heavily pretreated, the use of cells from healthy donors may not reflect the cellular distribution of the pathologic condition⁷⁷.

Table 2 summarizes the most widely used assays to analyze cytokine release, examples of controls utilized in these assays as well as their limitations.

Table 2: *In vitro* test systems for cytokine release

Test system	Possible read-outs	Controls (examples)	Limitations	Purpose
Co-culture of tumor and immune cells (e.g., CAR T cells)	ELISA Multiplex Cytokine Analysis Flow Cytometry	- Cell culture medium - PMA/ionomycin - Tumor cells lacking antigen expression	Bystander (immune) cells missing	Efficacy testing. Useful for assessment of CAR T cell MoA and KE1/2 (Table 1)
PBMC assay (including high cell density culture "RESTORE" protocol ^{78,79})	ELISA Multiplex cytokine analysis Flow Cytometry	- Cell culture medium - Vehicle controls - PMA/ionomycin - LPS/SEB - Isotype controls - Anti-CD3 (e.g., OKT3) - Anti-CD28 (e.g., TGN1412)	Described for cytokine release of mAb. Might be difficult to translate to preclinical testing of CAR T cells	Efficacy and safety testing of mAb. Could possibly be adapted for CAR T cells to assess KE2/3 (Table 1)
Co-culture assay of e.g., BOEC or HUVEC and PBMC ^{63-65,80}	ELISA Multiplex cytokine analysis Flow cytometry	- Cell culture medium - Vehicle controls - PMA/ionomycin - Isotype controls - Anti-CD3 (e.g., OKT3) - Anti-CD28 (e.g., TGN1412)	Confounding results due to HLA-mismatch might occur if cells are obtained from more than one donor	Safety testing possible. Could be adapted for CAR T cells to assess MoA,

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				KE1/2/3 (Table 1)
Whole Blood Assay 67-69	ELISA Multiplex cytokine analysis Flow cytometry	- Cell culture medium - Vehicle controls - PMA/ionomycin - LPS/SEB - Isotype controls - Anti-CD3 (e.g., OKT3) - Anti-CD28 (e.g., TGN1412)	Described for cytokine release of mAb. Might be difficult to translate to preclinical testing of CAR T cells	Safety testing for mAb possible. Might be useful in an adapted version for monitoring of patients during CAR T cell clinical trials.

BOEC, bone marrow-derived endothelial cells; CAR, chimeric antigen receptor; ELISA, enzyme-linked immunosorbent assay; HUVEC, Human umbilical vein endothelial cells; KE, key event; LPS, lipopolysaccharide; mAb, monoclonal antibody; MoA, mode of action; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; SEB, staphylococcal enterotoxin B

Apart from the limitations of such assays in general, additional consideration should be taken into account when using these assays for non-clinical safety assessment of CAR T cell products. Unlike other immune therapeutics such as monoclonal antibodies CAR T cells are a "living drug", i.e., they have the ability to expand and also contract within the patient's body and can also potentially form a memory pool^{2,9,81}. This poses the risk of recurrent CRS in case of tumor relapse or on-target / off-tumor toxicity if the targeted antigen is not strictly tumor-specific.

Furthermore, there are also other factors to consider which are (in part) unique for CAR T cells. The antibody fragment (often a scFv of a monoclonal antibody of murine origin) which confers specificity to the CAR, also impacts the affinity of the CAR to its target and therefore on the safety of the CAR (off-target/on-target off-tumor effects or low on/off binding rate, avidity of the CAR)⁴³. In addition, if not fully humanized, the non-human parts of the scFv might induce an immune response leading to premature elimination of CAR T cells rendering subsequent infusions difficult or even impossible^{82,83}.

The cellular composition of the final CAR T cell product may additionally impact the efficacy and hence also the safety of CAR T cell therapy. Turtle et al. conducted clinical trials with a defined ratio (1:1) of CD4⁺ and CD8⁺ CAR T cells. Here, they found, as also reported by others, that also the preconditioning regimen for lymphodepletion influences persistence, safety and immunogenicity of CAR T cell therapy^{7,8,52,53,82,84-86}.

Furthermore, Globerson Levin et al. reported that by using a mathematically guided approach they tested reducing the administered CAR T cell dose in a breast cancer model. This dose was still efficacious and could be safer as release of IFN- γ was reduced ⁸⁷.

All these factors presented above may have varying effects on the ability of CAR T cells to induce CRS and have to be taken into account regarding the use of test systems for CRS, i.e., that also controls should be adjusted accordingly. For cytokine release mediated by CAR T cells these controls should be adjusted for CAR design and the scFv targeting the antigen of interest as well as for antigen density on target cells (tumor cells with lower or high antigen expression).

Furthermore, most of the above mentioned *in vitro* test systems mainly focus on measuring released or intracellular cytokines without capturing effects of the interactions of CAR T cells with other blood cells, the endothelium and the target tissue, in other words, the microphysiological environment CAR T cells are experiencing *in vivo*. Here, new advanced *in vitro* test systems like organ-on-chips could be able to close this gap. Such systems are capable of measuring the cytokine release of endothelial cells under varying conditions, and contain a combination of 3D culture, multiple cell types, and flow ⁸⁸⁻⁹⁰.

4 Advanced *in vitro* test systems to assess cytokine release (MIE/MoA, KE1-KE4)

Organs-on-chips (OoC) are defined as "fit-for-purpose" microfluidic devices, containing living engineered organ substructures in a controlled microenvironment, that recapitulates one or more aspects of the organ's dynamics, functionality and (patho)physiological response *in vivo* under "real-time monitoring" ⁹¹. Using advanced bioengineering approaches, OoC mimic physiologically relevant tissue microenvironments and recapitulate the interaction between different human cell types and the interaction of different tissues. Most importantly, a completely controlled microenvironment that requires very few cell numbers allows for testing hypotheses in the presence or absence of specific cell types and for comparing patient-specific reactions relative to the physiological response of tissues from healthy individuals ⁹².

OoC development and use has grown exponentially over the past decade ⁹¹ and, in the field of small molecule toxicity, they have been shown to recapitulate key *in vivo* human features that were neither recapitulated in classical *in vitro* systems, such as multi-organ toxicokinetic studies ⁹³⁻⁹⁶ or immune cell recruitment ⁹⁷⁻⁹⁹ nor in animal models ¹⁰⁰⁻¹⁰⁴. The most common OoC systems tested for drug toxicity applications represent tissues such as liver, gut, lung and heart. More recently, several cancer-on-chip models have been developed ¹⁰⁵⁻¹⁰⁷ but the use of cancer-on-chip models combined with immune cells is still scarce ^{108,109}. However, for the study efficacy and safety of immunotherapies, models that include relevant features of the immune system as well as the vasculature are essential ^{97,109}. The endothelium is the first contact point for the circulating immune cells and their released cytokines. It, therefore, plays a key role in the mechanisms of toxicity of

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3 immunotherapies. Many OoC studies already employ methods that can cover the proposed irAOP
4 for CAR T cells from KE1 to KE4. The systemic inflammatory response to CAR T cell therapies
5 (KE4) is influenced by the changes that a treatment might cause on the activation of the
6 endothelium and the resulting cytokine release (KE2+3). Moreover, the adverse outcome on an
7 organismal level (hypotension, capillary leak, microthrombi) is caused for an important part by
8 endothelial dysfunction (e.g., loss of barrier function) (KE3). A number of OoCs have been
9 introduced focusing on recapitulating functional vascular endothelium ^{90,110-112}. Endpoints such as
10 the capacity to retain the controlled permeability to large molecules, the release of cytokines, the
11 presence of endothelial cell adhesion markers, and the adhesion of platelets and monocytes are
12 commonly analyzed while stimulating with stressors that are known to activate the endothelium
13 and/or to disrupt the membrane ⁸⁸⁻⁹⁰. Co-perfusion of tumor cells and monocytes through an OoC
14 model integrating a self-organized 3D vascularized network of endothelial cells has enabled the
15 investigation of the interplay between tumor cell and monocytes during extravasation ¹¹³. The
16 co-perfusion with immune cells is also a key feature necessary to study CAR T cells' safety profile
17 and to bridge KE1 with KE2 and for studying the KE2/3 interplay. A different study co-cultured
18 immune cells in a tumor-on-chip model in a static, not perfused configuration and tested the effect
19 of trastuzumab on the immunosuppressive mechanisms of cancer associated fibroblasts (CAFs)
20 using time-lapse imaging, advanced video analysis and 3D live images ¹¹⁴. Those techniques allow
21 for a qualitative and quantitative assessment of the interaction of the immune cells with the cancer
22 cells and could be applied to study KE3/KE4. The microfluidic character of OoCs ensures a more
23 physiological cell-to-media-ratio essential to assure relevant concentrations of cytokines as well as
24 an effective and physiologically relevant paracrine signaling between different cells within the same
25 tissue. Overall, tailored OoC systems that allow the evaluation of barrier function, cell adhesion to
26 endothelium, cell migration and invasion as well as a relevant production of cytokines will allow the
27 study of CAR T cells' irAOP (Table 3). This can be done either in a stepwise approach, one device
28 tailored for each KE, or in a holistic model representing the KE1/2/3/4. The controlled nature of OoC
29 models would also enable studies elucidating the role of each individual cell type for a specific KE
30 and the final adverse outcome. A detailed mechanistic approach using tailored OoC models, at the
31 molecular level, will also support the discovery and validation of biomarkers in early stages
32 leveraging a human-relevant but controlled environment. However, very complex OoC models
33 reflecting the interaction between various immune cells and the respective cancer cells are still
34 under development ¹⁰⁹. The main limitation of applying OoC models for CAR T cell efficacy and
35 safety assessment is the possibility of missing off-target effects in organs not included in the study
36 design. Particularly, KE4, systemic inflammation and the KE relationships thereof, could be
37 incomplete. Here, animal models could allow a more explorative approach in the sense that all
38 organs can be analyzed; however, only if all relevant human targets are indeed expressed ¹⁰⁴.

Table 3: OoC systems for CAR T cell efficacy and safety assessment

Test system	Possible read-outs	Controls (examples)	Limitations	Purpose
Cancer-chip	Imaging (monitor cell migration) Multiplex Cytokine Analysis	- Cell culture medium, conditioned - Tumor cells lacking antigen expression - Healthy cells vs cancer cells (positive control)	Bystander (immune) cells missing	Efficacy testing. Useful for assessment of CAR T cell MoA and KE1/2 (Table 1)
Vascular-chip	Live imaging (monitor membrane integrity) Multiplex Cytokine Analysis Platelet/monocyte adhesion	- Cell culture medium, conditioned from cancer-chips - Membrane disruptors - Endothelial cells stressors (e.g., TNF-α, as positive control)	Cancer cells are missing	Safety testing possible. Could be adapted for CAR T cells to assess MoA, in a patient specific fashion KE2/3 (Table 1)
Vascularized cancer chip	Live imaging (monitor membrane integrity) Imaging (monitor cell migration) Multiplex Cytokine Analysis	Same as above	To evaluate the order of the observed events	Safety testing possible. Could be adapted for CAR T cells to assess MoA, in a patient specific fashion KE1/2/3 (Table 1)
Vascularized cancer chip connected to the vascular chip	Live imaging (monitor membrane integrity) Imaging (monitor cell migration) Multiplex	- Same as above - Combinations where the exposure occurs first on the vasculature and/or first on the cancer tissue		Safety testing possible. Could be adapted for CAR T cells to assess MoA, in a patient specific

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	Cytokine Analysis			fashion KE1/2/3/4 simultaneously (Table 1)
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CAR, chimeric antigen receptor; KE, key event; MoA, mode of action

5 Animal models (non-clinical assessment of CRS mediated by CAR T cells *in vivo*)

In recent years, several syngeneic mouse models for CD19 CAR T cell therapy have been described. In the model reported by Kochenderfer et al. murine CD19 CAR T cells were able to eradicate CD19-positive lymphoma as well as normal B cells (B cell aplasia). However, no side effects due to CAR T cell therapy were reported in this model ¹¹⁵. Davila and colleagues described a similar model using C57BL/6 mice. Here, murine CD19 CAR T cell therapy induced long-term remission and B cell aplasia in mice engrafted with acute lymphoblastic leukemia (B-ALL). However, similar to the Kochenderfer mouse model, no signs of CRS like weight loss or elevated cytokine levels were reported in CD19 CAR T cell treated mice ¹¹⁶. Pennell and colleagues reported recently a human CD19 transgenic mouse model where CD19 CAR T cells not only induced B cell aplasia but also toxicities associated with CAR T cell therapy. Mice treated with CD19 CAR T cells showed signs of CRS like weight loss which could be blunted with IL-6 neutralizing therapy ¹¹⁷. Hence, this model could be a useful tool for studying CD19 CAR T cell-associated toxicities.

However, as the murine immune system differs from the human one e.g., in CD28 downstream signaling ¹¹⁸, translation of experimental CAR T cell therapy in syngeneic/transgenic mouse models to the situation in patients might be difficult to achieve. Translation to the bedside could also be hampered if CAR vectors used to treat mice differ from CAR vectors used in human *in vitro* studies and/or clinical trials.

Use of established cell lines for tumor engraftment might also be a limiting factor as fresh tumor samples are more heterogeneous as cell lines passaged *in vitro* for a long time. Hence, patient-derived tumor xenograft models (PDX models) could be a useful tool for non-clinical CRS assessment ^{119,120}. Here, use of patient-specific organoids in advanced 3D models could also be a way to circumvent the use of established cell lines in tumor models ¹²¹.

Norelli and colleagues were able to mimic CRS in humanized triple transgenic NSG mice (HuSGM3) engrafted with a patient-derived CD19⁺ ALL-CM leukemia cell line. This model also elucidated the role of monocyte-derived IL-1 and IL-6 as mediators of CRS, which could be prevented by monocyte depletion ⁵⁶.

Recently, Giavridis and colleagues reported an improved SCID-beige mouse model mimicking CRS upon CD19 CAR T cell therapy ⁵⁷. As the incidence of CRS is also associated with tumor burden ^{4,81,82,121} they engrafted mice intraperitoneally with Raji lymphoma cells. Cells were allowed to grow

to vascularized solid tumor masses before injection of CD19-specific CAR T cells. Here, CRS developed within three days after CAR T cell treatment and was mediated by macrophages producing IL-1, IL-6 and NO ⁸⁷.

Pfeiffer et al. developed an NSG mouse model with *in vivo* generation of CD19 CAR T cells by using a CAR vector specifically targeting CD8⁺ T cells. Here, CAR expression in CD8⁺ T cells resulted in B cell depletion and in some mice signs of CRS could be detected ¹²². As this model relies on the use of a special CAR vector the translatability to other CAR targets might, however, be limited.

Van der Stegen and colleagues used a SCID-beige mouse model to evaluate the toxicity associated with a target that is also expressed in healthy tissues (off-tumor toxicity). They developed an ErbB CAR that could target human and mouse ErbB dimers also expressed in healthy tissues. Here, toxicity of CAR T cell therapy was associated with the release of IL-6 by macrophages. This was also depending on the site of CAR T cell administration ¹²³. Such a model might be a useful tool if the specific CAR is able to target both – the intended human target cells as well as healthy murine tissues expressing the target at physiological levels.

Taken together, currently available mouse models can only reflect the situation in patients suffering from CRS upon CAR T cell administration in a limited fashion. One major limitation is the use of immunocompromised mouse models like non-obese diabetic/severe combined immunodeficiency (NOD-SCID)- γ c⁻ (NSG) mice ^{124,125}. Such mouse models are a useful tool for efficacy testing of novel cell products as they tolerate the engraftment of tumor cells and other cell types like HSPCs of human origin ¹²⁶⁻¹²⁸. However, without engraftment of HSPCs or cells of similar origin mimicking at least in part the human immune system, such models do not allow studying possible immune-related adverse events of the administered therapy. Syngeneic models as exemplified above using immunocompetent mice can overcome this obstacle. Here, however, the CAR T cells need to be of murine origin, thereby possibly limiting the translatability to the human setting.

The use of dogs as a large animal model for CAR T cell therapy was also recently discussed as dogs were originally used to study the use of hematopoietic stem cell transplantation. The high incidence of spontaneous tumor development in outbred dogs could here be of advantage for testing of novel CAR T cell products ¹²⁹⁻¹³¹. As there however might be also (unknown) differences between the canine and human immune system the translatability of such studies might be limited.

The similarity between the human and non-human primate (NHP) immune system potentially allows for safety testing of novel CAR T cell products. However, human CAR T cells engrafted into NHP host are not only at the risk of graft rejection by the host, but also lead to graft versus host disease. To avoid such risk, autologous CAR T manufactured with NHP cells have been developed ¹³²⁻¹³⁵. However, the use of NHP CAR T cells is not relevant in predicting CRS risk by human CAR T cells.

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In addition, there are significant difficulties in generating functioning NHP CAR T cells by lentivirus, due to species-specific restriction factors such as rhesus TRIM5 α in NHP¹³²⁻¹³⁵. Moreover, NHP models are immunocompetent, and do not allow for engraftment of tumors. Therefore, the value of NHP models in predicting CRS risk by CAR-T therapies is limited, and NHP studies have not been leveraged as an integral part of CAR-T investigational new drug (IND)-enabling safety assessment packages¹³⁶.

Table 4 summarizes currently available animal models for CAR T cells and their limitations regarding translation and/or CRS assessment.

Table 4: Currently available animal models for CAR T cell therapy

Model	Description	Limitations	Purpose
Syngeneic mouse models ^{115,118}	Immunocompetent model. Tumor cells and CAR T cells of murine origin.	Translation to situation in patients might be limited if CAR	Efficacy and safety testing of CAR T cells (off-tumor toxicity). Assessment of MoA and KE1/2 possible (Table 1).
NSG mouse model ¹²⁴	Immunocompromised model lacking T, B and NK cells. Tumor cells are engrafted followed by CAR T cell administration (both of human origin).	Without engraftment of immune cells assessment of CRS not possible	Efficacy testing of CAR T cells (MoA, KE1). Safety testing possible if mice are additionally engrafted with human immune cells (MoA, KE1/2).
SCID-beige mouse model ⁵⁷	Engraftment with 3×10^6 Raji tumor cells (<i>i.p.</i> ; lymphoma cell line, human origin). After three weeks <i>i.p.</i> injection of 30×10^6 CAR T cells (human origin).	Artificial tumor site. Tumor engraftment in bone marrow ("physiological" tumor niche) might give different results.	Efficacy and safety testing of CAR T cells (CRS). Assessment of MoA, KE1-4 possible (Table 1).
HuSGM3 mouse model (PDX model) ⁵⁶	Sublethally irradiated newborn triple transgenic NSG mice engrafted with HSPCs and ALL cells of human origin. Four or seven weeks (low or high tumor burden) later administration of CAR T cells of human origin.	Availability of matched HSPCs and tumor cells might be limited.	Efficacy and safety testing of CAR T cells (CRS). Assessment of MoA, KE1-4 possible (Table 1).
Dogs ¹²⁹⁻¹³¹	Immunocompetent model. Tumor	Ethics, costs, only	Efficacy and safety

	cells and CAR T cells of canine origin.	specialized centers, smaller groups due to animal size. With dogs possibly limited translatability to humans	testing of CAR T cells (off-tumor toxicity). Assessment of MoA and KE1/2 possible (Table 1).
Non-human primates 134, 135	Immunocompetent model. CAR T cells of primate origin.	Ethics, costs, only specialized centers, smaller groups due to animal size. GVHD and graft rejection, if using human CAR T cells. Poor lentivirus CAR transduction of NHP cells and lack of relevance if using NHP CAR T cells. No tumor.	Efficacy and safety testing of CAR T cells (off-tumor toxicity). Assessment of MoA and KE1/2 possible (Table 1).

ALL, acute lymphoblastic leukemia; CAR, chimeric antigen receptor; GVHD, graft-versus-host disease; HSPEC, hematopoietic stem and progenitor cell; Ip., intraperitoneal; KE, key event; MoA, mode of action; NHP, non-human primate

As outlined in section 3, in all these models presented here, appropriate controls should also be taken into account for CAR design (persistence, production of cytokines), use of scFv (affinity, antigen density on target cells, possible off-target toxicity) and the composition of the infused CAR T cell product.

6 Biomarkers for toxicities in CAR T cell therapy

Following the Biomarkers Definition Working Group (Biomarkers Definitions Working Group 2001)¹³⁷, we define biomarkers for non-clinical safety assessment of immunotherapeutic interventions like CAR T cells as *measurable and evaluable biological characteristics* evaluating the MIE, the KEs or the adverse event (e.g. CRS or ICANS) of an irAOP. In addition, biomarkers support the evaluation of non-clinical models assessing safety of novel CAR T cell constructs. Non-clinical models for safety assessment of novel CAR T cell constructs should mimic as closely as possible immunological processes underlying the MIE, KE and/or adverse events of CAR T cells. In order to be able to evaluate this, non-clinical models must objectively measure related biomarkers in a repeatable and reproducible manner. Biomarkers for non-clinical development of CAR T cells are either indicators for the underlying immunological processes leading eventually to toxicities as well as indicators for the suitability of a non-clinical safety model. However, biomarkers may also be correlated with the occurrence of CRS without being involved directly in the CRS pathophysiology

but can be used to predict if there will be an increased risk of developing CRS. Thus, biomarkers support iterative improvement of AOPs and non-clinical safety models (Figure 4).

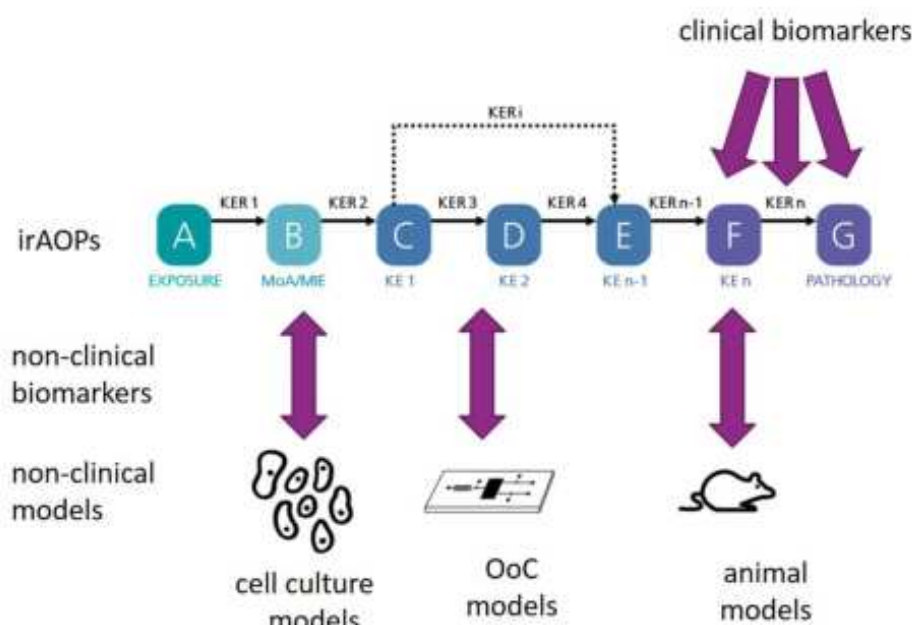


Figure 4 Schematic overview depicting biomarkers and connectors between irAOPs and non-clinical safety models to support iterative development of non-clinical safety models. Biomarkers are biological characteristics describing the underlying immunological processes leading eventually to toxicities and serve as indicators for the suitability of a non-clinical safety model. Abbreviations define irAOPs, immune-related adverse outcome pathways; KE, key event; KER, key event relationship; MI, molecular initiating event; MoA, Mode of action; OoC, organ-on-chip

The majority of reported biomarkers related to CRS and ICANS triggered by CAR T cells are clinical biomarkers, i.e., biological characteristics used for early prediction of adverse events in patients and used for patient management^{24,60,62,138}. Teachey et al. assessed 39 children and 12 adults with refractory/relapsed ALL treated with CTL019 using a preselected panel of 43 cytokines and compared cytokine levels between mild and severe grades of CRS. Among those, 24 cytokines (e.g., IFN- γ , IL-6, IL-8, sIL2R α , sgp130, sIL6R, MCP1, MIP1 α , MIP1 β , and GM-CSF) depicted statistically significant changes and combined in a selection of different statistical models (e.g., logistic regression and decision tree) to predict severe CRS. However, even though the serum biomarkers CRP and ferritin were related to CRS, they did not add informative value to models predicting severe CRS⁶². Hay et al. assessed 133 adult patients receiving CD19 CAR T cells to predict CRS using a preselected set of potential biomarkers⁶⁰. Among different clinical parameters,

like e.g., cyclophosphamide/fludarabine lymphodepletion, cytokine levels, serum angiopoietin-1 (ANG1), ANG2, and VWF levels were measured in a subset of patients. Higher levels of VWF and ANG2:ANG1 ratio were significantly associated with CRS grade ≥ 4 compared to grades < 4 . However, biological characteristics for early prediction of CRS grade ≥ 4 compared to grades < 4 comprise cytokines IFN- γ , IL-6, IL-8, IL-10, IL-15, MCP1, tumor necrosis factor receptor p55 (TNFRp55), and macrophage inflammatory protein-1b (MIP-1 β). A classification tree model for early identification of high-risk patients included fever and levels of MCP1 as parameters. The same model with the additional parameter IL-6 is predictive for high grade neurotoxicity⁶³. Rossi et al. used a panel of 32 secreted proteins to assess up to 2000 single cells of the preinfusion product of CD19 CAR T cells and defined five functional profiles of CAR T cells¹³⁹. Interestingly, polyfunctional CAR T cells (i.e., CAR T cell products with at least two proteins from different functional profiles to be co-secreted in single cells) were associated with grade ≥ 3 CRS.

Even though a plethora of molecules is known to be clinically related to toxicities triggered by CAR T cells (see above) and can partly be assigned to KEs of the related irAOP (see Table 1 and Figure 2), no standards are available to assess their suitability for application in non-clinical development of novel CAR T cell constructs. Hence, transferring reported clinical biomarkers to non-clinical assessment of novel CAR T cells requires addressing the following questions among others:

- (i) Which molecules are objectively measuring and evaluating the immunological processes underlying KEs leading eventually to CRS or ICANS, i.e., are reproducible indicators for these biological processes?
- (ii) Which of those described molecules are objectively measurable and evaluable as readouts of non-clinical models for CRS or ICANS triggered by CAR T cells?
- (iii) Which observed changes of molecule abundance of an upstream KE are critical leading to measurable and evaluable changes in the downstream KE, and thus eventually triggering harmful CRS or ICANS in first-in-human studies?
- (iv) Which molecules are robust and generalizable indicators for immunological processes independent from technical variations among different instances of the same non-clinical model?

Especially changes in (serum) cytokine levels are important biological characteristics as indicators for the risk of harmful CRS^{62,138}. However, the inter-individual variability of baseline cytokine levels as well as their association to diverse pathogenic processes make it difficult to identify specific set of cytokines for separate KEs and to define a threshold for toxicity¹⁴⁰, i.e., leading to measurable changes in downstream KEs.

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The majority of molecules herein described to be related to toxicities of CAR T cells (Table 1 and Figure 2) were detected based on studies assessing a preselected list of marker candidates. However, utilizing unbiased approaches for future biomarker discovery has the potential to reveal still unknown immunological characteristics leading to severe CRS or ICANS.

To explore CRS in an unbiased manner Norelli et al.⁵⁶ performed single-cell RNA sequencing (scRNA-seq) of whole human CD45⁺ leukocytes isolated from leukemic humanized mice treated with CAR T cells. Twelve major lymphoid and myeloid cell populations were identified based on cluster analysis at day 2 and day 7 after fever onset. They showed that on single-cell level, monocytes are the major source of inflammatory cytokines, including IL-1 β and IL-6 as well as CXCL8 (IL-8), CCL2 (MCP1), CCL8 and CXCL10. Besides monocytes, other cell types, including dendritic cells, also contributed to inflammatory cytokine production. Sheih et al. used single cell transcriptomics and TCRB sequencing to analyze the clonal diversity of CAR T cells at various times following infusion in patients with acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma and different grades of neurotoxicity and CRS. Unsupervised clustering of the scRNA-seq data identified two out of four clusters from clones at early time points after infusion that were enriched with genes associated to cytotoxicity and proliferation states, while at later time points after infusion proliferation states declined without enrichment of an exhaustion gene signature. However, they did not relate their findings to CRS or ICANS.¹⁴¹ Li et al. performed time series transcriptome profiling in one plasma cell leukemia patient (grade 3 CRS) treated with B-cell maturation antigen (BCMA) CAR T cells. They analyzed scRNA-seq datasets including infusion products, CAR T cells, and endogenous T cells at the peak and remission phases. Gene signatures of the infusion products were enriched in metabolically active, glycolysis and biosynthetic pathways. At the peak phase, two intermediate states were observed: a highly proliferative and a cytotoxic state, assuming a transition from proliferation to cytotoxic activity, whereas CAR T cells in remission phases were in a "resting primed" memory-like state.¹⁴² Moreover, they re-analyzed the single-cell datasets from Sheih et al and showed that BCMA- and CD19-directed CAR T cells have similar transcriptional characteristics, especially at the CAR T cell peak phase. Deng et al. performed whole transcriptome scRNA-Seq of infusion products for 24 patients with large B cell lymphoma.¹⁴³ In patients who were less responsive to treatment, they observed an enrichment for exhausted T cells, whereas those who achieved complete response showed enrichment for memory T cells. For patients with high-grade CRS, they observed a negative association with exhausted CD8 T cells and a positive association with exhausted CD4 T cells. Moreover, based on scRNA-seq and hybrid capture sequencing, they found cell populations with transcriptional expression pattern similar to myeloid lineage, which was associated with development of high-grade neurotoxicity. These ICANS associated cells expressed genes including IL1 β and CXCL8 (IL-8), which were related to CRS.⁵⁶ By performing a transcriptome analysis using human brain scRNA-Seq and bulk RNA-Seq datasets as well as immunohistochemistry,

Parker et al. showed that CD19, regarded as B cell specific, was expressed on mural cells, which line blood vessels and maintain the blood-brain barrier (BBB) integrity ¹⁴⁴. Based on mouse CD19-directed CAR T cells they indicated that neurotoxicity may be mediated by targeting CD19⁺ mural cells in mice due to increasing the BBB permeability. However, the study did not determine the precise contributions of CRS in this process. Fraietta et al. revealed by transcriptomic profiling, that CD19 CAR T cell products from complete responding patients with chronic lymphocytic leukemia were enriched in memory phenotype, including IL-6/STAT3 signatures, whereas those who did not respond showed enrichment for pathways including effector T cell differentiation, glycolysis, exhaustion and apoptosis ¹⁴⁵. Performing RNA-seq and miRNA-seq analysis of bone marrow cells from four patients with B-ALL before and after CD19 CAR T cell therapy, resulted in a global view of the transcriptome profiles and regulatory mechanisms in activated CAR T cells ¹⁴⁶. In one out of the four patients with severe CRS (the others achieved a molecular remission) up-regulated differentially expressed genes (DEGs) were enriched in cytokine-related and acute inflammatory response processes, while the down-regulated DEGs were enriched in the apoptosis and endocytosis related pathways.

Comprehensive single-cell or bulk transcriptome analyses using CAR T cells also provide an opportunity to guide the evaluation and optimization of CAR T cell manufacturing or correlate CAR T cell specific molecular signatures with clinical outcomes. To elucidate the underlying mechanisms of cytokine profiles, Xhangolli et al. used three different assays ¹⁴⁷. Co-cultured CAR T cells from healthy donors with Raji lymphoma cells were analyzed by single-cell 3'mRNA transcriptome sequencing, multiplexed single-cell cytokine secretion assay and live cell imaging of cytolytic activity. They illustrated the polyfunctional response of CAR T cells, whose activation states are highly mixed with T helper cell (TH) 1, TH2 and regulatory T cell (Treg) functions. Cytotoxic activities were equally observed in both CD4⁺ and CD8⁺ CAR T cells associated with a wide range of TH1 and TH2 signature cytokines (e.g., IFN- γ , TNF- α , IL-5, and IL-13). They showed that GM-CSF is highly prevalent in activated CD4⁺ CAR T cells independent of differentiation. Boroughs et al. showed that 4-1BB-based CAR T cells were enriched in a central memory phenotype compared with CD28-based CAR T cells which could have profound effects on the heterogeneity and functional state of CAR T cell infusion products ¹⁴⁸. Wang et al. performed single-cell profiling of APRIL CAR T cell products of three healthy donors in the presence and absence of antigen-specific stimulation and the resulting molecular signature for CAR T cell activation provides a resource for future dissection of underlying mechanisms ¹⁴⁹.

7 Conclusion & Outlook

The success of CAR T cell therapy in the treatment of relapse/refractory B cell malignancies has paved the way for the development of novel CAR T cell products targeting also e.g., multiple

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3 myeloma and solid tumors¹⁵⁰. The high remission rates, however, come with the price of potentially
4 fatal side effects like CRS.
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7 Currently available non-clinical *in vitro* as well as *in vivo* models often fail to reflect the complexity
8 of the human immune system and are therefore not able to accurately predict possible harmful side
9 effects like CRS. Furthermore, standardized biomarkers to measure or even predict the occurrence
10 of CRS are still missing. In addition, simplistic models with limited read-outs and endpoint analysis
11 might underestimate potential adverse effects of novel immune therapeutics.
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15 The IMI2/EU project imSAVAR develops immune-related adverse outcome pathways (irAOP)
16 which shall guide the refinement and/or development of non-clinical models to assess the safety of
17 novel immunomodulatory therapies¹⁵¹. With the help of the irAOP concept we aim at establishing
18 a platform with refined test systems and biomarkers to predict possible serious side effects of novel
19 immune therapies. Further, we aim at establishing a model grading system, which may be used as
20 a guide for researchers who want to include safety assessment in their non-clinical analysis. In
21 addition, the effects of established/novel interventional therapies could also be assessed on such
22 a test system platform.
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26 Furthermore, more sophisticated 3D *in vitro* models like organs-on-chips could in the future bridge
27 the gap between safety assessment in "classical/conventional" *in vitro* test systems and in animal
28 models thereby also reducing the costs for the development of novel immune therapeutics. This
29 could in future also lead to a replacement of animal models by complex OoC models with advanced
30 non-clinical biomarkers based on the irAOP. In complex OoC models the influence of additional cell
31 types, e.g., fibroblasts in solid tumors, on the development of CRS and other immune-related
32 adverse events could also be studied in more detail¹⁰⁹.
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41 Current clinical management of adverse outcomes of CAR T cell treatments builds on a minor
42 number of statistical models interrogating biomarkers and general indications (like fever), but are
43 mostly trained with a non-representative sample size and lack validation in independent patient
44 cohorts. Due to limited mechanistic understanding of the processes triggering adverse outcomes
45 of novel engineered T cell constructs, risk prediction of adverse outcomes early in development of
46 these constructs is still hard to achieve. The framework of irAOPs supports sharing and
47 continuously updating a common understanding of the molecular and cellular processes leading to
48 adverse outcomes, and thus supports systematic biomarker development. In this journal issue,
49 Mazein et al. take this approach even further by implementing the linear irAOP into an interactive
50 platform thereby enabling an extended exploration of the underlying molecular mechanisms.
51 Further, with increasing availability of advanced methods for examining molecular and cellular
52 states (e.g., multi-omics or multi-parametric flow cytometry, single-cell where needed) as well as
53 with new tools for integrative bioinformatics changes in the underlying processes of KEs and KERs
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3 can be easier described in the future. Therefore, we propose, to integrate advanced (unbiased)
4 assessment of molecular and cellular states into non-clinical development of novel CAR T cell
5 constructs. This will pave the way to an iterative approach to improve understanding of
6 pathogenesis and occurrence of adverse outcomes, to improve non-clinical safety models and to
7 define novel biomarkers predicting risk of adverse outcomes in first-in-human studies.
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Disclosure statement

The authors report there are no competing interests to declare.

