

Identification of Potential T cell Antigens for Small Cell Lung Cancer – Immunopeptidomics- based Target Identification and T cell Assay Optimization for Sensitive Detection of Antigen- specific T cells

Dissertation

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Summary

T cell-based immunotherapies such as peptide-based vaccines specifically activating or boosting vaccine induced T cell responses harbor great potential to fight cancer.

Part I of this work presents a mass spectrometry-based immunopeptidomics approach for the identification of potential T cell antigens that could be targeted in small cell lung cancer (SCLC). High numbers of HLA class I and HLA class II peptides were extracted from SCLC tumor samples of fresh patient material (cryorecanalization or biopsy) and xenograft models. Comparative analysis with datasets of peptides presented on healthy tissues identified tumor-exclusive HLA class I and II peptides in SCLC that were shared by different tumor samples and originated from cancer- or SCLC-associated proteins, among other proteins. Most interesting HLA class I ligands were selected on a patient individual basis or with the intent to create a warehouse of frequently shared antigens. Interestingly we were able to identify one mutated neoantigen. Additionally, we performed exploratory immunopeptidomics of soluble HLA from plasma samples of SCLC patients and identified patient-exclusive peptides. A comprehensive mass spectrometry dataset was acquired that is available to address further questions, including the analysis of other tumor-antigens like microbial-derived antigens. Selected peptides will now be validated in T cell assays like recall experiments using PBMCs of SCLC patients that were collected throughout this thesis.

Parts II and III of this work present two published articles of an optimized and further developed T cell assay that is based on the detection of activated β_2 -integrins to assess functional antigen-specific T cells. In the first article a protocol for simultaneous detection of activated β_2 -integrins on both CD4⁺ and CD8⁺ T cells was described using a conformation specific monoclonal antibody, clone m24. The combination with intracellular markers IFN- γ , TNF, IL-2 and CD154 allowed sensitive identification of low frequency antigen-specific T cells. The second article presents a simplified version of this method combining the extracellular staining of cell surface activation induced markers CD137 and CD154 with activated β_2 -integrins. Combinatorial gating of these extracellular markers allowed improved assessment of antigen-specific T cells by increasing the signal to noise ratio and highly correlated with production of intracellular cytokines IL-2, TNF or IFN- γ . These T cell assays can be used in recall experiments with PBMCs from SCLC patients for validation of SCLC-specific antigens.

Zusammenfassung

T-Zell-basierte Immuntherapien, wie Peptid-Impfstoffe, die neue oder vorhandene T-Zell-Reaktionen induzieren oder verstärken, bergen großes Potenzial in der Krebsbekämpfung.

Der erste Teil dieser Arbeit beschreibt die Analyse des Immunozeptidoms mittels Massenspektrometrie zur Identifikation potenzieller T-Zell Antigene im kleinzelligen Bronchialkarzinom (SCLC). Eine große Anzahl an HLA-Klasse I und II präsentierten Peptiden wurde aus SCLC-Tumorproben von frischem Patientenmaterial (Kryorekanalisation oder Biopsie) und von Xenograft-Modellen extrahiert. Durch eine vergleichende Analyse mit Datensätzen von Peptiden aus gesundem Gewebe konnten tumorexklusive HLA-Klasse I und II Peptide identifiziert werden, die in verschiedenen Tumorproben vorkamen und unter anderem von krebs- oder SCLC-assoziierten Proteinen abstammten. Interessante HLA-Klasse I-Liganden wurden, für einzelne Patienten oder mit der Absicht ein Warenhaus von häufig vorkommenden Antigenen zu erstellen, ausgewählt. Interessanterweise konnten wir ein mutiertes Neoantigen identifizieren. Zusätzlich analysierten wir das Immunozeptidom von löslichen HLA-Molekülen aus Plasmaproben und konnten SCLC plasmaexklusive Peptide identifizieren. Es wurde ein umfassender Massenspektrometrie-Datensatz gewonnen, der für die Beantwortung weiterer Fragen zur Verfügung steht, einschließlich der Analyse anderer Tumorantigene wie mikrobieller Antigene. Die ausgewählten Peptide werden nun anschließend in T-Zell-Tests validiert, z.B. mit Hilfe von PBMC-Proben der SCLC-Patienten, die im Rahmen dieser Arbeit gesammelt wurden.

In den Teilen II und III dieser Arbeit werden zwei Veröffentlichungen eines optimierten und weiterentwickelten T-Zell-Test vorgestellt, der auf dem Nachweis aktivierter β_2 -Integrine zur Analyse funktioneller antigenspezifischer T-Zellen beruht. Im ersten Artikel wurde ein Protokoll zum gleichzeitigen Nachweis von aktivierten β_2 -Integrinen auf CD4⁺ und CD8⁺ T-Zellen unter Verwendung eines konformationsspezifischen monoklonalen Antikörpers, Klon m24, beschrieben. Die Kombination mit den intrazellulären Markern IFN- γ , TNF, IL-2 und CD154 ermöglichte eine sensitive Identifizierung von niedrigfrequenten antigenspezifischen T-Zellen. Im zweiten Artikel wurde eine vereinfachte Version dieser Methode vorgestellt, bei der die extrazelluläre Färbung der Aktivierungsmarker CD137 und CD154 in Kombination mit aktivierten β_2 -Integrinen verwendet wurde. Die kombinatorische Analyse dieser Marker verbesserte das Signal-Hintergrund-Verhältnis. Die extrazelluläre Färbung korrelierte mit der Produktion von intrazellulären Zytokine IL-2, TNF oder IFN- γ in antigenspezifischen T-Zellen. Diese T-Zell-Tests können zur Validierung von SCLC-spezifischen Antigenen in PBMC-Proben verwendet werden.

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1 Publications and attended scientific meetings

1.1 Publications

The publications marked with * are presented in this thesis.

Research articles

***Schöllhorn A**, Maia A, Kimmerle F, Born J, Rammensee HG, Dimitrov S, Gouttefangeas C. Staining of activated β_2 -integrins in combination with CD137 and CD154 for sensitive identification of functional antigen-specific CD4+ and CD8+ T cells. *Front Immunol*. 2023 Jan 19;13:1107366. doi: 10.3389/fimmu.2022.1107366. PMID: 36741378.

Herold N, **Schöllhorn A**, Feile A, Gaißler A, Mohrholz A, Pawelec G, Löffler MW, Dimitrov S, Gouttefangeas C, Wistuba-Hamprecht K. Integrin activation enables rapid detection of functional V δ 1+ and V δ 2+ $\gamma\delta$ T cells. *Eur J Immunol*. 2022 May;52(5):730-736. doi: 10.1002/eji.202149682. Epub 2022 Feb 21. PMID: 35133647.

***Schöllhorn A**, Schuhmacher J, Besedovsky L, Fendel R, Jensen ATR, Stevanović S, Lange T, Rammensee HG, Born J, Gouttefangeas C, Dimitrov S. Integrin activation enables sensitive detection of functional CD4+ and CD8+ T cells: application to characterize SARS-CoV-2 immunity. *Front Immunol*. 2021 Mar 29;12:626308. doi: 10.3389/fimmu.2021.626308. PMID: 33854501.

Review articles

Maia A, **Schöllhorn A**, Schuhmacher J, Gouttefangeas C. CAF-immune cell crosstalk and its impact in immunotherapy. *Semin Immunopathol*. 2023 Mar;45(2):203-214. doi: 10.1007/s00281-022-00977-x. Epub 2022 Dec 8. PMID: 36480035.

Richardson JR, **Schöllhorn A**, Gouttefangeas C, Schuhmacher J. CD4+ T Cells: multitasking cells in the duty of cancer immunotherapy. *Cancers (Basel)*. 2021 Feb 3;13(4):596. doi: 10.3390/cancers13040596. PMID: 33546283.

During master's degree:

Wichers JS, Tonkin-Hill G, Thye T, Krumkamp R, Kreuels B, Strauss J, von Thien H, Scholz JA, Smedegaard Hansson H, Weisel Jensen R, Turner L, Lorenz FR, **Schöllhorn A**, Bruchhaus I, Tannich E, Fendel R, Otto TD, Lavstsen T, Gilberger TW, Duffy MF, Bachmann A. Common virulence gene expression in adult first-time infected malaria patients and severe cases. *Elife*. 2021 Apr 28;10:e69040. doi: 10.7554/eLife.69040. PMID: 33908865.

During bachelor's degree:

Brilisauer K, Rapp J, Rath P, **Schöllhorn A**, Bleul L, Weiß E, Stahl M, Grond S, Forchhammer K. Cyanobacterial antimetabolite 7-deoxy-sedoheptulose blocks the shikimate pathway to inhibit the growth of prototrophic organisms. *Nat Commun*. 2019 Feb 1;10(1):545. doi: 10.1038/s41467-019-08476-8. PMID: 30710081.

1.2 Attended scientific meetings

Virtual 18th CIMT Annual Meeting, Europe's cancer Immunotherapy Meeting, 2021

Poster A flow-cytometry-based method for assessment of functional antigen-specific CD4⁺ and CD8⁺ T cells by staining activated integrins

iFIT "Image-Guided and Functionally Instructed Tumor Therapies" Retreat 2021, Tübingen (Germany)

Poster Staining of activated β_2 -integrins for assessment of antigen-specific CD4⁺ and CD8⁺ T cells

19th CIMT Annual Meeting, Europe's cancer Immunotherapy Meeting, 2022, Mainz (Germany)

Oral presentation and Poster Staining of activated β_2 -integrins combined with extracellular activation markers for sensitive assessment of antigen-specific CD4⁺ and CD8⁺ T cells by flow-cytometry

21st International Conference on Progress in Vaccination Against Cancer PIVAC22, 2022, Turin (Italy)

Poster Immunopectidomics of small cell lung cancer for identification of T cell targets

1st International Conference of the iFIT Cluster of Excellence "Image-Guided and Functionally Instructed Tumor Therapies", 2023, Zell am See (Austria)

Poster Immunopectidomics of small cell lung cancer

20th CIMT Annual Meeting, Europe's cancer Immunotherapy Meeting, 2023, Mainz (Germany)

Poster Identification of T cell targets by immunopectidomics of small cell lung cancer

Society for Immunotherapy of Cancer's (SITC) 38th Annual Meeting, 2023, San Diego (CA, USA)

Poster Immunopectidomics of small cell lung cancer reveals numerous tumor-specific HLA ligands as T cell antigen candidates

2 Introduction

2.1 Immunotherapy for cancer

Cancer develops from normal cells that undergo transformation into malignant cells by accumulation of genetic mutations leading to uncontrolled proliferation. The hallmarks of cancer describe characteristic features and capabilities that tumors acquire during malignant transformation (1-3). Eight hallmarks for the development and progression of cancer are currently recognized: sustained proliferative signaling, evading growth suppressors, resisting cell death, replicative immortality, inducing or accessing vasculature, invasion & metastasis and more recently cellular metabolism and avoiding immune destruction were included (2). Two emerging hallmarks, senescent cells and unlocking phenotypic plasticity, and two enabling characteristics, non-mutational epigenetic reprogramming and polymorphic microbiomes, are proposed that should be discussed and considered within the next years (2).

The role of the immune system in tumor elimination is recognized in the hallmarks of cancer by addition of “avoiding immune destruction”, and immunotherapeutic approaches are meanwhile standard treatments for many types of cancer. Modern attempts to use the immune system to fight cancer began in 1891, when William Coley described a link between infection and cancer regression and used the injection of toxic bacterial products to treat sarcoma (4, 5). That the immune system is able to protect from cancer development was postulated more than 50 years ago by Burnet as the concept of cancer immunosurveillance (6, 7). This gave later rise to the concept of cancer immunoediting, that describes the process by which immune cells shape the immunogenic properties of cancer cells (8). Cancer immunoediting comprises three phases: elimination, equilibrium, and escape, as depicted in Figure 1 (8-11). In the elimination phase, both the innate and adaptive immune systems jointly destroy malignant cells, which ideally results in tumor suppression equal to the immunosurveillance concept. If malignant cells have not been eliminated, tumor development and tumor control by the immune system can reach an equilibrium. In the equilibrium phase, tumor growth is mainly controlled by T cells and may also represent a final step in cancer development preventing the tumor from spreading. Genetically unstable malignant cells under the pressure of the immune system, may however develop escape mechanisms that lead to tumor outgrowth that is no longer controlled by the immune system and results in the development of clinically relevant disease. Escape mechanisms include antigen or human leukocyte antigen (HLA) loss, secretion of inhibitory factors by the tumor, like interleukin (IL)-6, IL-10, transforming growth factor (TGF)- β , expression of inhibitory receptors molecules, like cytotoxic T lymphocyte antigen (CTLA)-4 or programmed cell death protein (PD)-1 on immune cells

(see Figure 1). Both the tumor suppressing and tumor promoting effects of the immune system are covered by this concept.

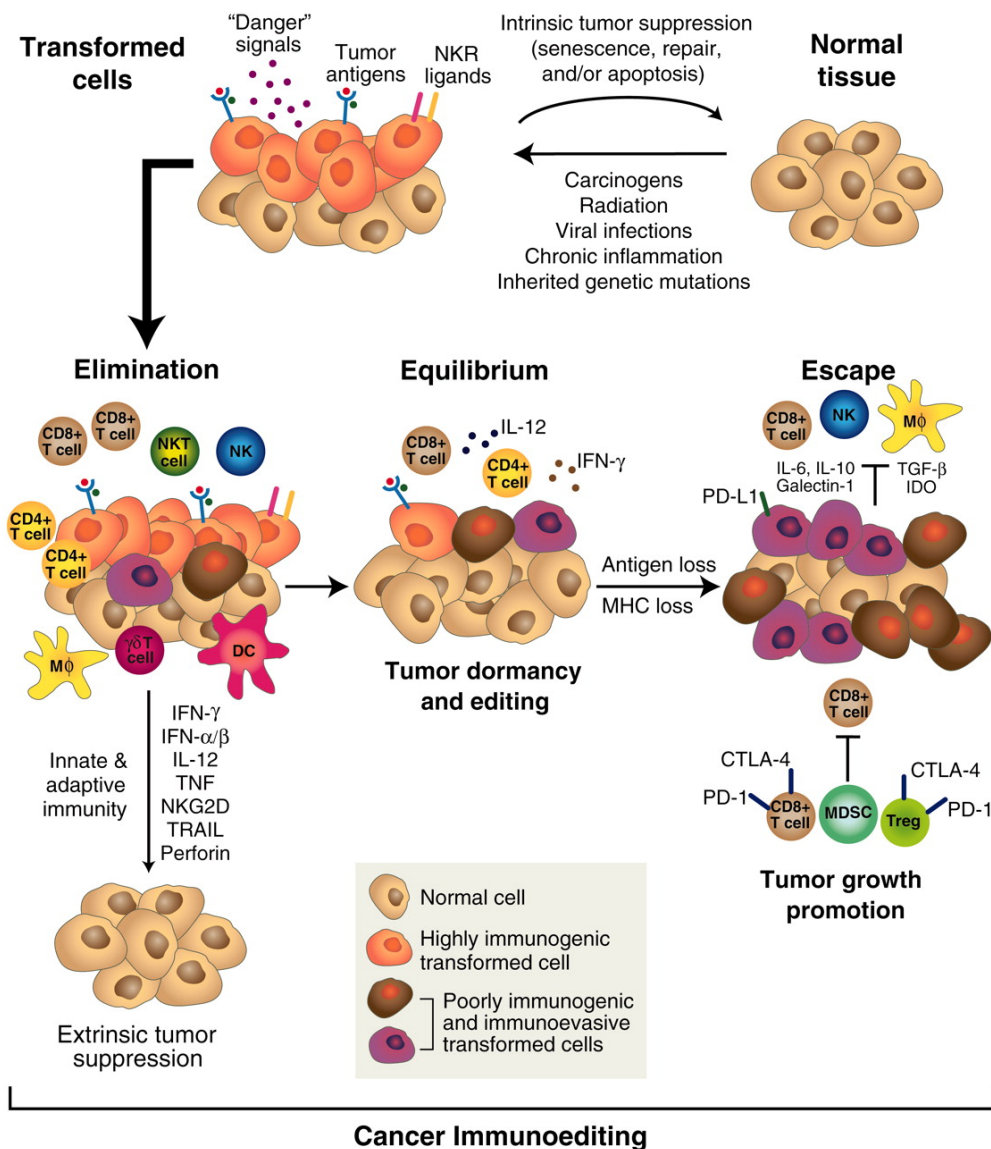


Figure 1. The concept of cancer immunoediting. Cancer immunoediting comprises three phases: elimination, equilibrium, and escape. Normal cells may undergo malignant cell transformation due to carcinogens, radiation, viral infections, chronic inflammation, or genetic mutations. Transformed cells can be recognized and eliminated by the immune system, involving different immune cells, such as CD8⁺ and CD4⁺ T cells, γδ T cells, natural killer (NK) cells, NKT cells, dendritic cells (DC), macrophages (MΦ) with different effectors, such as secreted cytokines interferon (IFN)-γ, IL-12 or tumor necrosis factor (TNF). Successful elimination ends with tumor suppression. A malignant cell that was not destroyed in the elimination phase can enter the equilibrium phase. In the equilibrium phase, tumor growth is mainly controlled by CD8⁺ and CD4⁺ T cells and cytokines like IL-12 and IFN-γ, this may also represent the final step in cancer development and prevent the tumor from spreading. Tumor growth is promoted when malignant cells escape by evasion mechanisms including antigen or MHC loss, secretion of inhibitory factors by the tumor, like IL-6, IL-10, TGF-β, expression of inhibitory receptors molecules, like CTLA-4 or PD-1 on immune cells. Reused from Schreiber et al. 2011 (10). Reprinted with permission from AAAS.

It is not deniable that the interplay of many cells of the immune system play a role to fight cancer, however, the following paragraphs will focus on T cells that are key players in anti-cancer immunity. The success of immune checkpoint inhibitors (ICI), such as antibodies

against CTLA-4 or PD-L1/PD-1, and their results in clinical trials additionally highlighted the role of T cells (12, 13). ICIs release the brake from T cells by blocking inhibitory receptors and will be discussed more detailed in section 2.2.1.

2.1.1 Basics of the immune system with a focus on T cells

The human immune system comprises the innate and adaptive immunity that differ by speed and specificity. The innate immune system represents a fast and broad first line defense against pathogens. It is composed of physical or chemical barriers, cell types such as natural killer (NK) cells, macrophages, dendritic cells (DCs) or neutrophils, as well as humoral factors. Defense mechanisms include killing of infected cell, phagocytosis, activation of the complement system, formation of reactive oxygen species or nitric oxide, secretion of cytokines and activation of DCs (14). Cells of the innate immunity discriminate between self and non-self by different germline encoded receptors. NK cells express a variety of activating and inhibitory receptors, e.g. natural killer group 2D (NKG2D) or killer immunoglobulin-like receptors (KIR), respectively, and integration of activating and inhibiting signals determines activation for target cell killing (15). Other innate immune cells, including DCs, express pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs), that recognize pathogen-associated molecular patterns (PAMPs) of different classes of pathogens leading to activation and induction of effector functions (16). The adaptive immune system reacts delayed but highly specific and has the ability for memory formation. It comprises T cells and B cells with their specific and variable antigen receptors, the T cell receptor (TCR) and the B cell receptor (BCR), respectively. By distinguishing between self and non-self, the immune system is not only able to protect the body from infections, but also to recognize and fight malignant cells, with T cells playing a key role in anti-cancer immunity. The innate and adaptive immune systems are linked by DCs, that are professional antigen presenting cells (APCs) with a central role for T cell activation (17). PRR recognition leads to DC activation, resulting in increased antigen uptake, processing and presentation, and migration to lymph nodes.

2.1.1.1 Antigen presentation

Antigens are presented in the form of peptides on major histocompatibility complex (MHC) molecules, in humans referred to as human leukocyte antigen (HLA) molecules. Together they form a peptide-HLA complex (pHLA). There are two main classes of HLA molecules in human each encoded by three highly polymorphic genes, HLA class I by HLA-A, -B and -C and HLA class II by HLA-DR, -DP and -DQ.

HLA class I molecules consist of a polymorphic α heavy chain non-covalently bound to the non-polymorphic β_2 -microglobulin light chain. Almost all nucleated cells express HLA class I molecules. With some exceptions, HLA class I peptides are typically 8 to 12 amino

acids (aa) long and fully located in the binding groove (18). Peptides presented by HLA class I molecules mainly derive from endogenous cytosolic or nuclear proteins that are mainly processed by the proteasome and transported into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP). After assembling with the HLA class I molecules, pHLA complexes are transported via the Golgi apparatus to the cell surface for presentation to cluster of differentiation (CD) 8⁺ T cells (19). Exogenous proteins taken up by phagocytosis can also be processed by the proteasome and loaded on HLA class I molecules for presentation to CD8⁺ T cells, a process called cross presentation (20).

HLA class II molecules consist of two chains, an α chain and a highly polymorphic β chain. HLA class II is expressed by professional APCs, like DCs, B cells or macrophages, but the expression on malignant cells has been reported (21, 22). HLA class II molecules present peptides with a variable length of mainly 12 to 25 aa where the core is located in the binding groove with the ends protruding (18). Peptides presented by HLA class II molecules derive from the endosomal-lysosomal compartment, mainly from exogenous proteins internalized by phagocytosis, endocytosis or micropinocytosis, but also endogenous cytoplasmic proteins deriving from autophagy are presented by HLA class II molecules (19). In a mature endosomal compartment the invariant chain, stabilizing the HLA class II molecule, gets degraded leading to the generation of a class II-associated invariant chain peptide (CLIP) bound to HLA class II. CLIP is replaced by peptides deriving from proteins that were processed in the endosomal compartment and pHLA class II complexes are transported to the cell surface for presentation to CD4⁺ T cells (19, 23).

HLA genes are highly polymorphic resulting in an enormous number of different HLA allotypes (24). Main differences are found in the peptide binding groove therefore each HLA allotype has a different peptide binding profile, resulting in different peptide motifs that can bind to the HLA allotype. Peptides presented by HLA class I molecules have specific binding motifs generally with two dominant anchor positions, represented by two amino acids that are mainly responsible for binding to the respective HLA class I molecule (18, 25). Anchor positions were also identified for HLA class II peptides, however binding of HLA class II peptides is more promiscuous (26).

2.1.1.2 T cells and their role in the immune system

A minority of T cells express a TCR that consist of a γ and a δ chain, called $\gamma\delta$ T cells, however most T cells have a TCR that consists of an α and β chain, each with a constant and a variable region and the complementarity-determining region (CDR) formed by the variable regions of the TCR is responsible for antigen recognition. $\alpha\beta$ T cells recognize peptides presented by HLA molecules with their TCR, which is referred to as HLA restriction (27). The TCR is expressed on the cell surface as a complex with CD3 $\epsilon\gamma$, CD3 $\epsilon\delta$, and CD3 $\zeta\zeta$

dimers responsible for signaling (28). T cells derive from hematopoietic stem cells in the bone marrow. The enormous diversity of TCRs is achieved during T cell development in the thymus by somatic recombination of V(D)J gene regions, where different variable (V) and joining (J) gene segments are assembled that together encode for the α chain, accordingly different variable (V), diversity (D), and joining (J) gene segments encode for the β chain. Pairing of different α and β chains adds an additional level of diversity. Each T cell expresses several ten thousand copies of one TCR specific for one antigenic peptide. T cells undergo positive and negative selection in the thymus, selecting for TCRs that are able to recognize self-HLA molecules, but depleting TCRs that recognize self-peptides presented by HLA, thereby generating the central tolerance (29). Due to central tolerance, there should be no T cells or only T cells with low-affinity TCRs in the periphery that recognize self-peptides presented by HLA and thus prevent autoimmune reactivity. Two main types of T cells can be distinguished, CD8⁺ and CD4⁺ T cells, based on the expression of the co-receptors CD8 and CD4, interacting with HLA class I and II molecules, respectively.

T cell priming and activation

Naïve T cells traffic to secondary lymphoid organs such as lymph nodes and scan APCs, mainly DCs, in search of their cognate antigen.

T cell priming and activation requires the formation of a stable immunological synapse between the T cell and an activated APC. During the scanning process, temporary binding of T cell and APC is mediated by adhesion molecules, CD2 or β_2 -integrins such as leukocyte function associated antigen (LFA)-1 on the T cell surface and intercellular adhesion molecule (ICAM)-1 or ICAM-2 among others on the DC surface. Following specific TCR-mediated recognition of the pHLA complex an immunological synapse is formed, in which cell adhesion such as LFA-1/ICAM-1 interactions, TCR-pHLA interactions, as well as stimulatory and inhibitory signals are integrated within the supramolecular activation cluster (SMAC). The SMAC is built within minutes after TCR-pHLA interaction and can be stable for hours (30, 31). TCR-mediated signaling leads to an activation and clustering of LFA-1, a process called inside out signaling (32), that is a crucial step for adequate cell-cell contact within the immunological synapse.

Three signals are required for successful priming of naïve T cells. Signal one is the specific interaction of the TCR and the coreceptor CD4 or CD8 with the pHLA complex. Signal two is a costimulation typically mediated by CD28 on the T cell and CD80 or CD86 on the APC providing a survival and proliferation signal. Both signals are required simultaneously for effective T cell priming. Signal one without signal two leads to anergy and signal two without signal one shows no effect on the T cell. Activated T cells upregulate more costimulatory molecules like CD154 (CD40L) and inducible costimulator (ICOS), but also

coinhibitory receptors such as CTLA-4. They secrete IL-2 and express the high affinity IL-2 receptor leading to clonal T cell expansion. Signal three is provided by cytokine secretion of the APC promoting T cell differentiation. Activated effector T cells exit the lymph node and express high amounts of adhesion molecules like LFA-1, which is important to enter the site of infection as well as for cell-cell interactions to exert their effector functions.

T cell differentiation

Naïve CD8⁺ T cells differentiate into cytotoxic T cells (CTLs) and exert effector functions against pathogen-infected or malignant cells. Direct cytotoxicity is mediated by cell-cell contact leading to apoptosis of the target cell using Fas-Fas ligand interactions and release of perforin and granzyme B. Indirect cytotoxicity is mediated by production of cytokines like interferon (IFN)- γ and tumor necrosis factor (TNF) leading to macrophage activation, apoptosis induction of target cells and increased expression of HLA class I and II (33). CTLs are the main population of CD8⁺ T cells, but other rare subsets of CD8⁺ T cells have been described in the context of different autoimmune or infectious diseases and cancer, such as CD8⁺ regulatory T cells (Treg), IL-17 or IL-9 producing CD8⁺ T cells (34). CD4⁺ T cells were shown to be important for proper CD8⁺ T cell memory formation and for induction of effector functions by licensing APCs to activate CD8⁺ T cells (35, 36).

Differentiated CD4⁺ T cells are highly diverse. The cytokines secreted by DCs during naive CD4⁺ T cell priming is defining the polarization into specific effector CD4⁺ T helper (Th) cell subsets, each having distinct functions in the immune system. CD4⁺ cell subsets comprise Th1, Th2, Th9, Th17, Th22, Tregs and follicular helper T cells (Tfh). Polarizing cytokines are IL-12 and IFN- γ for Th1, IL-4 for Th2, IL-4 and TGF- β for Th9, IL-6, IL-1 β and TGF- β for Th17, IL-6 and IL-23 for Th22 and TGF- β and IL-2 for Tregs (37-39). The different CD4⁺ T cell subsets are characterized by the expression of lineage-defining transcription factors, promoting their characteristic phenotype, cytokine profile and effector functions (38). Th1 cells mainly exert effector functions against intracellular pathogens by production of IFN- γ , IL-2 and TNF that promotes effector functions of CD8⁺ T cells and leads to activation of macrophages. Th2 cells are associated with protection against parasites and stimulation of tissue repair by secretion of IL-4, IL-5 and IL-13 with effects on eosinophils, goblet cells, mast cells, B cell secretion of immunoglobulin (Ig)E and M2 macrophage polarization (40). Th17 are important for immunity against extracellular pathogens and mediate their pro-inflammatory function by secretion of IL-17 and IL-22 (41). Under certain conditions, they can convert to a hybrid Th17/Th1 cell type with self-renewing capacity (41). Th9 and Th22 cells are described to secrete IL-9 and IL-22 but not IFN- γ , IL-4 or IL-17 (39). Tregs are required to mediate immune tolerance for T cell homeostasis and protection from autoimmunity. Two types of Tregs are distinguished, natural (n)Tregs and induced (i)Tregs.

nTregs develop in the thymus from T cells with high affinity to self-antigens, whereas iTregs develop in the periphery from naïve CD4⁺ T cells under certain conditions, like chronic inflammation (42). These Tregs express the transcription factor forkhead box P3 (FoxP3) and CD25, the α chain for the high affinity IL-2 receptor. They mediate their suppressive function through sequestering IL-2 by expression of CD25, secretion of IL-10 and TGF- β or by inhibitory checkpoint molecules like T cell immunoreceptor with Ig and ITIM domains (TIGIT) and CTLA-4, among others (41). Tfh cells are specialized for mediating help to B cells and secrete IL-21 (43). Cytotoxic CD4⁺ T cells were first reported in the context of viral infections (44, 45). But also in the context of cancer, they perform HLA class II-restricted target cell killing by secretion of granzymes and perforin or in a death-receptor dependent manner with Fas-FasL (CD95-CD95L) or TNF-related apoptosis-inducing ligand (TRAIL)-TRAIL receptor interactions (38, 46).

The generation of an immune memory is characteristic for the adaptive immune system. After successful pathogen clearance, long-lived memory T cells remain in the body. The most common memory T cell populations are central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). Effector memory T cells re-expressing CD45RA (T_{EMRA}) and most recently stem like memory T cells (T_{SCM}) are also described (47, 48). T cells can express different isoforms of CD45 (CD45RA and CD45RO), a cell surface protein expressed on hematopoietic cells, which can be used in combination with the expression of the lymph node homing receptor CC-chemokine receptor 7 (CCR7) to distinguish naïve from memory T cells. Naïve T cells express CD45RA and CCR7 but lack CD45RO, whereas memory T cells lack CD45RA. Originally, two major memory subsets were differentiated based on the expression of CCR7: CD45RA⁻ CCR7⁺ T_{CM} cells homing to the lymph nodes and tissue located CD45RA⁻ CCR7⁻ T_{EM} cells (49). T_{CM} have a high proliferation potential but limited immediate effector functions, whereas T_{EM} traffic in the periphery and can exert immediate effector functions including secretion of IFN- γ and perforin. Within the CD8⁺ T cells a CCR7⁻ T cell population re-expressing CD45RA (T_{EMRA}) was described that contains pre-loaded perforin but harbors low expansion potential (39, 48). In the context of cytomegalovirus (CMV) and dengue virus infections CD4⁺ T_{EMRA} cells were found and associated with viral control (50, 51). More recently, T_{SCM} with self-renewal capacity were described as antigen experienced cells displaying a mainly naïve phenotype with expression of memory markers, including Fas (CD95), and the ability to reconstitute the full memory compartment (52). In a proposed hierarchical model of T cell differentiation, T_{SCM} are directly generated from T_N cells, and progressively differentiate into T_{CM}, T_{EM} and terminally differentiated effector T cells that can be distinguished by combinatorial expression of different markers (47).

Cytotoxic CD8⁺ T cells and CD4⁺ T cells are important to fight malignant cells by mediating direct cytotoxicity. The various CD4⁺ T cells also contribute indirectly to anti-tumor immunity by secreting cytokines that lead to the recruitment and licensing of CD8⁺ T cells, the activation of B cells, the inhibition of angiogenesis, activation of macrophages and induction of senescence in malignant cells (38).

2.1.2 The T cell-centered fight against cancer

In a simplified way, the development of anti-cancer immunity can be seen as circular self-amplifying steps described as the cancer-immunity cycle (Figure 2) (53, 54).

The cancer-immunity cycle describes the ideal scenario of an anti-cancer T cell response leading to malignant cell killing. But there are many different stimulatory, but also inhibitory factors influencing the outcome. Negative interference at any step of the cancer-immunity cycle prevents the development of an adequate anti-tumor immune response in cancer patients (53, 54). The release of antigens can be negatively regulated by the induction of a tolerogenic cell death of cancer cells (53). The cytokine milieu can be tumor promoting or stimulate an anti-tumor immune response, with prominent cytokines such as TGF- β and IL-10 or IFN- γ , IL-1 and TNF, respectively (55). Cytokines can directly affect the antigen presentation in a positive (TNF, IL-1, IFN- α) or negative (IL-10, IL-4, IL-13) way (53). Additionally, antigens could be considered as self and not foreign, leading to Treg activation. T cells may also not properly traffic and infiltrate into tumor site, influenced by expression of chemokines and adhesion molecules or inhibitory factors in the tumor microenvironment (TME) (53, 54).

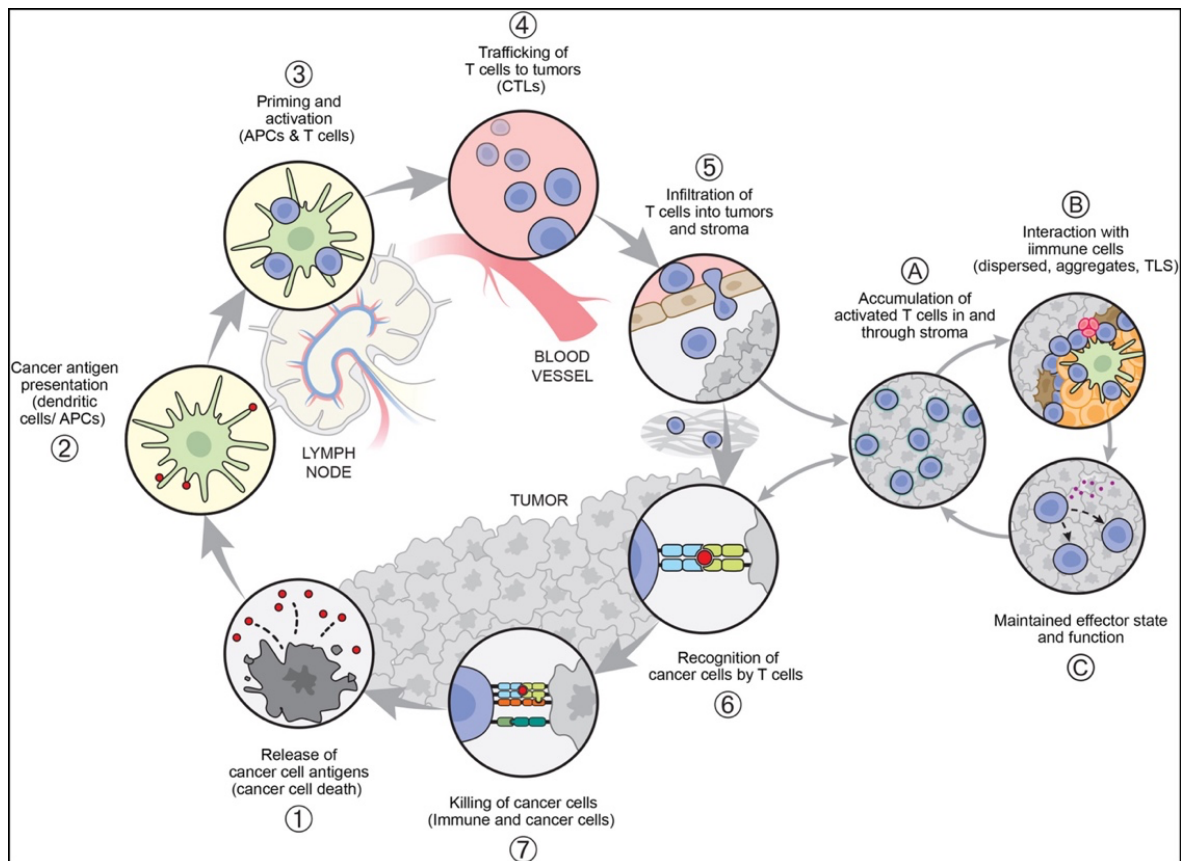


Figure 2. The cancer-immunity cycle. Cancer cell death is leading to the release of cancer-associated antigens [1], and proinflammatory cytokines such as TNF, IL-1 or other factors, like endogenous adjuvants cyclic dinucleotides (CDN) or adenosine triphosphate (ATP), for APC activation. Following antigen uptake and presentation on HLA molecules by APCs [2], T cells get primed and activated in the lymph nodes [3] to mediate an immune response against cancer-specific antigens that are considered foreign or for which central tolerance was incomplete. Activated T cells migrate to the tumor site [4] and infiltrate the tumor [5]. The stromal barrier that needs to be infiltrated and overcome before entering the tumor was added in the latest version of the cancer-immunity cycle [5]. Upon specific antigen recognition [6], T cells exert their cytotoxic activity against cancer cells, leading to cancer cell killing [7], starting the cycle from the beginning. The TME influencing the anti-cancer immunity is gaining importance and a subcycle describing the interactions in the TME was added to the cancer-immunity cycle. Activated T cells accumulate in the stroma [A], they interact with immune cells, mainly DCs [B] leading to maintained effector functions [C]. Reused from Mellman et al. 2011 (54) (CC BY NC ND).

Tumor infiltrating lymphocytes (TILs) and a pro-inflammatory TME are associated with improved clinical outcomes in a variety of tumor types (56). Based on the infiltration and presence of immune cells in the TME, different tumor phenotypes can be described in a simplified way (57, 58). In a cold tumor with an immune desert phenotype, no T cells are present in the TME and T cell priming fails. In an altered phenotype which can be immune excluded or immunosuppressed, direct contact of tumor cells and T cells is prevented by stromal barriers or immunosuppressive cell types, and checkpoint molecules and suppressive cytokines interfere with T cell functions. A hot or immune inflamed tumor is defined by high T cell infiltration, T cell activity and the presence of tertiary lymphoid structures (TLS) (54, 58). TLSs are organized lymphoid aggregates in non-lymphoid tissue that can develop at chronically inflamed sites including tumor sites (59). They mainly consist of DCs, T cells and B cells representing ideal structures for antigen presentation and

activation of effector functions. Cytotoxic CD8⁺ T cells (60) and different types of CD4⁺ T cells (37) are highly powerful anti-cancer effector T cells. The abundance of CD8⁺ T cells in the tumor was found to be a predictive marker for the effectivity of anti PD-1/PD-L1 checkpoint inhibition, more detailed discussed in section 2.2.1 (61). But CD4⁺ T cells are gaining more interest since they are able to mediate direct cytotoxic effects and indirect effector functions including the maintenance of CD8⁺ T cell responses (37, 38). Additionally CD4⁺ T cells were stated to be more effective in tumor rejection than CD8⁺ T cells (62). The infiltration of CD8⁺ T cells, Th1 cells or Tfh cells was found to be generally associated with a good prognosis in a comprehensive study summarizing studies across 17 cancer types (63). But effector T cells at the tumor site are often dysfunctional (64, 65) and found to be in an so called exhausted state with reduced effector functions and expression of inhibitory molecules, like CTLA-4, PD-1, Lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) or TIGIT due to chronic antigen exposure (65-67).

