

Department für Diagnostische Labormedizin
der Universität Tübingen
Institut für Pathologie und Neuropathologie

**Modeling immunotherapies in live 3D human cancer tissue
bioreactors and implications for functional
precision medicine**

**Thesis submitted as requirement to fulfill the degree
„Doctor of Philosophy” in *Experimental Medicine* (PhD)**

**at the
Faculty of Medicine
Eberhard Karls Universität
Tübingen**

presented by

Zhang, Yizheng

2026

Dean:	Professorin Dr. S. Y. Brucker
1st reviewer:	Professor Dr. C. Schürch
2nd reviewer:	Professor Dr. P. M. Loskill

Date of oral examination: 17.09.2026

COPYRIGHT NOTICE

This work, "Modeling immunotherapies in live 3D human cancer tissue bioreactors and implications for functional precision medicine", is a doctoral dissertation submitted to the Eberhard Karls University of Tübingen.

© Yizheng Zhang, 2026.

This work is licensed under the Creative Commons Attribution 4.0 International Public License (CC BY 4.0).

To view a copy of the full legal text of this license, please visit the following URL:

<https://creativecommons.org/licenses/by/4.0/legalcode>

Table of Contents

I List of Tables and Figures.....	4
II List of Abbreviations.....	7
1 Introduction.....	9
1.1 Cancer biology.....	9
1.1.1 Cancer development and cancer characteristics.....	9
1.1.2 Cancer heterogeneity and tumor microenvironment.....	12
1.2 Cancer treatments.....	14
1.2.1 Conventional cancer treatments.....	14
1.2.2 Targeted therapy.....	17
1.2.3 Immunotherapy.....	18
1.3 Precision medicine.....	23
1.4 Functional precision medicine.....	23
1.5 U-CUP perfusion bioreactor system.....	28
1.6 Lymph node structure and lymphoma biology.....	31
1.7 Research gap and objectives.....	34
1.8 Major findings and significance.....	35
2 Materials and Methods.....	38
2.1 Materials.....	38
2.1.1 Antibodies, reagents and equipments.....	38
2.1.2 Buffers and culture medium.....	41
2.1.3 Sample acquisition.....	45
2.2 Methods.....	47
2.2.1 Sample preparation and tissue culturing.....	47
2.2.2 Single-cell suspension cell count and flow cytometry analysis.....	49
2.2.3 Checkpoint inhibitor treatment.....	49
2.2.4 Generation of CAR T Cells.....	50
2.2.5 Generation of PI3K Enhanced CAR T Cells.....	50
2.2.6 CFSE labeling of CAR T Cells.....	51
2.2.7 Agarose-coated plate co-culture assay.....	51
2.2.8 Measurement of cytokines.....	52
2.2.9 Immunofluorescence (IF) staining and imaging.....	52
2.2.10 Hematoxylin and eosin (H&E) staining.....	53
2.2.11 Antibody conjugation.....	53
2.2.12 CODEX staining and imaging.....	54
2.2.13 Statistical analysis.....	56
3 Results.....	59

3.1 An optimized 3D perfusion bioreactor model to study human lymphoid tissues.	59
3.1.1 Study design and experiment workflow.....	59
3.1.2 Optimized agarose embedding enhances cell viability and retention in tonsil tissue explants.....	59
3.1.3 Agarose embedding improves the preservation of immune cell subsets in tonsil tissue cultures.....	64
3.1.4 Bioreactors outperform static culture for tissue explant culture.....	69
3.2 Bioreactor culture for lymph node tissue.....	71
3.2.1 Bioreactors preserve lymph node explant viability.....	71
3.2.2 Lymph node explants show superior viability to tonsil tissue in bioreactor culture.....	75
3.2.3 Preservation of cellular diversity and tissue architecture within 3D perfusion bioreactors.....	78
3.3 Modeling immune checkpoint inhibitor therapy.....	87
3.3.1 Anti-PD-1 therapy suppresses malignant tissue viability.....	88
3.3.2 Anti-PD-1 treatment promotes T cell activation.....	90
3.4 Modeling CAR T cell therapy.....	94
3.4.1 CAR T cells effectively eliminate target cells in bioreactor culture.....	94
3.4.2 Infiltration of CAR T cells within lymphoid tissue microenvironments.....	95
3.5 Evaluating novel CAR T products using the bioreactor system.....	99
3.5.1 Assess CAR T cell killing efficiency.....	99
3.5.2 Assess CAR T cell retention in tissue.....	100
3.5.3 Cytokine expression of CAR T cells.....	104
3.6 Targeting TRP-2 in melanoma using a bioreactor system.....	106
3.6.1 Tyrosinase-related protein 2 as a therapeutic target in melanoma.....	106
3.6.2 Anti-TRP-2 treatment reduces viability of melanoma cells in lymph node metastases.....	107
3.6.3 Anti-TRP2 treatment induces melanoma cell death via ADCC.....	108
3.7 Integrating 3D whole-organ light-sheet microscopy and 2D highly multiplexed microscopy.....	114
3.7.1 The concept of correlative LSM-CODEX imaging.....	114
3.7.2 Optimization of tissue clearing, antibody validation, and staining protocol for correlative spatial imaging.....	115
4 Discussion and Limitations.....	125
4.1 Discussion.....	125
4.2 Limitations.....	134
5 Summary.....	138
6 Zusammenfassung.....	140
7 References.....	142

8 Declaration of Contribution.....	156
9 Publications.....	158
10 Acknowledgment.....	159

I List of Tables and Figures

Table 1. Key resources.....	38
Table 2. Composition of buffers, working solutions and culture medium.....	41
Table 3. Patient samples and diagnosis.....	46
Table 4. Summary of Statistics.....	56
Table 5. Raw data of Fig. 6A.....	61
Table 6. Raw data of Fig. 6B.....	61
Table 7. Raw data of Fig. 6C.....	61
Table 8. Raw data of Fig. 6E.....	62
Table 9. Raw data of Fig. 7B.....	63
Table 10. Raw data of Fig. 7C.....	64
Table 11. Raw data of Fig. 8B.....	65
Table 12. Raw data of Fig. 8D.....	65
Table 13. Raw data of Fig. 9B.....	66
Table 14. Raw data of Fig. 9D.....	67
Table 15. Raw data of Fig. 10A.....	68
Table 16. Raw data of Fig. 10B.....	68
Table 17. Raw data of Fig. 10C.....	69
Table 18. Raw data of Fig. 10D.....	69
Table 19. Raw data of Fig. 11A.....	70
Table 20. Raw data of Fig. 11C.....	71
Table 21. Raw data of Fig. 12A.....	72
Table 22. Raw data of Fig. 12C.....	73
Table 23. Raw data of Fig. 13.....	74
Table 24. Raw data of Fig. 14.....	75
Table 25. Raw data of Fig. 15A.....	76
Table 26. Raw data of Fig. 15B.....	77
Table 27. Raw data of Fig. 15C.....	77
Table 28. Raw data of Fig. 15D.....	77
Table 29. Raw data of Fig. 15E.....	78
Table 30. Raw data of Fig. 19.....	82
Table 31. Raw data of Fig. 20.....	83
Table 32. Raw data of Fig. 21.....	84
Table 33. Raw data of Fig. 24.....	89
Table 34. Raw data of Fig. 25.....	90
Table 35. Raw data of Fig. 27.....	93
Table 36. Raw data of Fig. 28.....	95

Table 37. Raw data of Fig. 30.....	97
Table 38. Raw data of Fig. 31A.....	98
Table 39. Raw data of Fig. 31B.....	98
Table 40. Raw data of Fig. 32B.....	100
Table 41. Raw data of Fig. 33B.....	101
Table 42. Raw data of Fig. 36A.....	103
Table 43. Raw data of Fig. 36B.....	104
Table 44. Raw data of Fig. 37A.....	105
Table 45. Raw data of Fig. 37B.....	105
Table 46. Raw data of Fig. 38B.....	107
Table 47. Raw data of Fig. 40B.....	110
Table 48. Raw data of Fig. 42A.....	112
Table 49. Raw data of Fig. 42B.....	112
Table 50. List of CODEX-Conjugated Antibodies and Staining Evaluation.....	117
Figure 1. Schematic plot of CAR structure and the evolution of CAR generations.....	21
Figure 2. FPM in Cancer Management.....	26
Figure 3. Comparison of Cancer Models for FPM.....	30
Figure 4. Lymph node architecture and classification of major lymphoma entities.....	32
Figure 5. Experimental workflow for human lymphoid tissue perfusion culture and downstream immunotherapy analysis.....	48
Figure 6. Optimized agarose embedding enhances cellular retention and viability in ex vivo tonsil tissue cultures.....	61
Figure 7. Effect of embedding matrices on tissue viability.....	63
Figure 8. Agarose embedding preserves T cell subsets in ex vivo cultures.....	65
Figure 9. Agarose embedding preserves B cell subsets in ex vivo cultures.....	66
Figure 10. Effect of cytokine supplementation on T and B cell viability in bioreactor cultures.....	68
Figure 11. Evaluation of static plate culture versus 3D perfusion bioreactor platforms for tonsil tissue cultivation.....	70
Figure 12. Improving lymph node tissue culture via optimized bioreactor conditions.....	72
Figure 13. Preservation of T cell subsets in LN tissue.....	74
Figure 14. Preservation of B cell subsets in LN tissue.....	75
Figure 15. Differential immune cell viability in lymphoid tissues.....	76
Figure 16. Representative flow cytometry gating tree for immune cell subpopulations (Panel 1).....	79
Figure 17. Representative flow cytometry gating tree for immune cell subpopulations (Panel 2).....	80
Figure 18. Representative flow cytometry gating tree for immune cell subpopulations	

(Panel 3).....	80
Figure 19. 3D perfusion bioreactors sustain Th, Tc and NK cells.....	81
Figure 20. 3D perfusion bioreactors sustain pDCs, macrophages cells and DCs.....	83
Figure 21. 3D perfusion bioreactors sustain WBCs, non-immune and vascular cells....	84
Figure 22. Perfusion bioreactor culture preserves tissue architecture.....	87
Figure 23. Histological evaluation of tissue morphology pre- and post-cultivation.....	87
Figure 24. Modeling ICIs in lymphoma, metastatic lymph nodes, and benign lymph nodes.....	89
Figure 25. Anti-PD-1 treatment induces cytokine release in bioreactor cultures.....	90
Figure 26. Multiplexed imaging.....	92
Figure 27. Enhanced IFN- γ expression in CD4 ⁺ and CD8 ⁺ T cells.....	93
Figure 28. Anti-CD19 CAR T cells eliminate B cells in lymphoid tissue bioreactors.....	95
Figure 29. IF analysis of CAR T cell infiltration in tissues.....	96
Figure 30. Assessment of CAR T cell tissue infiltration by flow cytometry.....	97
Figure 31. Biocompatibility of agarose coating on CAR T cell viability and effector function.....	98
Figure 32. CAR T cell killing.....	100
Figure 33. CAR T cell infiltration.....	100
Figure 34. CAR T cell tissue persistence.....	101
Figure 35. Immune cells in CAR T cell treated tissue.....	102
Figure 36. Spatial analysis of PI3K-CAR T cell targeting.....	103
Figure 37. PI3K-CAR T cells induce an immune-activating cytokine profile.....	105
Figure 38. Anti-TRP2 Treatment of Melanoma.....	107
Figure 39. Representative CD15 IHC staining and scoring categories.....	109
Figure 40. Quantification of CD15 IHC staining results.....	110
Figure 41. Flow cytometry gating strategy for neutrophils.....	111
Figure 42. Neutrophils enhance anti-TRP2 antibody-mediated ADCC.....	112
Figure 43. Optimization of staining steps for cleared tissues.....	116
Figure 44. Comparison of signal intensity across different treated tissue.....	119
Figure 45. Representative multiplex fluorescence images of fresh and ECI-treated tissues.....	120
Figure 46. Representative multiplex fluorescence images of PFA fixed tissue and tissues processed with other clearing protocols.....	121
Figure 47. Additional representative multiplex fluorescence images of fresh and ECI-treated tissues.....	122
Figure 48. Additional representative multiplex fluorescence images of PFA fixed tissue and tissues processed with other clearing protocols.....	123

II List of Abbreviations

2D	two-dimensional
3D	three-dimensional
5-FU	5-fluorouracil
AI	artificial intelligence
AML	acute myeloid leukemia
BME	basement membrane extract
CAR	chimeric antigen receptor
CoC	Cancer-on-a-Chip
COI	cells of interest
CSC	cancer stem cell
EBRT	external beam radiation therapy
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial–mesenchymal transition
FOLFIRI	folinic acid, fluorouracil, and irinotecan
FPM	functional precision medicine
GNLY	granulysin
GZMA	granzyme A
H&E	hematoxylin and eosin
ICIs	immune checkpoint inhibitors
IF	immunofluorescence
IFN- γ	interferon- γ
IL	interleukin
LDH	lactate dehydrogenase
LLPS	liquid–liquid phase separation
LN	lymph node
LSM	light-sheet microscopy
NK	natural killer
NSCLC	non-small cell lung cancer

NT	non-transduced
PDX	patient-derived xenografts
PDOs	patient-derived organoids
PRF	perforin
TKIs	tyrosine kinase inhibitors
TME	tumor microenvironment
TNF- α	tumor necrosis factor- α
Tregs	regulatory T cells
VEGF	vascular endothelial growth factor
WBCs	white blood cells

1 Introduction

1.1 Cancer biology

Cancer is a heterogeneous group of diseases defined by uncontrolled proliferation and spread of abnormal cells with the ability to invade adjacent tissues and metastasize to distant organs via the circulatory and lymphatic systems (1). This dysregulation of cellular growth and death distinguishes cancer from benign conditions and is a significant medical problem. Cancer can develop in virtually any organ system and encompasses over 100 distinct types, each with unique genetic, molecular, and histopathological features (2).

Worldwide, cancer is one of the most serious public health concerns of the 21st century. Cancer accounted for about 20 million new cases and 9.7 million deaths last year, and was one of the three leading causes of death around the globe (3). The leading cancers are lung, breast, colorectal, prostate, and stomach cancers. Driven by a population that is aging, urbanization, exposure to environmental agents and lifestyle related risk factors, such as tobacco use, unhealthy diet, obesity, and physical inactivity, the burden of cancer continues to increase (3).

1.1.1 Cancer development and cancer characteristics

With the development of cancer is a multistep process resulting from increasing accumulation of genetic mutations, epigenetic derangement and malignant microenvironment alterations. These changes collectively disturb normal cellular homeostasis and allow malignant cells to gain characteristic functions such as persistent proliferative signaling and resistance to apoptosis, angiogenesis, tissue invasion, and immune evasion (4).

Tumorigenesis usually involves genetic changes initiated by a single cell. These mutations can happen spontaneously during DNA replication, be inherited through the germline, or be initiated by environmental carcinogens, such as tobacco smoke, ionizing radiation, or oncogenic viruses (5). Somatic mutations continue to accumulate

throughout life, most of which are leading to cell death or effectively detected and eliminated by immune surveillance systems. In contrast, mutations in critical regulatory genes, including oncogenes, tumor suppressor genes or DNA repair genes, can provide a selective growth advantage. The persistence and clonal expansion of cells harboring these driver changes allow for abnormal cellular processes that cause and maintain neoplastic proliferation (6).

A hallmark that characterizes cancer is the tumor cells to continue the state of constant proliferative signaling under sustained cellular persistence, so that they produce continuous signaling for cancer. Somatic mutations of these regulatory genes lead to constitutive activation of growth-promoting pathways, which often due to somatic alterations in important genes for growth-promoting activities, involving the most critical genes. For example, activating mutations in epidermal growth factor receptor (EGFR) are commonly found in non-small cell lung cancer (NSCLC), while mutations of KIT and PDGFRA are involved in gastrointestinal stromal tumors (7,8). These mutations often impact receptor tyrosine kinases and cause ongoing activation of downstream signaling pathways and initiate the induction of PI3K-AKT and RAS-RAF-MEK-ERK (mitogen-activated protein-kinase, MAPK) pathways by a variety of signal transducers (9,10). Such cascades drive up the nuclear translocation of transcription factors that control the transcription of genes associated with cell cycle advancement, proliferation, and differentiation. Mutations in downstream effectors such as SRC, AKT, and ERK also help drive amplification of proliferative signals, leading to unchecked growth of cancerous cells (11).

Under normal physiological conditions many tumor suppressor mechanisms work by preventing uncontrolled cellular proliferation, driving programmed cell death and maintaining tissue homeostasis. Tumor suppressors are responsible for cell cycle checkpoints, DNA damage sensors or apoptosis regulators, and inactivation of these genes is a frequent feature of cancer development. TP53, which encodes the p53 protein, is one of the best-known tumor suppressors. p53 is known to actively monitor genomic integrity by stopping the cell cycle in response to DNA damage and inducing apoptosis if the damage is irreparable. Retinoblastoma protein, a major tumor

suppressor, regulates cell cycle progression by inhibiting E2F transcription factors and preventing inappropriate entry into the S phase. Other tumor suppressors, including BRCA1, which is involved in DNA repair. NF2, which regulates contact inhibition, and members of the BCL2 family, which modulate apoptosis, also influence cellular growth regulation. Loss or mutation of these suppressors facilitates cancer cell bypass of regulatory checkpoints, leading to resistance to cell death and promotion of malignant progression (12).

Cancer cells achieve replicative immortality by evading telomere shortening to evade the usual division limitations. As the repetitive DNA sequences at chromosome ends, telomeres decrease in length with every cell cycle, in a process that can then promote cellular senescence or apoptosis on the part of normal cells. Cancer cells are able to sustain an infinitely long telomere length in comparison. This is done mainly by the reactivation of telomerase, a ribonucleoprotein enzyme that elongates telomeres. In another system, many tumors employ the alternative lengthening of telomeres pathway, a telomerase-independent route that maintains telomere structure. Adaptations allow cancer cells to avoid replicative exhaustion and remain in growth (13).

To support rapid growth, tumors need sufficient blood supply to obtain oxygen and nutrients. While growing, cancer cells produce pro-angiogenic factors, most notably vascular endothelial growth factor (VEGF), which stimulate development of new blood vessels (14). Hypoxia in the tumor microenvironment (TME) induces the upregulation of hypoxia-inducible factor, which further enhances VEGF expression (15). This angiogenic reaction not only drives tumor growth but it also allows invasion of adjacent tissues and a path to metastasis, contributing significantly to cancer development.

Local invasion and distant metastasis are hallmark features that distinguish malignant tumors from benign lesions. Under normal conditions, cells that detach from the primary tumor typically undergo anoikis, a form of apoptosis triggered by the loss of adhesion to the extracellular matrix (ECM) and disruption of cell–cell interactions. However, metastatic cancer cells develop mechanisms to resist anoikis, enabling survival in circulation (16). Another critical process in metastasis is epithelial–mesenchymal transition (EMT), during which epithelial cancer cells acquire mesenchymal

characteristics, enhancing their motility and invasiveness (17). Furthermore, cancer cells can actively remodel distant microenvironments, known as pre-metastatic niches, creating conditions that facilitate colonization and outgrowth at secondary sites. These adaptations underscore the complex, multi-step nature of metastatic progression.

Alongside the classical characteristics of cancer, new findings in cancer research have identified novel features involved in tumor formation and progression. These comprise deregulated cellular energetics, evasion of immune destruction, genomic instability and mutation, tumor-promoting inflammation, phenotypic plasticity, non-mutational epigenetic reprogramming, interactions with polymorphic microbiomes, and the influence of senescent cells within the TME (2). Together, these characteristics expand our understanding of the complexity of cancer biology and highlight the dynamic and adaptive nature of malignant transformation.

1.1.2 Cancer heterogeneity and tumor microenvironment

Tumor heterogeneity: Cancer cells display a wide variety of morphological and behaviour differences due to differences in gene expression, metabolism, motility, rate of proliferation and potential for metastasis. This heterogeneity can be interpatient heterogeneity that occurs between patients, intratumoral heterogeneity within different tumors in the same patient, and intratumoral heterogeneity that occurs even within a single tumor mass (18).

Two theories have been proposed for how tumor heterogeneity originates. The first is the clonal evolution model, which holds that cancer develops from a single mutated cell. With the tumor growing, more mutations will come to the subclones. Then those with a selective advantage, such as survival or proliferation, become dominant in these (19). The second is the cancer stem cell (CSC) model, which implies that some cancer cells possess self-renewal and ability to differentiate into various types of tumor cells resembling stem cells. In this model heterogeneity comes from hierarchical organization guided by CSCs (20).

Tumors, besides their innate heterogeneity of cancer cells, have microenvironment complexity, which is a concept that includes both cellular and non-cellular components, also at the microenvironmental level. Tumors comprise malignant as well as a mixture of

numerous stromal cells, including immune cells, endothelial cells, and fibroblasts in addition to ECM components. Collectively, these components make up the TME. Some tumors are very vascularized, while others are sparse or have poorly developed vasculature. Different tumors also have different types, abundance, and spatial distribution of infiltrating immune cells. Tumors, being three-dimensional (3D) structures, exhibit spatial heterogeneity in the stromal and malignant compartmentalization. Tumor cells may cluster or be diffusely distributed, while immune cells may stick to the borders or invade deep into the tumor core. Differences in cellular nature and spatial organization substantially contribute to tumorigenesis, therapeutic response, and clinical outcomes (21).

Cancer progression, therapeutic response, and clinical prognosis are significantly affected by heterogeneity of tumors. This heterogeneity is due to genetic and epigenetic alterations, as well as interactions with TME. Heterogeneous tumors have greater adaptability, allowing cancer cells to survive under selective pressures such as hypoxia or treatment (22). Subclones possessing advantageous qualities, such as invasiveness or drug resistance, that evolve via clonal evolution, may emerge to control and dominate others, driving disease progression and metastasis. Spatial and functional heterogeneity in TME, such as nutrient gradients and immune cell infiltration, also drives tumor behavior. Phenotypic diversity is also generated due to processes such as EMT that supports invasion and therapy resistance (23).

Heterogeneous subpopulations present a major obstacle to effective treatment. Certain cancer cells may have intrinsic resistance to a therapy, while others might develop resistance over the course of treatment, resulting in relapse and disease progression. Such variability frequently leads to therapeutic failure despite an initial positive response. Not to mention that the heterogeneity of the tumor makes it difficult to develop standardized treatment strategies, since one subpopulation may be eliminated by a targeted therapy but others may be spared. This includes immunotherapy, which is further confounded by heterogeneous tumors with the ability to evade immune response through mechanisms such as antigen loss or suppression of immune cell activity, restricting the effectiveness of therapy.

Tumor heterogeneity may lead to unpredictable disease progression and variable patient outcomes at the clinical level. This divergence between subclones' treatment response leads to difficulties in predicting prognosis and long-term survival. Recurrent, resistant subpopulations that remain long after the treatment of a tumor can drive the re-emergence of the tumor and metastasis further compounding its trajectory and worsening clinical outcomes. Consequently, patients with highly heterogeneous tumors frequently have shorter progression-free intervals and variable survival rates. A comprehensive understanding of tumor heterogeneity and its underlying mechanisms is crucial to better personalized cancer therapies and higher prognostic precision.

1.2 Cancer treatments

Treatment of cancer requires a multidisciplinary approach focused on the elimination of tumor cells with a goal to limit the progression of cancer and enhance the survival and quality of life of patients. Conventional treatments like surgery, chemotherapy, and radiation therapy remain central to cancer management. There have been huge breakthroughs over the past decades that have been used toward targeted therapies and immunotherapies, which are thus becoming more individualized and specific. Other treatments, such as hormonal therapy and stem cell transplantation, are also employed for the treatment of certain cancers. Which treatment is used varies according to the characteristics of cancer, from cancer type and stage to its molecular profile and also may vary based on a patient's general health status and goals for therapy (4).

1.2.1 Conventional cancer treatments

Surgical intervention is one of the most established and effective conventional cancer treatments, providing tumor resection, removal of relevant surrounding cancerous tissue, and surgical removal of lymph nodes (LNs). Surgery may serve several functions including diagnosis, curative management, symptom alleviation, and post-treatment reconstruction, depending on the disease type, location, and stage. Biopsy is one diagnostic procedure that helps confirm cancer presence and guide further management. Therapeutic surgery seeks to excise the tumor completely or reduce tumor mass when total removal is not possible. Reconstructive operations are intended

to restore appearance or function after treatment; breast reconstruction or esophageal reconstruction is a common example. By contrast, palliative surgery is primarily about reducing symptoms in advanced cancers, such as alleviating obstructions or controlling pain (24).

Advantages of cancer surgery can be considerable, but particularly so when tumors are localized and operable. Removal may reduce the tumor burden immediately, relieve symptoms such as pain or compression, and provide tissue samples for histopathological evaluation that can influence prognosis and guide therapy for the next phase of disease. Complete resection can be the cure in certain instances. But surgery is not appropriate in all cancers, especially some that have metastasized widely or in those patients for whom the risks outweigh the benefits. Usually, surgery and chemotherapy, radiation, and targeted therapy are also part of a multidisciplinary treatment regimen to get better results. Even though it provides tremendous advantages, surgery is associated with risks like infection, bleeding, and postoperative complications that should be managed carefully with patient selection, perioperative treatment, and rehabilitation (25). Tumor surgery is still an essential element of oncologic treatment, which emphasizes early detection and intervention to increase the efficiency of treatment.

Radiation therapy or radiotherapy is also a popular therapeutic method in cancer that uses very high-energy radiation, including X-rays, gamma rays and proton beams, to kill cancer cells, reduce tumors or relieve cancer symptoms. The therapeutic effect is accomplished by causing irreversible DNA damage to the cancerous cells, so that after the cancer replicates, the cells no longer will proliferate, ultimately causing the death of cells. Radiation therapy is mainly applied for local tumor control and it can be given alone or in combination with surgery, chemotherapy or immunotherapy and varies based on the type and stage of the tumor (26). There are two main types of radiation therapy: external beam radiation therapy (EBRT) and internal radiation therapy (brachytherapy). EBRT delivers targeted radiation from a machine positioned outside the body, often guided by advanced imaging technologies to maximize precision and minimize damage to surrounding healthy tissue. In contrast, brachytherapy involves

placing a radioactive source directly inside or adjacent to the tumor, allowing high-dose radiation delivery to the tumor while limiting exposure to normal tissues (27).

Radiation treatment provides advantages for several cancer types in precise local tumor treatment and preservation of organs, particularly in cancers where surgical removal could impair function or appearance. It is also often used in combination with other therapies to boost therapeutic effectiveness (28). But radiation therapy has its drawbacks. These encompass unintentional damage inflicted on the nearby healthy tissues, radiation-related complications, dosing constraints, and constraints due to size, location, and sensitivity of the tumor (29). Treatment needs to be careful and technological development to mitigate and maximize treatment can alleviate such issues.

Chemotherapy is a cornerstone conventional treatment for systemic cancer which uses cytotoxic agents to kill or inhibit the proliferation of rapidly dividing cells. Unlike local treatments such as surgery or radiotherapy, chemotherapy targets cancer cells that have spread throughout the body, making it particularly effective for metastatic and hematologic malignancies. Chemotherapeutic agents work through various mechanisms, including DNA damage, inhibition of mitosis, and disruption of metabolic processes essential for cell survival (30). Many classes of chemotherapeutic drugs, from alkylating agents, such as cyclophosphamide, to antimetabolites like methotrexate and 5-fluorouracil (5-FU), and anthracyclines such as doxorubicin and taxanes like paclitaxel. These drugs are usually integrated into a combination regimen to improve therapeutic effectiveness and attenuate resistance. Chemotherapy can be given as neoadjuvant (before surgery) to shrink the size of the tumor, adjuvant (after surgery) to eliminate residual disease, or as palliative treatment to suppress advanced cancer symptoms (31). Although effective, chemotherapy carries a high level of side effect risk due to its lack of selectivity, where it harms normal rapidly dividing cells such as those of the bone marrow, gastrointestinal tract, and hair follicles. Some commonly encountered adverse effects of chemotherapy include myelosuppression, nausea, alopecia, and fatigue (32).

Chemotherapy remains a key component of cancer management and in a multimodal approach, most clinical management would incorporate chemotherapy. It is effective differently for various cancers, stages, and individual tumor biology, with ongoing testing aimed at improving existing chemotherapeutic protocols and creating chemotherapeutically safer, more selective agents.

Following standard treatment options, there are also several specialized approaches used in specific cancer types or clinical scenarios. For example, some cancers, like prostate cancer and breast cancer, are hormone-dependent, meaning their growth is regulated by sex hormones. Hormone therapy, including surgical castration or hormone-suppressing medications, can effectively inhibit tumor progression (33,34). Hematologic malignancies such as acute leukemia are typically treated with hematopoietic stem cell transplantation, offering a potential cure by restoring healthy bone marrow function (35). A similar therapeutic option for hepatocellular carcinoma is liver transplantation in selected patients, particularly those with underlying cirrhosis or tumors unsuitable for surgical resection (36). As cancer treatment strategies continue to evolve, it is essential to select the most appropriate and individualized treatment plan to optimize clinical outcomes.

1.2.2 Targeted therapy

Targeted therapy is the latest development in cancer therapy, targeting molecular pathways that support tumor growth and survival. Targeted therapies as compared to traditional treatments that may affect cancer or healthy cells, focus on blocking or interfering with particular signaling pathways, receptors or genetic mutations which are distinct or overexpressed in cancer cells (37). This specificity enables the treatment to be much more efficient and minimally toxic.

In targeted therapy, small molecule inhibitors are a more common form of drug that enter cells and inhibit enzymes or proteins that control cancer signaling pathways. Examples are tyrosine kinase inhibitors (TKIs), imatinib (CML directed against BCR-ABL), and erlotinib (NSCLC therapy in those with EGFR mutations) (38,39). A further class of molecules include monoclonal antibodies targeting these molecules, and

the latter might be used to target the extracellular domain, such as HER2 in breast cancer with trastuzumab and VEGF in colorectal cancer with bevacizumab, thereby blocking tumor angiogenesis or receptor activation (40,41).

The efficacy of targeted therapy relies largely on molecular diagnostics for determining individuals who possess the relevant genes or biomarkers (42). Resistance, both intrinsic and acquired, is still an issue and often arises from secondary mutations, redundancy in the pathway, or compensatory signaling. Combination approaches are under active search to overcome resistance, which includes the pairing of targeted agents to chemotherapy or immunotherapy agents (43). More importantly, targeted therapy has revolutionized treatment for some malignancies with an enhanced course and quality of life. With ongoing genomic progress and precision methods of medicine, however, this is likely to increase the utility of targeted drugs in a wide variety of cancer subtypes.

1.2.3 Immunotherapy

Immunotherapies can harness a patients' own immune systems to recognize and destroy the cancer cells, making it one of the groundbreaking approaches for oncology. Unlike other drugs that take aim at the tumor, immunotherapy enhances or restores the body's ability to recognize and eliminate malignant cells, making it a powerful way to maintain long-term cancer control in selected patients (2).

Advanced malignancies remain a high burden of disease globally and they are among the leading causes of cancer deaths worldwide (44). Despite successful resection of localized solid tumors, patients presenting with advanced metastatic disease or hematological malignancies usually need systemic chemotherapy, radiotherapy, targeted treatment, and combination-based approaches (45). Although there has been significant progress in these traditional strategies, therapeutic resistance presents a major clinical issue. Many patients experience non-responsiveness to treatment or slowly develop resistance through treatment, ultimately resulting in disease progression and limited long-term survival benefits (46).

Within this context, cancer immunotherapy becomes a critical paradigm shift in oncology. Instead of attacking only the tumor cell, immunotherapeutic strategies are designed to restore or enhance antitumor immune responses and to help the immune system target and kill the tumor cells better. This strategy has shown durable clinical responses among multiple tumor entities, especially in patients with treatment-refractory or relapsed disease, thus making it a component of modern cancer therapy (47).

The clinical efficacy of cancer immunotherapy is closely related to the ability to elicit strong and long-lasting antitumor immune responses in the tumor microenvironment (48). Numerous immunotherapeutics, largely Immune checkpoint inhibitors (ICIs) and CAR T cell therapies, have dramatically changed the landscape of treatment for a number of malignancies over the last 10 years. These approaches have provided durable tumor control in certain patient populations with long-term remission and substantial general survival improvements that had previously been challenging to achieve with just conventional therapies (49,50).

ICIs have revolutionized cancer therapy by enhancing the immune system to eliminate tumor cells. Under physiological conditions, immune checkpoints are essential regulators of immune homeostasis that maintain self-tolerance and prevent excessive immune activation. However, many tumors exploit these inhibitory pathways to evade immune surveillance, resulting in impaired T cell activation and immune escape.

The two well characterized checkpoint pathways are the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1). CTLA-4 mostly controls T cell activation in the early priming of lymphoid organ cells by competing with the CD28 co-stimulatory receptor to bind CD80 and CD86 on antigen presenting cells. By contrast, PD-1 is expressed mostly in peripheral tissues and inhibits T cell proliferation, cytokine production and cytotoxic activities in the tumor environment due to interactions with its ligands (PD-L1 or PD-L2) (51). A variety of cancer cells upregulate PD-L1 expression as an adaptive strategy to block immune responses against cancer cells.

Monoclonal antibodies targeting these immune checkpoints restore T cell function by disrupting inhibitory signaling. Clinically approved agents include the anti-CTLA-4 antibody ipilimumab and the anti-PD-1 antibodies nivolumab and pembrolizumab, as well as anti-PD-L1 antibodies such as atezolizumab, durvalumab, and avelumab (52). The effectiveness of these therapies has been particularly impressive in melanoma, non-small cell lung cancer, renal cell carcinoma, and many other cancer types.

Despite these remarkable advances, only a minority of patients attain long-term clinical benefit. Resistance in primary and acquired forms is still a challenge, characterised by low infiltration of T cells in the tumor microenvironment, immunosuppressive cells, tumor heterogeneity, and insufficient antigen presentation.

Another major immunotherapeutic strategy is adoptive cell therapy (ACT), which involves the collection, *ex vivo* expansion or genetic modification, and reinfusion of autologous or allogeneic immune cells to enhance anti-tumor immunity. Among these approaches, chimeric antigen receptor (CAR) T cell therapy has emerged as one of the most successful forms of ACT. In CAR T cell therapy, T cells are isolated from the patient, genetically engineered to express synthetic chimeric antigen receptors that recognize specific tumor-associated antigens independently of major histocompatibility complex (MHC) presentation, expanded *ex vivo*, and subsequently reinfused into the patient (53).

CARs typically consist of an extracellular antigen-binding domain derived from a monoclonal antibody (single-chain variable fragment, scFv), a hinge region, a transmembrane domain, and intracellular signaling domains that activate T-cell proliferation, cytokine secretion, and cytotoxic function upon antigen recognition. This engineered receptor enables CAR T cells to directly identify and eliminate tumor cells expressing the target antigen in an MHC-independent manner (53). Since their development, CARs have evolved from first-generation constructs containing only the CD3 ζ signaling domain to second-generation CARs containing a single co-stimulatory domain, third-generation CARs containing two co-stimulatory domains, and fourth-generation CARs that additionally express inducible immune-modulating molecules, such as IL-12 (**Figure 1**) (54). Notably, second-generation CARs, which

typically incorporate either a CD28 or 4-1BB co-stimulatory domain, remain the foundation of all currently approved CAR-T therapies due to their proven clinical efficacy and were therefore used throughout this study.

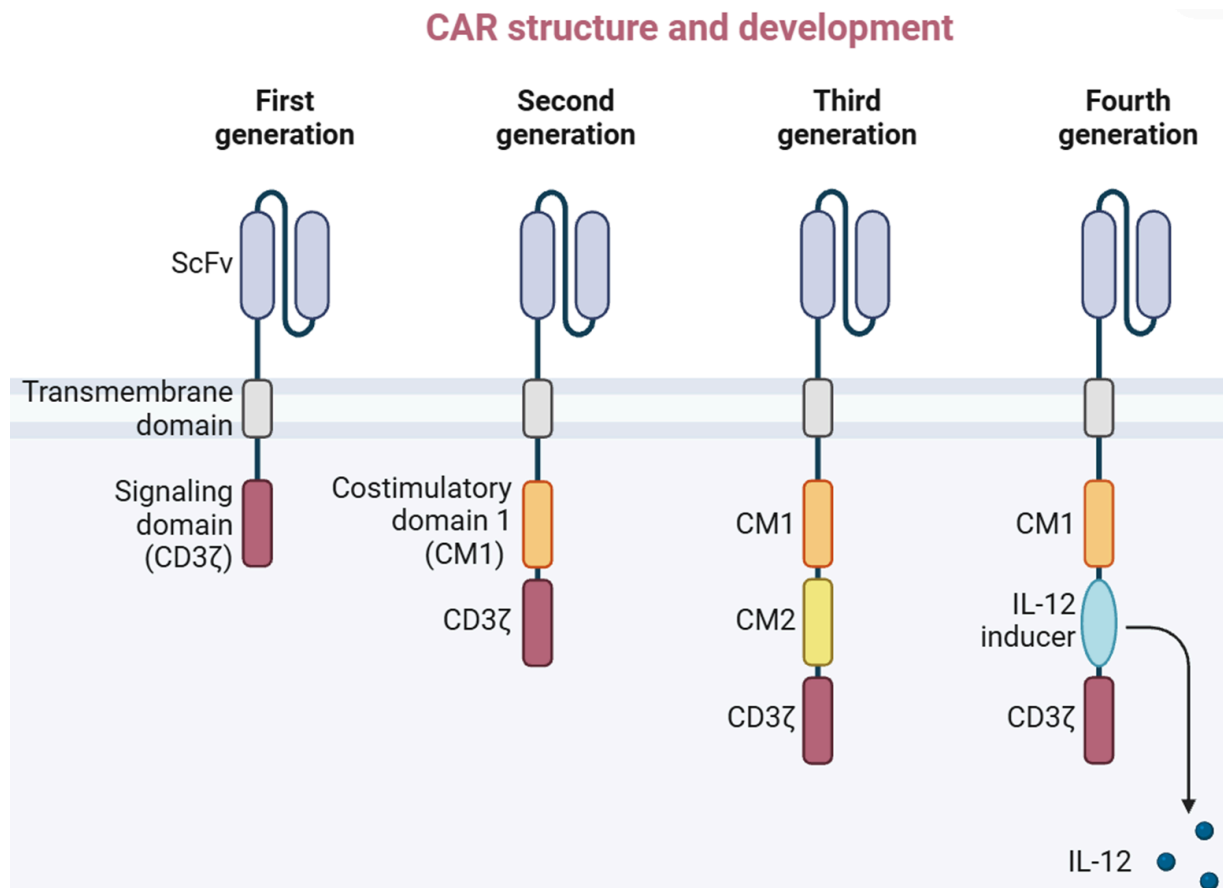


Figure 1. Schematic plot of CAR structure and the evolution of CAR generations.

(Figure created with BioRender.com)

CAR T cell therapy has demonstrated remarkable clinical efficacy in hematological malignancies, particularly B cell acute lymphoblastic leukemia, diffuse large B cell lymphoma, mantle cell lymphoma, multiple myeloma, and other B cell malignancies targeting CD19 or B cell maturation antigen. However, its application to solid tumors remains challenging due to antigen heterogeneity, limited T cell infiltration, an immunosuppressive tumor microenvironment, and treatment-associated toxicities such as cytokine release syndrome and immune effector cell-associated neurotoxicity

syndrome (55). Ongoing research aims to improve CAR design, identify novel target antigens, and enhance CAR T cell persistence and efficacy in solid tumors.

Solid tumors are not only composed of malignant cells but also include a complex and dynamic network of immune cells, stromal cells, vascular structures, as well as soluble mediators and ECM components, which are collectively known as the TME (56). This multicellular realm is critical in tumor initiation, progression, metastasis, and therapeutic responsiveness. In the TME, both innate and adaptive immune cell populations are located that can have antitumor cytotoxic or immunosuppressive functions depending on their phenotypic and functional states (57). However, during tumor evolution, malignant cells will form multiple resistance responses, enabling their survival and development of an immunosuppressive microenvironment. Such mediators include decreased expression of tumor-associated antigens, blockage of antigen presentation pathways, secretion of inhibitory cytokines, and recruitment of immunosuppressive cell populations which may lead to attenuated antitumor immunity (58–61). Growing evidence also indicates that the TME immunophenotypic composition is an important predictor of clinical outcome and therapeutic efficacy in cancer and for its prognostic/predictive value (56,62). Therefore, heterogeneity and dynamic nature of the TME pose major challenges for successful immunotherapy implementation.

Treatment response depends on many elements including tumor mutational burden, biomarker expression, and the immunological features of the TME. Those are the reasons why not all patients benefit from immunotherapy. In addition, the incidence rates of immune-related adverse events, e.g., colitis, pneumonitis, and endocrinopathies, should be closely followed and managed (63). In addition, beyond biologic and clinical limitation, the significant economic burden for sophisticated immune treatment strategies restricts access and places significant strain on global healthcare systems (64). As a result, there has been increasing interest in deciphering predictive biomarkers in order to enhance patient stratification and enhance immunotherapy efficacy.

Concurrently, there is a growing demand for robust preclinical platforms to evaluate novel immunotherapeutic agents and next-generation CAR T cell products. Such

platforms should not only enable the assessment of therapeutic efficacy but also provide detailed insights into how immunotherapies modulate tumor–immune cell interactions and reshape the TME. This information is essential for identifying mechanisms of response and resistance, discovering predictive biomarkers, and guiding the development of personalized immunotherapies and rational combination treatment strategies.

1.3 Precision medicine

Precision medicine, also called personalized medicine, individualizes prevention and treatment efforts based on the individual patient: their genetic makeup, environmental exposure and lifestyle, for instance. Unlike the long-standing “one-size-fits-all” approach, it strives to treat the right patient at the right time and dose with the most effective treatment. The core premise behind precision medicine is how the individual varies in genes and biology, resulting in different stages of disease and treatment response. By combining genomic, molecular and clinical features for the optimization of therapeutic efficacy and reduction of adverse effects, precision medicine is being developed (65). Precision medicine has driven dramatic changes especially in oncology. Identification of tumor-specific genetic mutations or molecular alterations allows targeted therapies, for example EGFR inhibitors in non-small cell lung cancer or HER2-directed therapies in breast cancer (39,40). As technology and genetics advance, precision oncology is likely to be incorporated in standard cancer care.

1.4 Functional precision medicine

This section is adapted from my first-author publication (66) :

Zhang Y., et al. From 2D cultures to 3D systems: evolving cancer models at the interface of functional precision medicine and theranostics, *Theranostics*, 2026, 16 (8), 4042.

With the dynamic interactions between various cellular states, accurate estimation of the behavior of cancer cannot be accomplished solely by static genomic, proteomic, or metabolic profiling (67). Genomic analyses may recognize accumulated mutations and

emerging therapeutic targets, but they fail to characterize functional effects of these alterations, or the time-dependent therapeutic response of tumor cells (68).

Functional precision medicine (FPM) as a method based on the *ex vivo* behavior of material derived from patients is a promising option for revealing therapeutic sensitivity, as it offers more direct, functionally relevant data. This has been clinically important, particularly in those patients who do not have any meaningful genomic changes or diseases which are not responsive to standard treatments (69).

As immuno-oncology progresses, the demand for experimental models that are able to preserve combined immunological activity between an individual tumor compartment and an integrated immune compartment is increasing. Such systems allow functional assessment of immunotherapy (checkpoint inhibitors, adoptive cell treatment and cancer vaccines) in a more realistic manner than the classical model of the native tissue. By this, FPM is evolving from a drug sensitivity screening platform to a broad, integrated approach for predictive immune theranostics.

The idea for FPM dates back several decades with functional assays that exposed patient-derived samples to therapeutics and measured cell death responses, at the time mainly directed at hematologic malignancies (70–72). Subsequent development of culture techniques and experimental protocols has significantly broadened the spectrum of FPM from drug sensitivity testing to a more holistic functional examination of tumor biology, including influences from the TME, immune cell interactions, and therapy-induced cellular adaptations (73,74).

Subsequent generations of the FPM platforms use more immune-competent co-culture systems, tumor explant models, and functional readouts including dynamic BH3 profiling, and lactate dehydrogenase (LDH) release assays. These methods permit a more comprehensive description of apoptotic priming, mitochondrial dependency, and treatment-facilitated cytotoxic responses at a functional level (75–77). Simultaneously, the inclusion of multimodal data sets in AI-enabled analytical pipelines aids in biomarker discovery and the identification of potential therapeutic targets, which can be further validated in patient-derived systems. As a whole, these advances further cement FPM

in a theranostic approach that connects functional prediction and therapeutic strategy (78,79).

Recent developments in machine learning have also facilitated automated and high-dimensional imaging data assessment, single-cell phenotyping, and even complex drug response signatures. To further stratify patients and predict their progression, therapeutic response, and risk, integrative computational models were integrated with multi-omics, clinical, and functional biological data. Taken together, these methods dramatically enhance clinical usefulness and translation potential of these FPM platforms (80,81).

A typical workflow for FPM includes patient enrollment, tumor sampling, and production of patient-derived two-dimensional (2D) or 3D model systems to validate *ex vivo* drug sensitivity. These functional data are then integrated with clinical parameters and molecular profiles and subsequently analysed in an FPM tumor board for therapeutic decision making. More recently, advanced 3D culture systems and tissue explant models designed to better preserve the structural and functional characteristics of native TME, including immune components, have been increasingly integrated with FPM pipelines. Meanwhile, microfluidic platforms and perfusion-based bioreactor systems have also been proposed to maintain immune–tumor interactions and to provide more physiologically relevant and potentially longitudinal functional readouts. Finally, the clinical utility of FPM is a matter of iterative validation, which involves regular comparison between model-derived predictions and actual patient treatment outcomes to improve predictive accuracy and translational relevance over time (**Figure 2**).

With the increasing variety of functional assay platforms, choice of model system has emerged as a crucial factor for technical feasibility and diagnostic relevance. In the context of theranostics, the model system itself can be regarded as a functional biosensor translating underlying biological complexity into quantifiable therapeutic response readouts. Accordingly, the sections below provide a comparative overview of commonly used model formats and discuss how their structural and functional design, from simplified 2D cultures to dynamic, perfused tissue explant systems, directly

influences their suitability for applications in personalized therapy and immuno-oncology.