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Publication [7]**Opportunities in using MPS for toxicity testing
of new drug modalities**

T. I. Maulana, N. Wevers, T. Kristoforus, M. Chandler, J. Joore, S. Kustermann, R. Villenaeve, P. Loskill, P. Vulto, K. Bircsak
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Abstract:

New drug modalities offer life-saving benefits for patients through access to previously undruggable targets. Yet these modalities pose a challenge for the pharmaceutical industry, as side effects are complex, unpredictable, and often uniquely human. With animal studies having limited predictive value due to translatability challenges, the pharmaceutical industry seeks out new approach methodologies. Microphysiological systems (MPS) offer important features that enable complex toxicological processes to be modeled in vitro including 1) an adjustable complexity of cellular components, including immune components, 2) a modifiable tissue architecture, 3) integration and monitoring of dynamic mechanisms, and 4) a multi-organ connection. Here we review MPS studies in the context of four clinical adverse events triggered by new drug modalities: peripheral neuropathy, thrombocytopenia, immune-mediated hepatotoxicity, and cytokine release syndrome. We conclude that while the use of MPS for testing new drug modality-induced toxicities is still in its infancy, we see strong potential going forward.

Title

Opportunities for microphysiological systems in toxicity testing of new drug modalities

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Abstract

New drug modalities offer life-saving benefits for patients through access to previously undruggable targets. Yet these modalities pose a challenge for the pharmaceutical industry, as side effects are complex, unpredictable, and often uniquely human. With animal studies having limited predictive value due to translatability challenges, the pharmaceutical industry seeks out new approach methodologies. Microphysiological systems (MPS) offer important features that enable complex toxicological processes to be modeled in vitro including 1) an adjustable complexity of cellular components, including immune components, 2) a modifiable tissue architecture, 3) integration and monitoring of dynamic mechanisms, and 4) a multi-organ connection. Here we review MPS studies in the context of four clinical adverse events triggered by new drug modalities: peripheral neuropathy, thrombocytopenia, immune-mediated hepatotoxicity, and cytokine release syndrome. We conclude that while the use of MPS for testing new drug modality-induced toxicities is still in its infancy, we see strong potential going forward.

Keywords: MPS, NAMs, new drug modalities, toxicity testing, in vitro, safety

1 The safety challenge posed by new drug modalities

New drug modalities have rapidly gained popularity over the past decade. These include antibody-drug conjugates (ADCs), chimeric antigen receptor (CAR)-T cells, multi-specific antibodies, nucleot(s)ide-based therapies, and gene therapy using adeno-associated viruses (AAVs) and lentiviral vectors (1). New modalities typically enable higher target specificity, improved stability, access to previously undruggable targets, and offer the possibility to leverage new mechanisms of action (e.g. modulating the host adaptive immune system). Today, portfolios of most pharmaceutical companies include multiple new modalities in addition to traditional small molecule drugs (2).

Despite their great potential as treatments for patients in need, these novel therapeutic modalities present new challenges by triggering complex and often unpredictable side effects, so called adverse events. Frequently observed adverse events include peripheral neuropathies induced by ADCs, thrombocytopenia induced by ADCs, AAVs, and oligonucleotide drugs, (immune-mediated) drug-induced liver injury triggered by AAVs, or cytokine release syndrome (CRS) as a result of treatment with T cell engagers and CAR-T cells. Due to the relative lack of clinical experience with new modalities, a thorough early nonclinical assessment is crucial to ensure patient safety in clinical trials.

In vivo animal models are considered the gold standard for classical safety testing. However, especially in the context of new drug modalities, safety assessment using these models may provide limited translatability. This has motivated the pharmaceutical industry to explore the use of new approach methodologies (NAMs) to complement or sometimes replace conventional nonclinical safety assessments (3). Particularly in cases where the immune system is involved, in vitro NAMs that comprise human immune system components are a promising option (4). Furthermore, in vitro NAMs enable mechanistic/investigative studies that cannot be performed in a similar manner in animal studies.

Microphysiological systems (MPS) are a sub-category of NAMs that deploy microengineering techniques for constructing composite, often perfused, tissues. The past decade saw tremendous advances in the MPS space to the level that they are recognized as a “game-changing technology” in the field of investigative toxicology (5) and explicitly mentioned in the 2022 FDA Modernization Act 2.0 as an alternative means for drug testing. Today extensive efforts are made in defining standardizing processes and methodologies for specific contexts of use (COU) (6). MPS offer unique features, setting them apart from simple 2D cell culture and animal models including adjustable complexity of cellular components, modifiable tissue architecture, integration and monitoring of dynamic mechanisms, and multi-organ connection. These features make MPS a powerful alternative to predict toxicities and understand the underlying mechanisms of new drug modalities (Figure 1). Here we review the use and the potential of MPS for evaluating adverse effects of new drug modalities. We will highlight design features, key adverse events triggered by new modalities, and the opportunities available for MPS to identify these clinical adverse events.

2 Key features of MPS for capturing complex toxicities of new drug modalities

2.1 Adjustable complexity of cellular components

A salient feature of MPS is their capacity to integrate multiple cell types and to recapitulate tissue-specific cellular organization in a "healthy" or "diseased" state, which can be tailored according to the experimental requirements and research questions (7). For nonclinical safety assessment, this feature can be valuable in dissecting the role of specific cell type(s) in initiating/perpetuating toxicity and recapitulating certain key events within adverse outcome pathway networks describing certain toxicity mechanisms (8).

In the case of new drug modalities, adverse effects can result not only from direct cell-drug interactions but also from a cascade of interactions between various specialized cell types and the drug within a specific microenvironment. One example is CRS after CAR-T cell therapy, where the interplay between tumor, adaptive immunity (T cells), innate immunity (monocytes, macrophages), and the vasculature is found to be crucial in the pathotoxicity mechanism (9). To this end, MPS increasingly integrate immune cells to recreate both innate and adaptive immune responses in a specific tissue. Some examples include tissue-resident macrophages in adipose tissue, Kupffer cells in liver tissue, microglia in neural tissue, tumor-associated macrophages in tumor tissue, and perfused circulating immune cells (PBMCs, monocytes, T cells, neutrophils) through blood vessel-like structures, which are also amenable for drug and inflammatory response studies (4, 10–17).

Another example of an adverse event that requires complex multicellular interactions is immune effector cell-associated neurotoxicity syndrome (ICANS) occurring after CAR-T cell and T cell bi-specific antibodies (TCB) therapy. Although the pathophysiology remains poorly understood, the interaction between the blood-brain-barrier (BBB), neurons, microglia, and immune effector cells seems to be an important driver

of toxicity (18, 19). Here, several MPS models have successfully replicated human BBB, some of which incorporate neural tissue as well. These models were used to assess BBB damage, immune cell recruitment, and neural inflammation, although they are not yet utilized for investigating ICANS specifically (20–26).

Combining cellular complexity with a careful cell sourcing strategy (animal or human origin, allogeneic or autologous) in MPS models presents the opportunity to understand the mechanisms of species-specific toxicity and de-risk toxicities before first-in-human studies. Such an approach is especially important when no pharmacologically relevant species are available for safety assessment (27).

2.2 Modifiable tissue microarchitecture

In addition to cellular complexity, the spatial distribution of the cells in a MPS model can be tailored to resemble the in vivo microarchitecture of a specific tissue. This feature supports numerous functions, including the recreation of tissue-specific barriers and the parenchyma. It serves as a valuable tool for real-time investigation of drug transport, cell trafficking, the effect of barrier damage in case of adverse drug events, among others.

For toxicity studies, epithelial and/or endothelial barriers established on MPS have been utilized in a wide range of applications. Some examples include the evaluation of pro-inflammatory cytokines typically detected in human plasma during CRS (TNF- α and IL-1 β) on BBB disruption and the resulting T cell infiltration and monocyte adhesion (11), uveitic side effects of TCBs in choroid tissue resulting from an inflammatory response of recruited PBMCs across the outer blood-retinal barrier (4), therapeutic IL-2-induced pulmonary oedema (28), drug-induced loss of gut barrier integrity (29), and cisplatin-induced damage of proximal tubule (30), among others. In investigating the toxicity mechanism of

immunotherapies and others that generate an immune response, integrating endothelium may be crucial as it serves as the initial point of contact for circulating immune cells and their released cytokines.

2.3 Integration and monitoring of dynamic mechanisms

Another feature of MPS is the capability to integrate dynamic mechanisms resembling *in vivo* conditions. This includes continuous perfusion of medium to enable controlled delivery of nutrients, removal of metabolic waste products and secretomes, and mimicked transendothelial/-epithelial delivery of therapeutic compounds as well as cell therapies (31–33). It can also include biophysical cues such as shear stress from the fluidic perfusion, and mimicked breathing and peristaltic motions in airway and gut tissues (34, 35). Relevant biophysical cues not only extend *in vitro* tissue culture duration to several weeks, but also better recapitulate physiological function and cellular responses to drug treatments (33). For example, alveolar-capillary barrier damage upon IL-2 therapy is significantly amplified when mechanical strain is applied in a lung MPS model, similar to *in vivo* (28). Another example is the effect of perfusion of human pluripotent stem cell-derived kidney organoids, which is beneficial for tissue vascularization. In this study, perfusion enables the circulation of PBMCs and the assessment of their extravasation and infiltration into 3D kidney tissue as an off-target effect of TCB therapy (36). Integrating dynamic mechanisms *in vitro* can therefore improve the translatability of the safety assessment findings to human settings.

Integrating dynamic mechanisms in MPS can be coupled with dynamic monitoring. For instance, linear fluidic perfusion of MPS provides the opportunity to dynamically sample the effluent (perfused medium) and analyze the release kinetics of signaling molecules, metabolic activity, and toxicity biomarkers in real-time at high temporal resolution and at different stages of the disease or treatment. This measurement can also be done through direct sensor integration (37). In addition, the optical accessibility of MPS allows

high-resolution real-time imaging to study spatiotemporal dynamics at the microscale level, such as cell-cell interactions, cellular migration, composition, morphology change, cell fate decisions, drug localization, and other aspects relevant to the toxicity assessment of the tissue of interest (38).

2.4 Multi-organ connection

Linking multiple MPS facilitates the recreation of systemic interactions between multiple organs, and has been demonstrated for the study of pharmacokinetics and off-target toxicities of various drug modalities (22, 31, 39–52). Applying such a system early in the drug discovery process can potentially reveal complex metabolic and toxicological relationships of a drug that are not observable by testing on a single organ model. For example, multi-organ connection of liver and heart MPS demonstrated the modulation of terfenadine-induced cardiotoxicity through liver metabolism, and additionally revealed human ether-a-go-go (hERG) liability of a small molecule drug through the formation of a hERG active metabolite (39). In the context of new drug modalities, especially biologics, recapitulating human immunity in multi-organ MPS is particularly important (27) and could allow the investigation of the mechanism of immune-mediated systemic toxicity, e.g. by elucidating the role of specific cell type(s) or related molecules in initiating the adverse event.

The following chapter will review specific clinical adverse events typically observed from the treatment of new drug modalities and the potential for MPS, that employ the key features described above, to elucidate toxicity mechanisms and improve toxicity prediction.

3 Opportunities for MPS to model clinical adverse events associated with new drug modalities

3.1 Cytokine release syndrome by CAR-T and bispecific T cell engager therapies

CRS is a systemic inflammatory reaction triggered by the induction of powerful immune effector responses, characterized by a high increase of proinflammatory cytokines in the peripheral blood, which can lead to systemic inflammation, organ damage, neurotoxicity, and even death in some patients (53). It is one of the most prevalent adverse events associated with advanced therapy medicinal products, such as CAR-T cells and TCB therapies for cancers (54–58). The occurrence is very challenging to predict (59). Despite being clinically manageable, CRS incidence, rate, and severity can vary greatly among patients (9) and the toxicity mechanism underlying this patient variability remains unclear. The pathophysiology of CRS is complex, involving T cells, tumor cells, endothelial cells, and "bystander" endogenous immune cells such as monocytes and macrophages at the local tumor site and in blood circulation (53). It is first initiated by a molecular initiating event (MiE, e.g. CAR-T cell recognizing the target antigen on tumor cells) followed by increased proinflammatory mediators and subsequent activation of monocytes, macrophages, and endothelial cells in the target tissue, leading to systemic inflammation (9, 60).

Nonclinical in vivo models have reliably failed to predict CRS partially due to the indistinguishable symptoms from graft-versus-host disease caused by human T cells attacking non-host tissues (61). The use of fully humanized mouse models also remains a challenge due to difficulties in achieving complete reconstitution of the human immune system, the risk of spontaneous rejection of injected human tumor cells, and high costs, among other factors (62). In a clinical trial involving a T cell engaging monoclonal antibody therapy, TGN1412, non-human primate (NHP) models failed to predict CRS in a first-in-human study due to a lack of CD28 expression on CD4+ effector memory T cells in macaques, which is required for T cell activation and cytokine production (63). Additionally, most of the currently available in vitro test systems for cytokine release lack the incorporation of relevant cell types, physiologically relevant ratio of

cells, and interaction of different tissues involved in CRS development (64). Thus, novel models are urgently needed to elucidate this mechanism and better predict patient responses.

MPS models have the potential to address this gap, as they can recapitulate aspects of the tumor microenvironment (32), systemic interaction between different organs, and integrate human immune components and thereby immune response, as described in the previous chapter. Many studies using MPS already demonstrate methods that can recapitulate the different stages of CRS development (Table 1). In CAR-T cell therapy, these include CAR-T cell circulation, extravasation towards the tumor, CAR-T cell-mediated tumor killing, cytokine release from CAR-T-tumor interaction, activation of vascular endothelium at the local tumor site, and vascular dysfunction in distant organs (e.g., loss of barrier function that could lead to organ damage such as brain edema). Early key events during CAR-T cell-tumor interaction have been demonstrated in MPS models. These systems were also utilized to assess the release of cytokines typically measured in the clinic to monitor potential CRS development in patients, such as IL-2, IL-6, IL-10, TNF- α , IFN- γ , and granzyme B (65, 66)(Figure 2A). The ability to recreate the cytokine release kinetics mimicking CRS development in one of these tumor MPS enabled the demonstration of a safety intervention strategy using dasatinib as a pharmacologic on/off switch to temporally control CAR-T cell activity (66).

Cytokine release in target tissue can lead to the activation of endothelium and subsequently leakage of the vasculature (64). MPS models have demonstrated methods to recreate and assess vascular endothelium, as reviewed elsewhere (67, 68). These include the assessment of endothelial barrier permeability (e.g. via transendothelial electrical resistance (TEER) measurement) and cell adhesion markers under flow, endothelial activation (e.g. ICAM-1, E-selectin), and adhesion of perfused platelets and monocytes, among others (25, 26, 69–73). Increasing cellular complexity of current tumor MPS could

potentially be applied to investigate the interplay between recruited (CAR-)T cells, monocytes, macrophages, and the tumor as well as their relative role in the release of CRS mediators. In terms of the recapitulation of patient-specific effects with regards to cytokine release upon CAR-T cell treatment, a study using tumor MPS included the integration of patient-derived tumor organoids to test ROR1-specific CAR-T cells and showed that inflammatory cytokine release was positively correlated with ROR1 expression level of the tumor organoids (66).

One of the CRS manifestations observed in patients is organ dysfunction, such as in the central nervous system (CNS). There is evidence of increased CAR-T cell recruitment into the CNS during CRS, which also coincides with ICANS development (53). However, the relative contributions of pro-inflammatory cytokines and recruited CAR-T cells in the CNS towards the development of ICANS are so far unknown. Nair et al. modeled the BBB damage induced by systemic circulation of pro-inflammatory cytokines TNF- α and IL-1 β and the resulting T cell recruitment (11). This MPS setup could well be extrapolated to assess the role of CAR-T cells in ICANS development. In addition, direct fluidic coupling of CAR-T cell- or TCB-treated tumor MPS with healthy organ models has the potential to systematically study immune-mediated adverse effects in “distant” organs in real time during the treatment, as certain clinical biomarkers of CRS are also produced systemically through inter-organ crosstalk. One example could be the connection of CAR-T cell- or TCB-treated tumor with a liver MPS to potentially detect C-reactive protein, as this is produced by hepatic tissue in response to tumor-site-derived proinflammatory cytokines.

3.2 Immune-mediated hepatotoxicity by AAV-based gene therapy

Hepatotoxicity is the most reported clinical adverse event for adeno-associated virus (AAV)-based gene therapies that are approved or in clinical trials. Outcomes range from elevated liver enzymes to acute liver failure and death (74–76). While the detailed underlying mechanisms of AAV-mediated hepatotoxicity are

largely unknown, it is hypothesized to be driven by both an adaptive as well as innate immune response (77, 78). With more than half of the systemic AAV-based gene therapy products approved in the last year and many more in clinical trials, there is an urgent need to predict and understand the mechanisms related to severe adverse events for subsequent methods of mitigation (79–81).

AAV tropism is often species-specific, limiting the value of nonclinical animal models for evaluating AAV-mediated hepatotoxicity (82–84). Humanized mouse models which replace the mouse liver (85, 86) and/or components of the immune system (85, 87) with respective human-specific cells may address a translational gap; however their low throughput, cost, and ethical considerations limit their relevance. One of the gold standards for in vitro hepatotoxicity evaluation is the primary human hepatocyte (PHH) sandwich culture. This setup is useful for estimating human hepatocyte-specific AAV transduction efficiency and comparison to murine or NHP hepatocyte monocultures for translation to animal data (83, 84, 88). However, the lack of liver microenvironment features including cellular complexity (immune cells, vasculature) and dynamic mechanisms (a blood flow mimic) limits their applicability for immune-mediated hepatotoxicity evaluation. To this end, several liver MPS which incorporate these features hold high potential for evaluating and predicting AAV-mediated hepatotoxicity (Table 2).

Many liver MPS incorporate Kupffer cells (89–94), the resident macrophages of the liver, which are important for the initial innate immune response to AAVs. All platforms are perfusable (89–97) and would be amenable to incorporating circulating immune cells. Vasculature is an important feature for liver MPS modeling AAV-mediated hepatotoxicity, as liver sinusoidal endothelial cells are known to be involved in inflammatory liver injury via multiple mechanisms including production of cytokines and adhesion of circulating immune cells (98). Of the models listed, only two have reported the incorporation of circulating immune cells (polymorphonuclear leukocytes or monocytes) and their adhesion to vasculature under

activated endothelium conditions (Figure 2B) (90, 94). The blood flow mimic combined with human circulating immune cells through vasculature mirrors physiologically relevant events that cannot be captured by in vitro models without perfusion.

For clinical translation, the ability to measure liver enzymes typically observed in patients including alanine transaminase (ALT), aspartate transaminase, alkaline phosphatase, and gamma-glutamyl transferase is critical. While secreted ALT is measured and reported by Feaver et al. (89) and Ewart et al. (93), most other systems do not report on clinically relevant liver enzymes. Authors instead focus on other markers of cellular injury including LDH release, albumin production, cytochrome P450 activity, steatosis, mitotoxicity, and cytokine production. The latter is used in most MPS studies in response to an immune-mediated challenge such as lipopolysaccharide (LPS) (89, 91–95, 99). While AAV clinical trials do not typically report on patient chemokine/cytokine levels, it should be noted that higher levels of IL-6, IL-8, and lower levels of CCL5 and IL-17 were reported in patients that died from acute liver failure due to drug-induced liver injury (100). Considering the interaction of AAVs with the immune system and the ability to measure cytokines in liver MPS, it will be feasible to utilize MPS to model clinically relevant levels of cytokine production. Such an approach may aid in identifying AAV-based gene therapies that trigger an immune-mediated hepatotoxicity response and to better understand the underlying mechanisms.

A number of improvements are foreseen that increase the utility of MPS for assessing AAV-mediated hepatotoxicity. Higher throughput capabilities will support library screens for the rational selection of AAV serotypes, promoters, and payloads for optimal transduction efficiency. Prolonged culture periods of up to 12 weeks will provide the timeframe in which the toxicities are observed in the clinic. Additionally, hepatotoxicity does not occur in all individuals, indicating the need for multi donor tests representative of a population. Finally, patients receiving AAV-based gene therapy may have underlying liver disease

and/or concomitant administration of hepatotoxic drugs which may provide a baseline level of hepatic injury which may additively or synergistically interact with the AAV. An immunocompetent liver MPS would not only be beneficial for capturing AAV-mediated hepatotoxicity and testing clinical mitigation strategies, but also for other drug modalities known to interact with the liver immune system including small molecule drugs (i.e., trovafloxacin (101), amoxicillin-clavuanate (102)) and immune check point inhibitors (i.e., infliximab, rituximab, nivolumab (103)).

3.3 ADC-induced peripheral neuropathy

A common side effect of chemotherapeutic drugs is peripheral neuropathy (PN), afflicting 30-40% of patients, resulting in pain and other symptoms that can impair normal daily activities and decrease therapy compliance. To reduce chemotherapy-induced PN, efforts are made to improve drugs' therapeutic impact while minimizing systemic toxicity. ADCs combine the cytotoxic effects of chemotherapy drugs with increased specificity via monoclonal antibodies that target cancer cells. However, PN is still a common side effect in patients treated with many currently approved ADCs (104). Efforts are being made to develop new ADCs that show high efficacy in targeting cancer cells but reduced PN side effects.

While animal models provide insights into PN, their translational relevance is hampered by species differences. Human in vitro models emerged to address this limitation. Initial efforts utilized neuronal-like cell lines for simplicity, but they lack the biological complexity of the cell types affected in PN (105). Models using primary cell sources better mimic tissue complexity, but face challenges in reproducibility and standardization (106). Finally, the commonly used 2D culture vessels fail to mimic the local cellular organization and tissue microarchitecture relevant to PN, e.g. the spatial separation of cell somas and axons, highlighting a need for more advanced models. A recent publication by Eldridge et al. (107)

provides a comprehensive overview of the requirements for a reliable in vitro model of chemotherapy-induced PN for mechanistic explorations needed to elucidate the underlying pathophysiology and assess novel therapies. In summary, the authors concluded that the field would benefit from more complex in vitro models, such as iPSC-derived, organoid-derived, or microfluidic models, that contain several relevant cell types, but still allow for sufficient throughput for drug testing.

Several models have been reported that hold high potential for assessing ADC-induced PN (Table 3). Work by Kramer et al. (108) describes the use of rat dorsal root ganglia (DRG) neurons in a single-chip device in which cells were seeded in a somal compartment and extended their neurites into a neurite compartment via microgrooves. They showed chemotherapy-induced PN evidenced by decreased nerve conduction velocity and peak amplitude. Also using rat DRG, Matsuda et al. (109) employed an MPS that allows parallel culture of 24 chips on one device. Using artificial intelligence-based image analysis, the authors showed morphological changes in cell somas and neurites following exposure to chemotherapy drugs known induce PN. The success of both models in assessing chemotherapy-induced PN indicate high potential in assessing PN-inducing effects of ADCs.

Other work describes the use of all-human in vitro models in MPS platforms. Klim et al. (110) and Melamed et al. (111) describe the differentiation of human embryonic stem cells or iPSCs to motor neurons. Motor neurons were seeded in 2D in the somal compartment of a microfluidic device and extended their axons towards an axonal compartment via microgrooves. Via knockdown approaches, the authors showed the essential role of the Stathmin-2 protein in mediating motor neuron growth and repair. While neither study focused on drug-induced PN, the microfluidic devices and presented readouts would allow for such an application.

Another microfluidic model employing motor neurons was published by Spijkers et al. (Figure 2C) (112). Similar to the work of Melamed et al. (111), this study describes the culture of iPSC-derived motor neurons in microfluidic chips that allow compartmentalization of somas and axons. In contrast to the chips used by Klim et al. (110) and Melamed et al. (111), the microfluidic platform used by Spijkers et al. (112) allows for increased throughput, in the form of 40 chips cultured in parallel within one 384-well plate. Furthermore, the cells grow embedded in extracellular matrix, mimicking the 3D structure found in vivo. Chemotherapy-induced PN was shown by exposure to the chemotherapeutic drug vincristine, which induced axonal damage in a highly reproducible dose-dependent manner. The response of this model to vincristine in combination with its relatively high throughput indicates suitability of this model for assessment of ADC-induced PN.

Sharma et al. (113) highlight the potential of co-culture of different cell types relevant to assess PN. The authors describe the generation of spheroids of human iPSC-derived motor neurons and human primary Schwann cells. These spheroids were placed in a single-chip MPS device, after which neurites grew into a somal compartment. This human co-culture model allowed for assessment of neurite outgrowth, myelination, and nerve conduction velocity. While displaying impressive biological complexity, the use of this model for assessment of ADC-induced PN may suffer from challenges in standardization of organoid cultures and the labor-intensive culture process.

At present, there are no published reports of in vitro studies on ADC-induced PN to the best of our knowledge. However, several presently reported models hold great potential for assessing peripheral neuron toxicity caused by novel drug modalities such as ADCs or AAVs (114). While certain models provide high biological complexity, others provide pragmatic solutions at higher throughput. Future model development would benefit from inclusion of human sensory neurons rather than motor neurons and

where necessary further increased complexity through the addition of supporting cell types. Importantly, a fit-for-purpose approach may be required to allow optimal assessment of novel drug modalities, depending on the hypothesized mechanism of action and required assay throughput.

3.4 Thrombocytopenia by ADCs, ASOs, and AAV vector gene therapies

Thrombocytopenia, or low blood platelet count, is an important adverse event to consider in patients treated with new drug modalities. It is the most common severe adverse event upon treatment with HER2-targeted ADCs (115). The toxicity mechanism is associated with impaired megakaryocyte production (116). Furthermore, thrombocytopenia was reported as one of the serious adverse events in two clinical trials with 2'-O-methoxyethyl-modified antisense oligonucleotides (ASOs) (117, 118). One patient receiving the ASOs had severe thrombocytopenia with a fatal outcome. Contrary to these studies, Crooke et al. (119) reported non-clinically relevant platelet reduction with ASO treatment, highlighting the lack of mechanistic understanding of the underlying toxicity. Thrombocytopenia as one of the features of thrombotic microangiopathy (TMA) and can also affect patients receiving AAVs for gene therapy. Although the occurrence is relatively rare with 9 cases out of more than 1,400 patients receiving AAV9 *SMN* gene therapy, the outcome can be fatal (120). TMA following AAV therapy was demonstrated to be dependent on anti-capsid antibodies (121).

Assessing drug toxicity on hematopoietic cells such as megakaryocytes in human bone marrow (BM) niches is challenging due to technical and ethical barriers in obtaining BM biopsies (122). Therefore, in vivo and in vitro models are used as a surrogate. Results from studies with NHP offered some insights into the mechanism of ASO-induced thrombocytopenia, but with limited translatability to humans (123). Mouse models offer limited value due to interspecies discrepancies in hematopoietic cell biology, immune systems, and drug sensitivity (124–126). Murine platelets lack FcγRIIA and protease-activated receptors

that are essential for antibody-dependent enhancement and thrombin activation, respectively (127–129). Humans have five-fold less circulating platelets than mice, but with longer platelet lifespan (8–12 days vs 4 days) (129). Humanized mouse models still have the limitations of murine cells' influence on the human BM niche (122). Moreover, in vitro 2D models lack the tissue architecture and cell-matrix interactions needed for the development of relevant phenotypes of hematopoietic stem cells (HSCs) (130). With adjustable cellular complexity, MPS enable the study of human HSCs and platelets in the context of drug-induced thrombocytopenia, in a more physiological tissue microarchitecture supported by dynamic mechanisms.

Current MPS that are relevant for the study of drug-induced thrombocytopenia range from BM-on-chip to vascular inflammation-on-chip (Table 4). BM-on-chips by Chou et al. (131) and Glaser et al. (132) mimic the BM niche by incorporating HSCs to achieve multilineage differentiation. Perfusion through an endothelium-lined vascular compartment provides an in vivo-like dynamic mechanism. These BM models were used to study drug toxicity in the BM niche. Toxicity to megakaryocytes in the context of drug-induced thrombocytopenia can potentially be assessed using these models. To model platelet production, Blin et al. (133) (Figure 2D) and Thon et al. (134) attempted to create platelet generator microfluidic models. These platforms utilized shear force for platelet formation from megakaryocytes. Although these systems are meant to address the shortage of platelet donation, they could potentially be used to evaluate drug toxicity on platelet formation. In the context of studying toxicity, the drug of interest can be added into the vascular channel and the effect on platelet production can subsequently be evaluated. Additionally, immune cells can be incorporated to better mimic the cellular complexity in the BM vascular system.

TMA is indicated by microvascular thrombosis as well as abnormal arterioles and capillary endothelia (135). Models by Hao et al. (136), Zhang et al. (137), and Barrile et al. (138) that include blood vessels and platelets can be employed to study thrombosis upon AAV administration. In the microfluidic device developed by Hao et al. (136), each microchannel of cancer cells and microvascular system received different doxorubicin concentrations from the gradient generator. With endothelium-lined vascular channels and platelet supply, this model allows the study of endothelial cell pathology and platelet-endothelial adhesion associated with thrombosis. For thrombosis, Zhang et al. (137) described a 3D bioprinted model with a hydrogel microchannel lined with endothelium and/or fibroblasts that was infused with whole blood. Another model by Barrile et al. (138) had an endothelium-lined vascular channel for the purpose of assessing drug-induced thrombosis. The vascular injury associated with TMA can be examined in a vascular-inflammation-on-chip model by Ehlers et al. (139). To induce vascular inflammation, PBMCs with or without cytokine stimulation were added into the endothelial cell tubules. The readouts include TEER and ICAM-1 and VE-cadherin expression on the endothelial cells as well as changes in endothelial morphology. In future applications, neutrophils can be incorporated into the system as they are shown to have an important role in vascular injury in TMA (140). As TMA in AAV gene therapy involves antibody-dependent complement activation, MPS modelling blood vessels can be leveraged by connecting them to lymph-nodes-on-chip for a multi-organ connection (141) enabling antibody production. Furthermore, antibodies against AAV capsid could be measured as a mediator that potentially leads to TMA.

4 Considerations for MPS in toxicity testing of new drug modalities

While transforming the way the pharmaceutical industry is developing drugs, new therapeutic modalities also bring challenges for safety assessment. In vitro NAMs and particularly MPS could address some of these challenges and improve shortcomings of existing conventional toxicology models.

Unique features of MPS are the adjustable cellular complexity and ability to combine aspects such as epithelia, stroma, vasculature, and immune cells together in a dynamic microenvironment. The incorporation of immune components in MPS is critical as many new drug modalities interact with or modulate the immune system, a crucial aspect where many current nonclinical models fall short. With this in mind, MPS could be tailored to key pharmaceutical industry questions or COU, for example, where the dynamic nature of inflammatory events such as the leukocyte recruitment cascade is central to the pathophysiological process, or the complex inter-cellular interactions of tissue-resident immune cells are important. However, for every COU, the chosen model's complexity should always be carefully balanced against throughput, cost, and robustness.

While moving from cell line-based monoculture systems to complex multi-cell-type patient-specific models, important logistical considerations in cell sourcing arise. In some use cases, where donor-matching is desirable, sourcing of autologous tissues may be challenging although HLA matching or progress in stem cell biology may alleviate some of these obstacles. Patient diversity and continuity of supply is another challenge. While organoid technology provides expandability (especially epithelia), not all cell types have unlimited supply. This means that cells derived from a single donor can be used for a limited period of time. This introduces a challenge for comparison with historical data. Guidelines need to be created to circumvent this, including multi-donor panels, as well as tight quality control standards for tissue sourcing, isolation, and banking.

Nonclinical safety testing is highly reliant on past experiences and has resulted in an intricate structure of procedures and regulation to ensure adverse effects are flagged before a drug enters clinical trials. As patient safety is of utmost importance, the space is inherently risk adverse, setting the standards very high for the replacement of existing regulations and procedures. With the advent of new drug modalities, the pharmaceutical industry and regulators enter a relatively unknown territory in which constant dialogue is necessary to improve and evolve the drug safety assessment process (142, 143). This new context provides ample space for innovative methodologies that help assess risks prior to dosing in humans. Opportunities include fields where gold standard models do not exist and where human-specific targets make most animal models non-predictive of the human outcome (144). Due to the urgency of delivering transformative medicine to patients in need, the field of cancer therapeutics is pioneering innovative in vitro model approaches for safety assessment and have showcased their predictive capabilities within this particular COU. With cancer therapeutics leading the way, MPS could play an important role yet to be defined by the regulatory framework.

MPS adoption by the pharmaceutical industry has been particularly slow in the context of regulatory safety, partially explainable by a lack of alignment between the different stakeholders in the MPS development process. While academic and commercial developers endeavor to mimic physiological tissues as closely as possible, the pharmaceutical industry is solely focused on the prediction of human responses to potential therapies. This divergence can lead to the development of in vitro models that may not have immediate pharmaceutical applications, slowing down the adoption of complex MPS technologies. To address these challenges, increased dialogue and education between industry and developers is crucial, including funding bodies, to direct grant funding towards questions with greater industry relevance. Developers could focus on innovation that aligns with industry needs and prioritize human translatability over increasing physiological relevance when it comes to safety modeling.

Commercial developers should work on commercializing and validating models in partnership with end users, something that is already increasingly happening. The pharmaceutical industry should also be more forthcoming in communicating its needs, and effort should be brought to manage expectations of the validity of in vitro tools to prevent the overstating of technological platforms, which could lead to mistrust and hinder adoption by end users.

The industry's perspective on NAMs is also evolving, with a growing understanding of the advantages and limitations of MPS for safety assessment. The initial adoption of MPS for safety assessment was seen as a relatively accessible and desirable goal, given the clear benefit of reducing reliance on animal models for drug testing. However, the complexity of drug-induced adverse events, coupled with a limited mechanistic understanding, was somewhat underestimated at the outset. The predictability of such events is often low, with certain adverse reactions being unique to specific new molecules, making it challenging to generalize findings across a larger set of compounds or modalities. In contrast, leveraging NAMs for disease modeling, where the underlying mechanisms are typically better understood, seems an easier goal to attain, facilitating more accurate in vitro predictions. By integrating safety endpoints as a secondary focus within these in vitro disease models, a more efficient and holistic assessment of a drug's profile can be achieved. This approach allows for the primary emphasis on disease-specific efficacy while still capturing relevant safety data. This trend is indicative of a maturing approach to drug development, where NAMs/MPS are increasingly recognized for their potential to streamline the discovery and evaluation process. The industry's shift towards such integrated models is a positive sign, reflecting a more nuanced appreciation of NAMs/MPS capabilities and their role in advancing pharmaceutical innovation (142).