The expression of stimulatory or inhibitory molecules at the tumor site or in the TME define the outcome of TCR signaling and influence the effector functions of T cells (68). Costimulatory molecules include ICOS, CD28, OX-40, CD154 (CD40L) or CD137 (4-1BB), and coinhibitory checkpoint receptors include the afore mentioned receptors CTLA-4, PD-1, LAG-3, TIM-3 or TIGIT (68). The latter ones are targets of checkpoint inhibition, discussed in section 2.2.1.

CD4⁺ T cell populations are not only anti-tumorigenic and can harbor tumor promoting effects reviewed in (37). Especially immunosuppressive Tregs are associated with bad prognosis in various cancer entities (63). But Th2 and Th17 cells have also been implicated with pro-tumorigenic effects and associated with bad prognosis in some cancer entities (63).

Not only T cells themselves, but also the composition of the TME shapes an anti-tumor immune response. The infiltration of B cells, DCs or macrophages of the M1 type were found to be associated with good prognosis (63). In contrast, other cell types that can be found in abundance in the TME are associated with immunosuppressive functions. Cancer-associated fibroblasts (CAFs) or myeloid cells, like myeloid derived suppressor cells (MDSCs) or tumor-associated macrophages (TAMs) of the M2 type, secrete anti-inflammatory cytokines and create a barrier to anti-tumor immunity (69, 70). The infiltration of M2 macrophages is associated with poor prognosis in many cancer types (63).

Mechanisms to escape an immune attack include the induction of an immunosuppressive TME, the presence of Tregs, the lack of costimulatory signals and the presence of inhibitory signals, as well as defects in the pathway of antigen processing and

presentation in tumor cells, such as loss or down regulation of cell surface HLA molecules, resistance to apoptosis in tumor cells or induction of apoptosis or anergy in T cells (71, 72).

The complement system was also found to be associated with cancer, having pro- and anti-tumorigenic effects (73). The association of the complement system with disease progression and poor prognosis and the interference with different steps of the cancer-immunity cycle were described (74). Different factors of the complement system can have negative effects on the antigen release by inducing a tolerogenic cell death or on the T cell priming and activation by inhibition of Th1 responses, induction of immunosuppressive cytokines or recruitment and activation of MDSCs or Tregs. At the tumor site the complement system can negatively interfere with the T cell infiltration, as well as with the killing of malignant cells by induction of Th2 cell responses and M2 macrophage polarization, or recruitment of MDSCs and Tregs (74).

The positive and negative regulators of the cancer-immunity cycle offer a wide range of targets that could be exploited by different therapies, including immunotherapy. Therapies are needed that induce tumor-specific T cell responses, and manipulate the immunosuppressive microenvironment and escape mechanisms.

2.2 Different immunotherapeutic approaches

Immunotherapy takes advantages of the natural mechanisms of the immune system to recognize and kill malignant cells. Different immunotherapeutic approaches are pursued: immunomodulators, oncolytic viruses, antibody-based immunotherapies, cell-based immunotherapies, and vaccines. T cells are able to specifically kill cancer cells and different immunotherapies that are exploiting the functions of T cells will be highlighted in this section.

2.2.1 Immune checkpoint inhibitors

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies that bind to inhibitory receptors of T cells or their ligands on APCs, thereby blocking their inhibitory function and releasing the functionality of T cells. A major breakthrough in immunotherapy was achieved with the approval of Ipilimumab targeting CTLA-4 by the food and drug administration (FDA) in 2011 for unresectable or metastatic melanoma (12). The approval of different ICIs targeting PD-1 (pembrolizumab, nivolumab, cemiplimab), or PD-L1 (atezolizumab, durvalumab, avelumab) was directly following. PD-1/PD-L1 represent the most frequently targeted inhibitory molecules for a wide range of different cancer entities (13). With the approval of the combination therapy targeting LAG-3 and PD-1 in untreated metastatic or unresectable melanoma in March 2022, a new target is exploited (75). A high number of cancer patients benefits from immune checkpoint blockade, especially patients with a high

mutational burden (76). ICIs have been a milestone in oncology with remarkable successes in cancer treatment. However, many cancer patients, especially those with low mutational burden cancers, do not benefit from ICI, which highlights the importance of the identification of potential predictive biomarkers, to identify patients that are more likely to respond to therapy. Additional therapies harnessing the immune system are needed that could also be combined to ICI.

2.2.2 Other antibody-based immunotherapies

Antibody-based cancer therapies comprise different categories that are characterized by different mode of action based on their natural properties by marking or blocking their target, including ICI, delivery of cytotoxic drugs or engagement of immune cells such as T cells (77). T cell engaging bispecific antibodies exist in different formats and are directed against CD3 and an antigen expressed on tumor cells, for example B cell maturation antigen (BCMA) as target in multiple myeloma, CD19 or CD20 expressed by B cells as target in B cell malignancies, or gp100 protein a melanoma antigen, with the intend to redirect the T cells to the tumor cells (78). Increasing numbers of FDA and European medicine agency (EMA) approved antibody-based therapies are available for different cancer entities, including T cell engaging antibodies: Blinatumomab (CD3/CD19), Teclistamab (CD3/BCMA), Tebentafusp (CD3/gp100), Mosunetuzumab (CD3/CD20) (79). There is an emerging number of new T cell engaging constructs that integrate several immune functions, such as bifunctional checkpoint-inhibitory T cell engagers (CiTEs), simultaneous multiple interaction T cell engagers (SMITEs), trispecific killer engagers (TriKEs) and BiTE-expressing chimeric antigen receptor (CAR) T cells (CART.BiTE cells), immune-mobilizing monoclonal TCRs against cancer (ImmTACs), bispecific constructs with T cell receptor (TCR)-derived antigen-recognition domains (80). Antibodies are dependent on cell surface antigens that are specifically expressed on tumor cells to mediate their mode of action. Identification of antigens that are not expressed on healthy tissue is important to prevent on target/off tumor effects. However, the loss of antigen expression on tumor cells will lead to therapy resistance.

2.2.3 Cell-based immunotherapies

Adoptive T cell therapy is a highly personalized cell-based immunotherapy that involves the transfusion of the patient's own T cells expanded *in vitro*. These T cells can be either naturally occurring tumor-specific T cells expanded from TILs or peripheral T lymphocytes, as well as T cells with genetically-modified chimeric antigen receptors (CAR) or TCRs (81). Adoptive cell transfer of autologous TILs after lymphodepletion was shown to mediate durable complete response in patients with metastatic melanoma (82). CAR T cells are genetically engineered to specifically target cell surface antigens on cancer cells in an HLA-independent manner. CAR-T cells targeting CD19 and BCMA have been approved by the FDA for the treatment of various hematologic malignancies. Their success is limited by antigen loss, on target/ off tumor effects, lacking tumor infiltration or an immunosuppressive environment, as well as associated toxicity (83). The treatment of solid tumors remains challenging and requires a new generation of CAR-T cells that overcomes antigen escape, enhances T cell function, overcomes toxicity and modulates the TME by providing immunostimulatory signals (83, 84).

2.2.4 Cancer vaccines

Therapeutic cancer vaccines are designed to induce new T cell responses and/or enhance existing T cell responses, but have so far achieved only limited therapeutic success in patients (85, 86). Critical components of a cancer vaccine include tumor antigens, formulations, adjuvants and delivery vehicles, with different approaches pursued for each of these components (see Figure 3 (85)). Tumor antigens can be generally classified into tumor-associated or tumor-specific and will be discussed in more detail in section 2.3. Different vaccine formats are tested, including DC-, tumor cell-, RNA-, DNA-, and peptide-based vaccines (85).

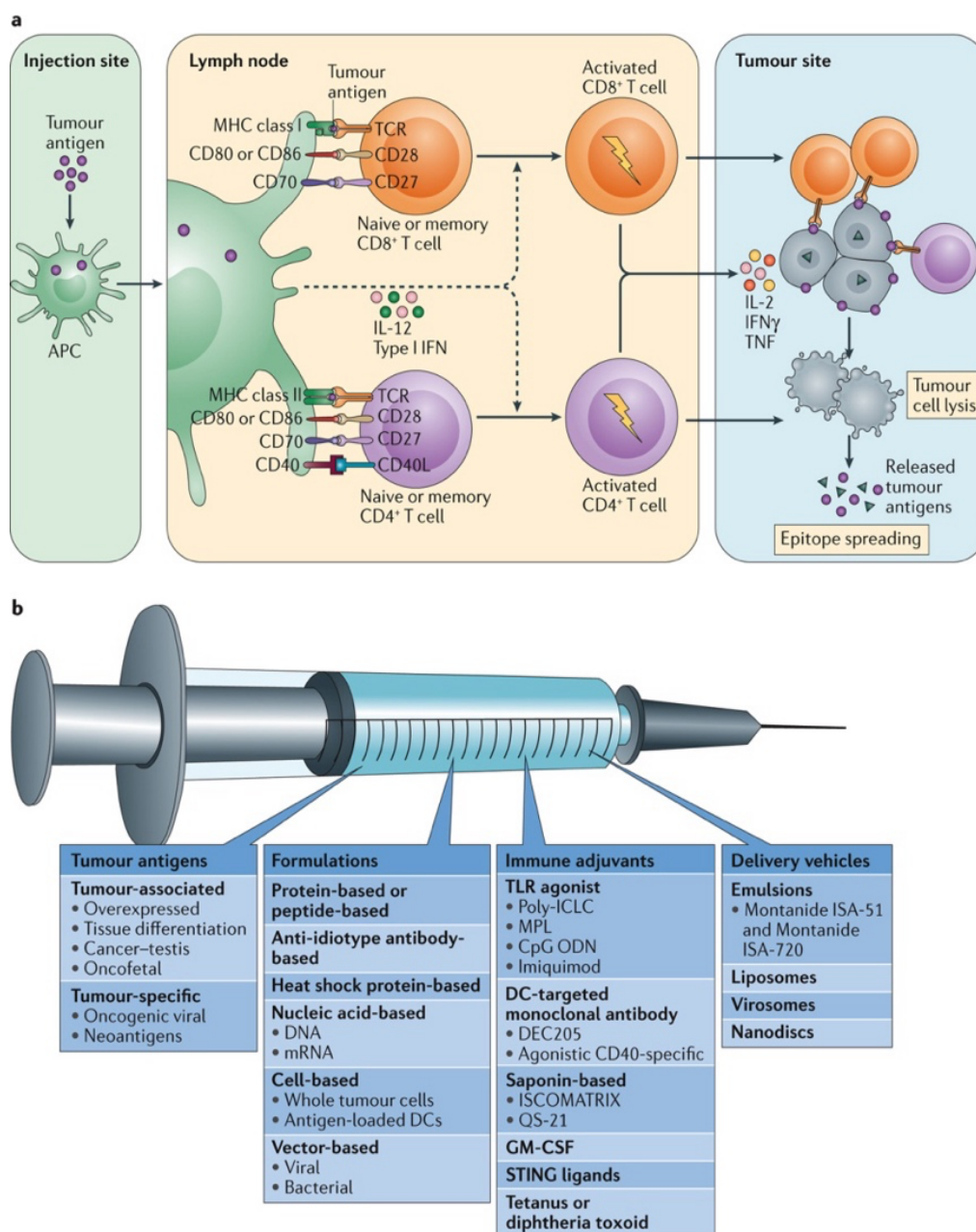


Figure 3. Mechanism and composition of a cancer vaccine. a) At the injection site tumor antigen is taken up by APCs. In the lymph nodes tumor antigen-specific CD8⁺ and CD4⁺ T cells (naïve and memory) get activated. They exert their effector functions at the tumor site leading to tumor lysis and epitope spreading. b) Critical components of a cancer vaccine include the tumor antigen, the formulation, the adjuvant and the delivery vehicle. Reused from Zhuting Hu et al. 2018 (84). Reproduced with permission from Springer Nature.

poly-ICLC, polyinosinic–polycytidylic acid with polylysine and carboxymethyl-cellulose; MPL, monophosphoryl lipid A; CpG ODN, CpG oligodeoxynucleotide; GM-CSF, granulocyte-macrophage colony-stimulating factor; STING, stimulator of interferon genes protein.

Autologous DCs are first differentiated and activated with cytokines before loading with peptides, proteins, virus, DNA, mRNA, lysate or tumor cells followed by injection into the patient (87). DC-based vaccines were tested in high numbers of clinical trials and have shown to be safe and immunogenic, in some cancer patients they were even capable of inducing tumor regression and clinical response (87). Sipuleucel-T for treatment of metastatic prostate cancer remains the so far only FDA approved cancer vaccine (88) and

derives from autologous peripheral blood mononuclear cells (PBMCs) that were incubated with prostatic acid phosphatase (PAP) fused to granulocyte-macrophage colony-stimulating factor (GM-CSF). The authorization in Europe was however withdrawn at the request of the marketing authorization holder.

An intrinsic feature of nucleic acids is their ability to stimulate innate immune responses as ligands of TLR 9, which recognizes bacterial CpG DNA (89) and TLR 3/7/8/13, which recognizes RNA (90). DNA and RNA vaccines can deliver multiple antigens in an HLA independent way and are able to induce cellular and humoral immunity (91). DNA vaccines were shown to be safe, however demonstrated poor immunogenicity and limited therapeutic effects (91). RNA vaccination gained recognition during the SARS-CoV-2 pandemic which led to the first approved mRNA vaccine (92), but the first clinical trial with a RNA vaccine against cancer was already published in 2008 (93). mRNA can be produced fast and easy, is highly stable in vitro, is safe and has a well-defined therapeutic window in patients (94). There are several different approaches how mRNA can be exploited for cancer immunotherapy that are tested in a high number of ongoing clinical trials, including non-formulated or formulated direct cancer vaccines (95).

A long-lasting expertise of the department of immunology in Tübingen is the design of peptide vaccines using immuno-peptidomics to identify naturally presented HLA ligands on tumor cells that could be used as T cell targets. This strategy has been applied over the last years for several cancer entities, including glioblastoma, ovarian carcinoma, hepatocellular carcinoma, chronic myeloid leukemia among others (96-104). Multiple clinical trials were testing different variations of peptide vaccines and exemplary studies are cited; vaccines using a single peptide (105) or multiple peptides against different antigens (96, 106, 107), vaccines using mutated neoantigens (108), or non-mutated antigens (96, 107, 109) or a combination of both (110). Nowadays peptide vaccines are mainly applied in a mix of HLA class I and HLA class II peptides (96, 106, 109, 110) or as elongated synthetic long peptides (108, 111, 112) to induce both CD4⁺ and CD8⁺ T cell responses. Long peptides need to be processed by APCs leading to DC activation and therefore optimal T cell activation, additionally they can contain overlapping CD4⁺ and CD8⁺ T cell epitopes (113). Peptide-based cancer vaccines using synthetic peptides have low side-effects and have been shown to induce cancer-specific immune responses in several studies, but clinical responses are so far limited to single patients (114). Studies with peptide vaccines conducted before 2004 showed an objective response rate of 2.9 % (86).

To generate a robust vaccine-induced T cell response and form an immunological memory, adjuvants are important to protect the antigen from degradation, mediate a prolonged antigen release and induce efficient antigen uptake and activation of APCs (115).

Adjuvants used for cancer vaccines include Montanide ISA™51, GM-CSF, TLR agonists such as Imiquimod, single-stranded RNA (TLR7/8), poly-ICLC (Hiltonol, TLR3), amphiphilic lipopeptide Pam3Cys-Ser-Ser (TLR1/2) (115), also listed in Figure 3 (85). A new TLR1/2 agonist called XS15, a water-soluble synthetic Pam3Cys-derivative designed and tested by Rammensee et al. harbors great potential in peptide-based vaccination (116).

Combination therapy integrating different immunotherapies or immunotherapy with standard of care treatment will direct the future, including therapies to induce tumor-specific T cell responses, induction of immunogenic tumor cell death, strategies to target an immunosuppressive TME, costimulation and induction of an pro-inflammatory environment reviewed in (117-119). Vaccines are nowadays frequently combined with ICIs and were tested in clinical studies (108, 120, 121). Several different therapy combinations are already FDA approved in individual cancer entities, the combination of chemotherapy to checkpoint inhibition, combination of different checkpoint inhibitors and combination of checkpoint inhibitors with targeted therapies (118). The identification of biomarkers especially in the context of combination therapy will help to select therapy approaches with best clinical impact for the patient, such biomarkers already include infiltration of lymphocytes, expression of checkpoint receptors or tumor mutational burden (TMB), however they are not always prognostic and their value might change under combination therapy (117).

2.2.5 Personalized immunotherapy

Different approaches achieve different levels of personalization in immunotherapy, stratification, warehouse-based personalization and patient-specific individualization (114, 122, 123). Stratification selects patients according to the assessment of predefined biomarkers like certain tumor antigens for treatment with a uniform drug product. human epidermal growth factor receptor 2 (HER-2) positive breast cancer or other HER-2 positive malignancies that are treated with targeted antibodies are one example for stratification. Warehouse-based personalization assembles a patient-adapted drug from pre-manufactured and pre-characterized antigens. The warehouse approach was already applied in peptide vaccination trials (110). Here a warehouse is generally built for one type of cancer containing frequently shared, pre-characterized and manufactured tumor-specific antigenic peptides for the most common HLA types in a population. A vaccination cocktail is then assembled based on the HLA type of the patient and if available on antigen expression analysis. Individualization is based on the identification, characterization and selection of patient-specific biomarkers and a patient-individual manufacturing of a drug product. By this approach patient-specific mutations can also be addressed with help of

next generation sequencing (NGS). The combination of a warehouse and an individualized approach can also be applied (110).

All immunotherapies have one thing in common: they are only as good as their targeted tumor antigen.

2.3 Tumor antigens for T cell-based immunotherapy

Tumor antigens can be broadly categorized into tumor-associated antigens (TAA), which are expressed by tumor cells but can also be present on other cells, and tumor-specific antigens (TSA), which are only expressed by tumor cells. TAAs include overexpressed or differentially expressed antigens, tissue specific antigens, cancer testis antigens or oncofetal antigens. TSAs include oncogenic viral derived antigens or mutated neoantigens (see also Figure 3, (85)).

Non-mutated overexpressed self-antigens, include proteins like HER-2 or telomerase reverse transcriptase (TERT). Tissue-specific antigens derive from proteins that are expressed in specific cell types and are shared by the cancer cells and their cell type of origin, e.g. the melanocyte-specific protein MelanA. Cancer germline antigens, like cancer-testis antigens or oncofetal antigens derive from proteins that are generally expressed in testes or during fetal development but are not expressed in most healthy adult tissues. Cancer germline antigens are often found on cancer cells after promotor demethylation leading to aberrant protein reexpression and represent a higher tumor specificity than other self-antigens (124, 125).

Using mass spectrometry (MS)-based approaches, non-mutated antigens from canonical proteins can be identified using database search against the reference human proteome. Increasing datasets of peptides that are naturally presented on benign tissue and cells (98, 99, 126, 127) enable a reliable identification of non-mutated tumor-associated self-antigens that are tumor-exclusive antigens. They are also referred to as tumor-specific due to the absence on healthy tissue and resemble potential targets for T cell-based immunotherapy. A major advantage of non-mutated TAAs, is their potential of being shared between different individuals which enables a broad applicability and can be utilized by the warehouse approach to design peptide vaccination (110, 123, 128). However, they need to overcome tolerance which could be achieved by using an appropriate adjuvant already discussed in 2.2.4 *Cancer vaccines* (115, 116). A different class of tumor-specific HLA-presented antigens resemble non-mutated self-antigens that were shown to be presented by TAP-deficient tumors (129).

Mutated neoantigens that derive from genomic alterations in cancer cells, are by definition tumor-specific and considered to be ideal T cell targets since they resemble foreign proteins against which no central tolerance has been developed. Mutated

neoantigens were found to be targeted under checkpoint inhibition in animal models (130). A correlation between a high tumor mutation load and therapy response to PD-1 blockade was first reported in non small cell lung cancer (NSCLC) and mutated neoantigen-specific CD8⁺ T cells were identified (76). Antigens derived from single-nucleotide variants are most commonly studied, but there are other sources of mutant neoantigens deriving from gene fusions or frameshift mutations such as insertions or deletions (124, 131). A vaccine with mutated neoantigens was shown to be immunogenic in melanoma (108). Some tumors including melanoma or small cell lung cancer (SCLC) bare exceptionally high numbers of mutations, whereas others hardly bare any mutations (132, 133). Other limitations of the widespread use of tumor-specific mutated neoantigens for immunotherapeutic purposes are the facts that they are tumor- and thus mainly patient-specific, and that only a small number of mutated peptides is found to be presented on HLA molecules (134-137). Immunoediting additionally decreases the expression of mutated antigens on malignant cells (138, 139). Combination of mass spectrometry and exome sequencing could be used to identify naturally presented mutated neoantigens (134).

Viral tumor antigens can originate from oncogenic viruses, such as hepatitis B virus (HBV), human papillomavirus (HPV) or Epstein-Barr virus (EBV) that are not cleared during infection and mediate malignant cell transformation (140). Such oncogenic viral antigens are tumor specific, but other microbial derived tumor antigens are also described (124). Other microbial antigens can derive from bacterial or viral proteins that are found at the tumor site. The tumor microbiome gains increasing interest and was shown to be composed of tumor type-specific intracellular bacteria (141). Not only bacteria, also fungi in the microbiome gain interest in the context of cancer (142, 143). Bacteria-derived peptides were shown to be presented by HLA and described to be immunogenic in glioblastoma (144) and melanoma (145).

Only a small fraction of the genome is protein-coding and non-canonical or cryptic antigens arising from translated unannotated open reading frames offer another growing source of possible tumor antigens (146-149). Additionally, proteins undergo posttranslational modification (PTM) including acetylation, methylation, glycosylation, or phosphorylation, and glycopeptides (150) and phosphopeptides (151) were shown to be presented by HLA molecules and described to be immunogenic. A comprehensive study analyzing posttranslational modified peptides including 29 different PTMs defined specific HLA binding motifs and identified thousands of shared tumor-specific antigens (152).

Mass spectrometry-based immunopectidomics, in some instances in combination with NGS, allows the identification of naturally HLA-presented tumor antigens of all the above-mentioned categories.

2.4 Immunopeptidomics

Nowadays, there are two main different approaches to identify HLA-presented tumor antigens, either based on the computational prediction of potentially HLA-presented peptides from NGS data or on the direct identification of naturally presented HLA ligands by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (123). Next generation exome or transcriptome sequencing data is translated into peptide sequences that are analyzed for potentially presented HLA ligands using HLA binding prediction tools mentioned later in this section. This results in a high number of potential HLA ligands that need to be analyzed with enormous effort for their relevance and tested in T cell-based assays.

LC-MS/MS remains the only method for direct identification of naturally presented HLA ligands, referred to as immunopeptidomics or HLA ligandomics. The field of immunopeptidomics was pioneered in the 1990s by sequencing peptides eluted from MHC molecules (25, 153). HLA-presented peptides can be isolated by immunoprecipitation and acidic elution or by mild acidic elution followed by ultrafiltration, subsequent chromatographic separation and sequencing by mass spectrometry (154). Immunopeptidomics is performed for identification of HLA ligands from tumor tissue (96-102), cell lines (155-157) or benign tissue (126, 127), as well as ligands from soluble HLA (sHLA) molecules that are present in plasma samples (158-161). For some cancer types there is only limited access to tumor tissue samples and biopsies are often very small, which is limiting the efficiency of peptide recovery. To circumvent this issue, xenograft models deriving from patients' biopsies or engrafted cell lines were recently proposed to be used for immunopeptidomics and identification of tumor antigens (162, 163). Small cell lung cancer (SCLC) was the cancer type studied throughout this thesis and will be discussed in detail in section 5.1.2.1 of results part I. Access to SCLC tissue samples is limited, since surgery is rarely performed (except for cryorecanalizations performed in Tübingen). We had access to SCLC samples that derived from such cryorecanalizations, which is a bronchoscopic therapy of stenoses of the respiratory tract that was invented in Tübingen by J. Hetzel and is essentially conducted in Tübingen and rarely performed at other places (164). Additionally, xenograft models were analyzed in this thesis, either patient-derived xenografts from biopsies or resections (PDX) or circulating tumor cell (CTC)-derived xenograft transplants (CDX) that are valuable models in SCLC (165). Especially for cancer entities where tumor tissue is hardly accessible, liquid biopsies such as plasma samples could be a potential source of tumor-associated antigens (161). Ligands of sHLA molecules from plasma samples of SCLC patients were additionally assessed throughout this thesis. Levels of sHLA were shown to be elevated in cancer patients and correlate with poor

prognosis (166-169). Immunopeptidomics of sHLA from plasma is proposed to be used as diagnostic approach and as cancer biomarker by identification of cancer associated antigens or analysis of peptide levels derived from sHLA in the plasma (159, 161).

Generally, the number of peptides that were identified in early immunopeptidomics was limited by technical sensitivity and manual spectrum annotation. Technological progress with development of Orbitrap or timsTOF mass analyzers, improved chromatographic performance but also integration and improvement of bioinformatic tools has meanwhile increased the numbers of identified peptides per sample to up to ten thousands (98, 126, 127). The annotation of MS spectra to peptide sequences is usually done by database search against a reference proteome, which is in standard conditions the reviewed human proteome, but can be extended by adding peptide sequences from NGS data, which allows for example the identification of naturally presented mutant neoantigens. However only a small number of mutated peptides is detectable with LC-MS/MS (134, 135, 137).

HLA binding prediction tools like SYFPEITHI (170) or netMHCpan-4.1 for HLA class I and netMHCIIpan-4.0 (171) for HLA class II are helpful tools to identify HLA-restricted T cell targets. HLA class II binder prediction is improving but remains challenging due to promiscuous binding of a single aa sequence to several different HLA types and highly variable peptide lengths (172, 173). HLA class II binding prediction was not performed within this thesis. However, binding prediction of peptides identified by immunopeptidomics allows exclusion of contaminants or peptides that derive from potentially unspecific eluted HLA molecules by using pan-HLA antibodies.

Besides being presented by HLA, a T cell target needs to be immunogenic (174). The immunogenicity of a peptide can be tested by *in vitro* priming of naïve T cells from healthy donors with autologous monocyte derived DCs or artificial APCs (aAPC) (175) or by *ex vivo* detection or *in vitro* stimulation of memory T cells from autologous or HLA-matched cancer patients using synthetic peptides. As a readout, different T cell assays can be used, such as enzyme linked immune spot (ELISpot), peptide-HLA multimer staining or intracellular marker staining (ICS), more detailed discussed in the following section.

2.5 Methods for identification of antigen-specific T cells

For the success of T cell-based immunotherapies, it is of high importance to first identify immunogenic antigens and later also to monitor e.g. vaccine-induced antigen-specific T cell responses in the patient. Either whole blood, but mainly isolated peripheral blood mononuclear cells (PBMCs) with the possibility of cryopreservation, are used for assessment of T cell responses. PBMCs include lymphocytes with B cells, T cells and NK cells, as well as monocytes and DCs, with T cells making up to 85 % of the PBMCs (176). Different activation markers can be employed for analysis of activated T cells after *in vitro*

contact with the antigenic peptides, including cytokines, surface markers or cytotoxic degranulation markers. Cytokines that are produced and secreted by T cells upon TCR-mediated antigen recognition include IFN- γ , TNF or IL-2. Surface markers such as CD154 (CD40L) (177, 178), CD137 (4-1BB) (179) or CD69 (180) and the cytotoxic degranulation marker CD107a (LAMP-1) (181) are upregulated rapidly upon activation and also frequently used for detection of antigen-specific T cells. Especially in the vaccination context against various pathogens, multifunctional T cells producing different cytokines at the same time were shown to be favorable for protection (182).

High efforts are made to achieve harmonization and drive reliability and inter-laboratory comparability of T cell assays, not only in the context of cancer (183-186). But harmonization and validation of T cell assays especially in the context of clinical trials is indispensable (187), which led to the conduction of proficiency panels by consortia like the European Association for Cancer Immunotherapy/ Cancer immunoguiding program (CIMT/ CIP) and the US Cancer Immunotherapy Consortium of the Cancer Research Institute (CIC of CRI) (188, 189).

There are sophisticated methods available to characterize T cells such as multiparametric assessment using CyTOF (190, 191) or TCR sequencing (192), however they are expensive, require specific devices and knowhow and are therefore not widely applicable for routinely use. Methods for detection of activated functional antigen-specific T cells should be simple, reliable, accurate, specific, sensitive, scalable and robust high-throughput immune assays with a fast read out. Fundamental methods that are routinely used include ELISpot and flow cytometry-based methods (193). ELISpot is a highly sensitive, robust, high-throughput assay for detection of functional cytokine secreting T cells on single cell level (194), however it is not possible to directly differentiate between the different cell types that potentially secreted the cytokines. The most simple assay is based on the enzymatic detection of one cytokine (generally IFN- γ for CD4⁺ and CD8⁺ T cells), whereas multicolor fluorescent-based ELISpot assays are able to identify multifunctional T cells and require less cells and antigens for more information (194). Flow cytometry-based assessment of T cells comprises different methods like HLA-peptide multimer staining, ICS or surface staining of activation-induced markers (AIM). Flow cytometry allows single cell analysis and the differentiation of individual immune cell subsets but requires higher amounts of cells than ELISpot. Peptide-HLA multimer staining is highly specific with a low detection limit and used to identify antigen-specific T cells based on their TCR specificity but gives no information on the functionality of the T cells. Additionally, it is mainly restricted to detection of CD8⁺ T cells due to challenging production of HLA class II multimers (193). ICS identifies functional T cells based on the intracellular detection of

cytokines such as IFN- γ , TNF or IL-2 or markers like CD154, however it does not maintain cell viability and requires the usage of protein transport inhibitors and permeabilization reagents (193). AIM assays are based on the staining of solely surface markers that do not require the addition of cell manipulating reagents which makes them easy applicable for identification of antigen-specific T cells with the benefit of preserving cell viability (195). Very recently, our research group and colleagues introduced activated β_2 -integrins as activation markers that can be exploited by flow cytometry to detect antigen-specific T cells *in vitro* (193, 196). β_2 -integrins such as LFA-1 are adhesion molecules that are expressed on circulating T cells in an inactive conformation. Upon TCR-mediated antigen recognition, β_2 -integrins change to an activated open high affinity form increasing their affinity and avidity (197). Activated β_2 -integrins can be detected by fluorescent multimers of their ligand ICAM-1 or conformational specific antibodies, like the antibody clone m24. The published method presents a fast read-out of antigen-specific CD8⁺ T cells after minutes of antigen stimulation using a single reagent, namely multimeric ICAM-1 (mICAM-1) to detect activated β_2 -integrins. The method maintained cell viability and allowed sorting of antigen-specific T cells, with the drawback of being limited to CD8⁺ T cells (196).

All the afore mentioned methods come with advantages and limitations (193). Further improvement, optimization, and development of novel T cell assays is a matter of research in the field. The optimization and further development of the detection of activated β_2 -integrins for identification of antigen-specific T cells is presented in results part II and III of this thesis.

3 Aim of the study

Immunotherapy harnesses the patient's own immune system to fight cancer and many studies have shown improvement of clinical outcomes in patients with various cancer types. Both CD4⁺ and CD8⁺ effector T cells are key players in killing malignant cells. T cell-based immunotherapies such as peptide-based vaccines specifically activating or boosting antigen-specific T cell responses harbor great potential to fight cancer. Therefore, identifying the tumor-derived target antigens that can be efficiently and specifically recognized by T cells is crucial for the development of such T cell-based immunotherapies.

Small cell lung cancer (SCLC) is the most aggressive subtype of lung cancer with early metastatic spread and a poor prognosis. (198). It is initially sensitive to chemotherapy, but most patients acquire resistance and relapse, leaving the patient with limited treatment options (199). Apart from the introduction of anti-PD-L1 immune checkpoint inhibitors, treatment options have poorly progressed in the last decades. In addition, many patients do not benefit from immune checkpoint inhibition (200). Therefore, novel clinically effective treatment options are needed. Specifically for T cell-based immunotherapies, SCLC associated T cell targets are hardly known so far and a broad knowledge of the antigenic landscape of SCLC is lacking. The first aim of this work was to characterize the antigenic landscape of SCLC and identify SCLC-associated T cell targets by assessment of naturally presented HLA-ligands using mass spectrometry-based immunopeptidomics. Comparative analysis with a huge dataset of HLA-ligands presented on healthy tissues was used to identify tumor-exclusive, also referred to as tumor-specific, peptides. Such tumor-specific peptides represent potential T cell antigens in SCLC and could be used for adoptive T cell therapy or peptide-based vaccination.

Identification of immunogenic peptides and monitoring of antigen-specific T cell responses in patients, e.g. after peptide vaccination requires specific, sensitive, and robust T cell assays. A T cell assay that is based on the detection of activated β_2 -integrins was previously published by our group and colleagues for sensitive assessment of antigen-specific CD8⁺ T cells. The second aim of this work was to optimize and further develop this assay for detection of both antigen-specific CD4⁺ and CD8⁺ T cells and to simplify the procedure so that it can be used more high throughput.

4 References

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5 Results

The results section of this thesis is separated into three parts.

Part I **“Identification of potential T cell targets for small cell lung cancer by mass spectrometry-based immunopeptidomics”** focuses on the identification of tumor-specific antigens in small cell lung cancer (SCLC) with the intended use for peptide vaccination or other T cell-based immunotherapies. Different tumor samples were analyzed: SCLC tissue samples from xenograft models and cryorecanalizations or biopsies and plasma samples obtained from SCLC patients. T cell responses were not assessed in the scope of this thesis, but PBMC samples of SCLC patients have been collected to this aim. The study was performed within the SFB1399 “Mechanism of drug sensitivity and resistance in SCLC”.

Not published

Part II **“Integrin activation enables sensitive detection of functional CD4⁺ and CD8⁺ T cells: application to characterize SARS-CoV-2 immunity”** and part III **“Staining of activated β_2 -integrins in combination with CD137 and CD154 for sensitive identification of functional antigen-specific CD4⁺ and CD8⁺ T cells”** present work on flow cytometry-based T cell assays for identification of functional CD8⁺ and CD4⁺ T cells by staining activated β_2 -integrins. These methods are based on a previously published work of S. Dimitrov, C. Gouttefangeas et al. “Activated integrins identify functional antigen-specific CD8⁺ T cells within minutes after antigen stimulation” published in Proceedings of the National Academy of Sciences (PNAS) in 2018.

Published articles

5.1 Part I:

Identification of potential T cell targets for small cell lung cancer by mass spectrometry-based immunopeptidomics

This study was performed in the research group of Cécile Gouttefangeas in the context of the SFB1399 “Mechanism of drug sensitivity and resistance in small cell lung cancer” in project C03 “Immunopeptidomics of small cell lung carcinoma” led and supervised by Cécile Gouttefangeas and Hans-Georg Rammensee.

Listed are all collaborations of this study:

Scientist	Affiliation	Contribution
Julie George	SFB1399, C01/B01, Department of Translational Genomics, University of Cologne	• Generation of xenograft models
Roman Thomas		• Sampling of biopsy and xenograft models
Lucia Torres Fernandez		• Contact person in Cologne • Data analysis of WES data and generation of mutated protein sequences/ FASTA inputs • 2D culture of xenograft-derived patient cells
Thorben Groß	Department of Medical Oncology and Pneumology, University Hospital Tübingen	• Sampling of cryorecanalizations and biopsy
Jürgen Hetzel		• Blood sampling of SCLC patients • Sampling of cryorecanalizations
Ana Marcu	Group of Prof. Stefan Stevanović, Department of Immunology University of Tübingen	• Immunopurification and mass spectrometry measurement of xenograft samples from 2017/2018 presented in Fig4A + biopsy S02243 • Teaching in immunopeptidomics • Mass spectrometry expert; discussion and support
Jens Bauer	Group of Prof. Juliane Walz, Department of Peptide-based Immunotherapy, University of Tübingen	• Mass spectrometry experts; discussion and support • Responsible persons/ maintenance of mass spectrometry devices
Marcel Wacker		
Naomi Hoenisch-Gravel		
Reinhild Klein and Team	Department of Hematology, Oncology, Clinical Immunology and Rheumatology, University Hospital Tübingen	• HLA typing

5.1.1 Abstract

Small cell lung cancer (SCLC) is the most aggressive subtype of lung cancer with early metastatic spread and a poor prognosis. It is initially sensitive to chemotherapy, but most patients relapse and acquire resistance to therapy, leaving the patient with limited treatment options. Apart from the introduction of anti-PD-L1 immune checkpoint inhibitors treatment options have not changed in the past. Despite a high mutational burden in SCLC, many SCLC patients fail to benefit from immune checkpoint inhibition. Therefore, new immunotherapeutic approaches, like peptide-based vaccination or cellular therapy, are needed. Since SCLC-associated T cell antigens are largely unknown, we aimed to analyze naturally presented HLA-ligands by extensive mass spectrometry-based immunopeptidomics of SCLC tumor tissue samples that were obtained from fresh/frozen patient material (cryorecanalizations or biopsies) and mouse xenograft models. HLA class I ligands were analyzed for both sample types and HLA class II peptides for cryorecanalization/biopsy samples only. We optimized the peptide identification and selection strategy for xenograft models to improve peptide recovery from xenografts and to exclude mouse-derived peptides. In total, we extracted >76,000 HLA class I ligands and >36,000 HLA class II peptides from 27 tumor samples. By comparative analysis with datasets of HLA class I and II peptides that were found to be presented on benign tissues, we identified 8,231 and 5,649 tumor-exclusive HLA class I and II peptides, respectively, that are also referred to as SCLC- or tumor-specific due to the absence on healthy tissues. These peptides were found to be shared by different tumor samples and on other malignancies and originated from cancer- or SCLC-associated proteins, such as MAGEA3, MAGEA6, TP53, ASCL1, CHGA or DLL3, among other proteins. Interestingly, one mutated HLA-ligand was also identified. Additional immunopeptidomics analysis of soluble HLA from plasma samples of SCLC patients identified patient-exclusive peptides with limited overlap to tumor samples. HLA class I ligands that were most frequently shared between SCLC samples and presented on common HLA class I types, as well as patient individual interesting ligands, have been selected to be tested for T cell recognition. Immunogenicity experiments with PBMCs of healthy donors (T cell priming) or PBMCs from SCLC patients (T cell recall), that were collected throughout this thesis, will further characterize SCLC-associated T cell antigens that could be useful for immunotherapeutic approaches to combat SCLC.