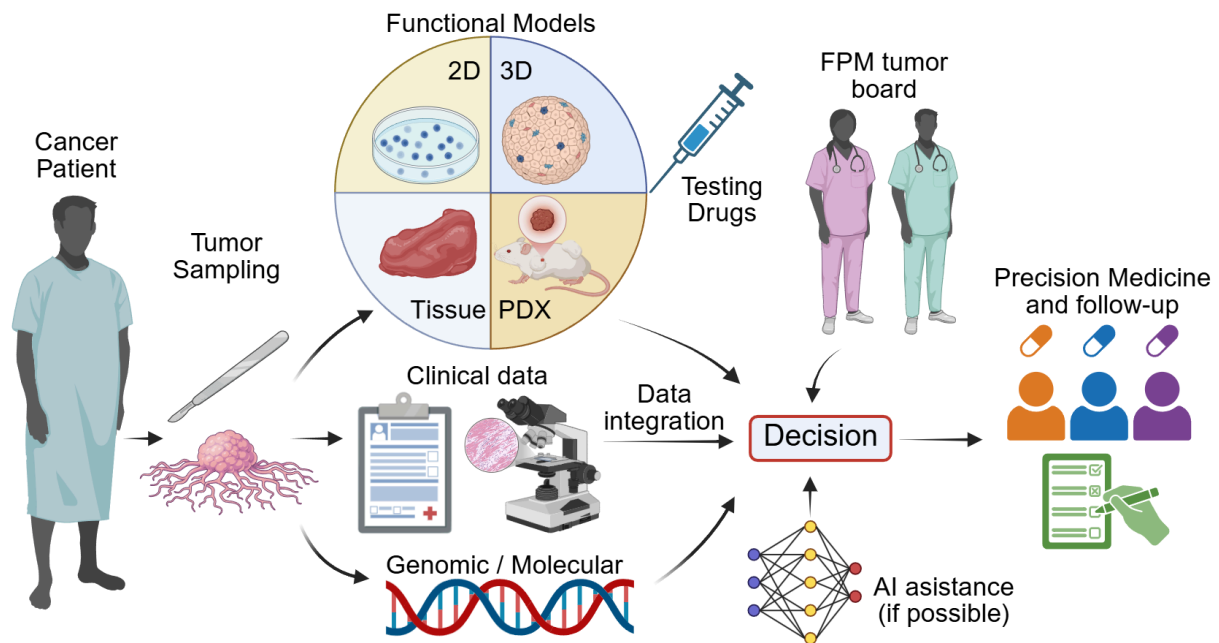


Figure 2. FPM in Cancer Management.

Generally, patients with advanced-stage cancer are admitted to treatment facilities where tumor samples are obtained through surgical procedures or biopsies. Subsequently, functional models are chosen based on the type of tumor and the urgency of the required treatment. The FPM tumor board evaluates drug sensitivity findings alongside clinical and genomic information, utilizing AI support to inform precision therapy and subsequent monitoring. Reproduced from (66). (Figure created with BioRender.com)

The application of patient-derived material in FPM also involves significant ethical and governance implications. In most clinical and research settings, participation requires written informed consent that clearly defines how freshly obtained tissue will be used for *ex vivo* functional testing, the extent to which results may be interpreted for clinical decision-making, and the conditions under which data may be stored or shared. All assay-derived datasets must be stored on secure, access-controlled institutional servers and processed in accordance with applicable local data protection regulations (82,83). Functional readouts are typically interpreted in the context of a multidisciplinary tumor board or comparable oversight structure, thereby integrating experimental findings alongside established clinical, pathological, and molecular information. Given the inherent variability and uncertainty in functional assays, findings reported to

clinicians and patients should use standardized and clearly defined terminology, avoiding overinterpretation or overstated predictive claims (84). These ethical, regulatory, and governance frameworks must be applied consistently to ensure responsible and clinically meaningful translation of FPM approaches.

FPM has now shown clinical utility and the rapid developments in culture techniques have increased its potential applications. Nonetheless, translating increasingly complex experimental cancer models into routine clinical FPM workflows continues to be a significant challenge. Importantly, I should not take greater model complexity as an inherent objective in itself, but rather something that needs to be harmonious with considerations such as feasibility, scalability, or clinical relevance.

While a number of novel systems, notably scaffold-based tumor models, patient-derived explants, and PDXs, can better mimic features of *in vivo* tumor biology, their technical demands, cost, and limited throughput restrict their application into clinical practice, although they are still highly promising in preclinical and translational research. In contrast, relatively easier platforms like 2D suspension cultures play an important role in FPM, especially for hematological malignancies due to their fast turnaround, cheapness, and proven predictive value in clinical contexts.

In solid tumors and in immunotherapy-related applications, the maintenance of stromal and immune constituents becomes pivotal, since therapeutic efficacy typically relies on intact TME interactions. 3D spheroid and organoid systems are currently the most established intermediate platforms for FPM studies, while *in vivo* validation using PDX models offers valuable complementary insights. More recently, hybrid 3D systems and perfused tissue explant and bioreactor platforms have further advanced these capabilities by permitting the maintenance of complex human tissues *ex vivo* for functional testing and mechanistic investigation.

In general, FPM is advancing quickly, and the increasing range of available cancer models opens a great deal of potential for better functional assessment of therapeutic responses, but also presents new problems in standardization and clinical integration. Developing harmonized standards for model selection, experimental design, and

analytical readouts will be critical in advancing these and other developments for routine precision oncology and theranostic applications.

1.5 U-CUP perfusion bioreactor system

In the past few decades significant advances have been achieved in *in vitro* development of cancer culture systems aimed at closely matching the cellular composition and spatial organization of a tumor. In this manner, model systems have gradually progressed from the traditional 2D monolayer culture to the more complex 3D platforms.

2D cultures are still commonly used as they have the advantage of fast processing, being cost-effective and operational simplicity which suit FPM workflow for clinical decisions that may not take years. However, their low structural complexity and absence of target immune context severely limit the utility and only provide indication on cytotoxic or targeted agent screening rather than immunotherapy assessment. In contrast, 3D culture systemologies offer biologically more representative tumor architecture and heterogeneity features. With the incorporation of several cell types and the ability to determine spatial gradients, these models are more versatile to assist in immune cell infiltration or heterogeneous drug distribution, these being growing requirements in immuno-oncology and for theranostic assay development. However, their sophistication, cost, and lengthy trial schedules could limit their use as routine tools, they are therefore, mainly seen as a utility tool for mechanistic researches, biomarker identification and selective FPM (69).

The U-CUP bioreactor system (CELLEC Biotek AG, Switzerland) is a perfusion-driven culture system designed for the cultivation of 3D patient-derived tissue specimens (85–87). As opposed to a static culture system, the U-CUP utilizes an external syringe pump for bidirectional perfusion driving the culture medium directly through the tissue for better nutrient homogenization, improved oxygen delivery and waste removal to minimize tissue loss of viability. This compact closed-system design also promotes reduction in culture volume, and reduces contamination and air bubble formation. Also, various other bioreactor platforms such as spinner flasks and rotating wall vessels,

microfluidic devices, and perfusion-based bioreactors have been utilized for 3D tissue culture for morphological, functional, and therapeutic analysis of responses (85,88–90).

Perfusion culture has been adapted well for tissue engineering studies and is popularly used in cancer research to maintain the viability and structure of patient-derived tumor tissues *ex vivo*. By maintaining constant delivery of nutrients and oxygen as well as the ability to exclude waste, perfusion bioreactors help protect vital parameters of the tumor microenvironment such as the stroma and immune cell populations.

Nevertheless, perfusion-oriented tissue explants are only one of many experimental models which are utilized to analyze tumor biology and therapeutic consequences. Some *in vitro* and *in vivo* models have been developed, with different advantages as well as drawbacks (**Figure 3**). Conventional 2D cell cultures are cheap, replicable, and feasible for high-throughput screening; however, they do not recapitulate the three-dimensional architecture and cellular complexity of the TME. Spheroids, organoids, scaffold-based cultures, and microfluidic systems of multiple dimensional models are closer to native tissues than 2D models, but ultimately lack the full stromal, vascular, and immune components of patient tumors. Furthermore, several of these models need long establishment times and may not faithfully preserve patient-specific cellular interactions.

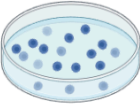
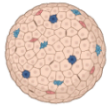
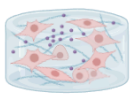
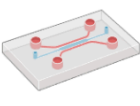
Models	Throughput	Cost	Speed	TME	Biobanking	Stage
 Cell Cultures 2D	High	Low	Fast	No	Yes (Standardized)	FPM application
 Spheroids 3D	Medium	Low	Moderate to Fast	No	Yes (Established)	Co-clinical study
 Organoids 3D	Medium	Medium	Moderate	Partial	Yes (Established)	Co-clinical study
 Scaffold-based 3D	Low to Medium	Medium	Moderate	Partial	Limited	Pre-clinical study
 Microfluidics 3D	Low to Medium	High	Slow	Partial	Limited	Pre-clinical study
 Tissue pieces 3D / ex vivo	Low	Medium	Moderate	Yes	Limited	Pre-clinical study
 Xenograft 3D / in vivo	Low	High	Slow	Yes	Yes (PDX banks)	FPM / co-clinical

Figure 3. Comparison of Cancer Models for FPM.

Models are evaluated based on several factors, including dimensionality, throughput, cost, speed of culture, TME preservation, biobanking capabilities, and their current phase in clinical application. The application stage of FPM signifies models that directly contribute to patient treatment choices. Conversely, the co-clinical study stage involves models utilized in conjunction with standard patient care to assess therapeutic responses. The preclinical study stage encompasses models employed for foundational and translational research purposes. Reproduced from (66). (Figure created with BioRender.com)

Among these models, patient-derived tissue explants uniquely preserve the native tumor architecture together with the heterogeneous cellular composition and spatial organization of the original TME. This enables investigation of tumor–immune interactions in a physiologically relevant setting while allowing rapid assessment of therapeutic responses using freshly resected patient specimens. Consequently, perfusion-based explant culture systems, such as the U-CUP bioreactor, provide a promising platform for FPM and the preclinical evaluation of novel anticancer therapies.

Importantly, the preservation of the intact TME also enables comprehensive spatial characterization by multiplex imaging techniques, allowing treatment-induced changes in immune cell composition, phenotype, and cellular interactions to be analyzed directly within their native tissue context.

1.6 Lymph node structure and lymphoma biology

The tissue that I selected for bioreactor culture is secondary lymphoid organs, mainly lymph nodes, which possess a well-defined architecture consisting of B cell follicles in the cortex, T cell-rich paracortical regions, and medullary cords and sinuses. This highly organized structure facilitates efficient immune cell trafficking, antigen presentation, and coordinated cellular interactions essential for adaptive immunity (**Figure 4A**). These organs are specialized microenvironments in which antigen presenting cells, T lymphocytes, B lymphocytes, dendritic cells, macrophages, and stromal cells communicate in an organized way to trigger and coordinate the activation of the immune system. Rather than simplified *in vitro* models, lymphoid tissues maintain the underlying spatial organization and cellular heterogeneity to communicate effectively both with other immune cells and immune partners (91). Therefore, *ex vivo* culture of lymphoid tissue offers a physiologically relevant tissue to study immune function with dynamic cell-cell and cell-matrix interactions.

Lymphomas are malignancies derived from lymphoid cells at various stages of differentiation and are intrinsically linked to the architecture and function of the lymphatic system. In contrast to most solid tumors, lymphomas arise and evolve within secondary lymphoid organs, particularly lymph nodes, which represent specialized microenvironments for immune cell activation, interaction, and selection. In many lymphoma entities, malignant transformation occurs within these physiological immune niches, especially germinal centers, where normal processes of clonal expansion and selection are subverted to drive tumor growth and immune escape (92).

Based on the classification of lymphoma, lymphomas are classified into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) by the World Health Organization (WHO). Non-Hodgkin lymphomas make up around 90% of all lymphomas and are

considered heterogeneous cancer cell types, obtained primarily from B cells but also T cell and NK cell based. B cell lymphomas comprise about 85–90% of NHL cases in western nations, while T/NK-cell lymphomas are rarer but are usually characterized by aggressive clinical presentation (93). NHL arising either from B lymphocytes or T lymphocytes, whereas HL is a distinct entity containing malignant Reed–Sternberg cells of B cell origin in a prominent inflammatory microenvironment. About 85–90% of all NHL cases are B cell lymphomas, which are common entities to include diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and marginal zone lymphoma (MZL). By contrast, T cell lymphomas are less common, and its main subtypes are peripheral T cell lymphoma (PTCL), anaplastic large cell lymphoma (ALCL), T cell large granular lymphocyte leukemia (T-LGL), follicular T cell lymphoma (F-TCL) and mycosis fungoides (MF) (94). These lymphoma manifestations vary markedly in their pathogenesis, biological behavior, and response to therapy (**Figure 4B**).

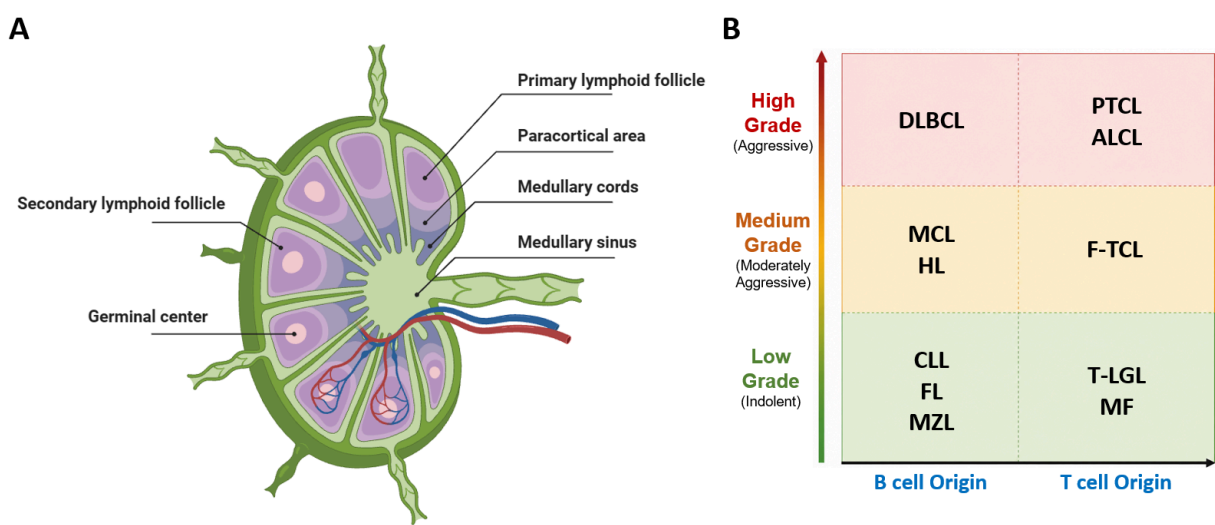


Figure 4. Lymph node architecture and classification of major lymphoma entities.

(A) Schematic illustration of the major anatomical compartments of the lymph node, including B cell follicles in the cortex, T cell-rich paracortical regions, and the medullary cords and sinuses (Figure created with BioRender.com). (B) Overview of representative lymphoma entities classified according to cell of origin (B cell or T cell) and clinical behavior (94).

Consequently, lymph nodes are not only the primary anatomical site of disease manifestation but also a key determinant of tumor–immune interactions and therapeutic response. This close biological relationship provides a strong rationale for the use of patient-derived lymph node tissue as a physiologically relevant model for *ex vivo* studies of lymphoma biology and treatment responses.

Lymph nodes are also one of the most frequent locations of tumor metastasis in many solid cancers (95). However, metastatic lymph nodes are also home to a plethora of tumor-exposed and invading immunologic populations, including cytotoxic T cells, regulatory T cells, B cells, dendritic cells, macrophages, and other stromal components that ultimately define the tumor microenvironment (96). Metastatic lymph nodes serve as a distinct *ex vivo* model that preserves both the malignant and immune compartments of individual patients, given that tumor progression and therapeutic response strongly depend on these cellular interactions. Compared to standard tumor cell cultures or organoids, the approach provides a more accurate representation of the patient-specific immune environment and functional evaluation of tumor-immune interactions in physiologically relevant conditions.

The cellular composition made by lymphoid tissue also makes it specific for characterizing cancer immunotherapies. Immune checkpoint inhibitors depend on recapitulating endogenous T cell responses within an intact immune microenvironment, which is not amenable for well-studied constructs using tumor cells alone. In metastatic lymph nodes, the integration of tumor cells into the host immune milieu affords a direct means to estimate immunologic activation, immune suppression, and antitumor responses after checkpoint blockade. Additionally, B cells constitutively express CD19, lymphoid tissue provides an intrinsic target population for evaluating CAR T cell activation, expansion, cytokine production, and antigen-specific cytotoxicity under physiologically relevant conditions. Taken together, these features render lymphoid tissue, specifically metastatic lymph nodes, a candidate *ex vivo* point of reference for functional precision medicine and preclinical characterization of emerging immunotherapies.

1.7 Research gap and objectives

A key gap in current laboratory approaches is the lack of physiologically relevant *ex vivo* models that can simultaneously preserve the native architecture and immune microenvironment of human lymphoid tissues while enabling efficient and scalable functional testing. Existing model systems each present important limitations. Conventional 2D cell cultures are inexpensive and well suited for high-throughput screening but fail to reproduce the three-dimensional architecture and cellular complexity of the tumor microenvironment. Tumor spheroids and organoids better recapitulate tissue organization; however, they generally lack the full complement of stromal and immune cell populations and often require prolonged establishment before therapeutic testing. PDX models preserve many aspects of tumor biology but are costly, labor-intensive, and require weeks to months to establish, making them impractical for time-sensitive functional precision medicine applications. Similarly, currently available *ex vivo* platforms, including precision-cut tissue slices, air–liquid interface cultures, and microfluidic tumor-on-a-chip systems, enable functional testing using freshly respected patient tissues but also have important limitations. Precision-cut tissue slices gradually lose tissue integrity and immune function during culture, air–liquid interface models rely primarily on passive diffusion and have limited the thickness of tissue slices for culture, while microfluidic systems often require specialized fabrication, complex operation, and substantial cost despite offering improved experimental control and scalability. Consequently, although these approaches provide valuable platforms for therapeutic testing, they frequently involve trade-offs between physiological relevance, throughput, technical complexity, and cost.

In this context, perfusion bioreactor-based systems provide a complementary approach by continuously supplying nutrients and oxygen throughout intact tissue explants while maintaining native tissue architecture and immune cell composition. Compared with conventional *ex vivo* culture methods, perfusion bioreactors offer improved preservation of tissue viability and immune functionality with relatively simple operation and clinically relevant processing times, although they generally have lower throughput than miniaturized screening platforms. Consequently, perfusion bioreactor-based culture

represents a valuable addition to functional precision medicine, bridging the gap between simplified *in vitro* systems and complex *in vivo* models while enabling more physiologically relevant investigation of tumor–immune interactions and therapeutic responses.

Thus, the objective of this thesis was to establish and validate a perfusion bioreactor-based 3D tissue explant culture platform to develop functional studies of patient-derived lymphoid tissues *ex vivo*. The platform was developed to preserve native tissue architecture, cellular heterogeneity and tumor–immune interactions in a dynamic perfusion-preserving environment. This system allows the systematic assessment of immune responses and therapeutic efficacy in intact human tissues through providing a physiologically relevant culture environment. This study also aimed to assess the potential of the platform for functional validation of clinically relevant immunotherapies such as immune checkpoint inhibitors and CAR T cell therapy. Through patient-derived tissue explants and functional investigations, this thesis aimed to provide an experimental model in which to study treatment-induced alterations in the tumor microenvironment and immune cell behavior.

1.8 Major findings and significance

In the current study, I used U-CUP 3D tissue perfusion bioreactors to develop a lymphoid tissue–derived immunotherapy model to aid in the early determination of responsive patients prior to treatment initiation. Through recreating the fundamental parameters of the lymphoid tissue microenvironment under dynamic perfusion, this system allows for an *ex vivo* assessment of treatment-induced functional and cellular responses and can also be used to search for candidate biomarkers related to therapeutic sensitivity. The bioreactor platform was designed to protect the viability and function of human lymphoid tissues, and provided an opportunity to systematically evaluate the efficacy of immunotherapeutics in a more realistic setting of the *in vivo* setting. Based on this, I showed that pembrolizumab selectively exerts cytotoxic effects on lymphoma and metastatic tumor cells of lymph node tissue while largely sparing adjacent non-malignant components. At the immunological level, I were able to

characterize cytokine expression and identified elevated expression of cytokine markers, IFN- γ , granzyme B, and perforin (PRF), and observed greater CD8⁺ T cell-mediated immune activity and tissue infiltration. I also demonstrated that CAR T cells with stimulation of PI3K signaling showed better penetration of tissues than standard CAR T cell products in this *ex vivo* system, which again suggested that regulated PI3K signaling plays a key role in fine tuning CAR T cell-based treatment strategy.

The design of tumor immunotherapies is only successful if experimental models can faithfully reproduce the complicated interactions among tumor cells and the immune system. Yet, current preclinical models do not generally replicate the native cellular composition, tissue architecture, and immune functionality of human tumors, limiting their capacity to predict clinical responses. By generating a highly efficient perfusion bioreactor to keep viable and functioning human lymphoid tissues *ex vivo*, this work fills a significant knowledge gap between traditional laboratories and patient physiology. Preserving tumor as well as immune cells in intact human tissue represents a more physiologically feasible means of understanding mechanisms of antitumor immunity and therapeutic response.

This platform possesses not only technical enhancements to tissue culture but has significant implications for cancer research. Maintaining healthy immune cell populations allows direct investigation of immune-tumor interactions in natural tissue microenvironments. As a result, the model also offers a platform to study mechanisms of immune activation, immune evasion, therapeutic resistance, and biomarker discovery which are very hard to study in conventional cell culture systems. Moreover, not limited to immune checkpoint inhibitors and CAR T cell therapy, its compatibility with other classes of immunotherapies suggests that it could become a versatile platform for preclinical evaluation of novel therapeutic modalities.

One of the most significant findings presented is probably the contribution made to the future of functional precision medicine. By keeping patient tissues alive *ex vivo*, one can directly monitor the reactions of individual therapeutics, preserving a patient's unique tumor microenvironment and immune landscape. Such functional testing might also

complement genomics and molecular profiling with phenotypic evidence of a therapy's effectiveness prior to the time of clinical application. A subsequent clinical validation of this approach might enable the patient-specific selection of treatment, prompt the development of personalized immunotherapy, diminish the need for more generalized preclinical models, and finally facilitate better clinical strategies for patients with lymphoid malignancies and metastatic solid tumors.

2 Materials and Methods

This section is adapted from my first-author publication (97) :

Zhang Y., et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors, *Theranostics*, 2026, 16 (8), 3928.

2.1 Materials

2.1.1 Antibodies, reagents and equipments

The key resources table details the sources and specific information for all antibodies, reagents, and equipment used (**Table 1**, reproduced from (97)).

Table 1. Key resources

RESOURCE	SOURCE	IDENTIFIER
Fluorophore-labeled antibodies		
APC anti-human CD3	BioLegend	300311
FITC anti-human CD3	BioLegend	300306
PE anti-human CD4	BioLegend	317410
BV510 anti-human CD8	BioLegend	344732
PE anti-human CD19	BioLegend	302208
FITC anti-human CD19	BioLegend	302206
PE-Cy7 anti-human CD56	BioLegend	362509
BV510 anti-human CD11c	BioLegend	301633
PE anti-human CD123	BioLegend	306006
PE-Cy7 anti-human CD68	BioLegend	333815
BV510 anti-human CD45	BioLegend	368525
APC-Cy7 anti-human CD31	BioLegend	303119
APC anti-mouse IgG	BioLegend	405308
PE anti-rabbit IgG	BioLegend	406421
Antibodies for CODEX or IF		
anti-CD3 (Oligo 20)	BioLegend	300402
anti-CD4 (Oligo 28)	BioLegend	357402
anti-CD5 (Oligo 75)	BioLegend	300602
anti-CD8 (Oligo 43)	BioLegend	344702
anti-CD11c (Oligo 44)	BD Biosciences	555391
anti-CD19 (Oligo 2)	BioLegend	302202
anti-CD20 (Oligo 48)	Novus	NBP2-54591

anti-CD31 (Oligo 68)	BioLegend	303102
anti-CD38 (Oligo 66)	BioLegend	356602
anti-Collagen 4 (Oligo 33)	Abcam	ab6586
anti-IFN- γ (Oligo 30)	Akoya biosciences	240081
anti-FITC (Oligo 6)	BioLegend	408301
Chemicals and biological reagents		
Pembrolizumab	Selleckchem	A2005
EDTA	ROTH	X986.2
DAPI	BioLegend	422801
APC-Annexin V	BioLegend	640920
DMEM / F12 with 15mM HEPES	ROTH	9031.1
Penicillin-Streptomycin	VWR-Biowest	L0022-100
Amphotericin B	ThermoFisher	15290026
Nicotinamide	Sigma-Aldrich	N0636
NAC	Sigma-Aldrich	A7250
Human serum	Sigma-Aldrich	H5422
Interleukin-2	Miltenyi Biotec	130-097-742
Interleukin-7	Miltenyi Biotec	130-093-937
Interleukin-15	Miltenyi Biotec	130-095-760
pCMV-AncBE4max	Addgene	112095
Agel-HF	NEB	R3552
T7 MEGAscript Kit	ThermoFisher	AMB13345
CleanCap AG	Tebubio	N-7113
PolyA-tailed	ThermoFisher	AM1350
sgRNA	IDT	N/A
IDTE pH7.5 buffer	IDT	11-05-01-05
NaCl	VWR Chemicals	27810.295
KCl	Merck	7447-40-7
MgCl ₂	ROTH	KK36.1
CaCl ₂	ROTH	CN92.1
HEPES	ROTH	HN77.3
CellTrace™ CFSE dye	ThermoFisher	C34554
Bovine serum albumin	BioSell	BSA.PF.0100
CFSE	ThermoFisher	C34554
Agarose	Biozym	840004
LEGENDplex™ Human CD8/NK Panel (13-plex)	BioLegend	741186
Fetal Calf Serum (FCS)	Sigma-Aldrich	F7524
PBS 10x	Sigma-Aldrich	D1408
DMSO	ROTH	7029.4
Trypan blue	Sigma-Aldrich	T8154
Triton X-100	ROTH	3051.3

Na ₂ HPO ₄	Sigma-Aldrich	S7907-500G
NaH ₂ PO ₄	Sigma-Aldrich	S9638-250G
Tris	ROTH	4855.2
BS ³	ThermoFisher	21580
mouse IgG	Biozol	JIM-015-000-003
rat IgG	Biozol	JIM-012-000-003
Sheared salmon sperm DNA	ThermoFisher	AM9680
Non-fluorescent oligonucleotides mix	Biomers	N/A
Poly-L-Lysine	Sigma-Aldrich	P8920-100ML
Acetone	neoFroxx	LC-4916.2
Methanol	Honeywell	32213
IDTE pH7.5	IDT	11-05-01-05
Hematoxylin	Sigma-Aldrich	1.09253
Eosin	Sigma-Aldrich	318906
Isopropanol	SAV Liquid Production	67-63-0
Ethanol	SAV Liquid Production	64-17-5
PFA 16%	ThermoFisher	0433368.9M
X-Vivo15 medium	Lonza	BEBP02-061Q
Cultrex BME	R&D	3432-005-01

Equipment and Consumables

Clean bench	Heraeus	HB2448K
Scalpel	Pfm medical	02.001.30.021
Petri dish	Greiner Bio-One	664160
Tissue-Tek Cryomold	Sakura	SA 62534-25
Tissue-Tek O.C.T. compound	Sakura	SA62550
Syringe 5 ml	B. Braun	4606051V
Falcon tube 50 ml	Cellstar	227261
Cell strainer 100 µm	pluriSelect	45-57100-51
Cell strainer 70 µm	pluriSelect	43-57070-51
Cell strainer 40 µm	pluriSelect	43-57040-51
20G needle	B. Braun	4657519
Centrifuge	Thermo Heraeus	Multifuge 1S-R
Incubator	Heracell	51013568
U-CUP bioreactor	Celtec Biotek	N/A
U-CUP rack	Celtec Biotek	N/A
Syringe pump	Harvard Apparatus	MA1 70-3xxx
Silicone adaptors Ø10mm x 4 mm	Celtec Biotek	URD10H04
Nylon meshes Ø10mm	Celtec Biotek	N/A
Collagen sponges	Celtec Biotek	N/A
Polystyrene tubes	Falcon	352058
Neubauer chamber	Hecht Assistant	N/A

Cytoseal	Epredia	8312-4
Coverslips 24 x 50 mm	Mariefeld Superior	0101222
Coverslips 22 x 22 mm	Mariefeld Superior	0101052
BONDIC pen	Bondic VIKO UG	SW10014
TOMO adhesion slides	Matsunami Glass	TOM-1190
LED Pads	Aibecy	A5 25 '000 Lux
Cryostat	Leica	CM3050S
All-in-one fluorescence microscope	Keyence	BZ-X810
LSR II Flow Cytometer	BD Biosciences	N/A
FACS Canto II	BD Biosciences	N/A
PhenoCycler (CODEX®)	Akoya Biosciences	N/A
Neon Transfection System	Thermo Fisher	N/A
24-well plates	Falcon	3003005
6-well plates	Falcon	3003055
Biological material		
Human lymphoid tissue	University Hospital Tübingen	
anti-CD19 CAR T cells	Düsseldorf University, Prof. Sascha Dietrich's Lab	
PI3K-CD19 CAR T & Control CAR T cells	Tübingen University, Prof. Judith Feucht's Lab	
SU-DHL-2 & TMD-8 cell lines	Tübingen University, Prof. Alexander Weber's Lab	

2.1.2 Buffers and culture medium

The information on composition of buffers, working solutions, and culture medium are shown in **Table 2** (reproduced from (97)).

Table 2. Composition of buffers, working solutions and culture medium

70% Ethanol		
Reagent	Final concentration	Amount
100% Ethanol		700 ml
ddH ₂ O		300 ml
Total	70%(v/v)	≈ 1000 ml
Store at RT		
1 x PBS		
Reagent	Final concentration	Amount
10 x PBS		100 ml
ddH ₂ O		900 ml
Total	1 x	1000 ml
Autoclave sterilization, store at RT		

EDTA-PBS		
Reagent	Final concentration	Amount
1 x PBS		1000 ml
EDTA	2 mM	744.5 mg
Total		≈ 1000 ml
Autoclave sterilization, store at RT		
PBS with 0.1% BSA		
Reagent	Final concentration	Amount
1 x PBS		10 ml
BSA		0.01 g
Total	0.1%	≈ 10 ml
Store at -20°C		
IL-2 aliquots		
Reagent	Final concentration	Amount
PBS with 0.1% BSA		1700 µl
IL-2 (1.7 x 10 ⁷ U/mg)		10 µg
Total		≈ 1700 µl
Aliquots	2000U / 20µl	20 µl x 85 vials
Store at -80°C		
IL-7 aliquots		
Reagent	Final concentration	Amount
PBS with 0.1% BSA		400 µl
IL-7 (2 x 10 ⁷ U/mg)		10 µg
Total		≈ 400 µl
Aliquots	10000U / 20µl	20 µl x 20 vials
Store at -80°C		
20 mM N-Acetylcysteine (NAC)		
Reagent	Final concentration	Amount
NAC		48.96 mg
ddH ₂ O		15 ml
Total	20 mM	≈ 15 ml
Store at -20°C		

20 mM Nicotinamide		
Reagent	Final concentration	Amount
Nicotinamide		36.63 mg
ddH ₂ O		15 ml
Total	20 mM	≈ 15 ml
Store at -20°C		

Culture medium aliquots		
Reagent	Final concentration	Amount
DMEM / F12 with 15mM HEPES		78 ml
Penicillin-Streptomycin	1 %	1 ml
amphotericin B	1 %	1 ml
20 mM NAC	1 mM	5 ml
20 mM Nicotinamide	1 mM	5 ml
Human serum	10 %	10 ml
Total		100 ml
Aliquots		10 ml x 10 tubes
Store at 4°C		

Final culture medium (used for bioreactors and static culture)		
Reagent	Final concentration	Amount
Culture medium aliquot		10 ml
IL-2 aliquots	200 U/ml	20 µl
IL-7 aliquots	1000 U/ml	20 µl
Total		≈ 10 ml
Freshly add ILs before use, every bioreactor needs 10 ml final culture medium		

0.5% Agarose		
Reagent	Final concentration	Amount
Agarose		0.1 g
PBS		20 ml
Total	0.5%	≈ 20 ml
Autoclave sterilization or boiling for 1h, store at RT		

CFSE staining reagent		
Reagent	Final concentration	Amount
CellTrace™ CFSE dye		1 vial
DMSO		18 µl

Total	5 mM	≈ 18 ml
-------	------	---------

Store at -20°C

Annexin V binding buffer

Reagent	Final concentration	Amount
HEPES	10 mM	2.38 g
NaCl	150 mM	8.77 g
KCl	5 mM	372.75 mg
MgCl ₂	1 mM	95.21 mg
CaCl ₂	1.8 mM	199.76 mg
ddH ₂ O		To 1000 ml
Total		1000 ml

Store at RT

S1 buffer

Reagent	Final concentration	Amount
EDTA	5 mM	1.46 g
10 x PBS	1 x	100 ml
BSA	5%	5 g
ddH ₂ O		900 ml
Total		≈ 1000 ml

Store at 4°C

S2 buffer

Reagent	Final concentration	Amount
1 x S1		500 ml
Na ₂ HPO ₄	61 mM	8.66 g
NaH ₂ PO ₄	39 mM	4.68
5 M NaCl	250 mM	50 ml
ddH ₂ O		450 ml
Total		≈ 1000 ml

Store at 4°C

S4 buffer

Reagent	Final concentration	Amount
1 x S1	0.9 x	900 ml
5 M NaCl	0.5 M	100 ml
Total		1000 ml

Store at 4°C		
H2 buffer (reaction buffer of codex multicycle)		
Reagent	Final concentration	Amount
1 M Tris pH= 7.5	0.1 M	10 ml
Triton X-100	0.1% (w/v)	1 ml
MgCl ₂	10 mM	0.95 g
5 M NaCl	150 mM	30 ml
ddH ₂ O		960 ml
Total		≈ 1000 ml
Store at RT		
Bleaching solution		
Reagent	Final concentration	Amount
1 x PBS		20 ml
30% H ₂ O ₂		3.6 ml
1M NaOH		0.64 ml
Freshly make before use		
CODEX blocking solution (also diluent for antibodies cocktail)		
Reagent	Final concentration	Amount
1 x S2		234 µl
mouse IgG	65 µg/ml	15 µl
rat IgG	65 µg/ml	15 µl
Sheared salmon sperm DNA	43 µg/ml	15 µl
Non-fluorescent oligonucleotides mix	0.5 mM	21 µl
Total		300 µl
Freshly make before use		

2.1.3 Sample acquisition

Human lymphoid tissue samples were freshly harvested from the University Hospital Tübingen and processed immediately after excision. Written informed consent was obtained from all patients, and the study was approved by the local ethics committee (022/2021BO2). Patient case descriptions and the associated pathologies are further presented in **Table 3** (reproduced from (97)). Because human tissues were obtained from different donors with varying pathological conditions, baseline differences in

cellular composition and viability were expected. To minimize the influence of inter-donor variability, freshly isolated tissue from the corresponding donor was used as the reference control wherever appropriate, and post-culture measurements of cell frequency and viability were normalized to the matched fresh tissue. When sufficient tissue was available, different explants from the same surgical specimen were allocated to multiple experimental conditions to enable paired analyses. This strategy was particularly important for lymph node specimens, which were available only in limited quantities. For example, in patients with melanoma lymph node metastases, tumor-involved lymph node tissue was used for immune checkpoint inhibitor studies, whereas adjacent non-tumor lymphoid tissue from the same specimen, when available, was used for bioreactor optimization experiments and CAR T cell studies. This experimental design minimized biological variability between experimental groups while maximizing the scientific information obtained from each valuable patient specimen.

Table 3. Patient samples and diagnosis

Case #	Tissue type	Pathological diagnosis	Disease Status and Prior Treatment
Case 1	adenoids	adenoid hypertrophy	benign
Case 2	adenoids	adenoid hypertrophy	benign
Case 3	tonsil	chronic tonsillitis	benign
Case 4	adenoids	adenoid hypertrophy	benign
Case 5	tonsil	chronic tonsillitis	benign
Case 6	tonsil	chronic tonsillitis	benign
Case 7	tonsil	chronic tonsillitis	benign
Case 8	tonsil	chronic tonsillitis	benign
Case 9	tonsil	chronic tonsillitis	benign
Case 10	tonsil	chronic tonsillitis	benign
Case 11	tonsil	chronic tonsillitis	benign
Case 12	lymph node	T cell lymphoma	primary disease with no prior treatment
Case 13	lymph node	chronic lymphadenitis	benign
Case 14	lymph node	classical Hodgkin lymphoma	primary disease with no prior treatment
Case 15	lymph node	melanoma metastasis	relapsed disease after surgery
Case 16	lymph node	reactively changed lymph nodes	benign
Case 17	lymph node	melanoma metastasis	primary disease with no prior treatment
Case 18	lymph node	nonspecific lymphadenopathy	benign
Case 19	lymph node	diffuse large B cell lymphoma	relapse after CHOP and stem cell transplant
Case 20	lymph node	classical Hodgkin lymphoma	primary disease with no prior treatment
Case 21	lymph node	melanoma metastasis	primary, prior pembrolizumab treatment

Case 22	lymph node	melanoma metastasis	primary disease with no prior treatment
Case 23	lymph node	melanoma metastasis	relapse, treated with nivolumab
Case 24	lymph node	Tumor-free lymph node	benign
Case 25	lymph node	chronic lymphadenitis	benign
Case 26	lymph node	follicular lymphoma	relapse, treated with obinutuzumab and CHOP
Case 27	lymph node	reactive plasmacytosis	benign
Case 28	lymph node	classical Hodgkin lymphoma	primary disease with no prior treatment
Case 29	lymph node	dermatopathic lymphadenitis	benign
Case 30	lymph node	follicular center lymphoma	primary disease with no prior treatment
Case 31	tonsil	chronic tonsillitis	benign
Case 32	adenoids	adenoid hyperplasia	benign
Case 33	tonsil	chronic tonsillitis	benign
Case 34	tonsil	chronic tonsillitis	benign
Case 35	tonsil	chronic tonsillitis	benign
Case 36	tonsil	chronic tonsillitis	benign
Case 37	lymph node	melanoma metastasis	Post-surgery Relapse
Case 38	lymph node	Tumor-free lymph node	benign
Some cases were utilized in multiple experiments			

2.2 Methods

2.2.1 Sample preparation and tissue culturing

Fresh tissues were rinsed twice with EDTA–PBS buffer to remove residual blood. A cylindrical tissue core of approximately 3 mm in diameter was first excised along the longest axis and subsequently sectioned into 3 × 3 × 3 mm fragments. For each bioreactor condition, four tissue fragments were either placed between two collagen sponges (Cellec Biotek) or embedded in 300 µL of 0.5% agarose (Biozym, 840004) maintained at 40 °C. The embedded constructs were then positioned between two adaptors within the chamber of the perfusion bioreactor (Cellec Biotek). Subsequently, 10 mL of pre-warmed culture medium was introduced into the chamber via the inlet valve. The bioreactors were connected to a syringe pump (Harvard Apparatus) and operated under a cyclic perfusion regimen consisting of repeated cycles of infusion (3 mL at 1 mL/min), a 5-second pause, withdrawal (3 mL at 1 mL/min), and a second 5-second pause, continuously maintained throughout the 3-day culture period at 37 °C in a humidified incubator with 5% CO₂.

Conventional static plate culture was then implemented as a control condition to compare tissue viability and functional preservation. Tissues were treated in the same way and assigned to both conditions for each patient sample. A total of four fragments were cultured in 6-well plates (Falcon, 3003055), each containing 3 mL of the same culture medium per well, each at the same incubation conditions. Tissues were harvested after the culture period for follow-up study. Two of the fragments were embedded in paraffin or Tissue-Tek O.C.T. compound (Sakura, SA62550) for histological and frozen section analysis respectively and the other two fragments were prepared into single-cell suspension for flow cytometry (**Figure 5A-D**).

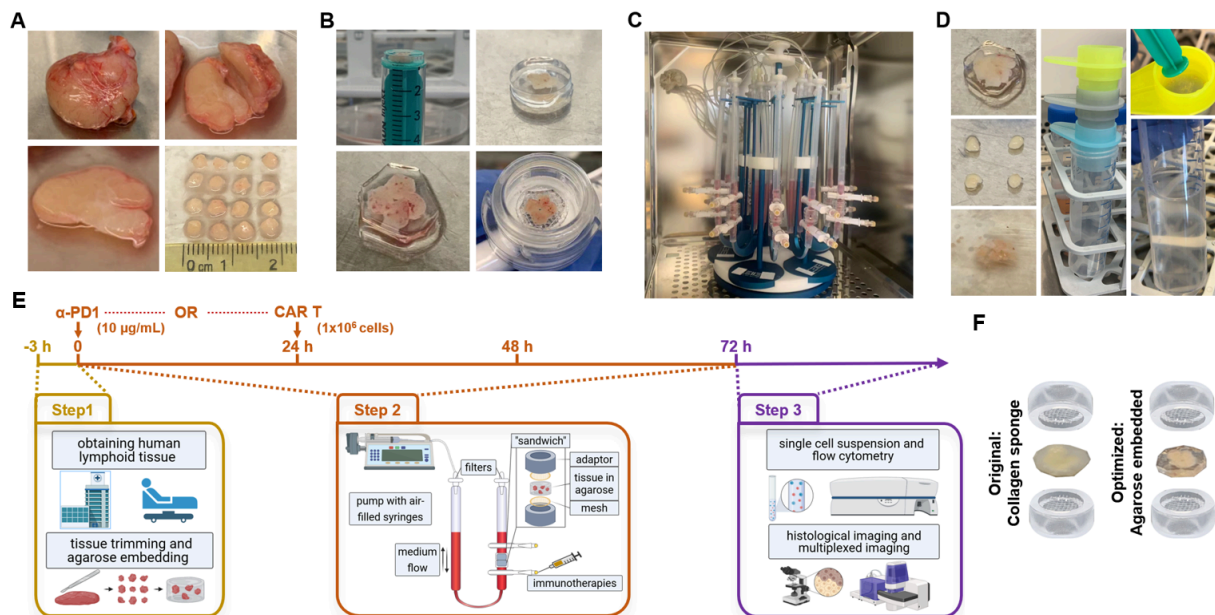


Figure 5. Experimental workflow for human lymphoid tissue perfusion culture and downstream immunotherapy analysis.

(A) Prepare lymphoid tissue pieces with the size of 3×3×3 mm by using scalpels. (B) Encapsulate the tissue pieces in 0.5% agarose within a cut-open syringe mold. (C) Culturing embedded tissue in the 3D perfusion bioreactor inside an incubator. (D) Harvesting tissue samples after culture for downstream analysis. (E) Schematic overview of the complete experimental workflow, illustrating tissue preparation, perfusion bioreactor culture, immunotherapy administration (including immune checkpoint blockade and CAR T cell treatment), and subsequent tissue processing and functional analyses (This figure is created with BioRender.com). (F) Comparison of perfusion chamber: original collagen sponge vs. optimized agarose embedding setup. Reproduced from (97).

2.2.2 Single-cell suspension cell count and flow cytometry analysis

The tissue fragments obtained were finely cut with a scalpel and mechanically dissociated with the aid of a syringe plunger followed by several steps of different cell strainers with decreasing pore sizes (100 μm , 70 μm , and 40 μm ; pluriSelect) to prepare single-cell suspensions. For cell determination, 10 μL of each suspension was combined with an equal volume of trypan blue (Sigma-Aldrich, T8154) and total viable and non-viable cells were counted by a Neubauer hemocytometer (Hecht Assistent). To obtain the sample yield per bioreactor on the basis of cells, two of four cultured tissue fragments were separately dissociated and counted. These values were summed and extrapolated by a factor of two to obtain a more likely-to-be cellular expression for the same tissue fragments. Two freshly collected tissue fragments were processed in the same manner prior to culture for baseline measurements. The rest of the cell suspensions were fractionally transferred into polystyrene tubes (Falcon, 352058) and incubated in addition to fluorophore-conjugated primary antibodies and a DAPI/Annexin V staining mixture (30 minutes at 4 $^{\circ}\text{C}$). Step cleaning and antibody dilution were carried out with Annexin V binding buffer. Samples were analyzed by flow cytometry with a BD LSR II Flow Cytometer (BD Biosciences).

2.2.3 Checkpoint inhibitor treatment

Lymphoid tissues were cultured in bioreactors following the previously described protocol. On the first day of culture, the PD-1 inhibitor pembrolizumab (Selleckchem, A2005) was introduced into the culture medium at a final concentration of 10 $\mu\text{g/mL}$, with untreated tissues maintained under the same conditions serving as control samples. After 72 hours of culture, the tissues were collected for subsequent assessments of viability and immune profiling. Flow cytometry was conducted to determine tissue viability through Annexin V and DAPI staining and to analyze T cell-related markers. Additionally, fluorescence imaging was employed to evaluate overall tissue structure and visualize changes associated with treatment within the tumor microenvironment following exposure to pembrolizumab (**Figure 5E**).

2.2.4 Generation of CAR T Cells

CD19-CAR T cells were produced following established protocols (98,99). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained through leukapheresis and subsequently activated using anti-CD3/CD28 antibodies (MACS GMP Pure, Miltenyi Biotec). The transduction process utilized the RV-SFG.CD19.CD28.4-1BBzeta retroviral vector, which was supplied by Prof. Malcolm Brenner from Baylor College of Medicine in Houston, TX, USA. This vector includes an anti-CD19 scFv (FMC63). After transduction, the cells were maintained in a culture medium enriched with 10 ng/mL IL-7 and 5 ng/mL IL-15 (Miltenyi Biotec) for a duration of 13 days, with media changes occurring on days 7 and 10. Flow cytometry was employed to evaluate transduction efficiency, and the cells were cryopreserved once sterility was confirmed.

2.2.5 Generation of PI3K Enhanced CAR T Cells

PI3K-enhanced CAR T cells were produced utilizing base-editing techniques (Bucher et al. (100)). Briefly, the base editor plasmid pCMV-AncBE4max (Addgene #112095) was linearized with AgeI-HF (New England Biolabs, NEB, R3552) and subsequently transcribed *in vitro* using the T7 MEGAscript Kit (ThermoFisher, AMB13345). The resulting transcripts were capped with CleanCap AG (Tebubio, N-7113), appended with a PolyA tail (ThermoFisher, AM1350), and purified through overnight precipitation with LiCl (included in the T7 MEGAscript Kit). T cells were isolated, activated, and cultured following previously established protocols (103). After a 48-hour period, 3 million T cells were resuspended in 100 μ L of buffer T and electroporated with 10 μ g of AncBE4max mRNA and 5 μ g of each synthetic sgRNA (Integrated DNA Technologies, IDT) in IDTE pH 7.5 buffer (IDT, 11-05-01-05) using the Neon Transfection System (Thermo Fisher) set to 1400 V for 3 pulses of 10 ms each. For comparison, mock-edited T cells received a scrambled sgRNA sequence GCACTACCAGAGCTAACTCA. The E81K point mutation was introduced using the sgRNA sequence GCTCTTGCTGCTCCGCTGTC. Post-electroporation, the cells were allowed to rest in antibiotic-free X-Vivo15 medium (Lonza, BEBP02-061Q) supplemented with IL-7 and IL-15 at a concentration of 5 ng/mL for a duration of 24 hours. The retroviral transduction of the RV-SFG-CD19-4-1BB-CD3 ζ CAR construct was then carried out as outlined in previous studies (101–103).

Transduced CAR T cells underwent a resting phase of at least 48 hours prior to their application. The PI3K enhanced CAR T cells and the mock CAR T cells used in this study were provided by Prof. Dr. Judith Feucht and her colleagues.