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Figures and Legends

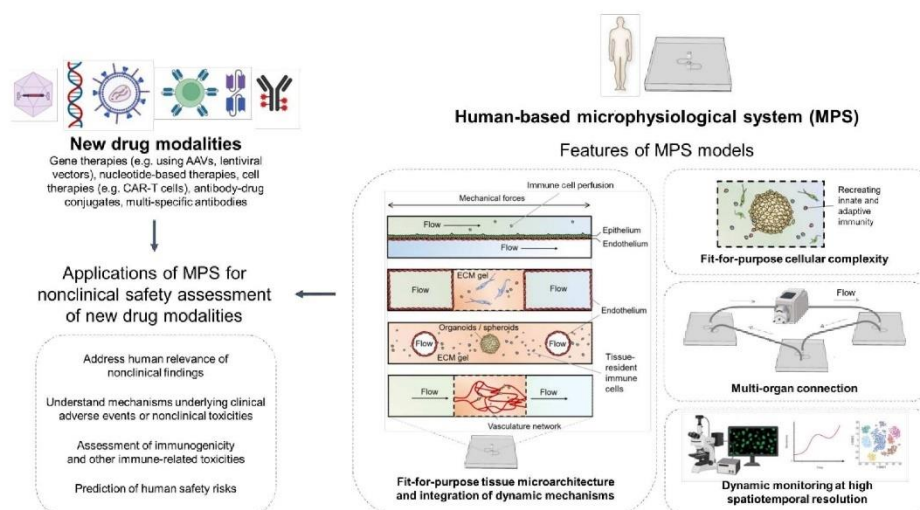
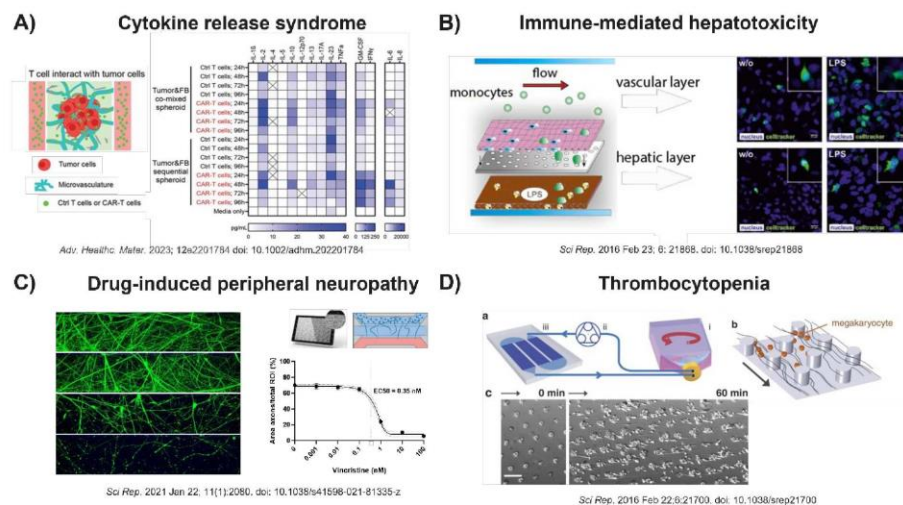


Figure 1. Features of MPS models applied for nonclinical safety assessment of new drug modalities. MPS models offer fit-for-purpose cellular complexity, tissue microarchitecture, integration of dynamic mechanisms and the possibility for multi-organ connection, all compatible with dynamic monitoring at high spatiotemporal resolution. These features offer the ability to predict new drug modality-mediated toxicities not captured by conventional safety models. Together, it is possible to address human relevance, understand mechanisms underlying clinical adverse events and nonclinical or immune-related toxicities, and predict human safety risks. Created partly with BioRender.com.



microfluidic production device. Within the device, serpentine channels (dark blue) were covered with (b) pillars arranged in a hexagonal lattice to promote contact with megakaryocytes (orange). (c) Top-view via SEM of pillars before and 60 mins after perfusion. Scale bar = 100 μm . Figure adapted from Blin et al., 2016 (133) (<https://www.nature.com/articles/srep21700>). All figures republished under the creative commons license (CC BY-NC: A; CC BY 4.0: B, C, D, <https://creativecommons.org/licenses/by/4.0/>).

Tables

Table 1. Summary of MPS capable of modeling key adverse events during CRS development

Author	CRS key events according to Alb et al. (64)	MPS model	Readouts applicable for toxicity testing
Wan et al. (65)	MiE, immune cell recruitment and activation, tumor-killing, increased proinflammatory mediators	Tumor-chip: Tumor spheroids + lung fibroblasts + HUVECs microvascular network + perfused (CAR-)T cells	Cytokine release assay (e.g. IL-2, IL-23, IL-6, GM-CSF, IFN- γ), (CAR-)T cell recruitment
Maulana et al. (66)	MiE, immune cell recruitment and activation, tumor-killing, increased proinflammatory mediators	Tumor-chip: Tumor aggregates/patient organoids + primary microvascular endothelial cells + perfused (CAR-)T cells	Cytokine release assay (IL-2, IL-6, IL-10, TNF- α , IFN- γ , and granzyme B), on- and off-target (CAR-)T cell infiltration
Lee et al. (73)	MiE, immune cell recruitment, tumor-killing, endothelial activation	Tumor-chip: Endothelial cells + melanoma cells + perfused T cells (all murine)	Activation (ICAM-1, E-selectin) and permeability of endothelium at the tumor site
Kerns et al. (145)	Off-target MiE, T cell activation, increased proinflammatory mediators	Alveolus lung-chip: alveolar epithelial cells + microvascular lung endothelial cells + PBMCs Colon intestine-chip: patient-derived colon organoids + large intestine microvascular endothelial cells + PBMCs	T cell recruitment, phenotyping of perfused T cells via flow cytometry (e.g. CD69 expression), cytokine release assay (granzyme B, IFN- γ)
Sasserath et al. (146)	Activation of further inflammatory cells (monocytes), systemic inflammation	Multi-organ chip: Liver (primary hepatocytes), cardiac (iPSC-derived cardiomyocytes), skeletal muscle (primary myoblasts) + THP-1 monocytes	Cytokine release assay (e.g. IL-6, IL-8, MIP-1, MCP-1, TNF- α), monocytes infiltration, cardiac functionality (beat frequency, conduction velocity), skeletal muscle force measurements
Vatine et al. (25)	Systemic inflammation: Vascular leakage through pro-inflammatory cytokines, inflammation of "distant" tissue	BBB-chip: iPSC-derived brain microvascular endothelial cells + astrocytes + neurons + blood perfusion	Inflammatory cytokine-induced BBB leakage, LDH release from neural cells
Nair et al. (11)	Systemic inflammation: Vascular leakage through pro-inflammatory cytokines, inflammation of "distant" tissue	BBB-chip: Primary brain microvascular endothelial cells, primary T cells, THP-1 monocytes	Inflammatory cytokines (TNF- α , IL-1 β)-induced BBB damage (TEER measurement), endothelial activation (ICAM-1, VCAM-1), monocytes adhesion, T cell transmigration

iPSC: induced pluripotent stem cell

Table 2. Summary of liver MPS capable of modeling immune mediated hepatotoxicity by AAVs

Author	Cell types			Other features			Long-term culture (4-12 weeks)
	Kupffer cells	Vasculature	Circulating immune cells	Perfusion	Measure clinically relevant liver enzymes	Measure clinically relevant chemokines/ cytokines	
Feaver et al. (89)	Y	N	N	Y	Y	Y	N.R.
Groger et al. (94)	Y	Y	Y	Y	N.R.	Y	N.R.
Li et al. (90)	Y	Y	Y	Y	N.R.	Y (Verneti et al., 2016)	4 weeks
Bircsak et al. (91)	Y	Y	N	Y	N.R.	Y	N.R.
Rubiano et al. (92)	Y	N	N	Y	N.R.	Y	N.R.
Azizgolshani et al. (95)	N	Y	N	Y	N.R.	Y	N.R.
Bonanini et al. (96)	N	Y	N	Y	N.R.	N.R.	N.R.
Ewart et al. (93)	Y	Y	N	Y	Y	Y	N.R.
Rajan et al. (147)	N	N	N	Y	N.R.	N.R.	N.R.
Ferrari et al. (97)	N	Y	N	Y	N.R.	N.R.	N.R.

N.R.: Not reported

Table 3. Summary of MPS capable of modeling ADC-induced peripheral neuropathy

Author	Species	Cell type(s)	MPS	2D/3D	Assay
Kramer et al. (108)	Rat	Dorsal root ganglia neurons	1-channel microfluidic chip, single-chip device	3D, hydrogel embedded	Effect of chemotherapy drugs on nerve conduction velocity and peak amplitude
Matsuda et al. (109)	Rat	Dorsal root ganglia neurons	1-channel microfluidic chip, 16 chips on one device	2D	Effect of chemotherapy drugs on soma- and neurite morphology
Klim et al. (110)	Human	embryonic stem cell-derived motor neurons	3-channel microfluidic chip with microgrooves, single-chip device	2D	Role of STMN2 in neurite outgrowth and axon regeneration
Melamed et al. (111)	Human	iPSC-derived motor neurons	2-channel microfluidic chip with microgrooves, single-chip device	2D	Role of STMN2 in neurite outgrowth and axon regeneration
Spijkers et al. (112)	Human	iPSC-derived motor neurons	3-channel microfluidic chip, 40 chips on one device	3D, hydrogel embedded	Effect of chemotherapy drug vincristine on axon outgrowth
Sharma et al. (113)	Human	iPSC-derived motor neurons and primary Schwann cells	1-channel microfluidic chip, single-chip device	3D, hydrogel embedded	Neurite outgrowth, myelination, and nerve conduction velocity

Table 4. Summary of MPS capable of modeling thrombocytopenia

Author	Species	Cell type(s)	MPS	Applications
Chou et al. (131)	Human	CD34+ cells, BM-derived MSCs, ECs	2-channel chip: hematopoietic and vascular channels	Hematopoiesis, BM dysfunction
Glaser et al. (132)	Human	CD34+ cells, ECs, BM stromal cells, fetal osteoblast, cancer cells	3-chamber chip: endosteal and perivascular niches; alternate cell of interest (cancer cells)	Effects of doxorubicin and G-CSF on BM niche, metastasis
Blin et al. (133)	Human	MKs differentiated from CD34+ cells	Chip with micropillars	Platelet production
Thon et al. (134)	Human, mouse	hiPSC-derived MKs Mouse primary MKs	2-channel separated by columns	Platelet production
Hao et al. (136)	Human	Tumor cells, ECs, platelet	Microfluidic system of drug injection site, gradient generator, tumor culture, vascular channel	Effects of chemotherapy on ECs, platelet-ECs adhesion
Zhang et al. (137)	Human	ECs, fibroblasts, cells from whole blood	Microchannel with ECs on its wall	Thrombosis, thrombolysis, fibrosis
Barrile et al. (138)	Human	ECs, cells from whole blood	2-chamber chip: lower channel lined with ECs	Drug-induced thrombosis
Ehlers et al. (139)	Human	PBMCs, ECs	3-channel chip within microtiter plate (64 chips)	Vascular inflammation

BM: bone marrow, ECs: endothelial cells, G-CSF: granulocyte-colony stimulating factor, hiPSC: human induced pluripotent stem cell, MKs: megakaryocytes, MSCs: mesenchymal stromal cells, PBMCs: peripheral blood mononuclear cells

Publication [8]**Breast cancer-on-chip for patient-specific efficacy and safety testing of CAR-T cells**

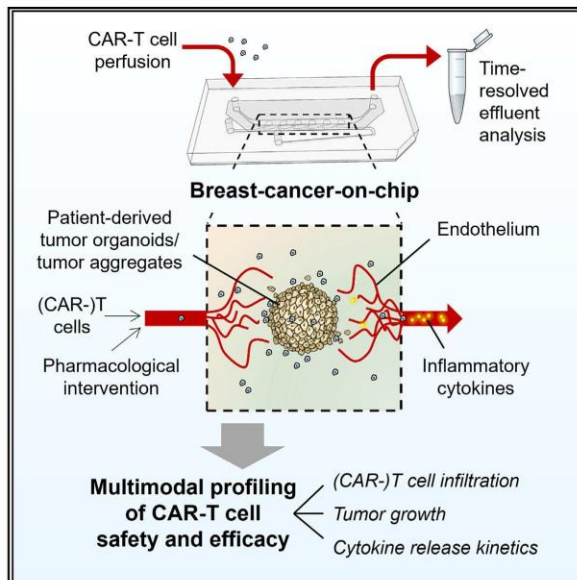
T. I. Maulana, C. Teufel, M. Cipriano, L. Lazarevski, F. E. van den Hil, V. Orlova, A. Koch, M. Hudecek, #M. Alb, #P. Loskill
Cell Stem Cell (2024), <https://doi.org/10.1016/j.stem.2024.04.018>

Abstract/Summary:

Physiologically relevant human models that recapitulate the challenges of solid tumors and the tumor microenvironment (TME) are highly desired in the chimeric antigen receptor (CAR)-T cell field. We developed a breast cancer-on-chip model with an integrated endothelial barrier that enables the transmigration of perfused immune cells, their infiltration into the tumor, and concomitant monitoring of cytokine release during perfused culture over a period of up to 8 days. Here, we exemplified its use for investigating CAR-T cell efficacy and the ability to control the immune reaction with a pharmacological on/off switch. Additionally, we integrated primary breast cancer organoids to study patient-specific CAR-T cell efficacy. The modular architecture of our tumor-on-chip paves the way for studying the role of other cell types in the TME and thus provides the potential for broad application in bench-to-bedside translation as well as acceleration of the preclinical development of CAR-T cell products.

Breast cancer-on-chip for patient-specific efficacy and safety testing of CAR-T cells

Graphical abstract



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In brief

Maulana et al. developed an advanced tumor-on-chip model that recreates chimeric antigen receptor (CAR)-T cell infusion, recruitment, and infiltration into solid tumors. Perfused culture enables the monitoring of patient-specific safety and efficacy parameters for over 1 week. The model provides novel opportunities for mechanistic studies of CAR-T cell function in a human-relevant setting.

Highlights

- Tumor-on-chip recapitulating 3D microenvironment with vasculature-like perfusion
- Model for CAR-T cell infusion, recruitment, and infiltration into solid tumors
- Allows for monitoring of dynamic cytokine release over more than 1 week
- Enables *in vitro* assessment of safety and efficacy in a patient-specific manner

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Breast cancer-on-chip for patient-specific efficacy and safety testing of CAR-T cells

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SUMMARY

Physiologically relevant human models that recapitulate the challenges of solid tumors and the tumor micro-environment (TME) are highly desired in the chimeric antigen receptor (CAR)-T cell field. We developed a breast cancer-on-chip model with an integrated endothelial barrier that enables the transmigration of perfused immune cells, their infiltration into the tumor, and concomitant monitoring of cytokine release during perfused culture over a period of up to 8 days. Here, we exemplified its use for investigating CAR-T cell efficacy and the ability to control the immune reaction with a pharmacological on/off switch. Additionally, we integrated primary breast cancer organoids to study patient-specific CAR-T cell efficacy. The modular architecture of our tumor-on-chip paves the way for studying the role of other cell types in the TME and thus provides the potential for broad application in bench-to-bedside translation as well as acceleration of the preclinical development of CAR-T cell products.

INTRODUCTION

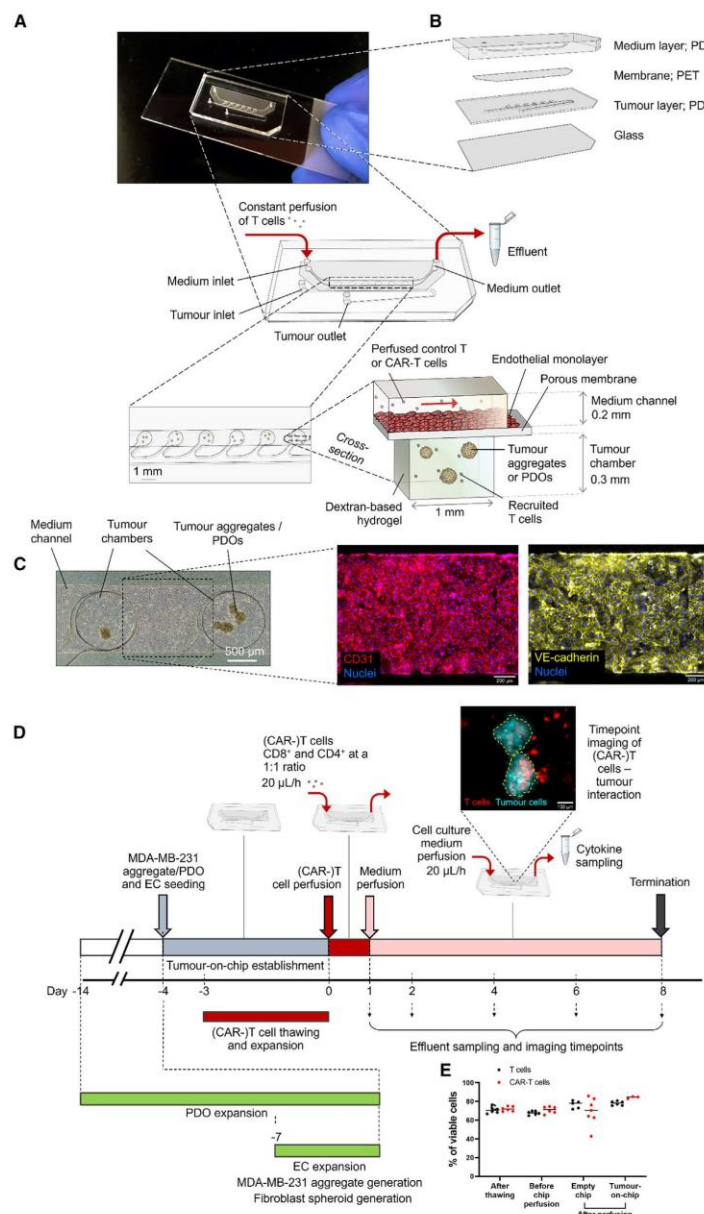
Chimeric antigen receptor (CAR)-T cells are an innovative cancer immunotherapy that has shown impressive remission rates for the treatment of B cell leukemia and lymphoma and has recently also been approved for the treatment of multiple myeloma.^{1,2} The use of this cellular immunotherapy for the treatment of solid tumor entities such as breast cancer has so far been challenging. The main hurdles include the much more heterogeneous expression of relevant target antigens and the physical barriers induced by tumor vasculature and stroma as well as by the abundant presence of immunosuppressive cells and soluble mediators as part of the tumor microenvironment (TME).^{3,4} Therefore, models that not only incorporate features of the TME but also recapitulate at least partly the three-dimensional (3D) architecture of solid tumors may not only allow to study the efficacy and safety of cancer immunotherapies but may also be able to provide mechanistic insights, e.g., for combinatorial therapy approaches. Additionally, compared with more simplistic two-dimensional (2D) coculture assays, these models might also be able to capture interactions of cellular immunotherapies with

the tumor vasculature and other cells within the tumor stroma. Further, incorporation of primary tumor material (e.g., patient-derived tumor organoids [PDOs]) may enable studying the efficacy (and safety) of cellular immunotherapies in a patient-specific manner. Such a specific approach is highly desired, as existing (mouse) models often only poorly recapitulate the TME of human tumors as well as the human immune system.^{5,6} Moreover, these models are also unsuitable to be implemented in the clinical setting to predict patient response, as their establishment takes months to achieve.⁷ Treatment with CAR-T cells and/or other (cellular) immunotherapies can additionally lead to adverse events such as on-target off-tumor effects and may also lead to excessive release of proinflammatory mediators both by the therapeutic as well as bystander (immune) cells.⁸ Thus, to investigate the mechanisms of adverse reactions and therefore broaden the applicability of this therapy, complex fit-for-purpose *in vitro* models are needed. Such models should generate results that reflect the human immune system and recapitulate both tumor heterogeneity and the TME; importantly, models with these characteristics should be able to better predict clinical safety and efficacy. Organ-on-chip technologies



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have already demonstrated the potential to fill this translational gap, specifically by recapitulating aspects of the TME⁹ and integrating human immune components.^{10,11} This type of model can also be established much faster compared with animal models, making it more feasible for direct clinical applications. However, to our knowledge, no organ-on-chip models are currently available to specifically address questions related to CAR-T cell efficacy and safety for patient-specific solid tumors. As part of the imSAVAR project (Immune Safety Avatar; www.imsavar.eu), we develop models to recapitulate the relevant molecular and cellular mechanisms (e.g., key events [KEs] and KE relationships within a specific immune-related adverse outcome pathway [irAOP]) and conditions (e.g., the TME) for an improved prediction of side effects of immunomodulatory therapies.

Here, we introduced a breast cancer-on-chip that recapitulated the initial events occurring upon CAR-T cell administration in patients (CAR-T cell perfusion through the vasculature and extravasation toward the tumor) as well as the effects that manifest over the following week (CAR-T cell infiltration and specific lysis of tumor cells as well as cytokine release [CR]). This enabled us to test varying treatment regimens of dasatinib as a pharmacologic safety switch to control CAR-T cells during the therapy.¹² Lastly, we integrated PDOs with varying receptor tyrosine kinase-like orphan receptor 1 (ROR1) expression levels from metastatic breast cancer patients to demonstrate their applicability in modeling patient-specific effects and revealed a target antigen density-dependent CAR-T cell response.¹³ This tumor-on-chip can serve as a standardized, scalable, and explorative platform to evaluate CAR-T cell therapy regarding its efficacy and cytokine-related safety aspects for solid tumors *in vitro* that may have the potential to predict clinical outcomes.

RESULTS

Tumor-on-chip model with defined vasculature channel enables multi-cell-type tumor tissue generation and immune cell perfusion

To mimic a perfused solid TME, we generated a tumor-on-chip model in which tumor aggregates from a triple-negative breast cancer cell line MDA-MB-231 expressing GFP or PDOs obtained from metastatic breast cancer can be placed on an adjacent endothelialized microchannel (medium channel). Specifically, the tu-

mor aggregates/PDOs were embedded in a dextran-based hydrogel and loaded into six cylindrical tumor chambers (1 mm diameter, 0.3 mm height) that are located underneath a medium channel (0.2 mm height). The medium channel is separated from the tumor chambers by a porous polyethylene terephthalate (PET) membrane (5 μ m pore size) and additionally endothelialized to mimic the tumor vasculature (Figure 1A). The MDA-MB-231 cells were aggregated and cultured until they reached $\sim$ 120 μ m in size before being loaded into the chip (Figures S1A and S1B). The injection method resulted in approximately 3 tumor aggregates per chamber and 15 per chip, respectively (Figures S1C and S1D). The stacked polydimethylsiloxane (PDMS) channels are microstructured via soft lithography and bottomed with glass to allow high-resolution microscopy (Figure 1B). This arrangement allows for constant (CAR-)T cell perfusion without disturbing the loaded tumor aggregates/PDOs and subsequent (CAR-)T cell migration into the underlying tumor chambers. The perfusion was initiated once a tight endothelial barrier had formed in the medium channel, as indicated by CD31 and vascular endothelial (VE)-cadherin expression as well as barrier permeability for macromolecules (Figures 1C and S3A).

The typical experimental timeline was designed to mimic the first week of CAR-T cell therapy (Figure 1D), as adverse effects such as CR syndrome (CRS) generally occur within the first week after CAR-T cell infusion in patients.⁶ Following the target cells' injection into the tumor chambers, either primary microvascular (allogeneic)- or human-induced pluripotent stem cell (hiPSC)-derived endothelial cells (ECs) generated from the same (CAR-)T cell donor (isogenic) were seeded into the medium channel and allowed to adhere overnight under static conditions before connecting the chips to constant medium perfusion (20 μ L/h) for 1–4 days. All tumor-on-chip systems included ECs unless indicated otherwise. Afterward, the tumor-on-chip systems were perfused with either ROR1-targeting CAR-T cells or untransduced T cells from the same healthy donor as a control (referred to as "control T cells" hereafter). CD19-specific CAR-T cells, generated from the same donor, were used in several experiments as an additional control condition. The (CAR-)T cells were linearly perfused for 20 h with a constant flow rate (20 μ L/h), which was followed by cell culture medium perfusion for over 1 week. Here, we first tested perfusion of CAR-T cells at three different concentrations (100,000, 500,000,

Figure 1. Concept and design of the tumor-on-chip and established timeline for CAR-T cell treatment on chip

(A) Schematic representation of the tumor-on-chip model. Tumor aggregates, patient-derived tumor organoids (PDOs), or fibroblast spheroids were embedded in a dextran-based hydrogel and cultured in the cylindrical tumor chambers of the chip, with six chambers in total per chip. The tumor chambers are located underneath a medium channel, which is lined with an endothelial monolayer. Control T cells or CAR-T cells were perfused through the medium channel and can migrate into the tumor chambers.

(B) A magnified view of the individual chip layers and materials. The PDMS medium layer contains the medium channel and the fluid inlets and outlets. The PDMS tumor layer contains the tumor channel and chambers, which are separated from the medium channel by a PET membrane and sealed at the bottom with a glass coverslip to allow high-resolution microscopy.

(C) Brightfield image of two tumor chambers and the medium channel on day 0 before (CAR-)T cell perfusion. The medium channel is covered by a tight monolayer of endothelial cells, as visualized by CD31 and VE-cadherin immunofluorescence staining.

(D) The typical experimental timeline for CAR-T cell efficacy and safety assessment. Target cells—either tumor aggregates, fibroblast spheroids, or PDOs—and endothelial cells were expanded and generated off-chip prior to chip seeding. Cells were seeded and cultured in the tumor-on-chip system for 1–4 days in parallel with the thawing and expansion of control T cells and CAR-T cells. On day 0, either control T cells or CAR-T cells were perfused through the chip for 20 h, followed by medium perfusion. Chips were imaged, and effluents were collected every 1 or 2 days up to day 8.

(E) Viability evaluation via flow cytometry of the control T cells and CAR-T cells after thawing, before, and after chip perfusion (20 h) through empty chip and tumor-on-chip setups (collected from the chip effluents); $n = 3-7$.

See also Figure S1.



and 1,000,000 T cells/mL) while keeping the number of MDA-MB-231 tumor aggregates relatively constant. We selected the values based on the range of peripheral T cell counts in patients who had CRS after receiving CD19-CAR-T cell therapy and proceeded with 500,000 T cells/mL for subsequent experiments, as this concentration, combined with 20 μ L/h flow rate setting, produced clinically relevant and detectable cytokine levels (Figure S1E).^{14,15} Moreover, concentrations higher than 500,000 T cells/mL often led to T cell clumping in the medium channel, which consequently disrupted the fluid flow. In all experiments, day 0 indicates the starting point of (CAR-)T cell perfusion. The perfusion process and parameters assured 70%–80% T cell viability in both the acellular chip and tumor-on-chip systems (Figure 1E). In summary, we have established an *in vitro* model, the experimental setup, and the timeline that allows the placement and culture of solid tumor (aggregates/PDOs) near an endothelialized medium channel and the perfusion of (CAR-)T cells without affecting their viability.

CAR-T cells migrate to, infiltrate, and lyse tumor-on-chip

To monitor the lytic capacity of CAR-T cells in the tumor-on-chip, the recruitment of control T cells and CAR-T cells was measured in the presence of a microvascular EC (mvEC) barrier. On day 1, both control T cells and CAR-T cells migrated to the GFP-expressing MDA-MB-231 tumor aggregates, with CAR-T cells showing a higher (2.5-fold) fluorescent intensity within the tumor region. Over the 8-day chip culture period, CAR-T cells persisted in/on the tumor aggregates, whereas the control T cells were eventually mostly excluded from tumor aggregates (Figures 2A, 2B, and S2A). Indeed, 3D imaging analysis showed a significantly higher density of infiltrating CAR-T cells (13-fold) within the tumor mass on day 8 when compared with control T cells (Figures 2C and S2B). CAR-T cells were also able to lyse tumor aggregates, as marked by the changes in tumor area and GFP intensity, whereas the tumor aggregates continued to grow in the control T cell condition (Figures 2D and 2E). In addition, on day 8, more dead cells could be observed within the tumor aggregates in the CAR-T cell condition compared with both control T cell and untreated condition (Figure S2C). We observed a higher number of CAR-T cells in the tumor chambers and a stronger tumor growth inhibition both in control T and CAR-T cell conditions when ECs were absent from the system, indicating an inhibitory effect of the endothelium (Figures S3B–S3D). When (CAR-)T cells were perfused at a lower concentration, i.e., 100,000 cells/mL, we observed less (CAR-)T cell infiltration into the tumor aggregates (Figure 2F). However, the number of CAR-T cells that migrated into the tumor chamber remained significantly higher than the number of control T cells. Additionally, at this lower concentration, no (CAR-)T cell migration was observed when no tumor aggregates were present (Figure 2G).

To assess if this CAR-T cell response is target specific, CAR-T cells were perfused through chips containing nonmalignant ROR1⁺ fibroblast spheroids instead of ROR1⁺ MDA-MB-231 tumor aggregates (Figure 2H). Despite migration of the CAR-T cells into the bottom chamber, the fluorescence intensity of CAR-T cells within the fibroblast spheroid region remained low over 1 week compared with that in the MDA-MB-231 aggregates (Figures 2A and 2B). In another set of experiments, we perfused the tumor-

on-chip models with CD19-specific CAR-T cells, which resulted in a continuous tumor aggregate growth, similar to the control T cell condition (Figure 2E). This demonstrated ROR1-specific CAR-T cell reactivity and the utility of the model for evaluating possible off-target effects by integrating cells from nonmalignant tissue.

Assessment of CAR-T cell CR kinetics in the tumor-on-chip model

In patients, CAR-T cell therapy is often accompanied by increased proinflammatory cytokine levels in the peripheral blood (including interleukin 6 [IL-6]), which may induce CRS. In such cases, patients can be treated with multiple doses of IL-6 receptor-blocking antibodies and/or glucocorticoids or even vasopressors in cases of higher-grade CRS.¹⁶ The molecular and cellular steps that may result in CRS can be described using the AOP concept, which is widely used in immunotoxicology.¹⁷ Within the EU project imSAVAR, we established an irAOP describing the key molecular and cellular events that lead to CRS mediated by CAR-T cells. To model the early KEs of this irAOP, in particular KE1 (activation/proliferation of CAR-T cells, release of proinflammatory mediators),¹⁸ and monitor the levels of clinically relevant (pro)inflammatory cytokines in the tumor-on-chip, we initially tested whether the cytokines originate from the CAR-T cell-tumor interaction. Here, we perfused either CAR-T cells or control T cells through acellular (empty), EC barrier (mvECs) only, or complete tumor-on-chip models, which included both mvECs and MDA-MB-231 tumor aggregates. We ensured that the mvECs expressed no ROR1 to exclude on-target off-tumor effects (Figure S1F). Compared with those in the control T cell condition, the levels of nearly all quantified cytokines—namely IL-2, IL-6, tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), and serine protease granzyme B—were higher (ranging from 6- to 100-fold) in the CAR-T cell condition on day 1 when tumor aggregates were present, whereas the level of anti-inflammatory cytokine IL-10 remained low in all conditions. The cytokine levels remained relatively lower in conditions without tumor aggregates, showing that cytokine secretion was mainly induced by target antigen recognition (Figure 3A).

In patients, however, CRS onset occurs beyond 1 day and develops over multiple days.⁸ Understanding the effect of potential mitigation strategies to manage the release kinetics of proinflammatory mediators and investigate the role of different cell types in perpetuating this proinflammatory cycle therefore requires a model that is viable and functional for this specific period. Here, linear perfusion of the CAR-T cell-treated tumor-on-chip allows such “real-time” monitoring of the cytokine level by measuring it on the chip effluents. By assessing tumor-on-chip perfused culture for up to day 8, our data showed that the peak cytokine concentration in the CAR-T cell condition occurred on either day 1 or 2 for all measured cytokines listed above. Cytokine concentrations in the CAR-T cell condition were consistently higher than those in the control T cell condition at almost all measured time points (Figure 3B). Moreover, both the perfusion of ROR1-CAR-T cells in chips containing ROR1⁺ fibroblast spheroids and CD19-CAR-T cells in chips containing ROR1⁺ MDA-MB-231 tumor aggregates resulted in lower cytokine levels compared with ROR1-CAR-T cells and ROR1⁺ MDA-MB-231 combination at most time points, showing a target-dependent cytokine response (Figures S4A and S4B).

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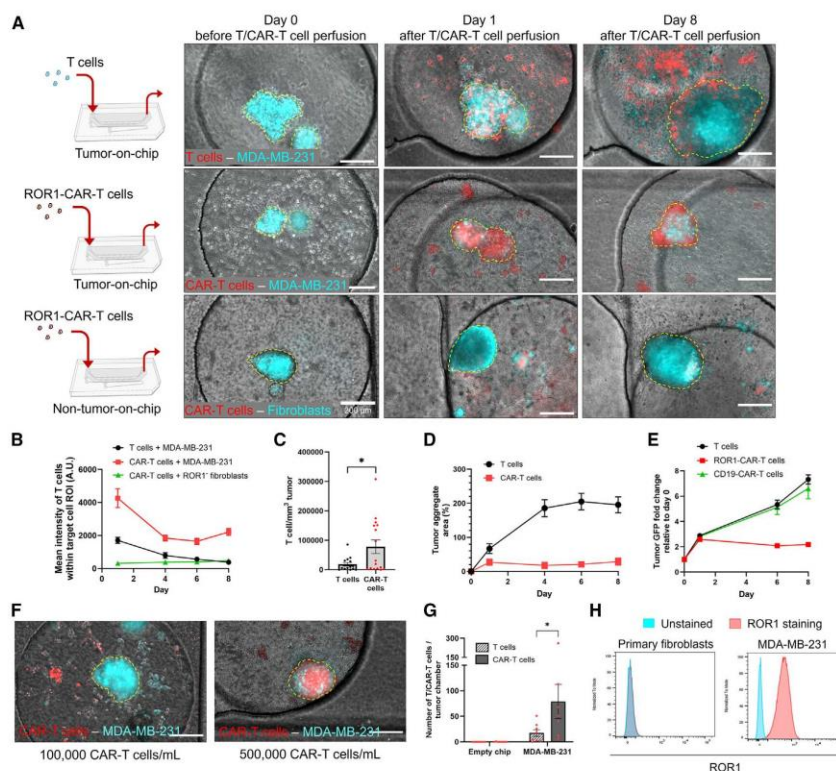


Figure 2. CAR-T cells infiltrated the tumor aggregates and hampered their growth in a target-specific manner on chip

(A) Representative images of MDA-MB-231 aggregates or fibroblast spheroids on day 0 (before [CAR-T] cell treatment), and on days 1 and 8 after tumor-on-chip perfusion with control T cells or CAR-T cells. MDA-MB-231 tumor cells express GFP and are pseudocolored cyan. Fibroblasts—representing nonmalignant tissue as a control—were labeled with CellTracker 5-chloromethylfluorescein diacetate (CMFDA) and pseudocolored cyan. Control T cells and CAR-T cells were labeled with CellTracker deep red. The yellow dashed line marks the region of interest of each tumor aggregate or fibroblast spheroid. MVECs were present in all chips. Scale bars, 200 μ m.