5.1.2 Introduction

5.1.2.1 Small cell lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide and ranked 6th among the leading causes of death, with smoking being responsible for approximately 85 % of the cases (1, 2). Lung cancer statistics in Germany in 2020 reported 56,690 new cases, counting for 11.5 % of all new cancer cases, and 44,817 deaths counting for 19.5 % of all cancer deaths (3). Lung cancer can be classified in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), accounting for approximately 85 % and 15 % of all lung cancer cases, respectively (4). SCLC and some types of NSCLC, such as large cell neuroendocrine carcinoma (LCNEC), are classified by “the 2015 world health organization (WHO) classification of tumors of the lung” into one group of neuroendocrine tumors, with SCLC being the most common neuroendocrine subtype (5). SCLC is a highly aggressive cancer with a poorer prognosis compared to NSCLC and a 5-year survival rate of 7.7 % for 2009-2013 in Germany (6) and 8.2 % for 2013-2019 in the US (7). The poor prognosis together with a high proliferation rate and early metastatic spread are characteristics of SCLC (8). SCLC is described to be of epithelial neuroendocrine (NE) origin and characterized by the expression of NE markers, such as chromogranin A (CHGA), synaptophysin, neural cell adhesion molecule 1 (NCAM or CD56), or insulinoma-associated protein 1 (INSM1), except for approximately 20 % of cases showing low or absent NE markers (9). Non-NE SCLC cells were found to support growth and survival of NE SCLC (10) and there is evidence from mouse studies for alternative lung epithelial cells of origin, that are not well characterized yet (11, 12). Different subtypes of SCLC are described (see section 5.1.2.1.2) and if they derive from different cells of origin is a matter of research in the field. Circulating tumor cells (CTCs) are highly abundant in SCLC patients and can be used as a prognostic factor (13), however this is rarely exploited in clinical practice (8). As for all lung cancers, smoking is the main risk factor in SCLC, only 2 % of SCLC cases appear in patients who never smoked (14). The universal loss of the tumor suppressors *TP53* and *RB1* is described as a hallmark in SCLC, leading to high genomic instability resulting in a high tumor mutational burden (TMB) (8, 15, 16).

Alternative staging systems are used in SCLC, either based on the Veterans Administration Lung Cancer Study Group (VALSG) classifying SCLC into limited stage (LS) and extensive stage (ES) disease, where limited disease is confined to one hemithorax and can be treated with a single radiotherapy portal, or based on the tumor-node-metastasis (TNM) staging system (8). With help of the TNM classification tumors are grouped into stages according to Union for International Cancer Control (UICC) criteria (17-19), presented in Table 1.

5.1.2.1.1 Therapy of SCLC

Most patients diagnosed with SCLC have symptoms at first presentation and approximately 70 % have metastatic stage IV ES-SCLC disease at diagnosis with the intension of palliative treatment (8, 20). Treatment of SCLC is based on the tumor staging and first line therapy options for the different stages are indicated in Table 1 (8, 20).

Table 1: Tumor staging and TNM classification with corresponding therapy approaches (8, 19, 20)

Staging			TNM T N M			Therapy approach		
							First line treatment	
Stage 0			Tis	N0	M0			
Stage I	IA1	Very limited	T1a	N0	M0	curative intention	Surgery (if amenable) + Adjuvant chemotherapy (Cisplatin and Etoposide) + if necessary: PCI	No surgery amenable Chemoradiotherapy: platinum-based and Etoposide + irradiation + PCI at remission (CR/PR)
			T1a	N0	M0			
	IA2		T1b	N0	M0			
	IA3		T1c	N0	M0			
IB	T2a		N0	M0				
Stage II	IIA	T2b	N0	M0				
	IIB	T1a-c	N1	M0				
Stage III	IIIA	T2a-b	N1	M0				
		T3	N0	M0				
		T1a-c	N2	M0				
		T2a-b	N2	M0				
		T3	N1	M0				
		T4	N0	M0				
	IIIB	T4	N1	M0				
		T1a-c	N3	M0				
		T2a-b	N3	M0				
		T3	N2	M0				
IIIC	T4	N2	M0					
	T3	N3	M0					
		T4	N3	M0				
	Stage IV	IVA	Extensive	any T	any N	M1a-b	palliative intention	Chemoimmunotherapy platinum-based and Etoposide and anti-PD-L1 (Atezolizumab or Durvalumab) + maintainance of immunotherapy (anti-PD-L1) + (P)CI (MRI controlled: CI at proof of brain metastasis, without MRI control: PCI at remission (CR/PR))
IVB		any T		any N	M1c			

T, tumor; N, node, M, metastasis; PCI, prophylactic cranial irradiation; CI, cranial irradiation; MRI, magnetic resonance imaging; CR, complete remission; PR, partial remission.

For many years, there were no substantial improvements for the treatment of SCLC with the standard of care treatment based on chemoradiotherapy combining radiotherapy and the usage of platinum-based drugs together with etoposide which is still the case for LS-SCLC (21, 22). However, for ES-SCLC, treatment changed with the FDA approval of anti-PD-L1 checkpoint inhibitors atezolizumab and durvalumab in combination with etoposide and platinum-based therapy in 2019 and 2020, respectively (23-25), also authorized by the EMA

(26, 27). Authorization followed the results of the clinical trials IMPower133 (28, 29) and CASPIAN (30), that improved median overall survival about 2 months (approx. from 10 to 12 months) and increased the percentage of patients that were still alive after 18 months by 10 %. The afore mentioned immunochemotherapy treatments led to significant but moderate increase in overall survival that is limited to a subset of patients, however a predictive biomarker is lacking (25, 31). In general, SCLC is initially sensitive to chemotherapy and immunotherapy with an response rate of 60-70 %, but in most cases relapses occur within several months with subsequent resistance to therapy (32). Other immunotherapies that are currently tested for SCLC will be discussed in section 5.1.2.1.5.

Approved second line therapy of recurrent metastatic SCLC is based on topotecan treatment, however other cytotoxic agents are available as options in treatment guidelines, such as irinotecan or paclitaxel, and retreatment with first line therapy is also considered (8, 20). The chemotherapeutic drug lurbinectedin, a synthetic alkaloid analogue that inhibits transcription by covalent binding to DNA, received accelerated FDA approval as second line treatment in metastatic SCLC (33), besides in Switzerland (October 2023) there is no approval for lurbinectedin in Europe (34).

Due to the aggressive properties of SCLC with the fact that most patients are not diagnosed in early phases of the disease, surgery of SCLC is quite rarely performed. However, retrospective analyses show survival benefits in early but also stage III SCLC patients (35, 36).

5.1.2.1.2 SCLC subtypes and inter- and intratumoral heterogeneity

SCLC is treated as one homogenous disease, however there is growing evidence for inter- and intra-tumoral heterogeneity. The definition of SCLC subtypes changed with time from classic/variant (37, 38) to neuroendocrine (NE)/ non-neuroendocrine (non-NE) (39) to transcription factor-based subsets (12, 40). The most prominent differentially expressed NE transcription factors that were first described to play crucial roles in SCLC were ASCL1 (Achaete-scute homolog 1) and NEUROD1 (Neurogenic differentiation 1), which reveal heterogeneity in SCLC and regulate distinct genes that contribute to neuronal function (41). POU2F3 (POU domain, class 2, transcription factor 3) was later identified as an additional third transcription factor defining a non-NE tuft cell-like variant of SCLC (42).

Transcriptomic studies in primary human tumors, patient-derived xenograft models, cell lines and mouse models, defined four different subtypes of SCLC by the differential expression of the transcription factors ASCL1, NEUROD1, YAP1 (Yes-associated protein 1) and POU2F3, resulting in the proposed nomenclature of SCLC-A, SCLC-N, SCLC-Y and SCLC-P, respectively (12). The different subtypes can be separated in neuroendocrine SCLC with high expression of ASCL1 (classic SCLC) or NEUROD1 (variant SCLC) and non-

neuroendocrine SCLC with low levels of ASCL1 and NEUROD1, but high levels of YAP1 or POU2F3 (12). Following these findings, a study of immunohistochemistry staining in SCLC patient samples revealed that ASCL1 and NEUROD1 can be expressed by the same tumor, however 69 % of cases were ASCL1-dominant, 17 % were NEUROD1-dominant, 14 % lack both markers of which 7 % expressed POU2F3 (43). In this studies, YAP1 was not shown to define a distinct subtype of SCLC but was found to be associated with low expression of neuroendocrine markers and elevated in ASCL1/NEUROD1 double-negative samples (43). SCLC-A is suggested to be separated into SCLC-A and SCLC-A2 by expression of different factors like HES1 (44) and another rare subtype that appeared in CDX models was defined by expression of *ATOH1* and was amplified for *MYCL* (45). Analysis of previously published transcriptomics data describes, in addition to the SCLC-A, SCLC-N and SCLC-P subtypes, the SCLC-I subtype with low expression of ASCL1, NEUROD1, POU2F3 and an inflamed gene signature including gene expression of HLA, immune checkpoint molecules or CD8 (40). Dependent on the analyzed dataset different subtype distributions were observed, but SCLC-A and SCLC-N represent the most frequent subtypes. These studies could not confirm that YAP1 exclusively defines a subgroup of SCLC, but is rather expressed by SCLC-P and SCLC-I (40).

The amplification of transcription factors MYC, MYCL and MYCN was described in different subsets of SCLC (8) and seems to play a role in the behavior of SCLC (45-48). *MYCL* and *MYC* were reported to be more amplified in SCLC-A and SCLC-N, respectively (41). The non-NE subtypes were described to express *REST*, to present activated Notch, Hippo and TGF- β pathways, as well as *MYC* amplification, and to undergo epithelial mesenchymal transition (EMT) (39). *MYCN* expression was found to be more associated with NEUROD1 in CDX models (45). The different subtypes and their characteristic features are presented in Figure 1.

loss of tumor suppressors RB1 and TP53					
neuroendocrine			non-neuroendocrine		
SCLC-A2	SCLC-A	SCLC-N	SCLC-P	SCLC-Y?	SCLC-I?
ASCL1		NEUROD1	POU2F3	YAP1	inflamed gene signature
MYCL		MYC/MYCN	MYC		
Notch mutant			NOTCH signaling		
HES1			REST		
"cold"			"hot"		
low immune gene signature			higher immune gene signature		

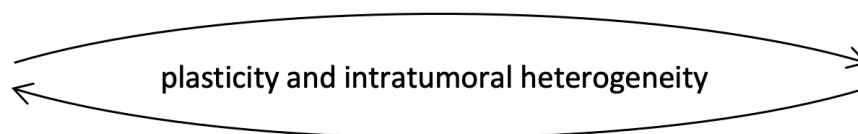


Figure 1. Proposed nomenclatures of SCLC subtypes with characteristic features. Loss of RB1 and TP53 is a common feature of SCLC. SCLC subtypes can be divided in neuroendocrine SCLC-A2, SCLC-A and SCLC-N expressing transcription factors (TF) ASCL1 and NEUROD1 and non-neuroendocrine SCLC-P, SCLC-Y and SCLC-I defined by TF POU2F3 and YAP1 or an inflamed gene signature. The definition of subtypes SCLC-Y and SCLC-I are still a matter of debate and marked by the question mark. The subtypes are further characterized by other features, like expression of MYC family members, Notch signaling, or expression of REST and HES1. The immune gene signature is described to be low in neuroendocrine SCLC and higher in non-neuroendocrine SCLC. SCLC exhibits plasticity and intra-tumoral heterogeneity that is still a subject of research.

Not only inter-, but also intra-tumoral heterogeneity is notable in SCLC, and different transcriptional subtypes of SCLC may be present within an individual tumor (43, 45, 48, 49). Studies of Myc overexpressing mouse models and transcriptomic data of human SCLC identified MYC as a driver for the transition from a highly neuroendocrine ASCL1 subtype to a low-neuroendocrine NEUROD1 subtype to a non-neuroendocrine YAP1-positive subtype by activating Notch signaling (46, 48). The mechanisms leading to SCLC plasticity are complex and several signaling pathways are implicated, including CREBBP/EP300, RAS/MEK/ERK pathway, MYC and NOTCH/REST signaling among others (9).

Aspects of this heterogeneity are associated with tumor evolution, metastasis and acquired resistance to therapy (8). Therapy vulnerabilities were identified for each of the different subtypes.

5.1.2.1.3 Additional molecular features and vulnerabilities of SCLC

As already mentioned, loss of the tumor suppressors *TP53* and *RB1* is as a hallmark of SCLC that leads to high genomic instability resulting in a high TMB (8, 15, 40). MYC, MYCL and MYCN were found to be amplified in SCLC. In addition, amplification of the transcription factor SOX2 (SRY-Box transcription factor 2) drives SCLC and promotes the neuroendocrine ASCL1-high SCLC subtype (50, 51). Extensive genomic and transcriptomic

profiling of SCLC primary tumors, as well as patient-derived and CTC-derived xenograft models confirmed these findings and identified additional alterations to be common in SCLC (15, 16, 51, 52). These include inactivating mutations or deletions, activating mutations, amplification or overexpression of multiple genes involved in cell cycle and apoptosis, epigenetic regulation, kinase signaling among others, such as *TP53*, *RB1*, *CREBBP*, *EP300*, *PTEN*, *PIK3CA* and *NOTCH1* (listed in Supplementary Table 1).

MYC, MYCL and MYCN represent potential therapeutic targets in lung cancer and different promising inhibitory strategies are followed (53). SCLC with high MYC expression was found to be vulnerable to aurora kinase and Checkpoint kinase 1 (CHK1) inhibition in mice (46, 47). Different aurora kinase and CHK1 inhibitors are tested in clinical trials in SCLC. The aurora kinase inhibitor alisertib however showed modest efficacy in a phase II trial in combination with paclitaxel in SCLC patients, but indicated increased efficacy for patients with MYC expression (54). Recently, it was reported in xenograft models that aurora kinase inhibition leads to a more inflamed SCLC subtype that is targetable with PD-L1 checkpoint inhibitors and led to durable response and improved overall survival (55). Several different molecular and cellular pathways are affected in SCLC and therapeutic targets include cell cycle and DNA damage repair, apoptosis pathways, neuroendocrine signaling, transcription inhibition, tumor development, epigenetic regulation, tumor immunity and angiogenesis with novel targets such as Poly [ADP-ribose] polymerase 1 (PARP1), B-cell lymphoma 2 (BCL-2), WEE1, Enhancer of zeste homolog 2 (EZH2), and Delta-like 3 (DLL3), among others (56, 57).

In vitro drug response studies of SCLC cell lines assessed subtype specific vulnerabilities and identified SCLC-P to be most sensitive and SCLC-N and SCLC-I to be resistant to cisplatin chemotherapy, whereas SCLC-A presented a range of sensitivities (40). Patient derived xenograft models of the SCLC-A subtype that were treated with cisplatin shifted towards a SCLC-I subtype, representing a potential mechanism of acquired resistance in SCLC (40). In addition, SCLC-P cell lines were found to be sensitive to PARP inhibition, SCLC-A models most sensitive to BCL-2 inhibitors, SCLC-N and SCLC-P sensitive to aurora kinase inhibitors and SCLC-I most sensitive to Bruton's tyrosine kinase (BTK) inhibitors (40).

ASCL1, one of the transcription factors associated with SCLC regulates different genes in the NOTCH pathway, including DLL3 (41) which is involved in the regulation of proliferation, migration and invasion of SCLC and represents a potential therapeutic target (58), exploited in several clinical trials in SCLC (see 5.1.2.1.5 *Immunotherapy of SCLC*). Another potential target is ganglioside GD2, for which the expression was shown to be high in SCLC cell lines and inhibition of GD2 led to decreased proliferation of cells and the induction of apoptosis (59).

Novel vulnerabilities in SCLC were identified when non-NE SCLC was shown to be sensitive to ferroptosis induction while NE ASCL1-high SCLC was ferroptosis resistant and instead dependent on the thioredoxin pathway (60, 61). However, targeting of both ferroptosis and thioredoxin pathways achieved anti-tumor activity in heterogenous SCLC xenografts, genetically engineered mouse models of SCLC and patient-derived cells (60).

Recently, neuronal pathways were reported to be associated with SCLC and SCLC cell line co-cultures with neurons presented increased proliferation (62, 63). SCLC cells were shown to form synapses with glutamatergic neurons *in vitro* and *in vivo* in mouse models and drugs interfering with glutamatergic signaling slowed tumor growth in mice (62).

Supplementary Table 1 list genes or proteins that have been associated with SCLC based on a literature search without guarantee of completeness. This list of genes was used for selection of potentially interesting SCLC T cell targets presented in this thesis.

5.1.2.1.4 Immune evasion in SCLC

Early studies in SCLC cancer cell lines described a deficient expression of HLA class I and defects in antigen processing (64, 65), as well as the lack of β_2 -microglobulin expression in SCLC tumor tissue (66). IFN- γ gene transfer in HLA deficient SCLC cell lines was however shown to restore the expression of cell surface HLA class I, which led to presentation of tumor antigens (67). More recently, HLA class II molecules were reported to be expressed on NSCLC, but not detectable on SCLC (68), however in other studies a subset of SCLC patients expressed HLA class II on tumor cells and TILs (69, 70). High HLA class I and class II expression was observed in the so called SCLC-I subtype with immune inflamed phenotype which was associated with increased benefit from checkpoint inhibition (40). In line with this observations, expression of HLA class I was shown to be increased in patients with non-NE SCLC that were particularly better responding to immune checkpoint inhibitors, whereas HLA class I expression was suppressed by epigenetic silencing of TAP1 in neuroendocrine SCLC (71). EZH2 inhibition in NE SCLC cell lines promotes a non-NE cell state with recovered MHC class I expression and an enhanced T cell recognition and tumor rejection was observed by treating mice with EZH2 inhibitors followed by stimulator of interferon genes (STING) agonists (71). A role of EZH2 in regulating MHC class I expression in SCLC and other cancer types was already reported in human and mice (72, 73).

B7-H3, an immune checkpoint from the same family as PD-L1, was shown to be highly expressed in SCLC (74), and was reported to be an attractive target for antibody based therapy in other malignancies, such as NSCLC (75). Clinical trials exploiting B7-H3 are ongoing (see 5.1.2.1.5 *Immunotherapy of SCLC*)

In addition to tumor intrinsic mechanisms, the TME plays a role in evading an immune attack. APCs were found to be suppressed by neuropeptides secreted by SCLC (76).

Secretion of IL-15 is implicated in the induction of CD4⁺ Tregs in SCLC and the presence of Tregs in SCLC biopsies negatively correlates with patient survival (77). TAMs were reported to be the most abundant cell type in the TME of limited stage SCLC patients and differences in the TME between NE-high vs NE-low tumors were observed. NE low tumors are associated with increased numbers of M2 macrophages and the expression of potentially immunosuppressive molecules like ANXA1, CXCR2, CD70 and ITGB6 on tumor cells (78). Furthermore, the accumulation of intratumoral M2 macrophages in SCLC biopsies was found to be associated with an unfavorable prognosis of patients (79).

5.1.2.1.5 Immunotherapy of SCLC

SCLC is listed among the cancer types with highest number of somatic mutations (80, 81). A high TMB leading to enhanced presentation of mutated neoantigens of tumor cells to T cells could be exploited by immunotherapy (82). T cell infiltration, especially CD8⁺ T cell infiltration, was found to be quite low in SCLC (74), however patients with higher numbers of tumor infiltrating immune cells were shown to have a more favorable outcome and presented higher overall survival (83-85). With the introduction of anti-PD-L1 immune checkpoint inhibitors (ICIs) atezolizumab and durvalumab in combination with established chemotherapy as first line standard therapy for ES-SCLC, immunotherapeutic approaches moved into the spotlight of SCLC therapy. However, these remain the only FDA and EMA licensed immunotherapies in SCLC. Of note, neither PD-L1 expression nor a high TMB in tumors of SCLC patients was consistently shown to be predictive for therapy response to anti-PD-L1 ICIs and still only a limited number of patients is benefitting from treatment with response rates at 60-65 % with or without checkpoint inhibitors (31). Ongoing clinical trials and their NCT numbers, which are discussed in the following paragraphs, were obtained from <https://clinicaltrials.gov>.

So far, the clinical results of other ICIs are disappointing in SCLC. The anti-CTLA-4 antibody ipilimumab vs placebo, both combined to etoposide plus either cisplatin or carboplatin, in patients with ES-SCLC could not show significantly improved overall survival or differences in the response rates (86). Regarding blockade of the PD-1 receptor, first positive results in early phase studies in ES-SCLC led to the accelerated FDA approval of nivolumab (CheckMate 032 (87)) and pembrolizumab (KEYNOTE-028 and -158 (88)), however follow up clinical phase III studies could not meet their primary endpoints and overall survival was not significantly increased (CheckMate 451 (89, 90), CheckMate 331 (91, 92) KEYNOTE-604 (93)), resulting in the voluntary withdrawal of the accelerated FDA approval for SCLC in 2021 (94). A phase III trial using anti-TIGIT tiragolumab combined to atezolizumab, carboplatin and etoposide in ES-SCLC did also not meet the expected response rates (95). Other anti-TIGIT antibodies are further investigated in different clinical

trials in SCLC (NCT04952597 NCT05224141, NCT04672356). Combination therapy of different checkpoint inhibitors and chemotherapy with other targeted therapies is also investigated. Some examples include the combination of nivolumab, ipilimumab and plinabulin, a small molecule inhibiting the polymerization of tubulin, in recurrent SCLC (Phase I/II, NCT03575793), and, as first line treatment in ES-SCLC, the combination of pembrolizumab, lenvatinib, a tyrosine kinase inhibitor, and chemotherapy (phase II, NCT05384015) or the combination of monalizumab (anti-NKG2A) with durvalumab, etoposide and platinum-based chemotherapy (Phase II, NCT05903092). NKG2A is an immune checkpoint that is expressed by CD8⁺ T cells and NK cells (96) and NK cells are also implicated in the control of SCLC development (9). Most studies focus on ES-SCLC, but a clinical phase III study investigates the combination of chemoradiation therapy with pembrolizumab followed by pembrolizumab and olaparib, a PARP inhibitor, in newly diagnosed LS-SCLC (NCT04624204).

In addition to ICI, other antibody-based immunotherapies, like T cell engaging antibodies or antibody drug conjugates, as well as cell-based immunotherapies, immunomodulators and vaccines are considered for SCLC treatment and tested in ongoing clinical trials.

T cell engaging antibodies, like tarlatamab, a BiTE construct targeting DLL3 and CD3 showed promising results in a phase II study (NCT 05060016) (97), and will be tested in a clinical phase III study in relapsed ES-SCLC (NCT05740566), but also after chemoradiotherapy in LS-SCLC patients (NCT06117774). Other antibody formats engaging T cells and targeting DLL3 are tested, such as the tri-specific T cell activating construct HPN328 (Phase I/II, NCT04471727) demonstrating first anti-tumor activity in SCLC (98), or a bispecific antibody like molecule, BI764532, which is tested in several different trials for dose escalation or combined to other therapies (NCT05963867 (Phase I), NCT05990738 (Phase I), NCT05879978 (Phase I/II), NCT06077500 (Phase I), NCT04429087 (Phase I), NCT05882058 (Phase II)).

Several antibody drug conjugates are under investigation in SCLC (99). Rovalpituzumab tesirine, a DLL-3 directed DNA cross-linking agent was not successful in clinical phase II studies in ES-SCLC (100, 101), but sacituzumab govitecan, a trophoblast cell-surface antigen 2 (Trop-2)-directed topoisomerase I inhibitor antibody drug conjugate, demonstrated promising antitumor activity as second-line treatment of ES-SCLC, according to first results from the phase II TROPiCS-03 trial (NCT03964727) (102). Different antibody drug conjugates targeting B7-H3 are also tested in ES-SCLC (Phase II, NCT05280470 and NCT06052423) or in relapsed SCLC (Phase III, NCT06203210).

Cell based approaches like CARs are also tested in phase I clinical trials, with CAR T cells targeting DLL3 (Phase I, NCT05680922), GD2 (early Phase I, NCT05620342), or other

antigens including B7-H3 (Phase I, NCT03198052), but also CAR NK cells targeting DLL3 (Phase I, NCT05507593). Immunomodulators tested in SCLC include the TLR7 agonist BNT411 that is expected to induce broad innate and adaptive immune reactions in combination with chemotherapy and checkpoint blockade (a phase I/II trial in combination with atezolizumab, carboplatin and etoposide, NCT04101357). N-803, an IL-15 agonist, is tested in patients that relapsed after checkpoint inhibition in several cancer types including SCLC (phase II, NCT03228667), and preliminary results show low toxicity and promising efficacy in stopping progression and in maintaining durable stable disease (103).

Several vaccination approaches, including dendritic cell (DC)-based vaccines or peptide vaccines, against different targets, including ganglioside GD3, fucosyl GM1, polysialic acid, and p53, were already exploited in the past in SCLC (4). Older studies of keyhole limpet hemocyanin conjugate vaccines targeting fucosyl GM1 and polysialic acid were shown to induce antibody responses in SCLC patients (104, 105). Results of a follow up clinical trial (NCT01349647) was not reported. In a clinical phase III study, vaccination with bacillus Calmette-Guerin (BCG) adjuvant and BEC-2, an anti-idiotypic antibody that mimics GD3 with the intend to induce anti GD3 antibodies, showed no improvement for SCLC patients with LS diseases after chemotherapy and chest radiation (106, 107). In ES-SCLC, a DC-based vaccine expressing p53 was shown to induce T cell responses and in combination with chemotherapy tumor regression was observed in a number of patients, however a clinical phase II study in recurrent SCLC could not improve overall response rates compared to paclitaxel (108-110). A personalized peptide vaccine was tested in a phase II clinical study in advanced SCLC. Four peptides were selected out of a pool of 31 peptides according to HLA type and pre-existing IgG responses against these peptides and administered emulsified in Montanide ISA™ 51 in different regiments. Specific IgG titers against peptides contained or not contained in the vaccine were found to be increased after vaccination (111). T cell responses were not assessed in this study, the authors argue that they have previously shown that peptide-specific IgG responses correlate with peptide-specific CD8⁺ T cell responses. A peptide cancer vaccine containing HLA-A*24:02-presented cell division cycle-associated protein 1 (CDCA1) and kinesin family member 20A (KIF20A) peptides emulsified in Montanide was tested in advanced SCLC in Japan, however no results are reported (phase I completed 2019, NCT01069653). The authors have previously reported that CDCA1 is involved in the development of NSCLC, but was also found to be expressed on SCLC (112, 113). Only two clinical trials investigating vaccination approaches in SCLC are currently recruiting patients. One is a personalized neoantigen DNA vaccine in combination with durvalumab following a combined carboplatin, etoposide and durvalumab treatment in ES-SCLC (phase II, NCT04397003), and the other one an autologous DC-based vaccine in

combination with atezolizumab as maintenance treatment in ES-SCLC (phase I/II, NCT04487756).

In summary, besides anti-PD-L1 immune checkpoint inhibitors, no other immunotherapeutic treatment found its way into the clinics so far, despite promising results in preclinical and early clinical phase studies. Still, there is lack of substantial improvement for the treatment of LS- and ES-SCLC. Giving the high anti-tumor potential of T cells, new T cell-based immunotherapies are promising that should also be considered in combination with other therapies to overcome immune evasion mechanisms or manipulating the immunosuppressive TME. Only a few T cell antigens for SCLC have been described so far, but knowledge of tumor antigens is a requirement for the development of further T cell therapies. Here, we aim to identify SCLC-associated T cell targets by assessment of natural HLA-presented peptides using mass spectrometry-based immunopeptidomics, to design T cell-based immunotherapies with a focus on peptide vaccination.

5.1.3 Materials and methods

5.1.3.1 Study subjects and patient characteristics

SCLC tumor tissue samples from xenograft models and cryorecanalization or biopsy (cohort 1), as well as blood samples from SCLC patients (cohort 2) were collected at the Department of Translational Genomics, University of Cologne (tissue samples) and at the Department of Medical Oncology and Pneumology, University Hospital Tübingen (blood and tissue samples). The study was approved by the ethics committee of the University of Cologne (project number 19-1164) and the ethics committee of the University of Tübingen (project number 526/2021BO2).

Except for one snap frozen biopsy (S02243), all SCLC tumor tissue samples collected in Cologne derived from xenograft models. SCLC xenograft models, either patient-derived xenografts from biopsies or resections (PDX) or circulating tumor cells (CTC)-derived xenograft transplants (CDX), were established at the Department of Translational Genomics and maintained at the animal facility of the pathology department in Cologne. In brief, a biopsy or resection of patient tissue is surgically implanted, or CTCs isolated by a ficoll gradient method (114) are s.c. injected, in the flanks of immunocompromised NSG mice and passaged over time. Mice develop tumors within several months, the exact time is highly variable dependent on the patient sample. Tumor samples were collected and snap frozen. One sample analyzed in this study derived from a 2D-culture that was generated from a xenograft-derived tumor (S03381_cells). The tumor was digested to single cells (Tumor Dissociation Kit, Miltenyi Biotec) and maintained at standard conditions in HITES medium (DMEM/F12 with 5 µg/ml insulin, 10 µg/ml transferrin, 10 nM β-oestradiol, 30 nM sodium selenite and 10 nM hydrocortisone) supplemented with 10 % FCS and 1 % Penicillin-Streptomycin (Pen/Strep) solution. 80 Mio cells were harvested and washed with phosphate buffered saline (PBS) before cryopreservation at -80 °C. All the afore mentioned steps were performed by our cooperation partner in Cologne.

SCLC tumor tissue samples collected in Tübingen derived from snap frozen cryorecanalization or biopsy. Cryorecanalization is a bronchoscopic therapy of stenoses in which tumor tissue gets frozen at the tip of a cryoprobe and removed from the surrounding tissue by retraction (115). In addition, EDTA-blood at different timepoints was collected from SCLC patients at the study site in Tübingen, either from patients with a collected matching tumor sample (part of cohort 1) or from patients under therapy without a matching tumor sample (cohort 2). Sample collection, preparation and analysis are illustrated in Figure 2.

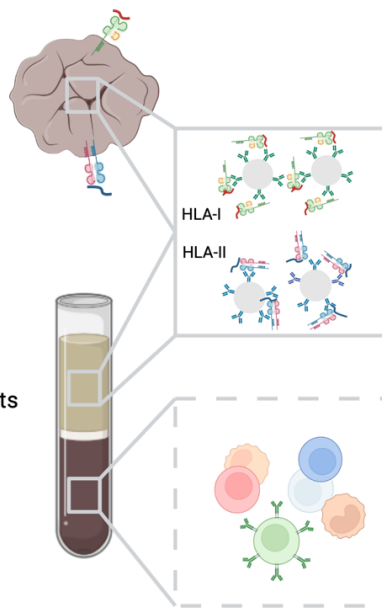
Sample collection

SCLC tumor samples

- Cryorecanalization/Biopsy
- Xenograft models

Blood samples from SCLC patients

- with matching tumor
- without matching tumor



Sample preparation and analysis

Immunopeptidomics

- HLA from SCLC tumors
- soluble HLA in plasma of SCLC patients

PBMCs of SCLC patients

- immunophenotyping
- immunogenicity testing

Figure 2: Schematic overview of SCLC patient sample collection with sample preparation and analysis. SCLC tumor samples from cryorecanalization or biopsy and from xenograft models were collected for immunopeptidomics analysis. Blood samples were collected from patients with collected matching tumor samples or from patients under therapy without matching tumor samples. Plasma and PBMCs were isolated from blood samples. Plasma was collected and frozen for immunopeptidomics analysis of soluble HLA (sHLA). PBMCs were collected and frozen for later analysis. Sample collection and immunopeptidomics analysis was performed throughout this study (solid line, black fond), analysis of PBMCs will be performed in follow up studies (dotted line, grey fond). Created with BioRender.com.

Patient characteristics are listed in Supplementary Table 2. Patient ID, sampling site, sex, age, HLA type, clinical histology, TNM classification with cancer stage (UICC), pre-treatment at sampling, smoking background, collected samples and whether immunopeptidomics was performed is indicated. In total, 27 tissue samples were collected from 24 different SCLC patients (cohort 1). Cohort 1 has a median age of 64 (range: 48 - 88) and a female to male ratio of 12:12. 15 samples derived from xenograft models (13 different patients) with a median age of 64 (range: 48-88) and a female to male ratio of 5:8. 12 samples derived from cryorecanalization or biopsy (11 different patients) with a median age of 64 (range: 57-85) and a female to male ratio of 7:4. Blood samples were taken from 10 patients with available cryorecanalization or biopsy (n=19 blood samples). Cohort 2 comprises 45 different SCLC patients for which only blood samples were collected (n=77 blood samples). The median age is 66 (range: 46-82) and the female to male ratio 22:23. The HLA class I distribution of all SCLC samples is presented in Supplementary Figure 1A. The most frequent HLA class I types are listed and the frequency within the German population is indicated in brackets (retrieved from allele frequencies.net, Germany DKMS – German donors n=3,456,066 (116)): A*02:01 (28.4 %), A*03:01 (15.1 %), B*08:01 (10.2 %), B*35:01 (5.8 %), C*04:01 (11.5 %), C*07:01 (14.7 %) and C*07:02 (13.9 %), covering 92.59 % and 89.67 % of the German and European populations, respectively.

5.1.3.2 Sample preparation

All SCLC tumor tissue samples were stored at -80 °C until HLA immunoaffinity purification was performed. EDTA-blood was handled on the day of sample collection. 200 µl full blood were frozen at -20 °C for HLA typing. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated for soluble HLA (sHLA)-based ligandomics and immunological assays, respectively.

Isolation of plasma for sHLA-based ligandomics

Plasma was separated from the cell fraction by centrifugation (10 min, 440 x g, RT, without brake). Processing of the cell fraction is described in the next paragraph. Plasma was collected and centrifuged for a second time (10 min, 380 x g, RT). 500 µl aliquots of plasma were collected for possible future analyses like cytokine quantification, remaining plasma was supplemented with protease inhibitor (stock used at 1:25, see below) and cleared by ultracentrifugation (10 min, 12,000 x g, at 4-10 °C). 100 µl aliquots of plasma were collected for possible future HLA quantification by enzyme-linked immunosorbent assay (ELISA), remaining plasma is used for HLA immunoaffinity purification. All plasma samples were stored at -80 °C until use.

One tablet of cOmplete™ Protease Inhibitor Cocktail (Roche) was resuspended in 2 ml PBS and stored at -20 °C before use.

Isolation of PBMCs

The remaining cell fraction after plasma separation was collected and diluted with PBS (w/o Ca²⁺/Mg²⁺, Gibco or in-house produced). PBMCs were isolated by ficoll cell density centrifugation (Lymphosep, Biowest or Leucosep tubes, Greiner Bio-One). After two washing steps with PBS, PBMCs were counted with an automated cell counter (NucleoCounter, Chemometec) using Solution 18 (AO/DAPI, Chemometec). Cell aliquots (8-12 Mio cells/ml) were frozen at -80 °C in 90 % heat-inactivated fetal bovine serum (FBS; Capricorn Scientific) containing 10 % DMSO (Merck) using freezing containers and stored in liquid nitrogen until use.

HLA typing

Genomic DNA was purified from 200 µl EDTA-blood or tumor lysate (left over from HLA immunoaffinity purification) using PureLink Genomic DNA Kits (Invitrogen). 4-digits HLA Typing of HLA class I (HLA-A, -B, and -C) and HLA class II (HLA-DR, HLA-DQ and HLA-DP) was performed by next generation sequencing at the HLA-laboratory of the Department of Hematology, Oncology, Clinical Immunology and Rheumatology at the University Hospital Tübingen.

Whole exome sequencing

Whole exome sequencing was performed for the majority of samples that were collected in Cologne (xenograft models and one biopsy (S02243)) at the Department of Translational Genomics, University of Cologne as previously described (15, 117). Whole exome sequencing was not performed for the samples collected in Tübingen (cryorecanalizations and one biopsy). If sequencing data was available, this is indicated in Supplementary Tables 3 and 4, and in Figure 5. Annotation of mutations to protein sequences was performed at the Department of Translational Genomics, Cologne. FASTA inputs for database search of mass spectrometry data were partially created at the Department of Translational Genomics, Cologne or by me.

5.1.3.3 Immunopectidomics workflow

HLA immunoaffinity purification

Isolation of HLA class I and HLA class II molecules from snap frozen tumor tissue, cell pellets or plasma was performed by standard immunoaffinity purification (118, 119). The pan-HLA class I-specific antibody W6/32 (120), and the pan-HLA class II-specific antibody Tü39 (121) combined to the HLA-DR-specific antibody L243 (122), all mouse IgG2 and produced in-house, were employed.

Antibodies were coupled to CNBr-activated sepharose (Sigma-Aldrich) at a ratio of 40 mg sepharose per 1 mg of antibody in 0.5 M NaCl, 0.1 M NaHCO₃, pH 8.3 at 4 °C. Remaining activated CNBr reaction sites were blocked using 0.2 M glycine. Sepharose coupled antibodies were stored in PBS at 1 mg antibody/ ml at 4 °C until use.