2.2.6 CFSE labeling of CAR T Cells

The *CellTrace*[™] carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher, C34554) dye was utilized to track the localization of anti-CD19 CAR T cells within the bioreactor system. Each bioreactor received an injection of 1×10^6 T cells for each condition, which included non-transduced (NT) T cells, conventional CAR-T cells, and PI3K-CAR-T cells. While the total cell count in each lymph node (LN) explant varied, the overall post-culture count averaged around $\sim 7.5 \times 10^6$ cells per bioreactor. Given an estimated B cell frequency of approximately 15% based on flow cytometric analysis, this resulted in an effector-to-target (E:T) ratio close to $\sim 1:1$. In brief, aliquots of CAR T cells that had been preserved in liquid nitrogen were rapidly thawed using a 37 °C water bath. When available, freshly expanded CAR T cells were employed directly. Prior to labeling, the cells underwent two washes with PBS. CFSE was added at a concentration of 1 μ L per 1 mL of cell suspension and incubated at 37 °C for a duration of 10 minutes. The staining reaction was then halted by adding 100 μ L of fetal calf serum, followed by a single wash with PBS. The CAR T cells were subsequently resuspended in the final culture medium and transferred into the bioreactor system. Administration of CAR T cells occurred 24 hours after the initiation of bioreactor culture, with the cells remaining in the system for an additional 48 hours for subsequent analyses (**Figure 5E**).

2.2.7 Agarose-coated plate co-culture assay

To investigate the effects of agarose coating on the viability and functionality of CAR T cells, 24-well plates were prepared either with a coating of 150 μ L of 0.5% agarose (in PBS) or without any coating. CAR T cells or non-transduced T cells (4×10^5 cells per well) were co-cultured with diffuse large B cell lymphoma cell lines SU-DHL-2 or TMD-8, serving as target cells (also at 4×10^5 cells per well), in bioreactor culture medium. The co-cultures were maintained for 48 hours at a temperature of 37 °C and an atmosphere

of 5% CO₂. Following this incubation period, the viability of T cells and the cytotoxic activity of CAR T cells against the target cells were evaluated using flow cytometry.

2.2.8 Measurement of cytokines

The LEGENDplex™ Human CD8/NK Panel (13-plex, #741186; BioLegend) was prepared following the manufacturer's guidelines. In brief, samples, standards, and assay buffer were placed in a 96-well plate, which was then enhanced with fluorescence-labeled capture beads specific to the targeted cytokines. The plate underwent incubation for 2 hours at room temperature with gentle shaking to facilitate analyte binding. Following this incubation period, the wells were washed to eliminate unbound materials, and detection antibodies along with streptavidin-PE were added for an additional hour of incubation at room temperature. After a second wash step, acquisition beads were resuspended in sheath fluid. Data acquisition was performed using a FACS Canto II flow cytometer (BD Biosciences), and cytokine concentrations were determined through LEGENDplex™ data analysis software (BioLegend).

2.2.9 Immunofluorescence (IF) staining and imaging

Tissue specimens were embedded in Tissue-Tek O.C.T. compound, rapidly frozen, and sectioned to a thickness of 5.0 µm utilizing a cryostat (Leica, CM3050S) at a temperature of -20 °C. The sections were either preserved at -80 °C or processed right away for immunofluorescence staining. For the staining procedure, the sections underwent an initial fixation in acetone (neoFroxx, LC-4916.2) for 10 minutes at room temperature, followed by a secondary fixation with 1.6% paraformaldehyde (PFA) in PBS for another 10 minutes. Once fixed, the sections were blocked using 5% bovine serum albumin (BioSell) in PBS for a duration of 30 to 60 minutes at room temperature to minimize non-specific binding. Subsequently, primary antibodies were introduced and incubated for 1.5 hours at room temperature. After washing, fluorophore-conjugated secondary antibodies were applied and incubated for one hour in darkness at room temperature. Nuclei were counterstained with DAPI (0.5 µg/mL; BioLegend) for 10 minutes in PBS. Imaging was conducted using an inverted fluorescence microscope (BZ-X810, Keyence), equipped with a 20× CFI Plan Apo λ objective (Nikon).

Fluorescence signals corresponding to DAPI, CFSE, PE, and APC were captured employing the respective DAPI, GFP, TRITC, and Cy5 filter sets.

2.2.10 Hematoxylin and eosin (H&E) staining

One of the well-preserved 2.5 μm FFPE sections was chosen for histological staining from each sample. The slides were first baked at 70 °C for one hour, followed by deparaffinization in xylene for three cycles of ten minutes each. They were then rehydrated through a series of ethanol gradients: 100% ethanol for two cycles of three minutes, 95% ethanol for two cycles of three minutes, 80% ethanol for three minutes, and finally, 70% ethanol for three minutes. After these steps, the slides were rinsed with double-distilled water for six minutes total (two cycles of three minutes). Subsequently, the sections were applied with hematoxylin (Sigma-Aldrich, 1.09253) for 45 seconds and rinsed with 30 °C double-distilled water for another 45 seconds. Eosin (Sigma-Aldrich, 318906) applied and waited for 30 seconds. A brief wash in water was performed for 15 seconds before re-immersing the slides in isopropanol (SAV Liquid Production, 67-63-0) again for 45 seconds. The final steps included transferring the slides to xylene for another 45 seconds and applying Cytoseal (Epredia, 8312-4) to cover and mount them (Marienfeld Superior, 0101222). For quantitative histological analysis, both the total tissue area and necrotic regions were evaluated using QuPath (v0.3.0) (104). The entire tissue area was defined initially using the “Create Threshold” function; necrotic tissue areas were annotated manually with the “Brush” tool under the guidance of a board-certified pathologist (C.M.S).

2.2.11 Antibody conjugation

Purified, carrier-free (no BSA) antibodies were conjugated to maleimide-activated oligonucleotides using a thiol–maleimide coupling strategy. All centrifugation steps were performed at room temperature, and low-retention filter tips were used throughout. Antibodies were first concentrated and buffer-exchanged using 50 kDa molecular weight cutoff (MWCO) centrifugal filter units (Amicon Ultra, Merck, UFC505096). Prior to use, filters were pre-blocked with 500 μL PBS containing 0.1% Tween-20 and centrifuged at

12,000 × g for 2 min. The antibody solution was then loaded onto the column and centrifuged at 12,000 × g for 8 min to concentrate the sample.

To generate reactive thiol groups, antibodies were reduced by adding 360 µL of freshly prepared reduction buffer (1× PBS supplemented with 2.5 mM tris(2-carboxyethyl)phosphine (TCEP, Merck, 646547) and 2.5 mM EDTA. Samples were briefly mixed and incubated for 30 min at room temperature. Following reduction, excess TCEP was removed by two sequential washes with 400 µL Buffer C (consisted of 2 mM Tris pH 7.0–7.5, 150 mM NaCl, and 1 mM EDTA in Milli-Q water) using centrifugation at 12,000 × g for 8 min each. Maleimide-modified oligonucleotides (thaw from -20 °C) were dissolved in 1× PBS (final volume 400 µL) under a nitrogen atmosphere to minimize oxidation. The oligonucleotide solution was immediately added to the reduced antibody in the filter unit at a mass ratio of 2:1 (oligo:antibody). The mixture was briefly vortexed and incubated for 2 h at room temperature to allow conjugation. Excess oligonucleotides were removed by washing the conjugate three times with 450 µL high-salt PBS (consisted of 1× DPBS supplemented with 1 M NaCl), each followed by centrifugation at 12,000 × g for 8 min. Finally, the antibody–oligonucleotide conjugates were recovered by adding antibody stabilizer solution (Candor Bioscience, 131 050) at a ratio of 2:1 (200 µL per 100 µg antibody). The solution was passed over the membrane multiple times to ensure complete recovery and centrifuged at 3,000 × g for 2 min into a fresh collection tube. The final conjugates were stored at 4 °C until further use.

2.2.12 CODEX staining and imaging

As described before, CODEX staining and multicycle imaging (107) were performed. A 5 µm-thick section with intact, unfolded tissue morphology was taken per sample for analysis. Fresh-frozen sections on 22 × 22 mm Poly-L-Lysine-coated coverslips (Sigma-Aldrich; Marienfeld Superior, 0101052) were air-dried at room temperature for 2 min, fixed in acetone for 10 min, and lightly dried again. Surrounding each area of the sample, I created a hydrophobic barrier using a UV-curable glue pen (Bondic VIKO UG). Sections were rehydrated in S1 buffer and then fixed in 1.6% paraformaldehyde (PFA) for 30 min. To minimize autofluorescence, the samples were incubated twice in

bleaching solution (**Table 2**) , under light-emitting LED (Aibecy A5 Ultra Bright 25,000 Lux LED Pads) for 90 min, and fluid exchange after 45 min. Sections were washed in S1 buffer (2 × 2 min), blocked with a CODEX blocking solution with mouse and rat IgG (Biozol), salmon sperm DNA (Thermo Fisher), and non-fluorescent CODEX oligonucleotides (Biomers) for 1 h at room temperature. Primary antibody cocktails (**Table 1**) were added and incubated overnight at 4°C. Following 2 x 2 min washes in S2 buffer and fixation with S4 buffer containing 1.6% PFA for 10 min, the sections were subjected to ice cold methanol (Honeywell) for 5 min and fixed with freshly prepared 4 mg/mL bis(sulfosuccinimidyl)suberate (BS³, ThermoFisher) fixative in PBS for 20 min. After final PBS washes, sections were stored in buffer S4 until imaging. The detailed recipes for all the buffers mentioned above are provided in **Table 2**. CODEX multiplexed imaging was conducted using a Keyence BZ-X810 inverted fluorescence microscope equipped with a Nikon 20x CFI Plan Apo λ objective and a PhenoCycler system (Akoya Biosciences). The detailed imaging settings and data acquisition methods have been previously described (105,106). In short, initial and final cycles were acquired without fluorescent oligonucleotides to capture and subtract the background signal. Two markers per cycle were imaged using the TRITC and Cy5 channels, while Hoechst and CFSE were detected in the DAPI and GFP channels, respectively. 9 z-planes were taken each cycle at a standard step size of 1.5 μ m. The images were taken in a 3 × 3 tile with 20× magnification to provide complete tissue coverage, with exposure times adjusted individually for each marker. The raw data was processed with CODEX Processor software (Akoya Biosciences) for background subtraction, composite imagery, nuclear-based cell segmentation, and single-cell expression matrix (CSV) export. Overlaying images were constructed using ImageJ (Fiji, v1.53t). Cell annotation/x–y coordinate extraction was performed in QuPath (v0.3.0). In this investigation, immune cell classification was achieved through threshold-based analysis using the workflow "Classify → Object Classification → Create Single Measurement Classifier," where thresholds were set in collaboration with a board-certified pathologist and normalized during experimental replicates within each group (see figure legends for values). Cell–cell distance analysis was conducted using a custom Python-based algorithm.

2.2.13 Statistical analysis

Statistical analyses were performed utilizing GraphPad Prism software version 8.0.2 (GraphPad Software). To evaluate differences among samples, various statistical tests were employed based on the distribution and pairing of the data, including t-tests, repeated measures one-way ANOVA (accompanied by Tukey's post hoc test), and non-parametric methods (Wilcoxon signed-rank test, Friedman test, or Kruskal–Wallis test with Dunn's post hoc test). Normality of the data was evaluated prior to selecting appropriate tests. Effect sizes were indicated using metrics such as R squared, eta-squared (η^2), Wilcoxon r, or Kendall's W when relevant. A summary of all statistical tests conducted, assessments of normality, corrections for multiple comparisons, and effect size calculations is provided in **Table 4 (Summary of Statistics**, reproduced from (97)). A p-value of less than 0.05 was deemed statistically significant.

Table 4. Summary of Statistics

Plot	Normality test	Distribution	Statistical Tests	p-value	Effect Size Metric	Effect Size	Multiple comparison
Fig. 6A	Shapiro-Wilk test	Non-normal	Friedman test	0.6543	Kendall's W	0.063	Dunn's test
Fig. 6B	Shapiro-Wilk test	Normal	Paired t test	0.0044	R squared	0.7094	not applicable
Fig. 6C	Shapiro-Wilk test	Normal	Paired t test	0.0001	R squared	0.8913	not applicable
Fig. 6E	Shapiro-Wilk test	Lognormal	Lognormal RM ANOVA	0.0006	R squared	0.8448	Tukey's test
Fig. 7B	Shapiro-Wilk test	Non-normal	Wilcoxon test	0.1562	Wilcoxon r	0.714	not applicable
Fig. 7C	Shapiro-Wilk test	Non-normal	Friedman test	0.5	Kendall's W	0.75	Dunn's test
Fig. 8B	Shapiro-Wilk test	Lognormal	Lognormal RM ANOVA	0.0013	R squared	0.7496	Tukey's test
Fig. 8D	Shapiro-Wilk test	Non-normal	Friedman test	<0.0001	Kendall's W	1	Dunn's test
Fig. 9B	Shapiro-Wilk test	Lognormal	Lognormal RM ANOVA	<0.0001	R squared	0.8933	Tukey's test
Fig. 9D	Shapiro-Wilk test	Non-normal	Friedman test	0.0012	Kendall's W	0.796	Dunn's test
Fig. 10A	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.7467	R squared	0.0928	Tukey's test
Fig. 10B	Shapiro-Wilk test	Non-normal	Friedman test	0.2417	Kendall's W	0.056	Dunn's test
Fig. 10C	Shapiro-Wilk test	Non-normal	Friedman test	0.4321	Kendall's W	0.0375	Dunn's test
Fig. 10D	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.4627	R squared	0.2226	Tukey's test
Fig. 11A	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.0227	R squared	0.9529	not performed
Fig. 11C	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.0209	R squared	0.6854	not performed
Fig. 12A	Shapiro-Wilk test	Non-normal	Kruskal-Wallis test	0.4806	eta-squared (η^2)	0.0276	Dunn's test
Fig. 12C	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.0754	R squared	0.2761	Tukey's test
Fig. 13B	Shapiro-Wilk test	Non-normal	Wilcoxon test	0.3125	Wilcoxon r	1.8	not applicable
Fig. 13D	Shapiro-Wilk test	Normal	Paired t test	0.5904	R squared	0.0786	not applicable
Fig. 14B	Shapiro-Wilk test	Normal	Paired t test	0.6311	R squared	0.0631	not applicable
Fig. 14D	Shapiro-Wilk test	Normal	Paired t test	0.6046	R squared	0.073	not applicable
Fig. 15A	Shapiro-Wilk test	Normal	Unpaired t test	0.0002	R squared	0.777	not applicable

Fig. 15B	Shapiro-Wilk test	Normal	Unpaired t test	0.7894	R squared	0.0075	not applicable
Fig. 15C	Shapiro-Wilk test	Normal	Unpaired t test	0.0125	R squared	0.4804	not applicable
Fig. 15D	Shapiro-Wilk test	Normal	Unpaired t test	0.0108	R squared	0.4938	not applicable
Fig. 15E	Shapiro-Wilk test	Normal	Unpaired t test	0.1294	R squared	0.2145	not applicable
Fig. 19A	Shapiro-Wilk test	Normal	Paired t test	0.1106	R squared	0.5106	not applicable
Fig. 19B	Shapiro-Wilk test	Normal	Paired t test	0.2997	R squared	0.2616	not applicable
Fig. 19C	Shapiro-Wilk test	Lognormal	Ratio paired t test	0.2132	R squared	0.3536	not applicable
Fig. 19D	Shapiro-Wilk test	Normal	Paired t test	0.3534	R squared	0.2158	not applicable
Fig. 19E	Shapiro-Wilk test	Lognormal	Ratio paired t test	0.0227	R squared	0.7645	not applicable
Fig. 19F	Shapiro-Wilk test	Normal	Paired t test	0.8094	R squared	0.0163	not applicable
Fig. 20A	Shapiro-Wilk test	Normal	Paired t test	0.1246	R squared	0.4844	not applicable
Fig. 20B	Shapiro-Wilk test	Normal	Paired t test	0.2139	R squared	0.3527	not applicable
Fig. 20C	Shapiro-Wilk test	Normal	Paired t test	0.9303	R squared	0.0022	not applicable
Fig. 20D	Shapiro-Wilk test	Normal	Paired t test	0.5402	R squared	0.1006	not applicable
Fig. 20E	Shapiro-Wilk test	Normal	Paired t test	0.0332	R squared	0.7178	not applicable
Fig. 20F	Shapiro-Wilk test	Non-normal	Wilcoxon test	0.625	Wilcoxon r	0.3	not applicable
Fig. 21A	Shapiro-Wilk test	Normal	Paired t test	0.9796	R squared	0.0002	not applicable
Fig. 21B	Shapiro-Wilk test	Normal	Paired t test	0.932	R squared	0.0021	not applicable
Fig. 21C	Shapiro-Wilk test	Normal	Paired t test	0.9672	R squared	0.0005	not applicable
Fig. 21D	Shapiro-Wilk test	Non-normal	Wilcoxon test	0.4375	Wilcoxon r	0.42	not applicable
Fig. 21E	Shapiro-Wilk test	Normal	Paired t test	0.0553	R squared	0.642	not applicable
Fig. 21F	Shapiro-Wilk test	Normal	Paired t test	0.6425	R squared	0.0591	not applicable
Fig. 24B	Shapiro-Wilk test	Normal	Paired t test	0.0011	R squared	0.9459	not applicable
Fig. 24D	Shapiro-Wilk test	Normal	Paired t test	0.0111	R squared	0.833	not applicable
Fig. 24F	Shapiro-Wilk test	Normal	Paired t test	0.6664	R squared	0.0512	not applicable
Fig. 27A	Shapiro-Wilk test	Normal	Paired t test	0.0023	R squared	0.9694	not applicable
Fig. 27B	Shapiro-Wilk test	Normal	Paired t test	0.9073	R squared	0.0053	not applicable
Fig. 27C	Shapiro-Wilk test	Normal	Paired t test	0.0444	R squared	0.7881	not applicable
Fig. 27D	Shapiro-Wilk test	Normal	Paired t test	0.9097	R squared	0.005	not applicable
Fig. 27E	Shapiro-Wilk test	Normal	Paired t test	0.0416	R squared	0.7965	not applicable
Fig. 28C	Shapiro-Wilk test	Normal	Paired t test	0.013	R squared	0.9742	not applicable
Fig. 28F	Shapiro-Wilk test	Normal	Paired t test	0.0316	R squared	0.8294	not applicable
Fig. 30B	Shapiro-Wilk test	Lognormal	Ratio paired t test	0.0138	R squared	0.6636	not applicable
Fig. 31A	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.0146	R squared	0.8942	Tukey's test
Fig. 31B	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.004	R squared	0.9255	Tukey's test
Fig. 32B	Shapiro-Wilk test	Normal	RM one-way ANOVA	0.0079	R squared	0.9094	Tukey's test
Fig. 33B	Shapiro-Wilk test	Lognormal	Ratio paired t test	0.0121	R squared	0.9086	not applicable
Fig. 36A	Shapiro-Wilk test Kolmogorov-Smirnov test	Lognormal	Ratio paired t test	0.0009	R squared	0.1353	not applicable
Fig. 36B	Shapiro-Wilk test	Lognormal	Ratio paired t test	0.0783	R squared	0.6975	not applicable
Fig. 37A	Shapiro-Wilk test	Lognormal	Ratio paired t test	0.0092	R squared	0.9234	not applicable
Fig. 38B	Shapiro-Wilk test	Normal	RM one-way ANOVA	<0.0001	R squared	0.9433	Dunnett's test
Fig. 40A	Shapiro-Wilk test Kolmogorov-Smirnov test	Non-normal	Mann Whitney test	0.0014	rank-biserial	0.29	not applicable
Fig. 40B	not applicable	Ordinal	Chi-square test	0.0029	Cramér's V	0.25	not applicable
Fig. 42A	Shapiro-Wilk test	Normal	Paired t test	0.0415	R squared	0.5978	not applicable

Fig. 42B	Shapiro-Wilk test	Normal	Paired t test	0.0110	R squared	0.9780	not applicable
----------	-------------------	--------	---------------	--------	-----------	--------	----------------

3 Results

3.1 An optimized 3D perfusion bioreactor model to study human lymphoid tissues

This section is adapted from my first-author publication (97):

Zhang Y., et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors, *Theranostics*, 2026, 16 (8), 3928.

3.1.1 Study design and experiment workflow

3D perfusion bioreactor systems for tissue explants are important platforms for mimicking key aspects of the TME (85–87,107). To study lymphoma-associated TME in a controlled *ex vivo* setting, I began by optimizing culture conditions with human lymphoid tissues obtained from the Waldeyer's ring, including palatine tonsils and pharyngeal tonsils (adenoids), which are readily accessible and provide a consistent source of non-malignant lymphoid tissue. Inside the perfusion chamber of the bioreactor, a support structure consists of two vertically interconnected hollow adapters and nylon mesh filters. In early studies using the manufacturer's original protocol, where tissue fragments are cultured between two collagen sponges (108,109) (**see Methods and Figure 5F**), I found limited preservation of tissue viability over time, accompanied by pronounced efflux of immune cells from the tissue into the surrounding culture medium.

3.1.2 Optimized agarose embedding enhances cell viability and retention in tonsil tissue explants

Agarose, a soft, thermosensitive, and biocompatible natural hydrogel, can partially recapitulate features of the cellular microenvironment (110). This 0.5% agarose matrix has been widely deployed in 3D culture systems for numerous tissue types such as lymphoid tissues, and is referred to as a supportive scaffold by which tissue architecture and function is preserved (111–113). I supplanted the need for collagen sponges with

direct embedding of tissue fragments in 0.5% agarose in order to enhance overall culture stability and viability (**Figure 5E** and **Figure 5F**). To determine the measurable magnitude of this modification, I initially determined the full cell number in each of the bioreactors before and after culture. Single-cell suspensions were prepared by mechanically mincing the tissue samples, followed by gentle dissociation and filtration. Cells released into the culture medium were collected by centrifugation. Total cell numbers were determined using a hemocytometer under a light microscope. (**Figure 6A**). In the original setup, I found by cell counting experiments that excessive cell efflux was observed, with more than half of the cells leaving the tissue explants and entering the culture medium. This significantly altered the cellular composition of the tissue and disrupted the integrity of the tumor microenvironment, thereby reducing the physiological relevance of the model for investigating immune–tumor interactions and evaluating immunotherapeutic responses. This process, followed by optimization, led to a significant decrease in cellular efflux into the culture medium and increased proportion of cells remaining in the tissue compartment (**Figure 6B**). Importantly, agarose-based embedding was also associated with significantly improved cell viability after 3 days of culture, as demonstrated by trypan blue dead/live cells counting assays and flow cytometry analysis (**Figure 6C-E**), indicating enhanced preservation of both tissue integrity and cellular function under these optimized conditions. The raw data for Figure 6 is presented in **Tables 5-8**.

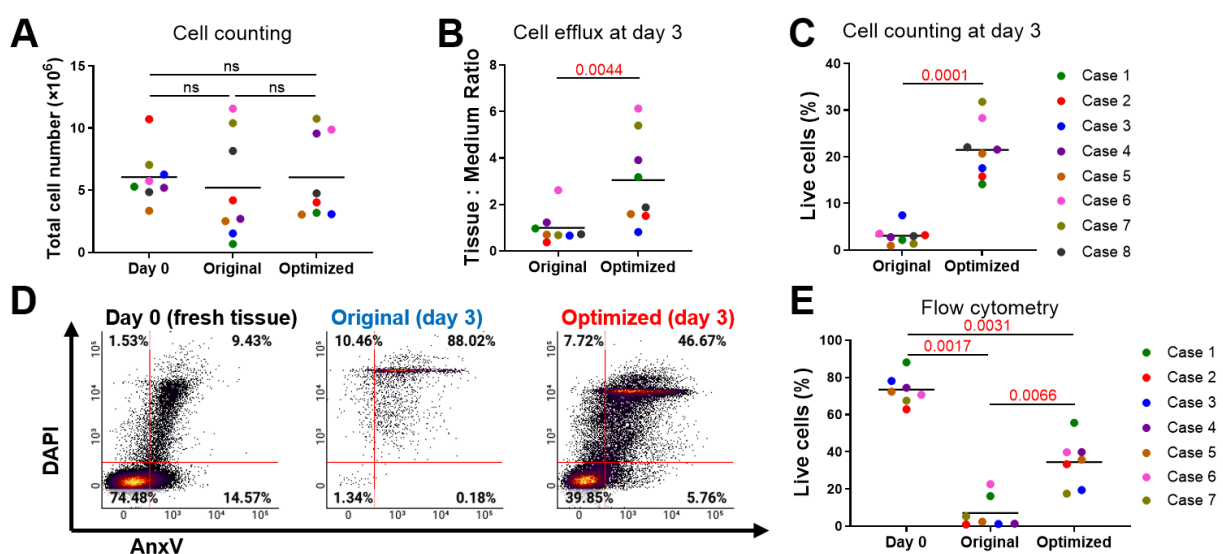


Figure 6. Optimized agarose embedding enhances cellular retention and viability in *ex vivo* tonsil tissue cultures.

(A) Quantification of total cell numbers per bioreactor pre- and post-culture, determined via direct cell counting (n = 8). (B) Efflux analysis demonstrating significantly reduced cell shedding into the culture medium from agarose-embedded tissues (n = 8). (C) Viability assessment via trypan blue exclusion staining, confirming superior tissue survival in agarose-embedded cases (n = 8). (D) Representative flow cytometry dot plots using DAPI and Annexin V to evaluate cellular viability. (E) Flow cytometric quantification validating enhanced retention of viable cells within the agarose-embedded tissue matrix (n = 7). Statistical analyses: (A) Friedman test followed by Dunn's post hoc test; (B, C) paired two-tailed t-test; (E) log-normal repeated measures one-way ANOVA with Tukey's post hoc test. Reproduced from (97).

Table 5. Raw data of Fig. 6A

	Day 0	Original	Optimized
Case 1	5.2972	0.6804	3.1968
Case 2	10.7146	4.1976	4.028
Case 3	6.2644	1.5272	3.078
Case 4	5.2072	2.7	9.5632
Case 5	3.358	2.52	3.0388
Case 8	4.8608	8.166	4.7616
Case 6	5.7424	11.5736	9.8832
Case 7	7.0432	10.41	10.756

Values in Table 5 indicate cell counting number (1×10^6).

Table 6. Raw data of Fig. 6B

	Original	Optimized
Case 1	0.973	3.176
Case 2	0.3787	1.507
Case 3	0.6628	0.8175
Case 4	1.2322	3.91015
Case 5	0.7064	1.591
Case 8	0.7225	1.8788
Case 6	2.6147	6.1325
Case 7	0.6837	5.3974

Values in Table 6 represent the ratio of cell counts in the tissue and the culture medium.

Table 7. Raw data of Fig. 6C

Original	Optimized
----------	-----------

Case 1	2.137437	14.12199
Case 2	3.191879	15.79157
Case 3	7.44879	17.54459
Case 4	2.73626	21.56124
Case 5	0.919167	20.6853
Case 8	2.969144	22.03337
Case 6	3.530774	28.32569
Case 7	1.351485	31.79183

Values in Table 7 indicate the percentage of live cells among all cells by cell counting.

Table 8. Raw data of Fig. 6E

	Day 0	Original	Optimized
Case 1	88.16	16.22	55.61
Case 2	62.84	0.92	33.34
Case 3	78.16	1.18	19.49
Case 4	74.48	1.34	39.85
Case 5	72.36	2.45	35.82
Case 6	70.73	22.59	39.78
Case 7	67.54	5.32	17.56

Values in Table 8 indicate the percentage of live cells among all cells by flow cytometry.

To further evaluate tissue preservation following culture, tonsil explants from each experimental group were fixed, paraffin-embedded, sectioned, and subjected to H&E staining. Histological examination demonstrated that agarose-embedded tissue fragments exhibited smaller necrotic areas than tissues cultured using collagen sponge support, indicating improved preservation of tissue morphology and viability after culture (**Figure 7A**). To objectively quantify these differences, the necrotic area and total tissue area were measured using QuPath software, and the proportion of necrosis was calculated as the ratio of necrotic area to total tissue area. Consistent with the qualitative observations, agarose embedding resulted in a lower percentage of necrotic tissue compared with the collagen sponge condition (**Figure 7B**).

As part of the optimization process, Basement Membrane Extract (BME), another ECM-based scaffold, was also evaluated as an alternative embedding material. However, BME did not improve post-culture tissue viability compared with either the

original collagen sponge support or the agarose-based system (**Figure 7C**). A possible explanation is that the soft and loosely structured nature of BME provides insufficient mechanical support to maintain tissue integrity during long-term perfusion culture. Therefore, BME was not investigated further. **Tables 9–10** contain the raw data for Figure 7.

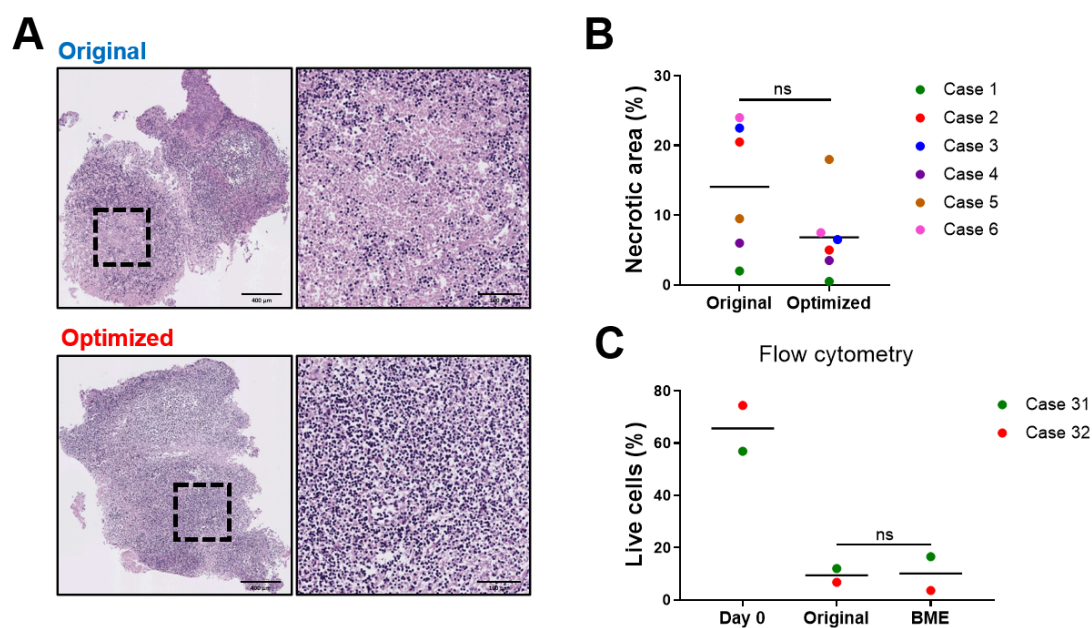


Figure 7. Effect of embedding matrices on tissue viability. (A) H&E staining illustrates necrotic regions within tissue sections. Scale bars represent 400 μ m under low magnification, and 100 μ m under high magnification. (B) Quantification of necrotic areas in post-culture tissue fragments maintained with or without agarose embedding (n = 6). (C) Evaluation of BME as an alternative ECM for tissue embedding (n = 2). Statistical analyses: (B) Wilcoxon signed-rank test; (C) Friedman test followed by Dunn's post hoc test. Reproduced from (97).

Table 9. Raw data of Fig. 7B

	Original	Optimized
Case 1	2.0	0.5
Case 2	20.5	5.0
Case 3	22.5	6.5
Case 4	6.0	3.5
Case 5	9.5	18.0
Case 6	24.0	7.5

Values in Table 9 indicate the percentage of necrotic area among the whole tissue area.

Table 10. Raw data of Fig. 7C

	Fresh tissue	Original	BME
Case 31	56.89	12.05	16.59
Case 32	74.42	6.76	3.6

Values in Table 10 indicate the percentage of live cells among all cells.

3.1.3 Agarose embedding improves the preservation of immune cell subsets in tonsil tissue cultures

Consequently, flow cytometric analysis showed that the major lymphoid immune cell populations were better preserved under agarose-based embedding conditions. Compared to collagen sponge support, the presence of agarose in this media resulted in significantly higher frequencies and viabilities of both CD3⁺ T cells (**Figure 8A-D**) and CD19⁺ B cells (**Figure 9A-D**). Notably, in cultured tonsil tissues, the viability and especially frequency of B cells was consistently lower than that of T cells across all experimental groups. Nevertheless, agarose embedding significantly improved B cell viability and frequency compared with the original bioreactor setup, indicating that the optimized culture conditions partially alleviated the loss of B cell viability during *ex vivo* culture. The results prove that our improved culture system better maintains survival of resident lymphocytes during perfusion culture under *ex vivo* conditions.

Interestingly, both T cell and B cell populations exhibited better preserved frequency and viability than those from the collagen sponge group or, at the least, maintained values comparable to or greater than those measured in freshly processed tissue immediately post-surgical resection. This suggests that the optimized agarose-based bioreactor system not only provides short term cell survival, but also stabilizes in-the-culture immune cell composition. **Tables 11–12** contain the raw data for Figure 8 and **Tables 13–14** contain the raw data for Figure 9.

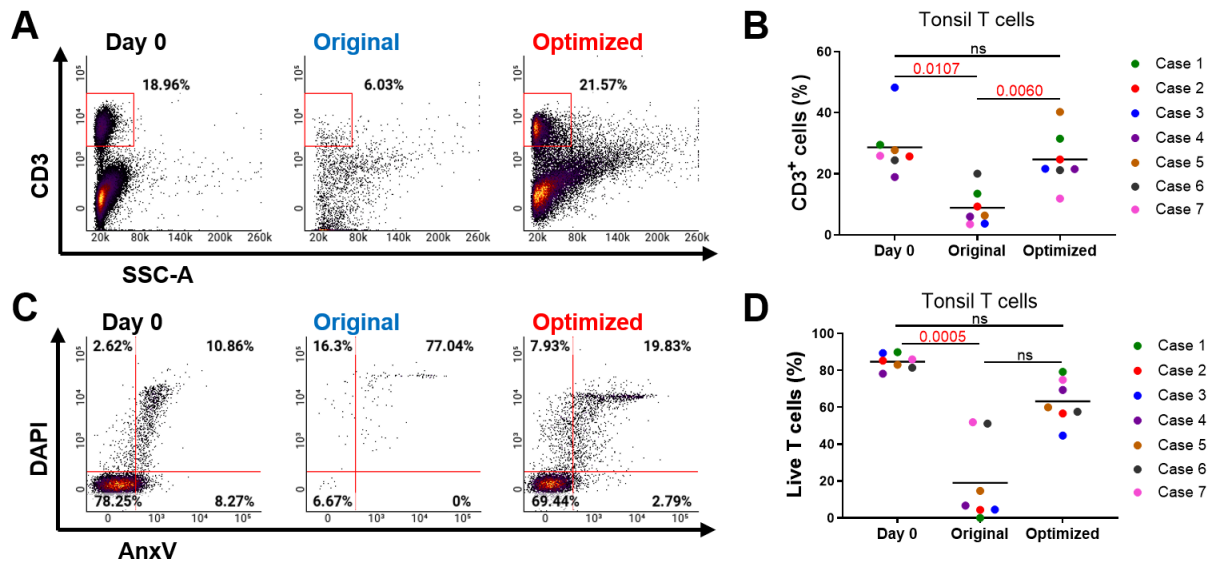


Figure 8. Agarose embedding preserves T cell subsets in ex vivo cultures.

(A) Gating strategy for CD3⁺ T cells shown via representative flow cytometry plots. (B) T cell frequency quantification across the different experimental conditions. (C) Representative DAPI/Annexin V dot plots evaluating T cell viability. (D) Flow cytometric data confirming an increase in T cell viability. (E) Representative plots showing CD19⁺ B cell populations. Statistical analyses: Data in (B) were analyzed by log-normal repeated measures one-way ANOVA with Tukey's post hoc test; data in (D) were analyzed using a Friedman test with Dunn's post hoc test. Reproduced from (97).

Table 11. Raw data of Fig. 8B

	Day 0	Original	Optimized
Case 1	29.51	13.51	31.5
Case 2	25.73	9.31	24.74
Case 3	48.25	3.72	21.7
Case 4	18.96	6.03	21.57
Case 5	27.76	6.38	40.29
Case 6	24.48	20.09	21.21
Case 7	25.94	3.52	11.9

Values in Table 11 indicate the percentage of CD3⁺ T cells among all cells.

Table 12. Raw data of Fig. 8D

	Day 0	Original	Optimized
Case 1	89.97	0	79.29
Case 2	85.39	4.4	56.67

Case 3	89.44	4.55	44.73
Case 4	78.25	6.67	69.44
Case 5	83.09	14.69	60.03
Case 6	81.51	51.17	57.65
Case 7	86.03	51.92	74.94

Values in Table 12 indicate the percentage of live CD3⁺ T cells among all CD3⁺ T cells.

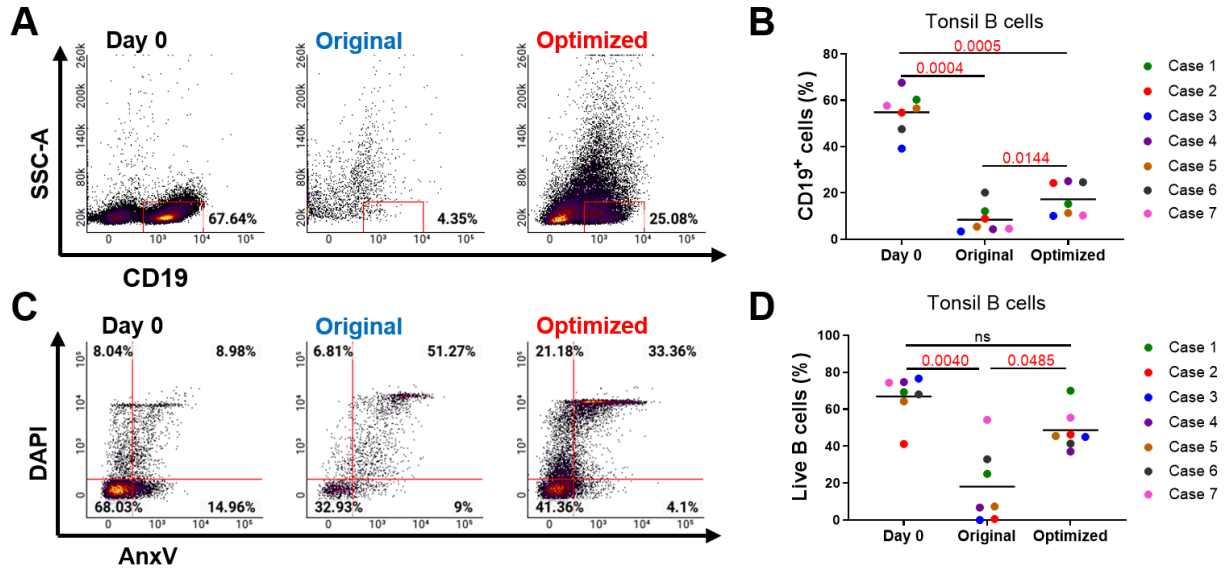


Figure 9. Agarose embedding preserves B cell subsets in ex vivo cultures.

(A) Representative flow cytometry plots identifying CD19⁺ B cells. (B) Quantification of B cell frequencies across conditions. (C) Representative DAPI/Annexin V dot plots evaluating B cell viability. (D) Quantitative analysis confirming changes in B cell viability (n = 7). Statistical analyses: Data in (B) were analyzed by log-normal repeated measures one-way ANOVA with Tukey's post hoc test; data in (D) were analyzed using a Friedman test with Dunn's post hoc test. Reproduced from (97).

Table 13. Raw data of Fig. 9B

	Day 0	Original	Optimized
Case 1	60.29	12.12	15.3
Case 2	54.71	8.9	24.32
Case 3	39.15	3.35	10.1
Case 4	67.64	4.35	25.08
Case 5	56.54	5.43	11.38
Case 6	47.59	20.16	24.63
Case 7	57.68	4.54	10.27

Values in Table 13 indicate the percentage of CD19⁺ T cells among all cells.

Table 14. Raw data of Fig. 9D

	Day 0	Original	Optimized
Case 1	69.28	25	70.03
Case 2	41.18	0.63	46.25
Case 3	76.61	0	45.08
Case 4	74.7	6.82	37.09
Case 5	64.3	7.38	45.57
Case 6	68.03	32.93	41.36
Case 7	74.37	54.26	55.49

Values in Table 14 indicate the percentage of live CD19⁺ T cells among all CD19⁺ T cells.

To investigate whether immune cell viability could be further improved during *ex vivo* perfusion culture, different cytokine supplementation strategies were evaluated in the bioreactor system. Different cytokine supplementation strategies in the bioreactor system were tested. Separate experimental groups were formed and supplemented with IL-2, IL-7, IL-4, IL-6, or combinations of these cytokines during the culture period. Flow cytometry showed that there was no significant increase in T cell or B cell viability in any of the tested cytokine conditions compared with the cytokine-free control (**Figure 10A-D**). While no differences were found, cultures supplemented with IL-2 or IL-7 consistently exhibited a modest trend toward higher T cell viability. Similarly, IL-4 and IL-6 showed little or no beneficial effect. By contrast, B cell viability did not significantly differ across all treatment groups, indicating that cytokine supplementation alone was insufficient to increase B cell survival under the current culture conditions. Based on the favorable trend observed for T cell preservation, IL-2 and IL-7 were selected for inclusion in the subsequent experiments. The raw data mapping to Figure 10 can be found in **Tables 15–18**.

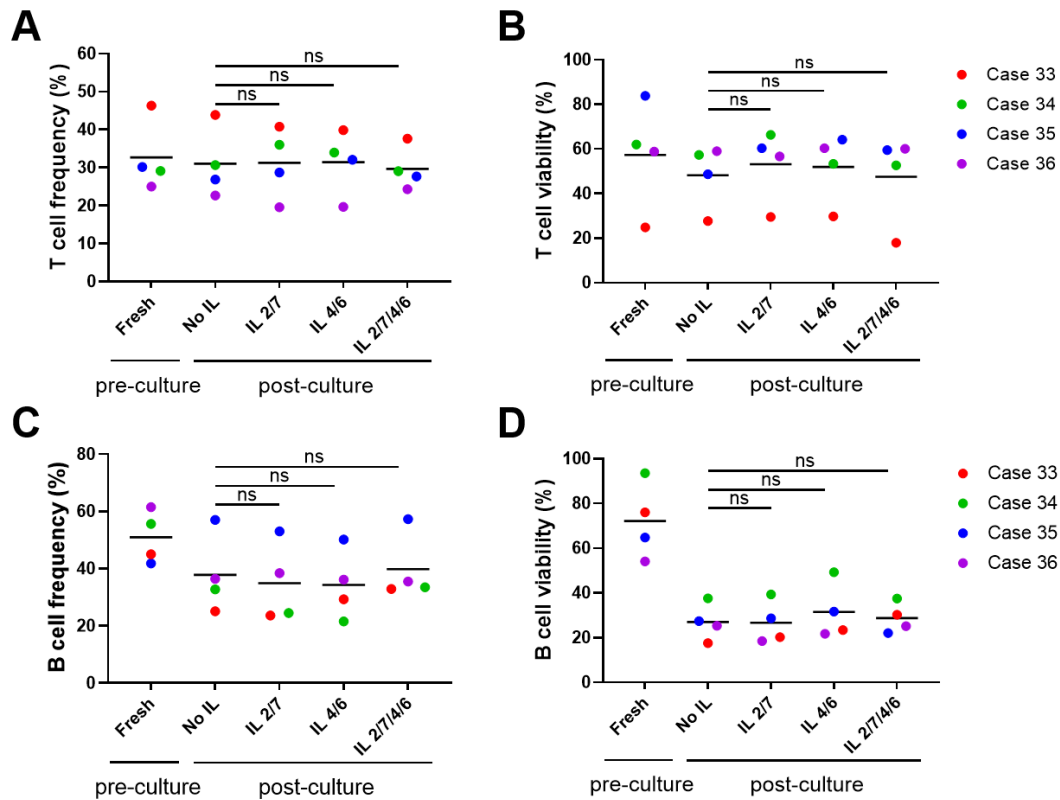


Figure 10. Effect of cytokine supplementation on T and B cell viability in bioreactor cultures.

(A–B) Flow cytometric evaluation of CD3⁺ T cell frequency and viability in human tonsil tissues following a 3-day perfusion bioreactor culture with varied cytokine combinations. (C–D) Flow cytometric tracking of CD19⁺ B cells under identical experimental conditions. Statistical analyses: Data in (A, D) were evaluated by repeated measures one-way ANOVA with Tukey's post hoc test; data in (B, C) were analyzed using a Friedman test with Dunn's post hoc test. Reproduced from (97).

Table 15. Raw data of Fig. 10A

	Fresh	No IL	IL 2/7	IL 4/6	IL 2/7/4/6
Case 33	46.32	43.85	40.76	39.86	37.62
Case 34	29.12	30.69	36.03	33.98	29.08
Case 35	30.19	26.88	28.74	32.08	27.67
Case 36	25.02	22.67	19.59	19.69	24.34

Values in Table 15 indicate the percentage of CD3⁺ T cells among all cells.

Table 16. Raw data of Fig. 10B

	Fresh	No IL	IL 2/7	IL 4/6	IL 2/7/4/6
Case 33	24.85	27.69	29.54	29.83	17.93

Case 34	62.05	57.34	66.35	53.38	52.72
Case 35	83.91	48.7	60.37	64.18	59.5
Case 36	58.85	59.03	56.71	60.38	60.16

Values in Table 16 indicate the percentage of live CD3⁺ T cells among all CD3⁺ T cells.

Table 17. Raw data of Fig. 10C

	Fresh	No IL	IL 2/7	IL 4/6	IL 2/7/4/6
Case 33	45	25.09	23.63	29.31	32.92
Case 34	55.63	32.76	24.47	21.59	33.5
Case 35	41.81	57.05	53.03	50.18	57.33
Case 36	61.47	36.43	38.43	36.15	35.53

Values in Table 17 indicate the percentage of CD19⁺ T cells among all cells.

Table 18. Raw data of Fig. 10D

	Fresh	No IL	IL 2/7	IL 4/6	IL 2/7/4/6
Case 33	76.07	17.63	20.29	23.42	30.32
Case 34	93.51	37.62	39.34	49.33	37.53
Case 35	64.82	27.47	28.67	31.7	22.12
Case 36	54.15	25.41	18.54	21.76	25.2

Values in Table 18 indicate the percentage of live CD19⁺ T cells among all CD19⁺ T cells.

3.1.4 Bioreactors outperform static culture for tissue explant culture

In order to examine the relative advantages of dynamic perfusion over standard static culture, tonsil explants were cultured under four different conditions: static plate culture with or without agarose embedding, and perfusion bioreactor culture with either collagen sponge or agarose support. Cell viability was then compared for all experimental groups.

Cell viability was highest among all conditions, where tissue seeded in the agarose-embedded perfusion bioreactor gained the highest viability (**Figure 11A-C**). In comparison to both collagen sponge-supported bioreactor and more typical static plate cultures, dynamic perfusion with agarose embedding resulted in higher tissue viability, suggesting that the combined effect of an optimized embedding strategy and continuous perfusion resulted in increased preservation of lymphoid tissue during *ex vivo* culture. On the other hand, static culture was found to display consistently lower cell viability

irrespective of embedding material, demonstrating the limitation of diffusion-dependent cultivation systems to maintain intact tissue explants.

While no clear signals of nutrient loss were noted by static cultures, it should be noted that culture medium volume was different between systems (3 mL vs 10 mL in the perfusion bioreactor). Thus, the superior performance of the perfusion system can probably be due to better mass transport and dynamic nutrient exchange; however, a possibility for medium volume is also not excluded. The raw data mapping to Figure 11 can be found in **Tables 19–20**.