(B) Quantification of the mean intensity values of the control T cells and CAR-T cells within each tumor aggregate or fibroblast spheroid region of interest at different time points after (CAR-T) cell perfusion; $n = 7$ –21 MDA-MB-231 tumor aggregates/fibroblast spheroids from 3–4 chips. Data are presented as the mean $\pm$ SEM.

(C) Quantification of infiltrating (CAR-T) cells per mm^3 of tumor volume on day 8 after (CAR-T) cell treatment; $n = 15$ –16 MDA-MB-231 tumor aggregates from 3 chips. Data presented as the mean $\pm$ SEM. * $p < 0.05$; two-tailed unpaired t test.

(D) MDA-MB-231 tumor aggregate growth after CAR-T cell treatment in comparison with (control) T cell treatment, as measured by quantifying the percentage change in each MDA-MB-231 aggregate area between certain time points and the initial area before (CAR-T) cell perfusion; $n = 12$ –16 aggregates from 4 chips. Data are presented as the mean $\pm$ SEM.

(E) Quantification of the fold change in the mean GFP intensity of MDA-MB-231 tumor aggregates in each tumor chamber on days 1–8 after the perfusion of (control) T cells, ROR1-CAR-T cells, or CD19-CAR-T cells relative to day 0; $n = 24$ tumor chambers from 4 chips. Data are presented as the mean $\pm$ SEM.

(F) Representative images on day 1 after perfusing CAR-T cells (red) through the tumor-on-chip setup containing MDA-MB-231 aggregates (cyan), with a CAR-T cell concentration of either 100,000 or 500,000 cells/mL. The focal plane was set on the aggregates for all acquisitions. Scale bars, 200 μ m.

(G) Quantification of control T cells or CAR-T cells per tumor chamber on day 1 after the tumor-on-chip was perfused at a concentration of 100,000 (CAR-T) cells/mL. An empty chip was used as a control; $n = 7$ –8 chambers from 3 chips. Data are presented as the mean $\pm$ SEM. * $p < 0.05$; Bonferroni's multiple comparisons test.

(H) Flow cytometry histograms showing ROR1 expression (red) in MDA-MB-231 cells and fibroblasts compared with the unstained control (cyan). See also Figures S2 and S3.



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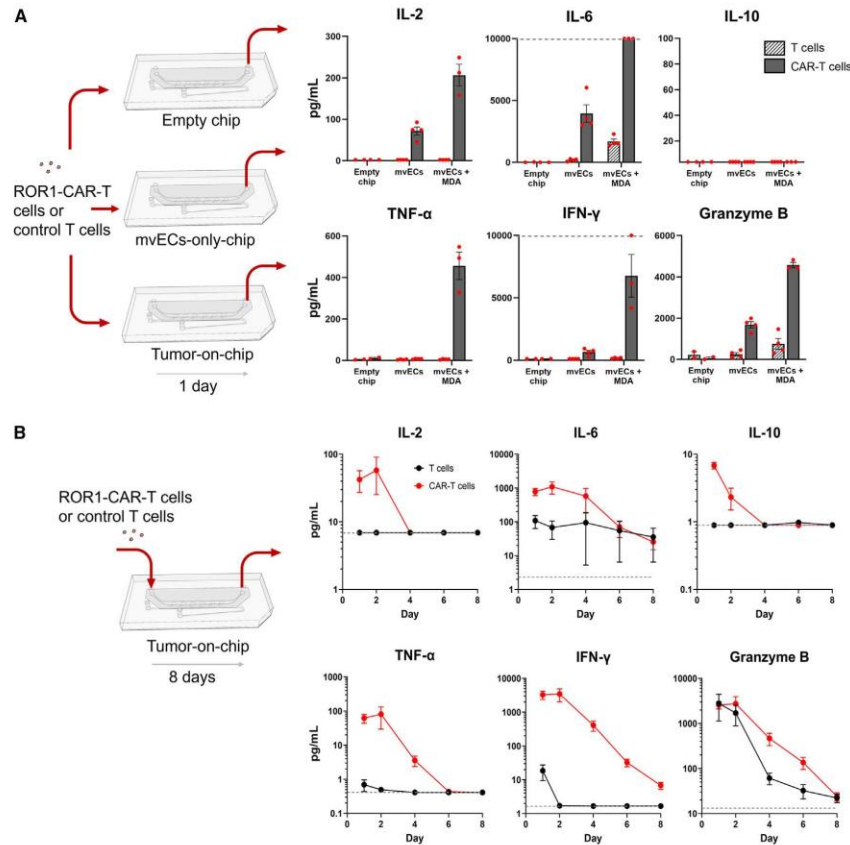


Figure 3. CAR-T cell-mediated cytokine concentrations and release kinetics in the tumor-on-chip model

(A) CAR-T cell-treated tumor-on-chip systems were assessed with regards to the secretion of cytokines IL-2, IL-6, IL-10, TNF- α , IFN- γ , and granzyme B on day 1 (20 h) after CAR-T cell perfusion. To ensure that the cytokines originate from CAR-T cell-tumor interaction, either control T cells or CAR-T cells were perfused through either acellular (empty), mvEC-only, or complete tumor-on-chip setups (mvECs + MDA); $n = 2-4$ chips. Data are presented as the mean $\pm$ SEM. Each red dot represents one chip. The dashed line indicates the upper detection limit.

(B) Release kinetics of the abovementioned cytokines from days 1 to 8 after control T cell or CAR-T cell perfusion from days 0 to 1. Samples were collected every 1–2 days; $n = 4$ chips. Data are presented as the mean $\pm$ SEM. The dashed line indicates the lower limit of detection. See also Figure S4.

By using this setup, we were able to recapitulate clinically relevant cytokine levels and kinetics, mimicking the initial surge in inflammatory cytokine secretion at the tumor site that was induced by target antigen recognition, which could induce CRS in patients undergoing CAR-T cell therapy.

On/off functional control of CAR-T cells in the tumor-on-chip model

As clinical CR kinetics were successfully mimicked using the CAR-T cell-treated breast cancer-on-chip models, we next tested whether the CR kinetics could be pharmacologically altered

to model therapeutic intervention and prevention of CRS onset. The occurrence of CRS is very challenging to predict,¹⁹ and modeling release kinetic of key CRS mediators requires a tailored human-relevant setup that includes relevant cell types in a perfused microenvironment. Once it occurs, a pharmacological intervention that enables a temporary “pause” in CAR-T cell activity upon the onset of this immune-related adverse event could save patients from severe adverse reactions.²⁰ Dasatinib is a Food and Drug Administration (FDA)-approved tyrosine kinase inhibitor that reversibly inhibits the phosphorylation of CD3 ζ and ζ -chain of T cell receptor-associated protein kinase 70 (ZAP70) in CAR constructs

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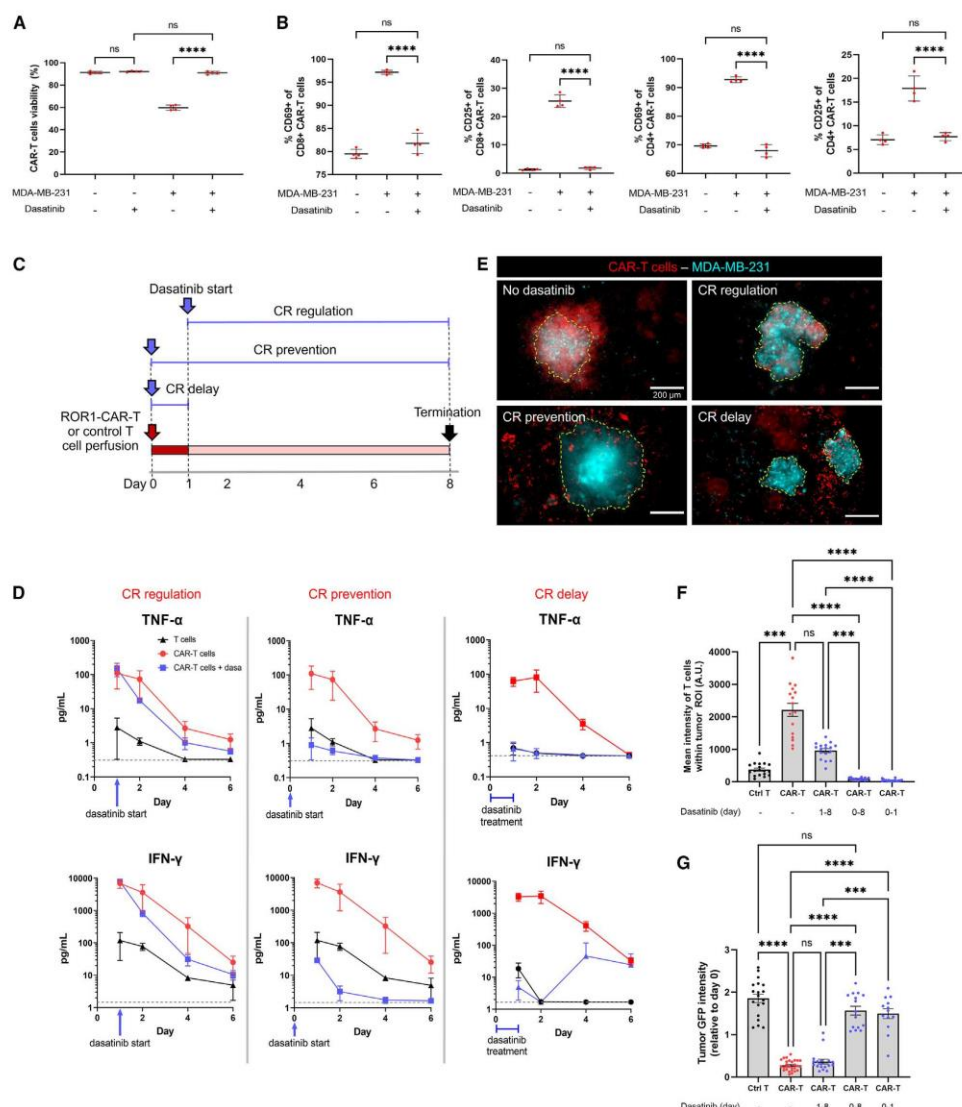


Figure 4. Dasatinib treatment regimens to model intervention strategies on CAR-T cell-treated tumor-on-chip models
(A) CAR-T cell viability after 24 h of dasatinib (50 nM) treatment either in the presence of MDA-MB-231 cells (effector-to-target cell ratio, 10:1) or without, cultured in a well-plate format.
(B) Percentage of CD8⁺ (early activation) and CD25⁺ (late activation) CAR-T cells within the CD8⁺ and CD4⁺ subset after 24 h of dasatinib (50 nM) treatment either in the presence of MDA-MB-231 cells (effector-to-target cell ratio, 10:1) or without, cultured in a well-plate format. Data were acquired from flow cytometry analysis for both (A) and (B); $n = 4$ wells. Data are depicted as mean with $\pm$ SEM. Each red dot represents one well. ns, not significant; **** $p < 0.0001$; Tukey's multiple comparisons test.

(legend continued on next page)
Cell Stem Cell 31, 1–14, July 5, 2024 7



containing either CD28_CD3 ζ or 4-1BB_CD3 ζ activation modules. In experiments using cell culture plates, we initially confirmed that dasatinib (50 nM) did not negatively affect CAR-T cell viability and successfully inhibited the upregulation of early (CD69) and late (CD25) activation markers after 24 h of interaction with MDA-MB-231 cells (Figures 4A and 4B). Afterward, we quantified the effects of dasatinib that was applied either throughout the CAR-T cell treatment period on the tumor-on-chip models (days 0–8; CR prevention regimen), before the peak of cytokine secretion (days 0–1; CR delay regimen), or after the peak of cytokine secretion (days 1–8; CR regulation regimen) (Figure 4C).

Applying dasatinib after the peak of cytokine secretion (days 1–8; CR regulation) resulted in a rapid drop in TNF- α and IFN- γ levels relative to those in the untreated CAR-T cell condition (Figure 4D), thereby mimicking acute management of early release of CRS mediators.²¹ This treatment scheme also enabled infiltration of CAR-T cells into the tumor within the first 24 h (before dasatinib treatment) but not subsequent excessive infiltration, as indicated by the lower fluorescence intensity (~2-fold) compared with that in the untreated CAR-T cell condition (Figures 4E and 4F). Furthermore, the tumor GFP signal intensity on day 8 dropped to a level similar to that observed in the CAR-T cell condition without dasatinib (Figure 4G), indicating the maintenance of the baseline level of antitumor cytotoxicity.

Dasatinib administration throughout the CAR-T cell treatment period (days 0–8; CR prevention) prevented the CR, as shown by the lower TNF- α and IFN- γ levels compared with those observed with untreated CAR-T cells and control T cells (Figure 4D). However, this treatment scheme also prevented CAR-T cells from infiltrating the tumor, and the signal intensity in the GFP-expressing MDA-MB-231 tumor aggregates changed only minimally, with no significant difference compared with that in the control T cell condition (Figures 4F and 4G). Therefore, this approach defeats the purpose of CAR-T cell treatment. The day 0–1 treatment approach delayed the IFN- γ peak but not the TNF- α peak, revealing the transient effect of dasatinib (Figure 4D). However, this approach also prevented CAR-T cells from infiltrating and lysing the tumor aggregates as seen on day 8 (Figures 4E–4G).

By investigating different intervention time points and durations using dasatinib in the tumor-on-chip models, we demonstrated the feasibility of the model to investigate a suitable intervention strategy by controlling the secretion of CAR-T-cell-derived CRS mediators without diminishing CAR-T cell efficacy.

PDOs on chip demonstrate antigen-dependent, potent antitumor efficacy of ROR1 CAR-T cells

ROR1 expression levels are tumor and patient specific.^{22,23} To evaluate therapeutic efficacy within the context of this heterogeneity, we integrated PDOs derived from two female patients with metastatic breast cancer that had high or low ROR1 levels (Figure 5A; Table S1) and compared them with MDA-MB-231 aggregates as a positive control condition. To evaluate tumor- and patient-specific effects of the PDOs, possible alloreactive responses of T cells toward ECs—e.g., when primary allogeneic mvECs were employed—were excluded by integrating hiPSC-derived ECs sourced from the same donor as the (CAR-)T cells.

In the semiautologous tumor-on-chip setup, CAR-T cell infiltration into the PDOs (Figures 5B–5D), PDOs' growth (Figure 5E), cytokine secretion (Figures 5F and 5G), and release kinetics (Figure 5G) varied according to ROR1 expression of the PDOs. Consistent with the respective target expression levels, CAR-T cells infiltrated ROR1-high PDOs in significantly higher numbers (9-fold) than ROR1-low PDOs. The highest CAR-T cell infiltration rate was observed in MDA-MB-231 aggregates, with an infiltration rate 2-fold higher than that in ROR1-high PDOs (Figure 5C). Additionally, ROR1-low PDOs continued to grow despite CAR-T cell treatment, whereas the growth of ROR1-high PDOs was restricted (Figures 5D and 5E). In another set of experiments, we integrated PDOs derived from two additional female patients and observed a similar trend (Figures S5A–S5C; Table S1).

Notably, despite the absence of CAR, control T cells were still recruited into the tumor chamber, which might indicate a baseline allogeneic response toward the PDOs. However, control T cells mostly remained excluded from the PDOs after the culture period (Figure 5B) and exhibited much lower cytokine levels on day 1 than the respective CAR-T cell condition (Figure 5F). In summary, our results indicate that the tumor-on-chip can also capture a patient-specific response by integrating tumor target cells obtained directly from patients to assess both the efficacy and CRS-related safety aspects.

DISCUSSION

This breast cancer-on-chip demonstrates the feasibility to evaluate the efficacy (T cell infiltration, tumor growth, and lysis), safety (abundant CR that could lead to CRS), as well as CR intervention strategies of CAR-T cell therapy for solid tumors *in vitro*. We

(C) Timeline of dasatinib treatment regimens with varying starting time points (blue arrows) and durations (blue bar lengths) during tumor-on-chip culture to control the release of CRS mediators. Tumor-on-chip systems containing MDA-MB-231 aggregates were perfused on days 0–1 with either control T cells or CAR-T cells and then with cell culture medium from days 1–8. Dasatinib (50 nM) was administered through the tumor-on-chip models on days 1–8, 0–8, or 0–1 to model cytokine release (CR) regulation, prevention, or delay, respectively.

(D) TNF- α and IFN- γ release kinetics in the control T cell, CAR-T cell, and CAR-T cell + dasatinib conditions with three different treatment schemes: CR regulation (left), prevention (middle), and delay (right). Cytokines were measured from the tumor-on-chip effluents. Data are shown as the mean $\pm$ SEM. The dashed line indicates the lower limit of detection. Data points from day 8 are excluded as the values are mostly below the detection limit. $n = 4$ chips per condition.

(E) Representative images of MDA-MB-231 aggregates (cyan) on day 8 after tumor-on-chip perfusion with CAR-T cells (red), showing different degrees of T cell infiltration into the aggregate after applying different dasatinib treatment schemes compared with an image of untreated cells. Conditions are indicated at the top left of each image. The yellow dashed line marks the region of interest of one tumor aggregate. Scale bars, 200 μ m.

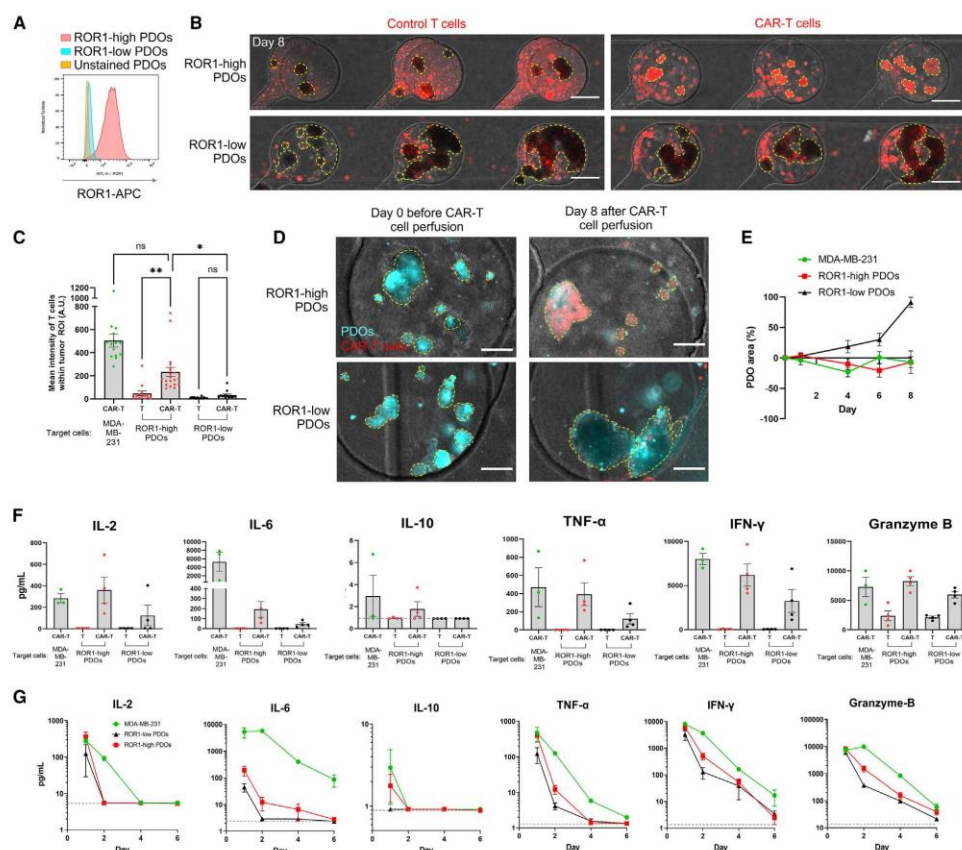
(F) Quantification of the mean intensity values of the control (Ctrl) T cells and CAR-T cells within each MDA-MB-231 tumor aggregate region on day 8 after or without dasatinib treatment; $n = 15$ –18 MDA-MB-231 aggregates from 3–4 chips. Data are presented as the mean $\pm$ SEM. ns, not significant, *** $p < 0.001$, **** $p < 0.0001$; Dunn's multiple comparisons test.

(G) Quantification of the fold change in the mean GFP intensity for each MDA-MB-231 tumor aggregate on day 8 relative to the mean GFP intensities of the aggregates on day 0 to assess tumor lysis. Each dot represents one MDA-MB-231 aggregate. Data are shown as the mean $\pm$ SEM; $n = 13$ –28 aggregates from 3–4 chips. ns, not significant, *** $p < 0.001$, **** $p < 0.0001$; Dunn's multiple comparisons test.

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demonstrated the feasibility and power of modeling aspects of early KEs at the tumor site upon therapy administration in a physiologically relevant coculture model and applied clinically relevant readouts. Bottom-up approach in designing the tumor microarchitecture, perfusion setup, and modularity in cellular and microenvironmental components allowed us to adjust the model complexity and exclude off-target effects while ensuring human and clinical relevance, thereby addressing several limitations of existing model systems to evaluate CAR-T cell performance.

First, our data showed that the tumor-on-chip successfully captured differences in T cell infiltration into the tumor, tumor growth, and cytokine response that depended on CAR expression on engineered T cells and ROR1 expression on the target cells during 8 days of perfused tumor-on-chip culture. By perfusing CAR-T cells or control unmodified T cells through the blood vessel-like channel, we could assess their recruitment to the tumor site and observed that CAR-T cells infiltrated the MDA-MB-231 aggregates at a higher density and persisted longer than control T cells. We observed the exclusion of unmodified control T cells from the tumor aggregates after 1 week of culture—a clinical phenomenon reported to predict the subset of cancer patients who are not responsive to cancer immunotherapy.^{24,25} Moreover, including ECs in the tumor-on-chip resulted in fewer recruited CAR-T cells in the tumor chamber and reduced tumor growth control (Figures S3B–S3D). The vasculature of tumors indeed poses a barrier for T cell trafficking, which has long been proposed as a therapeutic target in cancer immunotherapy.^{26,27} Future studies using tumor-on-chip or similar models could investigate strategies to overcome this inhibitory effect of the tumor vasculature and therefore improve CAR-T cell homing into the tumor. Moreover, such inhibitory effects of the TME could be underestimated in the well-plate setting, as we saw a significantly higher T cell and CAR-T cell infiltration and lower tumor GFP intensity values on day 1 compared with those in the chip setup (Figures S2D and S2E).

Furthermore, the ability to integrate the PDOs directly from patients and establish the tumor-on-chip model faster compared with murine models (in a week instead of several months) may allow for utilizing the model to assist clinical decision-making, which often requires a short time scale. In our setup, we found that relative ROR1 expression on PDOs positively correlated with the persistence of CAR-T cells within the tumor bulk, which aligned with recently published data showing that the antitumor killing of breast tumor-derived PDOs within the first 24 h positively correlates with the ROR1 expression level.²⁸ Integrating patient-specific PDOs in our system helps to reconstitute the TME surrounding the PDOs and therefore the physiological relevance, thereby improving existing *in vitro* models with PDOs that focused mainly on assessing treatment efficacy.^{29–33}

Next, we observed abundant release of proinflammatory cytokines in our models, which allowed us to study intervention strategies using dasatinib as a functional on/off switch of CAR-T cells. On either day 1 or 2 of CAR-T cell administration, we consistently detected peaks in cytokine levels. Cytokine levels typically decreased afterward, but whether this was caused by activation-induced T cell death following eradication of the tumor cells or T cell exhaustion remains to be investigated. This drop of cytokine levels might also result from the ongoing lysis of MDA-MB-231 tumor aggregates by the CAR-T cells, as the tumors stopped

growing in this condition, as shown in previous efficacy test runs. Additionally, replacing the non-recruited CAR-T cells with cell culture medium in the perfusate from day 1 may limit cytokine secretion from only the recruited CAR-T cells on subsequent days. Nevertheless, the presence of granzyme B was still detected on day 8 of culture in both the CAR-T cell and control T cell conditions, indicating an ongoing T cell-tumor interaction. Early secretion—up to day 3 after CAR-T cell administration—of IFN- γ was shown to predict the development of severe CRS.³⁴ Circulating IFN- γ levels have been associated with CRS in 12 out of 13 trials.³⁵ The concentration of IFN- γ typically found in CRS patients (>10 pg/mL) is indeed much lower than that detected in chip experiments (>3,000 pg/mL).^{15,35} However, a 100-fold increase in IFN- γ serum concentration was detected in patients suffering from severe CRS,⁸ which could also be observed in our tumor-on-chip even after 4 days of culture. Yet, it is essential to emphasize that the model primarily replicates cytokine levels at the local tumor site, which may facilitate the recruitment of bystander immune cells, rather than cytokine levels in the bloodstream.

Interestingly, we noticed differences in cytokine concentrations depending on the CAR-T cell donor used for perfusion, although the fold change values typically decreased to below 20 on day 6 for both CAR-T cell donors tested (Figure S4C). This also demonstrated the applicability of this model for safety evaluations of different CAR-T cell products before administering them to patients, as cell-intrinsic effects, such as the transcriptional features of CAR-T cells, have been associated with adverse effects.³⁶ Furthermore, our data show a similar pattern of onset and kinetics for IL-2, IL-10, and TNF- α secretion, as observed in patients suffering from high-grade CRS, although on a shorter time scale.^{8,37} Further tests and extensive benchmarking with clinical data (“real-world datasets”) may elucidate whether the absolute and fold change values obtained in the chip model can be graded as “toxic” or “non-toxic.”

Another cytokine, IL-6, that has been identified as one of the key mediators in CRS development,³⁸ was also shown to be elevated on day 1 or 2 after CAR-T cell perfusion in our tumor-on-chip models. High serum IL-6 levels are frequently detected in patients who suffer from severe CRS, and blocking IL-6 signaling (e.g., with tocilizumab) together with systemic immunosuppression via corticosteroids is the current standard of care in CRS management.^{8,39–45} Although we observed an increase in IL-6 production in the CAR-T cell condition at almost all time points and for different donors, importantly, our model did not include endogenous innate immune cells such as macrophages and monocytes, which are the main sources of IL-6 that eventually induces systemic inflammation.⁴⁶ Incorporation of these cells into the model in the future will therefore be crucial for further understanding their role in the amplification and perpetuation of the proinflammatory cycle that may lead to CRS. For this reason, tocilizumab is arguably not the best option to test CRS intervention in our tumor-on-chip, and interfering directly with CAR-T cell function, e.g., with dasatinib, provides a better alternative. In this regard, such an on/off switch that modulates activation and proliferation of CAR-T cells would be a CRS mitigation strategy that acts directly on the cells that induce CRS in this setting.⁴⁷ Dasatinib temporarily inactivates CAR-T cells without negatively affecting their viability¹² and thus can be applied to manage acute toxicity.²¹ By applying dasatinib at different

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time points and for varying durations, we identified an optimal strategy to control CR while maintaining CAR-T cell efficacy throughout a 1-week period.

In the experiments that incorporated the PDOs, we refined the model by integrating isogenic hiPSC-ECs. Our cytokine secretion data showed higher fold changes in TNF- α , IFN- γ , and granzyme B levels when T cells were cocultured with mvECs from multiple donors than when T cells were cultured with isogenic hiPSC-ECs. The fold change in cytokine levels in the isogenic setting remained similar to that in the condition without ECs (Figure S3E). HiPSC-ECs were confirmed to express CD31 prior to integration into the chip (Figure S3F). Despite enabling human leukocyte antigen (HLA)-matched autologous systems, hiPSCs-ECs expressed a certain level of ROR1 (Figure S3G), which induced IFN- γ and granzyme B secretion in our plate assay (Figure S3H). Indeed, ROR1 is not only a tumor antigen but also a known marker of stem cell derivatives.⁴⁸ Despite the resulting background activation, patient-specific effects due to relative ROR1 expression by the PDOs could be detected in the tumor-on-chip models. This would not be relevant for different types of CARs, and future advances in our understanding of EC maturation should allow us to minimize this effect for ROR1-CAR-T cells.

In conclusion, our model provides the foundation for studying different roles of cell types and composition as well as biophysical barriers in altering CAR-T cell safety and efficacy for solid tumors *in vitro*, while it also facilitates the examination of patient-specific effects, thereby offering new opportunities for advancing both mechanistic research and clinical translation of safer CAR-T cell products.

Limitations of the study

In terms of efficacy, our model does not yet fully mimic an immunosuppressive TME with stromal cells, immunosuppressive myeloid cells, and increased extracellular matrix deposition, which are among the factors of why CAR-T cells remain ineffective for solid tumors.⁴⁹ Also, fully recapitulating mechanisms of CRS development over several days/weeks as it occurs in patients would require increasing the complexity of the current model, which is beyond the scope of the study. As already mentioned, this model does not include macrophages and monocytes, which have central involvement in initiating and perpetuating CRS.^{38,46,50} Assessing additional systemic CRS biomarkers (e.g., C-reactive protein, ferritin) would require fluidic coupling with other organ-on-chip models (e.g., liver-on-chip), as certain biomarkers are produced systemically through inter-organ crosstalk.⁵¹ Perfusing the (CAR-)T cells for only 20 h and subsequently removing them from the circulation from days 1–8 may also disregard the role of circulating T cells in the release of inflammatory mediators. Moreover, the clinical value of integrating PDOs in the tumor-on-chip needs to be further validated, as PDOs from only 4 patients were tested in this proof-of-concept pilot study. Predicting the risk of CRS development as well as CAR-T cell efficacy using this model would require the testing of large number of additional patient samples.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
 - Tumor-on-chip concept, design and fabrication
 - Tumor and fibroblast aggregates generation
 - Patient-derived tumor organoids generation and culture
 - Microvascular endothelial cells isolation and culture
 - Human induced pluripotent stem cells generation from PBMCs
 - Human induced pluripotent stem cells-derived endothelial cells generation and culture
 - Generation and culture of ROR1- and CD19-specific CAR-T cells
 - Aggregates/spheroids/PDOs seeding on-chip
 - Endothelial cells seeding, chip culture, and characterization
 - T cell perfusion and chip culture
 - Dasatinib treatment
 - Allogeneic / isogenic endothelial cells – (CAR-)T cells coculture for cytokine assay
- QUANTIFICATION AND STATISTICAL ANALYSIS
 - Image acquisition and analysis
 - Cytokine quantification assay
 - Flow cytometry staining and analysis
 - Statistics and reproducibility

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.stem.2024.04.018>.

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AUTHOR CONTRIBUTIONS

T.I.M. performed the chip design and fabrication as well as cell culture and chip experiments with assistance from C.T., J.R., L.L., and M.C. C.T., L.L., and L.S.

Cell Stem Cell 37, 1–14, July 5, 2024 11



performed the well-plate assays of preliminary 2D experiments. C.T. and A.K. performed the flow cytometry and analyzed the data. A.K. provided the organoids. M.A. and M.H. provided the (CAR)-T cells, tumor cell line MDA-MB-231 GFP-ffluc, PBMCs for hiPSC generation, and intellectual expertise on CAR-T cell engineering. F.E.v.d.H. and V.O. provided the hiPSCs and hiPSC-derived endothelial cells and performed their characterization. T.I.M., M.C., M.A., and P.L. designed the study. T.I.M. performed the imaging and image data analysis. T.I.M. analyzed the data from the chip experiments. T.I.M. wrote the manuscript with support from P.L., M.C., C.T., A.K., V.O., M.A., and M.H. This work was jointly supervised by P.L. and M.A., who share senior authorship. All authors have read the manuscript and provided input and agree with its submission.

DECLARATION OF INTERESTS

M.H. is an inventor on patent applications and has been granted patents related to CAR technology, licensed in part to industry. M.H. is a cofounder and equity owner of T-CURX GmbH, Würzburg. M.H. receives speaker honoraria from BMS, Janssen, Kite/Gilead, and Novartis and research support from BMS.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ROR1 Antibody, anti-human, REAfinity (fin.conc. 1:100)	Miltenyi Biotec	130-118-015; RRID: AB_2733448
CD31 Antibody, anti-human, REAfinity (fin.conc. 1:50)	Miltenyi Biotec	130-110-808; RRID: AB_2657282
PE/Cyanine7 anti-human CD3 Antibody (fin.conc. 1:400)	BioLegend	317334; RRID: AB_2561452
APC/Fire 750 anti-human CD4 Antibody (fin.conc. 1:100)	BioLegend	357426; RRID: AB_2716183
PerCP anti-human CD8a Antibody (fin.conc. 1:50)	BioLegend	300922; RRID: AB_1575072
Brilliant Violet 421 anti-human CD25 Antibody (fin.conc. 1:50)	BioLegend	302630; RRID: AB_11126749
FITC anti-human CD69 Antibody (fin.conc. 1:50)	BioLegend	310904; RRID: AB_314839
CD31 Antibody, anti-human (fin. conc. 1:50)	DAKO	M0823; RRID: AB_2114471
VE-cadherin Antibody, anti-human (fin. conc. 1:50)	Invitrogen	36-1900; RRID: AB_2533243
Goat anti-Mouse IgG (H+L), Superclonal Recombinant Secondary Antibody, AlexaFluor 647 (fin. conc. 1:100)	Invitrogen	A28181; RRID: AB_2536165
Goat anti-Rabbit IgG (Heavy chain), Superclonal Recombinant Secondary Antibody, Alexa Fluor 555 (f.c. 1:100)	Invitrogen	A27039; RRID: AB_2536100
Biological samples		
Primary tumor materials to generate PDOs	Women's Hospital, University of Tübingen, Germany	Table S1
Resected skin tissue to isolate mvECs	Klinik Charottenhaus, Stuttgart, Germany	See experimental model and subject details
PBMCs to generate CAR-T cells and hiPSCs	DRK Frankfurt, Germany	See experimental model and subject details
Chemicals, peptides, and recombinant proteins		
SYLGARD 184 silicone elastomer	Dow Corning	5498840000
Hydrosil 1:1	SILADENT	101301
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2 Select	Bio-Techne	3533-005-02
TrypLE Express	Gibco	12604013
TrypLE Select	Gibco	12563-029
GlutaMAX	Thermo Fisher	35050-038
HEPES	Thermo Fisher	15630-056
Gentamicin	Thermo Fisher	15710049
Hoechst 33342	Thermo Fisher	62249
B27 Supplement	Thermo Fisher	17504-044
Heregulin β -1	PeproTech	100-03
FGF7	PeproTech	100-19
FGF10	PeproTech	100-26
EGF	PeproTech	AF-100-15
A83-01	Tocris	2939
Y-27632	STEMCELL Technologies	72034

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SB-202190	Sigma Aldrich	S7067
N-acetyl-cysteine	Sigma Aldrich	A9165
Nicotinamide	Sigma Aldrich	N0636
Human serum from platelet poor plasma	Sigma Aldrich	P2918
Fibronectin	Sigma Aldrich	F1141
Propidium iodide	Sigma Aldrich	P4170
DAPI	Sigma Aldrich	D8417
bFGF	Miltenyi Biotech	130-093-564
VEGF	Miltenyi Biotech	130-109-396
Dispase	Merck KGaA	D4693
EDTA	Invitrogen	15575020
CellTracker CMFDA	Invitrogen	C7025
CellTracker Deep Red	Invitrogen	C34565
3-D Life Dextran-CD Hydrogel SG	Cellendes GmbH	G93-1
FibriCol	Advanced Biomatrix	5133
Dasatinib	Selleckchem	S1021
Zombie aqua dye	BioLegend	423102
BD Fc Block	BD Biosciences	564220
ROTHistofix 4%	Carl Roth GmbH	P087.6
Critical commercial assays		
Legendplex Human CD8/NK Mix and Match Subpanel	BioLegend	741187
Experimental models: Cell lines		
MDA-MB-231	ATCC	HTB-26
Software and algorithms		
ImageJ-Fiji	https://imagej.nih.gov/ij/	Schindelin et al. ⁵²
Imaris 9.5	https://imaris.oxinst.com/versions/9-5	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Loskill (peter.loskill@uni-tuebingen.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The data reported in this paper will be shared by the [lead contact](#) upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

MDA-MB-231 cells were purchased from ATCC and were lentivirally transduced to express GFP-firefly luciferase fusion protein. The cells were cultured in RPMI-1640 (11835030, Thermo Fisher Scientific) supplemented with 10% (v/v) FCS (SH30066.03, HyClone FetalClone II Serum; Cytiva), 1X GlutaMAX (35050061; Thermo Fisher Scientific), and 100 U/mL penicillin-streptomycin (15140122; Thermo Fisher Scientific). Primary human fibroblasts were cultured in DMEM (41965039; Gibco) supplemented with 10% (v/v) FCS and 100 U/mL penicillin-streptomycin.