Tumor tissues and cell pellets were homogenized in lysis buffer containing one tablet of cOmplete™ Protease Inhibitor Cocktail (Roche) and CHAPS (Panreac AppliChem) in PBS. Frozen tissue was cut into small pieces with a scalpel, transferred into an appropriate reaction tube and mashed with a micropistille or potter device, the lysis buffer volume was adjusted according to the amount of sample used. The cell pellet was thawed, and lysis buffer was added according to the pellet volume. Samples were incubated for 1 h on a shaker at 4 °C before and after sonification (3x 20 sec at output control of 50 % and duty cycle of 5 or in an ultrasound bath, with 20 sec breaks on ice), followed by clearance using centrifugation (45 min, 4 °C, maximal speed (4,000 rpm or 13,000 rpm depending on the centrifuge used). Plasma samples were thawed at 4 °C and cleared by centrifugation (45 min, 4,000 rpm, 4 °C), followed by filtration with a 5 µm filter unit (Merck Millipore). Tissue mass, number of cells and plasma volume used for immunoaffinity purification are indicated in Supplementary Tables 3-5.

Two different columns were employed for HLA class I and HLA class II precipitation containing W6/32 or equal amounts of Tü39 and L243, respectively. 1 mg of antibody for

1 g of tissue, 1.5 mg of antibody for 100 Mio cells, or 1 mg of antibody for 10 ml of plasma, but never less than 1 mg of antibody, were used. Samples ran circularly overnight at 4 °C. Columns were washed for 30 min with PBS, followed by 1 h with ddH₂O. Elution of peptides was performed by incubation with 200 µl of 0.2 % trifluoroacetic acid (TFA) on a shaker; elution was repeated for a total of four times and fractions were pooled. Peptides were separated from the HLA molecules using Amicon Filter units (Merck Millipore) with a size of 3 kDa and 10 kDa for HLA class I and HLA class II, respectively. Amicon filter units were washed with MS grade H₂O, 0.1 N NaOH and 0.2 % TFA before sample application, followed by a recovery step with 32.5 % ACN/0.2 % TFA. All samples were concentrated by lyophilization. Concentrated peptide solutions obtained from plasma samples were additionally purified using ZipTip Pipette Tips with C18 resin (Merck Millipore), sample was desalted in 0.1 % TFA and eluted in 32.5 % ACN/0.2 % TFA. This step was skipped for SCLC tissue or the cell pellet due to small sample sizes, except if tissue was embedded in Tissue-Tek® (Sakura Finetek, USA) (sample S02730_03). After vacuum centrifugation of all samples, intended volume was adjusted with 1 % ACN/0.05 % TFA (5 µl/ replicate) and samples were stored at -80 °C until LC-MS/MS analysis.

Cryorecanalization and plasma samples were adjusted to a volume of 35 µl for MS measurement with two different devices (orbitrap-based/ timsTOF). Xenograft samples were adjusted to a volume of 25 µl for initial MS measurement with the QE. After QE measurements, samples were diluted and filled up again to a volume of 17 µl for timsTOF measurements. Detailed information on the different MS devices is given in the next paragraph.

Mass spectrometry data acquisition

Mass spectrometry analysis was performed on Orbitrap-based instruments and a trapped ion mobility separation (tims)-based time-of-flight (TOF) instrument as described before (119, 123, 124). Three technical replicates were acquired with each device in data-dependent acquisition (DDA) mode.

Orbitrap-based mass spectrometers included the Orbitrap Fusion Lumos and the Q Exactive HF (Thermo Fisher Scientific, Waltham, MA, USA), from now on called Lumos and QE. In brief, separation and elution of peptides was performed using an Ultimate 3000 RSLC nano UHPLC (Thermo Fisher Scientific) with a 50 µm x 25 cm PepMap RSLC column. A 90 min linear gradient from 3 % to 30 % (QE) or 40 % (Lumos) of solvent B (80 % ACN/ 0.15 % FA in water), followed by an increase to 95 % within 1 min for additional 5 min with a flow rate of 0.3 µl/ min was applied. Both Orbitrap devices were equipped with a Nanospray Flex ion source (Thermo Fisher Scientific). HLA class I peptides were fragmented by collision-induced dissociation (CID, Lumos) or higher-energy collisional dissociation

(HCD, QE) with a mass range of 400-650 m/z (mass to charge ratio) restricted to a charge state of 2-3. HLA class II peptides were fragmented by higher-energy collisional dissociation (HCD, Lumos and QE) with a mass range of 400-1000 m/z restricted to a charge state of 2-5. Maximum injection time for MS1 and MS2 spectra was set to 50 ms and 150 ms, respectively. The dynamic exclusion was set to 7 s for HLA class I and 10 s for HLA class II. First samples were analyzed with the Lumos, whereas the majority of samples was analyzed with the QE (see Supplementary Tables 3 and 4). Samples from xenograft models of batches 2 and 3 were analyzed with an adapted method, in which the maximum injection time for MS2 spectra was increased to 200 ms and the dynamic exclusion decreased to 1 s for HLA class I and 3 s for HLA class II.

All samples, if amenable (see Supplementary Tables 3 and 4), were additionally analyzed with the timsTOF equipped with a CaptiveSpray ion source (Bruker Daltonics). In brief, separation and elution of peptides was performed with the nanoElute LC system (Bruker Daltonics) and a 75 μ m x 25 cm Aurora Series emitter column (IonOpticks). A 60 min multi step gradient ranging from 0 % to 95 % Solvent B (ACN with 0.01 % FA in water) with a flow rate of 0.3 μ l/min was applied. HLA class I and HLA class II peptides were fragmented by CID employing parallel accumulation-serial fragmentation (PASEF) with a mass range of 600-2000 m/z for a charge state of 1 and 100-2000 m/z for a charge state of >2.

Database search

Data was processed with PEAKS Studio 10.6 (Bioinformatic Solutions Inc.) and searched against a reference database depending on the sample type. For cryorecanalization, biopsy and plasma samples, the reviewed human Swiss-Prot database downloaded on 14.10.2020 containing 20,383 protein entries was used. For samples from xenograft models, a combined reference database of the before mentioned human database and the reviewed mouse Swiss-Prot database downloaded on 03.02.2021 containing 17,492 protein entries was used. If available, corresponding mutated protein sequences were added to the reference database.

Database search was performed without enzyme specificity and with methionine oxidation as variable modification and three possible modifications allowed per peptide. Precursor peptide mass error tolerance was set to 5 ppm or 20 ppm for Orbitrap or timsTOF data, respectively, and 0.02 Da for fragment ions. The false discovery rate (FDR) was set to 1 % based on the calculation of a decoy database search. Peptides were filtered for a length of 8 to 14 amino acids (aa) for HLA class I and 12 to 30 aa for HLA class II.

Data presented in Figure 4A represents an exception and was processed with Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific, USA) using the SEQUEST HT search engine (125) and the reviewed human Swiss-Prot database downloaded on 05.12.2018

including 20,408 protein entries. This figure is the result of an initial analysis of 2020 using the database search just described, which was not used for the final data analysis.

HLA binding prediction

Peptides extracted from HLA class I molecules were additionally annotated to be ligands using binder prediction tools SYFPEITHI 1.0 (126) and netMHCpan4.1 (127). All peptides with a SYFPEITHI score ≥ 60 or a netMHC binding rank < 2 , were considered as ligands. NetMHC differentiates between strong < 0.1 and weak < 2 binders. Only HLA class I ligands were further analyzed. For HLA class I, the purity (ligands/ peptides*100) was calculated, indicated in Supplementary Tables 3-5. To the timepoint of data analysis, results from HLA typing of HLA class II were not yet available. All HLA class II peptides were further analyzed.

Comparative analysis

For identification of tumor-exclusive antigens from HLA class I and II, comparative analysis was performed with published benign HLA ligand datasets. These included benign tissue from the HLA ligand atlas (123), benign tissue analyzed with the timsTOF (124), hematological cells (128) and tonsils (129) with approx. 992,000 HLA class I ligands and 1,119,000 HLA class II peptides from > 30 different tissues or cell types, e.g. lung, brain, bladder, heart, skin, PBMCs, among others, of different donors. Testis was excluded from these datasets. After comparative analysis, all remaining peptides are referred to as tumor-exclusive or tumor-specific, as they are absent in healthy tissue. Datasets of peptides extracted from sHLA molecules of plasma from healthy donors (HD) were additionally included in the analysis of peptides from sHLA of SCLC patient's plasma samples (unpublished timsTOF analysis, Department of Peptide-based Immunotherapy + (119)). "Tumor-exclusive" antigens that were found in plasma samples of SCLC patients are referred to as patient-exclusive peptides.

Analysis strategy of HLA ligands from SCLC tumor samples

A flowchart of the workflow and analysis strategy of all peptide sequences obtained from the immunoprecipitation of tumor tissue samples is presented in Figure 3. For xenograft samples, which might contain both human- and mouse-derived peptides, database search was performed with the combined mouse and human proteomes (described in section *Database search*). Peptides were categorized into sequences that exclusively derived from human protein ("human only"), that could be derived from either human or mouse proteins ("human/mouse") and into sequences that exclusively derived from mouse proteins ("mouse only") according to the protein annotation of PEAKS. "Mouse only" peptides were directly excluded. HLA binding prediction (described in section *HLA binding prediction*) was performed on both „human only“ and „human/mouse“ datasets

from xenograft models, as well as all peptides from cryorecanalization/biopsy (see 3rd panel Figure 3 “*in silico* data processing”). Comparative analysis (described in section *Comparative analysis*) of HLA class I ligands was performed on all HLA class I ligands that were eluted from cryorecanalization or biopsy plus HLA class I ligands from xenograft samples categorized as „human only“ to identified tumor-exclusive HLA ligands (see 4th panel Figure 3 “comparative analysis”). After identification of tumor-exclusive ligands these sequences were compared with the dataset of “human/mouse” ligands to rescue these sequences from the dataset and identify the overall frequency within all samples. In order to reduce the number of peptides that will be later tested in T cell immunogenicity assays, different selection criteria were afterwards applied, including the comparison with additionally unpublished datasets of benign tissue analyzed with the timsTOF and plasma from healthy donors that was assessed in a previous publication (119) (see 5th panel Figure 3 “peptide selection”). The source proteins of all tumor-exclusive ligands were analyzed and checked whether they were associated with SCLC (see Supplementary Table 1), cancer-related or not found to be represented on published HLA-ligandomic datasets of benign tissue. Generally, we were following two different approaches to selected interesting SCLC ligands that will be tested in T cell immunogenicity assays, a warehouse approach, and a patient individual approach (see also section *Personalized immunotherapy*). In the warehouse approach frequently shared tumor-specific antigenic peptides are selected for the most common HLA types of a population. We therefore selected tumor-specific ligands that were shared with highest frequency by different SCLC tumor samples. Additionally, we selected frequently shared ligands of HLA class I types (A*01:01, A*02:01, A*03:01, A*11:01, A*24:02, B*07:02 and B*08:01), that are commonly shared in the European population and for which suitable pHLA monomer constructs are available for T cell priming experiments with artificial APCs (aAPCs, (130)). In the individualized approach ligands are selected based on patient individual analysis. In this approach ligands of patients with available autologous PBMCs were selected based on their source protein, whether it is associated with SCLC (see Supplementary Table 1), otherwise cancer-related or was not presented on benign tissue (Table 3). All sequences were additionally compared with in-house data of previously identified ligands eluted from other malignancies. Of special interest were other malignancies of the lung and the brain due to the neuroendocrine features of SCLC, including NSCLC, anaplastic astrocytoma, atypical teratoid rhabdoid tumor, ependymoma, glioblastoma, meningioma, neuroblastoma, oligodendroglioma, paraganglioma and subependymoma. The human protein atlas was used to check for potential tissue specificity or cancer association of proteins (<https://www.proteinatlas.org>).

Final lists of all selected peptides (n=25 frequently shared ligands and n=34 patient individual selected ligands) are presented in Table 2 and Table 3.

Population coverage of HLA class I alleles

Population coverage of HLA class I alleles in German and European populations was predicted using the online tool from IEDB (131) (<http://tools.iedb.org/population/>).

Synthetic peptides and spectrum validation

For the experimentally eluted peptides that were selected to be tested in T cell assays, synthetic peptides were produced for MS spectrum validation (6th panel Figure 3 “spectrum validation”). Peptides were synthesized in-house with the automated peptide synthesizers Liberty Blue (CEM) or Activo-P11 (Activotec) using the 9-fluorenylmethyl-oxycarbonyl/tert-butyl strategy (123) or purchased at Intavis Peptide Services (Tübingen, Germany) or JPT Peptide Technologies (Berlin, Germany). Synthetic peptides were spiked into a sample matrix at a concentration of approximately 100 fmol/ μ L per peptide (n= 59) and analyzed with the same LC-MS/MS instruments than the corresponding naturally presented peptides. MS spectra and annotated b- and y-ions were exported from PEAKS. Spectrum validation was performed by calculating the spectral correlation (R^2) between the overlapping intensities of annotated b- and y-ions and plotting the spectra of the naturally presented and the synthetic peptides. The identity was directly validated with a R^2 value >0.8 . A sequence was rejected with R^2 values <0.6 . For sequences with R^2 values between 0.6 and 0.8, the retention time was compared and visual examination of the MS spectra was performed.

5.1.3.4 Graphs and statistical analysis

All graphs and statistical analyses were generated using Prism 9.5.1 (GraphPad Software) or MS Office Excel 2019. Figures 2 and 3 were partially created with the help of BioRender.com. Overlap diagrams were created using BioVenn (132) or jvenn (133). Word clouds were prepared using Wordclouds.com. Mann-Whitney test and simple linear regression were applied and a p value of < 0.05 was considered statistically significant.

5.1.4 Results and discussion

5.1.4.1 Immunopeptidomics of SCLC tumor tissue samples

Two main different sample types were assessed, SCLC tissue samples directly derived from cryorecanalizations or biopsies (also referred to cryorec/biopsy and used interchangeably) without further manipulation, or tissue samples derived from xenograft models of biopsies (PDX) or circulating tumor cells (CDX). Throughout this thesis, samples from cryorecanalization or biopsy are always represented with an orange color code and samples from xenograft models with a blue color code. The workflow for identification of T cell targets in SCLC is presented in Figure 3.

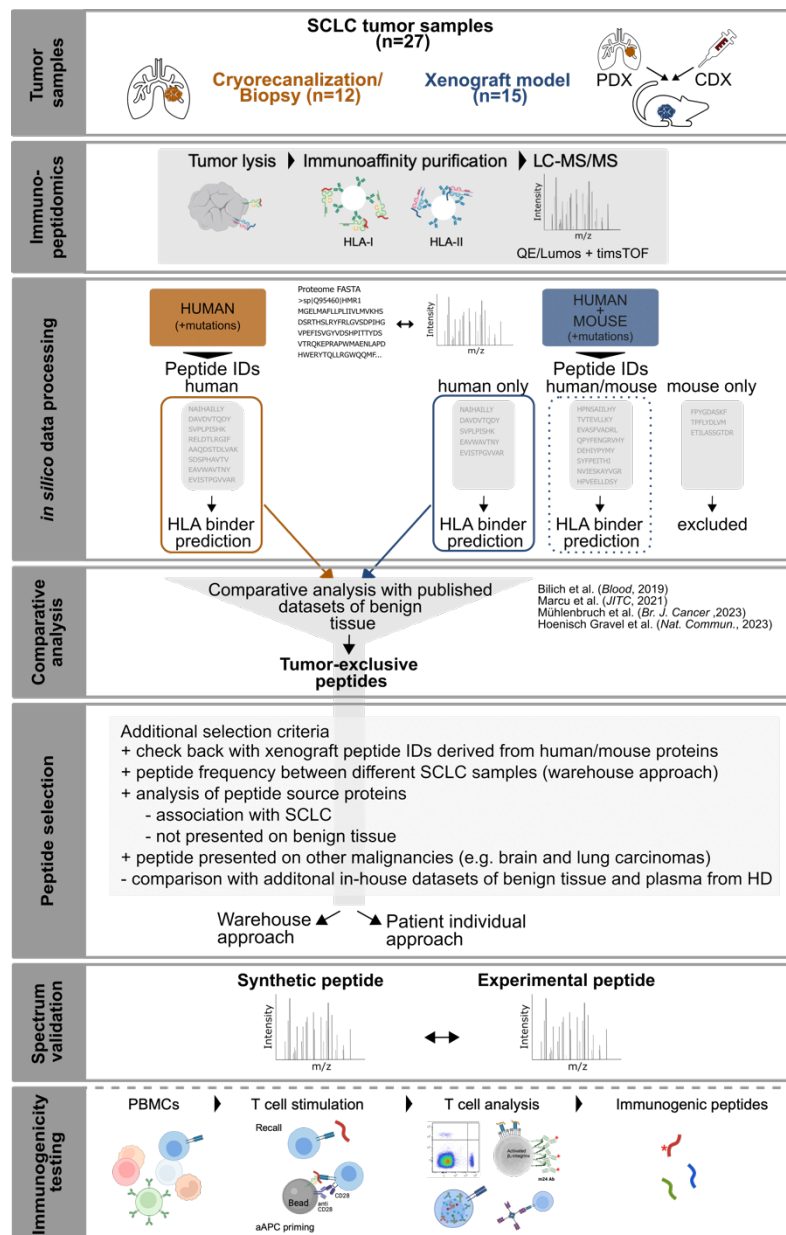


Figure 3: Workflow for identification of T cell targets in SCLC. The sequential steps presented in the different boxes are presented underlined in the figure legend.

SCLC tumor samples from cryorecanalization or biopsy and from xenograft models (CDX/PDX) were collected. Immunopeptidomics,

immunoaffinity purification followed by LC-MS/MS analysis was performed. Depending on the sample type, in silico data processing was performed with the human or the combined human and mouse proteome and available mutated protein sequences were added. HLA class I peptides were analyzed for both types of samples and *in silico* binding prediction was performed. HLA class II peptides were only analyzed for cryorecanalization/biopsy samples and no binding prediction was performed. Tumor-exclusive peptides were identified by comparative analysis with datasets of benign tissues and cells published by our institute. Additional selection criteria were applied to select interesting peptides for testing in T cell assays. Selected peptides were validated by spectrum validation with synthetic peptides. As final step for T cell target identification, immunogenicity testing with T cells from PBMCS of SCLC patients and healthy donors will be performed. The solid squares indicate steps that were performed throughout this study, immunogenicity testing, marked by the dotted square, will be performed in future studies. Partially created with BioRender.com.

Optimized peptide yields and adapted analysis strategy for SCLC tumor samples from xenograft models

It was recently reported that patient derived xenografts can be used for the identification of tumor antigens by immunopectidomics (134). Xenografts were shown to reflect the HLA ligandome of patients biopsies (134) or cell lines (135). Access to SCLC tissue samples is limited, since surgery is rarely performed (with the exception of cryorecanalization performed in Tübingen) and biopsies are often very small, which is limiting the efficiency of peptide recovery (see introduction section 2.4 *Immunopectidomics*). Therefore, the approach of HLA-ligandome analyses of samples from xenograft models is highly interesting for SCLC. SCLC xenograft models from patient-derived biopsies or resections (PDX) and from CTCs (CDX) (162) were established by our cooperation partner in Cologne and immunopectidomics analysis was performed throughout this study.

We were initially confronted to the problem that only very low peptide numbers could be retrieved from xenograft models. Different measures gradually improved peptide yields from xenograft samples presented in Figure 4A. Data presented in Figure 4A was analyzed with an Orbitrap-based MS device (Lumos or QE) and data processing was performed with Proteome Discoverer using a human proteome reference (see exception in 5.1.3.3, *Database search*). No binder prediction was performed due to missing HLA typing of the samples at that timepoint of analysis. Sample batches marked with an asterisk were handled by Ana Marcu. With a pilot sample in 2017, we were able to show the feasibility of extracting and identifying HLA class I peptides from SCLC xenograft models (196 peptides). Follow up samples in 2018 showed a very low peptide yield, 91 peptides in mean per sample. These samples were embedded in Tissue-Tek®, which contains water-soluble glycols and resins that can interfere with the ionization efficiency in mass spectrometry analysis (data not shown, (136)). A following batch in 2020 showed an improved mean in peptide yields (518 IDs). Three of the four samples were previously analyzed (marked with a symbol on top of the bar), but now not embedded in Tissue-Tek®. Improvement was achieved for two out of three samples. Reported low HLA expression of SCLC samples (64) and a low sensitivity of the mass spectrometer were further considered to be causal for low peptide numbers. The second batch in 2020 was selected for a high HLA class I expression (verified by our cooperation partner in Cologne, data not shown). In addition, the MS method was adapted, the dynamic exclusion was decreased to 1 s and the maximal injection time was increased to 200 ms, with the intent to increase the sensitivity of peptide detection in samples with low peptide yields (see 5.1.3.3, *Mass spectrometry data acquisition*). These measures jointly led to improved peptide numbers of more than 1,500

peptides in mean per sample. Of note, one sample with high HLA expression was embedded in Tissue-Tek® but still retrieved high peptide yields.

Subsequent analysis of the extracted peptides revealed sequences that potentially derived from mouse proteins. Most frequently identified peptides were only shared between xenograft samples and not found on any of the cryorecanalization or biopsy samples that were analyzed to that point (data not shown). One of these peptide sequences was SYFPEITHI, which is the first peptide sequence that was described and eluted from mouse MHC H2-Kd (137). For the intended goal of using the results of this study for the treatment of human patients, it is important to identify potentially mouse-derived peptides and handle them with care. Therefore, all tumor samples from xenograft models were processed with the combined human and mouse proteome (see 3rd panel Figure 3) with the drawback that increasing the search space could lead to higher numbers of false identified peptides.

To check whether this increased search space is leading to increased false identified mouse-derived peptides the samples from cryorecanalization or biopsy that should not contain any mouse contaminations were retrospectively also processed with the combined proteomes. All data presented in Figure 4B was therefore processed with the combined human and mouse proteome as described in 5.1.3.3, *Database search*. After data processing peptides were categorized into sequences that derived from “human only”, “human/mouse” and “mouse only” (see 5.1.3.3 *Analysis strategy of HLA ligands from SCLC tumor samples* and 3rd panel Figure 3). The percentage of “human only”-, “human/mouse”- and “mouse only”-derived peptide sequences were plotted for xenograft models (n=26 MS measurements) and cryorecanalization/biopsy samples (n=22 MS measurements) before (peptides) and after (ligands) HLA class I binder prediction (Figure 4B, left diagram). The right diagram of Figure 4B zooms on the “mouse only”-derived sequences, showing significantly increased percentages of mouse-derived peptide sequences for samples from xenograft models compared to samples from cryorecanalization/biopsy both at the peptide (8.3 % vs 2.0 %) and ligand (7.5 % vs 1.3 %) levels (Mann-Whitney test, $p < 0.0001$). Concluding that the increased search space did not especially generate false identified peptides or ligands for the xenograft samples. These findings underline the advantage of using the combined proteome for peptide sequence annotation in xenograft-derived samples to identify and exclude mouse-derived peptides. A similar strategy was also used in a previous immunopeptidomics study of xenograft samples (134).

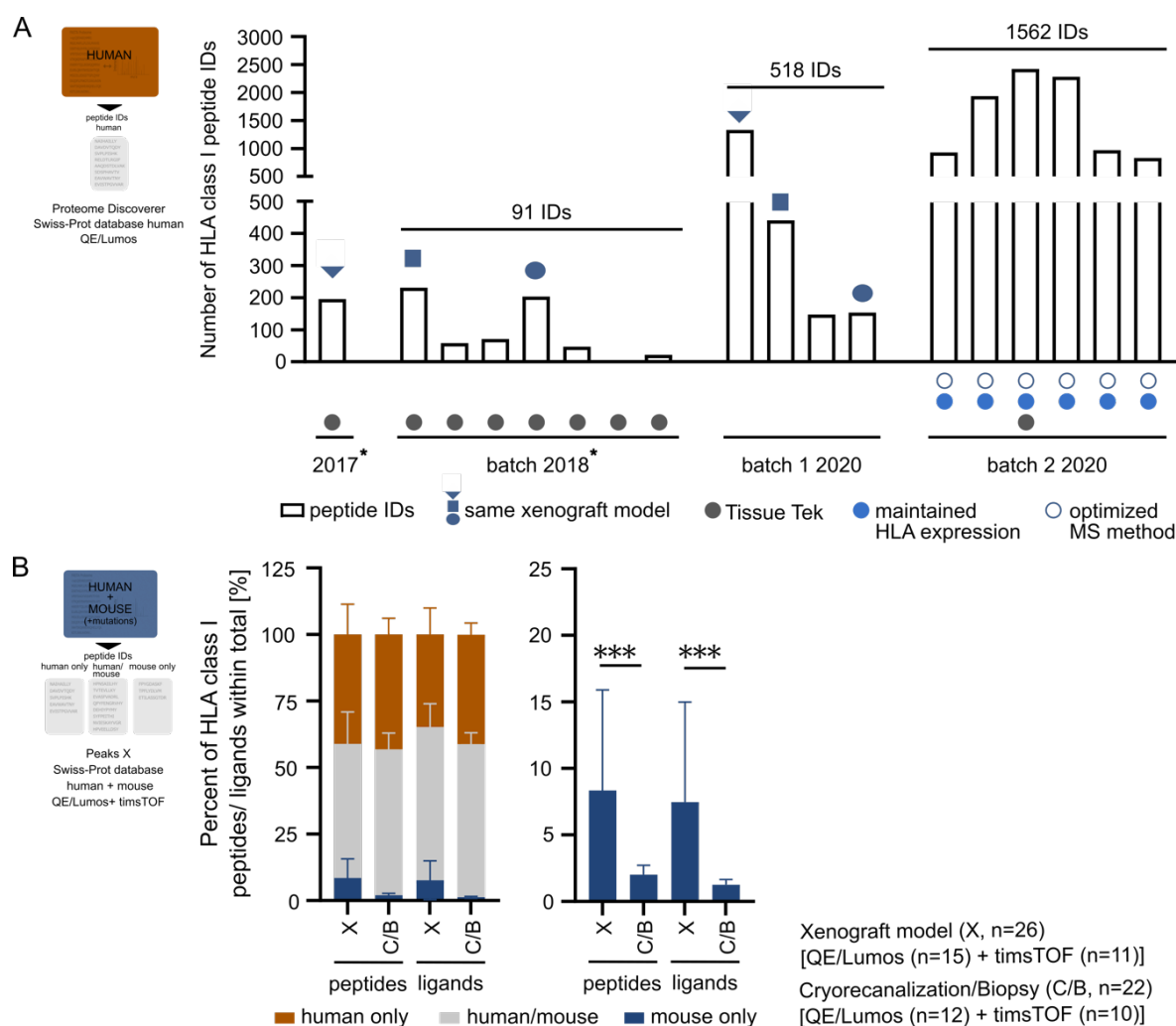


Figure 4. Optimization steps for immuno-peptidome analysis of xenograft models. (A) Number of HLA class I peptides extracted from SCLC samples of xenograft models. Different sample batches were analyzed. Samples from batches 2017 and 2018 were embedded in Tissue-Tek® (grey circle). Batch 1 2020 included three samples from previously analyzed xenograft models, now without Tissue-Tek®. Related samples are indicated with the dark blue symbols on top of the bar. In batch 2 2020, SCLC samples were selected for a high HLA class I expression and analyzed with an optimized MS method (filled and open blue circles). No binder prediction was performed. The mean of peptide IDs for each batch is indicated. MS analysis was performed with Orbitrap-based MS devices (QE or Lumos), data processing was performed with Proteome Discoverer using the SEQUEST HT search engine and the human Swiss-Prot database. Samples marked with an asterisk (*) were handled by Ana Marcu. (B) Comparison of the fraction of mouse-derived peptide sequences in SCLC samples from xenograft model (X) and cryorecanalization/ biopsy (C/B) processed with the combined human and mouse proteome. Percentages of “human only”, “human/mouse” or “mouse only”-derived sequences (left diagram) and a zoom on the “mouse only”-derived sequences (right diagram) on peptide or ligand (after HLA binding prediction) levels are plotted. Plots are showing combined data of $n=26$ and $n=22$ MS measurements from samples of xenograft models and cryorecanalization/ biopsy, respectively. MS analysis was performed with Orbitrap-based MS devices (Lumos or QE) and the timsTOF device, data was processed with PEAKS and the combined reviewed human and mouse Swiss-Prot proteome. Mann-Whitney test, $p < 0.05$ (***) < 0.001). X: xenograft model, C/B: cryorecanalization/ biopsy.

We can only speculate on the reasons for mouse-derived peptide contaminations. The peptides could be eluted from tumor infiltrated mouse cells by coelution of mouse MHC molecules with cross reactive anti-human HLA antibodies (data not shown). However, we were informed by our cooperation partners that mouse cell infiltration in xenograft tumors is low. Additionally, after binder prediction, the number of ligands derived from “mouse only” should substantially decrease compared to the number of peptides before binder

prediction, which is not distinctly the case (8.3 % vs 7.5 %, Figure 4B). Similar binding motifs between some mouse and human MHC allotypes could explain this observation. The mouse protein-derived peptides could also be acquired, processed, and presented by human tumor cells, explaining the human HLA binding motifs. 2 % and 1.3 % of sequences extracted from cryorec/biopsy were annotated to derive from mouse proteins on peptide and ligand levels (binder prediction to patients HLA allotype), respectively, either representing the frequency of false identified peptides or contamination from e.g. mouse anti-human HLA antibodies used for immunoprecipitation. At least 20 % of mouse-derived ligands in the cryorecanalization or biopsy samples were indeed assigned to derive from mouse immunoglobulins. The presence of mouse contamination in xenograft samples was described before, and similar explanations were discussed including coelution of mouse MHC, presentation of mouse peptides by human cells, proteolysis of mouse monoclonal antibodies or false identified peptides (134).

Taken together, we improved the number of HLA class I peptides extracted from SCLC samples of xenograft models and adapted the analysis strategy of xenograft samples by data processing with a combined proteome of human and mouse to identify and exclude potential mouse-derived peptides.

Descriptive analysis of peptides extracted from SCLC tumor samples

Peptide yields of SCLC samples are presented in Figure 5 by plotting the numbers of HLA class I ligands and HLA class II peptides from cryorecanalization or biopsy (Figure 5A) and xenograft models (HLA class I only, Figure 5B). Sample ID, tissue mass, sample source, whether patients were treated prior to sampling (pre-treatment) and whether sequencing data was available is indicated. Peptides identified with the Orbitrap-based MS, the timsTOF or with both devices are color coded. For xenograft samples, all HLA class I ligands after subtracting the “mouse only”-derived ligands are plotted.

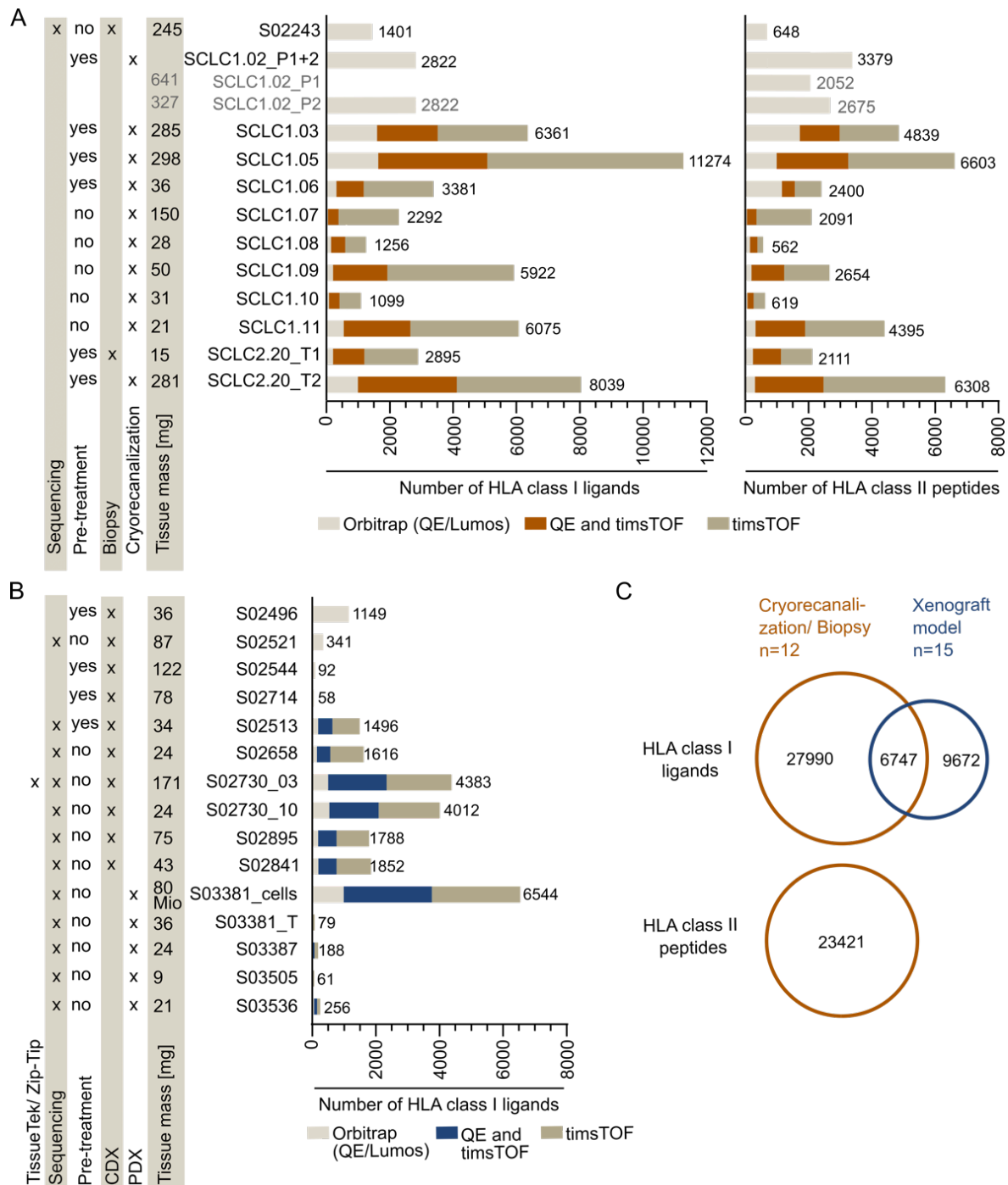


Figure 5. Peptide yields from tissue samples of SCLC patients (orange: cryorecanalization/biopsy, blue: xenograft models). Bar plots indicating the number of HLA class I ligands and HLA class II peptides extracted from samples of cryorecanalization/ biopsy (A) or xenograft models (B) analyzed with two different MS devices. Whether samples were embedded in Tissue-Tek® resulting in a Zip-Tip purification step, whether sequencing data was available, whether treatment of the patient was applied before sampling (pre-treatment), sample specifics (CDX, PDX, cryorecanalization or biopsy), tissue mass in mg (or cell number in Mio) and sample ID are indicated (left tables). Ligands eluted from xenograft samples include sequences derived from “human only” and “human/mouse” protein sources. Peptides identified with the Orbitrap-based MS (QE/ Lumos), the timsTOF or with both devices are color coded (light gold, dark gold, orange/blue, respectively). (C) Total number of different peptide sequences extracted from SCLC tissue samples. HLA class I ligands and the overlap between samples from cryorecanalization/ biopsy and xenograft model (top). HLA class II peptides from cryorecanalization/ biopsy samples (bottom). P, piece; T, tumor; CDX, circulating tumor cell derived xenograft; PDX, patient (biopsy) derived xenograft.

HLA class I ligands

A total of 76,732 HLA class I ligands were eluted from all SCLC samples (n=27). 48,623 ligands were shared between at least two samples, comprising 16,300 different sequences. 28,109 sequences were not shared but uniquely found in one or the other sample. This results in 44,409 different HLA class I ligand sequences extracted from SCLC samples, with 14.6 % (6747 ligands) being shared between SCLC samples from both cryorecanalization/biopsy and xenograft models (Figure 5C, top).

More HLA class I ligands were eluted from cryorec/biopsy samples than from samples of xenograft models with 52,817 (mean: 4,401/sample; range: 1,401 to 11,274; n=12) vs 23,915 (mean: 1,594/sample; range: 58 to 6,544; n=15) HLA class I ligands. The number of eluted peptides varies between the samples. This could result from different levels of HLA expression on tumor samples (not assessed for cryorec/biopsy samples) or the tumor size. Concerning the xenograft models that are plotted in panel B of Figure 5, the first four samples were analyzed before MS optimization, the following six samples were selected for high HLA expression and analyzed with the adapted MS method, the last five samples were not specifically selected for high HLA expression but analyzed with the adapted MS method, which could also contribute to variable peptide yields. Of note, one xenograft sample which was not a tumor piece, but cells cultured from a xenograft-derived tumor (S03381_cells) presented exceptionally high numbers of HLA class I ligands compared to its counterpart tumor piece (S03381_T) and to most other xenograft samples. The peptide length distribution of HLA class I ligands is comparable between both sample types and meets the expectations with highest frequency of HLA class I ligands having a peptide length of nine aa (Figure 6A).

HLA class II peptides

A total of 36,609 HLA class II peptides were eluted from cryorecanalization or biopsy SCLC samples (n=12). 20,070 peptides were shared between at least two samples, comprising 6,882 different sequences. 16,539 sequences were not shared but uniquely found in one or the other sample. This results in 23,421 different HLA class II peptide sequences (Figure 5C, bottom). In mean, 3,051 HLA class II peptides were eluted per sample ranging from 562 to 6,603. HLA class II peptides from xenograft samples were not analyzed, as the peptides reflected an unusual length distribution (Supplementary Figure 1B) compared to samples from cryorecanalization and biopsy (Figure 6B), which is a sign for contaminations instead of true HLA derived peptides. In addition, according to our cooperation partner in Cologne, the xenograft samples showed little to no HLA class II expression (data not shown).