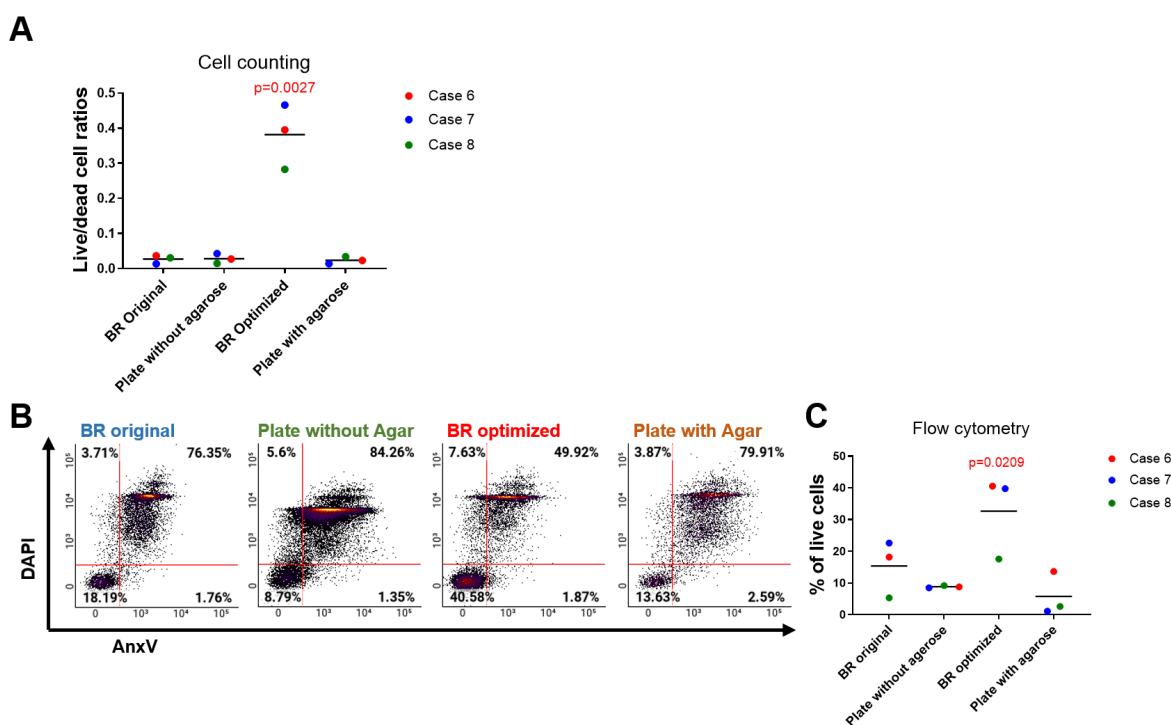


Figure 11. Evaluation of static plate culture versus 3D perfusion bioreactor platforms for tonsil tissue cultivation.

(A) Direct cell counting quantification and (B, C) flow cytometric analysis comparing the bioreactor system against static plate culture setups, evaluated in both the presence and absence of agarose embedding (n = 3). Statistical analysis: Repeated measures one-way ANOVA. Reproduced from (97).

Table 19. Raw data of Fig. 11A

	BR original	Plate without agerose	BR optimized	Plate with agarose
Case 6	0.0366	0.0272	0.3952	0.023

Case 7	0.0137	0.0425	0.4661	0.0139
Case 8	0.0306	0.0149	0.2826	0.0339

Values in Table 19 indicate the ratio of live cell number to dead cell number by cell counting.

Table 20. Raw data of Fig. 11C

	BR original	Plate without agerose	BR optimized	Plate with agarose
Case 6	18.19	8.79	40.58	13.63
Case 7	22.59	8.47	39.78	1.12
Case 8	5.32	9.17	17.56	2.59

Values in Table 20 indicate the percentage of live cells among all cells by flow cytometry.

3.2 Bioreactor culture for lymph node tissue

This section is adapted from my first-author publication (97) :

Zhang Y., et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors, *Theranostics*, 2026, 16 (8), 3928.

3.2.1 Bioreactors preserve lymph node explant viability

After optimization of culture conditions with tonsils, the bioreactor platform was then utilized on lymph node (LN) samples to determine the appropriateness of the bioreactor for the application to clinically relevant tissues. Given that lymph nodes were the main tissue in the subsequent immunotherapy studies, it was necessary to ascertain if comparable tissue viability and immune cell preservation could be maintained under optimized culture conditions.

For preliminary evaluation, enumeration of the total number of viable cells retrieved from each bioreactor was performed pre- and postculture (**Figure 12A**). Cell recovery was comparatively constant during the culture period, suggesting that the optimal perfusion conditions were able to preserve overall tissue integrity with minimal cellular loss.

Flow cytometric analysis also showed that LN explants retained good cell viability during the culture period (**Figure 12B-C**). Even though cell viability decreased gradually on days 3 to 5, tissue culturing on day 3 had similar viability values compared to freshly

isolated samples taken immediately upon operation (day 0). At day 5, a slight decrease in viability occurred, indicative of early tissue degradation during prolonged culture. Depending on these observations, a culture duration of 3 days was chosen across all following immunotherapy trials as this resulted in the best compromise between tissue preservation and adequate experimental treatment. The raw data for Figure 12 is presented in **Tables 21-22**.

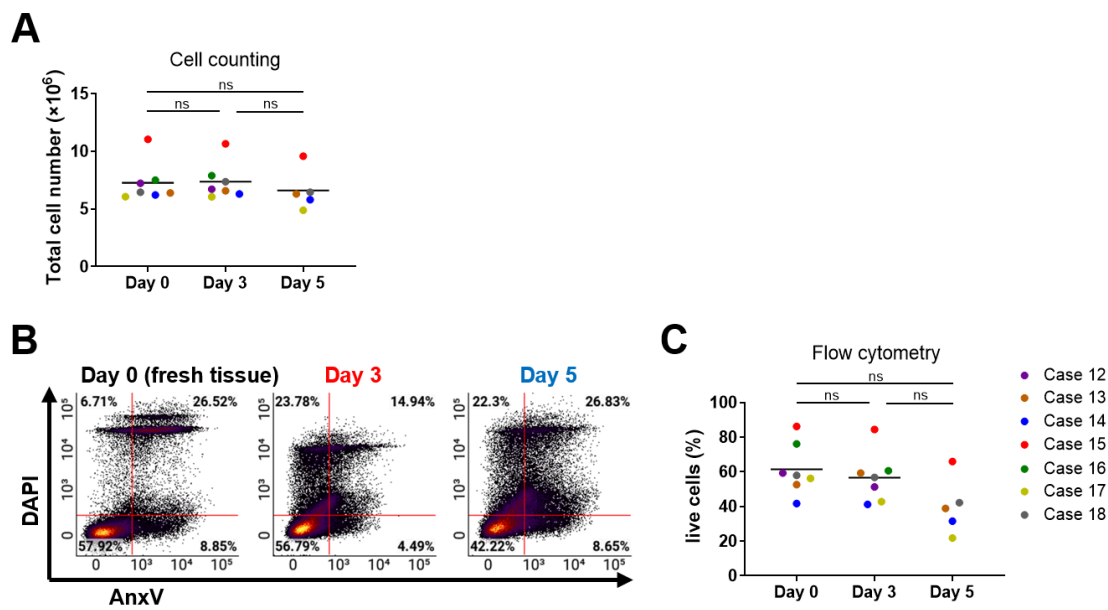


Figure 12. Improving lymph node tissue culture via optimized bioreactor conditions.

(A) Total cell counts were estimated in each bioreactor prior to and following the culture period, based on cell counting ($n = 5-7$). (B) Flow cytometry plots are presented as representatives, while (C) viability assessments of LN tissues were conducted at day 0 and after 3–5 days of culture ($n = 5-7$). Statistical Analysis: For (A), the Kruskal–Wallis test was used along with Dunn’s multiple comparisons; for (C), one-way ANOVA was employed with Tukey’s post hoc test. Reproduced from (97).

Table 21. Raw data of Fig. 12A

	Fresh tissue	Day 3	Day 5
Case 13	6.392	6.578	6.306
Case 14	6.214	6.304	5.81
Case 15	11.042	10.652	9.586
Case 17	6.066	6.058	4.894
Case 18	6.456	7.37	6.462

Case 12	7.234	6.714
Case 16	7.502	7.894

Values in Table 21 indicate cell counting number (1×10^6).

Table 22. Raw data of Fig. 12C

	Fresh tissue	Day 3	Day 5
Case 13	52.63	59.33	38.84
Case 14	41.71	41.18	31.54
Case 15	86.19	84.42	65.99
Case 17	56.2	42.78	21.83
Case 18	57.92	56.79	42.22
Case 12	59.25	51.26	
Case 16	76.12	60.59	

Values in Table 22 indicate the percentage of live cells among all cells by flow cytometry.

In order to better characterize the preservation of immune cell populations in cultured lymph node explants, flow cytometry of T and B lymphocytes was performed at 3 days of perfusion culture for frequency and viability analysis. CD3⁺ T cells were also the predominant immune cell population in this culture, which manifested frequencies (40% to 60%) and viabilities (60% to 80%). These results show that under the optimized perfusion culture conditions, both the abundance and viability of T cells were preserved across *ex vivo* culture (**Figure 13A-D**).

A similar preservation profile was noticed for CD19⁺ B cells. The frequency and viability of B cells also remained relatively constant at 3 days of culture with only minor changes from the freshly processed lymph node samples (**Figure 14A-D**). Collectively, these data show that the optimized bioreactor system preserves the primary lymphoid immune cell populations while preserving their viability, offering a viable platform for subsequent immunotherapy studies. **Tables 23–24** contain the raw data for Figure 13-14.

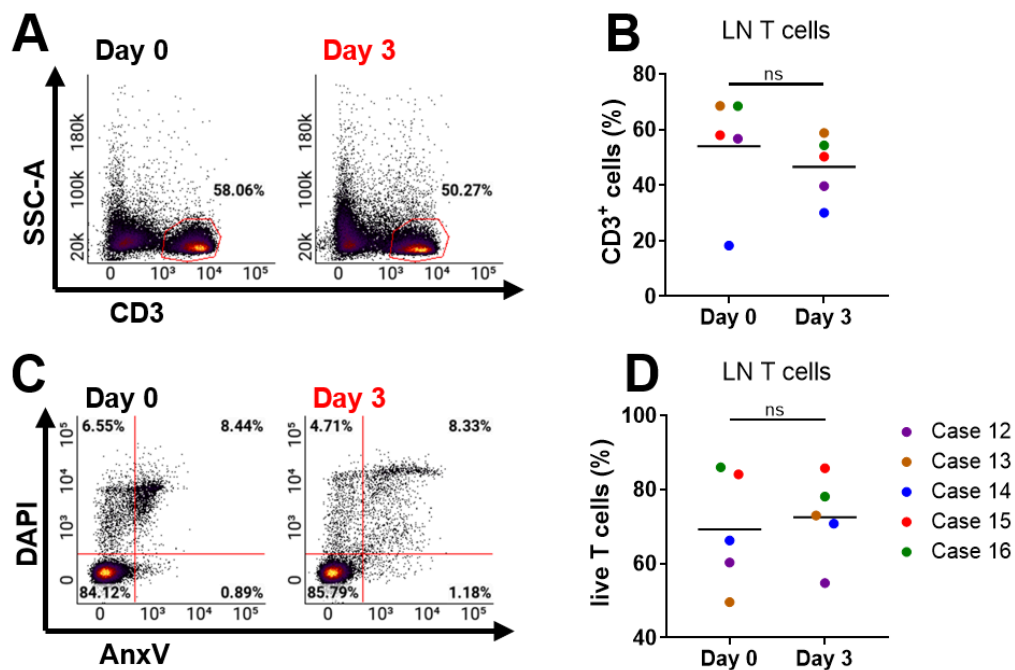


Figure 13. Preservation of T cell subsets in LN tissue.

(A–B) Frequencies and (C–D) viabilities of T cells monitored in LN cultures between day 0 and day 3 (n = 5). Statistical analysis: (B) Wilcoxon matched-pairs signed-rank test; (D) two-tailed paired t-test. Reproduced from (97).

Table 23. Raw data of Fig. 13

Fig.B	Fresh tissue	Day 3	Fig.D	Fresh tissue	Day 3
Case 16	68.52	54.35	Case 16	86.02	78.12
Case 15	58.06	50.27	Case 15	84.12	85.79
Case 14	18.29	30.13	Case 14	66.3	70.84
Case 13	68.54	58.84	Case 13	49.63	73.03
Case 12	56.76	39.73	Case 12	60.31	54.76

Left: Values indicate the percentage of CD3⁺ T cells among all cells. Right: Values indicate the percentage of live CD3⁺ T cells among all CD3⁺ T cells.

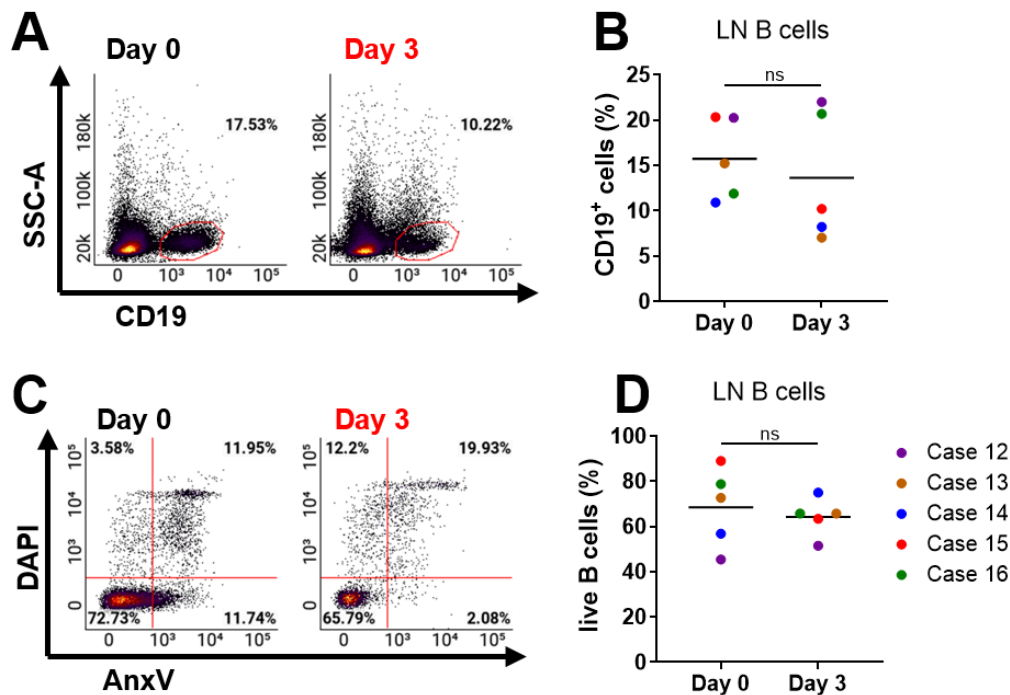


Figure 14. Preservation of B cell subsets in LN tissue.

(A–B) B cell frequencies and (C–D) viabilities over the identical culture period (n = 5). Statistics: Paired t-test. Reproduced from (97).

Table 24. Raw data of Fig. 14

Fig.B	Fresh tissue	Day 3	Fig.D	Fresh tissue	Day 3
Case 16	11.91	20.35	Case 16	78.75	65.8
Case 15	17.53	10.22	Case 15	89.08	63.45
Case 14	10.92	8.24	Case 14	56.85	75.07
Case 13	15.23	7.05	Case 13	72.73	65.79
Case 12	20.26	22.01	Case 12	45.49	51.51

Left: Values indicate the percentage of CD19⁺ T cells among all cells. Right: Values indicate the percentage of live CD19⁺ T cells among all CD19⁺ T cells.

3.2.2 Lymph node explants show superior viability to tonsil tissue in bioreactor culture

The viability and immune cell composition of cultured tonsil and LN explants were directly analysed to assess whether a particular lymphoid tissue is appropriate for *ex vivo* culture compared. Due to the heterogeneities in their initial cellular composition and

viability, all post-culture measurements of cell frequency and viability were adjusted to the freshly processed tissue from the same donor.

Overall, LN explants better maintained tissue viability compared to tonsil explants after perfusion culture (**Figure 15A**). Even though CD3⁺ T cell frequencies maintained the same between the two tissues (**Figure 15B**), LN samples exhibited significantly higher T cell viability (**Figure 15C**). Furthermore, LN tissues demonstrated markedly improved preservation of the B cell compartment. In contrast to tonsil explants, in which B cell viability declined substantially during culture, LN explants maintained both a higher frequency of CD19⁺ B cells (**Figure 15D**) and a trend toward improved B cell viability (**Figure 15E**). The raw data for Figure 15 is presented in **Tables 25-29**.

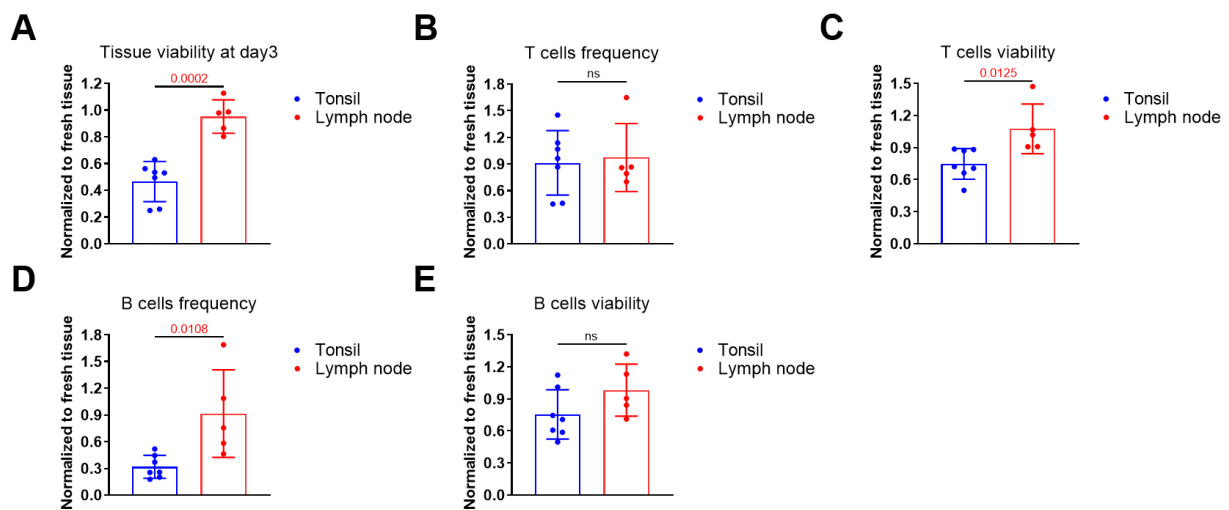


Figure 15. Differential immune cell viability in lymphoid tissues.

(A) Overall tissue viability, (B) T cell frequency, (C) T cell viability, (D) B cell frequency, and (E) B cell viability was compared between LN and tonsil tissues, normalized to fresh tissue values (n = 5-7). Statistics: unpaired t-test. Reproduced from (97).

Table 25. Raw data of Fig. 15A

Tonsil		Lymph node	
Case 1	0.630785	Case 14	0.987293
Case 2	0.530554	Case 15	0.979464
Case 3	0.24936	Case 13	1.127304
Case 4	0.535043	Case 18	0.803204

Case 5	0.495025	Case 12	0.865148
Case 6	0.56242		
Case 7	0.259994		

Values represent tissue viability (live cell number / total cell number) normalized to the corresponding fresh tissue.

Table 26. Raw data of Fig. 15B

Tonsil		Lymph node	
Case 1	1.067435	Case 14	0.792761
Case 2	0.961524	Case 15	0.865828
Case 3	0.449741	Case 13	1.647348
Case 4	1.137658	Case 18	0.858477
Case 5	1.451369	Case 12	0.699965
Case 6	0.866422		
Case 7	0.458751		

Values represent T cell frequency (T cell number / total cell number) normalized to the corresponding fresh tissue.

Table 27. Raw data of Fig. 15C

Tonsil		Lymph node	
Case 1	0.881294	Case 14	0.909123
Case 2	0.663661	Case 15	1.019853
Case 3	0.500112	Case 13	1.068477
Case 4	0.887412	Case 18	1.471489
Case 5	0.72247	Case 12	0.907975
Case 6	0.707275		
Case 7	0.871091		

Values represent T cell viability (live T cell / total T cell) normalized to the corresponding fresh tissue.

Table 28. Raw data of Fig. 15D

Tonsil		Lymph node	
Case 1	0.253773	Case 14	1.687602
Case 2	0.444526	Case 15	0.583001
Case 3	0.257982	Case 13	0.754579
Case 4	0.370787	Case 18	0.462902
Case 5	0.201273	Case 12	1.086377
Case 6	0.517546		

Case 7 0.178051

Values represent B cell frequency (B cell number / total cell number) normalized to the corresponding fresh tissue.

Table 29. Raw data of Fig. 15E

Tonsil		Lymph node	
Case 1	1.010826	Case 14	0.842499
Case 2	1.123118	Case 15	0.712281
Case 3	0.588435	Case 13	1.320493
Case 4	0.496519	Case 18	0.904579
Case 5	0.708709	Case 12	1.132337
Case 6	0.607967		
Case 7	0.746134		

Values represent B cell viability (live B cell / total B cell) normalized to the corresponding fresh tissue.

Altogether, these findings suggest that lymph node tissue is a more appropriate explant model for subsequent immunotherapy studies. Lymph nodes are more clinically relevant to cancer immunology than tonsil tissue, since they are the primary site of many lymphoid malignancies and a common site of tumor metastasis and immune activation. Coupled with their better preservation of overall tissue viability and better maintenance of both T and B cell populations, lymph nodes afford a more physiologically relevant immune microenvironment for investigating immunotherapeutic responses.

3.2.3 Preservation of cellular diversity and tissue architecture within 3D perfusion bioreactors

To comprehensively characterize the immune cell composition preserved within cultured lymph node explants, single-cell suspensions were analyzed by multiparametric flow cytometry using three independent antibody panels. A standardized gating strategy was applied to exclude doublets and cellular debris, followed by sequential identification of the major immune cell populations based on their surface marker expression. As shown in **Figure 16**, CD3⁺ T cells were further subdivided into CD4⁺ helper T cells, CD8⁺ cytotoxic T cells, and CD3⁻CD56⁺ natural killer (NK) cells. **Figure 17** illustrates the gating strategy used to identify CD19⁺ B cells, CD68⁺ macrophages, CD123⁺

plasmacytoid dendritic cells (pDCs), and CD11c⁺ conventional dendritic cells (cDCs). Finally, **Figure 18** shows the gating strategy used to distinguish CD45⁺ white blood cells (WBCs) from CD45⁻ non-immune cells. Together, these standardized gating strategies enabled robust and reproducible identification of the major immune cell populations throughout the subsequent functional analyses.

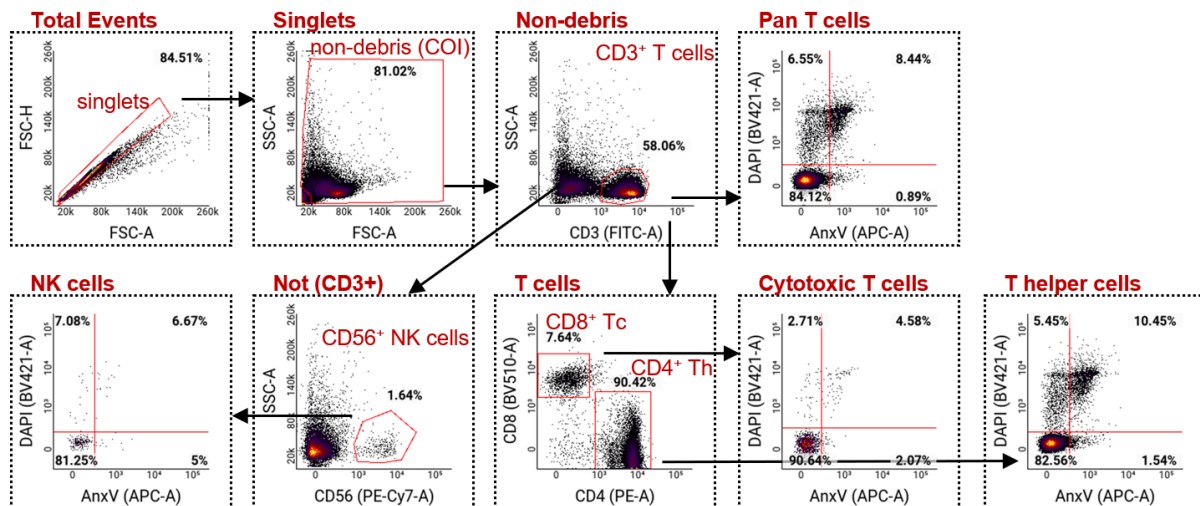


Figure 16. Representative flow cytometry gating tree for immune cell subpopulations (Panel 1).

After excluding doublets and cell debris, I applied a gating strategy to identify CD3⁺ T cells, CD4⁺ T helper cells, CD8⁺ cytotoxic T cells, and CD3⁻CD56⁺ NK cells. Reproduced from (97).

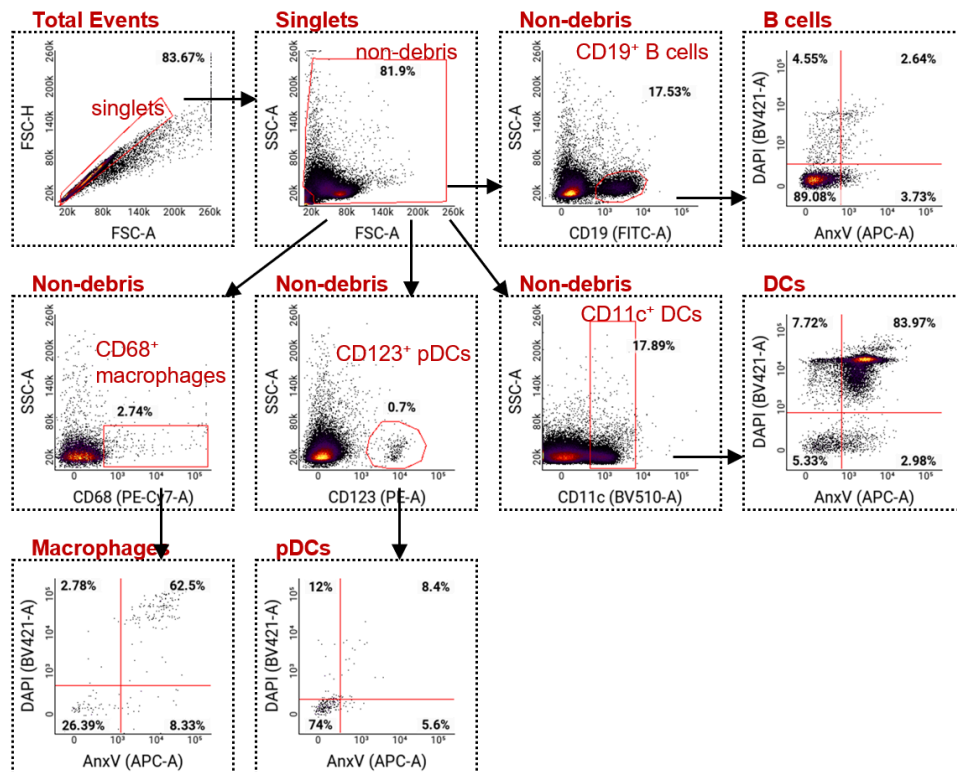


Figure 17. Representative flow cytometry gating tree for immune cell subpopulations (Panel 2). Gating strategy used to identify CD19⁺ B cells, CD68⁺ macrophages, CD123⁺ pDCs, and CD11c⁺ cDCs. Reproduced from (97).

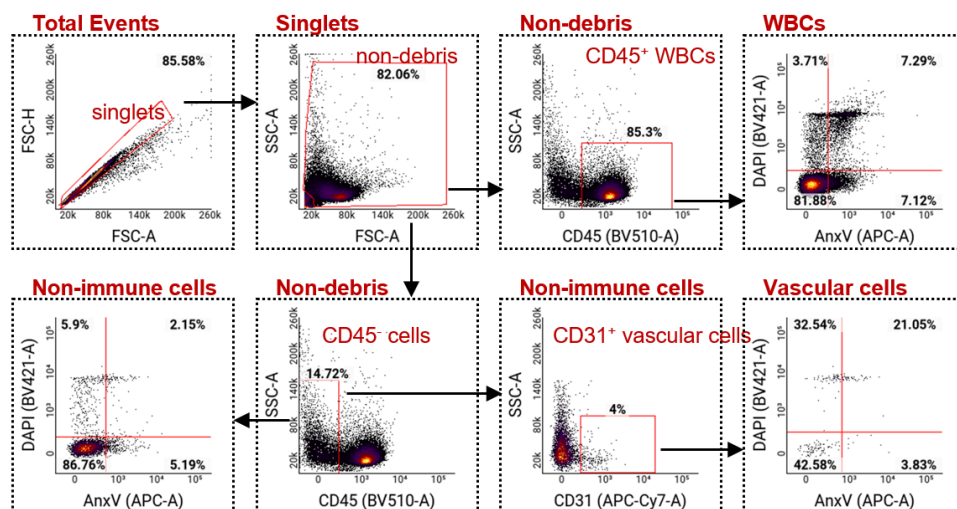


Figure 18. Representative flow cytometry gating tree for immune cell subpopulations (Panel 3). Gating strategy used to separate CD45⁺ WBCs and CD45⁻ non-immune cells. Reproduced from (97).

Comparing the frequencies and viability of the CD4⁺ T helper cells, the CD8⁺ cytotoxic T

cells, and the CD56⁺ natural killer (NK) cells over the three days post perfusion compared freshly isolated lymph node tissue (day 0) and cultured samples (day 3), I had to conclude whether the optimized bioreactor system was used in preserving the major lymphocyte subsets during *ex vivo* culture.

CD4⁺ T helper cells had consistent cellular frequencies and viability after culture that is not significantly different from freshly isolated tissue (**Figure 19A-B**). CD8⁺ cytotoxic T cells also maintained similar frequencies and viabilities between day 0 and day 3 (**Figure 19C-D**), suggesting that the major adaptive T cell compartments were maintained well during optimized perfusion culture. The relative frequency of CD56⁺ NK cells was modestly reduced following culture (**Figure 19E**), but NK-cell viability was similar to that of freshly isolated tissue, demonstrating that although the fraction of NK cells decreased during culture, the remaining NK-cell population retained viability (**Figure 19F**). **Table 30** shows the raw data for Figure 19.

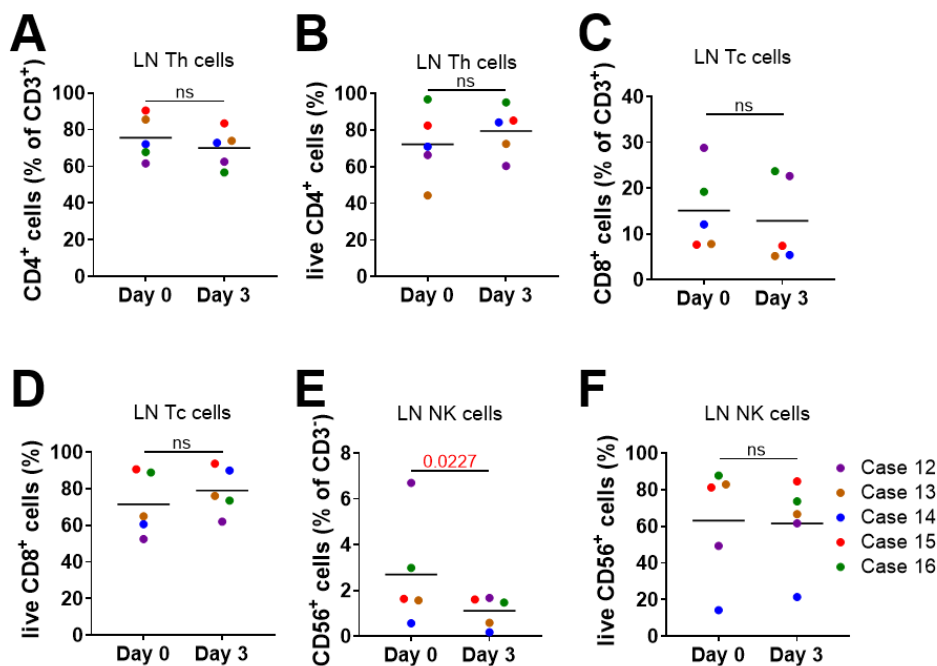


Figure 19. 3D perfusion bioreactors sustain Th, Tc and NK cells.

(A–F) Comparison of cell frequencies and viabilities between fresh tissue (day 0) and lymph node (LN) tissue cultured for 3 days ($n = 5$). (A–B) T helper cell frequency and viability. (C–D) Cytotoxic T cell

frequency and viability. (E–F) NK cell frequency and viability. Statistical analyses: Evaluated via a paired t-test or ratio paired t-test, where applicable. Reproduced from (97).

Table 30. Raw data of Fig. 19

Fig.A	Day 0	Day 3	Fig.B	Day 0	Day 3	Fig.C	Day 0	Day 3
Case 16	67.75	56.72	Case 16	96.87	95.21	Case 16	19.2	23.72
Case 15	90.42	83.49	Case 15	82.56	85.27	Case 15	7.64	7.41
Case 14	72.16	72.89	Case 14	71.07	84.29	Case 14	12.09	5.41
Case 13	85.57	73.99	Case 13	44.41	72.55	Case 13	7.82	5.19
Case 12	61.64	62.54	Case 12	66.46	60.45	Case 12	28.81	22.64
Fig.D	Day 0	Day 3	Fig.E	Day 0	Day 3	Fig.F	Day 0	Day 3
Case 16	88.89	73.54	Case 16	2.99	1.48	Case 16	87.75	73.72
Case 15	90.64	93.71	Case 15	1.64	1.61	Case 15	81.25	84.59
Case 14	60.61	89.94	Case 14	0.57	0.18	Case 14	14.29	21.43
Case 13	64.93	76.13	Case 13	1.57	0.59	Case 13	82.96	66.67
Case 12	52.57	62.07	Case 12	6.7	1.68	Case 12	49.34	61.74

Values representing the composition or viability of Th, Tc or NK cells.

The expression rate and the viability of CD123⁺ plasmacytoid dendritic cells (pDCs) (**Figure 20A-B**) and CD68⁺ macrophages (**Figure 20C-D**) among the antigen presenting cell populations also remained relatively stable, indicating that innate immune structures within the system have been maintained. A little increase in the frequency of dendritic cells with CD11c⁺ was registered post culture, but their cell viability was not changed (**Figure 20E-F**). **Table 31** shows the raw data for Figure 20.

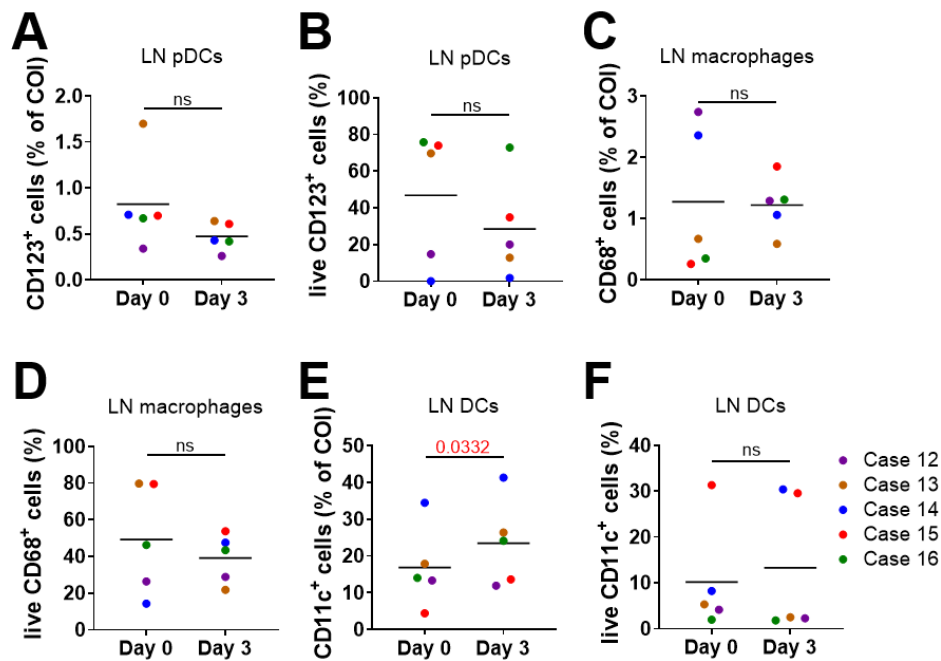


Figure 20. 3D perfusion bioreactors sustain pDCs, macrophages cells and DCs.

(A–B) Frequency and viability of pDCs. (C–D) Frequency and viability of macrophages. (E–F) Frequency and viability of conventional DCs. Statistical analyses: Data in (F) were analyzed using a Wilcoxon matched-pairs signed-rank test; all other comparisons were evaluated via a paired t-test or ratio paired t-test, as appropriate. Reproduced from (97).

Table 31. Raw data of Fig. 20

Fig.A	Day 0	Day 3	Fig.B	Day 0	Day 3	Fig.C	Day 0	Day 3
Case 16	0.67	0.42	Case 16	75.91	72.93	Case 16	0.35	1.31
Case 15	0.7	0.61	Case 15	74	34.85	Case 15	0.26	1.85
Case 14	0.71	0.43	Case 14	0	1.75	Case 14	2.36	1.06
Case 13	1.7	0.64	Case 13	69.79	12.9	Case 13	0.67	0.59
Case 12	0.34	0.26	Case 12	14.81	20	Case 12	2.74	1.29

Fig.D	Day 0	Day 3	Fig.E	Day 0	Day 3	Fig.F	Day 0	Day 3
Case 16	46.32	43.48	Case 16	14.07	24.14	Case 16	1.97	1.81
Case 15	79.57	53.81	Case 15	4.43	13.65	Case 15	31.36	29.57
Case 14	14.29	47.52	Case 14	34.48	41.31	Case 14	8.24	30.43
Case 13	79.79	21.75	Case 13	17.89	26.4	Case 13	5.33	2.5
Case 12	26.39	28.9	Case 12	13.36	11.95	Case 12	4.15	2.28

Values representing the composition or viability of pDCs, macrophages cells or DCs.

CD45⁺ white blood cells (WBCs) (**Figure 21A-B**), CD45⁻ non-immune cells (**Figure 21C-D**), and CD31⁺ vascular endothelial cells (**Figure 21E-F**) showed no significant differences in frequency or viability from newly processed tissue. Collectively, these findings suggest that the bioreactor system maintains main immune, stromal, and vascular cellular compartments of lymphoid tissue throughout culture without significant alteration in the total tissue composition. **Table 32** shows the raw data for Figure 21.

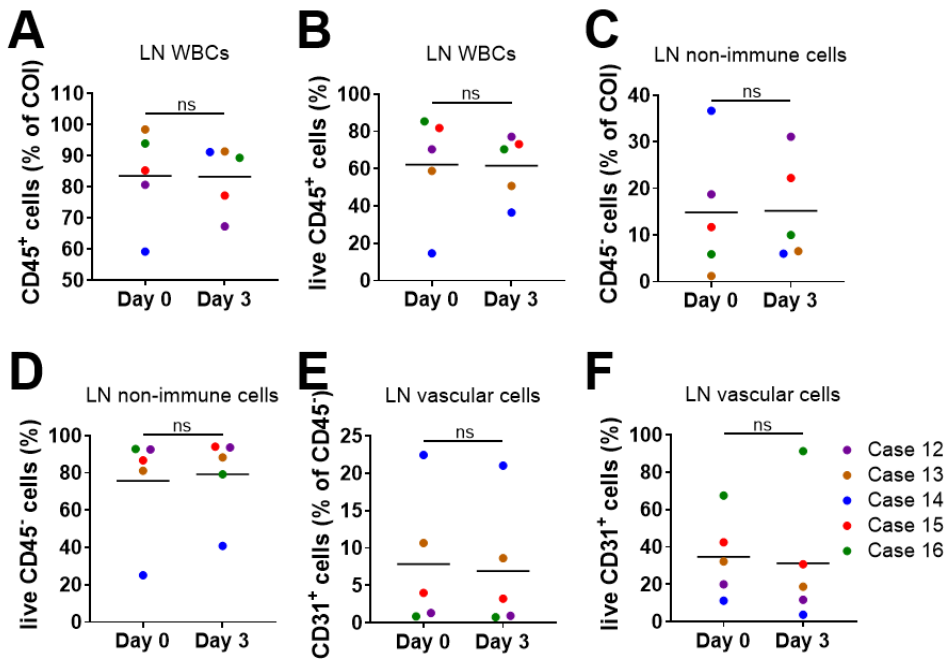


Figure 21. 3D perfusion bioreactors sustain WBCs, non-immune and vascular cells.

(A–B) Frequency and viability of white blood cells (WBCs). (C–D) Frequency and viability of non-immune cell types. (E–F) Frequency and viability of vascular cell types. Statistics: Utilized a paired t-test or ratio paired t-test as applicable. Reproduced from (97).

Table 32. Raw data of Fig. 21

Fig.A	Day 0	Day 3	Fig.B	Day 0	Day 3	Fig.C	Day 0	Day 3
Case 16	93.92	89.32	Case 16	85.44	70.45	Case 16	5.88	10.01
Case 15	85.3	77.21	Case 15	81.88	73.19	Case 15	11.72	22.24
Case 14	59.2	91.17	Case 14	14.61	36.53	Case 14	36.68	6.01
Case 13	98.44	91.39	Case 13	58.85	50.79	Case 13	1.2	6.56
Case 12	80.67	67.33	Case 12	70.43	77.22	Case 12	18.74	31.13

Fig.D	Day 0	Day 3	Fig.E	Day 0	Day 3	Fig.F	Day 0	Day 3
-------	-------	-------	-------	-------	-------	-------	-------	-------

Case 16	92.85	79.33	Case 16	0.86	0.75	Case 16	67.57	91.3
Case 15	86.76	94.14	Case 15	4	3.24	Case 15	42.58	30.8
Case 14	25.16	40.87	Case 14	22.45	21.03	Case 14	11.25	3.77
Case 13	81.17	88.35	Case 13	10.68	8.66	Case 13	32.26	18.77
Case 12	92.65	93.68	Case 12	1.32	0.95	Case 12	20	11.76

Values representing the composition or viability of WBCs, non-immune or vascular cells.

In order to determine if whole tissue architecture was maintained throughout *ex vivo* culture, IF staining and low magnification imaging were used on slices of the tissue on day 0 and day 3. Staining for CD3 and CD20 indicated that T and B cell compartments were spatially held during the culture and that core lymphoid compartmentalization survived. Concurrently, collagen IV and CD31 were stained and sustained ECM structures as well as vascular-linked networks were observed (**Figure 22**). Complementary histological analysis by H&E stain confirmed that overall tissue morphology (cellular density, total structure) remained quite well preserved after 3 days' culture (**Figure 23**). These data indicate that most critical structural and microarchitectural components of the lymphoid tissue microenvironment are retained in the optimized bioreactor environment during the culture period.

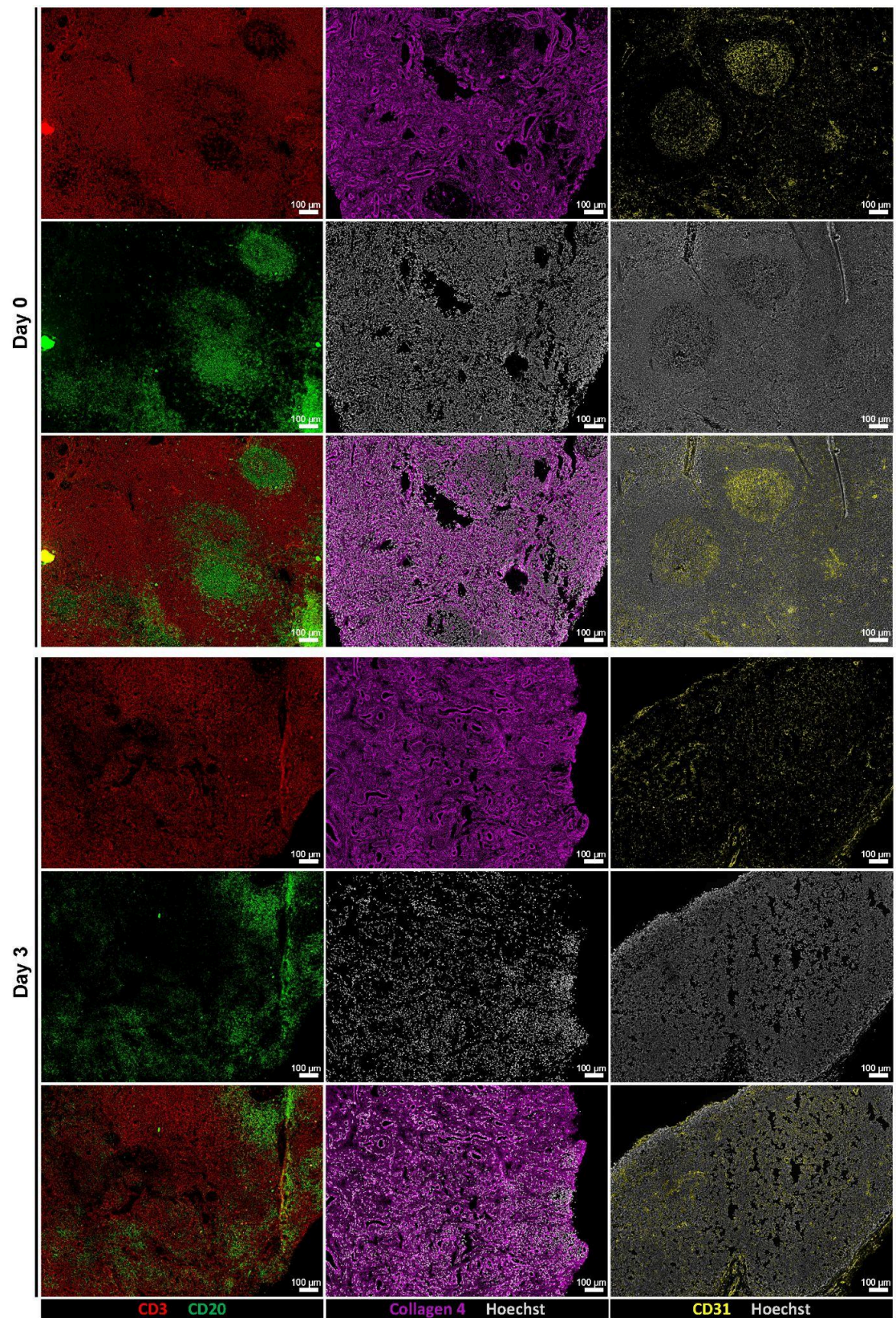


Figure 22. Perfusion bioreactor culture preserves tissue architecture.

Low-magnification immunofluorescence images compare tissue sections at Day 0 (top) and after 3 days of *ex vivo* culture (bottom). Left to right: CD3 (red) and CD20 (green) staining show intact lymphoid compartmentalization. Collagen IV (magenta) reveals a preserved extracellular matrix. CD31 (yellow) highlights stable vasculature. Scale bars: 100 μ m. Reproduced from (97).