Patient-derived organoids (PDOs) were established from specimens obtained from four advanced breast cancer female patients (age 52–63) treated at the Department of Women's Health in Tübingen (detailed information is provided in [Table S1](#)). All patients gave

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informed consent and the study was approved by the Ethical Committee of the Eberhard Karls University Tübingen (No. 150/2018BO2 and 662/2022BO2).

Human microvascular endothelial cells (mvECs) were isolated from resected skin tissue from plastic surgeries received from Dr. Ulrich E. Ziegler (Klinik Charlotenhof, Stuttgart, Germany) as described previously,¹¹ and derived from 5 donors (4 females in the age of 30, 34, 67 and unknown, and one 50 years old male). The study was approved by the local medical ethics committee: Patients gave an informed consent according to the permission of the "Landesärztekammer Baden-Württemberg" (IRB#: F-2020-166; for normal skin from elective surgeries). Sex- and gender-based analysis were not considered in the study design due to the logistical complexity of the 3 cell types cultured simultaneously, hence the experiments were conducted based on donors availability.

(CAR)-T cells were generated from two healthy female donor PBMCs obtained via apheresis procedure at the DRK Frankfurt, Germany, following informed consent for research purposes. iPSCs were also generated from these PBMCs using episomal vectors without TP53 shRNA⁵³ according to standard protocols at the Leiden University Medical Center (LUMC) iPSC core facility.

All cells cultures were maintained in a humidified incubator at 37 °C, 5% CO₂ and 95% rH. Cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and verified to be mycoplasma-free before experiments.

METHOD DETAILS

Tumor-on-chip concept, design and fabrication

The tumor-on-chip consists of two stacked microfluidic channels separated by an isoporous, semipermeable polyethylene terephthalate (PET) membrane (5 μm poresize: $r_p = 5 \mu\text{m}$; $p_p = 6 \times 10^5$ pores per cm²; TRAKETCH PET 5.0 p S210 x 300, SABEU GmbH & Co. KG, Northeim, Germany), which was functionalized by a plasma-enhanced, chemical vapor deposition (PECVD) process as previously described.⁵⁴ The tumor chambers (1 mm in diameter, 0.3 mm in height, six chambers per chip) branch off a main injection channel (0.2 mm in height) at a 45° angle and a high-resistance channel towards the outlet port, which forces the aggregates/organoids to sequentially enter the tumor chambers during the injection process, utilizing an injection mechanism previously successfully established in other organ-on-chip model.¹¹ This concept and design enable the integration of any aggregate/organoid below 0.2 mm in size. The tumor channel is designed to have an outlet channel that possesses a fluidic resistance of 1.2×10^{12} Pa.s/m³, which is at least twice higher than the fluidic resistance of the membrane and the medium channel combined. This ensures the placement of the cells into the tumor chambers during the injection instead of flowing out toward the outlet. The medium channel is situated right above the tumor chambers and is separated by a porous PET membrane that serves multiple functions: during the cell injection step, it enables the entrapment of the tumor aggregates/PDOs and provides a growth surface for the endothelial barrier formation, whereas during the culture period, its 5 μm pore size allows T cell trafficking and passive diffusion of diluted species.¹¹

The tumor and medium layers consisting 200–300 μm high channels were microstructured via replica molding of polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, USA) on two differently patterned master wafers fabricated via photolithographic processes.⁵⁵ The SU-8 structures on the tumor layer wafer have two heights: 300 μm for the round tumor chambers and 200 μm for the rest of the channels. For the replica molding, PDMS was homogeneously mixed in a 10:1 (elastomer base:curing agent) mass ratio and then degassed in a desiccator to remove air bubbles. Afterwards, two different replica molding approaches were conducted as previously described.¹¹ Standard molding approach was used by pouring the PDMS prepolymer solution onto the silicon wafer master mold to obtain 3 mm thick PDMS pieces with channel structures for the medium layer. PDMS was cured at 60 °C for 4 h. After curing, the PDMS were cut to the size of the chip and ports were pierced using a biopsy punch (Disposable Biopsy Punch, 0.75 mm diameter; 504 529; World Precision Instruments, Friedberg, Germany) to access the chips (for both cell injection and medium perfusion). The exclusion molding approach was used to fabricate the bottom tumor layer: here, PDMS prepolymer solution was poured onto the silicon wafer master mold, which was then clamped against a 5 mm-thick PMMA disk to produce a 0.3 mm thin layer with through hole channel structures. PDMS was cured at 60 °C for 2 h. Once the PDMS parts were cured, they were cleaned using isopropanol followed by deionized water and blow-dried with nitrogen pistol. The microfluidic chips were assembled in three consecutive bonding steps: (i) tumor layer to glass coverslip, (ii) medium layer to the membrane, (iii), and (iii) the tumor layer to medium layer. In all steps, bonding was achieved by oxygen plasma activation (75 W, 0.2 cm³m^{−1} O₂; Diener Zepto, Diener electronic GmbH & Co. KG, Ebhausen, Germany) for 24 s. Bonded parts were baked at 60 °C for at least 30 min after each bonding step, and overnight after the entire chip was assembled. All chips were O₂-plasma treated (75 W, 0.2 cm³m^{−1} O₂) for 5 min to sterilize and hydrophilize the PDMS surface before cell injection.

Tumor and fibroblast aggregates generation

Aggregates were formed using agarose microwells-based approach.⁵⁶ Briefly, 2.5 g of Hydrosil silicone components (1:1 wt) (101301; SILADENT) was added into each well of 6-well microwell culture plate (AggreWell400; STEMCELL Technologies) followed by centrifugation at 55 x g for 1 min and curing at 60 °C for 1 h. The cured molds were then carefully removed, cut into circular segments (d = 20 mm), and glued to a polymethyl methacrylate (PMMA) holder that has circular cutouts that match the surface of a 24-well plate (d = 15.5 mm, h = 2 mm). These cured molds were used as a reusable master mold for the agarose gel.

Prior to aggregate formation, the master molds were disinfected with 70% (v/v) ethanol and rinsed three times with PBS without Ca and Mg (indicated as PBS[−] in the following) (P04-36500; PAN-Biotech). Afterwards, 3% (w/v) agarose solution in DMEM (41965039; Gibco) was liquified by preheating in a microwave and 650 μL was added into each master mold and left for 10 min at room temperature (RT) to allow agarose gelation. Afterwards, the agarose molds were transferred into the wells of 24-well plate with the patterned

Cell Stem Cell 37, 1–14.e1–e9, July 5, 2024 e3



structures facing upwards. The wells were filled with 1 mL of PBS[−] followed by centrifugation at 1300 x g for 3 min to remove trapped air bubble below the agarose mold. PBS[−] was removed before adding the cells.

To generate the MDA-MB-231 aggregates, the cells were detached by incubation for 3 min with TrypLE Express Enzyme (12604013; Gibco) at 37 °C, pelleted by centrifugation at 200 x g for 5 min and resuspended in RPMI-1640-based cell culture medium described above. The cell suspension was then mixed with basement membrane extract (BME) (Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2 Select, 3533-005-02; Bio-technie) in 1:1 (v/v) ratio on ice and pipetted into each agarose well with 200 μ L mixture solution containing 100,000 cells. The 24-well plate was immediately centrifuged at 4 °C at 900 x g for 10 min, followed incubation for 30 min at 37 °C, 5% CO₂ and 95% rH for BME gelation. Afterwards, each well was supplemented with 1 mL of cell culture medium for 3 days of culture until chip injection.

To generate the fibroblast aggregates, a similar approach as described above was followed: fibroblasts were detached using TrypLE Express Enzyme for 3 min at 37 °C, pelleted by centrifugation at 200 x g for 5 min and resuspended in cell culture medium. 100,000 cells in 1 mL medium were pipetted into each agarose well. The 24-well plate was then centrifuged at RT at 900 x g for 10 min, and incubated at 37 °C, 5% CO₂ and 95% rH for 3 days until chip injection.

Patient-derived tumor organoids generation and culture

Pleural effusion (PE) samples from four metastatic breast cancer patients were collected by thoracentesis and processed as followed. PE samples were centrifuged at 500 x g for 10 min. Cell pellets were pooled and when necessary, red blood cells were lysed with 10 mL of RBC lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 100 μ M Na₂EDTA in H₂O, pH 7.4) on ice for 5 min. Cells were diluted in DPBS (Dulbecco's Phosphate Buffered Saline, P04-36500; Pan Biotech GmbH) and centrifuged at 500 x g for 10 min. The final cell pellet was resuspended in AdvDMEM/F12+++ (Advanced DMEM/F-12 (12534010), 1% Pen/Strep (15140122), 1x GlutaMAX (35050-038), 10 mM HEPES (15630-056); all from Thermo Fisher). For patient-derived organoid (PDO) culture setup, the desired amount of cell suspension was mixed with Basement Membrane Extract (BME; Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2 Select, Bio-technie, 3533-005-02) at a ratio of 30% cell suspension to 70% BME. 20 μ L droplets were plated into wells of a 48-well plate and placed upside-down in an incubator (37 °C, 5% CO₂) to solidify for 30 min. Afterwards, 280 μ L of culture medium (AdvDMEM/F12+++ supplemented with 10% (v/v) conditioned medium from L-WRN cells (ATCC-CRL-3276),⁵⁷ 5 nM Heregulin8-1 (100-03; PeproTech, NJ, USA), 5 ng/mL fibroblast growth factor 7 (FGF7) (100-19; PeproTech), 20 ng/mL fibroblast growth factor 10 (FGF10) (100-26; PeproTech), 5 ng/mL epidermal growth factor (EGF) (AF-100-15; PeproTech), 500 nM A83-01 (2939; Tocris, Wiesbaden, Germany), 5 μ M Y27632 (72034; STEMCELL Technologies), 500 nM SB-202190 (S7067; Sigma-Aldrich), 2% B27 Supplement (17504-044; Thermo Fisher Scientific), 1.25 mM N-acetyl-cysteine (A9165; Sigma-Aldrich), 5 mM nicotinamide (N0636; Sigma-Aldrich)) was added to each well. The medium was changed every 3–4 days.

The PDOs were passaged based on the confluency of the culture, ranging from 5–20 days. PDOs were recovered from the wells by resuspending the BME-droplets in ice-cold DPBS containing 5 μ M Y-27632 (DPBS/Y-27632). This organoid suspension was incubated with 1 mL of TrypLE Express Enzyme (1X; Thermo Fisher, 12604013) at 37 °C in a water bath for 5 min, followed by mechanical dissociation via 5 times pulling and dispensing the solution using a syringe with 27G needle (302200; Becton Dickinson). The suspension was then centrifuged at 500 x g for 10 min and the supernatant removed. For further culture, the desired amount of cell pellet was resuspended in AdvDMEM/F12+++ and mixed with BME at a ratio of 30% cell suspension to 70% BME and cultured as described above.

Prior to chip injection, the PDOs were expanded in a droplet of basement membrane extract hydrogel until they reached ~120 μ m in diameter, typically after ~10 days. To anticipate patient-specific differences in growth speed, the PDOs culture was monitored daily to better estimate the appropriate loading day. To retrieve the PDOs, gel droplets were first washed with PBS[−] before introducing 500 μ L of 2 mg/mL cold dispase II solution followed by pipetting up and down to break the gel droplet. The organoid cell suspension was then incubated for 10 min at 37 °C, 5% CO₂ and 95% rH. Afterwards, each well was resuspended with 500 μ L of AdvDMEM/F-12+++ and the retrieved PDOs were centrifuged at 500 x g for 5 min. Each droplet of PDOs on day 10 of culture was used to load 3 chips. After pelleting the PDOs, they were incubated in 10 μ M of CellTracker CMFDA solution (dissolved in phenol red-free DMEM/F-12 medium) for 20 min at 37 °C, 5% CO₂ and 95% rH. The organoids were then washed twice by adding phenol red-free DMEM/F-12 medium up to 10 mL and centrifuged at 500 x g for 5 min for each washing cycle. The organoid pellet was then resuspended in dextran-based hydrogel as will be described in the next section.

Microvascular endothelial cells isolation and culture

From resected skin tissue from plastic surgeries, subcutaneous fat, visible blood vessels, and connective tissue were removed, and the remaining skin tissues were cut into strips of ~4 cm length and ~1 mm width, followed by incubation in 10 mL of 2 U/mL dispase solution (D4693; Merck KGaA) at 4 °C overnight. Next, the epidermis layer was peeled off and discarded, and the remaining dermis layer was washed twice in PBS[−]. The mvECs were isolated from the dermis by incubating the dermis strips in 0.05% trypsin/EDTA (59417C; Sigma) for 40 min at 37 °C, followed by stopping the trypsinization with cell culture medium supplemented with 10% FCS and transferring the strips into a petri dish containing pre-warmed PBS[−]. Next, the dissociated cells were scraped out with a scalpel and the cell suspension was strained through a 70 μ m strainer (542070 ; Greiner Bio-One) and collected. The cell suspension was then centrifuged at 209 x g for 5 min. Lastly, the cell pellet was resuspended in 10 mL of Endothelial Cell Growth Medium (ECGM, C-22010; PromoCell GmbH) supplemented with 10 mg/mL Gentamicin (15710049; Thermo Fisher Scientific). The cells were seeded

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into two T25 cell culture flasks (690175; Greiner Bio-One) and incubated at 37 °C, 5% CO₂ and 95% rH. The cells were further expanded in T75 (658175; Greiner Bio-One) and used in passage 2 or 3 for this study.

Human induced pluripotent stem cells generation from PBMCs

hiPSCs were routinely cultured on Vitronectin XF in TeSR-E8 (all from Stem Cell Technologies) according to the manufacturer's protocol. Standard characterization of hiPSCs was performed as described previously.^{58–60} Pluripotency of the hiPSC clones was confirmed by flow cytometry analysis for OCT3/4, SSEA-4, NANOG and spontaneous differentiation towards three germ lineages. G-banding analysis was conducted at the Laboratorium voor Diagnostische Genoomanalyse (LGDA), LUMC according to standard procedures. A total of 20 metaphases were analyzed for each line. Cell line authentication was performed by the Department of Human Genetics, LUMC, by using the PowerPlex Fusion System 5C autosomal STR kit (Promega) as previously described.⁶¹ Both clones from two hiPSC lines had normal karyotypes, showed expression of pluripotency markers and underwent tri-lineage differentiation (data not shown). Endothelial cells were generated (see below) from the following clones (donor-matched for both patients) and used in subsequent experiments: LUMC0228iCTRL03 p11 and LUMC0229iCTRL01 p11.

Human induced pluripotent stem cells-derived endothelial cells generation and culture

Endothelial cells were differentiated from hiPSCs through mesoderm specification under defined culture conditions followed by cryopreservation until chip culture, as previously described.⁶² Cryopreserved hiPSC-derived endothelial cells were thawed in a water bath at 37 °C and transferred into a 15 mL tube containing 7 mL of Human Endothelial SFM medium (11111044; Thermo Fisher Scientific). The cells were centrifuged at 300 x g for 3 min and the pellet was resuspended in Human Endothelial SFM complete medium: Human Endothelial SFM medium supplemented with 20 ng/mL bFGF (130-093-564; Miltenyi Biotec), 30 ng/mL VEGF (130-109-396; Miltenyi Biotec), and 1% (v/v) human serum from platelet poor plasma (P2918; Sigma-Aldrich) and seeded into a T75 cell culture flask that was previously coated with 0.1% (w/v) gelatine solution for 1 h at 37 °C. The cells were expanded for 3–4 days prior to seeding into the tumor-on-chip and used only at passage 1.

Generation and culture of ROR1- and CD19-specific CAR-T cells

PBMCs were isolated by density gradient centrifugation from leukocyte apheresis of two different healthy donors using a separating solution with a density of 1.077 g/mL (Pancoll human; PAN Biotech). All donors provided their written informed consent.

CD4⁺ and CD8⁺ T cells were then isolated from PBMCs by magnetic associated cell sorting (MACS) using the CD4⁺ or CD8⁺ human T cell isolation kit (130-096-533 or 130-096-495; Miltenyi Biotec), respectively. Purity of isolated T cell fractions was verified by staining with fluorophore-conjugated antibodies (all from Biolegend, San Diego, CA, USA) and 7-AAD staining solution (130-111-568; Miltenyi Biotec) for dead cell exclusion on a MACS Quant 10 analyzer (Miltenyi Biotec). The following antibodies were used: anti-human CD3 PE (clone UCHT1), anti-human CD4 APC (clone RPA-T4), anti-human CD8 FITC (clone SK1).

T cells were seeded in 48-well plates (Costar plates; Corning) in CTL medium (RPMI 1640 supplemented with 1% (v/v) Penicillin/Streptomycin, 1X GlutaMAX-I, 0.1% (v/v) 2-Mercaptoethanol [all from Thermo Fisher Scientific, Darmstadt, Germany] and 10% (v/v) pooled human serum [Bavarian Red Cross Center, Wiesentheid, Germany]) and activated using Dynabeads Human T-Activator CD3/CD28 Beads and 50 U/mL rhIL-2 (Miltenyi Biotec).

The next day (day 1), two thirds of the medium was removed from all wells and T cells were treated with 5 ng/mL polybrene (Merck, Darmstadt, Germany). Lentiviral particles (MOI=3) encoding the ROR1 CAR construct (ROR1_41BB_CD3zeta_EGFRt)⁶³ or CD19 CAR construct (CD19_41BB_CD3zeta_EGFRt) were added to the cells (untransduced control: polybrene only) and centrifuged at 800 x g for 45 minutes at 32 °C. Afterwards, cells were incubated for 4 h at 37 °C, 5% CO₂ and 95% rH. Then, CTL medium supplemented with 50 U/mL rhIL-2 was added to all wells and cells were fed every other day by removing half of the medium from each well and adding CTL supplemented with 100 U/mL rhIL-2 (final conc. 50 U/mL rhIL-2). On day 7, CD3/CD28 Beads were magnetically removed and transduction efficacy was analyzed by staining with fluorophore-conjugated antibodies (all from Biolegend) and 7-AAD staining solution (Miltenyi Biotec) for dead cell exclusion on a MACS Quant 10 analyzer (Miltenyi Biotec). The following antibodies were used: anti-human EGFR Alexa Fluor 488 (clone AY13), anti-human CD4 APC (clone RPA T4), anti-human CD8 Pacific Blue (clone SK1). On day 9, CAR-modified (that is, EGFRt-positive) T cells were enriched by MACS using an in-house biotinylated anti-EGFR antibody (Cetuximab, Eli Lilly and Company, Indianapolis, IN, USA) and anti-Biotin Microbeads (Miltenyi Biotec). Purity of enriched CAR modified T cells was analyzed as described above. On the following day, enriched CAR modified as well as untransduced T cells were subjected either to an antigen-independent expansion protocol using irradiated CD19⁺ feeder cells as well as irradiated third-party donor PBMCs (ROR1-specific CAR-T cells) or to an antigen-dependent expansion protocol using irradiated CD19⁺ feeder cells (CD19-specific CAR-T cells). 14 days later, purity of expanded CAR-modified T cells was analyzed as above. Then, cells were counted using a Countess Counting II FL Device (Thermo Fisher Scientific) and cryopreserved at 10 million cells/mL in Cryo SFM freezing medium (C-29910; PromoCell GmbH).

Three days before the perfusion through the chips, each subset (i.e. CD4 and CD8) of cryopreserved control T and CAR-T cells were thawed, centrifuged at 300 x g for 8 min at 8 °C, counted and seeded in 24-well cell culture plates at a concentration of 3.0 x 10⁵ cells per well. T cells were cultured for 3 days in X-VIVO 15 medium (BE02-060F; Lonza) supplemented with 5 ng/mL of rhIL-15 (130-093-955; Miltenyi Biotec), 1X Glutamax and 100 U/mL penicillin-streptomycin.



Aggregates/spheroids/PDOs seeding on-chip

After chips sterilization and hydrophilization with O₂-plasma, the channels are flushed using 100 μ L pipette with 70% ethanol followed by rinsing three times with PBS⁻. The chips were kept at RT until cell seeding. The cells were seeded into the chip at two to four days before T cells perfusion.

To retrieve the MDA-MB-231 aggregates from the plate, cell culture medium was firstly removed from the wells before introducing 500 μ L of 2 mg/mL cold dispase II solution followed by pipetting up and down to break the gel. The plate was then incubated for 10 min at 37 °C, 5% CO₂ and 95% rH. For the fibroblast spheroids, this gel digestion step was skipped as no gel was used for the spheroids generation. Afterwards, each well was resuspended with 1 mL of cell culture medium and the retrieved aggregates/spheroids were centrifuged down at 500 x g for 5 min. The pellet was then resuspended in dextran hydrogel (3-D Life Dextran-CD Hydrogel SG; Cellendes GmbH) supplemented with RGD peptide (09-P-001; Cellendes GmbH) with a final concentration of 0.5 mmol/L. Aggregates/spheroids from one well were used to load 3 chips.

To retrieve the PDOs from the plate, gel droplets were first washed with PBS⁻ before introducing 250 μ L of 2 mg/mL cold dispase II solution followed by pipetting up and down to break the gel droplet. The PDO suspension was then incubated for 10 min at 37 °C, 5% CO₂ and 95% rH. Afterwards, each well was resuspended with 500 μ L of advDMEM/F-12 +/- and the retrieved PDOs were centrifuged down at 500 x g for 5 min. The pellet was resuspended in dextran hydrogel supplemented with 0.5 mmol/L RGD peptide. Each droplet of PDOs on day 10 of culture was used to load 3 chips.

In experiments that required PDOs/spheroids fluorescence-labeling, PDOs/spheroids pellet was resuspended in 10 μ M of CellTracker CMFDA dye solution (C7025; Invitrogen) dissolved in phenol red-free DMEM/F-12 medium and incubated for 20 min at 37 °C, 5% CO₂ and 95% rH. The cells were then washed twice by adding phenol red-free DMEM/F-12 medium up to 10 mL and centrifuging at 500 x g for 5 min for each washing cycle. Afterwards, the pellet was resuspended in dextran hydrogel supplemented with 0.5 mmol/L RGD peptide.

Once the cells were suspended in the hydrogel, 10 μ L was immediately injected into each chip via the tumor inlet. In the “empty chip” condition, blank hydrogel was injected without cells. Afterwards, the inlet and outlet of the medium channel were plugged using a metal wire with 0.7 mm diameter (45473; Menzani) and the main tumor channel was flushed via negative pressure to remove any remaining cells. Then, plugs from the medium channel inlet and outlet were carefully removed and inserted into the tumor channel inlet and outlet. The medium channel was subsequently flushed three times with 100 μ L of cell-specific culture medium to remove any deposited hydrogel that might disrupt the subsequent endothelial cells attachment and fluidic flow. The chips were then incubated at 37 °C, 5% CO₂ and 95% rH for 30 min to allow hydrogel crosslinking.

Endothelial cells seeding, chip culture, and characterization

MvECs were harvested by first removing the cell culture medium from the flask, rinsing with PBS⁻, followed by the insertion of 2 mL TrypLE Select (12563-029; Gibco). The flask was incubated for 3 min at 37 °C, 5% CO₂ and 95% rH to detach the cells. The trypsinization was stopped by adding 200 μ L of FCS into the solution, followed by transferring the solution into 15 mL tube. The cells were centrifuged at 200 x g for 5 min and resuspended in ECGM to achieve 9×10^6 cells/mL concentration for chip seeding. In case of hiPSC-ECs, the cells were harvested in a similar way with minor differences: the trypsinization process was done for 5 min at RT, and the cells were lastly centrifuged at 300 x g for 3 min at RT.

Endothelial cells were seeded into the chip on the same day of tumor cell loading and after the hydrogel in the tumor chambers was crosslinked. First, the medium channel of each chip was coated with 100 μ L solution containing 100 μ g/mL collagen-I (FibriCol, #5133; Advanced Biomatrix) and 20 μ g/mL fibronectin (#F1141; Sigma Aldrich) for 1 h at 37 °C, 5% CO₂ and 95% rH. The medium channel was then flushed three times with ECGM. Afterwards, 5 μ L of cell suspension (concentration 9×10^6 cells/mL) was injected into the medium channel inlet, followed by incubation for 4 h at 37 °C, 5% CO₂ and 95% rH to allow cell attachment on the membrane. Filter pipet tips filled with 100 μ L ECGM each were added into the inlet and outlet of the medium channel for 24 h of static culture. The chips were cultured for 4 days in push mode until control T and CAR-T cells perfusion. This culture duration was shortened to 1-2 days in some cases if stable endothelium was observed earlier. The details of the perfusion setup are described in the section below. Prior to the T cell perfusion, random chip samples were picked for immunofluorescence staining of the CD31 expression. Briefly, CD31 antibody (anti-human, APC, REAfinity; 130-110-808; Miltenyi Biotec) [in 1:35 working concentration] and Hoechst 33342 [in 1:250 working concentration] were diluted in ECGM. 50 μ L of the solution was gently introduced into the chip's medium channel followed by incubation for 30 min at 37 °C, 5% CO₂ and 95% rH. The staining solution was rinsed gently three times with ECGM before image acquisition.

For terminal immunofluorescence staining, cells were fixed by introducing ROTIHistofix 4% (P087.1; Carl Roth) into the chip's medium channel and incubated for 30 min at RT. Subsequently, the chip's medium channel was washed with PBS⁻ and blocking solution (3% BSA in PBS⁻) was incubated for another 30 min at RT. The primary antibody solution was prepared by diluting CD31 (anti-human, M0823; DAKO) and VE-Cadherin (anti-human, 36-1990; Invitrogen) 1:50 in antibody diluent (S0809; DAKO). This solution was incubated in the chip's medium channel overnight at 4 °C. On the next day, the primary antibody solution was replaced by PBS⁻ and unspecifically bound antibodies were washed off by rinsing the medium channel twice with PBS⁻. The secondary antibody solution was obtained by diluting the antibodies (Goat anti-Rabbit IgG (Heavy chain), Superclonal Recombinant Secondary Antibody, Alexa Fluor 555 and Goat anti-Mouse IgG (H+L), Superclonal Recombinant Secondary Antibody, Alexa Fluor 647) 1:100 in DAKO antibody diluent and adding DAPI [1 μ g/mL working concentration]. This solution was incubated in the chip's medium channel for 1 h at RT. Chips were washed twice with PBS⁻ prior to imaging. Thorough characterization of the endothelium has been performed in our previous studies employing the same seeding and culture method.^{10,11}

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For the barrier permeability assay, 4 kDa FITC- and 40 kDa-TRITC dextran were perfused through the medium channel either through chips containing mvEC barrier and MDA-MB-231 aggregates or blank chips. Fluorescence intensity was measured in the tumor tissue channel and normalized to the maximum fluorescence intensity in the medium channel. Images were taken every 3.7 min for up to 2 h using an epifluorescence microscope (Axio Observer 7, Carl Zeiss). The microscope chamber temperature was set to 37 °C during the acquisitions.

T cell perfusion and chip culture

To prepare the T cells, each subset (CD4 and CD8) of the cultured control T and CAR-T cells were retrieved from the 24-well plate and pipetted through a 70 μ L strainer to filter out cell clumps. Cells were centrifuged at 300 $\times$ g for 8 min at 8 °C followed by cell counting. To mimic the CAR-T cell product formulation in clinical trials, the typical 1:1 CD4⁺/CD8⁺ T cell ratio was selected throughout our experiments,^{14,15} as it has shown a superior antitumor reactivity in an in vivo study and their cooperation is essential to exert potent and long-lasting antitumor activity.^{64–66} Prior to the perfusion, both subsets were mixed and the concentration was set to 0.5 $\times$ 10⁶ cells/mL. The concentration was set to 100,000 cells/mL and 1,000,000 cells/mL only in preliminary experiments to determine the optimal perfused T cell concentration (Figure S1E). In experiments that required fluorescence-labeling of T cells, T cell pellet was resuspended in 2 μ M of CellTracker Deep Red dye solution (C34565; Invitrogen) dissolved in phenol red- and serum-free DMEM/F-12 medium and incubated for 15 min at 37 °C, 5% CO₂ and 95% RH. The cells were then washed twice by adding fully supplemented X-VIVO 15 medium up to 10 mL and centrifuging at 300 $\times$ g for 8 min at 8 °C for each washing cycle. Afterwards, the final cell pellet was resuspended in ECGM basal medium (C-22210; PromoCell GmbH) supplemented with 2% FCS, 12 μ g/mL ECGS, 90 μ g/mL heparin, 0.1 ng/mL EGF, 1 ng/mL bFGF (all from the Growth Medium SupplementPack C-39210; PromoCell GmbH) – this will be referred to as “coculture medium” in the following.

Day of control T and CAR-T cell perfusion was defined as day 0 for all experiments. Starting from day 0, the chips were connected to constant medium perfusion via an external syringe pumping system (LA-190, Landgraf Laborsysteme HLL GmbH, Langenhagen, Germany). The chips were connected to the syringe pump using blunt 21 GA stainless steel needles (made from the dispensing needles by removing the plastic hub after dissolving the glue overnight in a 70% ethanol solution) connected to Tygon tubings (0.51 mm inner diameter, Tygon ND 100-80 Medical Tubing, Saint-Gobain Performance Plastics Pampus GmbH, Willich, Germany), 21 GA stainless steel plastic hub dispensing needles (e.g., KDS2112P, Weller Tools GmbH, Besigheim, Germany) and Luer Lock style syringes. For (CAR-)T cell perfusion through the medium channel, the inlet of the channel was equipped with a 100 μ L-pipette tip reservoir holding the 400 μ L of T cell suspension (200,000 T cells/chip). The syringe pump was set to the withdraw mode at 100 μ L/h to ensure steady flow before introducing the (CAR-)T cells, thereby preventing them to precipitate on the inlet area. Once the (CAR-)T cells were suspended in the pipette tip reservoir, the flow rate was set to 20 μ L/h and the whole setup was transferred into an incubator at 37 °C, 5% CO₂ and 95% RH. After 20 h, the chip effluents and plate control supernatants were collected for further analysis and the perfusion setup was changed to push mode where coculture medium was dispensed from the syringes. The chips further linearly perfused in this mode at 20 μ L/h flow rate until up to day 8. Medium in the syringes was refilled after 3 days of perfusion. Before chip perfusion, medium is warmed-up to 37 °C and simultaneously degassed (vacuum source -70 kPa) and filter-sterilized using a Steriflip conical filter unit for 20 min. Effluent samples from each chip were collected every one or two days, centrifuged at 200 $\times$ g for 5 min to remove perfused cells, then at 10,000 $\times$ g for 10 min to remove debris and stored at -80 °C until further analysis. Terminal dead cell staining was done by introducing 20 μ g/mL propidium iodide (P4170; Sigma Aldrich) solution into the media channel followed by static incubation of the chips for 5 min at 37 °C. The medium channels were rinsed three times with PBS[−] before image acquisition.

For plate assay controls, the same amount of tumor aggregate per chip were plated into each well (48-well plate format), followed by the addition of 400 μ L of T cell suspension (200,000 control T or CAR-T cells (1:1 ratio of CD8⁺ and CD4⁺) per well). Cells were cocultured overnight in coculture medium. Supernatants from day 1 were collected, centrifuged the same way as the chip samples and stored at -80 °C until cytokine analysis (see “cytokine quantification assay” method section).

Dasatinib treatment

To model CRS intervention, dasatinib (S1021; Selleckchem) with a final concentration of 50 nM was supplemented in the coculture medium and perfused through the medium channel of the chips either from day 0-1, 0-8, or 1-8. This concentration was found to be potent to render CD19-CAR-T cells temporarily inactive.¹² In a preliminary experiment, CAR-T cells were cultured either with or without MDA-MB-231 cells (effector-to-target cell ratio, 10:1) for 24 h, with or without dasatinib supplementation (50 nM final concentration) in the coculture medium. In the coculture condition, MDA-MB-231 cells were seeded as a monolayer and CAR-T cells (1:1 ratio of CD8⁺ and CD4⁺) suspension was added into the well. CAR-T cells were collected on the next day for flow cytometry staining with anti-human CD69 (early activation marker), anti-human CD25 (late activation marker). Viability was assessed also during flow cytometry by adding Zombie aqua dye (423102; BioLegend) into the staining solution (see “flow cytometry staining and analysis” method section).

Allogeneic / isogenic endothelial cells – (CAR-)T cells coculture for cytokine assay

To demonstrate the differences of allogeneic and isogenic setup in terms of cytokine release by the T cells, we selected five mvECs donors for 24 h coculture with T cells. HiPSC-ECs was used in the isogenic setup. Briefly, endothelial cells were plated (10,000 cells/well, 96-well plate format) and cultured overnight. On the next day, 200,000 control T or CAR-T cells (1:1 ratio of CD8⁺ and CD4⁺) were

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added into each well (effector-to-target cell ratio, 20:1). Cells were cocultured overnight and coculture medium was used: see method section “[T cell perfusion and chip culture](#)” for the composition. Supernatants were collected and stored at -80°C until cytokine analysis (see “[cytokine quantification assay](#)” method section).