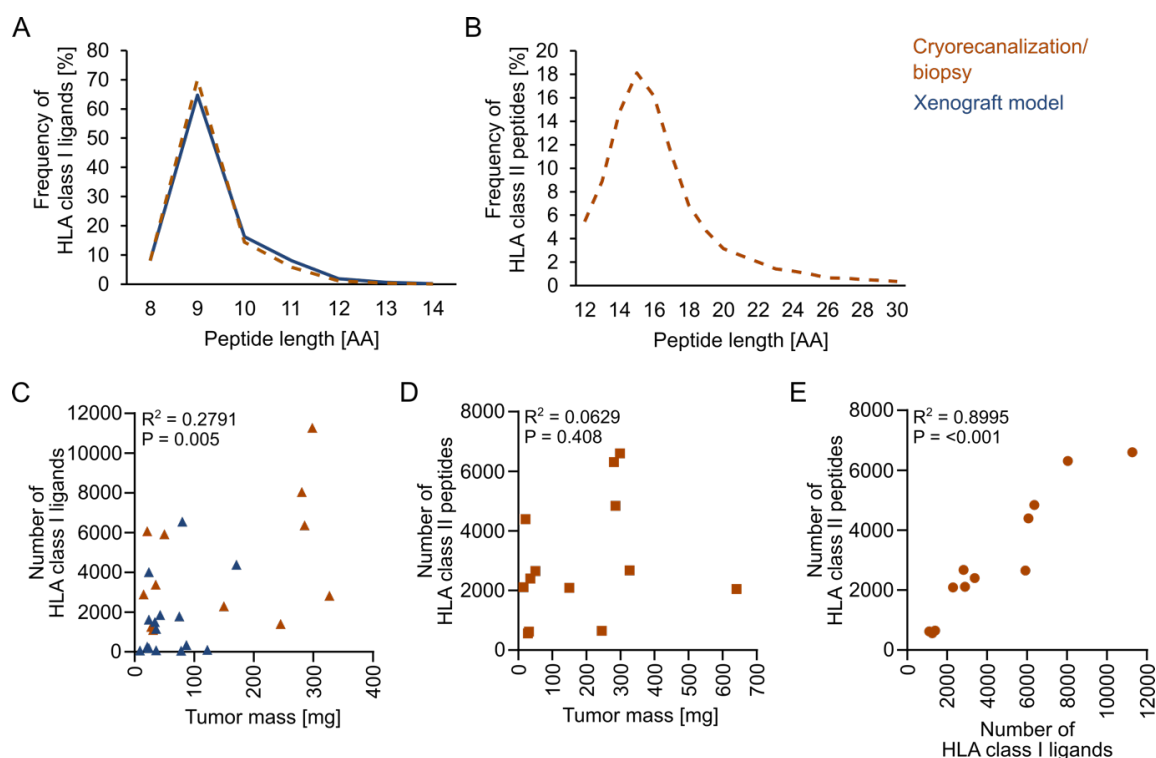


Figure 6. Characteristics of peptides eluted from SCLC tumor samples. Cryorecanalization/ biopsy samples are plotted in orange and samples from xenograft models in blue. (A, B) Line plots representing the length distribution of eluted peptides. Frequency of HLA class I ligands (A) and HLA class II peptides (B) plotted for the respective peptide lengths. (C, D) Scatter plot illustrating the relationship between the number of HLA class I ligands (C) or HLA class II peptides (D) and the tumor tissue mass used for HLA immunoprecipitation. (E) Scatter plot illustrating the relationship between the number of HLA class I ligands and the number of HLA class II peptides eluted from cryorecanalization/ biopsy samples. Each dot represents one sample. R^2 and the respective P value are indicated.

Tumor mass vs peptide numbers

The tissue mass of the samples from cryorecanalization/biopsy and the xenograft models ranged from 15 to 641 mg and 9 to 171 mg, and the mean number of HLA class I ligands per 10 mg of tissue was 744 (range: 57 to 2893) and 315 (range: 7 to 1693), respectively. The mean number of HLA class II peptides per 10 mg tissue was 461 (range: 26 to 2,093). In Figure 6C the numbers of HLA class I ligands eluted from cryorecanalization/biopsy and xenograft models are plotted against the tissue mass. A higher tissue mass not necessarily led to a clear increase in peptide numbers ($R^2=0.2791$, $P=0.005$). Cells that were cultured from a xenograft-derived tumor (S03381_cells) are not depicted in Figure 6C, since only a cell number and no tumor mass is available. The correlation for HLA class II peptides and tissue mass is not significant (Figure 6D, $R^2=0.0629$, $P=0.408$). However, the number of HLA class I ligands and HLA class II peptides extracted from cryorecanalization or biopsy samples correlates (Figure 6E, $R^2=0.8995$, $P<0.001$). This could originate from patient SCLC1.02, for which two different tumor pieces P1 and P2 were analyzed (see Figure 5A). For SCLC1.02_P1, no HLA class I peptides and a small number of HLA class II peptides were identified from a tumor piece of >600 mg, which is why SCLC1.02_P2 was additionally analyzed and HLA class I and II peptides were identified.

SCLC1.02_P1 is shown in Figure 6D, but not in Figure 6E as there is no pair of HLA class I and HLA class II peptides. SCLC1.02_P2 is depicted, as all other cryorec/biopsy samples with a pair of HLA class I and II datasets, in Figures C, D and E.

timsTOF vs Orbitrap-based MS measurements

Comparing timsTOF and Orbitrap-based MS for each sample, more peptides were identified with the former one (Figure 5A and B, Supplementary Tables 3 and 4). Summing up the numbers of peptides identified in each sample, timsTOF vs Orbitrap-based MS identified 62,217 (n=21) vs. 38,159 (n=27) HLA class I ligands and 27,433 (n=10) vs. 20,842 (n=13) HLA class II peptides, respectively. Regarding the mean peptide numbers per sample timsTOF vs Orbitrap-based MS identified 2,963 vs. 1,413 HLA class I ligands and 2,743 vs. 1,737 HLA class II peptides per sample, respectively. In mean, approx. 32 % of HLA class I ligands and HLA class II peptides within each sample were identified with both devices (plotted in Figure 5A and B). In total 16,828 of 44,409 different HLA class I sequences and 8,424 of 23,421 different HLA class II sequences were identified with both devices (Supplementary Figure 1C). Different MS devices lead to a distinct but overlapping peptide repertoire, which supports the usage of both devices to generate a complete picture of the HLA ligandome.

All in all, despite a low tissue mass, we were able to extract high numbers of HLA class I ligands from both SCLC tissue sources, xenograft models and cryorecanalizations or biopsies, and HLA class II peptides from cryorecanalizations or biopsies only.

Identification of SCLC specific tumor-exclusive HLA class I ligands

Ideally a T cell target should be cancer specific and therefore absent on benign tissue.

Comprehensive datasets of HLA-ligands that are presented on benign tissues, including lung, brain, bladder, heart, skin and PBMCs among others, were published by our institute (123, 124, 128, 129). These datasets were used for comparative analysis to identify tumor-exclusive SCLC ligands, that are referred to as tumor-specific due to the absence on healthy tissues (see 5.1.3.3. *Comparative analysis* and Figure 7). All HLA class I ligands from cryorec/biopsy and “human only” HLA class I ligands from xenograft models were assessed to identify tumor-exclusive ligands (see 5.1.3.3. *Analysis strategy of HLA ligands from SCLC tumor samples* and 3rd/4th panel Figure 3).

Out of 38,931 HLA class I ligands (cryorec/biopsy + “human only” xenografts), 8,231 tumor-exclusive HLA ligands were identified after overlapping the datasets of benign (grey, n=407 samples) and SCLC tissues (yellow, n=27 samples) (Figure 7A). The waterfall plot presented in Figure 7B illustrates the frequency of single HLA class I sequences within the two datasets, on top ligands from SCLC samples (yellow) and below ligands from healthy tissues (grey); all SCLC derived sequences but only benign data that was overlapping in

Figure 7A are plotted. Tumor-exclusive HLA class I ligands that were only found in SCLC samples and not in the benign dataset appear on the left side of the graph, are marked with a square and illustrated enlarged. Four ligands were found with highest frequency in 22 % (6 of 27) of the samples.

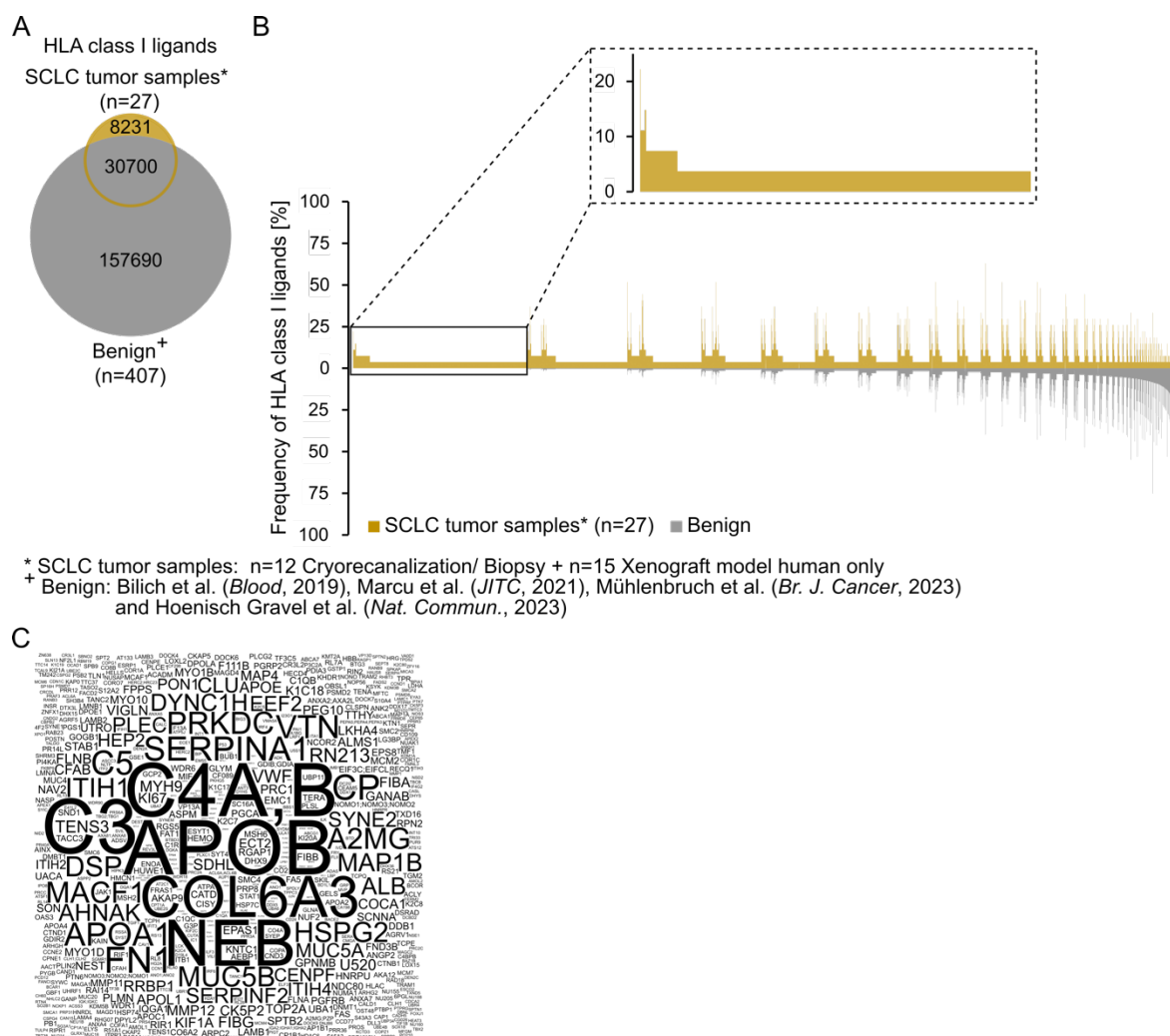


Figure 7. Comparative analysis of HLA class I ligands from SCLC tissue samples and benign tissues. The SCLC dataset (*SCLC tumor samples, yellow, n=27) presented in this graph comprises all HLA class I ligands extracted from cryorecanalization or biopsy samples (n=12) and HLA class I ligands extracted from xenograft models (n=15) that were assigned to derive from human protein sources only. The benign dataset (+ Benign, grey, n=407) comprises all HLA class I ligands that were published in the indicated manuscripts. Venn diagram representing the overlap (A) and waterfall plot representing the frequency of the different peptide sequences (B) of HLA class I ligands identified on SCLC samples (yellow) and on benign tissue (grey). The waterfall plot includes all SCLC derived sequences but only benign data that was overlapping in A. One bar represents one peptide sequence, the higher the bar the more often the sequence was found within the respective dataset. Tumor-exclusive HLA class I ligands that were only found in SCLC samples and not in the benign dataset are marked with a square and illustrated enlarged. (C) Word cloud representing the protein sources of the SCLC tumor-exclusive HLA class I ligands. Only proteins that were represented by at least three different ligands are plotted. The font is proportional to the number of ligands, the bigger the font the more often the protein was represented.

The 8,231 tumor-exclusive HLA class I ligands derived from more than 4,400 different proteins. All proteins that were represented by at least three different tumor-exclusive HLA class I sequences are illustrated as a word cloud in Figure 7C, the more often a protein was presented the bigger the font. Apolipoprotein B-100 (APOB), Complement C3 (C3),

Complement C4 A and C4 B (C4A/C4B), Nebulin (NEB) and collagen type VI α -3 (COL6A3) were represented most often in decreasing order by 74, 55, 53, 46 and 41 different tumor-exclusive ligand sequences, respectively. Inhibition of APOB was reported to reduce the risk for SCLC in data from genome-wide association studies and APOB inhibition was additionally found to increase neutral ceramidases that were themselves associated with a reduced risk of lung cancer (138). Peptides that derived from complement could be potentially interesting since the complement system was generally found to be associated with cancer progression and poorer prognosis (139, 140). The COL6A3 derived HLA*A2:01 restricted peptide FLLDGSAHV was recently described as a pan-cancer T cell epitope and affinity enhanced TCRs against this epitope led to tumor elimination *in vitro* and *in vivo* in mice (141). The peptide FLLDGSAHV was also presented on three SCLC samples, but also found to be present on benign tissue (spleen, HLA atlas (123) and timsTOF data (124)). Proteins associated with SCLC (see Supplementary Table 1) do not rank first, while centrosome-associated protein ALMS1 is represented by nine ligands and was shown to be frequently mutated in SCLC (15).

In a next step, all identified tumor-exclusive HLA class I ligands were compared with the list of xenograft-derived ligands that could derive from either human or mouse proteins ("human/mouse"). 241 ligands that were found to be tumor-exclusive in cryorecanalization or biopsy could also be found in the "human/mouse" dataset. In total, from the 8,231 tumor-exclusive ligands identified above, 994 were shared by at least two samples, 4,357 were previously found on other malignancies including brain and lung tumors (in-house database) and 162 derived from proteins that are known to be associated with SCLC, such as ASCL1, DLL3, Cyclin D1 (CCND1) and P53 (literature search presented in Supplementary Table 1).

Overall, we were able to identify a substantial number of tumor-specific HLA class I ligands that are absent on healthy tissues but shared between different SCLC samples and derived from potentially interesting proteins, including SCLC associated proteins.

Selection of potentially interesting HLA class I T cell targets

Whether a peptide is immunogenic and able to elicit a T cell response can be tested in recall experiments with T cells (PBMCs) from SCLC patients or in priming experiments with T cells (PBMCs) of healthy donors. Recall experiments of memory T cells will be performed with autologous or HLA-matched SCLC patient samples that have been collected throughout this thesis. T cell priming will be performed in samples of healthy donors with help of artificial APCs (aAPCs, (130)).

Only a limited number of peptides can be tested in such T cell assays due to a limited number of patient samples and high efforts and resources required for aAPC primings.

Therefore, a workflow including several selection criteria was established to reduce the 8,231 tumor-exclusive ligands to a suitable number of peptides (5th panel Figure 3). This workflow included the comparison with internal unpublished data of more healthy tissues and sHLA-derived ligands from plasma samples of healthy donors ((119)+ unpublished).

We were following two main strategies, a warehouse approach with frequently shared tumor-specific peptides from common HLA types (142-144) and an individualized approach of SCLC-associated or cancer-related tumor-specific ligands (see *Analysis strategy of HLA ligands from SCLC tumor samples* in section 5.1.3.3 and 5th panel Figure 3). For the warehouse approach, we selected tumor-specific peptides that were frequently shared between different SCLC samples and among common HLA types. All selected peptides are listed in Table 2. Peptides P1-P10 were selected due to highest frequency in SCLC samples independent of their HLA type. Additionally, P11-P25 were selected, which are most frequently shared ligands of HLA class I types (A*01:01, A*02:01, A*03:01, A*11:01, A*24:02, B*07:02 and B*08:01), that are commonly shared in the European population and for which suitable pHLA monomer constructs are available for T cell priming experiments with artificial APCs (aAPCs, (130)).

Table 2: List of frequently shared ligands. Most frequently shared sequences in different SCLC samples are listed on top, followed by frequently shared sequences among common HLA types with available monomer constructs for aAPC primings.

Peptide ID	Sequence	Source protein	HLA restriction	Total (CB/Xm/Xhm)	Frequency Total (n=27)	Frequency HLA-matched	Comment	On other malignancies (Total/Brain/NSCLC)	Spectrum validation timsTOF	Immunogenicity testing (planned)
P1 B ¹ *08	DYQALRTSI	CLH1/KIAA0034	B*08:01/A*24:02/C*07:04	6 (1/0/5)	22.22%	66.67%		7/7/0	+	aAPC/Recall
P2 A ¹ *02	ALGPSVAAA	FBLN2	A*02:01	6 (6/0/0)	22.22%	66.67%	Protein not on benign	0/0/0	?	aAPC/Recall
P3 A ¹ *03	SINKPTSER	NDC80	A*03:01	6 (2/4/0)	22.22%	60.00%		76/7/0	ongoing	aAPC/Recall
P4 mix	SGALHVVSL	PHLD	B*08:01/C*07:01/C*03:03/C*03:04	6 (0/6/0)	22.22%	31.58%		11/1/0	+	Recall
P5 A ¹ *02	VLGGPPAVA	SLIT3/KIAA0814	A*02:01	5 (5/0/0)	18.52%	55.56%		2/0/0	+	aAPC/Recall
P6 A ¹ *03	KSKEYFSKQK	PRDX1	A*03:01	5 (1/0/4)	18.52%	50.00%		18/1/0	ongoing	aAPC/Recall
P7 mix	EVFGRSEQY	ZIC2	A*26:01/A*25:01	5 (3/0/2)	18.52%	83.33%		19/11/1	+	Recall
P8 mix	VPSEIDAAF	MMP11	B*35:03/B*35:01/B*35:02	5 (4/0/1)	18.52%	62.50%		9/0/2	+	Recall
P9 mix	MYDGPVVEV	DPYL1	C*04:01	5 (2/0/3)	18.52%	62.50%		2/1/0	+	Recall
P10 mix	TIIDKSKSV	HMCS1	A*02:01/C*12:03/C*03:03/C*02:02	4 (1/0/3)	14.81%	23.53%		27/1/0	+	Recall
P11 A ¹ *01	EVDPIGHLY	MAGE3	A*01:01	2 (0/2/0)	7.41%	40.00%	Protein not on benign, Cancer related	10/0/2	+	aAPC
P12 A ¹ *02	ILLDVKTRL	KRT14/KRT16/KRT17	A*02:01	4 (4/0/0)	14.81%	44.44%		4/1/0	+	aAPC
P13 A ¹ *02	LLLPAPVPGA	MEGF6/KIAA0815	A*02:01	4 (4/0/0)	14.81%	44.44%	Protein not on benign	4/0/0	+	aAPC
P14 A ¹ *02	GLAEVQRL	AIXX/NEF5	A*02:01	4 (3/0/1)	14.81%	44.44%		7/4/0	+	aAPC
P15 A ¹ *03	ALNSGVSQK	TOP2A	A*03:01	4 (0/4/0)	14.81%	40.00%		10/1/1	ongoing	aAPC
P16 A ¹ *03	VLGGPSGTGK	NAV1	A*03:01	4 (2/0/2)	14.81%	40.00%		8/1/0	+	aAPC
P17 A ¹ *03/A*11	KSSDVFSK	RASF6	A*03:01/A*11:01	4 (0/4/0)	14.81%	28.57%		2/0/0	ongoing	aAPC
P18 A ¹ *03/A*11	STAKHVEK	KNTC1	A*03:01/A*11:01	4 (0/4/0)	14.81%	28.57%		13/3/0	ongoing	aAPC
P19 A ¹ *11	ATAQFKINKK	NUF2	A*11:01	3 (0/3/0)	11.11%	75.00%		6/0/0	+	aAPC
P20 A ¹ *11/A*03	GLAPNTPGK	PRC1	A*11:01/A*03:01	3 (0/3/0)	11.11%	21.43%		2/1/0	+	aAPC
P21 A ¹ *24/A*23	EYLDNRNTE	P53	A*24:02/A*23:01	4 (2/2/0)	14.81%	100.00%	SCLC, Cancer related	6/3/0	+	aAPC
P22 B ¹ *35/B*07	SPLDTSSVL	PGFRB	B*35:01/B*35:03/B*07:02	4 (4/0/0)	14.81%	33.33%		0/0/0	+	aAPC
P23 B ¹ *07	VLPAGGGTV	GRP	B*07:02	2 (2/0/0)	7.41%	40.00%	SCLC	0/0/0	+	aAPC
P24 B ¹ *51/B*07	YPRISVNNV	CATD	B*51:08/B*07:02	2 (2/0/0)	7.41%	33.33%		53/11/1	+	aAPC/Recall
P25 B ¹ *51/B*08	DGKKKISTV	SRP14	B*51:08/B*08:01	4 (2/2/0)	14.81%	57.14%		25/4/1	ongoing	aAPC

The peptide ID includes the HLA type. If a sequence is verified by spectrum validation, peptides will be tested with autologous and HLA-matched PBMC samples of SCLC patients (recall) or with aAPCs.

Abbreviations: CB, cryorecanalization/biopsy; Xh, “human only”-derived sequences from xenograft models; Xhm, “human/mouse”-derived sequences from xenograft models; “+”, $R^2 > 0.8$; “-”, $R^2 < 0.6$; “?”, R^2 between 0.6 and 0.8; aAPC, artificial antigen presenting cell.

After MS spectrum validation (see section 5.1.3.3 *Synthetic peptides and spectrum validation*), these peptides will be tested for immunogenicity either with autologous or HLA-matched SCLC PBMC samples (recall experiments) or with PBMCs obtained from healthy donors, depending on the availability of a monomer construct (aAPC priming experiments) indicated in the last column of Table 2. The selected peptides have a HLA population coverage of 99.66 % and 99.48 % for German and European populations, respectively. The peptide frequency within HLA-matched SCLC patients is also relevant. EYLDDRNTF that is restricted to A*24:02 and A*23:01 and derives from P53 was presented in all patient samples carrying this HLA types and therefore achieved a HLA-matched frequency of 100 %. 12 selected peptides are found with an HLA-matched frequency of more than 50 %.

For the individualized approach, ligands of patients with available autologous PBMCs were selected based on their source protein, whether it is associated with SCLC (see Supplementary Table 1), otherwise cancer-related, or not found to be represented by ligands on benign tissue, and additionally based on their presence on other malignancies. Selected peptides are listed in Table 3 and will be tested for immunogenicity using autologous and HLA-matched patient PBMC samples. Some are shared between different samples and listed on top (P26-P34). Others were found in individual patients (P35-P57). P58 with the sequence FLLDGSAHV (COL6A3) was also found to be shared in 33 % (3/9) of HLA A*02:01⁺ SCLC patients and included in the list since it was already found to be an interesting T cell epitope (141). Note, that this sequence was also found to be presented on healthy tissue (spleen, HLA atlas(123) and timsTOF data (124)), which would be an exclusion criteria according to our analysis strategy.

Table 3: List of individually interesting, selected peptides.

Peptide ID	Sequence	Source protein	HLA restriction	Total (CB/Xh/Xhm)	Frequency HLA-matched	Comment	On other malignancies (Total/Brain/NSCLC)	Spectrum validation times TOF	Immunogenicity testing (planned)
P26	SCLC1.06, S02730_0310	MAGSPVSSY	ASCL1	B*35:01	3 (1/0/2)	50.00%	SCLC	+	Recall
P27	SCLC1.06, 1.06, 1.09	AFDSSLVFI	COL6A3	C*04:01	3 (3/0/0)	37.50%	Cancer related	+	ongoing
P28	SCLC1.06, 1.09, S03381	TFDETVSTY	TPX2	C*04:01/A*01:01	3 (2/1/0)	15.79%	Cancer related	+	ongoing
P29	SCLC1.06, 1.06, 1.09	NFDFOVQKF	CFAP57	C*04:01	3 (3/0/0)	37.50%	Protein not on benign	+	Recall
P30	SCLC1.06, 1.06	VLLDKIKNL	COL6A3	A*02:01	2 (2/0/0)	22.22%	Cancer related	?	Recall
P31	SCLC1.06, 1.10	KPLDGSALY	COL6A3	B*35:01	2 (2/0/0)	33.33%	Cancer related	+	Recall
P32	SCLC1.06, S02895	ILPPAPSSYF	NFIB	A*24:02/B*15:01	2 (1/0/1)	66.67%	SCLC	+	Recall
P33	SCLC1.03, 1.09	RSAPVGLNK	CT085	A*03:01	2 (2/0/0)	20.00%	Protein not on benign	+	Recall
P34	SCLC1.06, 2.20	ALATGLVGG	KLK11	A*02:01	2 (2/0/0)	22.22%	Protein not on benign, Cancer related	+	Recall
P35	SCLC1.02	AARVPGSSSL	CXCL5	B*07:02	1 (1/0/0)		Protein not on benign, Cancer related	ongoing	Recall
P36	SCLC1.03	STYYWPRPR	GAGE (multiple)	A*03:01	1 (1/0/0)		Protein not on benign, Cancer related	ongoing	Recall
P37	SCLC1.03	KISGGPRISY	MAGEA6	A*32:01/A*03:01	1 (1/0/0)		Protein not on benign, Cancer related	+	Recall
P38	SCLC1.03	LPQKTFQSL	CFAP300	B*07:02	1 (1/0/0)		Protein not on benign	ongoing	Recall
P39	SCLC1.05	SPNNFLSY	CCND1	B*35:01	1 (1/0/0)		SCLC	+	Recall
P40	SCLC1.05	SLEAGLPLOV	CHGA	A*02:01	1 (1/0/0)		SCLC	+	Recall
P41	SCLC1.05	DAWPGTFSF	DLL3	B*35:01	1 (1/0/0)		SCLC	+	Recall
P42	SCLC1.06	FEDHNPPQL	PTEN	B*40:01/C*04:01	1 (1/0/0)		SCLC	ongoing	Recall
P43	SCLC1.06	DPFLNSGT	YAP1	B*35:01	1 (1/0/0)		SCLC	?	Recall
P44	SCLC1.06	GEEDSPSHI	STC1	B*40:01	1 (1/0/0)		Protein not on benign	ongoing	Recall
P45	SCLC1.06	IPLESTDGERL	NFIA/B/C/X	B*35:03	1 (1/0/0)		SCLC	+	Recall
P46	SCLC1.06	DLVKLKYL	MAGEB3	B*08:01	1 (1/0/0)		Protein not on benign, Cancer related	?	Recall
P47	SCLC1.10	LKPGAPLPAL	ALKL1	B*07:02	1 (1/0/0)		Protein not on benign	-	Recall
P48	SCLC1.11	EALLRVLL	DLK1	B*08:01	1 (1/0/0)		SCLC	-	Recall
P49	SCLC1.11	GVFELQHSF	DLL3	A*25:01	1 (1/0/0)		SCLC	+	Recall
P50	SCLC1.11	DVSSSVYSTW	KIT	A*25:01	1 (1/0/0)		SCLC	?	Recall
P51	SCLC2.20	FMKAITDRY	MAOM	A*29:02	2 (2/0/0)		Found on T1 and T2	+	Recall
P52	SCLC2.20	GEELFFDYRY	EZH1/EZH2	B*44:03	1 (1/0/0)		SCLC	?	Recall
P53	SCLC2.20	QETLSVLKF	N6MT1	B*44:02/B*44:03	1 (1/0/0)		SCLC	+	Recall
P54	SCLC2.20	SEVIEPPSL	PBRM1	B*44:03	1 (1/0/0)		SCLC	ongoing	Recall
P55	SCLC2.20	EEYDSIVFY	RB1	B*44:02/B*44:03	1 (1/0/0)		SCLC	+	Recall
P56	SCLC2.20	NEAGLVHGY	FJX1	B*44:03/B*44:02	1 (1/0/0)		Protein not on benign	ongoing	Recall
P57	SCLC2.20	SEVGSPPSY	LRAD3	B*44:03/B*44:02	1 (1/0/0)		Protein not on benign	+	Recall
P58	FLLDGSANV	FLLDGSANV	COL6A3	A*02:01	3 (3/0/0)	33.33%	Kim et al. 2022 (on benign)	?	Recall
P59	mut. R4M	KTMGFSFPR	PCDHB9_mut	A*11:01	1		mutated S02730_03	+	Recall

The peptide ID includes the sample in which the sequence was identified. Dependent on the spectrum validation, peptides will be tested with autologous and HLA-matched PBMC samples of SCLC patients (recall)..

Abbreviations: CB, cryorecanalization/biopsy; Xh, “human only” derived sequences from xenograft models; Xhm, “human/mouse” derived sequences from xenograft models; “+”, $R^2 > 0.8$; “-”, $R^2 < 0.6$; “?”, R^2 between 0.6 and 0.8.

Additionally, we identified one mutated peptide P59 K**T**MGFSFPR (PDCHB9_R4M) with an arginine to methionine mutation. The peptide was found in S02730_03 presented by A*11:01 and was validated with a synthetic peptide ($R^2=0.8318$, Figure 8). The spectral comparison of the synthetic and naturally presented peptide sequences is presented in Figure 8. Since T cells of this patient are not available, the immunogenicity of this peptide cannot be demonstrated.

P59 K**T**MGFSFPR_mutR4M timsTOF

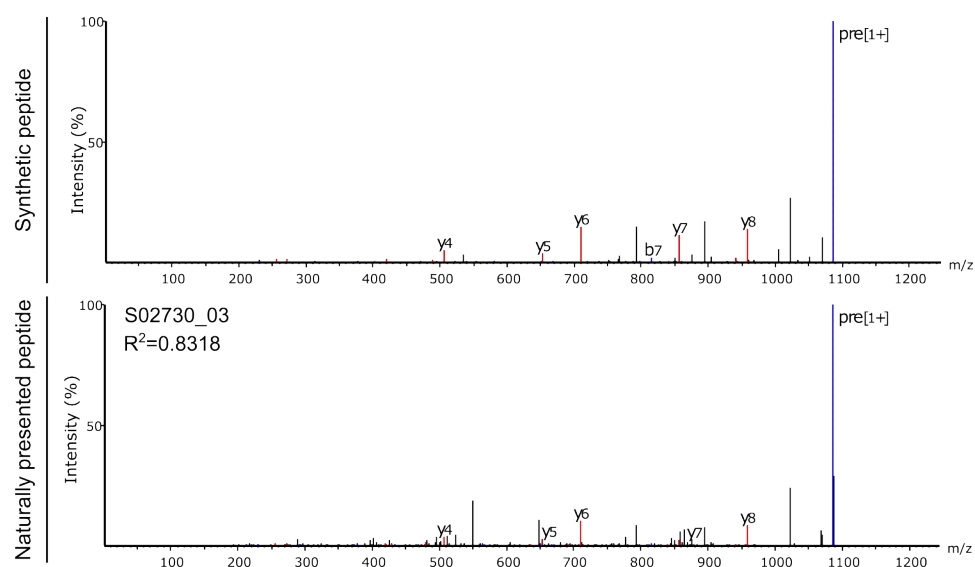


Figure 8. Spectral comparison of the synthesized and naturally presented mutated peptide KTMGFSFPR (PDCHB9_R4M, A*11:01, S02730_03) with timsTOF. Intensity [%] is plotted against the m/z ratio. Annotated b- and y-ions are indicated. The similarity between the synthetic and experimental spectrum was calculated (R^2).

For all selected peptide sequences presented in Table 2 and Table 3, MS spectrum validation with synthetic peptides and the timsTOF MS instrument was performed, validation with the QE is ongoing and will not be included in this thesis. The similarity between the spectra of the synthetic and all naturally presented peptides was computed (see 5.1.3.3 *Synthetic peptides and spectrum validation*). The correlation scores R^2 of the comparisons are listed in Supplementary Table 6 and 7 for the frequently shared and patient-individual peptides, respectively. A sequence was validated with a R^2 value >0.8 , indicated in green. A sequence was rejected with a R^2 value <0.6 , indicated in red. Spectra with a R^2 value between 0.6 and 0.8 were further analyzed for their retention time and visually inspected, indicated in yellow (Supplementary Table 6 and 7). For both lists, the top listed peptide (P1 and P26), as well as all peptide sequences that presented a R^2 value between 0.6 and 0.8, are illustrated in Supplementary Figure 2, 3, 4 and 5 and were additionally used for visual examination. Whether a sequence was validated is indicated in Table 2 and Table 3. Sequences that were directly confirmed with a R^2 value >0.8 are marked with “+”, sequences with a R^2 value <0.6 are marked with a “-”. Sequences with a

R^2 value between 0.6 and 0.8 are marked with a “?”, the retention time and the visual examination support their validation, however T cell test with autologous PBMCs would be needed to confirm the validation.

18 of 19 sequences that were found to be frequently shared between the samples were directly confirmed with the timsTOF. One sequence ALGPSVAAA (P2) presented R^2 values that ranged between 0.5541 and 0.7864, but the retention times were similar (see Supplementary Table 6). Visual examination of the spectra confirmed similarity (see Supplementary Figure 2). 13 sequences were identified with the timsTOF and the QE. Six sequences were solely identified with the QE.

18 of 26 sequences that were included in the list of individually interesting peptides were directly confirmed with the timsTOF. P29 NFDFQVQKF (Protein not on benign) was validated to be naturally presented by two samples (R^2 0.8833 and 0.9198) but gave no clear result for the third sample (R^2 0.7681, Supplementary Table 7). Visual examination of the spectra confirmed similarity (see Supplementary Figure 4). Two sequences P48 LKPGAPLPAL (ALKL1) and P49 EALLRVLLL (DLK1) could not be validated. Six sequences gave no clear results and would require validation with T cell assays. For P30 VLLDKIKNL, the similarity for one sample gave a R^2 of 0.79 but was clearly not confirmed for the other sample (R^2 0.0102, Supplementary Table 7). The MS spectra of P58 FLLDGSANV are “noisy”, but a similarity was visible (Supplementary Figure 5). R^2 values ranged between 0.5014 and 0.7017, however the retention times were comparable and ranged between 27 and 30 min (Supplementary Table 7). T cell assays could validate this peptide sequence that was, as discussed before, already described to be an interesting pan-cancer T cell epitope (141). Seven sequences were identified with the timsTOF and the QE. Eight sequences were solely identified with the QE. Synthetic peptide validation with the QE is ongoing but will not be included in the thesis.

Identification of SCLC specific tumor-exclusive HLA class II peptides

Equivalent to the already described procedure for HLA class I ligands, we performed comparative analysis for HLA class II peptides of SCLC samples from cryorecanalization or biopsy with the already mentioned published healthy datasets (123, 124, 128, 129) (see 5.1.4.1 *Identification of SCLC specific tumor-exclusive HLA class I ligands*, 5.1.3.3 *Comparative analysis* and Figure 9).

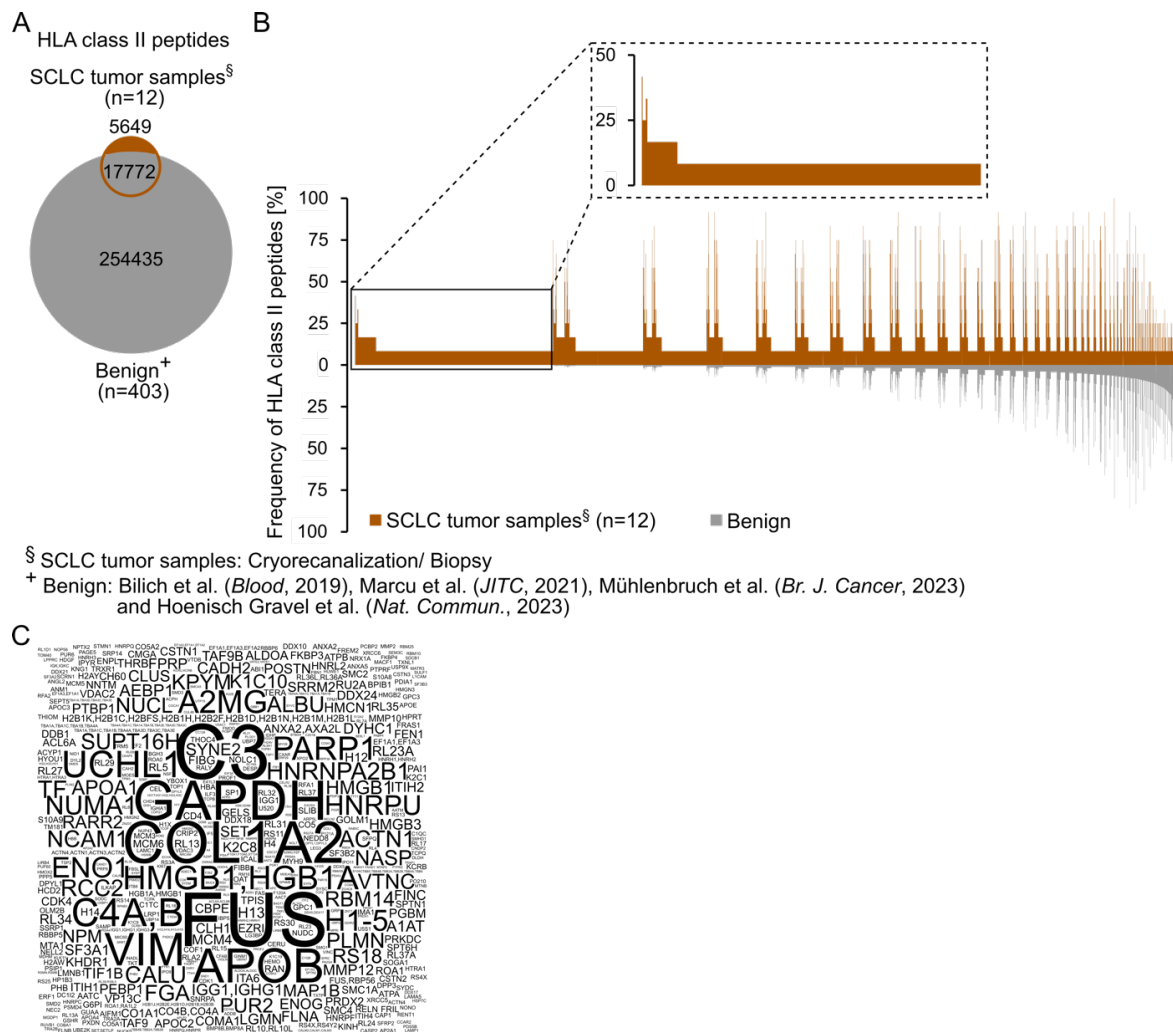


Figure 9. Comparative analysis of HLA class II peptides from SCLC tissue samples. The SCLC dataset presented in this graph comprises all HLA class II peptides extracted from cryorecanalization/ biopsy samples ([§]SCLC tumor samples, orange, n=12). The benign dataset (*Benign, grey, n=403) comprises all HLA class II peptides that were published in the indicated manuscripts. Venn diagram representing the overlap (A) and waterfall plot representing the frequency of the different peptide sequences (B) of HLA class II peptides identified on SCLC samples and on benign tissue. The waterfall plot includes all SCLC derived sequences but only benign data that was overlapping in A. One bar represents one peptide sequence, the higher the bar the more often the sequence was found within the respective dataset. Tumor-exclusive HLA class II peptides that were only found in SCLC samples and not in the benign dataset are marked with the square and illustrated enlarged. (C) Word cloud representing the protein sources of the tumor-exclusive HLA class II peptides. Only proteins that were represented by at least three different peptides are plotted. The bigger the font the more often the protein was represented.