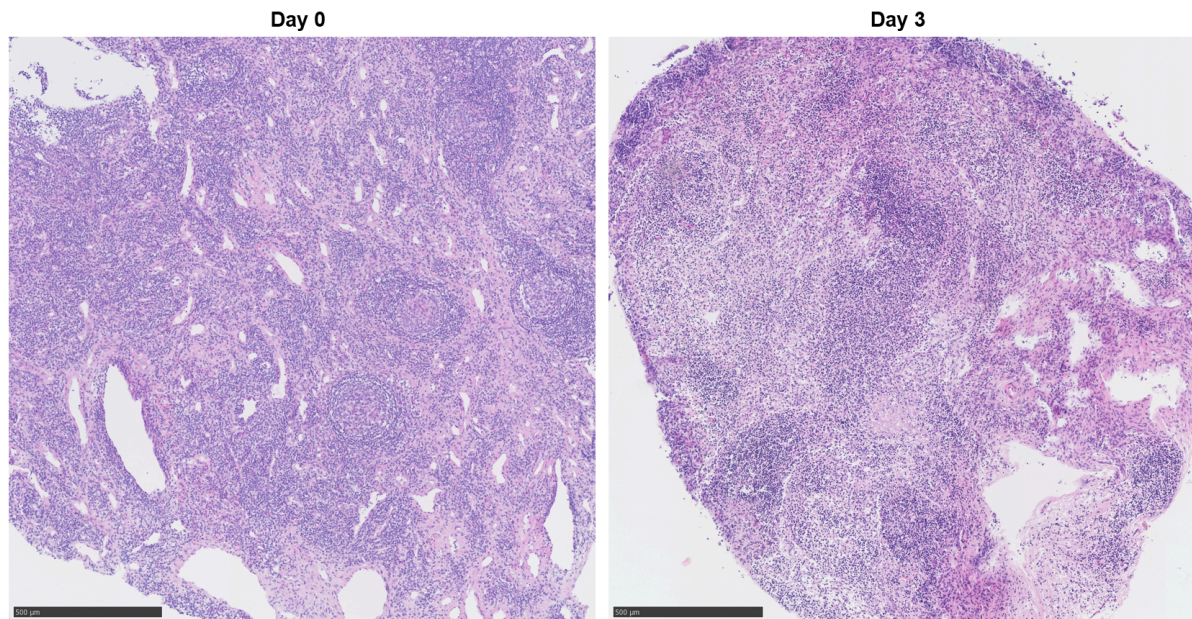


Figure 23. Histological evaluation of tissue morphology pre- and post-cultivation.

H&E stained tissue sections from baseline fresh tissue (Day 0) and cultured tissue samples harvested at Day 3. Reproduced from (97).

Together, these data demonstrate the significant power of the optimized bioreactor system to sustain long-term 3D culture of viable, structurally intact lymphoid tissues *ex vivo*. Preservation of cellular viability, immune composition, and tissue architecture emphasizes its role as a physiologically relevant *ex vivo* platform. Consequently, this system serves as a robust platform for improved functional studies in lymphoid biology and for increased specificity in the representation of immune–tumor interactions in translational research settings.

3.3 Modeling immune checkpoint inhibitor therapy

This section is adapted from my first-author publication (97) :

Zhang Y., et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors, *Theranostics*, 2026, 16 (8), 3928.

3.3.1 Anti-PD-1 therapy suppresses malignant tissue viability

After the optimized lymph node bioreactor platform was validated, the PD-1 immune checkpoint inhibitor pembrolizumab was used to test the suitability of the platform for functional immunotherapy. Patient-derived lymphoma tissues, melanoma metastatic lymph nodes, and benign lymph nodes were cultured in the presence or absence of pembrolizumab for 3 days, after which tissue viability was assessed by flow cytometry.

Pembrolizumab treatment induced a significant reduction in overall tissue viability in lymphoma explants compared with untreated controls (**Figure 24A-B**). A similar response was noted in malignant lymph nodes with melanoma metastases as pembrolizumab caused significant loss of tissue viability after 3 days of treatment (**Figure 24C-D**). The same treatment had no significant effect on benign lymph nodes despite pembrolizumab exposure (**Figure 24E-F**), demonstrating little effect in cells containing nonmalignant lymphoid tissue.

These results reflect the ability of the bioreactor system to determine the sensitivity pattern of tissue response to immune checkpoint blockade. Crucially, the selective reduction in tumor-immune viability experienced by malignant, but not benign, tissues indicates that the maintained TME allows functional study of clinically relevant immunotherapeutic responses in the *ex vivo* model.

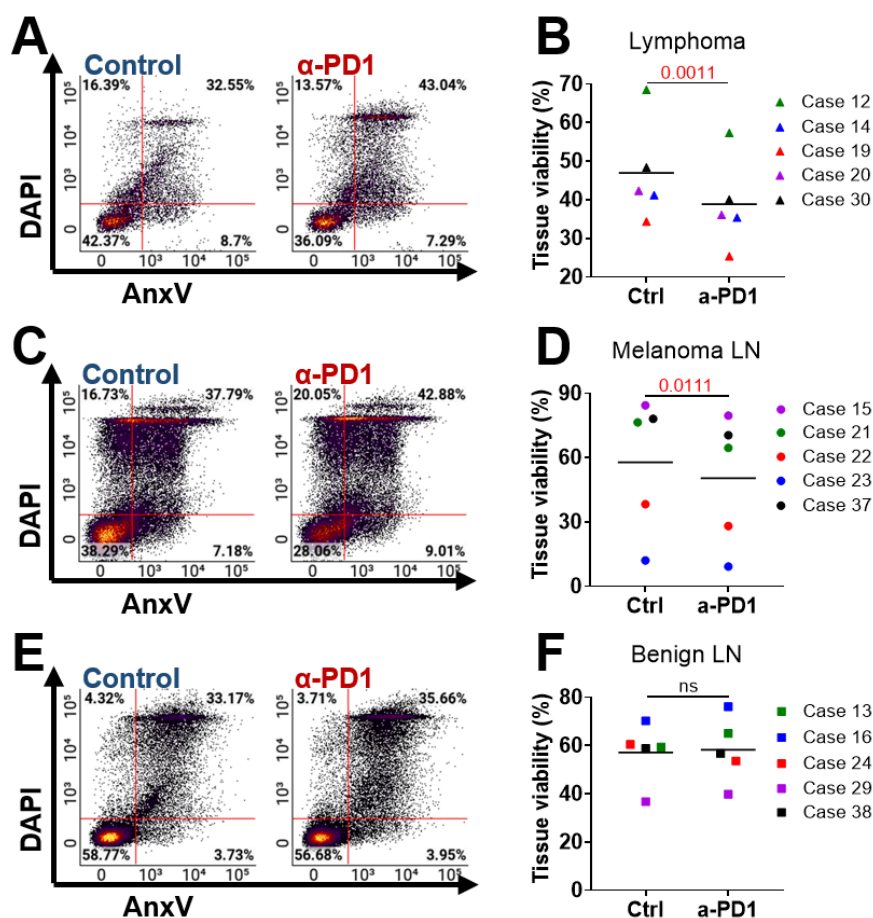


Figure 24. Modeling ICIs in lymphoma, metastatic lymph nodes, and benign lymph nodes.

Flow cytometry assessments conducted following three days of pembrolizumab therapy indicate: (A–B) alterations in lymphoma lymph node tissue (n = 5), (C–D) modifications in melanoma metastatic lymph node tissue (n = 5), and (E–F) shifts in immune cell populations within benign lymph node tissue (n = 5). Statistical analysis utilized a paired t-test. Reproduced from (97).

Table 33. Raw data of Fig. 24

Fig.B	Ctrl.	a-PD1	Fig.D	Ctrl.	a-PD1	Fig.F	Ctrl.	a-PD1
Case 12	68.53	57.37	Case 21	76.48	64.5	Case 13	59.33	65.05
Case 19	34.34	25.35	Case 22	38.29	28.06	Case 24	60.42	53.55
Case 14	41.18	35.38	Case 23	12.05	9.21	Case 16	70.2	76.13
Case 20	42.37	36.09	Case 15	84.42	79.6	Case 29	36.73	39.78
Case 30	48.4	40.06	Case 37	78.13	70.48	Case 38	58.77	56.68

Values represent tissue viability (live cells / total cells) in the three tissue types with or without anti-PD-1 treatment.

3.3.2 Anti-PD-1 treatment promotes T cell activation

Soluble immune mediators released from cultures upon the PD-1 blockade were further quantified in the culture supernatant after 3 days of pembrolizumab treatment. Treatment with pembrolizumab increased the production of various cytokines and cytotoxic effector molecules which mediate T cell activation, like IL-4, IL-6, IL-10, IL-17A, tumor necrosis factor- α (TNF- α), soluble Fas (sFas), soluble Fas ligand (sFasL), interferon- γ (IFN- γ), granzyme A (GZMA), granzyme B (GZMB), perforin (PRF), and granulysin (GNLY), whereas IL-2 levels remained mostly unchanged relative to control tissue after treatment (**Figure 25**). The collective elevation of these immune mediators also indicates the subsequent expansion of immune activation and cytotoxic effector function in the tumor microenvironment of the cultured environment after the blockade of PD-1.

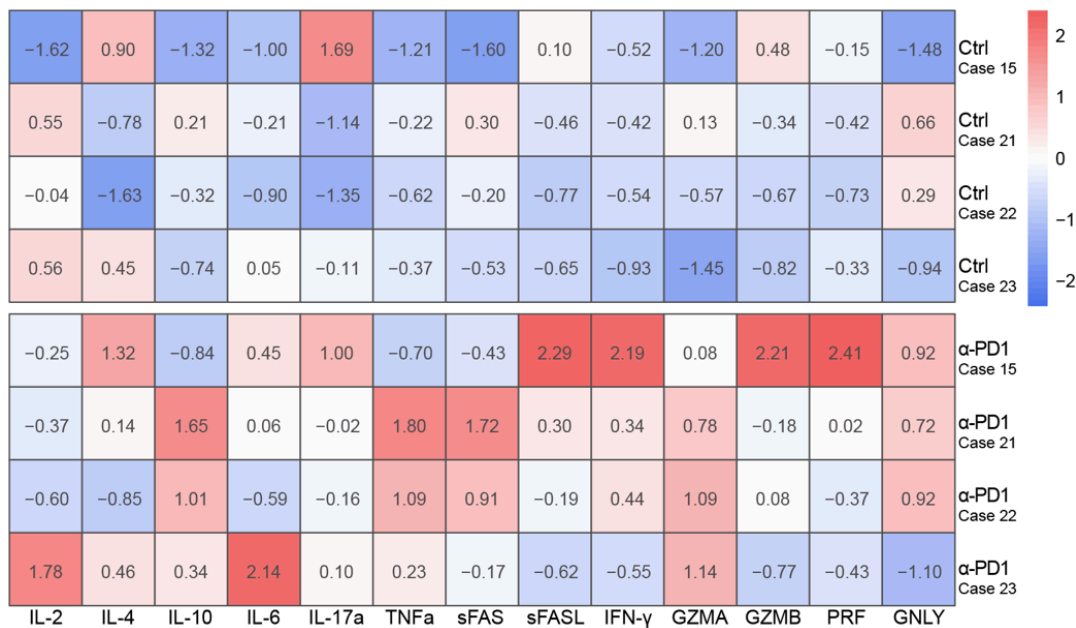


Figure 25. Anti-PD-1 treatment induces cytokine release in bioreactor cultures.

Heatmap representing relative soluble factor levels in culture media. Reproduced from (97). The raw data is shown in **Table 34**.

Table 34. Raw data of Fig. 25

	IL-2	IL-4	IL-10	IL-6	IL-17A	TNFA	sFas	sFasL	IFNG	GZMA	GZMB	PRF	GNLY
Con. 15	18.8	1.1	22.8	2484	31	12.7	23.4	39.9	867.7	1431.2	2824	1870.5	1316.1
Con. 21	120.5	0.4	152.4	3656.1	9.5	62.6	261.4	20.1	953.3	2977	1572.4	1533.4	6731.8

Con. 22	92.8	0	108.1	2639.6	7.9	42.5	198.3	9	845.4	2158.3	1058.4	1138.1	5807.9
Con. 23	121	0.9	72.5	4050	17.3	55.4	157.7	13.5	519	1138.7	839.7	1647.7	2685.2
Pem. 15	82.8	1.3	64	4643	25.8	38.8	170.1	117.3	3146.2	2913.4	5472.2	5128.1	7393.9
Pem. 21	77.4	0.8	275.3	4058.7	18	164.6	439.3	47	1590.1	3730.1	1820	2097.9	6877.5
Pem. 22	66.5	0.3	221.1	3105.1	17	129.2	337.9	29.7	1672.7	4087.3	2208.7	1592.2	7405.7
Pem. 23	178	0.9	163.5	7150	18.9	85.5	202.5	14.3	840	4142.7	908.2	1524.7	2267.9

Values represent the concentrations of cytokines in the culture medium (pg/mL) with or without anti-PD-1 treatment.

Multiplex CODEX imaging of cultured tissue sections was performed to verify these findings at the tissue level. Diverse immune cell populations remained well preserved throughout the culture period, corroborating the maintenance of the native tissue architecture and immune microenvironment (**Figure 26**). Moreover, enhanced IFN- γ staining was seen in pembrolizumab-treated tissues, offering spatial proof of local immune activation and partially confirming the cytokine profiling findings.

Multiplex imaging analysis further demonstrated that pembrolizumab primarily enhanced T cell function rather than altering T cell abundance. Firstly, I found that the overall IFN- γ signal intensity is increased (**Figure 27A**) The overall frequency of CD8 $^{+}$ T cells remained unchanged following treatment (**Figure 27B**), however, the proportion of IFN- γ producing CD8 $^{+}$ T cells increased significantly (**Figure 27C**), indicating functional activation rather than expansion of this population. A similar pattern was observed in the CD4 $^{+}$ compartment, where total CD4 $^{+}$ T cell frequencies remained stable, but the fraction of IFN- γ positive CD4 $^{+}$ T cells was elevated after pembrolizumab exposure (**Figure 27D–E**).

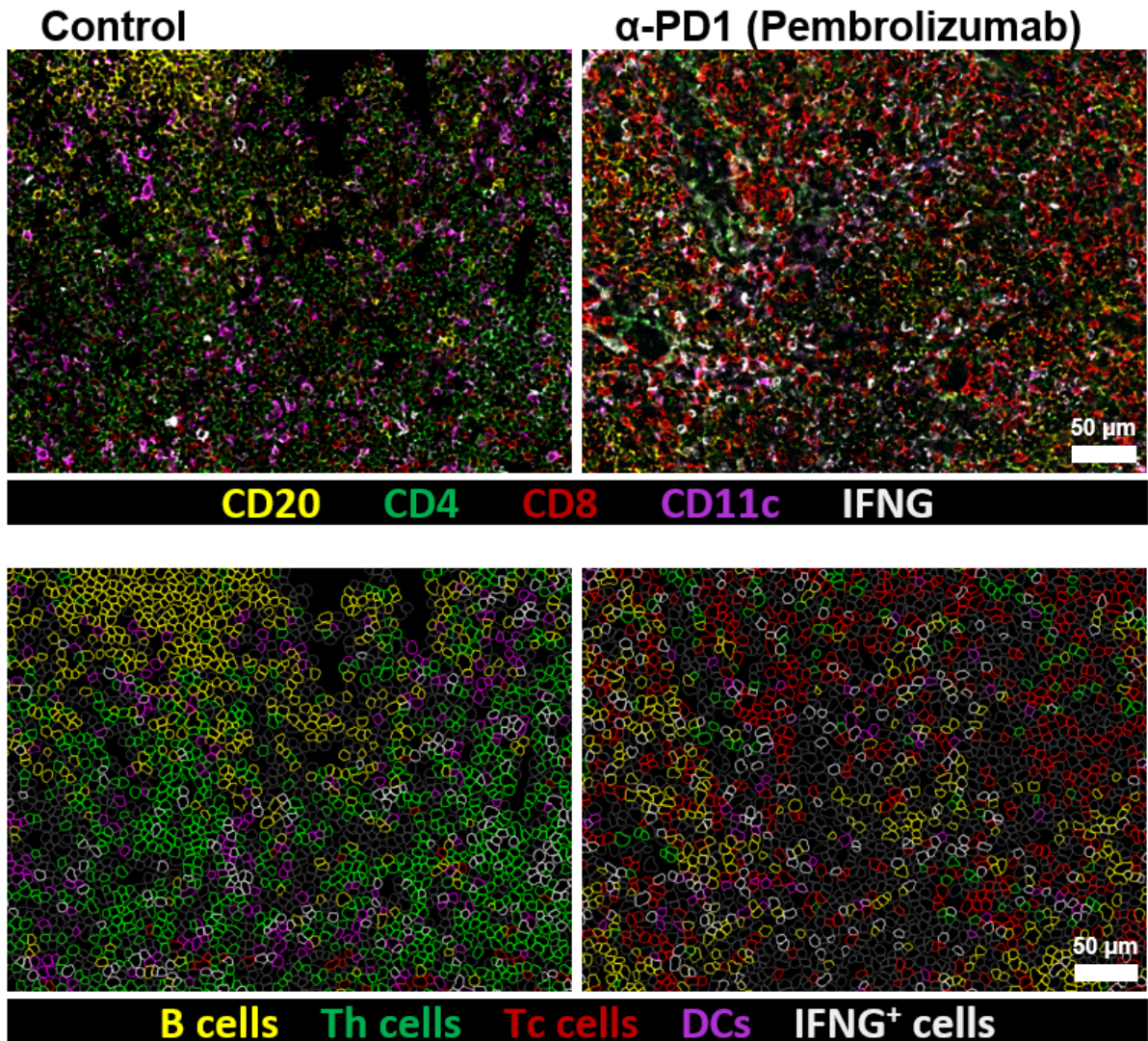


Figure 26. Multiplexed imaging.

CODEX images illustrating the expression levels of CD20, CD4, CD8, CD11c, and IFN- γ . Left: Original overlay images presented with pseudocolor; Right: Cell annotations derived from intensity thresholds (CD20: 1059; CD4: 2997; CD8: 5125; CD11c: 3532; IFN- γ : 4375). Reproduced from (97).

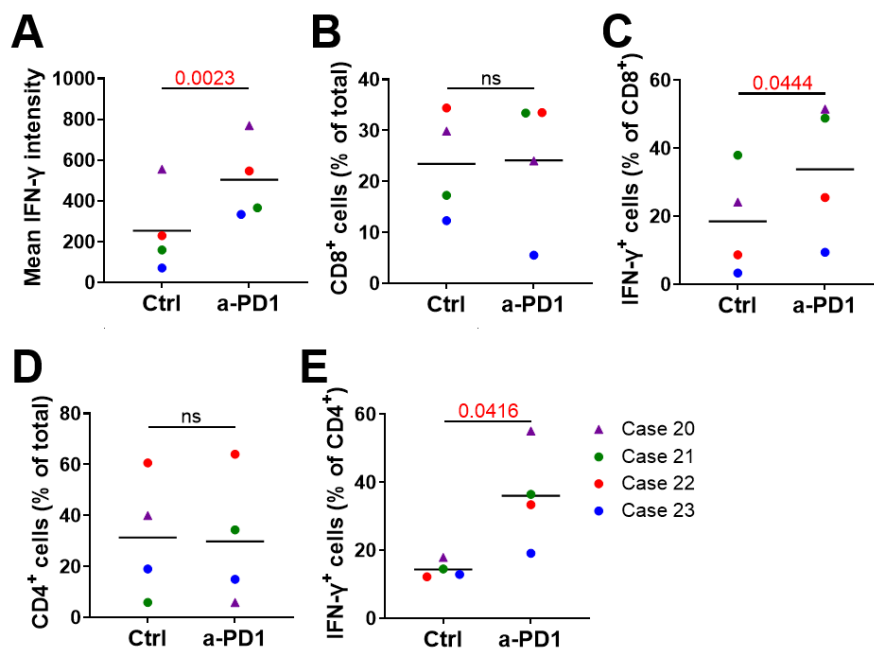


Figure 27. Enhanced IFN- γ expression in CD4 $^{+}$ and CD8 $^{+}$ T cells.

(A) Quantification of IFN- γ levels in samples through fluorescence intensity (n = 4). (B) Proportion of CD8 $^{+}$ Tc cells (n = 4). (C) Proportion of Tc cells that express IFN- γ (n = 4). (D) Proportion of CD4 $^{+}$ Th cells (n = 4). (E) Proportion of Th cells that express IFN- γ (n = 4). Statistics: paired t-test. Reproduced from (97). The raw data is shown in **Table 35**.

Table 35. Raw data of Fig. 27

Fig.A	Ctrl.	a-PD1	Fig.B	Ctrl.	a-PD1	Fig.C	Ctrl.	a-PD1
Case 20	555.5	769.5	Case 20	29.89	24.06	Case 20	24.17	51.51
Case 21	159.5	366.1	Case 21	17.27	33.42	Case 21	37.99	48.87
Case 22	229.8	547.3	Case 22	34.4	33.51	Case 22	8.69	25.54
Case 23	71.4	333.4	Case 23	12.3	5.56	Case 23	3.33	9.43

Fig.D	Ctrl.	a-PD1	Fig.E	Ctrl.	a-PD1
Case 20	39.98	5.88	Case 20	17.95	55.06
Case 21	5.9	34.34	Case 21	14.53	36.48
Case 22	60.65	64.03	Case 22	12.21	33.42
Case 23	19.06	14.99	Case 23	12.9	19.11

Values in Fig. A indicates the IFN- γ IF signal intensity value of stains. Values in Fig. B indicates Tc cell abundance (Tc cell number / total cell number). Values in Fig. C indicate the frequency of IFN- γ positive Tc cells (IFN- γ positive Tc cells / total Tc cells). Values in Fig. C and D indicate Th cell abundance and the frequency of IFN- γ positive Th cells.

Overall, these findings demonstrate that the bioreactor culture system effectively recapitulates key aspects of ICI treatment responses *ex vivo*. In particular, pembrolizumab treatment was associated with reduced viability of malignant lymph node tissues together with enhanced T cell–associated immune activation, supporting the ability of this platform to model clinically relevant antitumor immune responses within an intact tissue microenvironment.

3.4 Modeling CAR T cell therapy

This section is adapted from my first-author publication (97) :

Zhang Y., et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors, *Theranostics*, 2026, 16 (8), 3928.

3.4.1 CAR T cells effectively eliminate target cells in bioreactor culture

After demonstrating the success of the bioreactor platform for immune checkpoint inhibitor testing, it was then explored further for the potential to be utilized in CAR T cell immunotherapy, where tonsil and lymph node explants are rich sources of CD19⁺ B cells to be utilized as target cells to elicit anti-CD19 CAR T cell mediated cytotoxicity. Tissue explants were incubated with either anti-CD19 CAR T cells or non-transduced (NT) T cells under uniform perfusion conditions for 3 days, and the CD19⁺ B cells' frequency and viability were examined by flow cytometry.

Representative flow cytometry plots showed that a significant decrease of the proportion of viable CD19⁺ B cells in tissues treated with anti-CD19 CAR T cells occurred when compared with NT T cell controls (**Figure 28A-B**). Quantitative analysis validated the above findings in tonsil explants, where the CAR T cell treatment led to a pronounced loss of viable CD19⁺ B cells among the viable population compared to the control treatment patients (**Figure 28C**). Similar observations were noted in lymph node explants. CAR T cell treatment dramatically decreased both CD19⁺ B cell frequency and viability, and non-transduced T cell cultures of NT cells revealed no significant change in the B cell compartment (**Figure 28D-F**). **Table 36** contains the raw data for Figure 28.

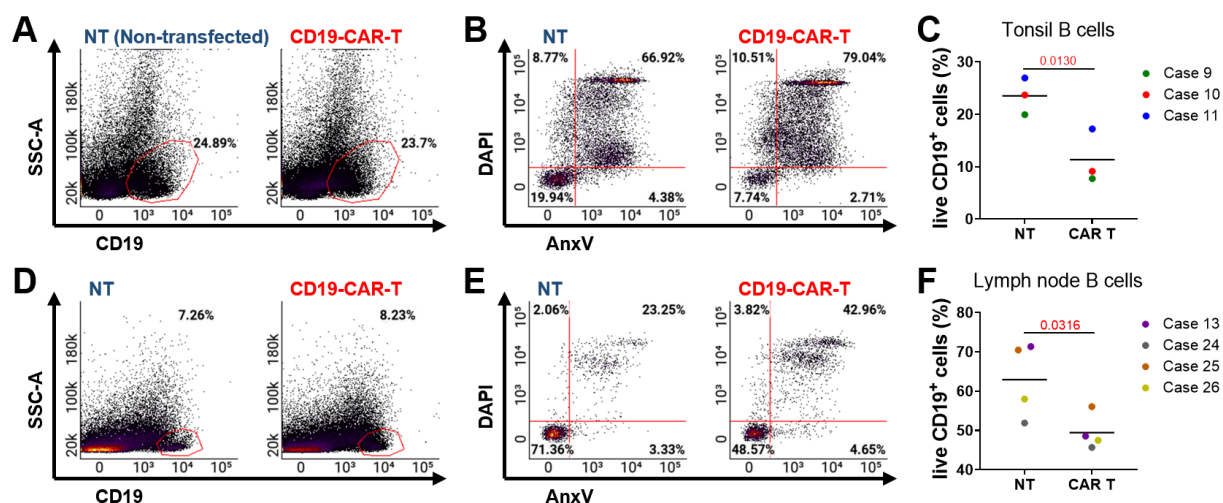


Table 36. Raw data of Fig. 28

Fig.C	NT	CAR T	Fig.F	Ctrl.	a-PD1
Case 9	19.94	7.74	Case 13	71.36	48.57
Case 10	23.69	9.14	Case 25	70.47	56.07
Case 11	26.93	17.22	Case 26	58.02	47.53
			Case 24	51.92	45.71

Values in Table 36 indicate B cell viability (live B cell number / total B cell number).

3.4.2 Infiltration of CAR T cells within lymphoid tissue microenvironments.

Whether CAR T cells can migrate into intact lymphoid tissue was inferred by monitoring tissue sections by immunofluorescence following co-culturing with NT or anti-CD19 CAR T cells. Representative IF images showed CAR T cells present within the parenchyma of the tissue, while the number of NT T cells showed that much fewer cells were apparent under the same culture conditions (**Figure 29**).

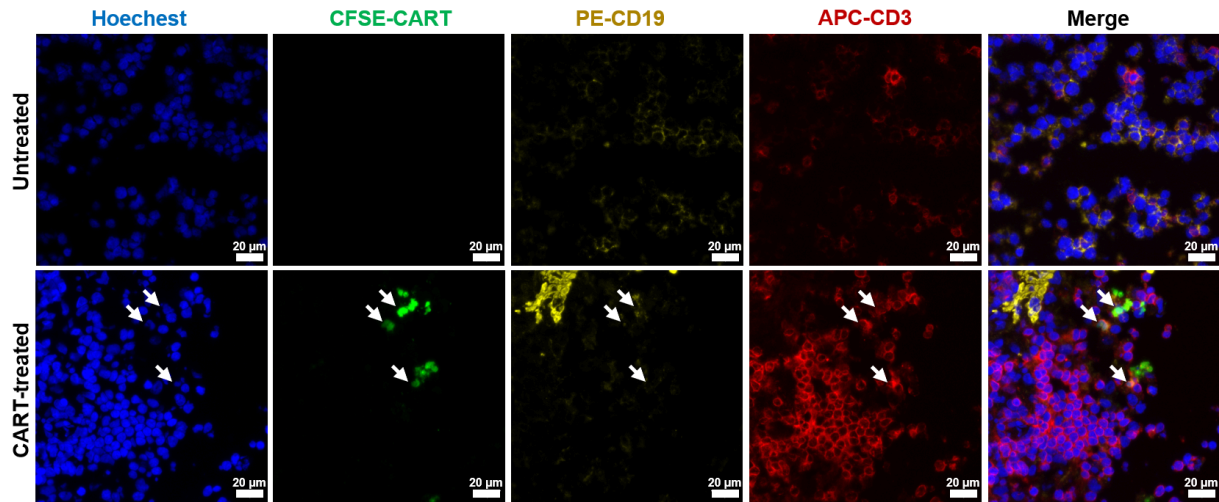


Figure 29. IF analysis of CAR T cell infiltration in tissues.

Representative image IF staining showing the presence of CAR T cells in lymphoid tissue. Scale bars: 20 µm. Reproduced from (97).

Quantitative analysis confirmed these observations, showing significantly greater infiltration of CAR T cells into the lymphoid tissue compared with NT T cell controls (**Figure 30A-B**). These findings indicate that the optimized perfusion bioreactor supports efficient migration and penetration of CAR T cells into preserved tissue structures while maintaining the three-dimensional architecture of the explants.

Together with the previously observed depletion of CD19⁺ B cells, these results demonstrate that the bioreactor system enables simultaneous evaluation of CAR T cell trafficking and target cell engagement within an intact tissue microenvironment. The raw data corresponding to Figure 30B are presented in **Table 37**.

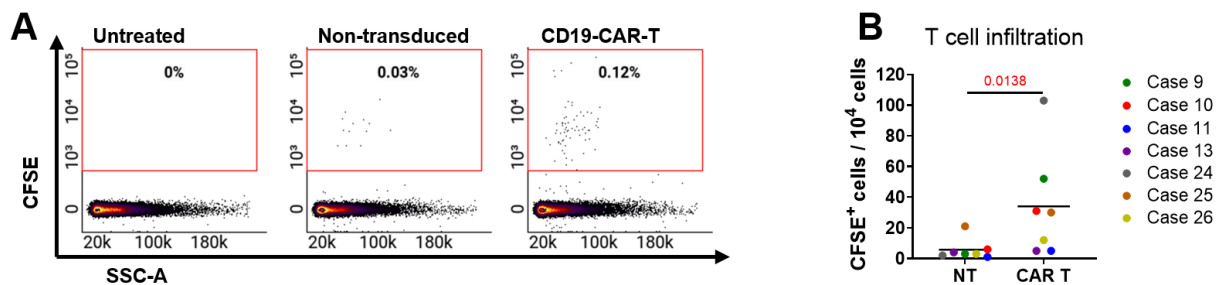


Figure 30. Assessment of CAR T cell tissue infiltration by flow cytometry.

(A) Flow cytometry plots illustrate the comparison between the frequency of CAR T cells and NT T cells in tonsillar and lymph node tissues. (B) A quantitative analysis of CAR T cells in relation to NT T cells is presented (n = 7). Statistical analysis utilized a paired t-test for ratios. Reproduced from (97).

Table 37. Raw data of Fig. 30

	Ctrl.	a-PD1
Case 9	3	52
Case 10	6	31
Case 11	1	5
Case 13	4	5
Case 25	21	30
Case 26	3	12
Case 24	2	103

Values in Table 37 indicates the CFSE⁺ cell number per 10000 cells (by flow cytometry).

Whether direct exposure to agarose itself might have any effect on the viability of CAR T cells or cytotoxic activity. To answer this question, complementary 2D co-culture experiments were carried out using anti-CD19 CAR T cells as well as CD19⁺ B cell lymphoma target cells in agarose-coated and agarose-free cultures.

CAR T cell viability was comparable between agarose-coated and uncoated cultures (**Figure 31A**). In a similar manner, quantitative analysis of CAR T cell cytotoxicity showed no statistical difference in target cell killing in the two groups tested (**Figure 31B**). These features demonstrate that agarose coating does not alter CAR T cell survival or inhibit antigen-based cytotoxicity of individual cells for the observed experimental conditions. Collectively these findings show that the agarose embedding scheme employed in the optimized bioreactor system is biologically compatible with CAR T cell performance and presumably is unlikely to compromise the functional immunotherapy studies achieved with the perfusion bioreactor. The corresponding raw data are displayed in **Table 38** and **Table 39**.

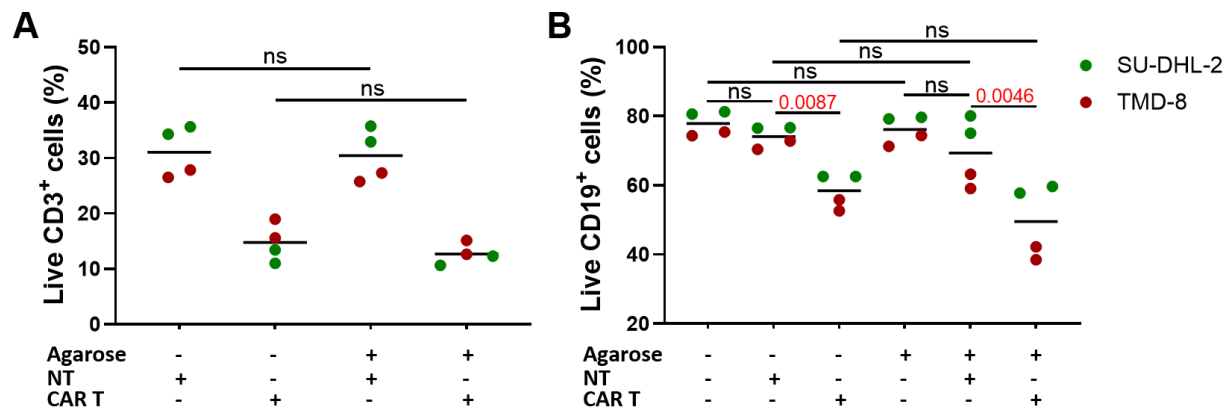


Figure 31. Biocompatibility of agarose coating on CAR T cell viability and effector function.

(A) Viability of NT control T cells and anti-CD19 CAR T cells following a 48-hour incubation in culture wells prepared with or without a 150 μ L coating of 0.5% agarose. (B) *In vitro* cytotoxic activity of CAR T cells directed against SU-DHL-2 and TMD-8 DLBCL cell lines, evaluated in the presence or absence of the agarose matrix (n = 4), representing two biological replicates, each performed with two technical replicates). Statistical analysis: Repeated measures one-way ANOVA with Tukey's multiple comparisons test. Reproduced from (97).

Table 38. Raw data of Fig. 31A

	Control+NT	Control+CAR T	Agarose+NT	Agarose+CAR T
SU-DHL-2	34.33	11.02	32.94	10.65
SU-DHL-2	35.63	13.45	35.75	12.3
TMD-8	26.5	15.61	25.79	12.65
TMD-8	27.86	18.97	27.33	15.16

Values indicate the viability of NT & CAR T cells from culture medium (live T cell / total T cell number).

Table 39. Raw data of Fig. 31B

	Control	Control+NT	Control+CAR T	Agarose	Agarose+NT	Agarose+CAR T
SU-DHL-2	81.29	76.71	62.57	79.69	80.13	57.79
SU-DHL-2	80.61	76.56	62.54	79.22	75.11	59.67
TMD-8	74.34	72.82	52.65	74.42	59.08	38.52
TMD-8	75.41	70.42	55.86	71.3	63.26	42.22

Values indicate the viability of lymphoma B cells (live B cell / total B cell number).

3.5 Evaluating novel CAR T products using the bioreactor system

This section is adapted from my first-author publication (97) :

Zhang Y., et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors, *Theranostics*, 2026, 16 (8), 3928.

3.5.1 Assess CAR T cell killing efficiency

To investigate whether the optimized bioreactor platform could distinguish functional differences between CAR T next-generation CAR T cell products, PI3K-19BBz CAR T cells, which harbor enhanced PI3K pathway signaling (100), were compared with conventional 19BB CAR T cells. Both CAR T cell products were co-cultured with lymphoid tissue explants under identical perfusion culture conditions, after which the frequency and viability of CD19⁺ B cells were evaluated by flow cytometry.

Representative flow cytometry plots indicated that similarly reduced population of CD19⁺ B cells was noted after treatment with conventional 19BB or PI3K-19BBz CAR T cells (**Figure 32A**). Quantitative validation confirmed that the proportion of viable CD19⁺ B cells in the two treatment groups did not differ significantly between the two groups (**Figure 32B**), which suggested that CAR T cell variants were involved in the same degree of antigen-specific cytotoxicity against CD19⁺ target cells under the same experimental conditions for the two types of cells. **Table 40** shows the raw data of Figure 32B.

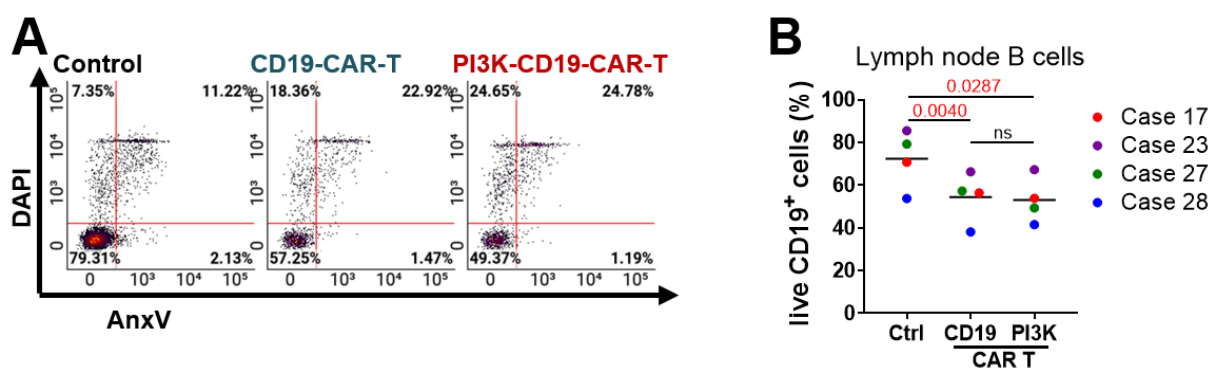


Figure 32. CAR T cell killing.

(A) Flow cytometry plots are presented as representatives, and (B) the quantification of viable CD19+ B cells within lymph node tissues after treatment with either CD19-CAR T or PI3K-CAR T ($n = 4$) is provided. Statistical analysis was conducted using one-way ANOVA accompanied by Tukey's post hoc test. Reproduced from (97).

Table 40. Raw data of Fig. 32B

	Control	CD19-CAR-T	PI3K-CAR-T
Case 27	79.31	57.25	49.37
Case 17	70.8	56.34	53.89
Case 28	53.77	38.09	41.5
Case 23	85.55	66.29	67.29

Values indicate the viability of B cells from co-cultured tissue (live B cell / total B cell number).

3.5.2 Assess CAR T cell retention in tissue

Although the PI3K-19BBz CAR T cells did not exhibit enhanced cytotoxic efficacy compared with conventional 19BB CAR T cells, they demonstrated markedly increased tissue infiltration. Specifically, the mean number of infiltrating CAR T cells was approximately two-fold higher than that observed with conventional 19BB CAR T cells. This was supported by flow cytometric quantification of recovered cells (**Figure 33A–B**) and further confirmed by fluorescence microscopy, which showed a greater density of CAR T cells within the tissue compartment (**Figure 34**). The raw data of Figure 33B is displayed in **Table 41**.

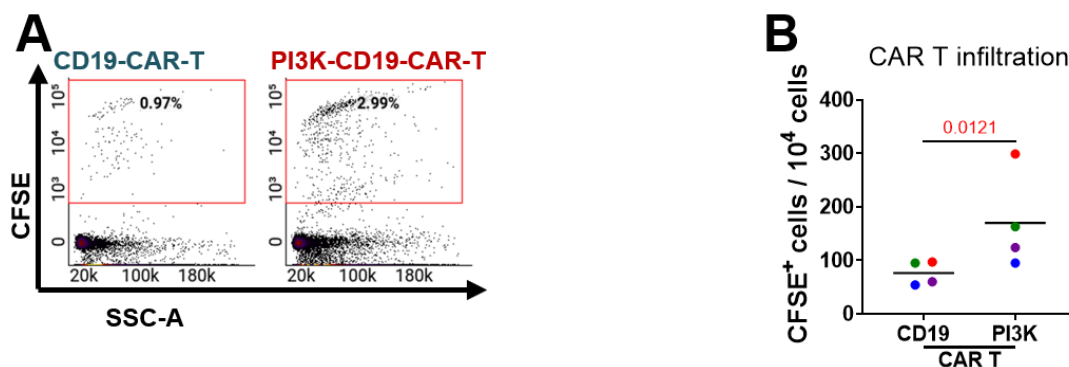


Figure 33. CAR T cell infiltration.

(A) Representative flow cytometry plots and (B) quantification of CD19-CAR T and PI3K-CAR T cell infiltration into tissues. Statistics: ratio paired t-tests. Reproduced from (97).

Table 41. Raw data of Fig. 33B

	CD19-CAR-T	PI3K-CAR-T
Case 27	95	163
Case 17	97	299
Case 28	54	95
Case 23	60	124

Values in Table 41 indicates the CFSE⁺ cell number per 10000 cells (by flow cytometry).

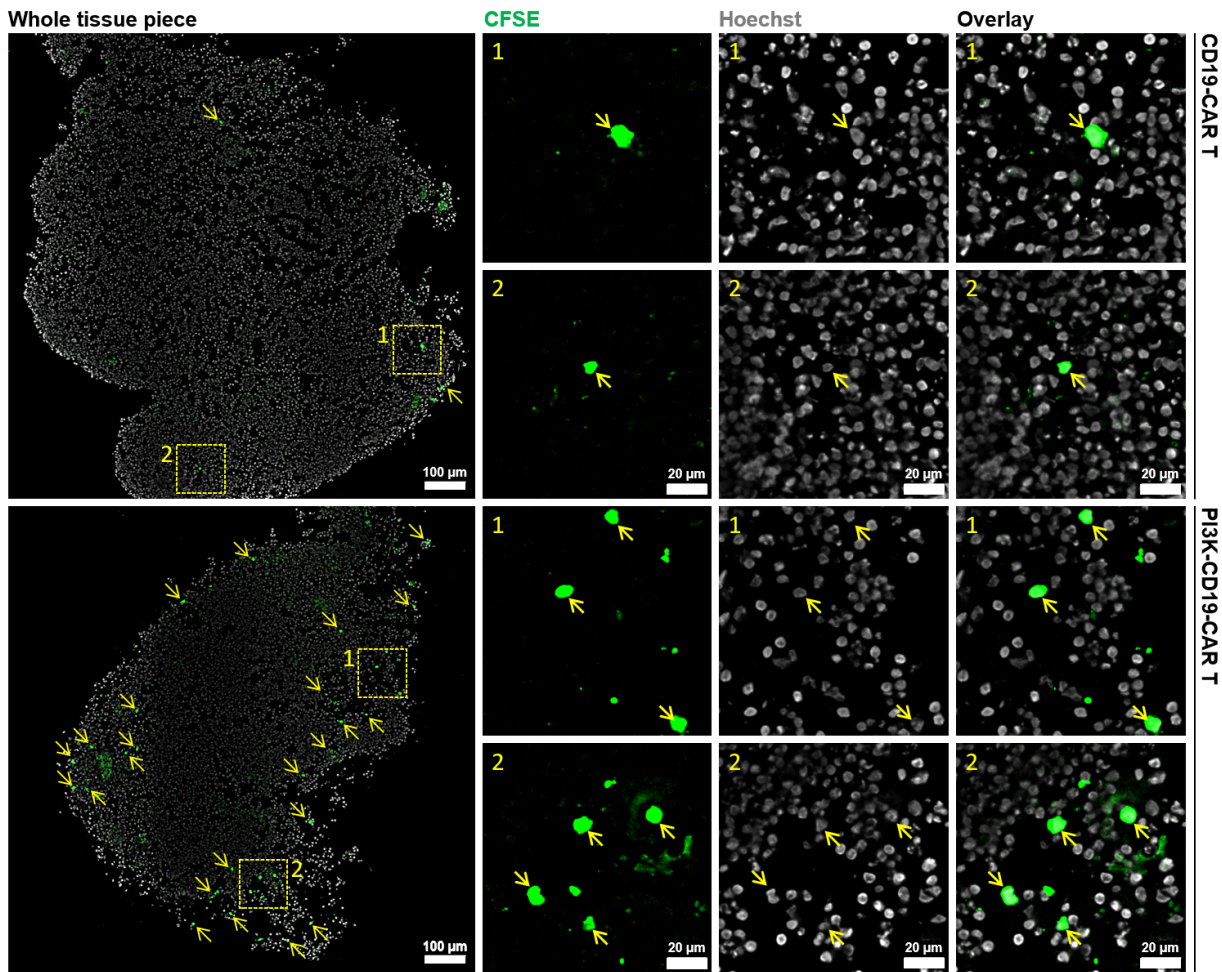


Figure 34. CAR T cell tissue persistence.

Representative fluorescence images depict the infiltration of CD19-CAR T cells and PI3K-CAR T cells into LN tissues. The CAR T cells are indicated by yellow arrows, with insets marked by rectangles. Scale bars are presented as 100 μ m for the zoomed-out view and 20 μ m for the zoomed-in view. Reproduced from (97).

To more precisely characterize the spatial distribution and functional status of CAR T cells within intact lymphoid tissues, CODEX multiplexed fluorescence imaging was conducted using markers such as CD3, CD20, CFSE, CD38, and IFN- γ . This technique allowed for imaging of localization and distribution of CAR T cells, activation-associated marker expression, while maintaining the three-dimensional tissue architecture.

Since PI3K-19BBz CAR T cells had consistently presented high tissue infiltration relative to conventional CAR T cells in the previous analyses, spatial characterization of this treatment group was performed mainly. As shown in **Figure 35**, CFSE-labeled CAR T cells were observed in the tissue parenchyma easily and in the nearness of CD20⁺ B cells, indicating successful migration to the lymphoid tissue and direct contact with target cells. Furthermore, the activation marker CD38 and the effector cytokine IFN- γ both presented in infiltrating CAR T cells suggests that these cells were functionally active even after they had been implanted in the tissue microenvironment.

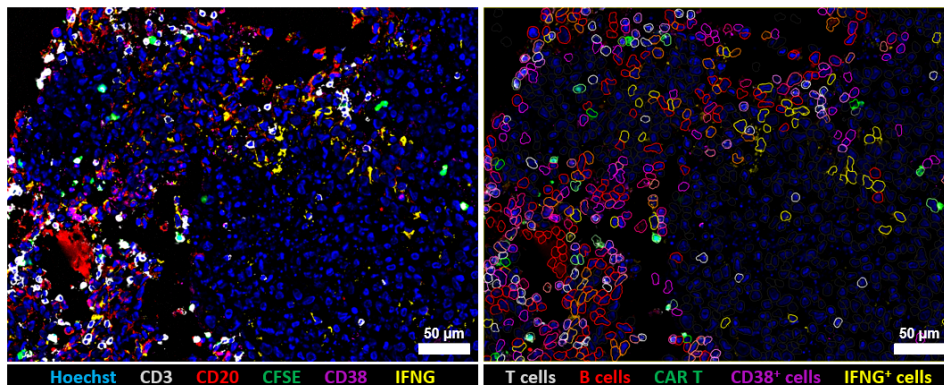


Figure 35. Immune cells in CAR T cell treated tissue.

A multiplex image alongside its segmented counterpart depicts the variety of cell types present. Left: the original overlay image displayed in pseudocolor; Right: cell classification determined by intensity thresholds (CD3: 737; CD20: 632; CFSE: 1561; CD38: 375; IFN- γ : 357). Scale bar: 100 μ m. Reproduced from (97).