QUANTIFICATION AND STATISTICAL ANALYSIS

Image acquisition and analysis

For the timepoint live imaging of T cell infiltration, GFP fluorescence intensity of the MDA-MB-231 tumor aggregates and tumor aggregate area, an epifluorescence microscope (Axio Observer 7, Carl Zeiss) was used and the focal plane was set to focus on the aggregate/spheroid/PDO. The microscope chamber temperature was set to 37°C during all live cell imaging acquisitions of the chips. Analyses of T cell infiltration, GFP fluorescence intensity of the MDA-MB-231 tumor aggregates and tumor aggregate area were conducted using ImageJ-Fiji software.⁵⁰ For T cell intensity measurements, the histogram of the fluorescent images was adjusted to remove the background signal, followed by quantification of the mean gray value of the CellTracker Deep Red-labeled (CAR-)T cells within the region of interest (ROI) of each tumor aggregate/fibroblast spheroid/PDO. Tumor aggregates/fibroblasts spheroids/PDOs smaller than $50\text{ }\mu\text{m}$ in diameter were considered too small and therefore excluded from the analysis. Tumor aggregate/fibroblasts spheroid/PDO ROI was defined by automated thresholding of the GFP signal (for MDA-MB-231 aggregates) or the CellTracker CMFDA signal (for fibroblasts spheroids and PDOs). Area of MDA-MB-231 aggregates or PDOs was measured after thresholding to analyze their growth upon control T or CAR-T cell treatment, and aggregates/PDOs area from day > 0 was compared with their initial area before control T or CAR-T cell perfusion. For GFP fluorescence intensity analysis of the tumor aggregate, the mean gray value of tumor aggregates was measured and compared with their initial mean gray value on day 0. To quantify T cells in the whole tumor chamber or within the tumor aggregate, a spinning disk confocal microscope (Cell Observer Z1, Carl Zeiss) was used. Here, confocal z-stacks imaging data were used to run the Spot Detection function in Imaris 9.5 software (Oxford Instruments). The estimated XY and Z diameter of T cells was set to $5\text{ }\mu\text{m}$ and $10\text{ }\mu\text{m}$, respectively. Tumor aggregate volume was rendered using the Surfaces function. The number of infiltrating (CAR-)T cells was quantified by counting the subset of (CAR-)T cells that have zero distance (colocalized) to the tumor aggregate. For all measurements, image data from a minimum of 3 chips were pooled for each condition for further statistical analysis. To measure MDA-MB-231 aggregates diameter after the aggregate generation method using the agarose microwells-based approach, a randomly selected area in the well (three wells in total) was imaged and further processed for automated thresholding and subsequent diameter measurement in ImageJ-Fiji to check for the diameter variability.

Cytokine quantification assay

Cytokines from undiluted samples from chips/plates were analyzed by fluorescent bead-based multiplex sandwich immunoassays (Legendplex Human CD8/NK Mix and Match Subpanel; BioLegend) containing capture beads targeting IL-2, IL-6, IL-10, TNF- α , IFN- γ and granzyme B and read by flow cytometry (BD LSR II or BD LSRFortessa, BD Biosciences) according to manufacturer's protocol. Flow cytometry data files were manually gated using LEGENDplex Cloud-Based Data Analysis Software Suite (BioLegend) to find the optimal differentiation between capture bead populations and subsequently applied to all datasets.

Flow cytometry staining and analysis

For the analysis of the expression of extracellular markers, the following antibodies were used depending on the experiment: anti-human ROR1 APC (130-118-015; Miltenyi Biotec), anti-human CD31 FITC (130-110-806; Miltenyi Biotec), anti-human CD3 PE/Cyanine7 (317334; BioLegend), anti-human CD4 APC/Fire750 (357426; BioLegend), anti-human CD8a PerCP (300922; BioLegend), anti-human CD69 FITC (310904; BioLegend) and anti-human CD25 BV421 (302630; BioLegend). Detailed information on the antibodies is summarized in the “[key resources table](#)”. 96-well V-bottom cell culture plate was used for staining and flow cytometry reading. For staining, cells in each well were firstly washed with flow cytometry staining buffer: 3% FCS with 2 mM EDTA (15575020; Invitrogen) in PBS⁺. Cells were then incubated in a blocking solution containing Human BD Fc Block (564220; BD Biosciences) diluted 1:25 (v/v) in PBS⁺ for 15 min at 4°C . Afterwards, cells were incubated for 30 min at 4°C in the antibody-mixture solution specific for each experiment. Zombie aqua dye (423102; BioLegend) was added 1:200 in the antibody-mixture solution to differentiate the viable cells during analysis. Cells were then washed twice with the staining buffer, fixed with ROTIHistofix 4% (P087.6; Carl Roth GmbH) for 10 min at RT, then washed and resuspended in the staining buffer. The samples were analyzed directly using flow cytometry (BD LSR II or BD LSRFortessa, BD Biosciences) or kept stored at 4°C for maximum overnight until analysis. Autofluorescence and isotype controls were included in every staining procedure. For the analysis, FSC-A and SSC-A properties were used to identify cells, cell doublets were excluded based on FSC-H vs. FSC-A. Dead cells were excluded from analysis. Primary cancer cells, cancer cell lines and endothelial cells were analyzed with established ROR1 marker by using unstained cells as cut-off. Same strategy was applied for CD31 staining on hiPSC-derived endothelial cells. For T cell analysis, T cell subpopulations were gated according to established markers and analyzed for activation marker expression by comparing activation marker signal in untreated vs. treated T cells.

Statistics and reproducibility

In all experiments, every chip is considered an independent biological replicate. For each run of chips, the same condition was performed at least twice. For graphs with a statistical analysis, the minimum sample size is 3. The sample size criteria depended on the

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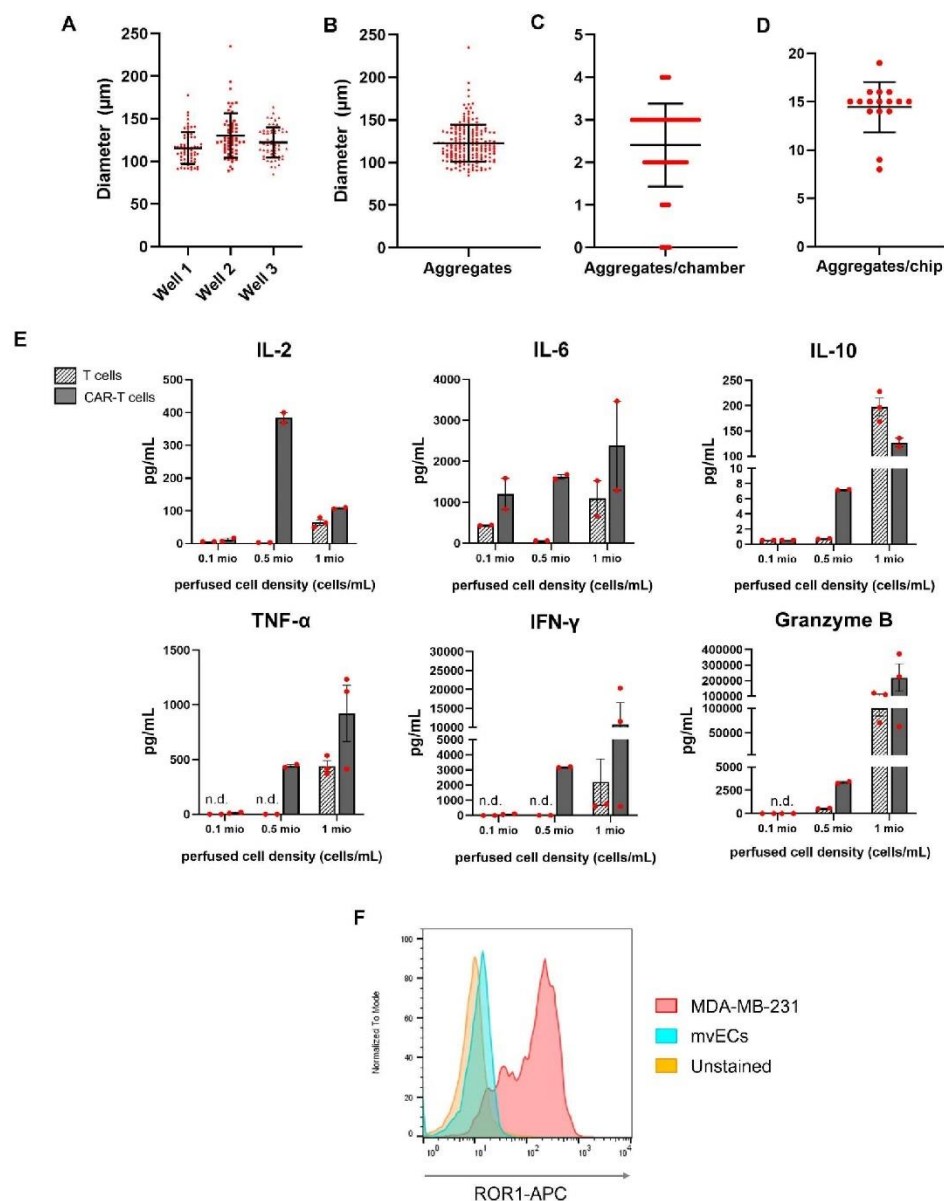
logistical complexity of the whole experiment, primary and iPS cells availability, distribution of donors to assure paired conditions, availability of pumping systems and technical issues. The criteria was the following: for proof of concept data, the sample size of 2 is the minimum; for the following experiments, a minimum sample size is 4. Every test was performed in parallel with the respective control conditions. Chips were randomly allocated into experimental groups using a numbering system for distinguishing them, which was kept until analysis. Chips were excluded from the analysis if technical issues with the microfluidic setup occurred (e.g. leakages, flow blockage due to bubbles). For the cytokine analysis, only chips with the correct effluent volume were considered. For the image analysis, aggregate/spheroid/organoid smaller than 50 μm in diameter were considered too small and therefore excluded from the downstream analysis. The number of biological replicates is described in each figure caption. For imaging-based quantification, n denotes the number of aggregates/spheroids/PDOs or tumor chambers, as indicated in each figure caption. For the effluent analysis, n denotes the number of chip replicates. Statistical analysis was performed using GraphPad Prism 9.2.0 software, and graphs are represented as average values $\pm$ SEM unless indicated otherwise. Detailed statistics are indicated in each figure legend.

Supplemental Information

**Breast cancer-on-chip for patient-specific
efficacy and safety testing of CAR-T cells**

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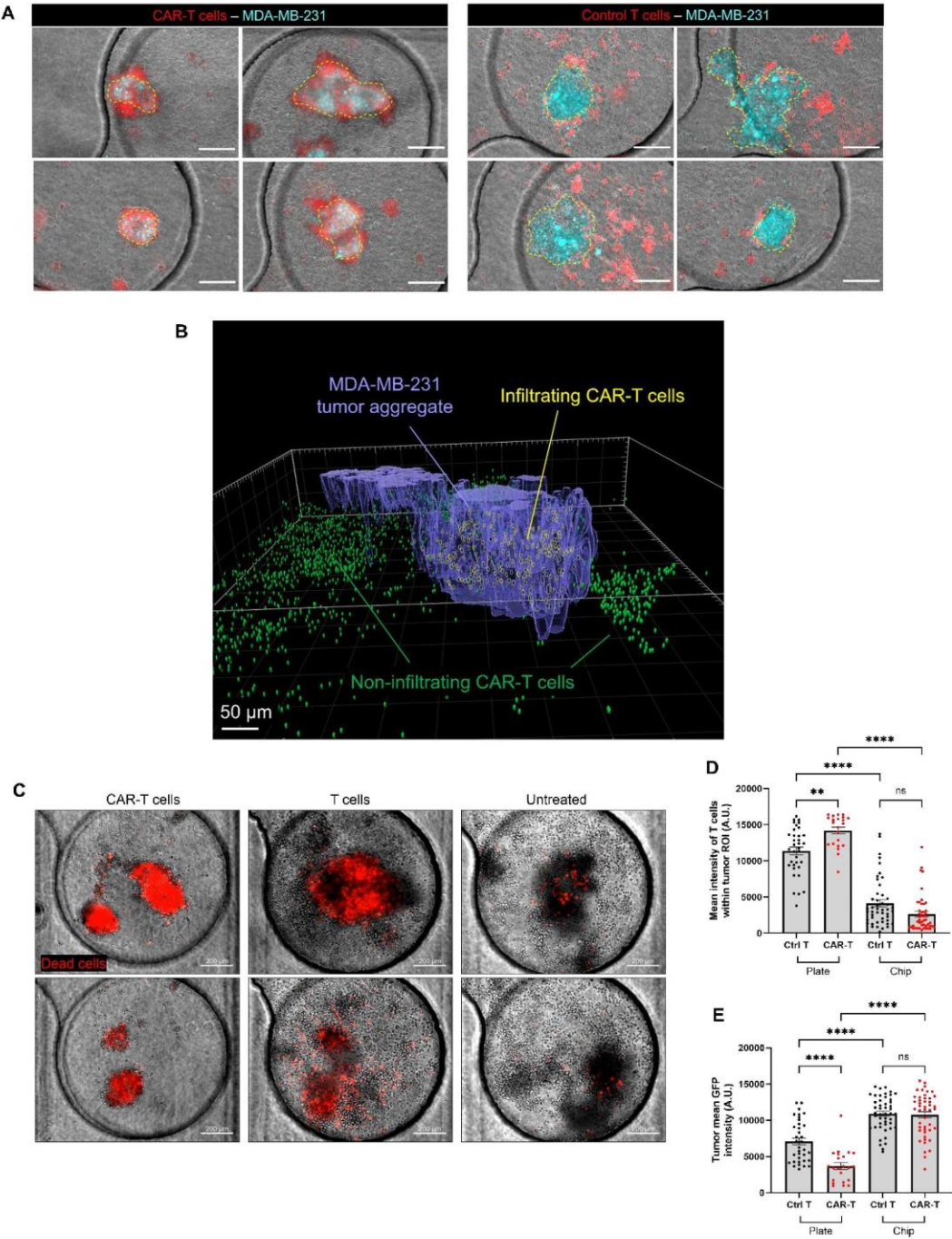
Supplementary Materials



Supplementary Figure 1: Tumor-on-chip initial setup characterization. Related to Figure 1.

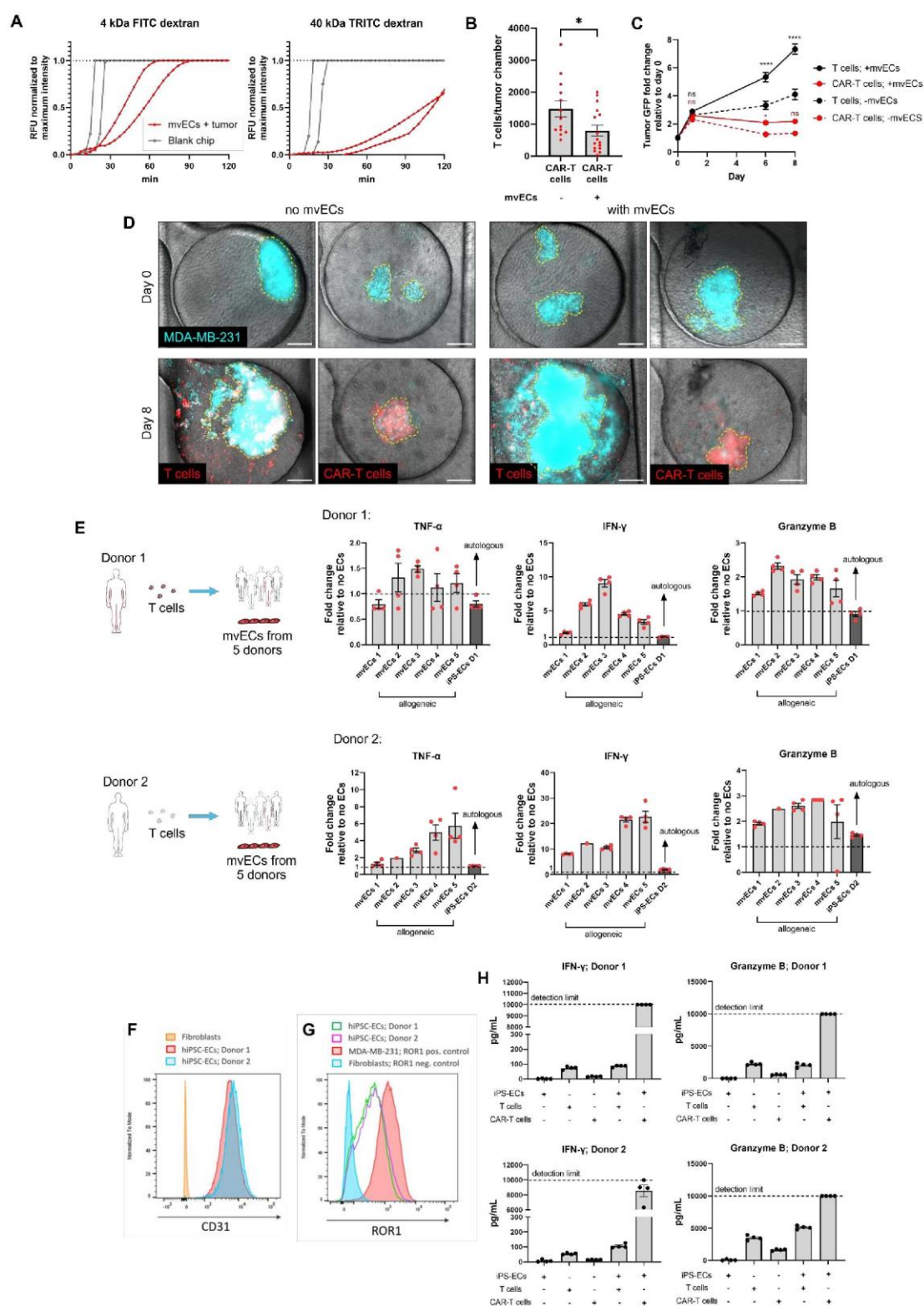
(A) Diameter of the MDA-MB-231 aggregates on day 3 after being generated using the agarose microwells-based approach. $n = 62$ -66 aggregates. Data depicted are mean $\pm$ SD. (B), Pooled data points from (a.); $n = 190$ aggregates. (C) Quantification of MDA-MB-231 aggregates per chamber

after chip loading; $n = 96$ chambers from 16 chips. **(D)** Quantification of MDA-MB-231 aggregates per chip after loading; $n = 16$ chips. **(E)** Quantification of the levels of cytokines IL-2, IL-6, IL-10, TNF- α , IFN- γ and granzyme B in the effluents of the chips after 20 h of (control) T or CAR-T cells perfusion through tumor-on-chips containing MDA-MB-231 aggregates. The concentration of the perfused cells was: 100,000, 500,000, and 1,000,000 cells/mL, respectively; $n = 2-3$ chips, indicated by the red dots. Data are depicted as mean with $\pm$ SEM. n.d.: not detected, values below the detection limit. **(F)** Flow cytometry histogram plots showing ROR1 expression in the MDA-MB-231 cells (red), mvECs (cyan) compared with the unstained control (orange).



Supplementary Figure 2: (CAR-)T cell infiltration positively correlates with tumor cell death and growth restriction. Related to Figure 2.

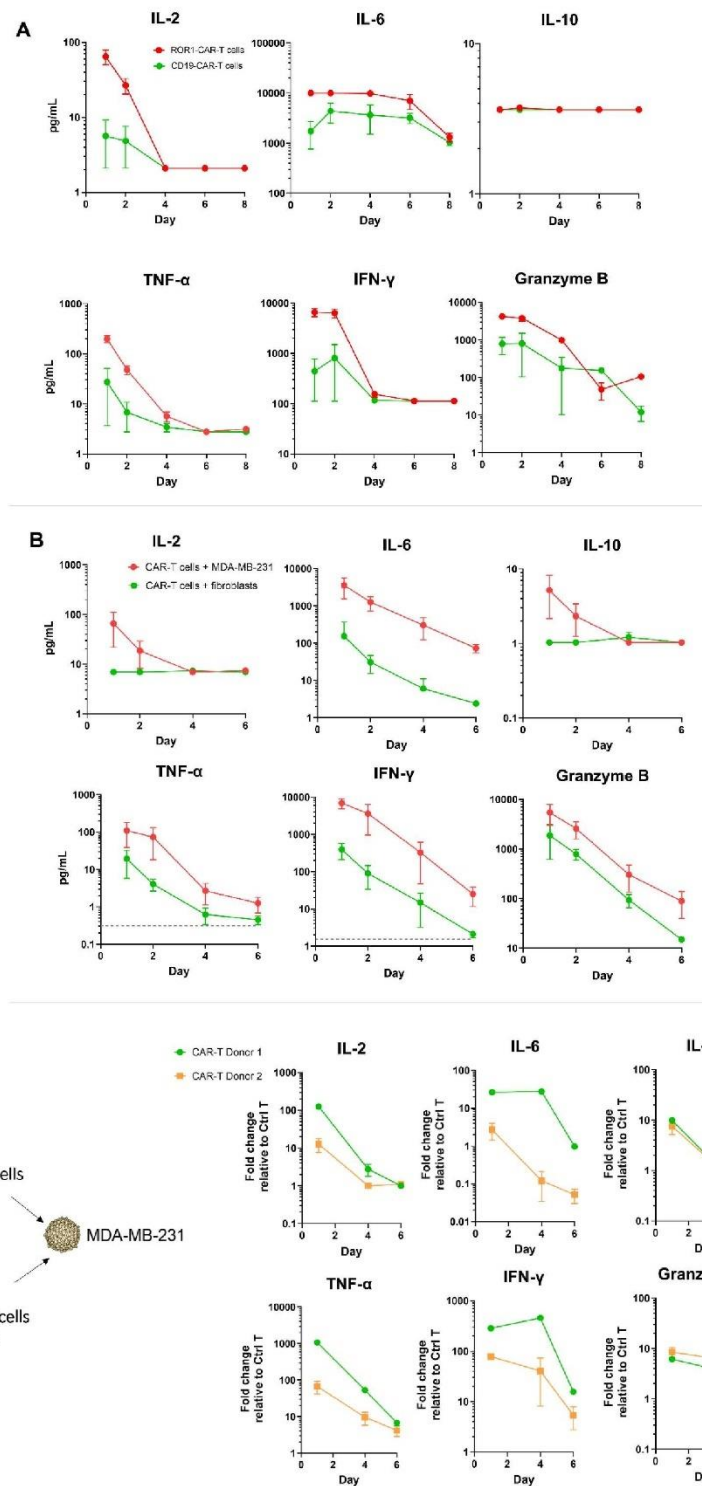
(A) Representative images from four different chips of infiltrating CAR-T cells (four images on the left) and excluded control T cells from the MDA-MB-231 tumor aggregates (four images on the right) on day 8. Scalebars: 200 μ m. **(B)** Representative 3D-rendered image of a tumor chamber, showing CAR-T cells within the interior (yellow) and exterior (green) of the MDA-MB-231 tumor aggregate (purple). **(C)** Representative images of dead cells in tumor chambers from two chips (in row) on day 8 after treatment with CAR-T cells, control T cells, or untreated. Dead cells were stained with propidium iodide. **(D)** Quantification of the mean intensity values of the control (Ctrl) T cells and CAR-T cells within each tumor aggregate region of interest on day 1 (20 h) after (CAR-)T cell treatment. Each dot represents one tumor aggregate ROI; $n = 22-47$ MDA-MB-231 tumor aggregates from 4 chips or wells. Data are presented as the mean $\pm$ SEM. ns: not significant, ** $p < 0.01$, **** $p < 0.0001$; Tukey's multiple comparisons test. **(E)** Quantification of the mean GFP intensity of each MDA-MB-231 tumor aggregate on day 1 (20 h) after control (Ctrl) T cell or CAR-T cell treatment. Each dot represents one tumor aggregate; $n = 22-47$ aggregates from 4 chips or wells. ns: not significant, **** $p < 0.0001$; Tukey's multiple comparisons test.



Supplementary Figure 3: The effect of the endothelial cells and their sourcing strategy on T cell recruitment and cytokine response. Related to Figure 2 and 5.

(A) mvEC barrier integrity determined by fluorescence macromolecule tracing of 4 kDa FITC-dextran and 40 kDa TRITC-dextran from two individual chips, either containing mvECs barrier and MDA-MB-231 tumor aggregates or blank chips. The fluorescence intensity of FITC and TRITC dextran was measured in the tumor tissue channel and normalized to the maximum fluorescence intensity in the medium channel. **(B)** Quantification of the recruited CAR-T cells in the tumor chamber on day 8 in chips with or without mvECs; $n = 13-16$ chambers from 3 chips. Data presented as the mean $\pm$ SEM. $*p < 0.05$; two-tailed unpaired t test. **(C)** Quantification of the fold change in the mean GFP intensity of MDA-MB-231 tumor aggregates in each tumor chamber on day 1-8 after the perfusion of (control) T cells or CAR-T cells through chips with or without mvECs relative to day 0; $n = 24$ tumor chambers from 4 chips. Data are presented as the mean $\pm$ SEM. ns: not significant, $*p < 0.05$, $****p < 0.0001$; Tukey's multiple comparisons test between with and without mvECs conditions. **(D)** Representative images on day 0 and 8 after perfusing T cells or CAR-T cells (red) through the tumor-on-chip setup containing MDA-MB-231 aggregates (cyan) with or without mvECs. Scale bars: 200 μm . **(E)** Fold change cytokine values of TNF- α , IFN- γ and granzyme B from T cells obtained from two different donors co-cultured for 24 h either in allogeneic or isogenic/autologous setting relative to the culture without endothelial cells. In the allogeneic setting, T cells were co-cultured with mvECs from 5 different donors. T cells were co-cultured with iPSC-ECs in autologous setting, which were differentiated from iPSCs previously reprogrammed from PBMCs of the same T cell donor. Each dot represents a data point from one cell culture well. Data are shown as mean with $\pm$ SEM; $n = 1-4$. **(F)** Flow cytometry histogram plots showing CD31 expression of hiPSC-ECs from two different donors used in the experiments integrating PDOs. Fibroblasts were used as the negative control. **(G)** Flow cytometry histogram plots showing ROR1 expression levels in the hiPSC-ECs from two different donors, as well as MDA-MB-231 and fibroblasts as the positive and negative control, respectively. **(H)** Quantification of the IFN- γ and granzyme B in the effluents of a plate culture experiment after 24 h of (control) T or CAR-T cells co-culture with or without autologous hiPSC-ECs. Single cultures

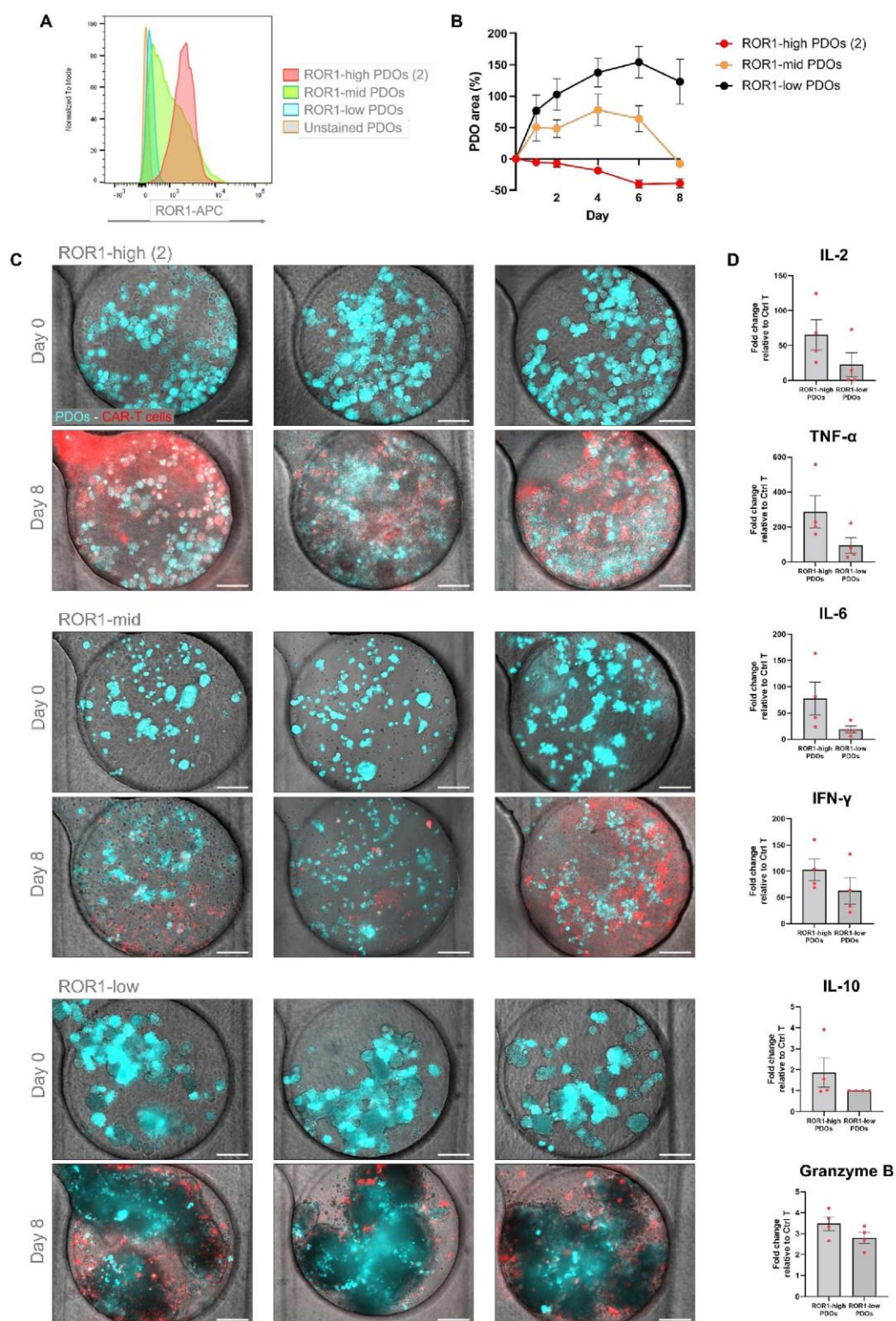
were used as the control conditions. Cells derived from two donors were tested; $n = 4$. Data are depicted as mean with $\pm$ SEM. Each red dot represents the cytokine value from one well.



Supplementary Figure 4: Target- and CAR-T cell donor-dependent cytokine release profile.

Related to Figure 3.

(A) Release kinetics of the cytokines IL-2, IL-6, IL-10, TNF- α , IFN- γ and granzyme B from days 1 to 8 after ROR1-CAR-T cell or CD19-CAR-T cell perfusion from days 0 to 1. Samples were collected every 1–2 days; $n = 4$ chips. Data are presented as the mean $\pm$ SEM. The dashed line indicates the lower limit of detection. **(B)** Release kinetics of the abovementioned cytokines from days 1 to 8 after ROR1-CAR-T cell perfusion from days 0 to 1 through chips containing either ROR1⁺ MDA-MB-231 aggregates or ROR1⁻ fibroblast spheroids. Samples were collected every 1–2 days; $n = 4$ chips. Data are presented as the mean $\pm$ SEM. The dashed line indicates the lower limit of detection. Data points from day 8 were excluded because the values were mostly below the detection limit. **(C)** Fold change cytokine values from day 1, 4 and 6 obtained from the tumor-on-chips containing mvECs barrier and MDA-MB-231 aggregates perfused with ROR1-CAR-T cells, relative to the respective control T cells condition. (CAR-)T cells were sourced from two healthy donors. Each dot represents effluent data collected from one chip. Data are shown as mean with $\pm$ SEM; $n = 2-3$.



Supplementary Figure 5: PDO growth and cytokine release from two additional patients with metastatic breast cancer. Related to Figure 5.

(A) Flow cytometry histogram plots showing ROR1 expression in high-ROR1-expressing patient-derived tumor organoids (PDOs) (red) [marked with “(2)” as this originates from a second patient with high ROR1-expressing tumor in this study], intermediate-ROR1-expressing PDOs (green), and low-ROR1 expression PDOs (cyan) from patients with metastatic breast cancer compared with the unstained control (orange). **(B)** PDO growth after CAR-T-cell treatment, as measured by quantifying the percentage change in PDO area in each tumor chamber at certain timepoints relative to the initial area before CAR-T cell perfusion; $n = 24$ chambers from 4 chips. Data are presented as the mean $\pm$ SEM. **(C)** Representative images from a tumor chamber from three individual chips (in column) of ROR1-high (2), ROR1-mid and ROR1-low PDOs (cyan) before CAR-T cell (red) perfusion on day 0 and on day 8 of the experiment. Scale bars: 200 μm . **(D)** Fold change cytokine values from day 1 obtained from the tumor-on-chips containing either ROR1-high- or ROR1-low-expressing PDOs perfused with CAR-T cells, relative to the respective control T cells condition, showing a trend of higher fold change in ROR1-high PDOs condition. Each dot represents effluent data collected from one chip. Data are shown as mean with $\pm$ SEM; $n = 4$.

Supplementary Table 1: Patient data for the PDOs used in the study. Related to Figure 5.

Line	Age	Primary tumor		Metastasis		Treatment		PDO
		Type	Receptor status	Type	Receptor status	Drainage	Prior therapy	Receptor status
(#10) PDO- ROR1-high	63	IBC (2020)	ER-/PR-/ HER2-	Lymphogenic and cutaneous metastasis	ER-/PR-/ HER2-	Pleural effusion	Carboplatin + Paclitaxel + Atezolizumab	ER-/PR-/ HER2-
(#44) PDO- ROR1-high (2)	56	NST (2019)	ER+/PR-/ HER2-	Cutaneous, Lymphogenic and osseus metastasis	ER+/PR-/ HER2-	Pleural effusion	Paclitaxel; Eribulin; Abemaciclib +Letrozole; Capecitabine	ER+/PR-/ HER2-
(#31) PDO- ROR1-mid	61	NST (2009)	ER+/PR+/ HER2-	Lymphogenic and hepatic metastasis	ER+/PR-/ HER2	Pleural effusion	Ribociclib + Letrozole; Palbociclib +Letrozole; Abemaciclib +Fulvestrant	ER+/PR-/ HER2-
(#43) PDO- ROR1-low	52	NST (2019)	ER+/PR+/ HER2-	Hepatic, osseus, and pulmonary metastasis	ER+/PR-/ HER2-	Pleural effusion	Letrozole + Goserelin + Abemaciclib; Paclitaxel	ER+/PR-/ HER2-

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**Breast cancer-on-chip integrating
white adipose tissue microenvironment
to study tumor-white adipose tissue crosstalk**

T. I. Maulana, L. Lazarevski, M. Cipriano, L. Christ, J. Rogal, A. Koch, P. Loskill
(2024)

Abstract:

Human models that accurately replicate the breast adipose tumor microenvironment (TME) are essential for studying the interactions between breast cancer (BC) and white adipose tissue (WAT) during tumor development. Here, we developed a breast cancer-on-chip model capable of integrating BC spheroids or patient-derived organoids and WAT-associated cellular components (mature adipocytes, stromovascular cells, and adipose tissue macrophages), enabling long-term co-culture to monitor BC-WAT interactions. This model demonstrated its utility in assessing BC's metabolic adaptation by inducing adipocyte lipolysis under glucose-deprived condition and examining the impact of WAT on BC lipid accumulation and growth. Additionally, the model revealed WAT's protective effect against the chemotherapeutic agent daunorubicin. The ability to study BC-WAT interactions in a physiologically relevant TME in this model can provide new insights into the mechanisms of cancer-WAT interaction beyond BC, offering broad potential for exploring cancer therapies targeting the WAT-cancer axis.