Out of 23,421 HLA class II peptides, 5,649 tumor-exclusive HLA class II peptides were identified after overlapping the datasets of benign tissue (grey, n=403) and SCLC tissue

(orange, n=12) (Figure 9A). The waterfall plot presented in Figure 9B illustrates the frequency of single HLA class II sequences within the two datasets, on top peptides from SCLC samples (orange) and below peptides from healthy tissues (grey); all SCLC derived sequences but only benign data that was overlapping in Figure 9A are plotted. Tumor-exclusive HLA class II peptides that were only found in SCLC samples and not in the benign dataset appear on the left side of the graph, are marked with a square, and illustrated enlarged. Eight peptide sequences were identified with highest frequency in 41.7 % (5 of 12) of the samples.

These 5,649 tumor-exclusive HLA class II peptides derived from more than 1,700 different proteins. All proteins that were represented by at least three different tumor-exclusive HLA class II sequences are illustrated as a word cloud, the more often a protein was presented the bigger the font (Figure 9C). Complement C3, RNA-binding protein FUS, collagen type I α -2 (COL1A2), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and apolipoprotein B-100 (APOB) were represented most often in decreasing order by 92, 71, 55, 52 and 45 different tumor-exclusive peptide sequences, respectively. C3 and APOB, and a different collagen type (COL6A3) were also found to be most frequently represented by tumor-exclusive HLA class I ligands. As mentioned before, the complement system was described to be associated with cancer progression and poorer prognosis (139, 140), and APOB inhibition was associated with a reduced risk for SCLC (138). GAPDH is a key enzyme in glycolysis and plays a role in cell survival and tumor development and progression (145). Among the first 20 proteins, the SCLC-associated proteins PARP1 and NCAM1 (see Supplementary Table 1) are listed with 31 and 22 different peptide sequences. PARP1 was shown to be higher expressed in SCLC than in other lung cancers (146, 147) and PARP1 inhibitors are tested in clinical trials in SCLC. NCAM1 is a neuroendocrine marker with a high expression in SCLC also used in histology staining for diagnosis (15). Of the 5,649 tumor-exclusive peptides, more than 141 derived from proteins that are known to be associated with SCLC (literature search, presented in Supplementary Table 1), 590 were shared by at least two samples and 2573 were previously found on other malignancies, including brain and lung tumors (in-house database).

Interestingly, two of the selected HLA class I ligands were found to be embedded in HLA class II derived peptides that were extracted from SCLC. KSKEYFSKQK is embedded in KPGSDTIKPDVQKSKEYFSKQK that was identified in one sample and derived from Peroxiredoxin-1 (PRDX1). SLEAGLPLQV is embedded in EDSLEAGLPLQV that was identified in two samples and derived from CHGA, which is found to be highly expressed in SCLC (see Supplementary Table 1). Since both CD4⁺ and CD8⁺ T cell responses are required for an efficient T cell-mediated anti-cancer response, these sequences are of great interest (148).

5.1.4.2 Immunopeptidomics of soluble HLA from SCLC patient-derived plasma samples

As already mentioned, surgery is rarely performed in SCLC patients, limiting the access to SCLC tissue samples. Especially for cancer entities where tumor tissue is hardly accessible, liquid biopsies such as plasma samples could be a potential source of tumor antigens (see introduction section 2.4 *Immunopeptidomics*). Therefore, besides the immunopeptidomics analysis of SCLC tissue samples, we performed explorative immunopeptidomics of ligands from soluble HLA (sHLA) molecules that were present in plasma samples of SCLC patients.

We collected plasma at different timepoints from two different SCLC patient cohorts, with and without a matching tumor tissue sample.

Descriptive analysis of peptides extracted from sHLA of plasma samples from SCLC patients

Peptide yields plotting the numbers of HLA class I ligands and HLA class II peptides extracted from sHLA that were present in plasma samples of SCLC patients are presented in Figure 10. Sample ID, sample volume and whether patients were treated prior to sampling (pre-treatment) is indicated. Peptides identified with the QE, the timsTOF or with both devices are color coded. For most patients we analyzed two different timepoints of plasma collection. A total of 130,507 HLA class I ligands and 34,089 HLA class II peptides were eluted from sHLA (n=32). In mean, 4,078 HLA class I ligands (range: 567 to 9754) and 1,065 HLA class II peptides (range: 32 to 2,843) were identified per sample. The number of peptides extracted from sHLA was variable between the patients and also between timepoints of the same patient.

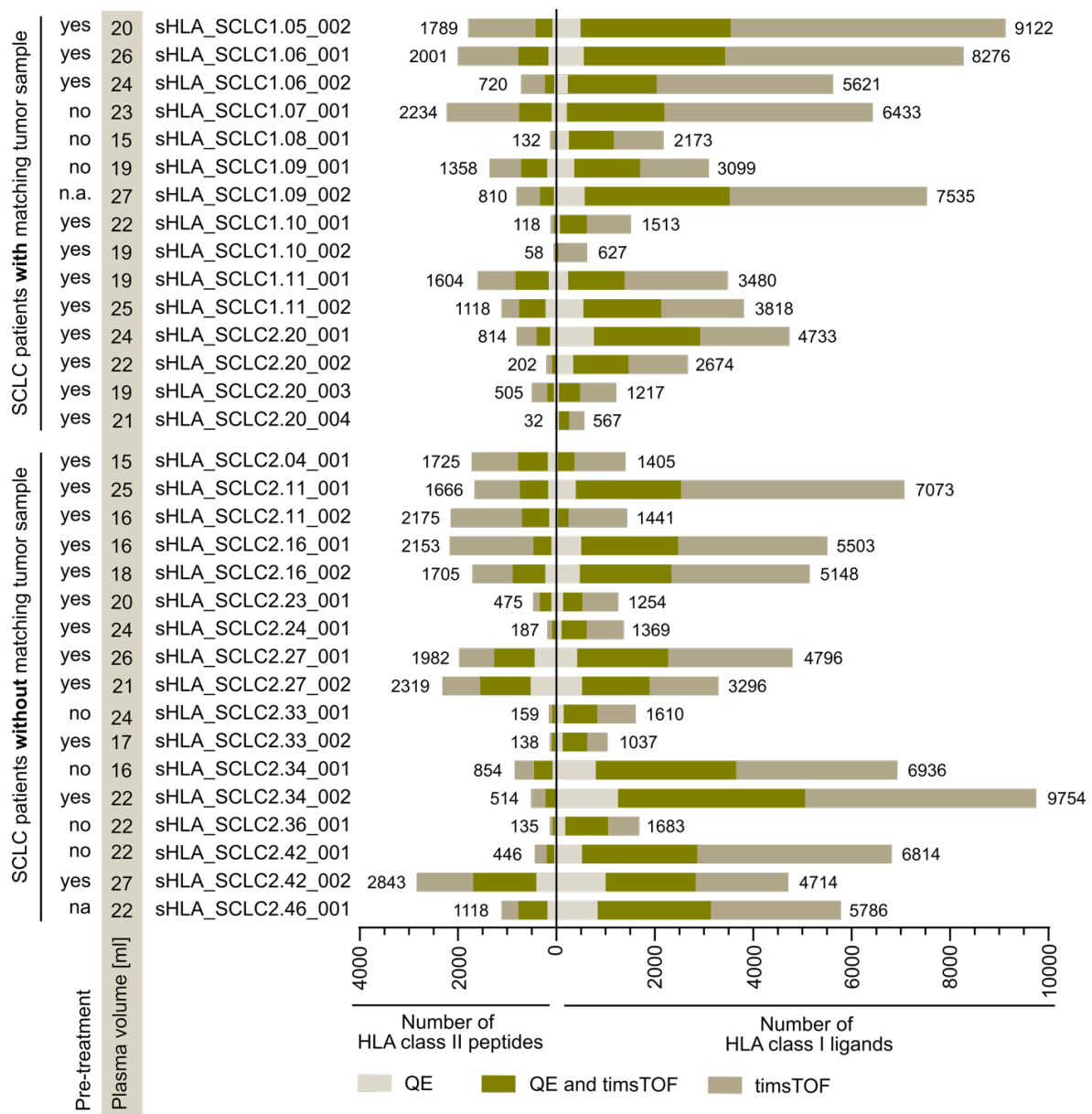


Figure 10. Peptide yields from plasma samples of SCLC patients. Bar plots indicating the number of HLA class I ligands and HLA class II peptides derived from soluble HLA (sHLA). On top samples of SCLC patients with a matching tumor sample and below samples of SCLC patients without a matching tumor sample are plotted. Whether treatment of the patient was applied before sampling (pre-treatment), plasma volume in ml and sample ID are indicated in the table (left). Peptides identified with the QE, the timsTOF or with both devices are color coded (light gold, dark gold, green, respectively).

The peptide length distributions are comparable between the plasma and the tissue samples for both HLA class I and II (compare Figure 11A, B and Figure 6A, B). As expected, most HLA class I ligands had a peptide length of nine aa (Figure 11A) and the majority of HLA class II peptides had a peptide length between 12 and 16 aa, with a peak at 15 aa (Figure 11B).

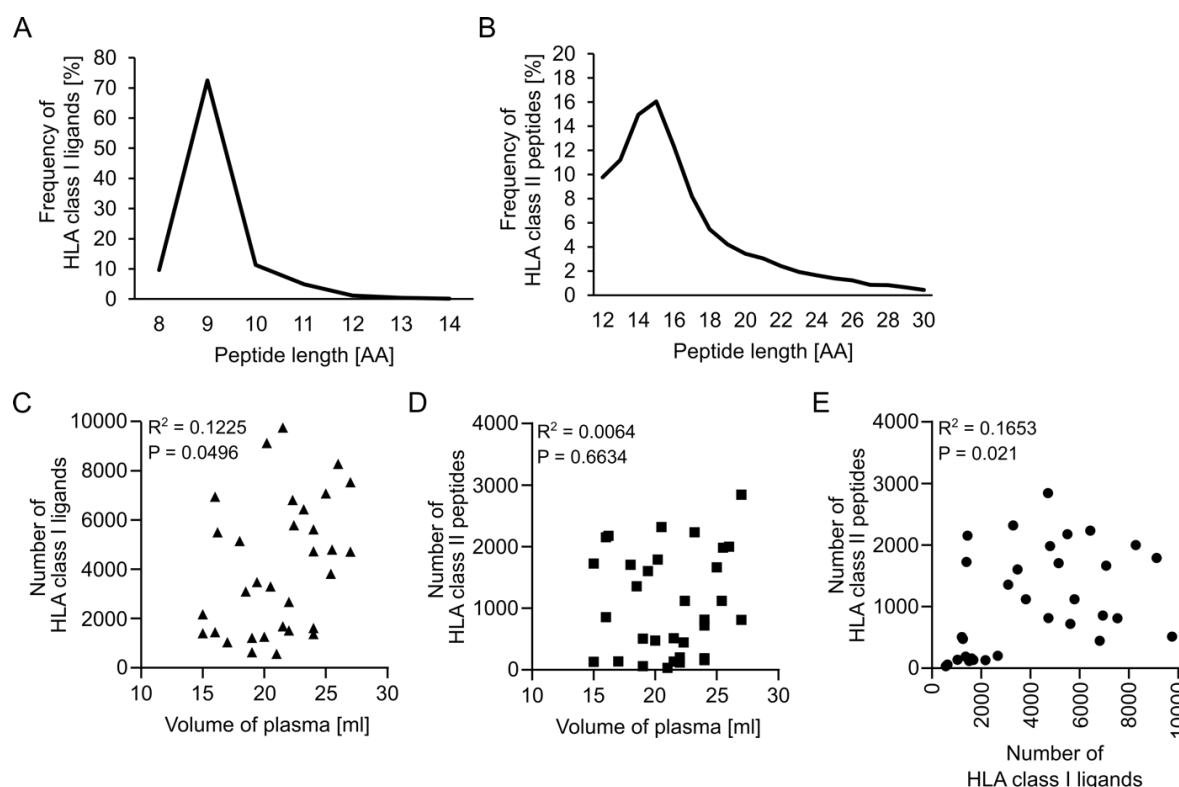


Figure 11. Characteristics of the peptides eluted from sHLA of plasma samples from SCLC patients. (A,B) Line plots representing the length distribution of eluted peptides. Frequency of HLA class I ligands (A) and HLA class II peptides (B) plotted for the respective peptide lengths. (C, D) Scatter plots illustrating the relationship between the number of HLA class I ligands (C) or HLA class II peptides (D) with the sample volume used for HLA immunoprecipitation. (E) Scatter plot illustrating the relationship between the number of HLA class I ligands and the number of HLA class II peptides extracted from plasma samples. R^2 and the respective P value are indicated.

In mean 1,908 HLA class I ligands and 516 HLA class II peptides were eluted from 10 ml of plasma. Higher plasma volume did not necessarily lead to a clear increase in HLA class I ligands ($R^2=0.1225$, $P=0.0496$, Figure 11C), while the correlation for HLA class II is not significant ($R^2=0.0064$, $P=0.6634$, Figure 11D). However, the number of HLA class I ligands and HLA class II peptides correlate with each other ($R^2=0.1653$, $P=0.021$, Figure 11E).

Generally, we were able to extract HLA class I ligands and HLA class II peptides from sHLA in plasma samples of SCLC patients. Higher numbers were identified for HLA class I than for HLA class II.

Comparative analysis of sHLA derived peptides from plasma samples of SCLC patients

We could show that a high number of peptides can be extracted from sHLA of plasma samples in SCLC patients. But it is not clear to which extent these ligands reflect a repertoire of tumor-specific peptides.

Similar to the comparative analysis performed for HLA-ligands of SCLC tumor tissue samples, we compared the peptides that were identified in plasma samples of SCLC patients with the already used published datasets of benign tissues (123, 124, 128, 129), but additionally included in-house datasets of peptides derived from sHLA of plasma from

healthy donors to identify “tumor-exclusive” peptides, that are referred to as patient-exclusive peptides for the plasma samples (see 5.1.3.3 *Comparative analysis*).

Comparative analysis of sHLA class I ligands

Comparative analysis identified 7,210 HLA class I ligands that appear to be patient-exclusive and not presented on healthy tissues or found in plasma of healthy donors (Figure 12A). The waterfall plot illustrates the frequency of sequences within the different datasets, on top HLA class I ligands from sHLA of plasma samples from SCLC patients (Figure 12B, green) and below ligands from plasma of healthy donors (light grey) and healthy tissues (dark grey); all SCLC derived sequences but only benign data that was overlapping in Figure 12A were plotted. SCLC patient-exclusive sequences that were only found in SCLC samples and not in the benign datasets appear on the left side of the graph, are marked with a square, and illustrated enlarged. NGFKSHAL that derived from complement C4 (C4A, C4B) was the most frequently shared HLA class I ligand in plasma samples and was found with a frequency of 37.5 % (in 12 of 32 SCLC plasma samples). Of interest, C4A and C4B were also highly represented in the dataset of tumor-exclusive HLA class I tumor ligands (see Figure 7C).

The dataset of SCLC patient-exclusive sequences of sHLA from plasma was compared with the dataset of tumor-exclusive sequences from SCLC tumor samples. However, for comparison reasons tumor-exclusive sequences of SCLC tumor samples were additionally subtracted with the dataset of plasma samples from healthy donors (in section 5.1.4.1, Figure 7, datasets of ligands from plasma samples of healthy donors were not subtracted). This resulted in 7,036 tumor-exclusive sequences from SCLC tumor samples for HLA class I (8,231 sequences identified in section 5.1.4.1 *Identification of SCLC specific tumor-exclusive HLA class I ligands* in Figure 7 and 7,036 ligands after subtracting data of sHLA from healthy donor plasma samples). These 7,036 tumor-exclusive sequences from SCLC tumor samples were compared to 7,210 patient-exclusive sequences from plasma samples of SCLC patients and identified 972 sequences that were shared between these two SCLC patient sample types (Figure 12C). Of note, three of the frequently shared sequences ALGPSVAAA, VPSEIDAAF and SPLDTSSVL (listed in Table 2) and three of the patient individual interesting sequences AFDSSLVFI, TFDENVSTY and IPLESTDGERL (listed in Table 3) were also found in either two or three plasma samples of SCLC patients.

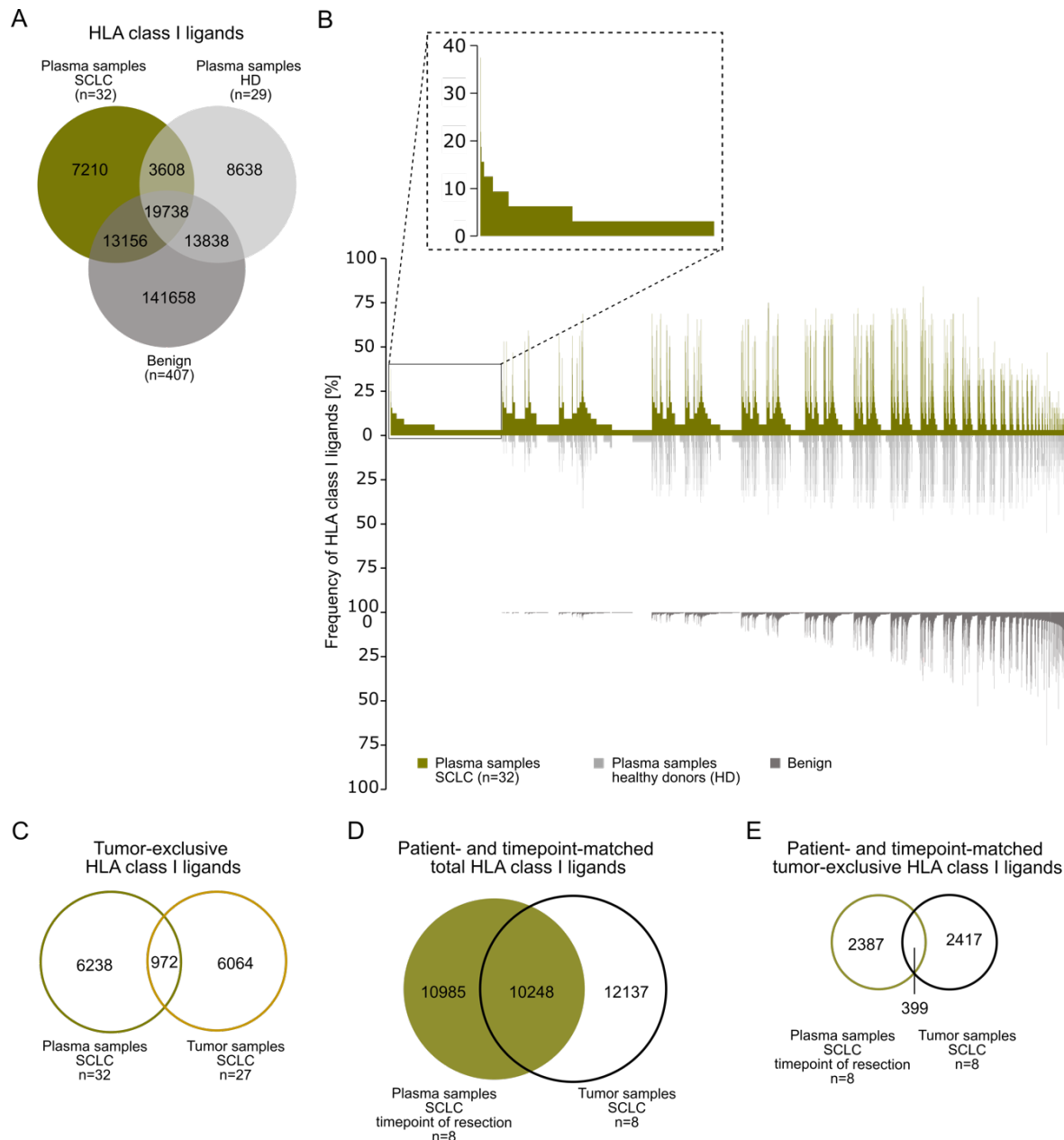


Figure 12. Comparative analysis of HLA class I ligands from sHLA of plasma samples obtained from SCLC patients. The SCLC dataset presented in this graph comprises all HLA class I ligands from sHLA of patients with and without a matching tumor sample (plasma samples SCLC, green, n=32). The benign datasets comprise in-house datasets of plasma samples from healthy donors (plasma samples HD, light grey, n=29) analyzed with the QE (n=20) and with the timSTOF (n=9) and published HLA class I ligands from benign tissue (benign, dark grey, n=407). A Venn diagram representing the overlap (A) and a waterfall plot representing the frequency of the different peptide sequences (B) of HLA class I ligands are plotted. The waterfall plot includes all SCLC derived sequences but only benign data that was overlapping in A. One bar represents one peptide sequence, the higher the bar the more often the sequence was found within the respective dataset. Patient-exclusive HLA class I ligands that were only found in plasma samples of SCLC patients and not on any healthy/ benign sample are marked with a square and illustrated enlarged. (C-E) Venn diagrams illustrating the overlap of HLA class I ligands from plasma samples or tumor samples of SCLC patients. (C) Overlap of patient-/tumor-exclusive ligands from plasma samples (green, identified in A and B, n=32) and tumor samples (yellow, n=27). Before the comparison, sequences that were found in plasma samples of healthy donors were additionally subtracted from the tumor-exclusive ligands of tissue samples that were identified in section 5.1.4.1 in Figure 7. (D) Overlap of ligands from patient- and timepoint-matched plasma (filled green, n=8) and tumor (black, n=8) samples. (E) Overlap of plasma/tumor-exclusive ligands from patient- and timepoint-matched plasma (green, n=8) and tumor (black, n=8) samples after subtraction of benign or healthy datasets.

The repertoire of the peptides found on sHLA in plasma samples might change under therapy. Therefore, a patient- and timepoint-matched comparison was performed (Figure 12D and E). Ligands extracted from the plasma samples at the timepoint of resection were compared to the ligands of their patient-matched tumor samples (n=8 timepoint matching samples) on a total ligandome level (Figure 12D) and on a plasma/tumor-exclusive level after subtraction of healthy datasets (Figure 12E). The subtraction of ligands from benign tissue or plasma of healthy donors decreased the overlap from 30.7 % (10,248 sequences, Figure 12D) to 7.7 % (399 sequences, Figure 12E) of HLA class I ligands.

Comparative analysis of sHLA class II peptides

Comparative analysis identified 4,153 HLA class II peptides that appear to be patient-exclusive and not presented on healthy tissues or found in plasma of healthy donors (Figure 13A). The waterfall plot in Figure 13B illustrates the frequency of sequences within the different datasets, on top HLA class II peptides from sHLA of plasma samples from SCLC patients (green) and below ligands from plasma of healthy donors (light grey) and healthy tissues (dark grey); all SCLC derived sequences but only benign data that was overlapping in Figure 13A were plotted. SCLC patient-exclusive sequences that were only found in SCLC samples and not in the benign datasets appear on the left side of the graph, are marked with a square, and illustrated enlarged. MVGIQGSDSVFSGFLLFPD that derived from complement C1q (C1Q) is the most frequently shared HLA class II peptide, with a frequency of 37.5 % (in 12 of 32 SCLC plasma samples).

The dataset of SCLC patient-exclusive sequences of sHLA from plasma was compared with the dataset of tumor-exclusive sequences from SCLC tumor samples. Again, for comparison reasons tumor-exclusive sequences of SCLC tumor samples were additionally subtracted with the dataset of plasma samples from healthy donors (which was not performed in section 5.1.4.1, Figure 9). This resulted in 5,390 tumor-exclusive sequences from SCLC tumor samples for HLA class II (5,649 sequences identified in section 5.1.4.1 *Identification of SCLC specific tumor-exclusive HLA class II peptides* in Figure 9 and 5,390 after subtracting data of sHLA from healthy donor plasma samples). These 5,390 SCLC tumor-exclusive sequences from SCLC tumor samples were compared to 4,153 patient-exclusive sequences from plasma samples of SCLC patients and 256 sequences were found to be shared (Figure 13C).

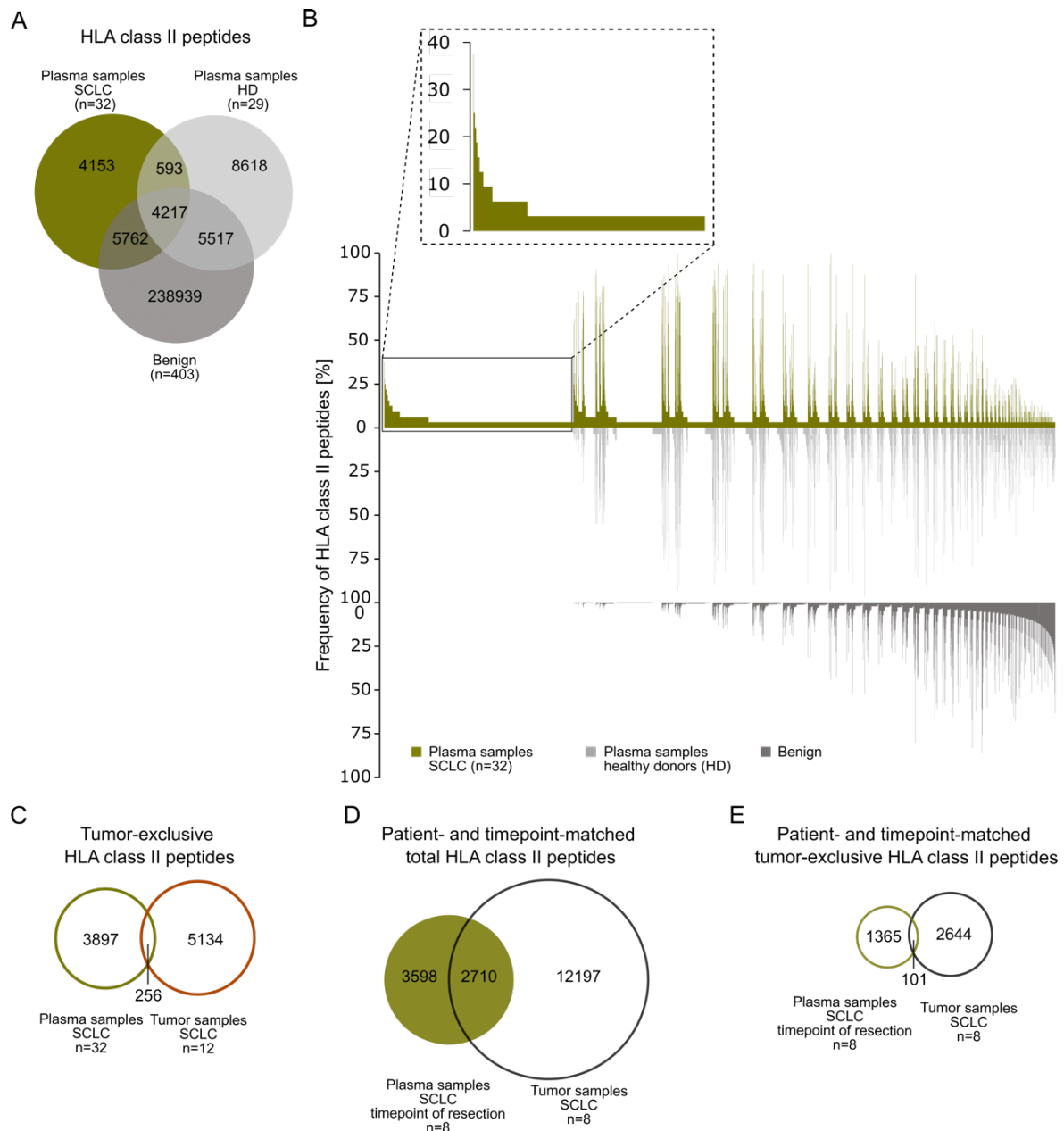


Figure 13. Comparative analysis of HLA class II peptides from sHLA of plasma samples that derived from SCLC patients. The SCLC dataset presented in this graph comprises all HLA class II peptides from sHLA of patients with and without a matching tumor sample (plasma samples SCLC, green, n=32). The benign datasets comprise in-house datasets of plasma samples from healthy donors (plasma samples HD, light grey, n=29) analyzed with the QE (n=20) and with the timsTOF (n=9) and published HLA class II peptides from benign tissue (benign, dark grey, n=403). A Venn diagram representing the overlap (A) and a waterfall plot representing the frequency of the different peptide sequences (B) of HLA class II peptides extracted from plasma samples of SCLC patients (green), plasma samples of healthy donors (light grey) and benign tissue (dark grey) are plotted. The waterfall plot includes all SCLC derived sequences but only benign data that was overlapping in A. One bar represents one peptide sequence, the higher the bar the more often the sequence was found within the respective dataset. Tumor-exclusive HLA class II peptides that were only found in plasma samples of SCLC patients and not on any healthy/ benign samples are marked with a square and illustrated enlarged. (C-E) Venn diagrams illustrating the overlap of HLA class II peptides from plasma or tumor samples of SCLC patients. (C) Overlap of tumor-exclusive peptides from plasma samples (green, identified in A and B, n=32) and tumor (red, n=12) samples. Importantly, before comparison sequences that were found in plasma samples of healthy donors were additionally subtracted from the tumor-exclusive peptides of tissue samples, identified in section 5.1.4.1 see Figure 9. (D) Overlap of all peptides from patient- and timepoint-matched plasma (filled green, n=8) and tumor (black, n=8) samples. (E) Overlap of patient/tumor-exclusive peptides from patient- and timepoint-matched plasma (green, n=8) and tumor samples (black, n=8) after subtraction of benign or healthy datasets.

As already mentioned, the repertoire of the peptides found on sHLA in plasma samples might change under therapy, peptides extracted from the plasma samples at the timepoint of resection were compared to peptides from their patient-matched tumor samples (n=8 timepoint matching samples) on a total ligandome level (Figure 13D) and on a patient-/tumor-exclusive level (Figure 13E), approx. 14.6 % (2710 sequences, Figure 13D) of sequences are overlapping, which is narrowed down to an overlap of 2.5 % after subtraction of healthy datasets (101 sequences, Figure 13E).

Overall, we could identify a high number of patient-exclusive peptides (7,210 and 4,153 HLA class I and II, respectively) in plasma samples of SCLC patients that were never found on any healthy tissue or in plasma samples of healthy donors. Patient-/tumor-exclusive peptides deriving from complement appear to be abundant in SCLC samples (plasma and tumor tissues, HLA class I and II) and not found to be presented on healthy tissues or in plasma samples of healthy donors (see also Figure 7C, Figure 9C), again suggesting that they are potentially interesting targets.

The overlap between patient-exclusive peptides from plasma samples of SCLC patients and tumor-exclusive peptides from SCLC tumor tissue (Figure 12C/Figure 13C) was however limited (7.3 % for HLA class I and 2.8 % for HLA class II). For eight SCLC patients, timepoint-matched sample pairs of tumor tissue and plasma from the timepoint of resection were available. Interestingly, the ratio between overlapping peptides and peptides that were particularly found in one (tumor tissue) or the other sample type (plasma sample) changed after subtracting the healthy datasets (compare Figure 12D to E, and Figure 13D to E). The overlap decreased from 30.7 % to 7.7 % for HLA class I and from 14.6 % to 2.5 % for HLA class II after subtracting the healthy datasets. These results indicate that peptides from sHLA of plasma samples partially reflect the repertoire of peptides from tumor samples (overlap of 30.7 % for HLA class I and 14.6 % for HLA class II), however, most of these peptides are also found in healthy tissues or plasma from healthy donors and are not suitable to be used as T cell targets in immunotherapy. Patient-/tumor-exclusive peptides that were never found on any healthy tissue or in plasma of healthy donors were shared to a much lesser extent between the plasma and the tumor samples (7.7 % in Figure 12E and 2.5 % in Figure 13E for HLA class I and II, respectively), suggesting that it is difficult to identify potential T cell antigens in the plasma samples that are directly reflecting the tumor antigen repertoire. However, we indeed identify many patient-exclusive peptides in plasma samples of SCLC patients that were never found in any healthy dataset. These may reflect interesting patient-specific antigens that require further in-depth analysis, including analysis of source proteins for potential SCLC or cancer associations. Bigger datasets of

plasma samples from healthy donors are needed for more comprehensive analysis of potentially tumor-specific antigens.

First analysis of two SCLC patients, comparing the sHLA ligandome of plasma samples that were collected at different timepoints, revealed an overlap of approx. 30 % between different plasma samples (data not shown). This indicates a similar overlap between plasma and tumor samples as well as plasma and plasma samples, which should be treated with caution at this stage. It should be noted that the plasma samples were collected under therapy, which might also lead to changes in the peptide repertoire between the different timepoints of plasma collection. Analysis of plasma samples from all collected SCLC patients, as well as comparison of the peptide repertoire between different timepoints of plasma collection after subtraction of peptide datasets obtained from healthy tissues or plasmas of healthy donors could be performed to obtain a more complete picture.

5.1.5 Conclusion and outlook

This is the first study that extensively analyzed potential T cell antigens in SCLC by mass spectrometry-based immunopeptidomics that could be used as T cell targets for T cell-based immunotherapy of SCLC. We exploited different SCLC samples, tumors obtained from fresh patient material (cryorecanalization or biopsy) and tumors obtained from xenograft models (PDX or CDX), and extracted in total >112,000 naturally presented HLA class I and II peptides from these samples. By comparative analysis with datasets of >2,000,000 HLA class I and II peptides that were found to be presented on benign tissues (published by our Department/ Institute of Immunology (123, 124, 128, 129)), we identified >13,800 tumor-exclusive HLA class I and II peptides, that are referred to as SCLC- or tumor-specific due to the absence on healthy tissues. These tumor-specific antigens represent potential T cell targets and could be applied in immunotherapeutic approaches such as peptide-based vaccination.

We were focusing on the identification of tumor-associated antigens deriving from non-mutated wild type protein sequences, however, if available, we included mutated amino acid sequences on patient individual basis for data analysis. Mass spectrometry measurements were performed with Orbitrap-based and timsTOF instruments, which expanded the repertoire of identified peptides and could be a beneficial strategy for future studies, analysis is however more labor-intensive. Despite the generally low tissue mass (mean: 118 mg, range: 9 to 641 mg) that was available for immunopeptidomics analysis, a considerable number of HLA-bound peptides was extracted. HLA class I ligands were analyzed for both sample types xenograft models and cryorecanalization/biopsy and HLA class II peptides for cryorec/biopsy only.

Xenograft models are valuable samples for immunopeptidomics since surgery is rarely performed in SCLC, limiting the access to tumor samples. First measures, including MS method adaptation, avoiding TissueTek and sample selection based on a high HLA expression, led to improved peptide recovery from xenograft samples. The identification of tumor-exclusive antigens from xenografts by comparative analysis with human datasets of published HLA ligandomics data from benign tissue should be performed with care and measures to identify and exclude potential mouse-derived contaminants are necessary. We adapted the analysis strategy by using the combined proteome of human and mouse for peptide sequence annotation. Of note, one xenograft sample from 2D-cultured cells of a xenograft tumor presented an exceptionally high number of HLA class I ligands and low mouse contamination. To identify tumor-exclusive antigens within the xenograft tumors, we were only considering peptides that were annotated to derive from human proteins only (see Figure 3). Tumor-exclusive ligands that were found in cryorec/biopsy samples were, however, compared to the xenograft-derived dataset of peptides that could either derive from human or mouse proteins, to check for the presence and the frequency of these ligands in this dataset. We were able to identify tumor-exclusive peptides as well as one mutated neoantigen in SCLC xenografts. However, in order to detect and exclude potential mouse contaminations, the mouse and human reference proteomes were combined for the database search in xenograft samples, resulting in an almost doubling of the search space. An increase in the search space can lead to an increase in false identified peptides. To test whether mouse-derived peptides in xenograft samples were only identified due to the increased search space, the cryorec/biopsy dataset was retrospectively also analyzed with the combined human and mouse proteomes. This showed that significantly more peptides were assigned to the mouse proteome in the xenograft samples than in the cryorec/biopsy samples suggesting that our strategy for xenograft samples does not substantially increase false identified peptides. One limitation of our analysis strategy for xenograft-derived peptides is that only peptides that have been exclusively assigned to human proteins are considered in the downstream analysis (see Figure 3). Thereby we might miss tumor-exclusive peptide sequences that originate from protein sequences that are identical in human and mouse proteins ("human/mouse"). We partially solved this problem by comparing the tumor-exclusive dataset from cryorec/biopsy with the dataset of "human/mouse" peptides from xenograft samples. However, "human/mouse" peptides that were not found in cryorec/biopsy samples will not be considered.