Quantitative spatial analysis of the average intercellular distances between PI3K-CAR T cells, B cells, and non-B cells demonstrated that PI3K-modified CAR T cells were significantly more closely associated with B cells than with non-B cells, consistent with preferential localization toward target cell populations (**Figure 36A**). In addition,

CODEX-based quantification confirmed a higher abundance of PI3K-CAR T cells within LN tissues compared with conventional CAR T cell conditions (**Figure 36B**), further supporting enhanced tissue infiltration and target-proximal positioning in the PI3K-modified setting. The raw data mapping to Figure 36 can be found in **Table 42** and **Table 43**.

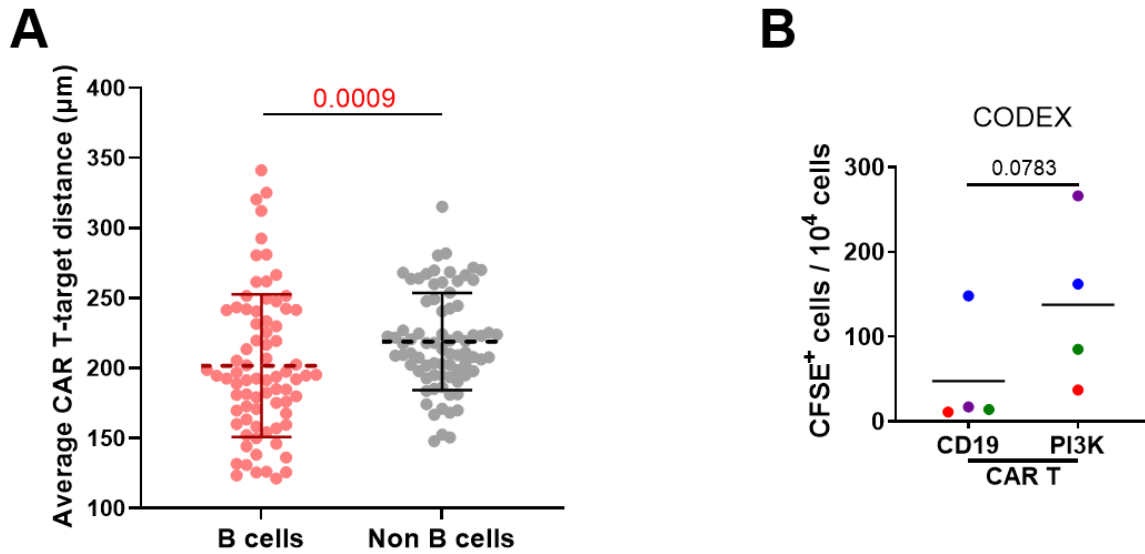


Figure 36. Spatial analysis of PI3K-CAR T cell targeting.

(A) The mean distances were measured between effector cells (CAR T cells) and target cells (B cells), as well as between effector cells and non-target cells. Data was gathered from four separate experiments, which identified 9, 14, 30, and 24 effector cells; 307, 64, 264, and 243 target cells; and 812, 881, 1307, and 886 non-target cells for the distance analysis. (B) The prevalence of CAR T cells within the tissue was assessed. Statistical methods employed included paired t-tests for analysis of ratios. Reproduced from (97).

Table 42. Raw data of Fig. 36A

CAR T to B cells				CAR T to Non B cells			
744.8407	666.5425	775.2753	475.0969	708.0895	450.3106	746.9149	595.6086
645.1779	480.9146	597.5674	479.1682	642.9566	391.8195	601.3212	710.267
386.9893	397.5381	639.8586	432.9513	445.8173	399.062	696.8509	711.6395
507.4014	849.1841	656.6568	483.4815	551.0089	589.8912	714.8692	743.143
464.5517	457.7879	641.4017	490.2168	516.9065	404.2963	698.9106	558.0277
444.5794	321.0593	581.3531	476.8191	526.0468	453.3252	584.2734	589.2508
489.4444	661.5404	544.3689	416.4395	554.4397	490.7812	578.0201	590.9133

452.939	743.3261	582.2054	694.0826	536.2016	537.5256	581.6037	486.733
535.5806	510.2325	574.4237	693.2618	597.0498	441.9273	576.0211	480.5155
	904.3712	548.117	382.432		656.5755	593.4175	549.8939
	424.333	526.4638	361.2267		720.1054	559.8284	592.2513
	327.1607	666.7273	422.9329		524.5768	637.4727	699.9415
	403.5193	619.0228	827.584		479.8531	594.0715	534.8013
	332.7483	706.3027	333.2562		536.0869	673.0131	553.3689
		523.1361	861.7275			566.2232	556.8879
		517.2828	366.5097			524.5248	461.8759
		513.8869	334.1919			554.9531	539.3772
		507.0472	408.7866			546.3134	706.0553
		516.1742	346.8655			516.5741	577.306
		640.1338	349.0627			695.0233	587.3754
		509.0321	419.2059			512.757	715.4275
		521.9055	449.7106			515.896	510.6602
		510.365	515.6053			504.7264	550.6036
		500.5107	565.9403			538.4864	835.3282
		536.7928				582.1231	
		637.5181				688.7784	
		642.5267				692.1051	
		466.5526				493.8658	
		613.819				660.3503	
		609.0612				648.1884	

Values in Table 42 indicate the average distance

Table 43. Raw data of Fig. 36B

	CD19-CAR-T	PI3K-CAR-T
Case 28	148	162
Case 23	17	266
Case 27	14	85
Case 17	11	37

Values in Table 43 represent the number of CFSE⁺ cells per 10,000 cells, determined by counting CFSE-positive cells following CODEX image segmentation.

3.5.3 Cytokine expression of CAR T cells

Multiplexed IF analysis revealed an increase in the proportion of PI3K-CAR T cells within the tissue that were expressing IFN- γ (**Figure 37A**), indicating enhanced effector

function at the single-cell level in situ. Cytokine profiling of culture supernatants from LN tissues treated with PI3K-CAR T cells demonstrated elevated concentrations of various T cell activation-related mediators, such as IL-4, IL-6, IL-17 α , TNF- α , and IFN- γ , in comparison to those treated with standard CAR T cells. Importantly, treatment with PI3K-CAR T cells correlated with reduced levels of IL-10, aligning with a transition away from an immunosuppressive cytokine environment. Additionally, IL-2 levels were also diminished, indicating a more complex alteration of the cytokine network rather than a simple upregulation of signals associated with T cells (**Figure 37B**). Collectively, these findings imply that PI3K-modified CAR T cells elicit a distinct and more functionally responsive immune response profile when compared to traditional CAR T cells within the tissue microenvironment.

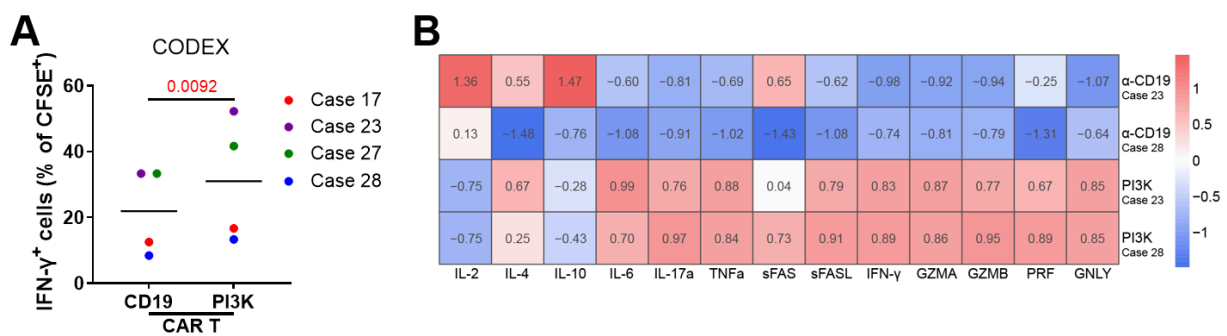


Figure 37. PI3K-CAR T cells induce an immune-activating cytokine profile.

(A) Proportion of CAR T cells that express IFN- γ (n = 4). (B) Heatmap displaying the comparative levels of cytokines observed following CD19-CAR T or PI3K-CAR T therapy. Statistical analysis: paired t-tests ratio. Reproduced from (97).

Table 44. Raw data of Fig. 37A

	CD19-CAR-T	PI3K-CAR-T
Case 28	8.41	13.33
Case 23	33.33	52.23
Case 27	33.33	41.67
Case 17	12.5	16.67

Values in Table 44 represent the IFN- γ positive rate of the CFSE⁺ CAR T cells.

Table 45. Raw data of Fig. 37B

	IL-2	IL-4	IL-10	IL-6	IL-17ATNFA	sFas	sFasL	IFNG	GZMA	GZMB	PRF	GNLY	
CD19-CAR-t.1	141.1	26.1	170.1	4263	40.2	157.3	288.2	61	1900.5	10702.8	32178.2	1695.9	10145.7

CD19-CAR-t.2	106.5	18.5	117.4	2811.8	39.1	114.6	173.1	30.6	2378.3	12147.3	37332.4	1066.7	11810
PI3K-CAR-t.1	81.7	26.5	128.7	9036.9	58.4	359.1	254.5	153.8	5455.4	32332	92394.1	2239.2	17525.9
PI3K-CAR-t.2	81.8	24.9	125	8152.5	60.8	354.2	292.9	161.3	5565	32216.7	98670.4	2371.3	17520.3

Values represent the concentrations of cytokines in the culture medium (pg/mL) with CD19-CAR T or PI3K-CAR T treatments.

Finally, it has been demonstrated that PI3K-CAR T cells possess a higher degree of tissue penetration and functional activation in the *ex vivo* lymphoid tissue model, suggesting that modulation of PI3K signaling may contribute to improved CAR T cell behavior and potentially enhance therapeutic performance. In addition, the 3D perfusion bioreactor system effectively recapitulates key aspects of CAR T cell activity within a physiologically relevant tissue microenvironment. This platform thereby provides a valuable tool for the functional evaluation, development, and optimization of next-generation CAR T cell therapies.

3.6 Targeting TRP-2 in melanoma using a bioreactor system

3.6.1 Tyrosinase-related protein 2 as a therapeutic target in melanoma

Tyrosinase-related protein 2 (TRP2), or dopachrome tautomerase (DCT), is a melanocyte differentiation antigen highly expressed in melanoma cells and involved in melanin biosynthesis (114). Owing to its restricted expression pattern and immunogenicity, TRP2 represents an appealing target for melanoma immunotherapy. TRP2-derived peptides can be presented by major histocompatibility complex (MHC) molecules, recognized by cytotoxic T lymphocytes enabling selective immune targeting of melanoma cells (115). Although TRP2 has been evaluated in cancer vaccines and adoptive T cell-based treatments, studies investigating TRP2-targeting antibodies remain limited.

To address this gap, we collaborated with professor Lukas Flatz, whose laboratory developed and synthesized anti-TRP2 antibodies. Using our bioreactor platform, I evaluated the therapeutic efficacy of these antibodies in human lymph node metastatic melanoma tissues. **In this section, the bioreactor experiments, data analysis, statistical evaluation, and figure preparation were performed by myself.**

3.6.2 Anti-TRP-2 treatment reduces viability of melanoma cells in lymph node metastases

I used human LN tissue with melanoma metastasis as the experimental model, as anti-TRP2 humanized antibodies may exploit resident immune cells within the LN to mediate tumor cell killing. Anti-TRP2 treatment was administered for 3 days using two antibody clones: IgG12 and IgG16 at 1 $\mu\text{g/mL}$. Following culture, tissues were dissociated and analyzed by flow cytometry, with cancer cells approximately identified by exclusion of CD45⁺ and CD31⁺ cells (**Figure 38A**). Cell viability analysis demonstrated that the IgG12 anti-TRP2 clone significantly reduced melanoma cell viability (**Figure 38B**). These results indicate that the bioreactor culture system provides a reliable platform for evaluating novel immunotherapeutic agents. The raw data of Figure 38B can be found in **Table 46**.

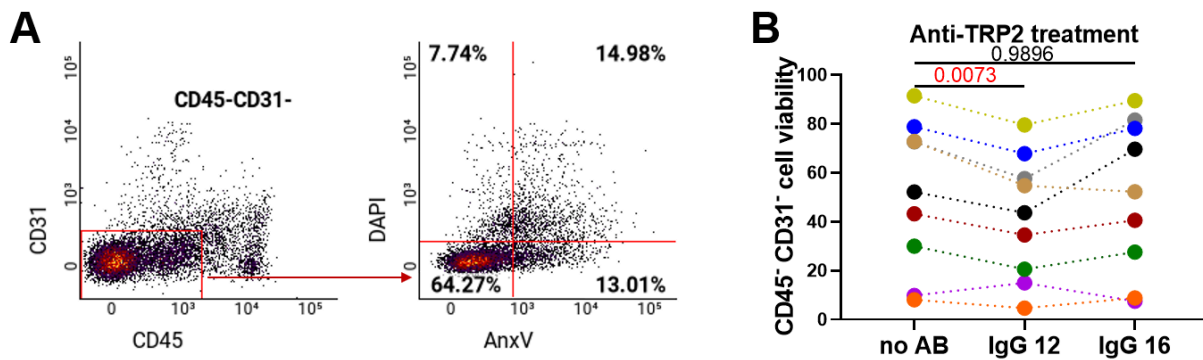


Figure 38. Anti-TRP2 Treatment of Melanoma.

(A) Gating strategy for identification of melanoma cells by flow cytometry, excluding CD45⁺ immune cells and CD31⁺ endothelial cells. (B) Viability of melanoma cells following 3-day treatment with anti-TRP2 antibody clones IgG12 and IgG16 (n = 9). Statistics: one-way ANOVA with Dunnett's test.

Table 46. Raw data of Fig. 38B

No AB	IgG 12	IgG 16
43.29	34.65	40.6
30.08	20.6	27.61
72.87	54.83	52.27
8.12	4.74	8.88
78.84	67.84	78.2

72.71	57.69	81.62
52.19	43.74	69.68
9.9	15.08	7.54
91.57	79.62	89.52

Values in Table 46 represent the percentage of live CD45⁺CD31⁺ cells to all CD45⁺CD31⁺ cells under IgG12 or IgG16 treatments.

3.6.3 Anti-TRP2 treatment induces melanoma cell death via ADCC

To investigate the mechanism underlying the therapeutic activity of anti-TRP2 antibodies, I examined whether antibody-dependent cell-mediated cytotoxicity (ADCC) contributes to melanoma cell killing. ADCC is an immune effector mechanism in which an Fc receptor expressing immune cells, such as neutrophils, recognizes and eliminates antibody coated target cells, including virus-infected or tumor cells. In this process, IgG antibodies bind to antigens on the surface of target cells, while Fc receptors on effector cells recognize the Fc region of the bound antibodies, triggering cytotoxic responses that can involve granzyme- and perforin-mediated lysis.

To evaluate the presence of antibody binding and potential effector cells in melanoma tissues, I analyzed human IgG and CD15 (a neutrophil marker) expression using a tissue microarray (TMA) provided by the laboratory of Lukas Flatz. Immunohistochemical (IHC) staining was performed using the BenchMark ULTRA platform (Ventana, Roche Diagnostics). Tissue sections were pretreated with BenchMark ULTRA CC1 solution (Ventana, Roche Diagnostics) for 64 minutes at 95 °C, followed by incubation with polyclonal rabbit anti-human IgG (A0423; Dako, Agilent, Santa Clara, CA; 1:30,000) and mouse anti-human CD15 antibody (MAB11295; Abnova, Taipei, Taiwan; 1:25) for 32 minutes at 37 °C. Slides were subsequently counterstained with Hematoxylin II (Ventana, Roche Diagnostics; 20 minutes at 37 °C) and Bluing Reagent (Ventana, Roche Diagnostics; 8 minutes at 37 °C). Signal detection was performed using the UltraView Red ISH DIG Detection Kit (Ventana, Roche Diagnostics) according to the manufacturer's instructions. Following staining and digital slide scanning, cores that were missing or morphologically distorted were excluded, leaving 219 TMA cores suitable for further analysis.

The tissue cores were subsequently divided into two groups based on IgG staining status (IgG-positive and IgG-negative). CD15-positive neutrophils were manually annotated, and each tissue core was scored according to the number of CD15-positive cells observed per core. Scores were assigned as follows: no CD15-positive cells (-), 1–5 positive cells (+), 6–10 positive cells (++), and more than 11 positive cells (+++) (Figure 39).

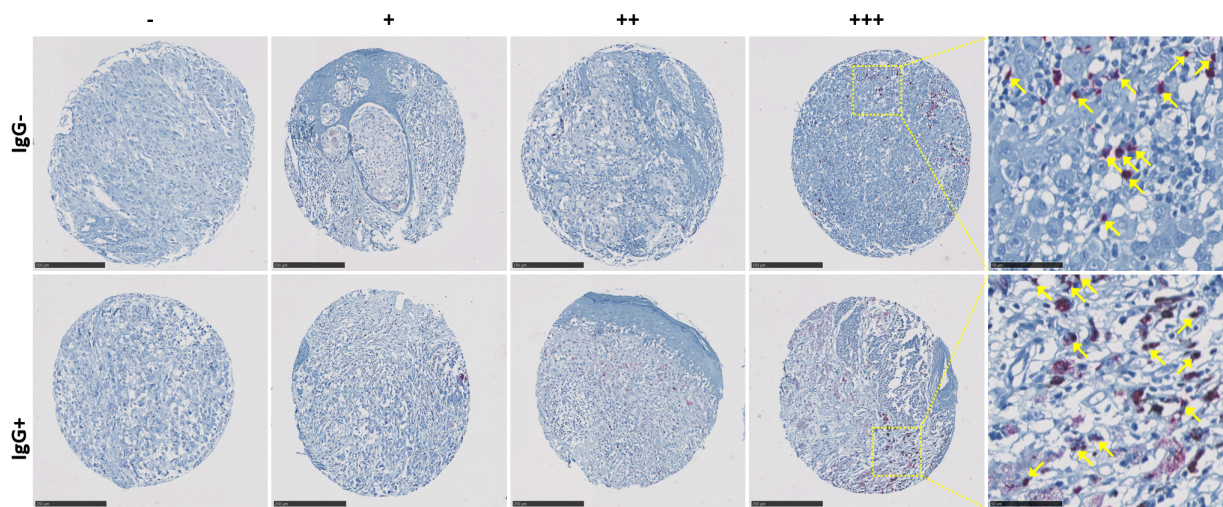


Figure 39. Representative CD15 IHC staining and scoring categories.

Representative images illustrating the scoring system based on the number of CD15-positive neutrophils per core: - (0 cells), + (1–5 cells), ++ (6–10 cells), and +++ (>11 cells)

To assess whether IgG deposition is associated with neutrophil infiltration, the number of CD15-positive cells per TMA core was compared between IgG-positive and IgG-negative samples. As the data did not follow a normal distribution (Kolmogorov–Smirnov test, $p < 0.05$), differences between groups were evaluated using a Mann–Whitney test. IgG-positive cores exhibited significantly different CD15-positive cell counts compared with IgG-negative cores (**Figure 40A**). I also evaluate this relationship using categorical scoring, CD15-positive neutrophils were grouped according to the predefined scoring system: no CD15-positive cells (-), 1–5 positive cells (+), 6–10 positive cells (++), and more than 11 positive cells (+++) per core. A chi-square test demonstrated a significant association between IgG status and neutrophil infiltration (**Figure 40B**, see raw data in **Table 47**). Together, these results indicate that IgG deposition is associated with increased neutrophil infiltration in

melanoma tissues, supporting a potential role for ADCC in mediating anti-TRP2 antibody–induced tumor cell killing.

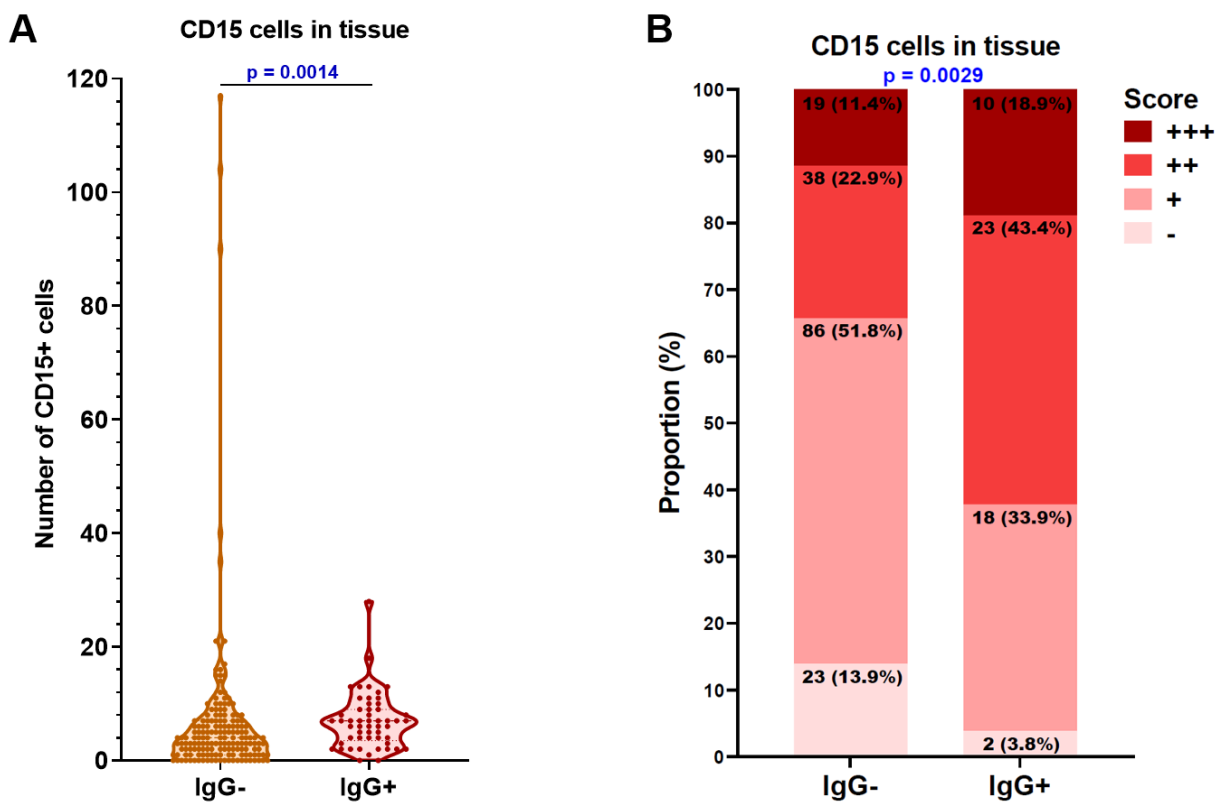


Figure 40. Quantification of CD15 IHC staining results.

(A) Distribution of CD15-positive cells per core in IgG-positive and IgG-negative samples. As the data were not normally distributed (Kolmogorov–Smirnov test $p < 0.05$), differences were analyzed using a Mann–Whitney test ($p = 0.0014$; $n_{\text{IgG-}} = 166$, $n_{\text{IgG+}} = 53$). (B) Categorical analysis of neutrophil infiltration using the scoring system (–: 0 cells; +: 1–5 cells; ++: 6–10 cells; +++: >11 cells per core) (chi-square test, $p = 0.0029$).

Table 47. Raw data of Fig. 40B

	IgG-	IgG+
+++	19	10
++	38	23
+	86	18
-	23	2

Values in Table 47 represent the number of cases in each scoring category for the control and treatment groups.

Next, given that neutrophils may contribute to the mechanism underlying anti-TRP2 induced melanoma cell killing, I investigated their potential role in our bioreactor culture system. To this end, exogenous human neutrophils were added to bioreactor cultures containing LN tissue with melanoma metastases. Briefly, peripheral blood from healthy donors was collected to isolate human neutrophils. Briefly, whole blood was mixed 1:1 with dextran solution (2% Dextran, 0.9% NaCl) in 50 mL Falcon tubes and allowed to sediment for approximately 30 minutes until two phases formed. The upper phase was carefully transferred onto 15 mL Pancoll (1.077 g/mL; PAN-Biotech, #P04-601000) in a new 50 mL Falcon tube (approximately 15–20 mL overlay) and centrifuged at 1600 rpm for 30 minutes at room temperature without brake. After centrifugation, the supernatant containing plasma, the PBMC layer, and Pancoll was removed. The remaining pellet was resuspended in 15 mL erythrocyte lysis buffer (Qiagen, #158103) and incubated for 10 minutes, followed by centrifugation at 1600 rpm for 10 minutes at room temperature. The cell pellet was washed with PBS and centrifuged again at 1600 rpm for 10 minutes. Finally, the neutrophil pellet was resuspended in culture medium.

The purity of the isolated neutrophils was assessed by staining with APC-Cy7–anti-CD15 (BioLegend, #323047) followed by flow cytometry analysis (**Figure 41**). A total of 3×10^6 neutrophils were then added to the bioreactor system containing melanoma LN tissue 24 h after the start of culture and the initiation of anti-TRP2 treatment.

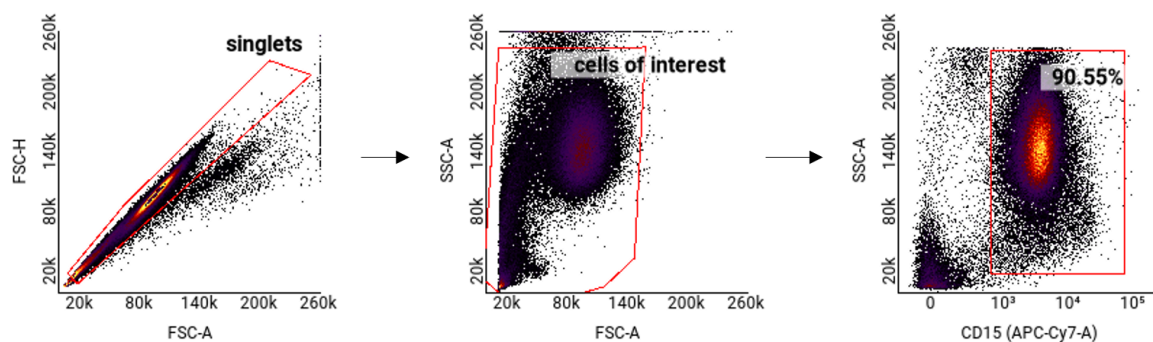


Figure 41. Flow cytometry gating strategy for neutrophils.

Gating strategy for CD15⁺ neutrophils.

Thereafter, I investigated the combined effect of neutrophils and anti-TRP2 antibodies (IgG12/16) on melanoma cell viability. The addition of neutrophils to the bioreactor culture significantly enhanced the tumor-killing efficacy of the IgG16 treatment group. A similar trend toward reduced melanoma cell viability was also observed in the IgG12 treatment group upon neutrophil supplementation (**Figure 42A**). Since ADCC requires interaction between the Fc region of antibodies and Fc receptors on effector cells, I next introduced an Fc receptor blocker (FcRB) to determine whether the observed effects were mediated through this mechanism. Blocking Fc receptor signaling attenuated the tumor-killing effect, resulting in increased melanoma cell viability compared with the neutrophil plus IgG12 treatment condition (**Figure 42B**).

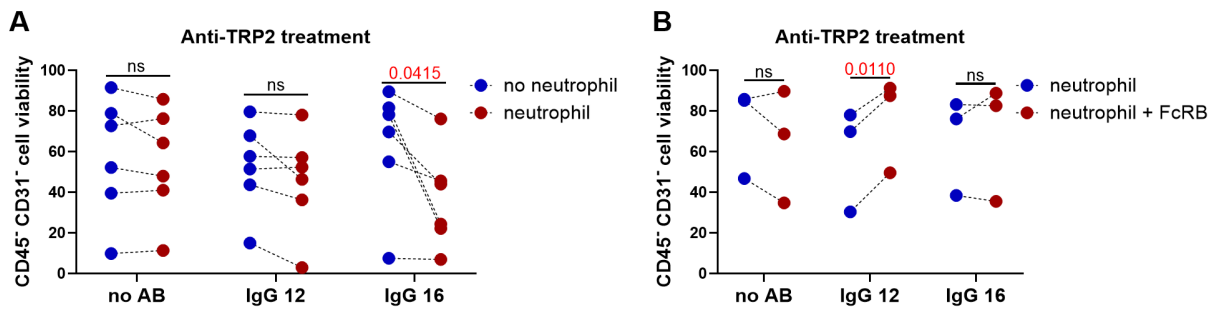


Figure 42. Neutrophils enhance anti-TRP2 antibody-mediated ADCC.

(A) Flow cytometric analysis of CD45⁺CD31⁻ melanoma cell viability after treatment with anti-TRP2 antibodies (no Ab, IgG12, IgG16) in the presence or absence of neutrophils (n = 6, paired t-test). (B) Flow cytometric analysis of anti-TRP2 antibodies (no Ab, IgG12, IgG16) combined with neutrophils, with or without FcRB (n = 3, paired t-test).

Table 48. Raw data of Fig. 42A

	No neutrophil					With neutrophil							
no AB	78.84	72.71	39.55	52.19	9.9	91.57	64.27	76.27	41.04	47.98	11.34	85.75	
IgG 12	67.84	57.69	51.49	43.74	15.08	79.62	46.34	57.13	52.41	36.3	2.99	78.01	
IgG 16	78.2	81.62	55.01	69.68	7.54	89.52	22.22	24.4	45.63	44.01	6.97	76.16	

Values in Table 48 represent the percentage of live melanoma cells (CD45⁺CD31⁻ cells to all CD45⁺CD31⁻ cells) under IgG12 or IgG16 treatments with or without adding neutrophils.

Table 49. Raw data of Fig. 42B

	Neutrophil only			Neutrophil + FcRB		
no AB	85.24	46.82	85.75	68.73	34.79	89.74

IgG 12	69.9	30.39	78.01	87.51	49.66	91.33
IgG 16	83.2	38.43	76.16	82.59	35.58	88.81

Values in Table 49 represent the percentage of live melanoma cells (CD45⁺CD31⁻ cells to all CD45⁺CD31⁻ cells) under the effect of FcRB.

Taken together, these findings indicate that neutrophils can enhance the anti-tumor activity of anti-TRP2 antibodies in the melanoma LN bioreactor culture system. The increased melanoma cell killing observed following neutrophil supplementation, together with the attenuation of this effect upon Fc receptor blockade, supports a mechanism involving Fc receptor–dependent ADCC. These results suggest that neutrophils may act as important effector cells contributing to anti-TRP2 antibody–mediated melanoma cell elimination. Importantly, these results further highlight the utility of the human tissue bioreactor culture system established in this study, which preserves key components of the tumor microenvironment and enables functional evaluation of immune-mediated therapeutic mechanisms in patient-derived tissues. This platform therefore represents a valuable experimental system for investigating immune effector mechanisms and for testing novel antibody-based cancer therapies in a physiologically relevant human tissue context.

However, multiple limitations need to be considered. There was a limited number of patient samples examined, and inter-patient heterogeneity may affect neutrophil function and antibody responsiveness. Furthermore, the *ex vivo* culture system cannot fully recapitulate systemic immune interactions, immune cell recruitment, or long-term therapeutic responses observed *in vivo*. Future studies should include larger patient cohorts, characterize the functional phenotypes of infiltrating neutrophils, and evaluate combinations with other immunotherapeutic strategies. Further optimization and validation of this bioreactor platform may facilitate its application in FPM for predicting individual responses to antibody-based immunotherapies.

3.7 Integrating 3D whole-organ light-sheet microscopy and 2D highly multiplexed microscopy

3.7.1 The concept of correlative LSM–CODEX imaging

Immunotherapy has the potential to induce durable cancer remission. However, therapeutic efficacy is frequently limited by heterogeneous responses across metastatic lesions (116). To systematically characterize tissue niches associated with immune evasion, I established a multiscale imaging workflow that integrates whole-organ light-sheet microscopy (LSM) with highly multiplexed spatial imaging using CODEX.

Whole-organ LSM enables visualization of heterogeneous immunotherapy responses across metastatic sites within the same organ or across different organs (117). However, due to limitations in the numbers of markers that are used for cell type identification, it cannot directly characterize the TME in detail. In contrast, multiplex immunofluorescence imaging provides high-resolution, high-dimensional cellular and spatial characterization of the tumor microenvironment. By correlating organ-scale imaging with single-cell spatial profiling, this platform aims to identify diverse immunosuppressive mechanisms and guide the development of more effective therapeutic strategies for metastatic cancer.

This collaborative project was conducted together with the group of Bettina Weigelin at the Werner Siemens Imaging Center, and is jointly led by PhD student Gina Dunkel and myself. Gina Dunkel was responsible for tissue clearing, 3D light-sheet microscopy imaging, CT-guided localization of regions of interest, tissue milling, and generation of tissue arrays. My contributions included antibody conjugation and validation, tissue sectioning, multiplex staining, CODEX imaging, and preliminary spatial data analysis. The project is currently in preparation for publication, and I am expected to serve as co-first author. **All data shown in this section were generated by me.**

3.7.2 Optimization of tissue clearing, antibody validation, and staining protocol for correlative spatial imaging

Since LSM requires intact 3D tissues, samples must undergo optical clearing protocols. These multi-step processes make the tissue transparent through permeabilization, decolorization, dehydration, clearing, rehydration, and embedding. These procedures involve exposure to various chemical reagents that may compromise antigen integrity and reduce the performance of conventional antibodies in processed tissues. To maximize the number of antibodies compatible with this workflow, I systematically compared different tissue-clearing protocols and selected conditions that minimally affected antibody binding in the tissue. In parallel, to ensure reliable identification of key cell populations within the TME, I generated and validated new antibody conjugates for highly multiplexed spatial imaging. In addition, I optimized the staining protocol to improve signal quality.

During the initial staining experiments with cleared tissues, I observed strong tissue autofluorescence, resulting in high background signal and relatively weak marker-specific staining. To optimize the staining protocol and improve signal quality, I investigated whether the addition of a bleaching step prior to antibody blocking could reduce background fluorescence. The bleaching solution consisted of 10 mL PBS, 1.8 mL 30% H₂O₂, and 0.32 mL 1 M NaOH, freshly prepared before use, and tissues were incubated twice for 45 min. In addition, I evaluated the effect of combining bleaching with antigen retrieval (pH 9, 10 min at 95°C).

To compare the different conditions, tissues were stained using an anti-CD45 CODEX-conjugated antibody with or without the additional optimization steps. The results showed that tissues processed without bleaching exhibited strong background fluorescence, making the CD45 signal difficult to distinguish clearly. In contrast, the inclusion of the bleaching step markedly reduced background staining and significantly improved signal-to-noise ratio. PFA fixation did not substantially affect staining intensity; however, it improved preservation of tissue morphology and was therefore retained in the protocol. In comparison, antigen retrieval negatively affected staining quality, as CD45 membrane localization was lost and replaced by nonspecific nuclear staining

(**Figure 43**). Based on these findings, the optimized staining workflow was established by maintaining PFA fixation and incorporating an additional bleaching step prior to antibody staining.

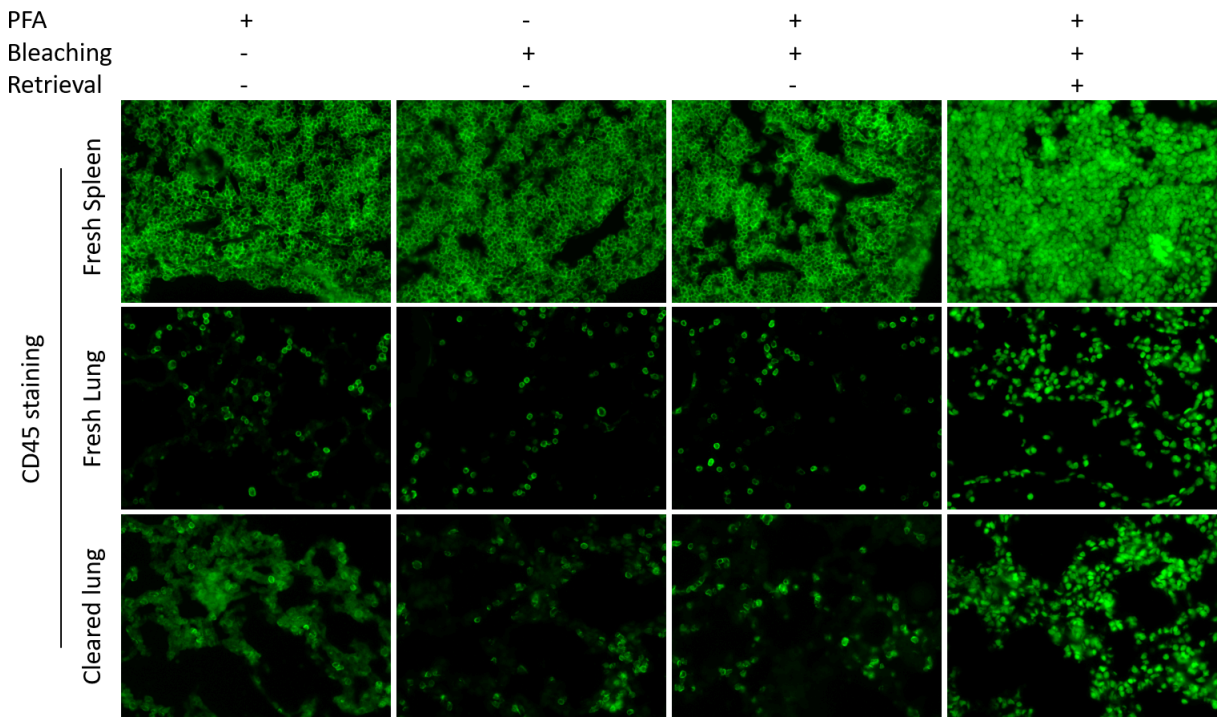


Figure 43. Optimization of staining steps for cleared tissues.
Representative fluorescence images of anti-CD45 CODEX staining in fresh spleen, fresh lung, and ECI-cleared lung tissues under various staining conditions, including bleaching, PFA fixation, and antigen retrieval optimization.

To find out which clearing method can best preserve antigen and adapt to CODEX, I applied in total of 85 oligo conjugated antibodies (have not been validated) on the staining of 10-spleen tissue microarray, which containing 1 fresh spleen, 1 PFA fixed spleen and 8 spleens cleared by different methods, including ethyl cinnamate (ECi), ECi + MeOH-H₂O₂, ECi + Urea/Collagenase, ECi + Zwittergent, ScaleS, EZ-Clear, (3D Imaging of Solvent-Cleared Organs) 3DISCO and Pegasos (118–124).

I evaluated the staining patterns of each marker across different tissue preparations. In the clearing ECi group, 49 markers showed positive (20 markers showed strong positivity, 13 were moderately positive, 16 were weakly positive) and 36 were negative.

This is comparable to the control tissues, where the fresh tissue had 45 positive markers and the PFA-fixed tissue had 48. However, the other seven groups had fewer positive markers, especially in the clearing Pegasos group, with the 53 markers showing negative, indicating reduced antigen avidity (**Table 50**).

Table 50. List of CODEX-Conjugated Antibodies and Staining Evaluation

Conjugates	Panel	Dilution	ET (ms)	Oligo	Clone	Catalogue #	Company	1	2	3	4	5	6	7	8	9	10
B220	1	1:100	350	3	RA3.3A1/6.1	BE0067	Bio X Cell	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CD21/35	1	1:50	500	72	7G6	553817	BD Bioscience	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sca-1	1	1:100	250	56	D7	108102	BioLegend	+++	+++	+++	++	+++	+++	+++	++	++	++
CD206	1	1:100	500	25	C068C2	C068C2	BioLegend	+++	+++	+++	+	++	++	+	+	+	+
CD68	1	1:100	500	48	FA-11	137002	BioLegend	+++	++	++	-	+	-	+	-	-	-
CD8	1	1:25	500	71	4SM16	14-0195-95	Invitrogen	+++	+++	+++	++	+++	+++	+	+++	+++	+
TNFR	1	1:50	500	32	LG.3A10	553777	BD Bioscience	+++	+++	+++	+++	+++	+++	++	++	+++	+
Ly6G	1	1:400	167	55	1A8	BE0075-1	Bio X Cell	+++	+++	+++	+++	+++	+++	++	+++	+++	+++
Ki-67	1	1:50	500	52	B56	556003	BD Bioscience	+++	+++	+++	+++	+	+++	+	+++	++	++
CD3	1	1:200	500	6	17A2	555273	BD Bioscience	+++	+++	+++	+	+++	+	+	-	-	-
IgM	1	1:50	500	33	II/41	14-5790-82	Invitrogen	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
Ly6C	1	1:100	250	41	HK1.4	BE0284	Bio X Cell	+++	++	+	+	-	-	+	+	+	-
Collagen IV	3	1:50	500	33	polyclonal	ab6586	Abcam	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
aSma	2	1:100	500	15	1A4	614852	BioLegend	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
HSP70	2	1:50	500	58	30A5	MA1-91639	Invitrogen	+++	+	++	++	++	++	++	++	+	+
F4/80	2	1:100	500	48	t45-2342	565409	BD Bioscience	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
MHC-2	2	1:400	200	36	M5/114.15.2	BE0108	Bio X Cell	+++	+++	+++	+++	+++	++	+++	++	++	++
CD45	2	1:200	500	62	30-F11	553076	BD Bioscience	+++	+++	+++	+++	+++	+++	+++	+++	++	+
CD31	2	1:50	500	66	MEC13.3	553370	BD Bioscience	+++	+	++	++	++	+	++	+	+	+
Ter-119	2	1:400	300	43	TER-119	553671	BD Bioscience	+++	+++	+++	+++	+++	+++	+++	++	+++	+
CD169	2	1:100	500	46	3D6.112	MCA884GA	Bio-Rad	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
CD106	1	1:100	350	76	429	553330	BD Bioscience	++	++	++	+	+	-	+	-	-	-
CD48	1	1:200	500	15	HM48-1	103402	BioLegend	++	++	++	+	++	+	+	+	++	+
CD11b	1	1:100	500	28	EPR1344	ab209970	Abcam	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
IBA	1	1:100	500	63	EPR16589	ab221790	Abcam	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CD38	1	1:200	500	44	90	102702	BioLegend	++	+	+	-	+	-	+	-	-	-
p16	1	1:50	500	38	F-12	sc-1661	Santa Cruz	++	+	+	-	-	-	-	+++	-	-
PD-1	2	1:200	500	23	29F.1A12	BE0273	Bio X Cell	++	+	+	-	-	-	-	-	-	-
CD41	2	1:400	250	75	MWREg30	133902	BioLegend	++	+	+	-	-	-	-	-	-	-
MPO	2	1:100	500	21	polyclonal	AF3667	R&D Systems	++	++	++	++	+	+	++	++	++	+
CD107a	2	1:100	500	11	1D4B	121602	BioLegend	++	++	+	-	++	+	++	+	-	-
Nkp46	4	1:50	500	45	29A1.4	AF2225	R&D Systems	++	++	++	++	++	++	++	++	++	++
Foxp3	4	1:50	500	53	d6o8r	12653F	Cell Signalling	++	+	+	+	+	+	+	++	+	+
PD-L1	4	1:50	500	81	10F.9G2	124302	BioLegend	++	++	+	++	++	+	++	++	+	+
CD150	3	1:100	167	24	polyclonal	NBP1-76555	Novus	+	+	++	+	+	+	+	+	+	+

CD135	1	1:50	500	61	113308	MAB7681	Novus	+	++	+++	+++	++	+++	+	+	+	++
Hifla	1	1:100	500	57	polyclonal	GTX127309	GeneTex	+	++	++	++	++	+++	++	+++	++	++
CD5	1	1:50	500	77	53-7.3	553017	BD Bioscience	+	+	++	++	++	++	+++	++	++	-
LYVE-1	3	1:50	500	49	ALY7	14-0443-82	Invitrogen	+	-	-	-	-	-	-	-	-	-
CD11c	2	1:50	500	26	HL3	553799	BD Bioscience	+	+	+	-	+	-	-	-	-	-
SIRPa	2	1:50	250	42	P84	144002	BioLegend	+	+	+	++	++	+	+	+	+	+
CD86	2	1:100	500	61	PO3	105102	BioLegend	+	-	-	-	-	-	-	-	-	-
CD11c	2	1:100	500	38	ap-mab0806	NB110-97871	Novus	+	-	-	-	-	-	-	-	-	-
VISTA	2	1:100	500	49	MH5A	143702	BioLegend	+	-	-	-	-	-	-	-	-	-
PNAd	4	1:50	500	17	meca-79	120802	BioLegend	+	+	+	+	+	+	+	+	+	+
Podoplanin	1	1:20	500	81	8.1.1	127402	BioLegend	+	++	++	-	+	++	+	+	+	+
ESAM	1	1:100	500	58	1G8	93892	BioLegend	-	-	+	+	-	++	+	++	++	++
Siglec H	1	1:100	500	20	551	129602	BioLegend	-	++	++	+	-	-	-	+	-	-
Caspase-3	1	1:100	500	43	5A1E	94530	Cell Signalling	-	+	++	+	+	+	-	-	-	-
CD127	1	1:100	500	74	A7R34	135002	BioLegend	-	+	+	-	-	+	+	-	-	+
CD137	1	1:50	500	75	4b4-1	555955	BD Bioscience	-	+	+	-	-	+	-	+	-	-
Tim-3	1	1:100	500	23	RMT3-23	MABF230	Sigma Aldrich	-	+	+	+	-	-	-	-	-	-
CD47	1	1:200	500	65	miap410	BE0283	Bio X Cell	-	+	+	-	+	+	-	-	+	-
CD34	1	1:20	250	59	RAM34	14-0341-82	Invitrogen	-	-	-	-	-	-	-	-	-	-
ICOS	1	1:20	350	60	C398.4A	313502	BioLegend	-	-	-	-	-	-	-	-	-	-
CTLA-4	1	1:100	500	80	F-8	sc-376016	Santa Cruz	-	-	-	-	-	-	-	-	-	-
Foxp3	1	1:100	500	53	150D	320002	BioLegend	-	-	-	-	-	-	-	-	-	-
Endomucin	1	1:100	500	21	polyclonal	AF4666	R&D Systems	-	-	-	-	-	-	-	-	-	-
CD16/32	1	1:100	500	45	93	101302	BioLegend	-	-	-	-	-	-	-	-	-	-
p21	1	1:50	500	46	EPR18021	ab232512	Abcam	-	-	-	-	-	-	-	-	-	-
CD105	2	1:20	250	69	MJ7/18	120402	BioLegend	-	-	-	-	-	-	-	-	-	-
CD49d	2	1:50	500	68	R1-2	103610	BioLegend	-	-	-	-	-	-	-	-	-	-
TCRgd	2	1:100	500	24	UC7-13D5	107502	BioLegend	-	-	-	-	-	-	-	-	-	-
MerTK	2	1:100	500	20	DS5MMER	14-5751-82	Invitrogen	-	-	-	-	-	-	-	-	-	-
FAS-L	2	1:50	500	80	NOK-1	556372	BD Bioscience	-	-	-	-	-	-	-	-	-	-
p53	2	1:100	500	14	DO-1	sc-126	Santa Cruz	-	-	-	-	-	-	-	-	-	-
CD15	2	1:100	500	5	MC-480	125602	BioLegend	-	-	-	-	-	-	-	-	-	-
FceRia	2	1:50	500	51	36951	134302	BioLegend	-	-	-	-	-	-	-	-	-	-
CD152	2	1:50	500	53	UC10-4B9	106302	BioLegend	-	-	-	-	-	-	-	-	-	-
CD90	2	1:100	500	29	30-H12	105302	BioLegend	-	-	-	-	-	-	-	-	-	-
CD127	3	1:50	500	79	polyclonal	PA5-47927	Invitrogen	-	-	-	-	-	-	-	-	-	-
CD47	2	1:100	500	65	B6H12	556044	BD Bioscience	-	-	-	-	-	-	-	-	-	-
CD42b	2	1:100	500	45	Sp219	ab240268	Abcam	-	-	-	-	-	-	-	-	-	-
Granzyme B	2	1:100	500	81	2C5	550558	BD Bioscience	-	-	-	-	-	-	-	-	-	-
p21	2	1:50	500	14	F-5	sc-6246	Santa Cruz	-	-	-	-	-	-	-	-	-	-
CD69	2	1:50	500	53	H1.2F3	104502	BioLegend	-	-	-	-	-	-	-	-	-	-
CD61	3	1:50	500	49	2C9.G2	104302	BioLegend	-	-	-	-	-	-	-	-	-	-
CD71	3	1:50	500	21	C2F2	15867108	Invitrogen	-	-	-	-	-	-	-	-	-	-
CD115	3	1:50	500	66	460615	MAB3818	R&D Systems	-	-	-	-	-	-	-	-	-	-
Fas/CD95	3	1:50	500	2	SA367H8	152602	BioLegend	-	-	-	-	-	-	-	-	-	-
CD90	3	1:50	500	63	G7	105202	BioLegend	-	-	-	-	-	-	-	-	-	-
CD19	3	1:50	500	8	1D3	553783	BD Bioscience	-	-	-	-	-	-	-	-	-	-
CD4	4	1:50	500	7	RM4-5	100506	BioLegend	-	-	-	-	-	-	-	-	-	-
CD25	4	1:50	500	30	3C7	101902	BioLegend	-	-	-	-	-	-	-	-	-	-
CD23	4	1:50	500	23	EPR26479-9	ab302526	Abcam	-	-	-	-	-	-	-	-	-	-

*1: Fresh tissue, 2: PFA fixed, 3: Clearing ECI, 4: Clearing ECI + MeOH-H₂O₂, 5: Clearing ECI + Urea/Collagenase, 6: Clearing ECI + Zwittergent, 7: Clearing ScaleS, 8: Clearing EZ-Clear, 9: Clearing DISCO, 10: Clearing Pegasos.