Breast cancer-on-chip integrating white adipose tissue microenvironment to study tumor-white adipose tissue crosstalk

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ABSTRACT

Human models that accurately replicate the breast adipose tumor microenvironment (TME) are essential for studying the interactions between breast cancer (BC) and white adipose tissue (WAT) during tumor development. Here, we developed a breast cancer-on-chip model capable of integrating BC spheroids or patient-derived organoids and WAT-associated cellular components (mature adipocytes, stromovascular cells, and adipose tissue macrophages), enabling long-term co-culture to monitor BC-WAT interactions. This model demonstrated its utility in assessing BC's metabolic adaptation by inducing adipocyte lipolysis under glucose-deprived condition and examining the impact of WAT on BC lipid accumulation and growth. Additionally, the model

revealed WAT's protective effect against the chemotherapeutic agent daunorubicin. The ability to study BC-WAT interactions in a physiologically relevant TME in this model can provide new insights into the mechanisms of cancer-WAT interaction beyond BC, offering broad potential for exploring cancer therapies targeting the WAT-cancer axis.

KEYWORDS

breast cancer, adipose tissue, organ-on-chip, cancer model, tumor microenvironment

INTRODUCTION

Breast cancer (BC) remains the most common type of cancer among women worldwide, and the global burden is predicted to increase ¹. Regions with low levels of socio-demographic index are predicted to have the largest disease burden by 2050, with metabolic risks being the leading risk factor for death ². BC itself is a heterogeneous group of diseases and is classified into more than 20 subtypes based on their differences in clinical, morphological, and genetic features ³. In addition to the heterogeneity of BC at the single-cell level, growing concerns now focus on the adipose tumor microenvironment (TME) ^{4,5}. The female breast is mainly composed of white adipose tissue (WAT), with BC typically developing in mammary ducts or lobules nearby. Being a metabolically active tissue, it can support tumor growth and influence treatment response through a multitude of mechanisms, including direct energy supply in the form of fatty acids ⁶⁻¹⁴, production of growth-supporting adipokines ¹⁵, hormones ^{16,17}, and exosomes ^{18,19}, catabolizing chemotherapeutic drugs ²⁰, and shaping the inflammatory microenvironment ^{5,21}. WAT itself is composed of mainly mature adipocytes (90% of volume, 50% of cellular content) ²², along with the stromal vascular fraction (SVF) that includes mesenchymal stem cells, adipocyte and vascular progenitors, endothelial cells, fibroblasts, and various types of immune cells ²³. The interaction between BC and WAT is therefore highly complex, and studying the disease requires a faithful recapitulation of human breast TME.

Current studies of BC and WAT interaction mostly rely on mouse models, which differ anatomically from the human breast microenvironment, including less branching of the mammary duct and lack of stromal interface between tumor and WAT, among others ⁴. With regard to cellular components, phenotypes, metabolism, and immunity, significant differences exist between humans and mice ^{24–29}. On the other hand, culturing mature white adipocytes in vitro can be challenging due to their buoyancy and fragility, let alone the integration of 3D tumor in close proximity. Although many in vitro models resulted in insights into cancer cells-adipocytes bidirectional crosstalk with regards to fatty acid transport mechanism, these models do not yet enable long-term co-culture for multiple weeks to monitor tumor growth in a three-dimensional, organotypic, and perfused TME ³⁰.

To bridge this gap, we developed a tailored breast cancer-on-chip model that integrates BC spheroids or patient-derived organoids (PDOs) and immunocompetent white adipose tissue (WAT) in a microfluidic system. We fit-for-purposely adjusted our previously published WAT-on-chip model ³¹ to mimic the primary breast TME during early tumor progression in patients ⁴. This enabled us to test the impact of BC cells on adipocyte lipolysis and WAT's presence on lipid accumulation, tumor growth, and chemotherapeutic efficacy. The breast cancer-on-chip model represents an advancement of engineered model systems by providing a robust platform for studying the complex interactions between BC cells and the adipose microenvironment, paving the way to enable the exploration of new therapeutic strategies targeting metabolic pathways and tumor-WAT interactions applicable beyond BC.

RESULTS AND DISCUSSION

Tailored breast cancer-on-chip enables the integration of BC spheroids/organoids and immunocompetent white adipose tissue

To mimic BC development within an adipose TME, we generated a breast cancer-on-chip model in which mature adipocytes along with the stromal vascular fraction (SVF) can be placed to

surround a cell-line derived BC tumor spheroid or PDO in two separated microfluidic chambers (**Figure 1A**).

The chip layers are composed of micropatterned layers of hybrid materials (**Figure 1B**). The tissue and medium layer (0.2 mm channel heights) consist of two stacked layers of micropatterned polyethylene terephthalate glycol (PET-G) and polydimethylsiloxane (PDMS), respectively. Both layers are separated by an isoporous, semipermeable hexamethyldisiloxane (HMDSO) coated polyethylene terephthalate (PET) membrane with a 3 μm pore size to allow the transport of nutrients and metabolic waste products. The adipocytes and SVF occupy the peripheral part of the tissue chamber (i.e. WAT area), which has an open-top middle part (1 mm diameter) to allow the placement of BC spheroids or PDOs through the tumor injection ports (**Figure 1C**). Thermoplastic PET-G material, instead of PDMS, was used to limit absorption of small hydrophobic molecules within the tumor-WAT interaction chamber. The upper PDMS layer accommodates the pipetting access for BC spheroids/PDOs injection as well as the insertion of fluidic connection on the medium inlet and outlet for medium perfusion. A top layer made of poly(methyl methacrylate) (PMMA) is used to support proper foil-sealing of the chip, thereby ensuring a sterile environment. On the bottom side, a glass coverslip is used to seal the medium channel and simultaneously allow high-resolution imaging.

To generate the model, a PDMS pin was inserted into each tumor injection port before the injection of WAT-associated cells through the WAT injection inlet (**Figure 1D**). This PDMS pin keeps WAT-associated cells from entering the middle part of the chamber during the loading process, thereby providing space for the subsequent injection of BC spheroids or PDOs upon its removal. The fluidic resistance of the resistance channel forces the collagen-I-embedded cells to fill the WAT area during the cell loading process. Collagen-I is the primary extracellular matrix (ECM) component in the breast adipose tumor microenvironment, where it increases in abundance and stiffness as the

primary tumor progresses³². Upon hydrogel gelation, the PDMS pins were carefully removed to allow the injection of BC spheroids/PDOs that were embedded in a hydrogel composed of denatured collagens and hyaluronic acids (HyStem®-C). In patients' BC tissue, hyaluronic acids specifically accumulate at the peri-tumor stroma during BC development^{33–35}. In experiments using cell lines, BC spheroids were formed for 2-3 days in cell culture well plates until they reached 150-250 μm in size before chip loading (**Figure S1**).

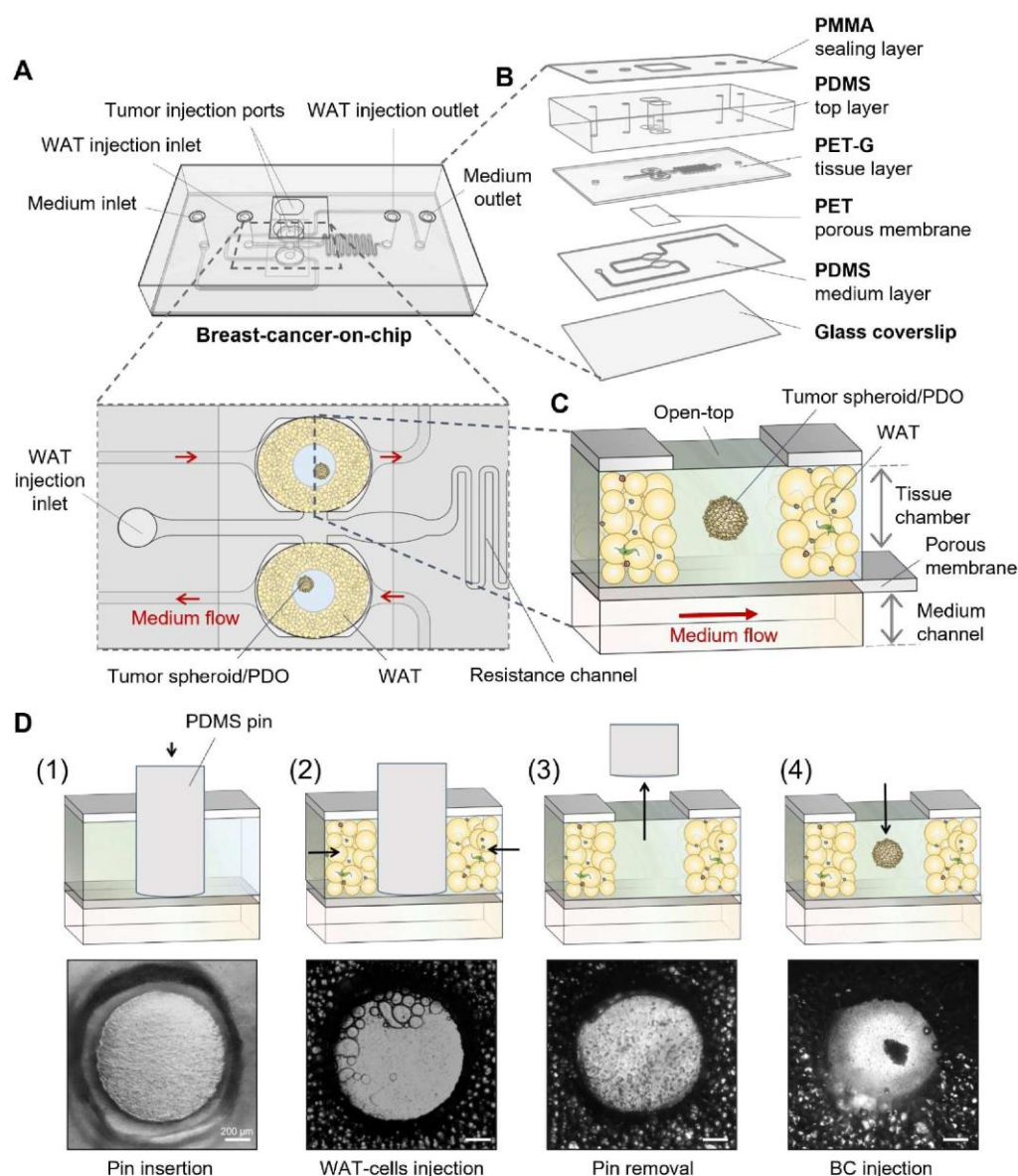


Figure 1: Breast cancer-on-chip concept, design, and tissue generation.

(A) Schematic representation of the breast cancer-on-chip model. Each chip consists of two tumor chambers containing BC spheroids or PDOs and WAT-associated cells. The tumor chambers are located above a medium channel for constant medium perfusion during culture. (B) A magnified view of the individual chip layers and materials. The PMMA sealing layer and PDMS top layer contain the tumor injection

ports, fluid inlets, and outlets. The PET-G tissue layer contains the tumor chambers, WAT injection channel, and resistance channel, which are separated from the medium channel of the PDMS medium layer by a PET membrane. The medium channel is sealed at the bottom with a glass coverslip to allow high resolution microscopy. **(C)** Cross-sectional view of the tumor chamber and underlying medium channel. **(D)** The injection sequence of WAT-associated cells and BC spheroids/PDOs. (1) Insertion of PDMS pin into the tumor injection port. The microfluidic channels were previously filled with PBS-, (2) Injection of collagen-I-embedded WAT-associated cells, (3) Removal of PDMS pin upon collagen-I gelation, (4) Injection of BC spheroids/PDOs through the open-top tumor injection port.

Breast cancer-on-chip enables co-culture of tumor and white adipose tissue

The typical experimental timeline for the BC-WAT co-culture on-chip experiments was designed to allow at least a two-week co-culture to monitor tumor growth, lipid accumulation, and the release of metabolites (**Figure 2A**). Thorough characterization of the WAT has been performed in our previous studies employing the same seeding and culture method^{31,36}.

Briefly, following the adipocytes and SVF cell isolation and injection, cells were cultured under static conditions overnight on-chip. On the next day, BC spheroids/PDOs were injected, followed by another static culture overnight. On day 1 of co-culture, the chips were connected to constant medium perfusion (20 μ L/h) for 13 days, with medium refreshment every 3-4 days. The culture methods and parameters maintained the viability of the vast majority of both PDO tumor cells and adipocytes, as seen from a calcein/PI-based live/dead-staining (**Figure 2B**). Lateral separation of the BC spheroids in the tumor region and adipocytes and SVF in the WAT region enabled the monitoring of tumor growth via brightfield or phase-contrast imaging (**Figure 2C**), while still enabling the perfusion of the whole tissue area. Moreover, different phenotypes of adipose tissue macrophages can be observed on day 14, including CD86 (M1-like), CD206 (M2-like), and Trem2 (lipid-associated macrophages) that are distributed throughout the three-dimensional space along with the adipocytes (**Figure 2D**). The formation of crown-like structures (clustering of macrophages surrounding a dying adipocyte) could also be observed (**Figure S2**), which is a sign of adipose-tissue inflammation and associated with poor breast cancer prognosis in patients³⁷. With this, we have established an in vitro model that allows the culture of BC spheroid/PDO and the surrounding WAT in a perfused microenvironment for up to two weeks with retained tissue structure. To our

knowledge, no in vitro models integrating human mature adipocytes, SVF (including tissue-resident immune components), and 3D tumor with organotypic breast tumor microarchitecture have yet been developed. The immune components in adipose tissue play a crucial role in shaping the tumor microenvironment towards anti- or pro-inflammatory which can modulate treatment response ^{5,38}, which can be studied using this breast cancer-on-chip model. There are indeed many existing breast cancer-on-chips for studying cancer-microenvironment crosstalk, as reviewed elsewhere ^{39,40}. However, to our knowledge, the integration of WAT microenvironment has never been reported so far. Next, we demonstrate the model's utilization to assess the impact of WAT on tumor growth, exemplified using breast cancer cell lines from different subtypes.

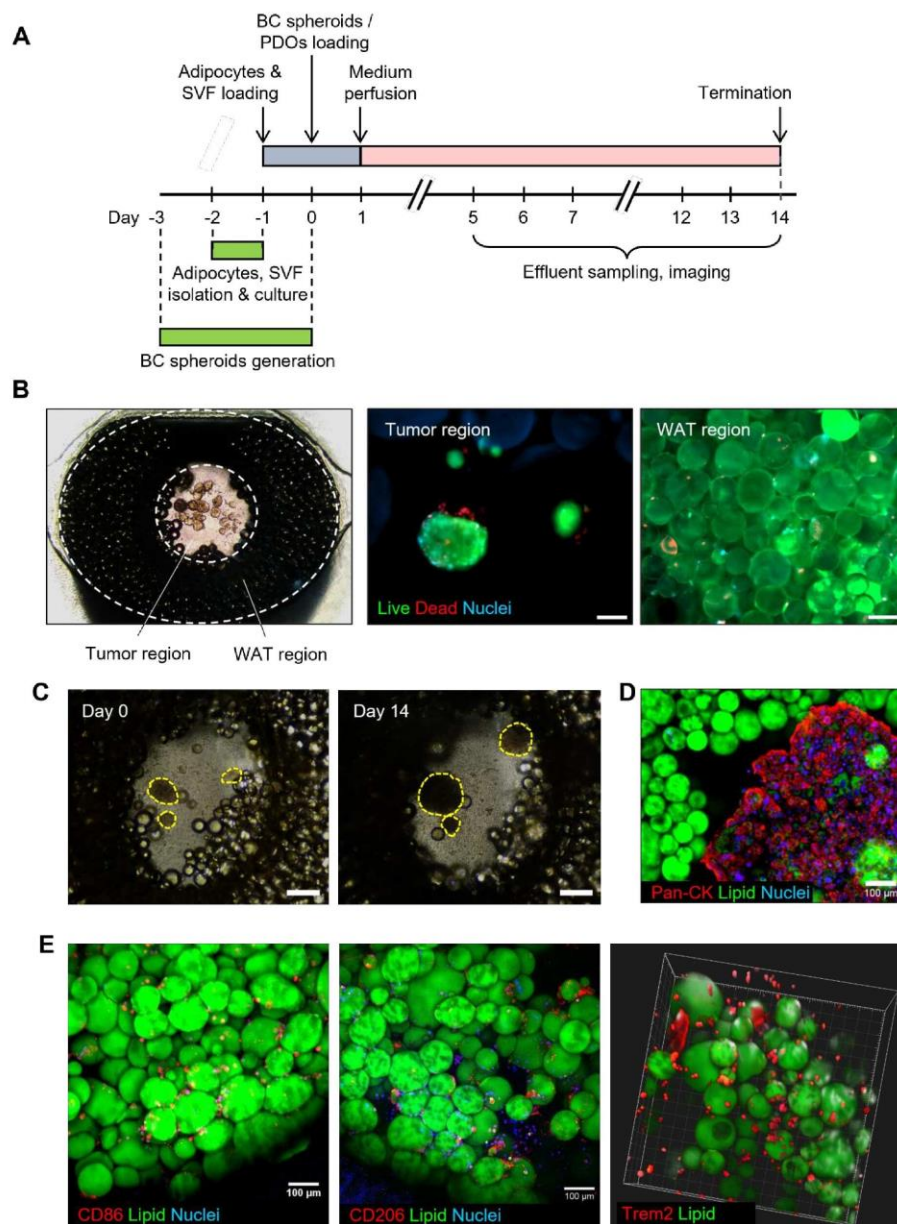


Figure 2: Breast cancer-on-chip culture timeline and characterization.

(A) The typical experimental timeline of breast cancer-on-chip culture. Adipocytes and SVF were isolated from a skin biopsy on day -2, cultured overnight, followed by seeding on day -1. BC spheroids were expanded and generated off-chip prior to chip seeding on day 0. On day 1, medium was perfused through

the chip. Chips were imaged, and effluents were collected at the end of the first and second week of culture. **(B)** Viability evaluation via live/dead staining with calcein AM and propidium iodide of the PDOs in the tumor region and adipocytes in the WAT region on day 14 of culture. Scale bars: 100 μm . **(C)** Representative images of a tumor region showing the growth of PDOs (marked with yellow dashed lines) upon two weeks of culture in the breast cancer-on-chip. Scale bars: 200 μm . **(D)** Pan-cytokeratin expressing BC spheroid in the tumor region interfaces the lipid-stained unilocular adipocytes in the WAT region on day 14 of culture. **(E)** Identification of adipose tissue macrophages by visualizing CD86 (left panel), CD206 (middle panel), and Trem2 (right panel) expression. Macrophages were distributed throughout the tissue along with the lipid-stained unilocular adipocytes.

Adipocytes secrete more glycerol in the presence of BC and low glucose environment

Cancer cells are known for their ability to adapt to any available resources to sustain their growth. Using this model, we first investigated whether BC cells' presence induces adipocyte lipolysis due to limited glucose availability as a way to obtain an alternative nutrient source. During lipolysis, triacylglyceride in adipocytes is broken down into three free fatty acid molecules and one glycerol molecule ⁴¹. Hence, glycerol was measured in the effluent to quantify lipolytic activity of adipocytes that were either co-cultured with BC spheroids or cultured alone. Although glycolysis is considered as a hallmark of cancer, cancer cells can undergo metabolic reprogramming from glycolysis to fatty acid uptake and β -oxidation in glucose-deprived conditions, which they rely on for survival ⁴². This phenomenon has been described in multiple cancer types other than BC ^{43–45}. To simulate TME with varying glucose availabilities, chips were perfused with serum-free medium containing either high (17.5 mM) or low (5 mM) glucose concentrations. A concentration of 5 mM represents the average fasting blood glucose level, thus more closely resembles physiological conditions ^{46,47}. Two BC cell lines were used to represent different BC subtypes, including BT474 and HCC70 for HER2+ and triple-negative subtypes, respectively.

In high glucose concentration, glycerol levels in the first week of culture were lower than the basal condition when BT474 and HCC70 spheroids were present, and gradually decreased over time. In contrast, we interestingly observed increased glycerol release at almost all measurement timepoints when BC spheroids were present and only when cells were cultured in low glucose environment (**Figure 3**). This indeed indicates that BC spheroids may induce adipocyte lipolysis to

potentially support their growth when not much glucose is available in the TME. In a previous study, higher glucose concentration (25 mM) has indeed been shown to stimulate lipogenesis and impair mammary adipocyte lipolytic function, whereas the opposite effect was observed when glucose concentration is low (5 mM)¹⁰. Moreover, fatty acid release by adipocytes may be inhibited due to their increased glucose uptake⁴⁸. In cancers such as melanoma, cancer cells can actively induce lipolysis in adipocytes through the secretion of hormone-sensitive lipase^{49,50}. In BC, the direct mechanism may involve the combination of hormone-sensitive lipase, monoacylglycerol lipase, adipose triglyceride lipase, and other factors, although it remains largely unknown and may highly differ between BC subtypes and inflammatory states^{11,51}. Future experiments using this model could explore the exact mechanism by which BC cells induced adipocytes' lipolysis. In addition, it is important to note that the amount of glycerol in the effluents typically decreased over time. Whether this was caused by an increased glycerol consumption by the BC spheroids, inhibition of lipolysis, or other factors remains to be investigated. Nonetheless, the glycerol concentration on the second week of culture falls within a similar range as detected in our previous WAT-on-chip model, which incorporates a comparable adipocyte chamber volume³¹.

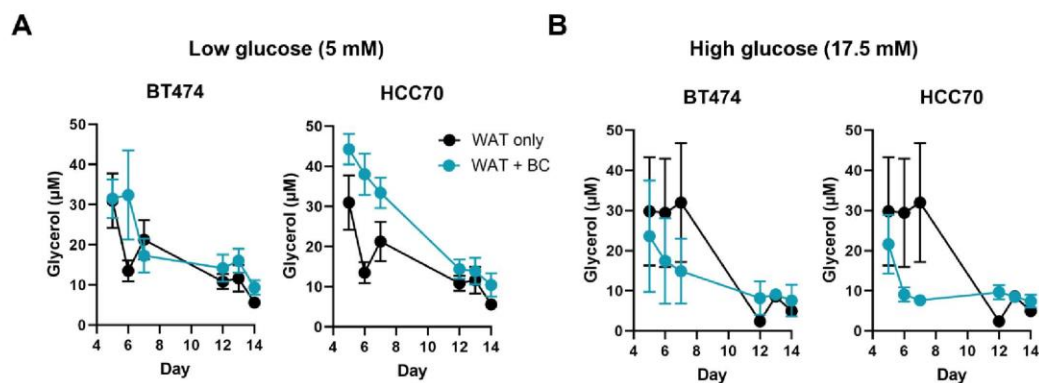


Figure 3: Glycerol release in low and high glucose conditions

Measured glycerol concentration in the chip effluents to assess the lipolytic activity of adipocytes. WAT-associated cells were either cultured alone or co-cultured with BC spheroids (BT474 or HCC70) under (A)

low glucose (5 mM) or (B) high glucose (17.5 mM) concentrations; n = 3-4 chips. Data are presented as the mean $\pm$ SEM.

White adipose tissue promotes lipid accumulation and growth of BC spheroids on-chip

A growing body of evidence has shown that adipose-derived fatty acids promote the progression of tumor types that typically develop near white adipose tissue, including prostate ^{6,7}, ovarian ^{8,9}, melanoma ⁵², and breast cancers ¹⁰⁻¹⁴. To test whether the breast cancer-on-chip model can demonstrate the tumor growth-promoting effect of WAT, we measured BC spheroid growth on-chip during the two-week of culture with or without WAT, as well as lipid accumulation in each BC spheroid using BODIPY 493/503 neutral lipid stain on day 14 of culture. In conditions without WAT, blank hydrogel was injected on day 0 into the tissue chambers without adipocytes and SVF.

Upon 14 days of culture, we were indeed able to detect lipids in the BC spheroids (**Figure S3**). Regardless of the glucose concentration, the results indicated that WAT induced lipid accumulation in both BT474 and HCC70 spheroids, with an overall greater accumulation observed at the high glucose concentration. However, the impact of WAT was more pronounced at low glucose levels, resulting in a 4.2-fold increase of BODIPY 493/503 mean fluorescence intensity compared to 2.4-fold for BT474 spheroids, and a 2.7-fold increase compared to 1.4-fold for HCC70 spheroids (**Figures 4A and 4C**). This indicates that BC cells may increasingly rely on exogenous fatty acids and switch their metabolism to fatty acid oxidation when glucose availability is low, seemingly aligned with the previous data where we detected an increased glycerol release by adipocytes in this condition. In terms of growth, we consistently observed the positive influence of WAT's presence on both BC types, an effect that was further amplified by higher glucose concentration (**Figures 4B and 4C**). This implies a synergistic effect of WAT-derived soluble factors and glucose on cancer cell proliferation.

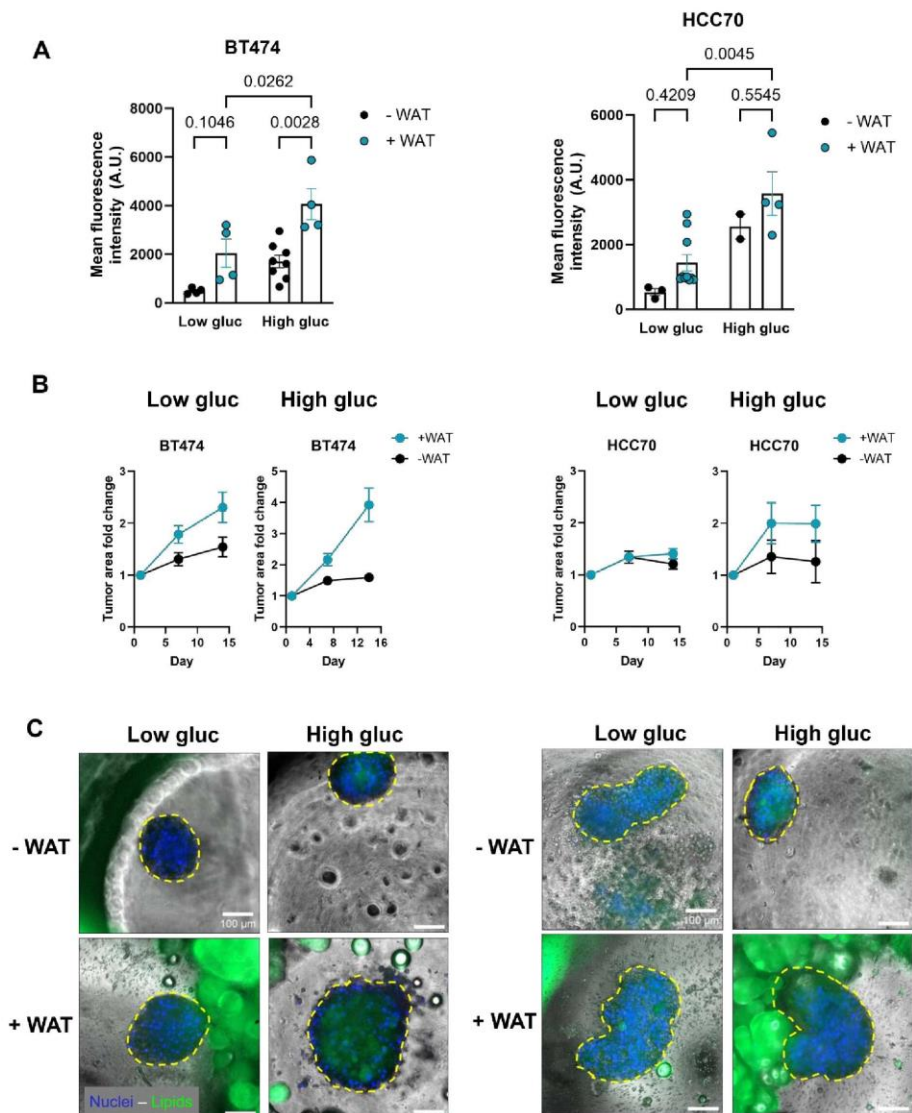


Figure 4: BC lipid accumulation and growth upon culture in WAT microenvironment

(A) Quantification of the mean intensity values of BODIPY 493/503 within each BC spheroid of BT474 (left graph) or HCC70 (right graph) on day 14, cultured alone or in a WAT microenvironment, and either in low glucose (5 mM) or high glucose (17.5 mM) conditions. $n = 2-10$ BC spheroids from 2–5 chips. Data are presented as the mean $\pm$ SEM. p values are indicated in the graphs; Tukey's multiple comparisons test. **(B)** Growth kinetics of BC spheroids cultured alone or in a WAT microenvironment, and either in low glucose (5 mM) or high glucose (17.5 mM) conditions. $n = 5-11$ BC spheroids from 3–5 chips. Data are presented as the mean $\pm$ SEM. **(C)** Representative images upon BODIPY 493/503 staining of BT474 (left panel) and

HCC70 (right panel) BC spheroids on day 14 after cultured alone or in a WAT microenvironment, and either in low glucose (5 mM) or high glucose (17.5 mM) conditions. The yellow dashed lines indicate BC spheroid region. Scale bars: 100 μ m.

As tumor growth seems to benefit from WAT's presence, targeting fatty acid transport pathways could potentially be combined with conventional treatments to improve treatment efficacy. Indeed, therapies targeting lipid metabolism have a substantial clinical interest, some of them are already undergoing clinical trials⁵³. Such therapeutic interventions could potentially be tested in this breast cancer-on-chip to evaluate treatment efficacy while monitoring tumor-WAT dynamics in "real-time". In our preliminary assay using MCF-7 and BT474 BC cell lines, we observed increased BC spheroid growth after the culture in adipocytes-conditioned medium for 4 days, but not when only supplemented with oleic acid (100 nM) as a fatty acid surrogate, regardless of the glucose concentration tested (**Figure S4**). It is therefore important to note that WAT can support breast tumor growth and progression through various mechanisms beyond just free fatty acid trafficking, including adipokine signaling¹⁵, hormone signaling^{16,17}, exosomes^{18,19}, and inducing inflammation^{5,21}, among others, which could potentially also be tested in this model.

WAT protects BC spheroids from chemotherapeutic daunorubicin

Another mechanism by which adipocytes may promote cancer progression occurs during treatment, for example by metabolizing chemotherapeutic drugs⁵⁴. Daunorubicin, for instance, was shown to be catabolized by adipocytes into a therapeutically less effective metabolite²⁰. This may therefore reduce the active drug concentration in the TME, consequently reducing the efficacy of this chemotherapy.

To assess the impact of WAT within this context in our breast cancer-on-chip system, we applied 1 μ M of daunorubicin throughout 4 days of culture either in the presence of WAT or without and quantified tumor cell death in BC spheroids via propidium iodide staining. This concentration was

previously determined by a dose-response assay in a well-plate setup and showed a potent antitumor effect within this timeframe (**Figure S5**). For this experiment, HCC70 was chosen as a representative BC. HCC70 is categorized as a triple-negative subtype lacking hormone and HER2 receptors, which is typically treated with chemotherapy alone, as targeted therapies are not yet well established ^{55,56}.

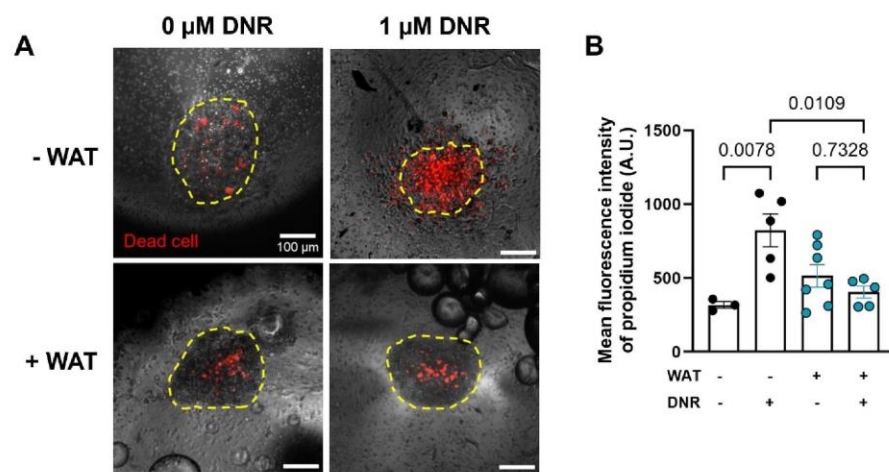


Figure 5: WAT impact on daunorubicin-induced BC cell death

(A) Representative images of HCC70 spheroids on day 4 upon dead cell staining using propidium iodide (red). HCC70 spheroids were either untreated or treated with 1 μ M daunorubicin (DNR), and either cultured alone or in WAT microenvironment. The yellow dashed lines indicate BC spheroid region. Scale bars: 100 μ m. **(B)** Quantification of the mean intensity values of propidium iodide within each BC spheroid on day 4, cultured alone or in a WAT microenvironment, and either untreated or treated with 1 μ M daunorubicin (DNR). $n = 3$ -7 BC spheroids from 3-4 chips. Data are presented as the mean $\pm$ SEM. p values are indicated in the graphs; Tukey's multiple comparisons test.

Upon 1 μ M daunorubicin treatment of HCC70 spheroids for 4 days in chips, a protective effect of WAT was indeed observed. Here, WAT incorporation resulted in no significant effect on BC cell death upon daunorubicin treatment, whereas BC cell death was increased by nearly 3-fold when WAT was not present (**Figure 5A and 5B**). The results align with a previous study showing the chemoprotective effect of adipocytes against daunorubicin ²⁰, although the exact mechanism

requires further investigation. The impact of adipocyte-rich microenvironment in reducing the active drug concentration has been shown for multiple chemotherapeutic drugs beyond daunorubicin, namely doxorubicin and also mitoxantrone ^{20,57}. As this protective outcome can be demonstrated using this breast cancer-on-chip model, it may also be applicable in testing combination therapies for efficacy evaluation in a more complex breast TME. On the other hand, the ability of adipocytes to absorb chemotherapeutic drugs can be utilized as a local drug delivery method. In this case, drugs can be released through cancer-induced lipolysis by for example activating the lipid metabolic pathway mediated by FABP4 ⁵⁸. Such local drug delivery methods that exploit cancer-WAT interaction may therefore be also tested in the breast cancer-on-chip system.

CONCLUSION

In conclusion, the tailored breast cancer-on-chip model presents an advancement of human-based models for studying BC within its adipose-rich tumor immune microenvironment. By integrating BC spheroids or patient-derived organoids with immunocompetent WAT, this model successfully mimics the complex cellular interactions that occur *in vivo*. The microfluidic design, composed of hybrid materials and layered structures, supports a dynamic and perfused environment for long-term co-culture, enabling detailed observations of BC growth, lipid accumulation, and drug response.

This model addresses the limitations of previous *in vitro* systems and animal models by closely replicating the human breast TME, which includes mature adipocytes and the stromal vascular fraction (SVF) with immune components. The separation of BC spheroids and WAT within the chip allows for precise monitoring and imaging, facilitating the study of metabolic crosstalk and potentially immune interactions. Moreover, this model can in principle integrate other tumor types beyond BC, where tumor-WAT interaction plays a central role in cancer progression and therapy.