Altogether, 8,231 and 5,649 SCLC-specific tumor-exclusive HLA class I and II peptides, respectively, were identified and found to be shared between several SCLC samples or by

other malignancies, derived from proteins that were associated with SCLC or cancer-related, or derived from proteins that were never found to be represented on published benign tissue, among other protein sources. Only a limited number of peptides can be further tested in T cell assays. Based on two different approaches, 58 tumor-exclusive HLA class I ligands were selected and will be tested for immunogenicity, which will be part of follow up studies. In the warehouse approach, frequently shared peptides were selected that were found to be presented by different SCLC tumor samples and on common HLA types, like A*02:01, A*03:01, B*07:02, B*08:01 among others (25 HLA class I ligands). A warehouse with pre-selected, pre-characterized and pre-manufactured peptides of common HLA types could be used in clinical trials, creating personalized vaccination cocktails by selecting peptides out of the warehouse according to the HLA type of the patient and optimally also on antigen expression of the individual tumors. In the individualized approach, for SCLC patients with available PBMC samples, we selected peptides that derived from proteins that were associated with SCLC, were cancer-related or were never found to be represented on published benign tissue (33 HLA class I ligands). All selected peptide sequences need to be tested in immunogenicity assays in recall experiments using autologous or HLA-matched PBMCs of SCLC patients or by aAPC priming with PBMCs from healthy donors.

Analysis of HLA class II presented peptides was performed throughout this study and tumor-exclusive peptides were identified. The origin of HLA class II bound peptides could be either the tumor cells themselves (40) or tumor infiltrating cells such as TAMs, CAFs, B cells or DCs, which all express HLA class II. Interestingly two of the interesting HLA class I sequences that were selected for immunogenicity screenings (Table 2 and 3) were found to be embedded in tumor-exclusive HLA class II peptides. HLA class II peptides require more in depth analysis and need to be filtered according to similar criteria as HLA class I ligands, to select a number of HLA class II peptides that could be tested in immunogenicity screenings with PBMC samples of SCLC patients or in monocyte derived DC primings.

As mentioned, access to SCLC tumor samples is limited and liquid biopsies such as plasma samples could be a potential source of tumor antigens (149). Therefore, we performed explorative immunopeptidomics of ligands from soluble HLA (sHLA) molecules that were present in plasma samples of SCLC patients. We extracted more than >160,000 HLA class I and II peptides from sHLA in plasma samples and identified >11,300 patient-exclusive peptides by comparative analysis. The overlap of tumor-exclusive peptides from tumor samples and patient-exclusive peptides from plasma samples was however limited. The patient-exclusive peptides in plasma samples of SCLC patients that were never found in any healthy dataset might still reflect interesting patient-specific antigens that require

further in-depth analysis, including analysis of source proteins for potential SCLC or cancer associations. The role of the peptidome from sHLA of plasma samples from SCLC patients therefore requires further investigation and bigger datasets of plasma samples from healthy donors are needed for more comprehensive analysis of potentially tumor-specific antigens.

Future sample collection should consider 2D-cultured cells from xenograft models as valuable sample source as well as IFN- γ treatment of cultured cells or xenograft models, which might lead to increased HLA expression on tumor cells thereby improving the peptide recovery from samples of xenograft models. Future immunopeptidomics studies in SCLC could include more samples of less frequent HLA types to increase the population coverage, different subtypes of SCLC to identify distinct but also overlapping targets on ligandome level, and the selective analysis of relapsed and therapy resistant SCLC tumors to combat rare tumor clones that are potentially involved in relapse. So far, we generated a highly valuable mass spectrometry dataset of naturally presented HLA-peptides in SCLC that is available for further examination, including analysis of other tumor-antigens such as cryptic peptides, microbe-derived peptides or posttranslational modified peptides.

As mentioned, all selected potential T cell antigens need to be tested in immunogenicity assays. T cell responses can be assessed by different read outs, including flow cytometry-based methods. The optimization and further development of a flow cytometry-based T cell assay using the detection of activated β_2 -integrins for identification of antigen-specific T cells is presented in the following result parts II and III.

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5.2 Part II

Integrin activation enables sensitive detection of functional CD4⁺ and CD8⁺ T cells: application to characterize SARS-CoV-2 immunity

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Supplementary data part II see pages 190 to 196.

The author of this thesis was involved in the conception of this study. The author performed experiments presented in Figure 10 and some of the experiments presented in Figures 7 and 8. In addition, the author performed an extensive series of experiments for assay optimization which are discussed in the manuscript, as well as a series of experiments during the revision process, which were not published but sent to reviewers only. The author gave intellectual input, was involved in the interpretation of the data, figure preparation, writing and finalizing the manuscript.

5.2.1 Abstract

We have previously shown that conformational change in the β_2 -integrin is a very early activation marker that can be detected with fluorescent multimers of its ligand ICAM-1 for a rapid assessment of antigen-specific CD8⁺ T cells. In this study, we describe a modified protocol of this assay for sensitive detection of functional antigen-specific CD4⁺ T cells using a monoclonal antibody (clone m24 Ab) specific for the open, high affinity conformation of the β_2 -integrin. The kinetics of β_2 -integrin activation was different on CD4⁺ and CD8⁺ T cells (several hours vs. few minutes, respectively); however, m24 Ab readily stained both cell types 4-6h after antigen stimulation. With this protocol, we were able to monitor *ex vivo* effector and memory CD4⁺ and CD8⁺ T cells specific for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and hepatitis B virus (HBV) in whole blood or cryopreserved peripheral blood mononuclear cells (PBMCs) of infected or vaccinated individuals. By costaining β_2 -integrin with m24 and CD154 Abs, we assessed extremely low frequencies of polyfunctional CD4⁺ T cell responses. The novel assay used in this study allows very sensitive and simultaneous screening of both CD4⁺ and CD8⁺ T cell reactivities, with versatile applicability in clinical and vaccination studies.

5.2.2 Introduction

One of the first events that can be detected upon T cell receptor (TCR)-mediated stimulation of T cells is the activation of membrane-bound β_2 -integrins. This activation involves a conformational change (from the bent, inactive form, to the open, high affinity conformation) and a clustering of integrin molecules on the cell membrane. Activated integrins are essential for ligand binding [e.g. the binding of the β_2 -integrin leukocyte function-associated antigen (LFA)-1 to intercellular adhesion molecule (ICAM)-1], for immunological synapse formation, and for downstream cell functions.

In cytotoxic effector CD8⁺ T cells (CTLs), β_2 -integrins participate in the interaction with target cells (e.g., virus-infected or cancer cells), facilitating downstream functions, such as directed granzyme, perforin, and cytokines secretion, and, ultimately, cell killing (1-4). A good correlate to these CD8⁺ T cell functions is, therefore, an integrin conformational change, as it can be detected earlier than the conventional flow cytometry cytotoxic markers, such as CD107a and tumor necrosis factor (TNF). Using fluorescent multimers of ICAM-1, we have previously shown that effector CD8⁺ T lymphocytes can be specifically and very rapidly detected upon antigen-specific T cell stimulation. We observed that the binding of ICAM-1 multimers to β_2 -integrins on CD8⁺ T cells identifies functional effectors that will later degranulate and produce effector cytokines. In addition to being a quick readout (approximately after 4-20 min stimulation in most cases) based on the use of a single detecting reagent, the assay preserves cell viability and can be used for the

enrichment of antigen-responding CD8⁺ T cells for an immediate analysis or for a post *in vitro* expansion analysis (5, 6).

CD4⁺ T helper cells primarily interact with major histocompatibility complex (MHC)-class II antigen-presenting cells and exert fundamentally different functions in comparison to CD8⁺ T cells. The main effector molecules of effector CD4⁺ T cells are cytokines and costimulatory receptors, such as CD154 (CD40L), all of which are routinely used for measuring CD4⁺ T cell immune responses (7, 8). The formation of immunological (of CD4⁺ T cells) and cytotoxic (of CD8⁺ cells) synapses differs substantially. The former takes hours to be established and is stable once formed, whereas the contacts formed by CD8⁺ T cells are brief and CD8⁺ T cells can establish multiple cytotoxic synapses over a short period of time (9, 10). The kinetics of β_2 -integrin activation on CD4⁺ T cells is not known so far, but based on the properties of synapse formation, we can expect it to be slower than that of CD8⁺ T cells. Still, the integrin activation should occur earlier than the expression of cytokines and CD154, and, thereby, might provide a fast and easy detectable surface marker to be used for monitoring and isolation of CD4⁺ T cells.

In the present study, we describe a modified protocol of the ICAM-1 multimer assay, which is also taking advantage of β_2 -integrin activation on T cells but was adapted for sensitive detection of antigen-specific CD4⁺ effectors. Instead of ICAM-1 multimers, a monoclonal antibody (Ab) specific for the open, high affinity conformation of the β_2 -integrin (Ab clone m24 (referred hereafter as m24 Ab); (11, 12)) combined with the ethylenediaminetetraacetic acid (EDTA) treatment to disrupt the cell conglomerates formed during CD4⁺ T cell activation, was used. The assay can be used for simultaneous screening of both CD4⁺ and CD8⁺ T cell reactivities and should be useful for high-throughput detection of antigen-specific T lymphocytes, epitope mapping studies, and the monitoring of clinical trials. Staining with m24 Ab together with CD154 enables sensitive and accurate detection of extremely low frequencies of polyfunctional CD4⁺ T cells. The study presents examples of antigen-specific CD4⁺ and CD8⁺ T cell detection in whole blood (WB) or cryopreserved peripheral blood mononuclear cells (PBMCs) obtained from cytomegalovirus (CMV)- and Epstein-Barr virus (EBV)-infected volunteers and from hepatitis B virus (HBV) vaccinees. In addition, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-directed T cells were readily detected in convalescents who have previously been tested positive for the virus and in an individual who received a SARS-CoV-2 peptide vaccine.

5.2.3 Materials and methods

Study subjects and blood samples

First, we obtained either PBMC concentrates or Na-heparinized blood from healthy individuals. Following donors were selected for the study: one person with detectable CD4⁺ T cells binding to the HLA-DRB1*11 multimer refolded with the CMV peptide HPTFTSQYRIQGKLE; two HLA-A*02⁺ people with detectable CD8⁺ T cells binding to the HLA-A*02:01 multimer refolded with the CMV NLVPMVATV; one HLA-A*02⁺ person with detectable CD8⁺ T cells binding to the HLA-A*02:01 multimer refolded with the EBV BRLF1 peptide YVLDHLIVV and a CD4⁺ T cell reactivity to an in house-made HLA-class II peptide-pool containing nine CMV, EBV and Flu-derived epitopes. Two individuals vaccinated with hepatitis B vaccine (Engerix-B, GlaxoSmithKline, London, UK) approximately 10 years ago were also included. PBMCs were isolated by cell density centrifugation (Biocoll, Biochrom AG, Berlin, Germany), were washed twice in phosphate buffered saline (PBS) without Ca²⁺/Mg²⁺, and were frozen in aliquots in 90% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA) + 10% dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany).

Na-heparinized blood was also obtained from three convalescent persons who had been recently diagnosed with the SARS-CoV-2 infection (either by PCR testing after nasopharyngeal swab or by serological Ab testing) and additionally from four uninfected control donors. Na- heparin plasma was isolated by centrifugation. Anti-CoV-2 Ab testing was performed with the SARS-CoV-2 ELISA from EUROIMMUN, (Lübeck, Germany). Tests were specifically conducted for detecting spike protein domain 1 (S1)-specific immunoglobulin G (IgG) and nucleocapsid (N)-specific IgG. ELISA was performed according to the instructions of the manufacturer. All three individuals developed serum IgG against S1 and/or N at the time of T cell testing, whereas unexposed control donors did not reveal any IgG reaction, as shown in Figures 7, 8. All studies were approved by the Ethics Committee of the University of Tübingen (approvals 156/2012B01, 713/2018B02, and 256/2020B02), and participants provided written informed consent.

A self-experimenting healthy volunteer was vaccinated with SARS-CoV-2-derived peptides, with details presented in a study by Rammensee et al. (13 Preprint). Briefly, the donor received subcutaneous injections of synthetic peptides emulsified in Montanide™ ISA (Seppic SA, Paris, France) together with the TLR 1/2 ligand XS15 as an adjuvant. This experimental vaccine contained, among others, the four following HLA-class II peptides: CMV/YQE (which had been already applied in 2017), as well as CoV/IGY, CoV/ASA, and CoV/FYV (“Peptides, stimuli, and pMHC tetramers” section). According to the results of an interferon-gamma (IFN-γ) ELISpot experiment performed ~3 weeks after vaccination, all

four peptides had induced a T cell response *in vivo* (13 Preprint). For the experiment shown in Figure 9, blood was obtained ~6 weeks after vaccination.

Characteristics of all donors (n=11 healthy individuals without history of CoV-2 infection, and n=3 CoV-2 convalescents) are summarized in Table 1.

Table 1. Demographic characteristics of the participants

Participants #	Age	Gender	Tested specificities	Corresponding Figures
#1	60	male	CMV, SEB	1, 3, 5, 12, S1, S3, S5
#2	32	male	CMV, SEB	2, 3, 4, S2
#3	30	male	HBV, SEB	3, 4, 6, 12, S3, S5
#4	49	male	HBV	3, 12, S4
#5	31	male	SEB	3, 4
#6	67	male	CoV-2	9, 12
#7	53	female	MHCII peptides, EBV	10
CoV-2 #1	51	male	CoV-2	7, 8, 12
CoV-2 #2	63	female	CoV-2	7, 8, 12
CoV-2 #3	50	female	CoV-2	7, 8, 12
UD #1	27	female	CoV-2	7, 8
UD #2	29	female	CoV-2	7, 8
UD #3	22	female	CoV-2	7, 8
UD #4	53	female	CoV-2	7, 8

Peptides, stimuli and pMHC tetramers

For antigen-specific stimulation, we used the following synthetic peptides, representing known T cell epitopes derived from virus-associated antigens: for CD4⁺ T cell stimulation, HLA-DRB1*11:01-restricted HPTFTSQYRIQGKLE peptide from CMV pp65, aa 366-380 (CMV/HPT, donor #1) and a mix of nine HLA-class II binders from various proteins of CMV (n=2), EBV (n=6) and flu (n=1) viruses (donor #7); for CD8⁺ T cell stimulation, the HLA-A*02-restricted NLVPMVATV peptide from CMV pp65, aa 495-503 (CMV/NLV, donor #2), and the HLA-A*02-restricted YVLDHLIVV peptide from EBV BRLF1, aa 109-117 (EBV/YVL, donor #7).

HLA-class II peptides used for experimental CoV-2 immunization were CMV pp65, aa 510-524 YQEFFWDANDIYRIF (CMV/YQE); SARS-CoV-2 nucleocapsid (N), aa 83-98 IGYRRATRRIRGGD (CoV-2/IGY) and aa 311-326 ASAFFGMSRIGMEVT (CoV-2/ASA), and SARS-CoV-2 envelope (Env), aa 56–70 FYVYSRVKLNSSRV (CoV-2/FYV, donor #6). All peptides were synthesized and dissolved as previously described (purity ≥ 80%) (14). We also used pools of overlapping 15-mer peptides spanning the entire HBV/Env (donors #3 and #4), SARS-CoV-2/Prot N, or SARS-CoV-2/Prot M or covering the immunodominant sequence domains of SARS-CoV-2/Prot S for activation. HBV/Env peptide pool (JPT, Berlin, Germany) was dissolved in 100% DMSO, whereas all SARS-CoV-2 pools (Miltenyi, Bergisch

Gladbach, Germany) in 10% DMSO. All were aliquoted and kept at -80 °C until further use. Staphylococcus enterotoxin B (SEB; Sigma-Aldrich, Darmstadt, Germany) was solved in PBS without Ca²⁺/Mg²⁺ and frozen at -20°C in 1 mg/ml aliquots. As a negative control, we used cells in medium alone (control experiments showed that the background activation was not significantly different between cells cultured alone, or in the presence of mock DMSO/water, or of control HLA-class II binding peptides derived from self or the HIV virus, data not shown).

We produced biotinylated pHLA-A*02:01 monomers (CMV A*02/NLV, donor #2) in-house by conventional refolding, as previously described (15). We generated fluorescent pHLA-A*02:01 tetramers by coincubating streptavidin-phycoerythrin (PE) or -allophycocyanin (APC) (BioLegend, San Diego, CA, USA and Thermo Fisher Scientific, Waltham, MA, USA) at a 1 (streptavidin):4 (pHLA-A2 monomer) molar ratio. Multimers were aliquoted and stored at -80°C in a Tris-buffered saline (TBS) buffer containing 16% glycerol (15). PE-labeled DRB1*11:01 CMV/HPT tetramer was kindly provided by NIH Tetramer Core Facility (Atlanta, GA, USA) and stored at 4°C until use (donor #1).

Production of human ICAM-1 multimers

Soluble fluorescent ICAM-1 complexes were produced as described previously by Dimitrov et al. (5). We generated fluorescent ICAM-1-Fc/anti-Fc multimeric complexes by co-incubating 200 µg/ml recombinant human ICAM-1-Fc [produced and purified as previously described (16) or bought from BioLegend (San Diego, CA, USA; cat. number 552906)] with 3.41× diluted polyclonal anti-human Fc-PE F(ab')₂ fragments (Jackson ImmunoResearch Labs Inc., West Grove, PA, USA) at 4°C for 24h while slightly rotating (15 rpm). For example, to prepare 100 µl of 200µg/ml ICAM-1 multimers using ICAM-1-Fc from BioLegend (San Diego, CA, USA), we mixed 24.4 µl ICAM-1-Fc (4.1× dilution, final concentration 200 µg/ml) with 29.3 µl Fc-PE F(ab')₂ fragments (3.41× dilution) and 46.3 µl sterile PBS. The stock solutions of the anti-human Fc-PE F(ab')₂ fragments, which came lyophilized, were prepared according to the recommendation of the manufacturer (diluted in 1ml Milli-Q water, Merck Millipore, Darmstadt, Germany). We confirm from experience the stability of the multimeric ICAM-1 complexes for at least 2 months when stored at 4°C. For longer storage, ICAM-1 multimers can be kept at -80°C, but not at -20°C, as this increases significantly the background staining.

Cell stimulation, m24 Ab or ICAM-1 multimer stainings

We used fresh heparinized blood or frozen PBMCs for the assays. WB was used within 2 h after collection. Cryopreserved PBMCs were thawed, washed, and rested overnight in T cell medium (TCM) [IMDM (Lonza) 10% heat-inactivated human serum and 100 U/ml penicillin/ 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 50 µM β-

mercaptoethanol (Merck)] containing 1 µg/ml DNase I (Sigma-Aldrich, St. Louis, MO, USA), and then they were washed and counted on the Neubauer chamber Thermofisher, Waltham, MA, USA) before stimulation and staining.

We stimulated WB (up to 1 ml per test) or PBMCs at 2×10^6 cells/ml (1000 µl/test) or 1×10^7 cells/ml (200 µl/test) in TCM, for the indicated times at 37 °C (water bath or incubator) in 5 ml or 15 ml Falcon tubes (BD Biosciences, Heidelberg, Germany) with the following peptides: 4 µg/ml of the single peptides CMV/HPT, CMV/HQE, CoV/IGY, CoV/ASA, CoV/FYV, EBV/GLC, a pool of EBV/YVL and 9 HLA-class II binders with 4 µg/ml per peptide; 2 µg/ml of the peptide pools HBV/Env, SARS-CoV-2/ S, CoV-2/ N, CoV-2/M; or 4 µg/ml SEB.

After stimulation, we left the tubes at room temperature (RT) for 1-4 min and stained the cells at RT with a pre-titrated amount (1:80 to 1:200) of the m24 Ab clone (BioLegend, San Diego, CA, USA) labelled with PE. After 15-min incubation, EDTA was added for another 10 min at RT at a final concentration of 4 mM. Whenever indicated, the cells were also stained with HLA-A*02/ pMHC multimers (0.6 µg/ml).

For alternative staining with ICAM-1 multimers after stimulation, we incubated the cells with ICAM-1 multimers for 4 min at 37 °C and left the tubes at RT for 5 min. Whenever indicated, the cells were incubated with EDTA for 10 min at RT at a final concentration of 4 mM or also stained with pA*02 multimers (0.6 µg/ml).

After m24 Ab or ICAM-1 multimers staining, we fixed the samples and lysed erythrocytes with the fluorescence-activated cell sorting (FACS)-Lysing solution (BD Biosciences, San Jose, CA, USA) for WB or fixed the samples with 1.5% Fix Solution (Polysciences, Inc., Warrington, PA, USA) for PBMCs, followed by washing with PBS/0.5% bovine serum albumin (BSA)/0.1% sodium azide. After centrifugation, we stained the cells for surface markers in order to identify CD4⁺ T cells or CD8⁺ T cells with Abs CD3-PerCP-Cy5.5, CD4-BV421, and CD8-BV605/APC in PBS/0.5% BSA/0.1% sodium azide for 15 min at RT. Cells were washed once and fixed in PBS/0.5% BSA/0.5% formaldehyde. All Abs used were from BioLegend (San Diego, CA, USA).

For MHC-class II tetramer staining (Figure 3), PBMCs were incubated with 18 µg/ml DRB1*11:01 CMV/HPT tetramer for 3 h at 37°C diluted in 200 µl PBS/0.5% BSA (Sigma-Aldrich, St. Louis, MO, USA), washed once, and then Ab CD4-FITC (in-house production), CD8-PE-Cy7 (Beckman Coulter, Brea, CA, USA), and Zombie Aqua (BioLegend, San Diego, CA, USA) were added in 50 µl PBS/0.5% BSA for another 15 min. The cells were washed once with PBS/0.5% BSA/0.1% sodium azide and fixed in PBS/0.5% BSA/1% formaldehyde.

m24 Ab staining combined to intracellular cytokine staining

For the assessment of intracellular CD154 and cytokine expression, we stimulated up to 1 ml WB or PBMCs at 37° C 2×10^6 or 1×10^7 cells/ml in TCM) for the indicated times and with

the indicated peptides and concentrations in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) and 5µg/ml monensin (BD Biosciences, San Jose, CA, USA)

After stimulation, we left the tubes at RT for 1–4min; then, extracellular staining with m24-PE Ab, together with Zombie Aqua staining when indicated, was performed for 15 min at RT, followed by incubation with 4 mM EDTA for 10 min at RT. Samples were fixed and erythrocytes were lysed with the FACS- Lysing Solution, followed by washing with PBS/0.5% BSA/0.1% sodium azide.

CD3-PerCP-Cy5.5, CD4-BV421, and CD8-APC- Cy7 Abs were added in PBS/0.5% BSA/0.1% sodium azide for 15min at RT. After one washing step in PBS/0.5% BSA/0.1% sodium azide, the cells were then permeabilized for 20 min at RT with the FACS-Perm2 Solution (BD Biosciences, San Jose, CA, USA) and washed with PBS/0.5% BSA/0.1% sodium azide, followed by intracellular staining with IFN-γ-FITC, IL-2-PE-Cy7, TNF-BV605, and CD154-APC Abs in PBS/0.5% BSA/0.1% sodium azide for 30 min at RT. The cells were washed once with PBS/0.5% BSA/0.1% sodium azide and fixed in PBS/0.5% BSA/0.5% formaldehyde. All Abs used in the study were purchased from BioLegend (San Diego, CA, USA).

Flow cytometry and data analysis

We acquired the data on an LSRFortessa (BD Biosciences, San Jose, CA, USA); analyses were performed with either the FACS DiVa version 8.0 software or Flow Jo 10.6.2 (BD Biosciences, Heidelberg, Germany) software. We collected between 50,000 and 400,000 CD4⁺ or CD8⁺ events for the antigen-specific assays. Gating strategies are shown in Figures 1, 2 and in Supplementary Figures 1, 2, 5. Results are presented as the percentage of cells within the parent populations.

5.2.4 Results

Antigen-specific CD4⁺ T cells can be best visualized with an Ab specific for activated β_2 -integrins

We have previously shown that antigen-specific CD8⁺ T cells are successfully detected by staining of activated β_2 -integrins with fluorescent ICAM-1 multimers (5).

We, therefore, investigated whether ICAM-1 multimers binding could also be used as a marker for the detection of antigen-specific CD4⁺ T cells. We selected a healthy donor with a high frequency of ex vivo detectable CD4⁺ T cells specific for the HPTFTSQYRIQGKLE epitope derived from the CMV pp65 protein (CMV/HPT). The WB cells were incubated with the CMV/HPT peptide, were incubated with SEB, or remained unstimulated for 4h, and ICAM-1 multimers were added for the final 4min of activation. Blood cells were further processed for the detection of intracellular TNF production (gating strategy is shown in Figure 1A).

CMV/HPT- and SEB- stimulated cells showed only modest staining for both TNF and activated integrins (Figure 1B, top). The result was unexpected since the frequency of CMV/HPT-reactive (TNF⁺) CD4⁺ T cells was found to be ~1.2% in prescreening experiments of this donor. To check whether the antigen-specific CD4⁺ T cells had aggregated during the activation, and were therefore excluded from the lymphocyte gate, we treated WB with 4 mM EDTA after (Figure 1B, middle), or at the time of (Figure 1B, bottom), ICAM-1 multimer staining.

With EDTA, much higher frequencies of CMV/HPT-specific or SEB-reacting TNF⁺ CD4⁺ T cells were detected (~1.2% and 8.7% and 1.2% and 9.8%, respectively). However, these TNF⁺ cells were mostly ICAM-1 multimer^{neg}, most probably because EDTA reversed (when used after the staining) or prevented (when used at the time of the staining) ICAM-1 multimers binding to activated β_2 -integrins by chelating Mg²⁺ and Ca²⁺ ions required for β_2 -integrin conformational change and for stable interaction with ICAM-1 multimers. To confirm that the stimulation leads to an aggregation of antigen-specific CD4⁺ T cells, which can be disrupted by EDTA treatment, we analyzed the distribution of TNF⁺ CD4⁺ T cells within the lymphocyte gate (FSC-A/SSC-A) or within singlet cells (FSC-A/FSC-H). Without EDTA treatment, 15% of the CMV/HPT-specific and 42% of the SEB-responding TNF⁺ cells were within the lymphocyte or singlet gates, whereas EDTA treatment increased these numbers to 84-88% (Supplementary Figures 1A–D).

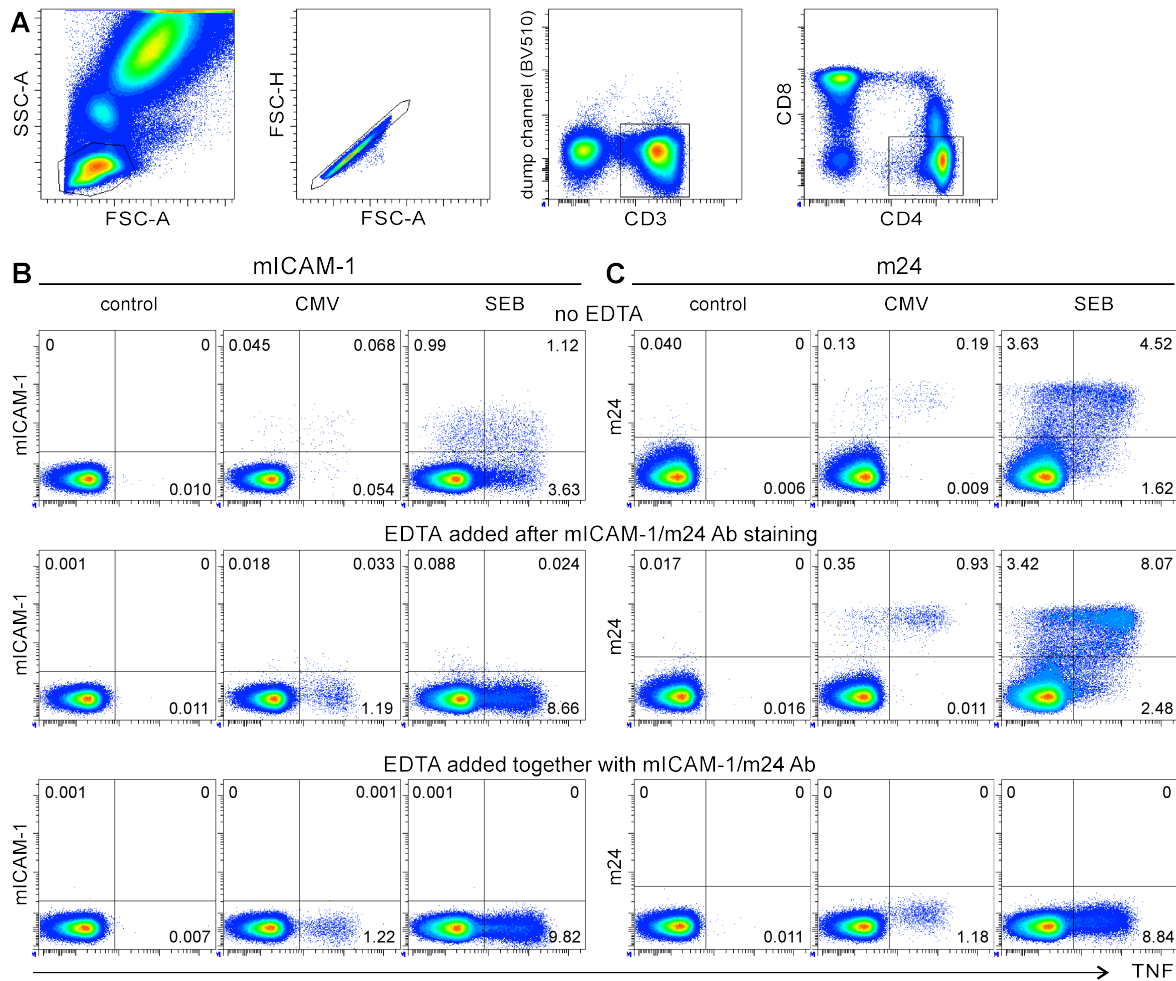


Figure 1. m24 Antibody (Ab) staining of activated β_2 -integrins followed by ethylenediaminetetraacetic acid (EDTA) treatment can be used to detect antigen-specific CD4⁺ T cells. Assessment of whole blood (WB) antigen-specific CD4⁺ T cells by staining of activated β_2 -integrins. (A) Gating strategy. From left to right, the lymphocyte gate, the FSC-A/H duplet exclusion, the gating of CD3⁺ events followed by CD4⁺ events. (B, C) Intercellular adhesion molecule-1 (ICAM-1) multimer and m24 Ab stainings under different EDTA treatment. WB from a donor having a large number of CMV/HPT-specific CD4⁺ T cells was cultured without stimulus (left, control), with the CMV/HPT peptide (middle) or with *Staphylococcus enterotoxin B* (SEB) (right) in the presence of brefeldin A and monensin. After 4 h, cells were either stained with multimers of ICAM-1 (mICAM-1) for 4 min (B) or with m24 Ab for 15 min (C), without (top), or with EDTA addition after (middle), or at the time (bottom) of the staining. The cells were subsequently labeled with CD3, CD4, and CD8 Abs, and for the intracellular expression of tumor necrosis factor (TNF) to identify CMV-specific CD4⁺ T cells. Numbers indicate frequencies among CD4⁺ T cells in %.

Because ICAM-1 multimers staining was not suitable for labelling activated β_2 -integrins on antigen-specific CD4⁺ T lymphocytes, we next tested staining of the cells with a monoclonal Ab that binds specifically to the extended/open high-affinity, but not to the resting, unactivated conformation of β_2 -integrins (m24 Ab). The WB cells from the same donor were either stimulated with the CMV/HPT peptide or with SEB or remained unstimulated for 4 h, and were then stained with m24 Ab for 15min, followed by the detection of intracellular TNF.

The unstimulated cells showed very low staining with m24 or anti-TNF Ab. Significant numbers of TNF⁺ CD4⁺ T cells were detected after the stimulation with CMV/HPT (0.2%) and SEB (6.1%), most of them being m24⁺ (Figure 1C, top); however, these were much less

than when ICAM-1 multimers and EDTA were used. Addition of EDTA after (Figure 1C, middle), or at the time of (Figure 1C, bottom), m24 Ab staining improved TNF detection to expected frequencies (~1 and 9% for CMV/HPT and SEB, respectively). When EDTA was added after m24 Ab staining, the TNF-producing cells were essentially m24⁺, indicating that once the Ab is bound, EDTA does not reverse the binding, as it does for ICAM-1 multimers. When EDTA was added at the time of m24 Ab staining, the cells were m24^{neg}, showing that EDTA blocked m24 Ab binding to activated β_2 -integrins. Without EDTA treatment, 18% of the CMV/HPT-specific and 50% of the SEB-stimulated TNF⁺ cells were within the lymphocyte or singlet gates, whereas EDTA treatment increased these numbers to 74-86% (Supplementary Figures 1E–H). Hence, m24 Ab staining of activated β_2 -integrins, followed by EDTA treatment, can be used to detect antigen-specific CD4⁺ T cells.

Antigen-specific β_2 -integrin activation on CD8⁺ T cells can be visualized by either ICAM-1 multimers or m24 Ab staining

In a previous study, antigen-specific CD8⁺ T cells were detected with ICAM-1 multimeric complexes (without EDTA) (5).

We, therefore, tested whether the staining of activated CD8⁺ T cells with m24 Ab would be comparable to that obtained with ICAM-1 multimers and to which extent EDTA influences detection. We selected an HLA-A*02⁺ donor with a high frequency of CD8⁺ T cells specific for the immunodominant epitope CMV pp65 NLVPMVATV (NLV). The WB cells were stimulated with the CMV/NLV peptide for 16min. ICAM-1 multimers and HLA-A*02 (referred hereafter as A*02) tetramers refolded with the CMV/NLV peptide were added for the final 4min of the (5), alternatively, m24 Ab and A*02/NLV tetramers were added after the stimulation, and the cells were incubated for 15min. The gating strategy is shown in Figure 2A. The combination of A*02/NLV and ICAM-1 multimers identified 0.92% A*02/NLV⁺ CD8⁺ T cells (from which nearly all, i.e., 0.88%, were ICAM-1 multimer⁺, Figure 2B, top left), whereas the combination of A*02/NLV and m24 Ab identified 0.99% A*02/NLV⁺ CD8⁺ T cells (0.95% were m24⁺, Figure 2B, top right). The treatment with EDTA after ICAM-1 multimer or m24 Ab staining only marginally increased the frequency of tetramer⁺ cells (1.1%, Figure 2B, middle).

Similar to what we had observed for CD4⁺ T cells, staining of activated integrins on CD8⁺ T cells was largely lost when EDTA was used after the staining with ICAM-1 multimers but not after the staining with m24 Ab. When EDTA was added together with ICAM-1 multimers or m24 Ab staining, the cells did not stain with either of the reagents (Figure 2B, bottom). To confirm that the stimulation did not aggregate the antigen-specific CD8⁺ T cells even without EDTA treatment, we analyzed the distribution of A*02/NLV⁺ CD8⁺ T cells on FSC-A/SSC-A and FSC-A/FSC-H plots (Supplementary Figures 2B,C, top). Without EDTA

treatment, 80–81% of the CMV/NLV⁺ cells were within the lymphocyte or singlet gates, whereas EDTA treatment slightly increased these numbers to 91–93% (Supplementary Figures 2B,C, bottom).

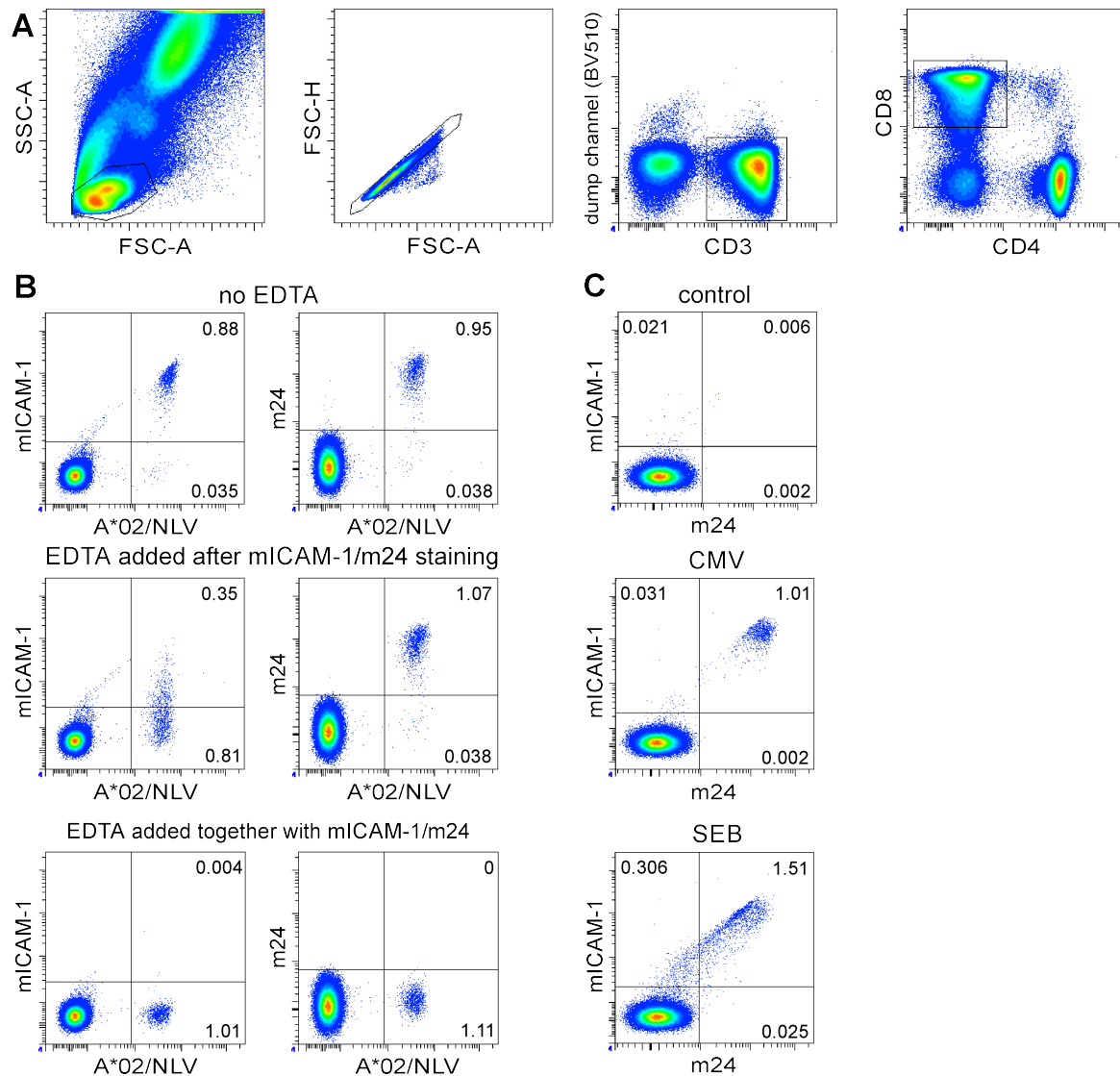


Figure 2. The antigen-specific β_2 -integrin activation on CD8⁺ T cells can be visualized by either ICAM-1 multimers (without EDTA) or by m24 Ab with or without EDTA treatment. Assessment of WB antigen-specific CD8⁺ T cells by staining of activated β_2 -integrins. (A) Gating strategy. From left to right, the lymphocyte gate, the FSC-A/H duplet exclusion, the gating of CD3⁺ events, followed by CD8⁺ events. (B) The WB from a selected HLA-A*02⁺ donor was incubated with the NLV peptide for 16 min, then either not treated (top) or treated with EDTA after (middle), or at the time of (bottom) the staining with multimers of ICAM-1 or with m24 Ab. Cells were simultaneously stained with A*02/NLV tetramers and thereafter with CD3, CD4, and CD8 Abs. (C) Staining with m24 Ab and ICAM-1 multimers together, without EDTA treatment. Numbers indicate frequencies among CD8⁺ T cells in %.