I found that 29 markers exhibited positive staining across all tissue groups. In the clearing ECI group, 16 of these 29 markers had fluorescence intensities above the average, and this group also showed the highest median fluorescence intensity among all samples. Meanwhile, the fresh tissue group exhibited the highest mean fluorescence intensity. In contrast, groups such as Clearing ECI + Zwittergent, Clearing ScaleS, Clearing EZ-Clear, and Clearing Pegasos showed both mean and median fluorescence intensities below average (**Figure 44**).

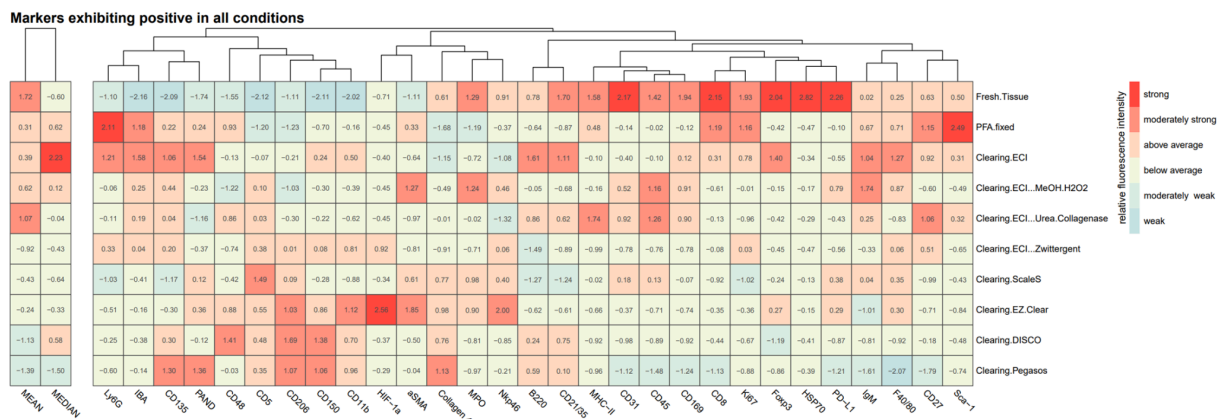


Figure 44. Comparison of signal intensity across different treated tissue.

Heatmap displaying relative fluorescence signal intensities across fresh tissue, PFA-fixed tissue, and tissues processed using various clearing methods. Data were normalized by column to highlight differences across experimental conditions.

I also presented representative 6-color fluorescence images demonstrating robust staining results in fresh tissue, Clearing ECI, and PFA-fixed tissue. Markers including B220 (green), CD27 (red), Ly6G (magenta), Ki-67 (yellow), and CD3 (cyan) were sharply defined with strong signal intensity, while tissue morphology remained well-preserved. In contrast, tissues treated with Clearing ECI + MeOH-H₂O₂, Clearing ECI + Zwittergent, Clearing EZ-Clear, and Clearing DISCO displayed weak or absent CD3 staining. Clearing Pegasos performed the worst, showing negative staining for CD3, CD27, and Ki-67 (**Figure 45** and **Figure 46**).

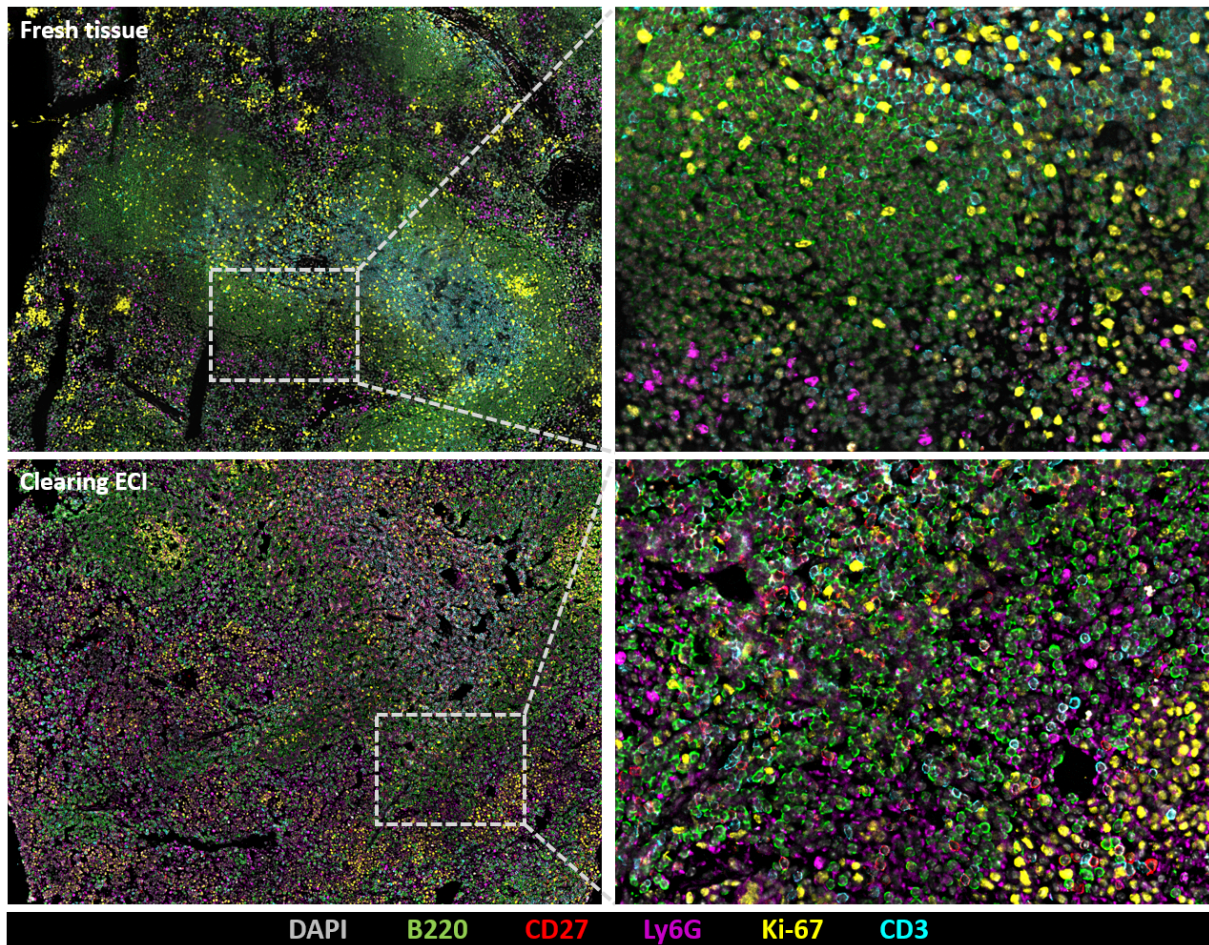


Figure 45. Representative multiplex fluorescence images of fresh and ECI-treated tissues.
 Markers include B220 (green), CD27 (red), Ly6G (magenta), Ki-67 (yellow), and CD3 (cyan).

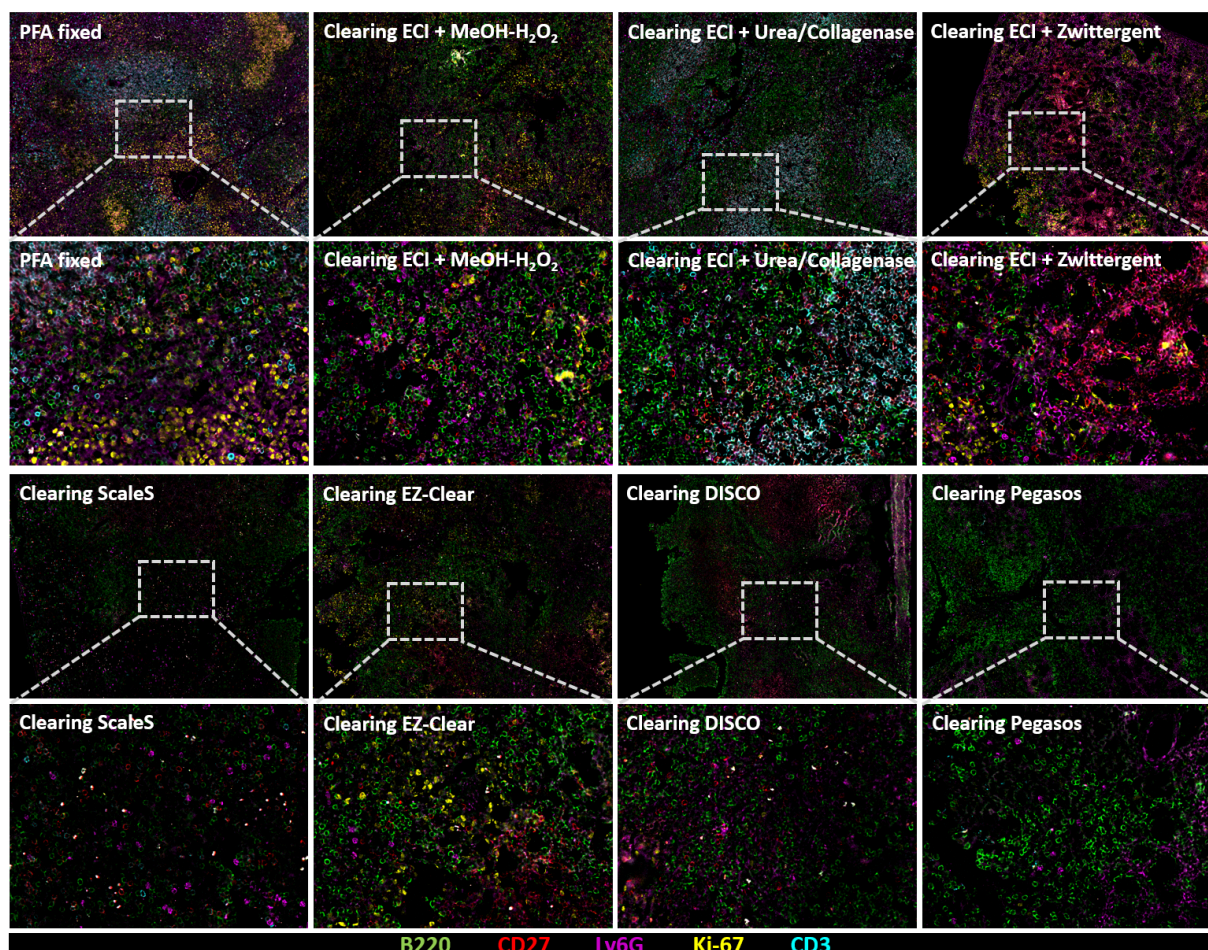


Figure 46. Representative multiplex fluorescence images of PFA fixed tissue and tissues processed with other clearing protocols.

Markers include B220 (green), CD27 (red), Ly6G (magenta), Ki-67 (yellow), and CD3 (cyan).

Similarly, additional multicolour images with markers including IgM (green), Sca-1 (red), CD21/35 (magenta), Ly6C (yellow), and IBA (cyan) also showed that Clearing ECI, similar to control fresh tissues and PFA fixed tissue, produced the best staining results (**Figure 47 and Figure 48**).

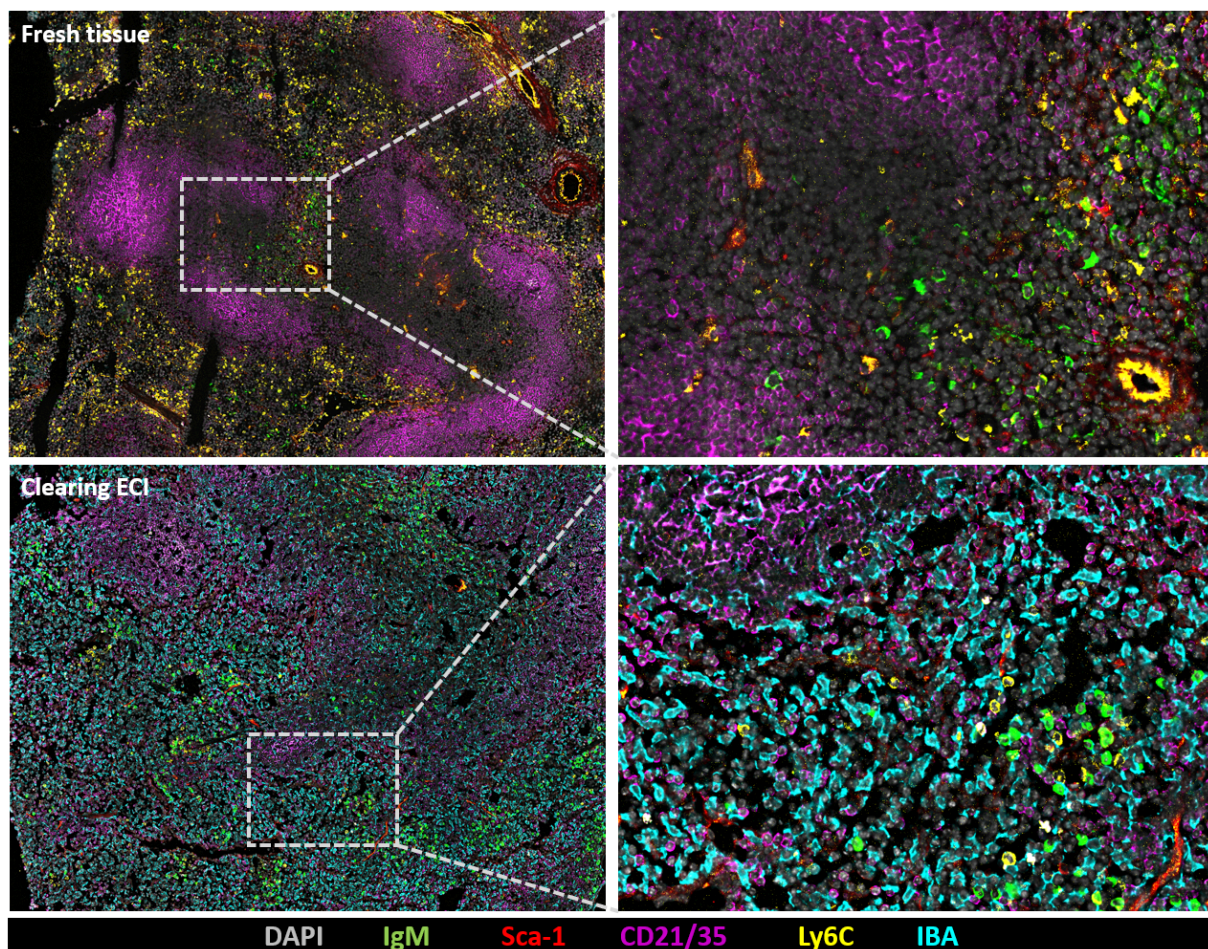


Figure 47. Additional representative multiplex fluorescence images of fresh and ECI-treated tissues.

Markers include IgM (green), Sca-1 (red), CD21/35 (magenta), Ly6C (yellow), and IBA (cyan).

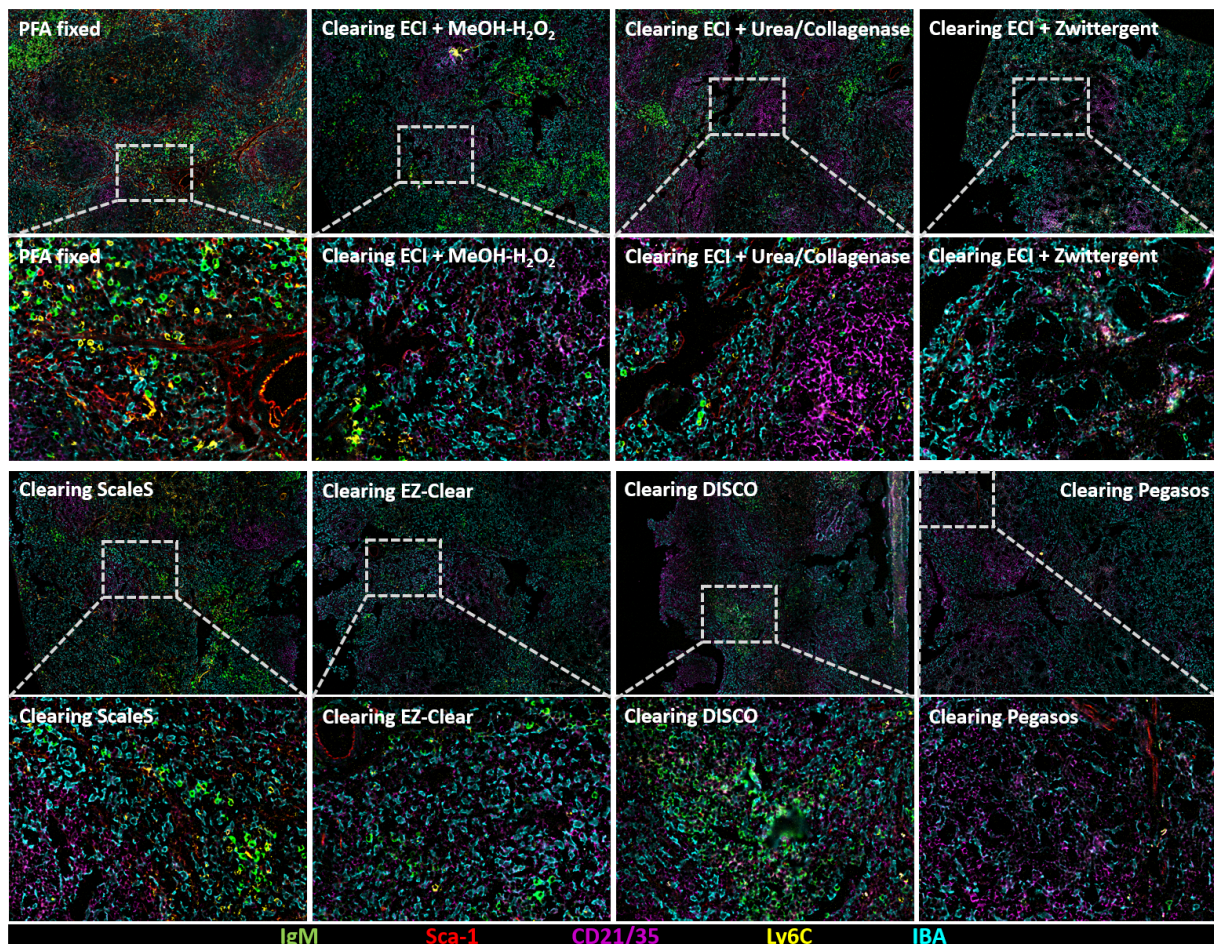


Figure 48. Additional representative multiplex fluorescence images of PFA fixed tissue and tissues processed with other clearing protocols.

Markers include IgM (green), Sca-1 (red), CD21/35 (magenta), Ly6C (yellow), and IBA (cyan).

Together, based on these results, I can identify that the ECI-based clearing protocol provides the best balance between tissue transparency, antigen preservation, and compatibility with highly multiplexed CODEX imaging among all tested clearing methods. Through systematic optimization of tissue clearing conditions, antibody conjugation and validation, as well as staining procedures, I established a workflow that preserves both tissue morphology and high-dimensional spatial information after whole-organ processing. Importantly, this optimization enables reliable integration of 3D whole-organ light-sheet microscopy with 2D highly multiplexed microscopy, allowing regions of interest identified at the organ scale to be further characterized at single-cell resolution within the tumor microenvironment.

Nevertheless, there are many challenges. As in the case of the tissue-clearing reagent, the duration of chemical treatment can additionally modulate the antigen integrity and antibody staining intensity impacting CODEX quality. In addition, the clearing efficiency and staining performance of specific tissues, specimens of different sizes, and fixation conditions may differ which in turn could impair the preservation of imaging consistency between tissue samples. This workflow, moreover, continues to be technically challenging and time-consuming and it may not be available routinely in studies or for clinical purposes. Future work should aim at standardizing sample preparation and clearing protocols, expanding existing antibodies to validated panels, and integrating automated image analysis and spatial quantification pipelines. Those refinements would improve reproducibility, scalability, and robustness, support comprehensive spatial characterization of the tumor microenvironment, and support more global translational use in cancer research and precision medicine.

4 Discussion and Limitations

4.1 Discussion

In the last ten years, cancer immunotherapy represents a shift in cancer therapy especially when targeted against patients with treatment-resistant malignancies, generating durable clinical responses (125). Newer evidence from TME has shown that therapeutic success cannot merely be attributed to underlying tumor characteristics, but that it is also deeply impacted by the TME, which modulates immune cell activation, treatment responsiveness, and eventually clinical outcome (56). Hence, the heterogeneity of tumor cells and their surrounding microenvironment poses this challenge as a major barrier for the advancement of immunotherapeutic strategies. Concurrently, the identification of patients who are the most probable to benefit from particular immunotherapies and the design of personalized treatment strategies are equally important outstanding challenges in the field. The magnitude of these challenges is important in the setting of advanced lymphoma and metastatic cancers, characterized by the spread of malignant cells resistant to therapy, which is a leading driver of cancer-related mortality. Under these late-stage disease conditions, traditional treatment approaches often fall short or are restricted by acquired resistance and systemic disease progression. Thus, immunotherapy has gained traction as an alternative therapeutic approach to address some of the limitations of traditional treatment modalities (126).

FPM combines direct *ex vivo* therapeutic testing of patient-derived tumor material with molecular and clinical information to support individualized treatment decision-making. In contrast to molecular profiling approaches, which infer potential drug responsiveness primarily from genomic or transcriptomic alterations (127), FPM evaluates the actual functional response of viable tumor cells to therapeutic agents under experimentally controlled conditions. By directly assessing treatment sensitivity at the cellular level, this strategy may provide a more immediate and biologically relevant representation of therapeutic efficacy.

This approach is particularly attractive in hematological malignancies, where rapid sample processing and short experimental turnaround times are technically feasible. Importantly, several prospective studies have demonstrated the clinical applicability of FPM and reported associations with improved treatment stratification and patient outcomes (72,128).

As anticancer therapies become more and more accessible, it is more and more vital to discover a patient-specific therapy which works well. Most therapeutic drugs have been shown to be effective in treating certain malignancies, yet response is variable in patients depending on tumor heterogeneity, variation in the TME, innate or acquired resistance pathways and many therapeutic agents. This makes the choice of the most effective therapy a complex matter in clinical oncology.

A similar trend has been noted in infectious diseases. Antimicrobials like penicillin have revolutionized treatment of such diseases and remained in use as evidence-based therapeutics, but as the number of antimicrobial agents expanded and antibiotic resistance developed, antimicrobial susceptibility testing became common practice, allowing for more targeted and personalized treatment selection (129). However, most oncological treatment approaches remain largely adhered to pre-set protocols with regard to tumor type and disease stage. Many patients are treated in an increasingly hierarchical fashion with first-line, second-line and third-line therapy following resistance or disease progression, in clinical practice. While this stepwise treatment program has achieved better survival and efficacy in some patients, it can prolong their access to potentially improved therapies and subjects them to nonresponsive interventions and unnecessary toxicities (130).

FPM may offer significant potential to redefine contemporary therapeutic choices, facilitating quick and direct evaluation of patient-centered drug responses in this framework. FPM enables functional assessment of living tumor tissues, potentially leading to more targeted treatment stratification and even to the development of genuinely personalized therapeutic strategies. Moreover, FPM's conjunction with the best and newest imaging tools and spatially-resolved analyses may ultimately support

the development of advanced theranostic platforms that can directly link predictive diagnostics and therapeutic interventions.

FPM has demonstrated considerable adaptability and translational potential in hematological malignancies, largely due to the relative accessibility of tumor material and the feasibility of establishing functional *ex vivo* testing platforms. Several prospective studies have already demonstrated that FPM-based approaches can improve treatment stratification and therapeutic efficacy, highlighting their potential for broader clinical implementation (72,128). However, extending these successes to solid tumor immunotherapy remains considerably more challenging. In contrast to hematological malignancies, solid tumors are characterized by highly complex TMEs composed of heterogeneous cellular populations, spatially organized immune interactions, stromal barriers, and dynamic signaling networks. As a result, accurate functional assessment of immunotherapeutic responses requires model systems capable of preserving not only tumor cell viability, but also the structural and immunological complexity of the native tissue environment. Addressing these challenges will be critical for advancing FPM toward clinically meaningful applications in solid tumor immunotherapy.

Standardization of experimental protocols, quality controls, and reproducible analytical readouts will further facilitate the integration of FPM in routine clinical oncology across diverse cancer entities. While great strides have been made in tackling hematological malignancies, the translation of FPM applications into solid tumors is highly complex. Yet to date, it has been tested in only small numbers of prospective clinical studies in this context, and there are critical issues regarding the need for tumor-specific model systems that are physiologically relevant, technically sound, and economically feasible for clinical application (131). However, mounting evidence from preclinical and co-clinical studies suggests that FPM in solid tumors is continuously moving towards translational and clinical applicability. Especially, development of immune-competent 3D and *ex vivo* tissue platforms retaining the core characteristics of the native TME constitutes a significant advance in the accurate functional assessment for therapeutic response. Consequently, further efforts must be directed toward the combination of such

sophisticated culture systems with quantitative spatial, cell- and function-based measurements. Such multidimensional platforms could allow for not only greater predictive power of therapeutic efficacy, but also yield mechanistic insight into treatment responses and resistance with potential to facilitate the development of truly theranostic means of precision oncology.

Our lymphoid bioreactor system expands the reach of FPM to allow functional characterization of immunotherapeutic responses in a preserved immune-competent TME. Preserving the structural organization and cellular heterogeneity of native lymphoid tissue, this platform allows direct tumor-associated effects and immune-mediated responses to be assessed simultaneously in the same physiological context. Importantly, this approach may complement existing FPM workflows by providing clinically actionable, patient-specific data to assist in immunotherapy decision-making. This is particularly relevant in lymphoma, where conventional drug-sensitivity assays are often insufficient to capture the complexity of immune–tumor interactions and, thus, may fail to fully predict therapeutic response.

Conventional 2D cell line models are extensively used in cancer research but they typically do not have structural and cellular characteristics of the 3D TME, that is, such important factors as immune cells, vasculature, fibroblasts, or ECM (132). While 3D culture mediums such as spheroids and organoids partially mitigate these drawbacks, they offer a partial solution to imitating the complexity of the tumor tissues due to a more accurate recapitulation of tumor structure, but do not entirely maintain the cellular heterogeneity, organization and dynamic interactions found in native tissues (133,134). *In vivo* models including PDXs are able to retain some tumor structure and tumor heterogeneity better but have limited potential in immunotherapy due to the absence of a fully functional human immune system (135). Furthermore, PDX models have a high cost, low throughput, and long experimental time cycles, restricting their relevance to functional precision oncology workflows. Together, these constraints suggest a long-lasting requirement for model systems that are physiologically specific and large enough to accurately recapitulate tumor biology and facilitate accurate evaluation of therapeutic targets in cancer studies (136).

Short-term bioreactor cultures with patient-derived tissues allow quick functional characterization of response to therapy to facilitate timely and personalized drug treatment planning. From a clinical point of view, the small surgical samples may be subjected to co-analysis with routine pathological examination, and *ex vivo* drug experiments may provide additional functional data for clinical therapy decisions. The native 3D architecture is better preserved by embedding lymphoid tissue in 0.5% agarose, as compared to collagen sponges or the static plate conditions; meanwhile, the medium exchange is more stable. This manner enables a preservation of immune, stromal, and tumor cell viability at levels comparable to freshly isolated tissue (111,137). Improvements in culture conditions to optimize long-term immune cell function in such systems may also be needed. Specifically, prolonged T and B cell survival might be improved by approaches to increased cytokine supplementation (besides IL-2 and IL-7), such as those with BAFF or IL-15 (138,139), that would enhance the physiological relevance of *ex vivo* models of immune tissue.

Dynamic perfusion improves tissue viability through continuous nutrient and oxygen supply and waste removal, and minimizes the occurrence of central necrosis, common in static culture systems (140). Such persistent medium circulation resembles the reality of physiologic fluid-flow environments in which cellular survival, differentiation, and immune–tumor interactions are modulated in native tissues. Given this, the enhanced survival of immune and tumor cell types in our model might also be attributable to the enhanced physiological milieu sustained during perfusion culture. As a result, the system more faithfully retains functional tissue states and better represents *in vivo* tumor–immune dynamics when compared to traditional 2D cultures or static 3D models.

While Bonaiti et al., developed a 3D perfusion bioreactor system for evaluating vaccine-induced immune responses using human tonsil explants (141), our platform is based on lymph node (LN) tissue, which provides a more physiologically relevant immunological context relevant to tumor–immune interactions and cancer immunobiology. The viability of tissue remained high using a 3-day culture period in the system, establishing a realistic, biologically relevant window for *ex vivo* drug screening. By doing so, I showed that it can be used to model immune checkpoint blockade and

CAR T cell therapy, where efficacy can be compared as well as immune-modulatory properties in the same physiologically realistic scenario I describe above. These data reinforce perfusion bioreactors being effective platforms for standardized and controlled assessment of immunotherapeutic approaches. The standard endpoint was selected as a 3-day culture duration as tissue viability was high and comparable to freshly isolated LNs at this point while viability exhibited deterioration by day 5. This decision is bolstered by previous observations suggesting appropriate *ex vivo* functional testing includes 3-day exposure windows for immune checkpoint inhibitors and around 2-day treatment time frames for CAR T cell assays (142,143).

One of the major technical issues facing the creation of the perfusion culture system was significant cellular efflux during the startup of the perfusion culture system from the tissue explants. In the original collagen sponge support-based model, a significant share of immune cells translocated out of the tissue and into the nearby culture medium and disrupted the native cellular composition, resulting in damage to the tissue microenvironment. In order to address this challenge I proposed a straightforward agarose embedding approach that led to a marked decrease in cellular loss and an absolute improvement in tissue viability. The advantageous action attributed to agarose is expected due to multiple processes. Specifically, the semi-solid matrix acts as mechanical support that stabilizes the tissue during continuous perfusion, so as to minimize its deformation and compression inside the bioreactor chamber. Second, agarose provides a physical barrier, which, at the same time, serves to narrow the passive leakage of cells and remains porous enough to promote the passage of nutrients, oxygen, cytokines, and therapeutic antibodies. Finally, lessening shear stress directly onto tissues, agarose embedding can thus be more effectively maintaining cell-cell contacts and tissue architecture, which are important for normal functional immune interactions. Taken together, the above results indicated that agarose is used to function mainly as a mechanical stabilising agent not as a biological scaffold and an effective treatment for maintaining intact lymphoid tissues in dynamic perfusion culture.

During optimization, other embedding materials were also tested, such as BME. While ECM-derived materials bear a closer resemblance to the native extracellular matrix,

neither material provided superior tissue preservation under dynamic perfusion conditions. Particularly, BME was soft, gel-like and weak in providing mechanical support and prone to deformation or displacement during the continuous medium flow. As a result, tissue viability was not enhanced compared with the agarose-based system. These findings indicate that mechanical stability might be favoured rather than biological mimicry for the choice of embedding materials in perfusion culture of lymphoid tissues. However, the best scaffold is likely to be tissue type-dependent. Although agarose was well adapted for lymphoid tissues, other solid tumors like hepatocellular carcinoma, breast cancer, pancreatic cancer, or brain tumors have different extracellular matrix compositions and mechanical properties. Based on the findings, tissue-specific biomaterials and hybrid scaffolds in subsequent trials can be developed to optimize perfusion culture for various tumor entities.

Comparison of the dynamic perfusion culture and conventional static plate culture further confirmed the importance of continuous medium exchange for preserving intact lymphoid tissues. Independent of the embedding material used, perfusion culture consistently resulted in significantly higher tissue viability than static culture. Continuous perfusion provides more efficient transport of oxygen and nutrients while facilitating removal of metabolic waste products, thereby creating a microenvironment that more closely resembles physiological tissue perfusion. Although differences in culture medium volume between the two systems cannot be completely excluded as a contributing factor, these findings strongly support the superiority of dynamic perfusion over conventional diffusion-dependent culture methods for maintaining viable patient-derived lymphoid tissues.

Moreover, expansion of the duration of culture indicated that in the first 3 day period of perfusion culture, lymphoid tissue viability and immune cell composition were best preserved, while after 5 days, there was a slow degradation. Several factors might contribute to this observation. Despite continuous perfusion, long-term *ex vivo* culture inevitably becomes involved in metabolic stress and accumulation of waste products, lack of vital survival factor supplies, and a lack of systemic physiological signals that are normally given by the circulation. Such alterations may ultimately drive apoptosis or

functional fatigue of immune cells. Importantly, however, 3 days of culture was enough time for clinically relevant immunotherapy studies to take place successfully, with a tissue viability and immune composition that approaches fresh tissue. Future optimization strategies may ultimately prolong the cultural period by increasing oxygen delivery, optimized perfusion states, specialized cytokine delivery, or including vascularized tissue engineering methods.

An interesting biological observation was the differential preservation of B cell populations between tonsil and lymph node tissues. While B cell viability in cultured tonsil explants was markedly decreased, frequency and viability of B cells in lymph node tissues were remarkably consistent. The exact mechanism is unclear, but a number of explanations are possible. The human tonsils have abundant germinal center B cells, highly proliferative and prone to apoptosis when there are no survival signals, such as those from follicular helper T cells and follicular dendritic cells (144). In contrast, lymph nodes may harbor unique distributions of B cell subsets and can sustain more specialized survival niches when cultured *ex vivo*. Moreover, differences in state of inflammation, immune activation, and architecture of tissue between tonsils and lymph nodes might influence the ability to sustain B cell homeostasis. Whatever the exact mechanism, lymph node tissue emerges as a highly preferred model for functional immunotherapy studies, especially for those focused on CD19-targeted CAR T cells.

Applying the optimized platform to immune checkpoint inhibition showed that the bioreactor recapitulates key features of clinically relevant anti-tumor immune responses. Pembrolizumab selectively reduced the viability of malignant lymphoma tissues and melanoma metastatic lymph nodes while exerting minimal effects on benign lymphoid tissues. It was also associated with increased production of inflammatory cytokines, elevated levels of cytotoxic effector molecules, and enhanced IFN- γ production by both CD4⁺ and CD8⁺ T cells. Taken together, the data presented here suggest that the preserved tissue microenvironment supports functional activation of endogenous immune cells following PD-1 blockade. However, the current study was based on a limited number of patient samples and thus the substantial inter-patient heterogeneity observed clinically was not sufficiently captured. By expanding the patient cohort, future

studies are likely to distinguish responders from non-responders, identify predictive biomarkers, and correlate *ex vivo* drug responses with clinical outcomes. Such investigations represent an important next step toward implementing this platform as a functional precision medicine tool for individualized immunotherapy selection.

Similarly, our CAR T cell studies indicate the bioreactor platform is favorable for functional evaluation of genetically modified T cell therapies in intact human lymphoid tissues. Tissue explants were penetrated by Anti-CD19 CAR T cells, which efficiently removed endogenous CD19⁺ B cells, demonstrating preservation of tissue accessibility and target-cell recognition. Additionally, analysis of PI3K-enhanced CAR T cells showed that the platform can assess next-generation CAR products more directly than traditional cytotoxicity assays. No significant difference between the B cell killing as a whole was seen, but PI3K-modified CAR T cells had better tissue infiltration and activation, suggesting the bioreactor is sensitive to function indicators that cannot necessarily be detected by target cell depletion. The current study, however, targeted only one CAR construct made of a CD19-targeting model and examined primary factors such as cytotoxicity, tissue infiltration and persistence. This platform should be more broadly extended in future studies to other CAR targets, including CD20, CD22, BCMA, Claudin18.2 or GPC3, as well as to study the added functional properties such as T cell exhaustion, metabolic fitness, serial killing capacity, cytokine secretion and long-term persistence. Multiplex spatial imaging, live-cell imaging, and multi-omics approaches will also improve mechanistic understanding of CAR T cell function in preserved human tissues.

An important consideration in this study is the inherent donor-to-donor variability associated with patient-derived tissues. In contrast to established cell lines, freshly resected human lymphoid tissues differ considerably in their cellular composition, immune activation status, pathological diagnosis, prior treatment history, and tissue quality. Such biological heterogeneity contributed to variability in several experimental readouts, particularly baseline cell viability and immune cell frequencies. The corresponding donor was selected as a matched reference control wherever appropriate to minimize these effects, and post-culture measurements were normalized

to the paired fresh tissue. Moreover, when sufficient material was available, multiple explants from the same surgical specimen were allocated to different experimental conditions to enable paired comparisons. Although the absolute values vary widely across donors, the overall trends of tissue preservation and immunotherapy response remained highly consistent across independent experiments. This biological heterogeneity does not solely represent a limitation but also reflects the diversity encountered in clinical practice.

Overall, these experimental results indicate that the engineered perfusion bioreactor maintains the immune tumor microenvironment structural integrity and is therefore capable of functional evaluation of clinically relevant immunotherapies. Retention of the native tissue architecture preserves spatially resolved cell–cell interactions such as immune cell infiltration patterns and tumor–immune cell proximity, which are lost during two-dimensional and static culture systems (106). The platform thus preserves spatial and functional characteristics and enables a better mechanistic grasp of immunotherapy-mediated immune responses, while establishing an experimental environment with physiologic relevance for therapeutic testing. Consequently, the bioreactor mediates the transition from simplified *in vitro* and *ex vivo* methods to more challenging *in vivo* systems by providing experimental control while preserving tissue biology of clinical relevance. Its potential to preserve individual patient tissue architecture and functional drug testing remains substantial as a translational platform for functional precision medicine. Based on upcoming studies that include larger patient cohorts, additional tumor entities and molecular biomarkers in addition to functional therapeutic studies, this will improve patient-specific treatment response prediction and ultimately support more informed clinical decision-making in cancer immunotherapy.

4.2 Limitations

Although the bioreactor system provides a convenient and physiologically relevant platform for *ex vivo* modeling of the human TME, several biological, technical, and logistical limitations need to be considered when interpreting its results and evaluating its translational applicability.

Biological and technical limitations: Since the platform relies on freshly resected human surgical specimens, availability, pathological composition, and baseline tissue quality are inherently subject to variation between individual donors. Such biological heterogeneity is a core feature of patient-derived tissue models and is indicative of the diversity we encounter in clinical practice. To restrict the potential effects of inter-donor variability, the reference control of freshly isolated tissue from the donor from which the tissue was obtained was applied, where appropriate, and post-culture measurements were normalized to match fresh tissue. Despite that, variability in baseline cell frequencies and viabilities was still present across donors.

Also, pre-analytical variables can introduce some experimental variability. Despite the efforts to transport, process, and initiate culture of freshly resected tissues as rapidly as possible, the time duration between surgical resection and culture initiation was of course dependent on the specimen. The seasonal variations in ambient temperature during transport and handling could also affect tissue conservation. These differences may have also driven the variation in baseline tissue viability between samples. Crucially, though absolute values of cell viability were different between donors, the general trend was most consistent for treatment responses: treated and control groups presented proportional changes. These findings indicated that the bioreactor platform may be capable of collecting biologically significant functional responses in a manner that retains patient-specific heterogeneity.

Lastly, the technical limitations of the optimized bioreactor system are inherent. While embedding of tissue explants in 0.5% agarose can effectively preserve three-dimensional tissue architecture, reduce cell efflux, and enhance overall tissue integrity, the embedding matrix will likely also change the nature of cellular behaviour by altering the nearby mechanical environment or diffusion properties. Therefore, slight effects on immune cell function or therapeutic response cannot be completely omitted, and they should also be taken into account when interpreting the scientific results.

Regarding viability determination, the analysis of mechanically dissociated single-cell suspensions was carried out by Annexin V/DAPI-based flow cytometry. Although this method allows quantitative and multiplexed analysis of apoptotic and necrotic cell

populations across distinct immune subsets, it can also be biased by susceptibility of fragile or already apoptotic cells to mechanical dissociation which may lead to an underestimation of cell death. In the future, incorporation of complementary in situ assays, such as cleaved caspase-3 or TUNEL staining, may be included to enable preservation of spatial features, as well as providing a more comprehensive and context-preserving evaluation of tissue viability.

Sampling and statistical issues: Imaging-based analyses were conducted with a small number of explants and tissue sections, which could limit the generalizability of spatially resolved observations, particularly due to potential inter-explant variability. While lymphomas are typically believed to present less intratumoral heterogeneity than most solid tumors (145), spatial variation in immune cell distribution and tumor architecture may still account for local response to therapy. In addition, the lack of multiregional sampling contributes to a partial capture of the residual spatial heterogeneity.

A secondary source of heterogeneity is the variation in donor-to-donor relationships, as patient-specific differences in immune composition, tumor phenotype, and prior treatment history might impact both experimental reproducibility and data interpretation. Moreover, cytokine profiling has been carried out on a small number of cases, limiting statistical robustness and requiring judicious interpretation of mechanistic inferences found from these data. The small and limited sample size and subset analyses reduce statistical power and need to be addressed in larger and more diverse patient cohorts to increase the validity and generalizability of the findings in future.

Immunophenotyping scope: The current study employed immunophenotypic analyses mainly in relation to T cells, such as CD4⁺ and CD8⁺ subsets. Nevertheless, other essential immune compartments, like regulatory T cells (Tregs; e.g., FOXP3⁺) and specific B cell subsets (including naïve, memory, germinal center, and plasma cell subpopulations), were not systematically characterized. This partial representation restricts our knowledge on the complete immune landscape of the lymphoid TME and its dynamic modulation in response to immunotherapeutic interventions.

Throughput and practical limitations: The throughput of the platform is relatively low, limiting the range of experimental conditions that can be measured from one tissue sample to the next. Combined with intrinsic tissue heterogeneity, this limitation reduces the scalability and experimental breadth of the system. To overcome these challenges, optimization in future may involve increasing throughput capacity, streamlining the processes of tissue processing, and developing strategies for the biobanking of viable tissue samples. These advances would improve experimental reproducibility, and as well facilitate mechanistic studies more broadly and also allow for more extensive retrospective studies across larger patient cohorts.

5 Summary

In this work, I described the evolution of cancer modeling techniques from traditional 2D cultures to advanced 3D systems, including their respective merits and drawbacks and emphasized the increasing significance of a systems approach for functional precision oncology and immuno-theranostics. Based on these principles, I implemented and optimized a 3D perfusion-based bioreactor culture technique that could effectively sustain the viability, integrity, and function of fresh human lymphoid tissues *ex vivo*. Relative to conventional plate-based cultures and previous systems described, our system is significantly better in terms of tissue viability as well as the presence of immune cells, thus providing a more physiologically relevant platform that retains important aspects of the native tumor microenvironment.

I showed in the present study using this platform the versatility of this platform for the modeling of multiple types of immunotherapies. The immune checkpoint inhibition of pembrolizumab led to decreased lymphoma and metastatic lymph node viability, increased CD8⁺ cell infiltration, and increased T cell activation-associated cytokines, indicating effective immune response in the preserved tissue structure. In addition, our bioreactor enabled the careful studies of CAR T cell therapies, to monitor tissue penetration, cytotoxicity, or tissue-specific responses. These observations demonstrate the utility of the platform for optimizing CAR T cell products in lymphoid malignancies as well as solid tumors. Furthermore, I performed the application of this system to the analysis of new antibody-based therapies and demonstrated that anti-TRP2 antibodies were therapeutically valuable in metastatic melanoma tissues, demonstrating the platform's potential to facilitate the early-stage assessment of an innovative immunotherapeutic approach.

Notably, the short-term and patient-specific characteristic of this *ex vivo* bioreactor system aligns with the principles of functional precision medicine by providing immediate and personalized evaluation of therapeutic response. This strategy has a potential influence on clinical decision-making, in a time frame that is clinically relevant. In this regard our results all together place this bioreactor-based human tissue culture

system in a position of flexibility, translational power and potential, as well as a potent tool for immunotherapy research, therapeutic optimization and the future development of personalized cancer treatment strategies.

6 Zusammenfassung

In dieser Arbeit beschreibe ich die Entwicklung von Krebsmodellierungstechniken von traditionellen 2D-Kulturen hin zu fortschrittlichen 3D-Systemen, einschließlich ihrer jeweiligen Vor- und Nachteile, und betone die zunehmende Bedeutung eines systemischen Ansatzes für die funktionelle Präzisionsonkologie und Immuntherapie. Basierend auf diesen Prinzipien implementierte und optimierte ich eine 3D-Perfusions-Bioreaktor-Kulturtechnik, die die Vitalität, Integrität und Funktion von frischem humanem lymphatischem Gewebe *ex vivo* effektiv erhalten kann. Im Vergleich zu herkömmlichen, plattenbasierten Kulturen und zuvor beschriebenen Systemen ist unser System hinsichtlich der Gewebevitalität sowie der Präsenz von Immunzellen deutlich überlegen und bietet somit eine physiologisch relevante Plattform, die wichtige Aspekte der natürlichen Tumormikroumgebung bewahrt.