With this model, we demonstrated that BC cells' presence can induce lipolysis in adipocytes, particularly under low glucose conditions, suggesting a metabolic adaptation that supports tumor growth. The study also demonstrates that WAT promotes lipid accumulation in BC spheroids, highlighting the role of adipose-derived fatty acids in cancer progression. Additionally, we demonstrated its use for testing a chemotherapeutic drug, where it shows that WAT provides a protective effect against daunorubicin, underscoring the potential for adipocytes to influence treatment outcomes by metabolizing drugs and reducing their efficacy.

Overall, the breast cancer-on-chip model offers a robust platform for investigating the intricate relationship between BC and the WAT. It holds promise for exploring new therapeutic strategies that target metabolic pathways and tumor-WAT interactions, ultimately contributing to more effective treatments for BC.

ACKNOWLEDGEMENTS

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AUTHORS CONTRIBUTIONS

T.I.M. and L.L. performed the chip design and fabrication, as well as cell culture and chip experiments with assistance from L.C. A.K. provided the breast cancer cell lines and patient-derived organoids as well as expertise in breast cancer. T.I.M., M.C., J.R. and P.L. provided intellectual contributions and designed the study. T.I.M. and L.L. performed the imaging and image

data analysis. T.I.M. and L.L. analyzed the data from the chip experiments. T.I.M. wrote the manuscript with support from L.L., L.C., P.L., J.R., M.C., and A.K.

METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

MCF-7, HCC70, and BT474 cells were kindly provided by Dr. André Koch (Department of Women's Health Tübingen, Eberhard Karls University, Tübingen, Germany). The cells were cultured in DMEM (1X) (41965-039; Gibco) supplemented with 10% (v/v) FCS (SH30066.03, HyClone™ FetalClone™ II Serum; Cytiva), and 100 U/mL penicillin-streptomycin (15140122; Thermo Fisher Scientific). Human breast epithelial cell line MCF-10A was purchased from Cytion (305026; Cytion) and cultured in DMEM/F12 (1:1, no phenol red) (11039-021; Gibco) supplemented with 5% (v/v) FCS, 20 ng/mL human recombinant EGF (GFH26-100; CellGS), 0.5 mg/mL Hydrocortisone-500 (C-31061; PromoCell), 10 µg Human Insulin Solution (I9278-5ML; Sigma-Aldrich), and 100 U/mL penicillin-streptomycin.

Patient-derived organoids (PDOs) were established from specimens obtained from four advanced breast cancer female patients treated at the Department of Women's Health in Tübingen. All patients gave informed consent and the study was approved by the Ethical Committee of the Eberhard Karls University Tübingen (No. 150/2018BO2 and 662/2022BO2).

Adipocytes and stromal vascular fractions were isolated from resected skin tissue from plastic surgeries received from Dr. Ulrich E. Ziegler (Klinik Charlottenhaus, Stuttgart, Germany) as described previously ³¹. The study was approved by the local medical ethics committee: Patients gave an informed consent according to the permission of the "Landesärztekammer Baden-Württemberg" (IRB#: F-2020-166; for normal skin from elective surgeries). Sex- and gender-based analysis were not considered in the study design due to the logistical complexity of the multiple cell

types/sources cultured simultaneously, hence the experiments were conducted based on donors availability.

All cells cultures were maintained in a humidified incubator at 37 °C, 5% CO₂ and 95% rH. Cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and verified to be mycoplasma-free before experiments.

METHOD DETAILS

Breast cancer-on-chip concept, design and fabrication

The Breast cancer-on-chip consists of two stacked microfluidic layers separated by an isoporous, semipermeable polyethylene terephthalate (PET) membrane (3 µm poresize: rP = 3 µm; pP = 8×10^5 pores per cm²; TRAKETCH PET 3.0 p S210 × 300, SABEU GmbH & Co. KG, Northeim, Germany), which was functionalized by a plasma-enhanced, chemical vapor deposition (PECVD) process as previously described ³⁶. and layer of micro-patterned polyethylene terephthalate glycol (PET-G). A top layer made of poly(methyl methacrylate) (PMMA) is used to support proper sealing of the chip to ensure a sterile environment. A thin glass coverslip (0.13 – 0.16 mm thickness) is the bottom layer of the chip, which supports high resolution imaging. The lower microfluidic layer serves as media compartment for constant media perfusion (0.2 mm in height), and is separated by a porous PET membrane. Its 3 µm pore size allows passive diffusion of diluted species ³¹ during culture period. The tissue chambers (1 mm in diameter, 0.2 mm in height, two chambers per chip) accommodate human WAT and any tumour aggregates below 0.3 mm in size. The tissue channel is designed to have an outlet channel that possesses a fluidic resistance at least twice higher than the fluidic resistance of the membrane and the medium channel combined. This ensures the placement of WAT-associated cells into the chambers during the injection instead of flowing out towards the outlet. The upper PDMS layer functions as a pipetting access to the tissue chambers through two open-top chambers.

The medium layers consisting 200 μm high channels were micro-structured via replica molding of polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, USA) on a patterned master wafer fabricated via photolithographic processes⁵⁹. The SU-8 structures on the medium layer wafer are 200 μm . A 3D-printed mold was used to mold the upper access layer. For the replica molding, PDMS was homogeneously mixed in a 10:1 (elastomer base:curing agent) mass ratio and then degassed in a desiccator to remove air bubbles. Afterwards, two different replica molding approaches were conducted as previously described²⁹. Standard molding approach was used by pouring the PDMS prepolymer solution into the 3D-printed master mold to obtain 3 mm thick PDMS pieces with hole to access the tissue layer. PDMS was cured at 60 °C for 4 h. After curing, the PDMS were cut to the size of the chip and ports were pierced using a biopsy punch (Disposable Biopsy Punch, 0.75 mm diameter; 504 529; World Precision Instruments, Friedberg, Germany) to access the chips (for both cell injection and medium perfusion). The exclusion molding approach was used to fabricate the bottom medium layer: here, PDMS prepolymer solution was poured onto the silicon wafer master mold, which was then clamped against a 5 mm-thick PMMA disk to produce a 0.2 mm thin layer with through hole channel structures. PDMS was cured at 60 °C for 2 h. Once the PDMS parts were cured, they were cleaned using isopropanol followed by deionized water and blow-dried with nitrogen pistol. The tissue layers consisting 200 μm high tissue chambers and a 100 μm high resistance channel were micro-structured via hot embossing. Chip-sized PDMS hot embossing stamps with the tissue layer design were placed onto a glass slide. Then, a 0.5 mm-thick sheet of PET-G was placed on top of the stamps together with another glass slide. The construct was clamped and hot embossed at 115 °C for 30 min. After embossing, the access holes were cut using a CO₂ laser (Universal Laser Systems). Once the PET-G parts were fabricated, they were cleaned using isopropanol followed by deionized water and blow-dried with nitrogen pistol.

The microfluidic chips were assembled in four consecutive bonding steps: (i) medium layer to glass coverslip, (ii) membrane and tissue layer to the medium layer, (iii) access layer to the tissue layer, and (iv) sealing layer onto the access layer. In all steps, bonding was achieved by oxygen plasma activation (75 W, $0.2 \text{ cm}^3 \text{ m}^{-1} \text{ O}_2$; Diener Zepto, Diener electronic GmbH + Co. KG, Ebhausen, Germany) for 24 s. Functionalization of PET-G parts and PMMA layers was achieved by APTES treatment prior to bonding. PET-G parts were O_2 -plasma activated (75 W, $0.2 \text{ cm}^3 \text{ m}^{-1} \text{ O}_2$) for one min before treatment with 4% APTES solution at 50 °C. PMMA layers were O_2 -plasma activated (75 W, $0.2 \text{ cm}^3 \text{ m}^{-1} \text{ O}_2$) for 1 min before treatment with 1% APTES solution at RT. Bonded parts were baked at 60 °C for at least 30 min after each bonding step, and overnight after the entire chip was assembled. All chips were O_2 -plasma treated (75 W, $0.2 \text{ cm}^3 \text{ m}^{-1} \text{ O}_2$) for 5 min to sterilize and hydrophilize the PDMS surface before cell injection.

PDMS pins for blocking the middle part of the tumor chamber was produced by molding PDMS pre-polymer solution (see above) in a 3D-printed Clear Resin mold fabricated via stereolithography (Formlabs), and cured as previously described. The cured PDMS pins were O_2 -plasma treated (75 W, $0.2 \text{ cm}^3 \text{ m}^{-1} \text{ O}_2$) for 1 min prior to their injection into the primed chips.

BC spheroids generation

Spheroids were formed using a BIOFLOAT™ 96-well plate with anti-adhesive surface (83.3925.400; Sarstedt). Briefly, cell culture flasks were washed with PBS- and BC cells were detached by incubation with TrypLE™ Express Enzyme for 3 min, while MCF-10A cells were incubated for 8 min. After stopping the enzymatic reaction by adding 10% (v/v) FCS, cells were pelleted by centrifugation at 200 x g for 5 min. BT474 cells were resuspended in culture media to have a final concentration of 2000 cells/mL, and MCF-7 and HCC70 cells were resuspended to contain 3000 cells/mL. MCF-10A cells contained 5000 cells/mL. To form spheroids, 100 μL of the

respective cell suspension was pipetted into a well and the plate kept in a humidified incubator at 37 °C, 5% CO₂ and 95% rH for three days. BC spheroids were formed until they reached 150-250 µm in size before chip loading

Patient-derived cancer organoids generation and culture

Pleural effusion (PE) samples from four metastatic breast cancer patients were collected by thoracentesis and processed as followed. PE samples were centrifuged at 500 x g for 10 min. Cell pellets were pooled and when necessary, red blood cells were lysed with 10 mL of RBC lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 100 µM Na₂EDTA in H₂O, pH 7.4) on ice for 5 min. Cells were diluted in DPBS (Dulbecco's Phosphate Buffered Saline, P04-36500; Pan Biotech GmbH) and centrifuged at 500 x g for 10 min. The final cell pellet was resuspended in AdvDMEM/F12+++ (Advanced DMEM/F-12 (12534010), 1% Pen/Strep (15140122), 1x GlutaMAX (35050-038), 10 mM HEPES (15630-056); all from Thermo Fisher). For patient-derived organoid (PDO) culture setup, the desired amount of cell suspension was mixed with Basement Membrane Extract (BME; Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2 Select, Bio-technie, 3533-005-02) at a ratio of 30% cell suspension to 70% BME. 20 µL droplets were plated into wells of a 48-well plate and placed upside-down in an incubator (37°C, 5% CO₂) to solidify for 30 min. Afterwards, 280 µL of culture medium (AdvDMEM/F12+++ supplemented with 10% (v/v) conditioned medium from L-WRN cells (ATCC-CRL-3276)⁶⁰, 5 nM Heregulinβ-1 (100-03; PeproTech, NJ, USA), 5 ng/mL fibroblast growth factor 7 (FGF7) (100-19; PeproTech), 20 ng/mL fibroblast growth factor 10 (FGF10) (100-26; PeproTech), 5 ng/mL epidermal growth factor (EGF) (AF-100-15; PeproTech), 500 nM A83-01 (2939; Tocris, Wiesbaden, Germany), 5 µM Y27632 (72034; STEMCELL Technologies), 500 nM SB-202190 (S7067; Sigma-Aldrich), 2% B27™ Supplement (17504-044; Thermo Fisher Scientific), 1.25 mM N-acetyl-cysteine (A9165; Sigma-Aldrich), 5 mM nicotinamide (N0636; Sigma-Aldrich)) was added to each well. The medium was changed every 3–4 days.

The PDOs were passaged based on the confluency of the culture, ranging from 5-20 days. PDOs were recovered from the wells by resuspending the BME-droplets in ice-cold DPBS containing 5 μ M Y-27632 (DPBS/Y-27632). This organoid suspension was incubated with 1 mL of TrypLE™ Express Enzyme (1X; Thermo Fisher, 12604013) at 37°C in a water bath for 5 min, followed by mechanical dissociation via 5 times pulling and dispensing the solution using a syringe with 27G needle (302200; Becton Dickinson). The suspension was then centrifuged at 500 x g for 10 min and the supernatant removed. For further culture, the desired amount of cell pellet was resuspended in AdvDMEM/F12+++ and mixed with BME at a ratio of 30% cell suspension to 70% BME and cultured as described above.

Prior to chip injection, the PDOs were expanded in a droplet of basement membrane extract hydrogel until they reached 150-250 μ m in diameter, typically after ~14 days. To retrieve the PDOs, gel droplets were first washed with PBS⁻ before introducing 500 μ L of 2 mg/mL cold dispase II solution followed by pipetting up and down to break the gel droplet. The organoid cell suspension was then incubated for 10 min at 37 °C, 5% CO₂ and 95% rH. Afterwards, each well was resuspended with 500 μ L of AdvDMEM/F-12+++ and the retrieved PDOs were centrifuged at 500 x g for 5 min. The organoid pellet was then resuspended in HyStem®-C hydrogel as will be described in the next section.

Adipocytes and stromal vascular cells isolation and culture

Isolation of adipocytes and SVF cells is based on our published protocol ³¹. Primary mature adipocytes were isolated from human subcutaneous WAT biopsy on the same day of surgery. In brief, the tissue was cut into pieces of approximately 1 cm³ while blood vessels and connective tissue were excluded, followed by an enzymatic digestion in collagenase solution (DMEM/F12 + GlutaMAX™ + 0.13 U/mL Collagenase NB 4 Standard Grade + 1% (w/v) fatty acid free BSA) for 60 min at 37 °C, 5% CO₂ and 95% rH on a rocking shaker (50 cycles/min). Finally, the digested

fat tissue was strained using a 380 μm mesh and washed three times with wash medium (DMEM/F12 no phenol red, 1% P/S) in a 1:1 ratio to remove residual digestive solution. For each washing step, adipocytes and wash medium were mixed and left to rest for 5 min to allow phase separation. The lower phase was aspirated before new wash medium was added. Washed adipocytes were transferred into a T75 flask together with 10 mL WAT base medium (DMEM/F12 no phenol red, 10% FCS, 1% P/S) and cultured overnight in a humidified incubator at 37 °C, 5% CO₂ and 95% rH.

To isolate SVF cells, WAT was cut into pieces of approximately 1 cm³ while blood vessels and connective tissue were included. After being shaken manually for 30 sec, the tissue pieces were enzymatically digested in adipose tissue digestion solution for 30 min at 37 °C on a rocking shaker. After digestion, the tissue pieces were shaken once again and further strained through a 380 μm mesh. After being allowed to stand at RT, adipocytes and non-buoyant cells in digestive solution were separated from each other, the pact layer of adipocytes was carefully removed, and the remaining cell suspension was centrifuged at 300 x g for 5 min. To lyse erythrocytes, supernatant was carefully removed, and the cell pellet resuspended in SVF base medium (DMEM/F12 no phenol red, 10 ng/mL GM-CSF, 10% FCS, 1% P/S) before Red Blood Cell lysis buffer (freshly prepared according to manufacturer's protocol, 130-094-183; Miltenyi Biotec) was added to incubate for 5 min at RT. Next, the suspension was passed through a 100 μm cell strainer, washed with wash medium, and centrifuged at 300 x g for 5 min. After centrifugation, supernatant was removed, and the remaining pellet was resuspended in SVF base medium and seeded into a T25 flask for overnight culture in a humidified incubator at 37 °C, 5% CO₂ and 95% rH.

Cell seeding on-chip

After chips sterilization and hydrophilization with O₂-plasma, the channels are flushed using 100 μL pipette with 70% ethanol followed by rinsing three times with PBS-. Then, PDMS blocking pins

were gently inserted into the tumor chambers until the PDMS pin's bottom surface is in contact with the PET membrane, ensuring complete blockage of the chamber's middle part. The chips were kept at RT until cell seeding. Adipocytes and SVF cells were seeded into the chip at one day before spheroid seeding.

Adipocytes were harvested by removing content from the T75 flask and allowing phases to separate in a 15 mL tube. The lower phase was aspirated, and wash medium was added to the remaining adipocytes. This washing step was repeated three times. Adherent SVF cells were collected by removing cell culture medium from the flask and rinsing it with PBS-. Detachment of cells was ensured by adding TrypLE™ and PBS- in a 1:1 ratio to the flask for 5 min at 37 °C, 5% CO₂ and 95% rH, followed by a 3 min long incubation, and finally an 8 min long incubation with TrypLE™. After each incubation step, the enzymatic reaction was stopped by adding 10% (v/v) FCS and collected in a 15 mL tube. The cells were centrifuged at 300 x g for 5 min and resuspended in 1 mL WAT base medium for counting.

For the seeding of ten chips, 500,000 SVF cells were resuspended in 30 µL of a FibrCol® collagen-I hydrogel, followed by adding 60 µL of washed adipocytes at RT with a pre-cut 100 µL pipette tip to reduce shear force during mixing. Collagen-I hydrogel was previously set to at a final concentration of 5 mg/mL on ice. pH value was set to ~7 with 0.1 M NaOH. Once the cells were homogeneously mixed in the hydrogel, 10 µL was immediately injected into each chip via the tissue inlet. In the "spheroid-only" condition, blank hydrogel was injected without cells. Afterwards, the inlet and outlet of the tissue layer were plugged using a metal wire with 0.7 mm diameter (45473; Menzanium) and the medium channel was subsequently flushed once with 100 µL of WAT base medium to remove any deposited hydrogel that might disrupt fluidic flow. The chips were then incubated at 37 °C, 5% CO₂ and 95% rH under static conditions to allow hydrogel crosslinking.

Spheroids were seeded onto the chips using the HyStem®-C hydrogel (HyStem®-C Kit, G21005; Advanced BioMatrix). HyStem®-C components (Glycosil and Gelin-S) were reconstituted according to manufacturer instructions. The crosslinker (Extralink) was reconstituted to have a final concentration of 2%. The hydrogel was formed upon adding Extralink to Glycosil and Gelin-S in a 1:2:2 volume ratio, respectively. Each spheroid was single-picked from the BIOFLOAT™ plate it was formed in and dispensed in a droplet of the prepared hydrogel. Next, a pipette was set to 5 µL and primed with gel. The spheroid was taken up to be located and the tip of the pipette tip, then inserted into the open-top chamber. To ensure that the spheroid was placed in the middle of the chamber, surrounded by WAT, the process was monitored under the microscope. The loading process was repeated for the second spheroid chamber and all remaining chips that required spheroids. Afterwards, 100 µL BC-WAT co-culture (either 5 mM or 17.5 mM glucose depending on the experiment) medium-filled tips were inserted into the media inlet and outlet, and the chip surfaces were dried and sealed with PCR foil. As a control, duplicates of all cell lines were placed in each a well of a 96-well plate and cultured in 100 µL of BC-WAT co-culture medium.

On-chip culture

Starting from day 1, the chips were connected to constant media perfusion via an external 12-channel syringe pumping system (LA-190, Landgraf Laborsysteme HLL GmbH, Langenhagen, Germany). The chips were connected to the syringe pump using blunt 21 GA stainless steel needles (made from the dispensing needles by removing the plastic hub after dissolving the glue overnight in a 70% ethanol solution) connected to Tygon tubings (0.51 mm inner diameter, Tygon ND 100-80 Medical Tubing, Saint-Gobain Performance Plastics Pampus GmbH, Willich, Germany), 21 GA stainless steel plastic hub dispensing needles (e.g., KDS2112P, Weller Tools GmbH, Besigheim, Germany) and Luer Lock style syringes. The syringes were filled with either DMEM/F12 (1:1) w: 1.2g/L NaHCO₃ (P04-41158; Pan Biotech) and 5 mM glucose solution (A24940-01; Gibco) to contain low glucose (5 mM) or DMEM/F12 (1:1) no phenol red (11039-021;

Gibco), to contain high glucose (17.5 mM). Both media were supplemented with 2.5 nM Recombinant Human Heregulin β -1 (100-03; PeproTech), 5 ng/mL Recombinant Human EGF (GFH26-100; Cell GS), 10 mM ROCK1 inhibitor, Y-27632 2HCl (S1049; Selleckchem), 60 pM Human Insulin Solution (I9278-5ML; Sigma-Aldrich), 0.1% (w/v) fatty acid free BSA (A1311.50; USBiologicals), and 50 μ g/mL primocin (Ant-pm-1; Invivogen) – this is referred to as “BC-WAT co-culture medium”. Once the chips were connected to the pumping system, the flow rate was set to 20 μ L/h in push mode and the whole setup was transferred into an incubator at 37 °C, 5% CO₂ and 95% rH. The chips were linearly perfused in this mode at 20 mL/h flow rate until up to day 14. Medium in the syringes was refilled every 3 to 4 days of perfusion. Before chip perfusion, medium is warmed-up to 37 °C and simultaneously degassed (vacuum source -70 kPa) and filter-sterilized using a Steriflip conical filter unit for 20 min. Effluent samples from each chip were collected on days 5, 6, 7, 12, 13, and 14 and centrifuged at 10,000 x g for 10 min to remove debris and stored at -80 °C until further analysis.

Daunorubicin treatment

To assess the impact of WAT on anti-tumor drug efficacy, daunorubicin (Daunorubicin (RP 13057) HCl, S3035; Selleckchem) with a final concentration of 1 μ M was supplemented in the co-culture medium and perfused through the medium channel of the chips for four days, starting the day after spheroids were seeded (marking day 0 of the experiment).

Preliminary media test

To assess the effect of different composed media on tumour growth and lipid accumulation, we cultured spheroids in respective medium for four days. Media included final concentrations of glucose at 5 mM, 15 mM, or 25 mM in either BC-WAT co-culture medium +/- 100 nM BSA-Oleate Monosaturated Fatty Acid Complex (Cay29557-5; Cayman Chemical) or adipocytes conditioned medium (CM). For the collection of adipocytes CM, 10 μ L of an adipocyte-HyStem®-C hydrogel

mixture was pipetted into the center of a 96-well plate well and left to gelify for 30 min at 37 °C, 5% CO₂ and 95% rH. Adipocytes were incubated in BC-WAT co-culture medium for four days, while spheroids were generated in the meantime. After four days, adipocytes CM was collected and spheroids cultured in respective media for four days at 37 °C, 5% CO₂ and 95% rH. Growth was monitored via BF imaging on days 0 and 4 of spheroid culture. BODIPY staining was conducted on day 4, as previously described.

Endpoint live/dead staining

Cell viability was determined after two weeks of co-culture. Briefly, chips were washed once with PBS- by flushing the media channels before fixing the tissue on-chip with 4% PFA for 1 hr on a rocking shaker. After fixation, the chips were washed once with PBS- and then stained for 30 min with 50 µL staining solution containing 1 µg/mL DAPI (D8417-1MG; Sigma-Aldrich), 25 µg/mL Calcein AM (C3099; Thermo Fisher Scientific), and 0.25 µM SYTOXTM Orange (S34861; Thermo Fisher Scientific) diluted in PBS+. After incubation, chips were washed three times with PBS+ before performing fluorescence imaging.

After daunorubicin treatment of the chips, live/dead staining was performed on the last day of the experiment. In brief, the chips were washed once with PBS- by flushing the media channels before fixing the tissue on-chip with 4% PFA for 1 hr. After fixation, the chips were washed once with PBS- and then stained for 30 min at 37 °C, 5% CO₂ and 95% rH with 100 µL staining solution containing 80 µM Hoechst 33342 (62249; Thermo Fisher Scientific) and 25 µg/mL Calcein AM diluted in PBS+. After incubation, chips were washed three times with PBS+ and further incubated with 50 µL of 135 µg/µL propidium iodide (PI) (P4170; Sigma-Aldrich) staining solution for 5 min at 37 °C, 5% CO₂ and 95% rH. Finally, chips were washed three times with PBS+ before performing fluorescence imaging.

DAPI was excited at 340 nm and the emission captured at 488 nm. Hoechst 33342 was excited at 361 nm and the emission captured at 497 nm. Calcein AM was excited at 494 nm and its emission captured at 517 nm. SYTOXTM Orange was excited at 547 nm and the emission captured at 570 nm. Finally, PI was excited at 535 nm and the emission captured at 617 nm.

Immunofluorescence staining

To stain neutral lipids, chips were washed once with PBS- by flushing the media channels before fixing the tissue on-chip with 4% PFA for 1 hr. After fixation, the chips were washed three times with PBS- and then stained for 1 hr in the dark with 50 μ L staining solution containing 1 μ g/mL DAPI and 1 μ g/mL BODIPY 493/503 dye (D3922; Thermo Fisher Scientific) diluted in antibody diluent (S3022; Agilent). After incubation, chips were washed three times with PBS- before performing fluorescence imaging.

When staining lipids on-plate, spheroids were fixed with 4% PFA for 15 min. After fixation, wells were washed with PBS- once, followed by centrifugation at 100 x g for 1 min to ensure that spheroids would be located at the bottom of the well. After repeating the washing once, 50 μ L was discarded from each well and 50 μ L of staining solution, containing 1 μ g/mL DAPI and 1 μ g/mL BODIPY 493/503 dye diluted in antibody diluent were added and left to incubate at RT for 1 hr in the dark. Finally, wells were carefully washed twice before fluorescently imaged.

For the IF staining of specific markers on chip, chips were washed with PBS+ by flushing the media channels and tissue was fixed for 1 hr with 4% PFA. All incubation steps were performed with chips on a rocking shaker at RT. After washing three times with PBS-, cells were permeabilized with 0.2% (w/v) Saponin in PBS+ for 20 min and washed three times for 5 min with wash buffer containing 0.1% (v/v) Tween in PBS-. To block unspecific binding sites, cells were incubated with 0.3% (v/v) Triton-X and 3% (w/v) BSA in PBS- for 30 min. All staining solutions were prepared with Antibody diluent (S3022; Agilent Technologies, Inc, Santa Clara, CA) and the concentrations of

primary and secondary antibodies are shown in the table below. Incubation with primary antibody solutions was performed overnight. After washing three times with wash buffer, the cells were incubated 1.5 hr with secondary antibody solutions containing 1 µg/mL DAPI (D8417-1MG; Sigma-Aldrich) and 1 µg/mL BODIPY 493/503 dye. Before fluorescence images were taken, the chips were washed three more times with wash buffer.

	Staining with unconjugated antibodies	
	Primary antibody	
Staining target	Antibody	Final concentration/ dilution
Cytokeratin	Mouse Recombinant Monoclonal Cytokeratin 1 antibody [C-11] (ab7753; Abcam)	5 µg/mL
CD206	Rabbit Recombinant Monoclonal Mannose Receptor antibody [EPR6828] (ab125028; Abcam)	3.6 µg/mL
CD86	Rabbit Recombinant Monoclonal Anti-CD86 antibody [EPR21962] (ab239075; Abcam)	11.38 µg/mL
Trem2	TREM2 Recombinant Rabbit Monoclonal Antibody (9H4L26) (702886; Thermo Fisher Scientific Inc.)	5 µg/mL
	Secondary antibody	
	Antibody	Final concentration/ dilution
	Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor™ 546 (A10036; Thermo Fisher Scientific Inc.)	1:100
	Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor™ 647 (A-31573; Thermo Fisher Scientific Inc.)	1:100

Glycerol assay

Glycerol-Glo™ assay (J3151; Promega) was used for glycerol detection in chip effluent as per manufacturer's instructions.

ATP assay

CellTiter-Glo® 3D cell viability assay (G9681; Promega) was used for determining cell viability in medium effluent as per manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image acquisition and analysis

For the imaging acquisitions (e.g. live/dead staining), an epifluorescence microscope (Axio Observer 7, Carl Zeiss) was used. The focal plane was set to focus on the BC spheroid/PDO for the quantitative analysis of lipid accumulation and tumor spheroid growth. The microscope chamber temperature was set to 37 °C for all live cell imaging acquisitions of the chips (e.g. live/dead staining). Analyses of lipid accumulation and tumor growth were conducted using ImageJ-Fiji software ⁶¹. BC spheroid/PDO ROI was defined using the brightfield image. The ROI was then used to quantify mean grey value or the BODIPY 493/503 or propidium iodide, depending on the experiment.

Statistics and reproducibility

In all experiments, every chip considered an independent biological replicate. Chips were excluded from analysis if technical issues with the microfluidic setup occurred (e.g. leakages, flow blockage due to bubbles). For size determination and imaging-based quantification of BODIPY, spheroids smaller than 50 µm in diameter were considered too small and therefore excluded from analysis. For measuring glycerol concentrations, only chips with the correct effluent volume were considered. Statistical analysis was performed using GraphPad Prism 9.5.2 software, and detailed statistics are indicated in the figure. All graphs are represented as average values ± SEM unless indicated otherwise.

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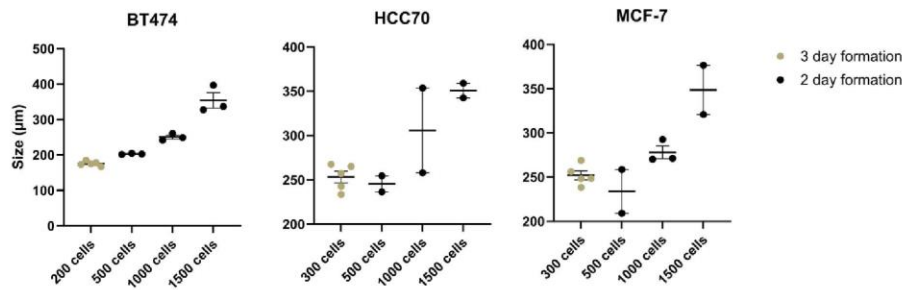
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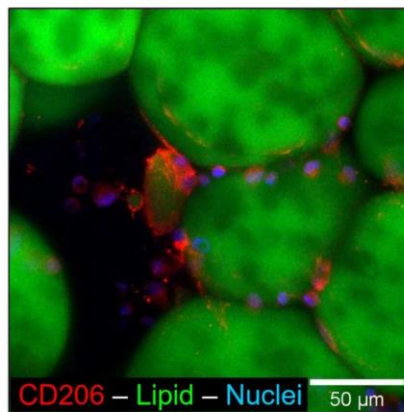
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SUPPLEMENTARY FIGURES



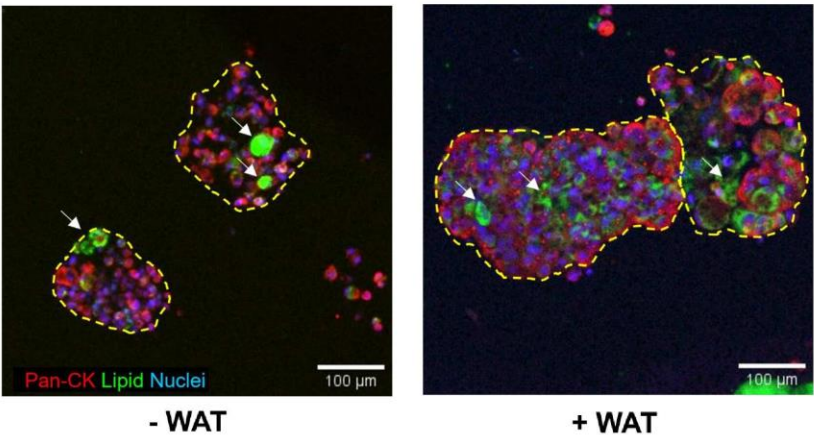
Supplementary Figure 1: BC spheroid generation

Measured BC spheroid sizes (μm) generated from 200-1500 cells after 48 and 72 h. $n = 2-5$ BC spheroids. Data are presented as the mean $\pm$ SEM.



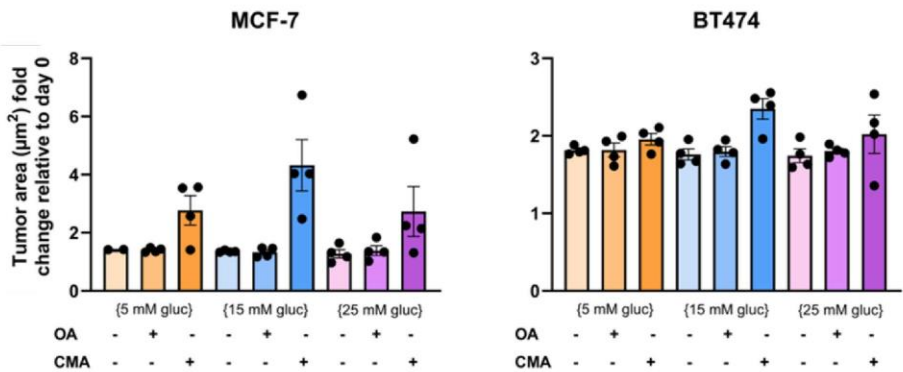
Supplementary Figure 2: Crown-like structures on chip

Formation of crown-like structures of adipose tissue macrophages visualized by CD206 expression enwrapping an adipocyte on chip.



Supplementary Figure 3: Lipid accumulation in BC spheroids

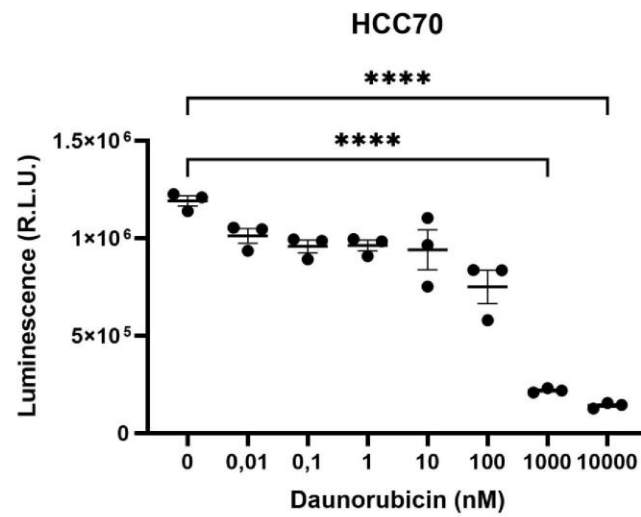
Representative images of BC spheroids upon on-chip culture either alone (-WAT) or in WAT microenvironment (+WAT). BC spheroids were stained with pan-cytokeratin. Lipids were stained with BODIPY 493/503. The dashed yellow line marks the region of BC spheroids.



Supplementary Figure 4: Effects of oleic acid supplementation and adipocytes-conditioned medium: Preliminary test

Quantification of BC spheroid growth (MCF-7 left graph, BT474 right graph) upon 4 days culture in varying glucose concentrations (5 mM, 15 mM, 25 mM), with and without oleic acid (100 nM) supplementation (OA) or conditioned medium from adipocytes culture (CMA) in well plate culture

setup. Each data point indicates one spheroid. n = 2-4 spheroids. Data are presented as the mean $\pm$ SEM.



Supplementary Figure 5: Daunorubicin dose-response

Quantification of ATP level (R.L.U: relative light unit) measured using CellTiter-Glo® 3D to assess cell viability upon 4 days treatment with daunorubicin at indicated concentrations. Each data point indicates one spheroid. n = 3 spheroids. Data are presented as the mean $\pm$ SEM. ****p<0.0001; Tukey's multiple comparisons test.