In der vorliegenden Studie demonstrierte ich anhand dieser Plattform deren Vielseitigkeit für die Modellierung verschiedener Arten von Immuntherapien. Die Immun-Checkpoint-Inhibition durch Pembrolizumab führte zu einer verringerten Vitalität von Lymphomzellen und Tumorzellen in metastasierten Lymphknoten, einer erhöhten Infiltration von CD8⁺-Zellen und einem Anstieg der mit der T-Zell-Aktivierung assoziierten Zytokine. Dies deutet auf eine effektive Immunantwort bei erhaltener Gewebestruktur hin. Darüber hinaus ermöglichte unser Bioreaktor die detaillierte Untersuchung von CAR-T-Zell-Therapien, um Gewebepenetration, Zytotoxizität und gewebespezifische Reaktionen zu untersuchen. Diese Beobachtungen belegen den Nutzen der Plattform zur Optimierung von CAR-T-Zell-Produkten bei lymphatischen Malignomen sowie soliden Tumoren. Weiterhin setzte ich dieses System zur Analyse neuer Antikörper-basierter Therapien ein und zeigte, dass anti-TRP2-Antikörper in Lymphknotengewebe mit metastasiertem Melanom therapeutisch wirksam sind. Dies unterstreicht das Potenzial der Plattform, die frühzeitige Bewertung innovativer immuntherapeutischer Ansätze zu erleichtern.

Bemerkenswert ist, dass die kurzfristige und patientenspezifische Charakteristik dieses *Ex-vivo*-Bioreaktorsystems den Prinzipien der funktionellen Präzisionsmedizin

entspricht, indem es eine unmittelbare und personalisierte Bewertung des Therapieansprechens ermöglicht. Diese Strategie hat das Potenzial, klinische Entscheidungen in einem klinisch relevanten Zeitrahmen zu beeinflussen. Meine Ergebnisse positionieren dieses Bioreaktor-basierte System zur Kultur humaner Gewebe in einer Position der Flexibilität, des translationalen Potenzials und der Wirksamkeit als potentes Werkzeug für die immuntherapeutische Forschung, die Therapieoptimierung und die zukünftige Entwicklung personalisierter Krebsbehandlungsstrategien.

7 References

1. Brown JS, Amend SR, Austin RH, Gatenby RA, Hammarlund EU, Pienta KJ. Updating the Definition of Cancer. *Mol Cancer Res.* 2023 Nov 1;21(11):1142–7. doi:10.1158/1541-7786.MCR-23-0411 PubMed PMID: 37409952; PubMed Central PMCID: PMC10618731.
2. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 2022 Jan;12(1):31–46. doi:10.1158/2159-8290.CD-21-1059 PubMed PMID: 35022204.
3. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024;74(3):229–63. doi:10.3322/caac.21834
4. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell.* 2011 Mar 4;144(5):646–74. doi:10.1016/j.cell.2011.02.013 PubMed PMID: 21376230.
5. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. *Nature.* 2013 Aug;500(7463):415–21. doi:10.1038/nature12477
6. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med.* 2004 Aug;10(8):789–99. doi:10.1038/nm1087
7. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med.* 2004 May 20;350(21):2129–39. doi:10.1056/NEJMoa040938 PubMed PMID: 15118073.
8. Lasota J, Miettinen M. KIT and PDGFRA mutations in gastrointestinal stromal tumors (GISTs). *Semin Diagn Pathol.* 2006 May;23(2):91–102. doi:10.1053/j.semdp.2006.08.006 PubMed PMID: 17193822.
9. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev.* 2004 Apr;30(2):193–204. doi:10.1016/j.ctrv.2003.07.007 PubMed PMID: 15023437.
10. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med.* 2020 Mar;19(3):1997–2007. doi:10.3892/etm.2020.8454 PubMed PMID: 32104259; PubMed Central PMCID: PMC7027163.
11. Wheeler DL, Iida M, Dunn EF. The Role of Src in Solid Tumors. *The Oncologist.* 2009 Jul;14(7):667–78. doi:10.1634/theoncologist.2009-0009 PubMed PMID: 19581523; PubMed Central PMCID: PMC3303596.

12. Kontomanolis EN, Koutras A, Syllaios A, Schizas D, Mastoraki A, Garmpis N, et al. Role of Oncogenes and Tumor-suppressor Genes in Carcinogenesis: A Review. *Anticancer Res.* 2020 Nov;40(11):6009–15. doi:10.21873/anticancer.14622 PubMed PMID: 33109539.
13. Shay JW, Wright WE. Telomeres and telomerase in normal and cancer stem cells. *FEBS Lett.* 2010 Sep 10;584(17):3819–25. doi:10.1016/j.febslet.2010.05.026 PubMed PMID: 20493857; PubMed Central PMCID: PMC3370416.
14. Ferrara N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer.* 2002 Oct;2(10):795–803. doi:10.1038/nrc909 PubMed PMID: 12360282.
15. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer.* 2003 Oct;3(10):721–32. doi:10.1038/nrc1187 PubMed PMID: 13130303.
16. Paoli P, Giannoni E, Chiarugi P. *Anoikis* molecular pathways and its role in cancer progression. *Biochim Biophys Acta BBA - Mol Cell Res.* 2013 Dec 1;1833(12):3481–98. doi:10.1016/j.bbamcr.2013.06.026
17. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell.* 2009 Nov 25;139(5):871–90. doi:10.1016/j.cell.2009.11.007 PubMed PMID: 19945376.
18. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer.* 2012 Apr 19;12(5):323–34. doi:10.1038/nrc3261 PubMed PMID: 22513401.
19. Nowell PC. The clonal evolution of tumor cell populations. *Science.* 1976 Oct 1;194(4260):23–8. doi:10.1126/science.959840 PubMed PMID: 959840.
20. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CHM, Jones DL, et al. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res.* 2006 Oct 1;66(19):9339–44. doi:10.1158/0008-5472.CAN-06-3126 PubMed PMID: 16990346.
21. Bilotta MT, Antignani A, Fitzgerald DJ. Managing the TME to improve the efficacy of cancer therapy. *Front Immunol.* 2022;13:954992. doi:10.3389/fimmu.2022.954992 PubMed PMID: 36341428; PubMed Central PMCID: PMC9630343.
22. Zellmer VR, Zhang S. Evolving concepts of tumor heterogeneity. *Cell Biosci.* 2014;4:69. doi:10.1186/2045-3701-4-69 PubMed PMID: 25937891; PubMed Central PMCID: PMC4417538.
23. Neelakantan D, Drasin DJ, Ford HL. Intratumoral heterogeneity: Clonal cooperation in epithelial-to-mesenchymal transition and metastasis. *Cell Adhes Migr.* 2015;9(4):265–76. doi:10.4161/19336918.2014.972761 PubMed PMID: 25482627; PubMed Central PMCID: PMC4594578.

24. Mintzer D. The changing role of surgery in the diagnosis and treatment of cancer. *Am J Med.* 1999 Jan 1;106(1):81–9. doi:10.1016/S0002-9343(98)00373-8
25. Ghaferi AA, Birkmeyer JD, Dimick JB. Complications, failure to rescue, and mortality with major inpatient surgery in medicare patients. *Ann Surg.* 2009 Dec;250(6):1029–34. doi:10.1097/sla.0b013e3181bef697 PubMed PMID: 19953723.
26. Chandra RA, Keane FK, Voncken FEM, Thomas CR. Contemporary radiotherapy: present and future. *Lancet Lond Engl.* 2021 Jul 10;398(10295):171–84. doi:10.1016/S0140-6736(21)00233-6 PubMed PMID: 34166607.
27. DeVita VT, Lawrence TS, Rosenberg SA. DeVita, Hellman, and Rosenberg's cancer: Principles & practice of oncology: Tenth edition. In: DeVita, Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology: Tenth Edition [Internet]. Wolters Kluwer Health Adis (ESP); 2015 [cited 2026 Jan 5]. p. 1–2280. Available from: <https://pure.johnshopkins.edu/en/publications/devita-hellman-and-rosenbergs-cancer-principles-amp-practice-of-o/>
28. Jabbour SK, Kumar R, Anderson B, Chino JP, Jethwa KR, McDowell L, et al. Combinatorial Approaches for Chemotherapies and Targeted Therapies With Radiation: United Efforts to Innovate in Patient Care. *Int J Radiat Oncol Biol Phys.* 2024 Apr 1;118(5):1240–61. doi:10.1016/j.ijrobp.2024.01.010 PubMed PMID: 38216094.
29. Barazzuol L, Coppes RP, van Luijk P. Prevention and treatment of radiotherapy-induced side effects. *Mol Oncol.* 2020 Jul;14(7):1538–54. doi:10.1002/1878-0261.12750 PubMed PMID: 32521079; PubMed Central PMCID: PMC7332214.
30. Zhang B. Overview of systemic anticancer treatments: conventional cytotoxics. *Drug Ther Bull.* 2025 Mar 3;63(3):37–43. doi:10.1136/dtb.2023.000059 PubMed PMID: 39904574.
31. Corrie PG. Cytotoxic chemotherapy: clinical aspects. *Medicine (Baltimore).* 2008 Jan 1;Oncology36(1):24–8. doi:10.1016/j.mpmed.2007.10.012
32. MacDonald V. Chemotherapy: Managing side effects and safe handling. *Can Vet J.* 2009 Jun;50(6):665–8. PubMed PMID: 19721789; PubMed Central PMCID: PMC2684058.
33. Puhalla S, Bhattacharya S, Davidson NE. Hormonal therapy in breast cancer: A model disease for the personalization of cancer care. *Mol Oncol.* 2012 Apr;6(2):222–36. doi:10.1016/j.molonc.2012.02.003 PubMed PMID: 22406404; PubMed Central PMCID: PMC5528370.
34. Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA Cancer J Clin.* 1972;22(4):232–40. doi:10.3322/canjclin.22.4.232 PubMed PMID: 4625049.

35. Wright-Kanuth MS, Smith LA. Hematopoietic stem cell transplantation. Clin Lab Sci J Am Soc Med Technol. 2001;14(2):112–7. PubMed PMID: 15625984.
36. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med. 1996 Mar 14;334(11):693–9. doi:10.1056/NEJM199603143341104 PubMed PMID: 8594428.
37. Lee YT, Tan YJ, Oon CE. Molecular targeted therapy: Treating cancer with specificity. Eur J Pharmacol. 2018 Sep 5;834:188–96. doi:10.1016/j.ejphar.2018.07.034 PubMed PMID: 30031797.
38. Waller CF. Imatinib mesylate. Recent Results Cancer Res Fortschritte Krebsforsch Progres Dans Rech Sur Cancer. 2014;201:1–25. doi:10.1007/978-3-642-54490-3_1 PubMed PMID: 24756783.
39. Steins M, Thomas M, Geissler M. Erlotinib. Recent Results Cancer Res Fortschritte Krebsforsch Progres Dans Rech Sur Cancer. 2014;201:109–23. doi:10.1007/978-3-642-54490-3_6 PubMed PMID: 24756788.
40. Oh DY, Bang YJ. HER2-targeted therapies - a role beyond breast cancer. Nat Rev Clin Oncol. 2020 Jan;17(1):33–48. doi:10.1038/s41571-019-0268-3 PubMed PMID: 31548601.
41. Jiao J, Wu Y, Wu S, Jiang J. Enhancing Colorectal Cancer Treatment Through VEGF/VEGFR Inhibitors and Immunotherapy. Curr Treat Options Oncol. 2025 Mar;26(3):213–25. doi:10.1007/s11864-025-01306-8 PubMed PMID: 40045029.
42. Sawyers CL. The cancer biomarker problem. Nature. 2008 Apr 3;452(7187):548–52. doi:10.1038/nature06913 PubMed PMID: 18385728.
43. Aldea M, Andre F, Marabelle A, Dogan S, Barlesi F, Soria JC. Overcoming Resistance to Tumor-Targeted and Immune-Targeted Therapies. Cancer Discov. 2021 Apr;11(4):874–99. doi:10.1158/2159-8290.CD-20-1638 PubMed PMID: 33811122.
44. Mani K, Deng D, Lin C, Wang M, Hsu ML, Zaorsky NG. Causes of death among people living with metastatic cancer. Nat Commun. 2024 Feb 19;15(1):1519. doi:10.1038/s41467-024-45307-x
45. Liu B, Zhou H, Tan L, Siu KTH, Guan XY. Exploring treatment options in cancer: tumor treatment strategies. Signal Transduct Target Ther. 2024 Jul 17;9(1):1–44. doi:10.1038/s41392-024-01856-7
46. Kuczyński EA, Sargent DJ, Grothey A, Kerbel RS. Drug rechallenge and treatment beyond progression—implications for drug resistance. Nat Rev Clin Oncol. 2013 Oct;10(10):571–87. doi:10.1038/nrclinonc.2013.158 PubMed PMID: 23999218; PubMed Central PMCID: PMC4540602.

47. Russo M, Chen M, Mariella E, Peng H, Rehman SK, Sancho E, et al. Cancer drug-tolerant persister cells: from biological questions to clinical opportunities. *Nat Rev Cancer*. 2024 Oct;24(10):694–717. doi:10.1038/s41568-024-00737-z
48. Zhang Y, Zhang Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. *Cell Mol Immunol*. 2020 Jul 1;17(8):807. doi:10.1038/s41423-020-0488-6 PubMed PMID: 32612154.
49. Pala L, Pagan E, Sala I, Oriecua C, Oliari M, Pas TD, et al. Outcomes of patients with advanced solid tumors who discontinued immune-checkpoint inhibitors: a systematic review and meta-analysis. *eClinicalMedicine*. 2024 Jul 1;73. doi:10.1016/j.eclinm.2024.102681 PubMed PMID: 39007061.
50. Cappell KM, Kochenderfer JN. Long-term outcomes following CAR T cell therapy: what we know so far. *Nat Rev Clin Oncol*. 2023 Jun;20(6):359–71. doi:10.1038/s41571-023-00754-1
51. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways. *Am J Clin Oncol*. 2016 Feb;39(1):98–106. doi:10.1097/COC.0000000000000239 PubMed PMID: 26558876; PubMed Central PMCID: PMC4892769.
52. Ribas A, Wolchok JD. Cancer Immunotherapy Using Checkpoint Blockade. *Science*. 2018 Mar 23;359(6382):1350–5. doi:10.1126/science.aar4060 PubMed PMID: 29567705; PubMed Central PMCID: PMC7391259.
53. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science*. 2018 Mar 23;359(6382):1361–5. doi:10.1126/science.aar6711 PubMed PMID: 29567707.
54. Rafei H, Upadhyay R, Sharma P. A guide to CAR T cell therapies: development, current status and future prospects - PubMed. 2026. doi:10.1038/s41577-026-01322-1
55. Zugasti I, Espinosa-Aroca, Lady, Fidyk K, Mulens-Arias V, Diaz-Beya M, Juan M, et al. CAR-T cell therapy for cancer: current challenges and future directions. *Signal Transduct Target Ther*. 2025 Jul 4;10(1):210. doi:10.1038/s41392-025-02269-w
56. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med*. 2018 May;24(5):541–50. doi:10.1038/s41591-018-0014-x
57. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. *Cell*. 2010 Mar 19;140(6):883–99. doi:10.1016/j.cell.2010.01.025
58. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of “tumor escape” phenotypes. *Nat Immunol*. 2002 Nov;3(11):999. doi:10.1038/ni1102-999 PubMed PMID: 12407407.

59. Rabinovich GA, Gabrilovich D, Sotomayor EM. IMMUNOSUPPRESSIVE STRATEGIES THAT ARE MEDIATED BY TUMOR CELLS. *Annu Rev Immunol.* 2007;25:267. doi:10.1146/annurev.immunol.25.022106.141609 PubMed PMID: 17134371.
60. Quatromoni JG, Eruslanov E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am J Transl Res.* 2012 Oct 10;4(4):376. PubMed PMID: 23145206.
61. Demoulin S, Herfs M, Delvenne P, Hubert P. Tumor microenvironment converts plasmacytoid dendritic cells into immunosuppressive/tolerogenic cells: insight into the molecular mechanisms. *J Leukoc Biol.* 2013 Mar;93(3):343–52. doi:10.1189/jlb.0812397 PubMed PMID: 23136258.
62. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science.* 2006 Sep 29;313(5795):1960–4. doi:10.1126/science.1129139
63. Postow MA, Sidlow R, Hellmann MD. Immune-Related Adverse Events Associated with Immune Checkpoint Blockade. *N Engl J Med.* 2018 Jan 11;378(2):158–68. doi:10.1056/NEJMra1703481 PubMed PMID: 29320654.
64. Okwundu N, Grossman D, Hu-Lieskovan S, Grossmann KF, Swami U. The dark side of immunotherapy. *Ann Transl Med.* 2021 Jun;9(12):12. doi:10.21037/atm-20-4750
65. Collins FS, Varmus H. A New Initiative on Precision Medicine. *N Engl J Med.* 2015 Feb 26;372(9):793–5. doi:10.1056/NEJMp1500523 PubMed PMID: 25635347; PubMed Central PMCID: PMC5101938.
66. Zhang Y, Payab N, Weigelin B, Schürch CM. From 2D cultures to 3D systems: evolving cancer models at the interface of functional precision medicine and theranostics. *Theranostics.* 2026 Jan 21;16(8):4042–57. doi:10.7150/thno.127053
67. Duan XP, Qin BD, Jiao XD, Liu K, Wang Z, Zang YS. New clinical trial design in precision medicine: discovery, development and direction. *Signal Transduct Target Ther.* 2024 Mar 4;9(1):1–29. doi:10.1038/s41392-024-01760-0
68. Zareian S, Sardari S. From Molecules to Medicine: Navigating the Challenges of Network Science in Precision Medicine. *J Mol Clin Med.* 2024 Dec 25;7(1):1. doi:10.31083/j.jmcm0701002
69. Flobak Å, Skånland SS, Hovig E, Taskén K, Russnes HG. Functional precision cancer medicine: drug sensitivity screening enabled by cell culture models. *Trends Pharmacol Sci.* 2022 Nov 1;43(11):973–85. doi:10.1016/j.tips.2022.08.009 PubMed PMID: 36163057.
70. Salmon SE, Hamburger AW, Soehnlen B, Durie BG, Alberts DS, Moon TE.

Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. *N Engl J Med.* 1978 Jun 15;298(24):1321–7. doi:10.1056/NEJM197806152982401 PubMed PMID: 77475.

71. Shoemaker RH, Wolpert-DeFilippes MK, Kern DH, Lieber MM, Makuch RW, Melnick NR, et al. Application of a human tumor colony-forming assay to new drug screening. *Cancer Res.* 1985 May;45(5):2145–53. PubMed PMID: 3986767.

72. Kornauth C, Pemovska T, Vladimer GI, Bayer G, Bergmann M, Eder S, et al. Functional Precision Medicine Provides Clinical Benefit in Advanced Aggressive Hematologic Cancers and Identifies Exceptional Responders. *Cancer Discov.* 2022 Feb 1;12(2):372–87. doi:10.1158/2159-8290.CD-21-0538

73. Turpin R, Peltonen K, Rannikko JH, Liu R, Kumari AN, Nicorici D, et al. Patient-derived tumor explant models of tumor immune microenvironment reveal distinct and reproducible immunotherapy responses. *Oncoimmunology.* 14(1):2466305. doi:10.1080/2162402X.2025.2466305 PubMed PMID: 39960413; PubMed Central PMCID: PMC11834457.

74. Magré L, Verstegen MMA, Buschow S, van der Laan LJW, Peppelenbosch M, Desai J. Emerging organoid-immune co-culture models for cancer research: from oncoimmunology to personalized immunotherapies. *J Immunother Cancer.* 2023 May;11(5):e006290. doi:10.1136/jitc-2022-006290 PubMed PMID: 37220953; PubMed Central PMCID: PMC10231025.

75. Friedman AA, Letai A, Fisher DE, Flaherty KT. Precision medicine for cancer with next-generation functional diagnostics. *Nat Rev Cancer.* 2015 Dec;15(12):747–56. doi:10.1038/nrc4015

76. Kropivsek K, Kachel P, Goetze S, Wegmann R, Severin Y, Hale BD, et al. P856: A SINGLE-CELL FUNCTIONAL PRECISION MEDICINE LANDSCAPE OF MULTIPLE MYELOMA. *HemaSphere.* 2022 Jun 23;6(Suppl):749–50. doi:10.1097/01.HS9.0000846304.52658.85 PubMed PMID: null; PubMed Central PMCID: PMC9429035.

77. Potter DS, Du R, Bhola P, Bueno R, Letai A. Dynamic BH3 profiling identifies active BH3 mimetic combinations in non-small cell lung cancer. *Cell Death Dis.* 2021 Jul 27;12(8):741. doi:10.1038/s41419-021-04029-4 PubMed PMID: 34315868; PubMed Central PMCID: PMC8316436.

78. Dinić J, Jovanović Stojanov S, Dragoj M, Grozdanić M, Podolski-Renić A, Pešić M. Cancer Patient-Derived Cell-Based Models: Applications and Challenges in Functional Precision Medicine. *Life.* 2024 Sep;14(9):9. doi:10.3390/life14091142

79. Drexler HG. Establishment and culture of leukemia-lymphoma cell lines. *Methods Mol Biol Clifton NJ.* 2011;731:181–200. doi:10.1007/978-1-61779-080-5_16 PubMed PMID: 21516408.

80. Dellamonica D, Ruau D, Griffiths B, Rossi G, Li BT, Razavi P, et al. The AI revolution: how multimodal intelligence will reshape the oncology ecosystem. *Npj Artif Intell.* 2025 Nov 7;1(1):40. doi:10.1038/s44387-025-00044-4
81. Hsu CY, Askar S, Alshkarchy SS, Nayak PP, Attabi KAL, Khan MA, et al. AI-driven multi-omics integration in precision oncology: bridging the data deluge to clinical decisions. *Clin Exp Med.* 2025 Nov 21;26(1):29. doi:10.1007/s10238-025-01965-9
82. Stenzinger A, Moltzen EK, Winkler E, Molnar-Gabor F, Malek N, Costescu A, et al. Implementation of precision medicine in healthcare—A European perspective. *J Intern Med.* 2023;294(4):437–54. doi:10.1111/joim.13698
83. Huang A. Ethical Dimensions of Artificial Intelligence in Personalized Medicine: Navigating Challenges and Shaping Future Policies. In: 2025 IEEE 49th Annual Computers, Software, and Applications Conference (COMPSAC). 2025. p. 895–900. doi:10.1109/COMPSAC65507.2025.00117
84. Joseph G, Lee R, Pasick RJ, Guerra C, Schillinger D, Rubin S. Effective communication in the era of precision medicine: A pilot intervention with low health literacy patients to improve genetic counseling communication. *Eur J Med Genet.* 2019 May 1;Evidence-Based Genetic Counselling62(5):357–67. doi:10.1016/j.ejmg.2018.12.004
85. Shekarian T, Zinner CP, Bartoszek EM, Duchemin W, Wachnowicz AT, Hogan S, et al. Immunotherapy of glioblastoma explants induces interferon- γ responses and spatial immune cell rearrangements in tumor center, but not periphery. *Sci Adv.* 2022 Jul;8(26):eabn9440. doi:10.1126/sciadv.abn9440
86. Hirt C, Papadimitropoulos A, Mele V, Muraro MG, Mengus C, Iezzi G, et al. “In vitro” 3D models of tumor-immune system interaction. *Adv Drug Deliv Rev.* 2014 Dec 15;Engineering of tumor microenvironments79–80:145–54. doi:10.1016/j.addr.2014.05.003
87. Hirt C, Papadimitropoulos A, Muraro MG, Mele V, Panopoulos E, Cremonesi E, et al. Bioreactor-engineered cancer tissue-like structures mimic phenotypes, gene expression profiles and drug resistance patterns observed “in vivo.” *Biomaterials.* 2015 Sep 1;62:138–46. doi:10.1016/j.biomaterials.2015.05.037
88. Wu Y, Chung YY, Chin YT, Lin CY, Kuo PJ, Chen TY, et al. Comparison of 2,3,5,4'-tetrahydroxystilbene-2-O- β -D-glucoside-induced proliferation and differentiation of dental pulp stem cells in 2D and 3D culture systems—gene analysis. *J Dent Sci.* 2022 Jan 1;17(1):14–29. doi:10.1016/j.jds.2021.09.021
89. Nakazato T, Kawamura T, Uemura T, Liu L, Li J, Sasai M, et al. Engineered three-dimensional cardiac tissues maturing in a rotating wall vessel bioreactor remodel diseased hearts in rats with myocardial infarction. *Stem Cell Rep.* 2022 May 10;17(5):1170–82. doi:10.1016/j.stemcr.2022.03.012

90. Sart S, Agathos SN, Li Y, Ma T. Regulation of mesenchymal stem cell 3D microenvironment: From macro to microfluidic bioreactors. *Biotechnol J*. 2016;11(1):43–57. doi:10.1002/biot.201500191
91. CI WM. Normal structure, function, and histology of lymph nodes. *Toxicol Pathol*. 2006;34(5). doi:10.1080/01926230600867727 PubMed PMID: 17067937.
92. Müller-Hermelink HK, Zettl A, Pfeifer W, Ott G. Pathology of lymphoma progression. *Histopathology*. 2001 Apr;38(4):285–306. doi:10.1046/j.1365-2559.2001.01120.x PubMed PMID: 11318894.
93. Polepole P, Mudenda VC, Munsaka SM, Zhang L. Spectrum of common Hodgkin lymphoma and non-Hodgkin lymphomas subtypes in Zambia: a 3-year records review. *J Health Popul Nutr*. 2021 Aug 23;40(1):37. doi:10.1186/s41043-021-00261-y
94. de Leval L, Jaffe ES. Lymphoma Classification. *Cancer J*. 2020 Jun;26(3):176. doi:10.1097/PPO.0000000000000451
95. Ji H, Hu C, Yang X, Liu Y, Ji G, Ge S, et al. Lymph node metastasis in cancer progression: molecular mechanisms, clinical significance and therapeutic interventions. *Signal Transduct Target Ther*. 2023 Sep 27;8(1):367. doi:10.1038/s41392-023-01576-4
96. Zhang Y, Wu D, Zhang Z, Ma J, Jiao S, Ma X, et al. Impact of lymph node metastasis on immune microenvironment and prognosis in colorectal cancer liver metastasis: insights from multiomics profiling. *Br J Cancer*. 2025 Apr;132(6):513–24. doi:10.1038/s41416-024-02921-2
97. Zhang Y, Foth I, Makky A, Bucher P, Grimm M, Bruch PM, et al. Modeling immunotherapies in live 3D human cancer tissue bioreactors. *Theranostics*. 2026 Jan 14;16(8):3928–45. doi:10.7150/thno.118298
98. Schubert ML, Schmitt A, Hückelhoven-Krauss A, Neuber B, Kunz A, Waldhoff P, et al. Treatment of adult ALL patients with third-generation CD19-directed CAR T cells: results of a pivotal trial. *J Hematol Oncol*. 2023 Jul 22;16(1):79. doi:10.1186/s13045-023-01470-0 PubMed PMID: 37481608; PubMed Central PMCID: PMC10363324.
99. Brinkmann BJ, Floerchinger A, Schniederjohann C, Roider T, Coelho M, Mack N, et al. CD20-bispecific antibodies improve response to CD19-CAR T cells in lymphoma in vitro and CLL in vivo models. *Blood*. 2024 Aug 15;144(7):784–9. doi:10.1182/blood.2023022682
100. Bucher P, Brückner N, Kortendieck J, Grimm M, Schleicher JT, Bartels K, et al. CAR-adapted PIK3CD base editing enhances T cell anti-tumor potency. *Nat Cancer*. 2026 Jan 6;1–16. doi:10.1038/s43018-025-01099-7
101. Feucht J, Sun J, Eyquem J, Ho YJ, Zhao Z, Leibold J, et al. Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. *Nat Med*.

2019 Jan;25(1):82–8. doi:10.1038/s41591-018-0290-5 PubMed PMID: 30559421; PubMed Central PMCID: PMC6532069.

102. Hamieh M, Dobrin A, Cabriolu A, van der Stegen SJC, Giavridis T, Mansilla-Soto J, et al. CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. *Nature*. 2019 Apr;568(7750):112–6. doi:10.1038/s41586-019-1054-1

103. Rivière I, Brose K, Mulligan RC. Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc Natl Acad Sci*. 1995 Jul 18;92(15):6733–7. doi:10.1073/pnas.92.15.6733

104. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. *Sci Rep*. 2017 Dec 4;7(1):16878. doi:10.1038/s41598-017-17204-5

105. Phillips D, Matusiak M, Gutierrez BR, Bhate SS, Barlow GL, Jiang S, et al. Immune cell topography predicts response to PD-1 blockade in cutaneous T cell lymphoma. *Nat Commun*. 2021 Nov 18;12(1):6726. doi:10.1038/s41467-021-26974-6

106. Schürch CM, Bhate SS, Barlow GL, Phillips DJ, Noti L, Zlobec I, et al. Coordinated Cellular Neighborhoods Orchestrate Antitumoral Immunity at the Colorectal Cancer Invasive Front. *Cell*. 2020 Sep 3;182(5):1341-1359.e19. doi:10.1016/j.cell.2020.07.005

107. Polak R, Zhang ET, Kuo CJ. Cancer organoids 2.0: modelling the complexity of the tumour immune microenvironment. *Nat Rev Cancer*. 2024 Aug;24(8):523–39. doi:10.1038/s41568-024-00706-6

108. Wendt D, Marsano A, Jakob M, Heberer M, Martin I. Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. *Biotechnol Bioeng*. 2003;84(2):205–14. doi:10.1002/bit.10759

109. Manfredonia C, Muraro MG, Hirt C, Mele V, Governa V, Papadimitropoulos A, et al. Maintenance of Primary Human Colorectal Cancer Microenvironment Using a Perfusion Bioreactor-Based 3D Culture System. *Adv Biosyst*. 2019;3(4):1800300. doi:10.1002/adbi.201800300

110. Mercey E, Obeïd P, Glaise D, Calvo-Muñoz ML, Guguen-Guillouzo C, Fouqué B. The application of 3D micropatterning of agarose substrate for cell culture and *in situ* comet assays. *Biomaterials*. 2010 Apr 1;31(12):3156–65. doi:10.1016/j.biomaterials.2010.01.020

111. Margolis LB, Fitzgerald W, Glushakova S, Hatfill S, Amichay N, Baibakov B, et al. Lymphocyte Trafficking and HIV Infection of Human Lymphoid Tissue in a Rotating Wall Vessel Bioreactor. *AIDS Res Hum Retroviruses*. 1997 Nov;13(16):1411–20. doi:10.1089/aid.1997.13.1411

112. Ravi M, Kaviya SR, Paramesh V. Culture phases, cytotoxicity and protein expressions of agarose hydrogel induced Sp2/0, A549, MCF-7 cell line 3D cultures. *Cytotechnology*. 2016 May 1;68(3):429–41. doi:10.1007/s10616-014-9795-z
113. Ise H, Takashima S, Nagaoka M, Ferdous A, Akaike T. Analysis of cell viability and differential activity of mouse hepatocytes under 3D and 2D culture in agarose gel. *Biotechnol Lett*. 1999 Mar 1;21(3):209–13. doi:10.1023/A:1005412129500
114. Avogadri F, Gnjjatic S, Tassello J, Frosina D, Hanson N, Laudénbach M, et al. Protein Expression Analysis of Melanocyte Differentiation Antigen TRP-2 (Tyrosinase-Related Protein-2). *Am J Dermatopathol*. 2016 Mar;38(3):201–7. doi:10.1097/DAD.0000000000000362 PubMed PMID: 26894771; PubMed Central PMCID: PMC5844230.
115. Ji S, Lee J, Lee ES, Kim DH, Sin JI. B16 melanoma control by anti-PD-L1 requires CD8+ T cells and NK cells: application of anti-PD-L1 Abs and Trp2 peptide vaccines. *Hum Vaccines Immunother*. 2021 Jul 3;17(7):1910–22. doi:10.1080/21645515.2020.1866951 PubMed PMID: 33522416; PubMed Central PMCID: PMC8189047.
116. Keenan BP, Yadav M, Anstas G, Fabrizio D, Murugesan K, Montesion M, et al. Intratumoral heterogeneity and immunotherapy resistance: clinical implications. *Ann Oncol Off J Eur Soc Med Oncol*. 2026 Mar;37(3):314–28. doi:10.1016/j.annonc.2025.10.1239 PubMed PMID: 41203206.
117. Taranda J, Mathew G, Watrud K, El-Amine N, Lee MF, Elowsky C, et al. Combined whole-organ imaging at single-cell resolution and immunohistochemical analysis of prostate cancer and its liver and brain metastases. *Cell Rep*. 2021 Nov 16;37(7):110027. doi:10.1016/j.celrep.2021.110027 PubMed PMID: 34788609; PubMed Central PMCID: PMC9159673.
118. Jing D, Zhang S, Luo W, Gao X, Men Y, Ma C, et al. Tissue clearing of both hard and soft tissue organs with the PEGASOS method. *Cell Res*. 2018 Aug;28(8):803–18. doi:10.1038/s41422-018-0049-z
119. Ertürk A, Bradke F. High-resolution imaging of entire organs by 3-dimensional imaging of solvent cleared organs (3DISCO). *Exp Neurol*. 2013 Apr;242:57–64. doi:10.1016/j.expneurol.2012.10.018 PubMed PMID: 23124097.
120. Masselink W, Tanaka EM. Ethyl Cinnamate-Based Tissue Clearing Strategies. *Methods Mol Biol*. 2023;2562:123–33. doi:10.1007/978-1-0716-2659-7_7 PubMed PMID: 36272071.
121. Hama H, Hioki H, Namiki K, Hoshida T, Kurokawa H, Ishidate F, et al. ScaleS: an optical clearing palette for biological imaging. *Nat Neurosci*. 2015 Oct;18(10):1518–29. doi:10.1038/nn.4107 PubMed PMID: 26368944.
122. Hsu CW, Cerda J, Kirk JM, Turner WD, Rasmussen TL, Flores Suarez CP, et al.

EZ Clear for simple, rapid, and robust mouse whole organ clearing. *eLife*. 2022 Oct 11;11:e77419. doi:10.7554/eLife.77419 PubMed PMID: 36218247; PubMed Central PMCID: PMC9555867.

123. Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell*. 2014 Nov 6;159(4):896–910. doi:10.1016/j.cell.2014.10.010 PubMed PMID: 25417164.

124. Biswas L, Chen J, De Angelis J, Singh A, Owen-Woods C, Ding Z, et al. Lymphatic vessels in bone support regeneration after injury. *Cell*. 2023 Jan 19;186(2):382-397.e24. doi:10.1016/j.cell.2022.12.031 PubMed PMID: 36669473; PubMed Central PMCID: PMC11913777.

125. Edsjö A, Holmquist L, Geoerger B, Nowak F, Gomon G, Alix-Panabières C, et al. Precision cancer medicine: Concepts, current practice, and future developments. *J Intern Med*. 2023;294(4):455–81. doi:10.1111/joim.13709

126. Rusch T, Bayry J, Werner J, Shevchenko I, Bazhin AV. Immunotherapy as an Option for Cancer Treatment. *Arch Immunol Ther Exp (Warsz)*. 2018 Apr 1;66(2):89–96. doi:10.1007/s00005-017-0491-5

127. Letai A. Functional precision cancer medicine—moving beyond pure genomics. *Nat Med*. 2017 Sep;23(9):1028–35. doi:10.1038/nm.4389

128. Malani D, Kumar A, Brück O, Kontro M, Yadav B, Hellesøy M, et al. Implementing a Functional Precision Medicine Tumor Board for Acute Myeloid Leukemia. *Cancer Discov*. 2022 Feb 1;12(2):388–401. doi:10.1158/2159-8290.CD-21-0410

129. Gajic I, Kabic J, Kekic D, Jovicevic M, Milenkovic M, Mitic Culafic D, et al. Antimicrobial Susceptibility Testing: A Comprehensive Review of Currently Used Methods. *Antibiotics*. 2022 Apr;11(4):4. doi:10.3390/antibiotics11040427

130. Bakker JL, Wever K, van Waesberghe JH, Beeker A, Meijers-Heijboer H, Konings IR, et al. What is the benefit of treatment with multiple lines of chemotherapy for patients with metastatic breast cancer? A retrospective cohort study. *Cancer Epidemiol*. 2015 Dec 1;39(6):848–53. doi:10.1016/j.canep.2015.09.010

131. Rocha AMA de la, Berlow NE, Azzam DJ. Functional precision medicine: the future of cancer care. *Trends Mol Med*. 2025 May 1;31(5):404–8. doi:10.1016/j.molmed.2024.10.015 PubMed PMID: 39567286.

132. Rodrigues J, Heinrich MA, Teixeira LM, Prakash J. 3D *In Vitro* Model (R)evolution: Unveiling Tumor–Stroma Interactions. *Trends Cancer*. 2021 Mar 1;7(3):249–64. doi:10.1016/j.trecan.2020.10.009

133. Hofer M, Lutolf MP. Engineering organoids. *Nat Rev Mater*. 2021 May;6(5):402–20. doi:10.1038/s41578-021-00279-y

134. Zhao Z, Chen X, Dowbaj AM, Sljukic A, Bratlie K, Lin L, et al. Organoids. *Nat Rev Methods Primer*. 2022;2:94. doi:10.1038/s43586-022-00174-y PubMed PMID: 37325195; PubMed Central PMCID: PMC10270325.
135. Chiorazzi M, Martinek J, Krasnick B, Zheng Y, Robbins KJ, Qu R, et al. Autologous humanized PDX modeling for immuno-oncology recapitulates features of the human tumor microenvironment. *J Immunother Cancer*. 2023 Jul 1;11(7):e006921. doi:10.1136/jitc-2023-006921 PubMed PMID: 37487666.
136. Abdolahi S, Ghazvinian Z, Muhammadnejad S, Saleh M, Asadzadeh Aghdaei H, Baghaei K. Patient-derived xenograft (PDX) models, applications and challenges in cancer research. *J Transl Med*. 2022 May 10;20(1):206. doi:10.1186/s12967-022-03405-8
137. Zeng T, Chen H, Yoshitomi T, Kawazoe N, Yang Y, Chen G. Effect of Hydrogel Stiffness on Chemoresistance of Breast Cancer Cells in 3D Culture. *Gels*. 2024 Mar;10(3):3. doi:10.3390/gels10030202
138. Smulski CR, Eibel H. BAFF and BAFF-Receptor in B Cell Selection and Survival. *Front Immunol*. 2018 Oct 8;9. doi:10.3389/fimmu.2018.02285
139. Saligrama PT, Fortner KA, Secinaro MA, Collins CC, Russell JQ, Budd RC. IL-15 maintains T-cell survival via S-nitrosylation-mediated inhibition of caspase-3. *Cell Death Differ*. 2014 Jun;21(6):904–14. doi:10.1038/cdd.2014.10
140. Cen W, Wang S, Umrath F, Reinert S, Alexander D. Impact of Fluid Dynamics on the Viability and Differentiation Capacity of 3D-Cultured Jaw Periosteal Cells. *Int J Mol Sci*. 2022 Apr 23;23(9):4682. doi:10.3390/ijms23094682 PubMed PMID: 35563073.
141. Bonaiti E, Muraro MG, Robert PA, Jakscha J, Dirnhofer S, Martin I, et al. Tonsil explants as a human in vitro model to study vaccine responses. *Front Immunol*. 2024 Sep 17;15. doi:10.3389/fimmu.2024.1425455
142. Goodman DB, Azimi CS, Kearns K, Talbot A, Garakani K, Garcia J, et al. Pooled screening of CAR T cells identifies diverse immune signaling domains for next-generation immunotherapies. *Sci Transl Med*. 2022 Nov 9;14(670):eabm1463. doi:10.1126/scitranslmed.abm1463 PubMed PMID: 36350984; PubMed Central PMCID: PMC9939256.
143. Forsythe SD, Erali RA, Sasikumar S, Laney P, Shelkey E, D'Agostino R Jr, et al. Organoid Platform in Preclinical Investigation of Personalized Immunotherapy Efficacy in Appendiceal Cancer: Feasibility Study. *Clin Cancer Res*. 2021 Sep 15;27(18):5141–50. doi:10.1158/1078-0432.CCR-21-0982
144. Bentebibel SE, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci U S A*. 2011 Aug 16;108(33):E488–97. doi:10.1073/pnas.1100898108 PubMed PMID: 21808017; PubMed Central PMCID:

PMC3158181.

145. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. *Biochim Biophys Acta*. 2010 Jan;1805(1):105. doi:10.1016/j.bbcan.2009.11.002 PubMed PMID: 19931353; PubMed Central PMCID: PMC2814927.

8 Declaration of Contribution

This work was conducted at the Department of Pathology and Neuropathology, University Hospital and Comprehensive Cancer Center Tübingen, under the supervision of **Prof. Dr. Christian Schürch**. The conceptualization of the project and optimization of experimental design were developed through close and continuous discussion between my supervisor and myself. Co-supervisors **Prof. Dr. Bettina Weigelin** and **Prof. Dr. Sven Becker** provided input on study conceptualization, manuscript preparation, and publication.

To clarify my individual contribution, all experimental work and data analyses presented in this thesis, were carried out independently by myself. Specifically, I designed the experimental strategy, including the selection of experimental approaches and optimization of methodological workflows. I independently carried out all procedures related to sample acquisition, transportation, and processing, including tissue preparation and *ex vivo* culture in the bioreactor system. I performed all single-cell suspension preparation, cell counting, and flow cytometry analyses. All experimental treatments, including immune checkpoint inhibitor treatment and CAR T cell treatment for comparative analyses, were conducted by myself. I also maintained and cultured all tumor cell lines used in this study. Furthermore, I performed all histological and imaging-related procedures, including cell staining, preparation of FFPE and frozen tissue blocks, tissue sectioning, and subsequent H&E, IF, and CODEX staining. **All experiments conduction, data generation, processing, statistical analyses, and figure generation presented in this thesis were conducted independently by myself.**

I would also like to acknowledge the collaborators and colleagues listed below for their contributions, including conceptual input, access to patient materials, analytical support, technical resources, and methodological guidance:

Human tissue samples were provided by **Prof. Dr. Sven Becker** (Department of Head and Neck Surgery, University Hospital Tübingen), **Prof. Dr. Lukas Flatz** (Department of Dermatology, University Hospital Tübingen), and their respective colleagues. CAR T cell

samples were generated and provided by **Prof. Dr. Judith Feucht** (Department of Pediatric Hematology and Oncology, University Hospital Tübingen) and **Prof. Dr. Sascha Dietrich** (Department of Hematology, Oncology and Clinical Immunology, University Hospital Düsseldorf), together with their colleagues. Lymphoma cell lines were provided by **Prof. Dr. Alexander Weber** (Department of Innate Immunity, University of Tübingen).

MD student **Ivan Foth** contributed to early experimental work, including establishment of standardized tissue processing protocols and optimization of culture media composition. PhD student **Ahmad Makky** provided support with cell–cell distance analysis, and **Dr. Ilona Hagelstein** assisted with cytokine analyses. Technical support was provided by **Karen Greif**, **Christine Beschorner**, and **Saumya Manmadhan** (Schürch Lab), including reagent procurement and histological slide scanning etc.. Additional assistance was occasionally provided by group members **Lanzhu Li**, **Jing Zhao**, **Yannick Palmowski**, **Mohammed Zidane**, and **Mingyuan Luan**.

AI tools were used solely for grammatical improvement where necessary and German language translation. I certify that I independently performed the experimental work, data collection, data analysis, figure generation, and interpretation of results in consultation with my supervisor, and this manuscript was written independently by myself. I declare that this thesis is based on two first-author peer-reviewed publications (66,97) authored by me (also see the next page, items 1 and 2 in the “Publications” section), together with the data I generated for 2 main side projects I participated in, which form the main body of my PhD project. All figures reproduced from these publications are properly cited and licensed under CC-BY 4.0. No sources other than those explicitly cited were used.

Tübingen, 04.07.2026

Yizheng Zhang

9 Publications

1. **Zhang, Y.**, Foth, I., Makky, A., Bucher, P., Grimm, M., Bruch, P.M., Hagelstein, I., Dietrich, S., Leibold, J., Flatz, L., Feucht, J., Becker, S., Schürch, C.M. (2026). Modeling immunotherapies in live 3D human cancer tissue bioreactors. *Theranostics*, 16(8), 3928-3945. DOI: 10.7150/thno.118298.
2. **Zhang, Y.**, Payab, N., Weigelin, B., & Schürch, C. M. (2026). From 2D cultures to 3D systems: Evolving cancer models at the interface of functional precision medicine and theranostics. *Theranostics*, in press. DOI: 10.7150/thno.127053.
3. Kaldjob-Heinrich, L., Nuciforo, S., Lemke, S., Stahl, A., Czermmel, S., Babaei, S., Blukacz, L., Meier, M.-A., **Zhang, Y.**, Schürch, C. M., Gonzalez-Menendez, I., Woelffling, P., Malek, N. P., Scheble, V., Nahnsen, S., Claassen, M., Templin, M., Bösmüller, H., Heim, M. H., Dauch, D., & Bitzer, M. (2025). Adenosine receptor 3 in liver cancer: Expression variability, epigenetic modulation, and enhanced histone deacetylase inhibitor effects. *Gastro Hep Advances*, 4(3), 100590. DOI: 10.1016/j.gastha.2024.11.006.
4. Wyss, N., Berner, F., Walter, V., Jochum, A.-K., Purde, M. T., Abdou, M.-T., Sinnberg, T., Hofmeister, K., Pop, O. T., Hasan Ali, O., Bauer, J., Cheng, H.-W., Lütge, M., Klümper, N., Diem, S., Kosaloglu-Yalcin, Z., **Zhang, Y.**, Sellmer, L., Macek, B., Karbach, J., König, D., Läubli, H., Zender, L., Meyer, B. S., Driessen, C., Schürch, C. M., Jochum, W., Amaral, T., Heinzerling, L., Cozzio, A., Hegazy, A. N., Schneider, T., Brutsche, M. H., Sette, A., Lenz, T. L., Walz, J., Rammensee, H.-G., Früh, M., Jäger, E., Becher, B., Tufman, A., Nuñez, N., Joerger, M., & Flatz, L. (2024). Autoimmunity against surfactant protein B is associated with pneumonitis during checkpoint blockade. *American Journal of Respiratory and Critical Care Medicine*, 210(7), 919–930. DOI: 10.1164/rccm.202311-2136OC.

10 Acknowledgment

First and foremost, I would like to express my deepest gratitude to my PhD supervisor, **Prof. Dr. Christian Schürch**, for his outstanding academic guidance throughout my doctoral studies. His scientific insight greatly shaped this work, while his mentorship extended far beyond research, offering inspiration and support in both professional development and life planning. I am also sincerely grateful for his constant willingness to help and for the invaluable immunological knowledge I gained under his supervision.

I would also like to thank my parents, **Ms. Lixian Wu** and **Mr. Xian Zhang**, for their unwavering support throughout my educational journey, which has spanned 24 years from primary school to the completion of this PhD. Their encouragement and trust have been fundamental to my perseverance and achievements.

I feel very fortunate to have met **Ms. Lin Zhu**. The time spent with you has been the happiest and most meaningful part of my life in Tübingen, and your companionship and support have meant more to me than words can express.

Finally, I would like to express my sincere gratitude to the **University of Tübingen** and the **China Scholarship Council (CSC)**. Without the opportunity and financial support they provided, the completion of my PhD studies would not have been possible.