Later, we assessed whether m24 Ab and ICAM-1 multimer stainings are interchangeable. The WB cells from the same donor were either stimulated with the CMV/NLV peptide or with SEB or remained unstimulated for 16 min; then, the cells were stained with ICAM-1 multimers and m24 Ab. Double stainings confirmed that both ICAM-1 multimers and m24 Ab essentially identify the same cells (Figure 2C).

Hence, antigen-specific β_2 -integrin activation on singlet CD8⁺ T lymphocytes can be visualized by either ICAM-1 multimers (without EDTA) or by m24 Ab with or without EDTA treatment, but for simultaneous detection of CD4⁺ and CD8⁺ T cells, m24 Ab and EDTA treatment should be combined.

Kinetics of β_2 -integrin activation and m24 Ab binding is different for CD4⁺ and CD8⁺ T cells

To determine the optimal duration for cell stimulation, we performed time courses of β_2 -integrin activation and m24 Ab binding on CD4⁺ and CD8⁺ T cells. The WB from one to four selected donors was stimulated with various antigens as indicated or remained unstimulated. Blood cells were harvested after different incubation times, followed by m24 Ab staining plus EDTA treatment. The kinetics is shown as % m24⁺ in Figure 3A (for CD4⁺ T cells) or in Figure 4A (for CD8⁺ T cells), representative examples are in Figures 3B,C (for CD4⁺ T cells) and Figures 4B,C (for CD8⁺ T cells).

A short-term activation with the CMV/HPT epitope in one donor induced only a weak m24 Ab staining on CD4⁺ T cells, whereas T cell response peaked after 4–6 h of stimulation (Figures 3A,B top). Costaining with HLA-DRB1*11 CMV/HPT tetramers and m24 Ab was not feasible because of strong TCR downregulation after peptide stimulation; however, we found comparable frequencies of specific cells with both methods [2% DRB1*11/HPT⁺ (Figure 3C) vs. 1.76% m24⁺ CD4⁺ T cells at the maximum of response after 6h of stimulation with the CMV/HPT peptide]. Reactivity to overlapping 15-mer peptides (HBV/Env) in two HBV- vaccinated donors also reached a maximum between 4 and 6 h (Figures 3A,B bottom). For all four tested donors, the percentage of m24⁺ CD4⁺ T cells increased earlier after SEB stimulation but peaked after 4–6 h (Figures 3A,B). All unstimulated cells showed weak staining with m24 Ab (0.01–0.05%).

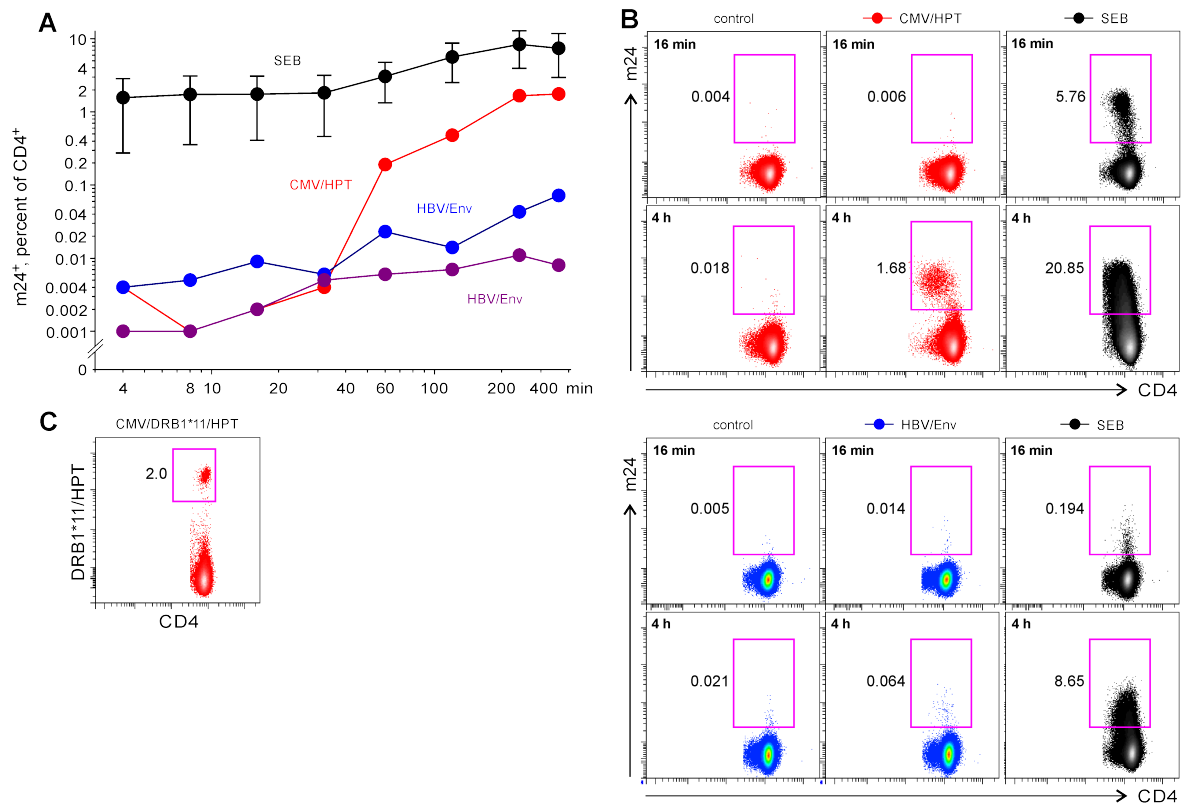


Figure 3. The flow cytometry assessment of antigen-specific CD4⁺ WB T cells by staining of activated β_2 -integrins with m24 Ab. The cells were stained with m24 Ab, treated with EDTA afterwards, and then stained with CD3, CD4, and CD8 Abs, gating strategy is displayed in Figure 1A. (A) The time course of m24 Ab staining following incubation of WB cells with SEB (black, four donors, presented as mean $\pm$ SEM), CMV/HPT peptide (red, one donor), or HBV/Env pool of overlapping peptides (blue and purple, 2 donors) for 4, 8, 16, 32, 60, 120, 240, or 360 min. Background from the relevant unstimulated sample was subtracted. (B) Examples of m24 Ab staining obtained after 16 min or 240 min, i.e., 4 h without stimulation (control) or in the presence of matched peptides or SEB of the donor with a CMV/HPT CD4⁺ reactivity (top panel). One of the two HBV-vaccinees (bottom panel). (C) DRB1*11/HPT multimer staining was performed without CMV/HPT peptide stimulation and was obtained from the same donor as in (B) (top panel). Numbers indicate frequencies among CD4⁺ T cells in %.

In contrast to antigen-specific CD4⁺ T lymphocytes, and as we previously observed with ICAM-1 multimers staining (5), responding CMV/NLV⁺ CD8⁺ T cells were very rapidly detected (immediate peak response of m24 Ab staining within only 4-16 min, Figure 4A). After prolonged activation with the CMV/NLV epitope (>1h), the percentage of m24⁺ cells diminished only slightly (1.45 vs. 1.37% after 16 min or 4 h of stimulation, respectively, with a more substantial decrease found in the intensity, Figure 4B), in contrast to the strong decline we had observed when using ICAM-1 multimers (5).

To confirm that m24 Ab selectively identifies antigen-specific T cells, we costained cells from the CMV-reactive HLA-A*02⁺ donor with A*02/NLV tetramers and m24 Ab. The majority of NLV-specific CD8⁺ T cells could be detected by m24 Ab binding, with maximal staining achieved within 4–16min of activation (Figures 4A,C, 97% of double-stained cells after 16min of stimulation). For CD8⁺ T cells, the frequency of m24⁺ cells after SEB stimulation peaked at 4 min in one case, whereas for the other two donors, it reached the

maximum at 4 h, but with decreased staining intensity (Figures 4A,B). All unstimulated cells showed weak staining with m24 Ab (<0.01 to 0.05%).

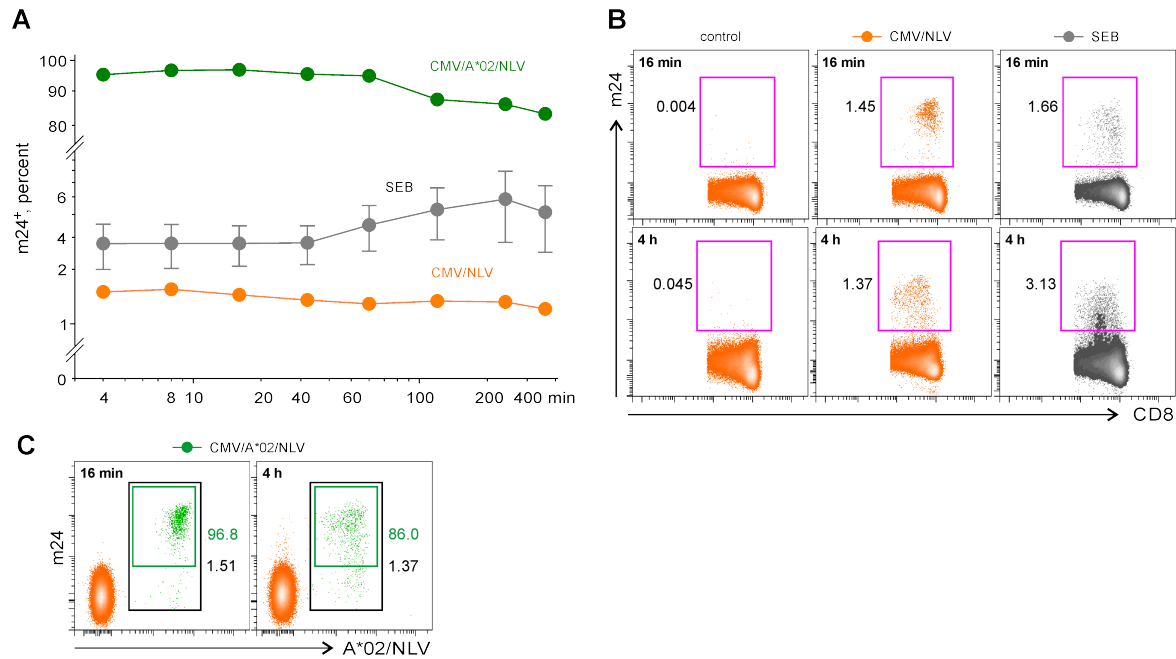


Figure 4. The flow cytometry assessment of antigen-specific CD8⁺ T cells by staining activated β_2 -integrins with m24 Ab. Cells were stained with m24 Ab, treated with EDTA afterwards, and then stained with CD3, CD4, and CD8 Abs; gating strategy is displayed in Figure 2A. Percentages of m24⁺ cells among total CD8⁺ T cells are indicated. (A) The time course of m24 Ab staining following incubation of WB cells with SEB (gray, three donors, presented as mean $\pm$ SEM), or CMV/NLV peptide (orange, one donor; green, the same donor, but additionally stained with A*02/NLV multimers), for 4, 8, 16, 32, 60, 120, 240, or 360 min. Background from the relevant unstimulated sample was subtracted. Numbers indicate frequencies among CD8⁺ T cells in %. (B) Examples of m24 Ab staining obtained after 16 or 240 min, i.e., 4 h without stimulation (control), in the presence of CMV/NLV peptide, or after stimulation with SEB. (C) Combined m24 Ab and CMV/NLV tetramer staining of the CMV-specific CD8⁺ T cells. Percentages of A*02/NLV⁺ within the CD8⁺ subset and of m24⁺ within the A*02/NLV⁺ cells are indicated in black and green, respectively.

We conclude that m24 Ab identifies antigen-responding CD4⁺ T cells, but in contrast to CD8⁺ T cells, several hours of stimulation are required to activate β_2 -integrins.

m24 Ab binding identifies functional antigen-specific CD4⁺ T cells

We next tested whether m24 Ab binding correlates with the functionality of antigen-specific CD4⁺ T cells. The WB from the DRB1*11⁺ donor was stimulated with the CMV/HPT peptide, was stimulated with SEB, or remained unstimulated. Blood cells were harvested after 1, 2, 3, 4, or 6h, followed by incubation with m24 Ab plus EDTA treatment and staining for intracellular CD154 and cytokine (TNF, IFN- γ , and IL-2) upregulation. Results of the CMV/HPT stimulation are shown in Figure 5A, and density plots after 6h of Incubation without or with CMV/HPT are shown in Figure 5B, top and middle, respectively. m24 Ab binding was the earliest event to be detected (after 1–2 h of stimulation, Figure 5A, opened bars), and upregulation of CD154 and cytokines was exclusively confined to the m24⁺ cell subset [positive (marker⁺ m24⁺ cells) vs. negative (marker⁺ m24^{neg} cells), Figure 5A, gray bars]. Almost all m24⁺ cells (85%) expressed CD154, TNF, or IFN- γ at 6h. Moreover, cytokine-producing cells were mainly m24⁺ CD154⁺. Only 15%, 12%, and 1% of the TNF-, IFN- γ -, or IL-2-producing cells did not express CD154, and virtually none were m24^{neg} (Figure 5B bottom). Some single stained m24⁺ or CD154⁺ events, but no double-stained m24⁺ CD154⁺ events, were observed in unstimulated cells (Figure 5B top, left). Comparable results were obtained for the SEB-stimulated cells in this individual, with a strong association between m24 Ab staining and TNF or IFN- γ production; still, a fraction of CD154⁺ or IL-2⁺ cells were m24^{neg}; however, we found that CD154⁺ m24^{neg} cells were predominantly also cytokine^{neg} (Supplementary Figures 3A, B).

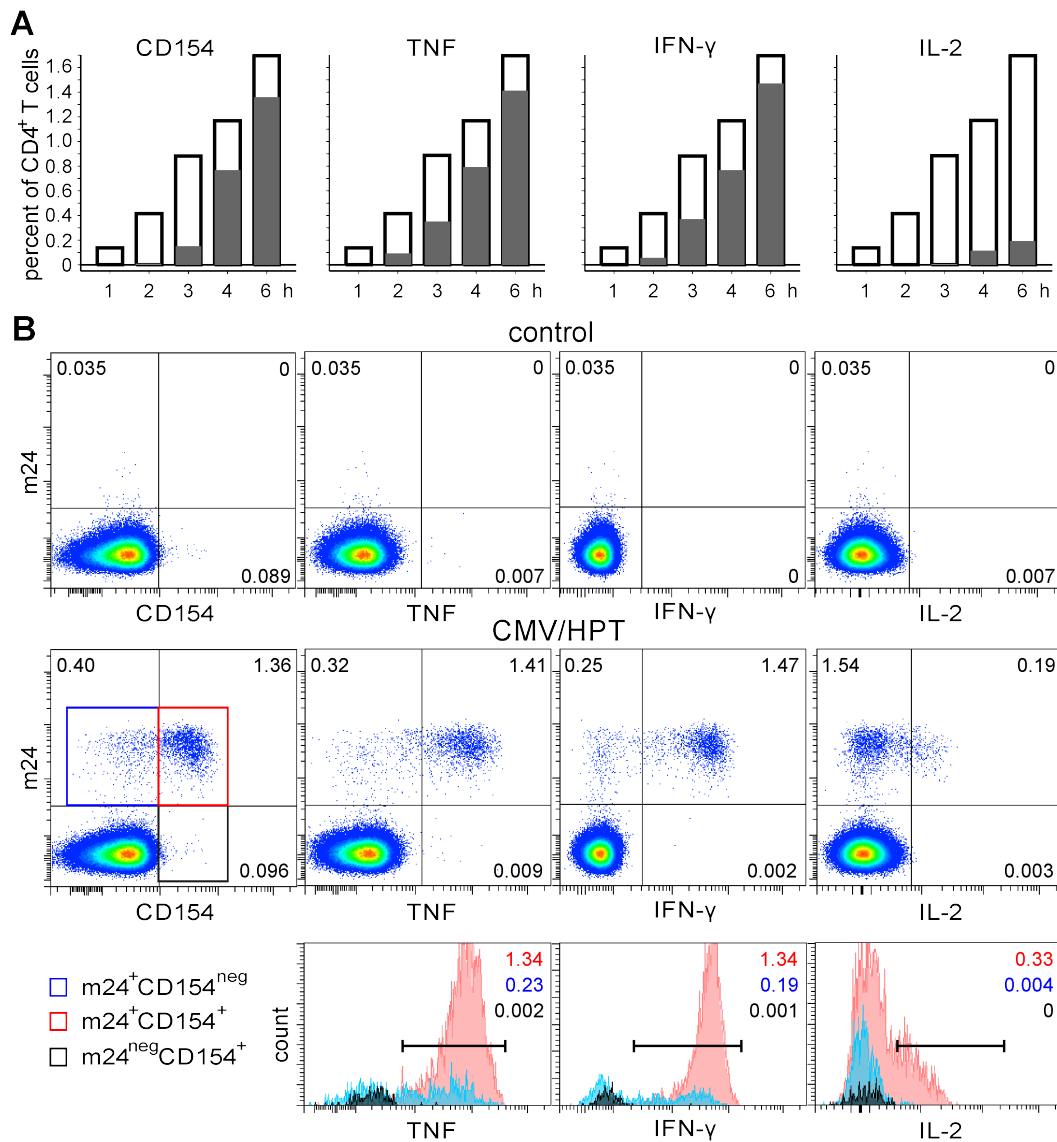


Figure 5. The functional profile of CMV-specific m24⁺ CD4⁺ WB T cells. WB of a DRB1*11⁺ donor was stimulated with CMV/HPT peptide. (A) Frequencies of m24 Ab staining, CD154, and cytokine expression within CD4⁺ T cells are shown as bar graphs (at the indicated times and after subtraction of the background assessed in the control test); empty bars represent total m24⁺ cells, positive gray bars represent marker⁺ (CD154, TNF, IFN-γ, or IL-2) m24⁺ CD4⁺ T cells, whereas negative gray bars (practically not visible) are marker⁺m24^{neg} CD4⁺ T cells. (B) Staining obtained after 6 h without stimulation (control, top) or in the presence of CMV/HPT (middle). Numbers on density plots indicate frequencies among CD4⁺ T cells. m24⁺ CD154^{neg}, m24⁺ CD154⁺ and m24^{neg} CD154⁺ subsets were gated (middle panel: blue, red and black frames, respectively) and further displayed as histograms according to their TNF, IFN-γ, and IL-2 expression (bottom). Numbers on histograms indicate frequencies of the respective color-coded population among CD4⁺ T cells (markers are shown). Gating strategy is as shown in Figure 1A.

To address specificities against further viruses, the WB from two HBV-vaccinated donors was stimulated with HBV/Env overlapping peptides, was stimulated with SEB (one donor only), or remained unstimulated. The cells were harvested after 1, 2, 3, 4, or 6 h, followed by m24 Ab staining, EDTA treatment, and staining for intracellular CD154 and cytokines upregulation. For the first donor, the time course of HBV/Env stimulation is shown in Figure 6A, and density plots after 6 h of incubation without or with HBV peptides are displayed in Figure 6B top and middle, respectively. Frequencies of HBV-specific cells were low ($\sim 0.1\%$ within the $CD4^+$ T cell subset), but readily detectable by m24 Ab staining. Similar to what we observed for CMV-specific cells, the CD154 expression and cytokine production were essentially confined to the $m24^+$ cell fraction [positive (marker $^+$ $m24^+$ cells) vs. negative (marker $^+$ $m24^{neg}$ cells), Figure 6A, gray bars]. However, a significant proportion of $m24^+$ cells (about 50%) did not upregulate CD154 or cytokines even after 6 h. Some $m24^+$ cells negative for the functional markers were also present in the unstimulated control. When we analyzed single and double $m24^+$ and $CD154^+$ cells, we found that cytokine-producing cells were mainly $m24^+$ $CD154^+$. Only 6%, 0%, and 0% of the TNF-, IFN- γ -, or IL-2-producing $CD4^+$ T cells, respectively, were $CD154^{neg}$, whereas 6%, 9%, and 7% were $m24^{neg}$ (Figure 6B bottom). In unstimulated cells, some cells were $m24^+$ or $CD154^+$, but no double-stained $m24^+$ $CD154^+$ events were observed (Figure 6B top, left). After SEB stimulation, a strong association between m24 Ab staining and TNF, IFN- γ , and IL-2 production was observed. Again, a significant proportion of the $CD154^+$ cells was $m24^{neg}$; however, those were predominantly cytokine neg (Supplementary Figures 3C,D).

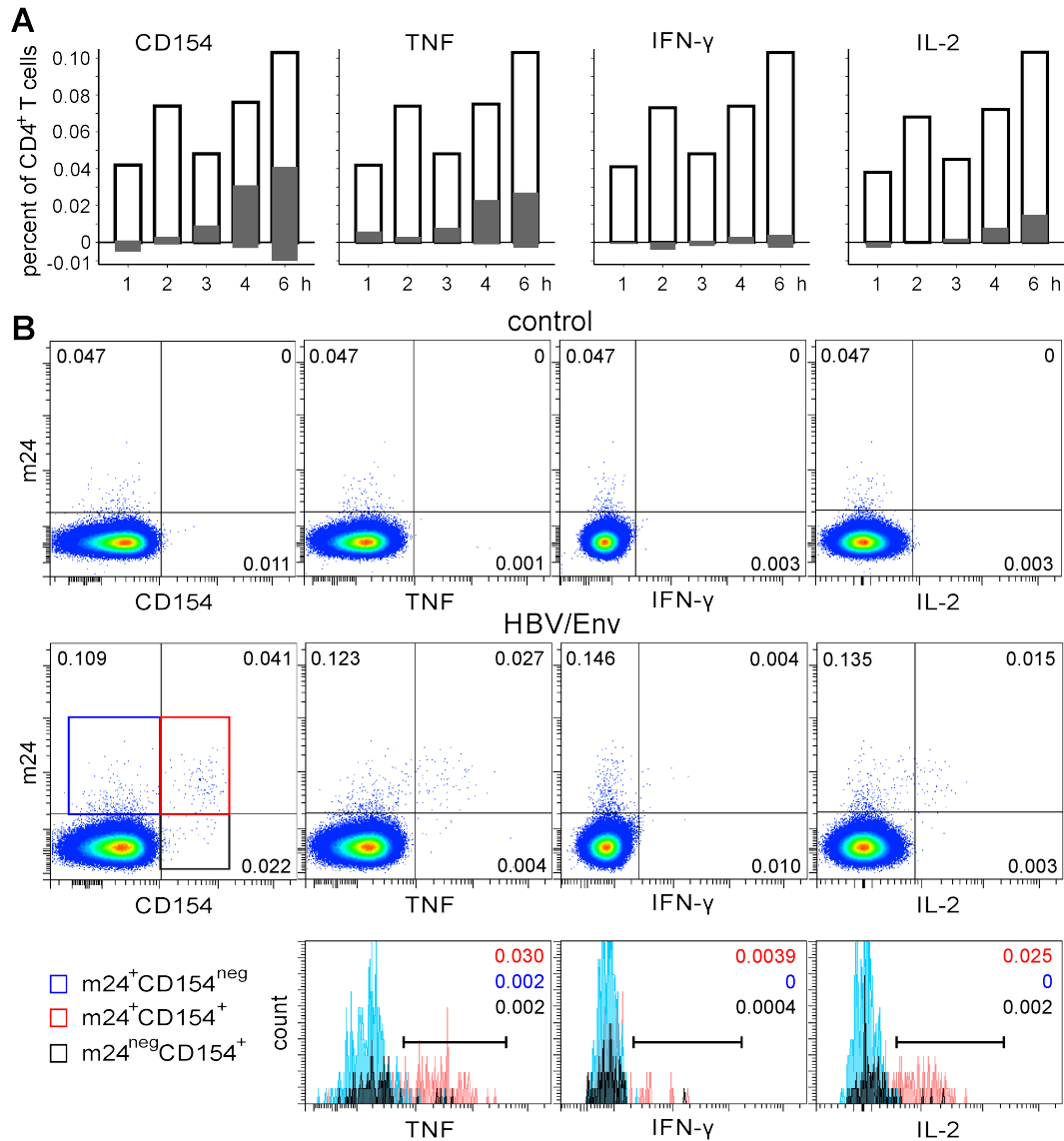


Figure 6. The functional profile of HBV-specific $m24^{+}CD4^{+}$ WB T cells. The WB from a donor who was vaccinated ~ 10 years ago against HBV was stimulated with HBV/Env overlapping peptides. (A) Frequencies of $m24$ Ab staining, CD154, and cytokine expression within $CD4^{+}$ T cells are shown as bar graphs (at the indicated times and after subtraction of the background assessed in the control test); empty bars represent total $m24^{+}$ cells, positive gray bars represent marker⁺ ($CD154$, TNF , $IFN-\gamma$, or $IL-2$) $m24^{+}CD4^{+}$ T cells, whereas negative gray bars are marker⁺ $m24^{neg}CD4^{+}$ T cells. (B) Staining obtained after 6 h without stimulation (control, top) or in the presence of the HBV/Env overlapping peptides (middle). Numbers on density plots indicate frequencies among $CD4^{+}$ T cells. $m24^{+}CD154^{neg}$, $m24^{+}CD154^{+}$ and $m24^{neg}CD154^{+}$ subsets were gated (middle panel: blue, red and black frames, respectively) and further displayed as histograms according to their TNF , $IFN-\gamma$, and $IL-2$ expression (bottom). Numbers on histograms indicate frequencies of the respective color-coded population among $CD4^{+}$ T cells (markers are shown). Gating strategy is as shown in Figure 1A.

Similar observations were made for a second HBV vaccinee (Supplementary Figure 4). Although some background staining with $m24$ or $CD154$ Abs was observed in the unstimulated test, the combination of both markers (0.001% of $m24^{+}CD154^{+}$ cells) enabled distinct detection of HBV-specific cells even at very low frequencies (Supplementary Figure 4B: 0.011, 0.008, 0.001, and 0.004% for $CD154$, TNF , $IFN-\gamma$, and $IL-2$ dot plots, respectively).

In conclusion, m24 Ab staining is suitable for detecting functional antigen-specific CD4⁺ T cells, and the combination of this marker with the measurement of CD154 upregulation can be used for the identification of extremely low frequencies of functional antigen-specific T cells.

Monitoring of SARS-CoV-2 specific T cell immunity

Later, we examined the feasibility of our assay to monitor SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells. Blood was obtained from CoV-2 convalescents (n = 3) and unexposed healthy donors (n = 4), as confirmed by Ab ELISA on the day of blood withdrawal. In accordance with our previous results for CMV- and HBV-specific CD4⁺ T cells, there were no m24⁺ cells producing CD154 or cytokines without peptide-specific stimulation (0–0.001%, exemplary donor CoV-2 #3 is shown in Figure 7A top, pink frames). The addition of overlapping peptides derived from the membrane (M), N, or spike (S) proteins induced a clear CD4⁺ T cell response in previously exposed individuals, detectable by costaining with m24 and CD154 Ab, even at very low frequencies (down to 0.005–0.006%; M-, N-, and S-specific CD4⁺ T cell responses from donor CoV-2 #3 occurring at frequencies of 0.050, 0.006, and 0.014% are shown in Figure 7A, pink frames). All three individuals responded to the three proteins, albeit at a different intensity. The strongest responses were directed at the protein M (up to ~0.05% of the CD4⁺ T cells in CoV-2 #2 and #3, Figure 7B). CoV-2 #1 responded weakly to protein M (0.005%) but strongly to proteins N and S (both 0.009%). The cytokine production (TNF, IFN- γ , and IL-2) was observed within m24⁺ cells. The T cell activation was virtually not detected in the four unexposed donors (0–0.003%), except for one of them (UD #2) who interestingly showed a distinct, but very low (possibly cross-reactive) response to the protein S alone (0.005% m24⁺ cells expressing either CD154, TNF, or IFN- γ , Figure 7B). Altogether, T cell reactivities against all three proteins tested were significantly increased in the convalescent vs. the non-exposed donor group when considering m24 and CD154 Ab staining together.

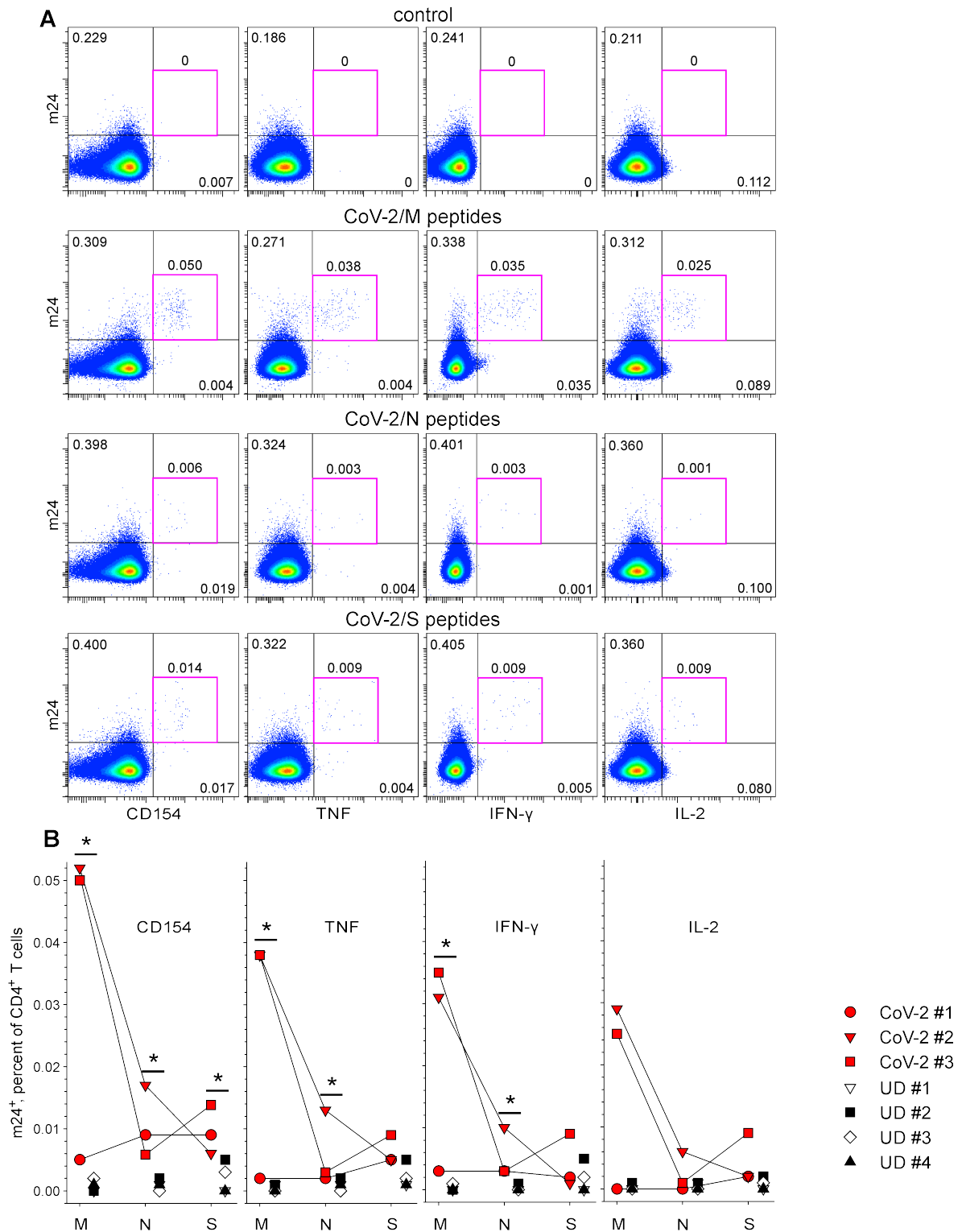


Figure 7. The functional profile of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific m24⁺ CD4⁺ WB T cells. m24 Ab staining in combination with CD154, TNF, IFN- γ or IL-2 expression after stimulation with pools of CoV-2 membrane (M)-, nucleocapsid (N)-, and spike (S)-derived overlapping peptides for 4 h. (A) Examples of m24 Ab staining obtained from CoV-2 #3 without stimulation (control) or in the presence of the M, N or S peptides. Numbers indicate frequencies among CD4⁺ T cells in %. (B) The results for marker⁺ m24⁺ CD4⁺ T cells obtained from SARS-CoV-2 convalescents ($n=3$, CoV-2 #1 to #3) and unexposed healthy controls ($n=4$, UD #1 to #4) are shown as graphs (after subtraction of background assessed in the control test). Asymptotic significance was calculated using two-tailed Mann-Whitney U -tests, $*p < 0.05$. Numbers indicate frequencies among CD4⁺ T cells in %. Gating strategy is as shown in Figure 1A.

The anti-SARS-CoV-2 CD8⁺ T cell reactivity was assessed simultaneously to the CD4⁺ T cell measurement (m24 Ab staining after 4h of stimulation). There were no m24⁺ cells producing cytokines without antigen-specific stimulation (0– 0.001%, shown for CD8⁺ T cells from CoV-2 #1 in Figure 8A, top). Interestingly, only protein N was recognized in two out of three convalescents, and m24⁺ cells produced TNF (0.017 and 0.006% for CoV-2 #1 and CoV-2 #3, respectively) and IFN- γ (0.023 and 0.011%) (Figure 8B) but not IL-2 ($\leq 0.001\%$). None of the four unexposed volunteers reacted to any of the CoV-2 peptides (0–0.002%), except again for UD #2 who had a response to the N-derived peptide pool (0.010% m24⁺ CD8⁺ T cells expressing either TNF or IFN- γ).

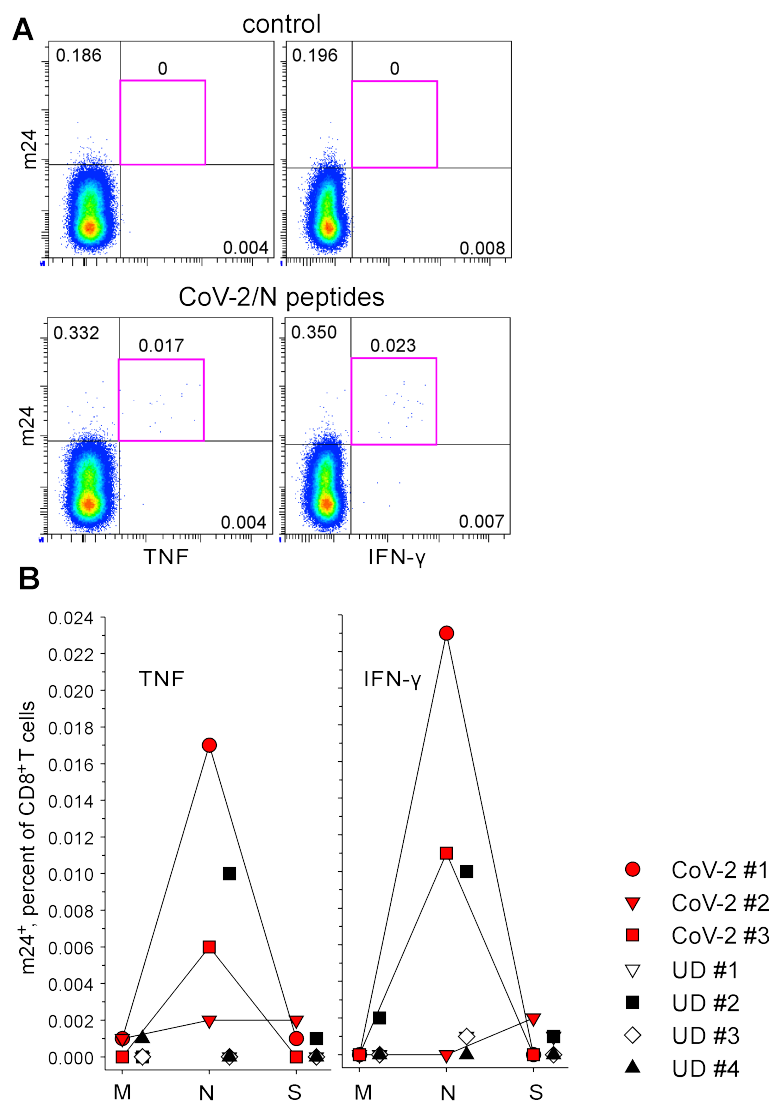


Figure 8. The functional profile of SARS-CoV-2-specific m24⁺ CD8⁺ WB T cells. m24 Ab staining in combination with TNF or IFN- γ expression after 4 h of stimulation with pools of SARS-CoV-2 M, N, and S-derived overlapping peptides. (A) Examples of m24 Ab staining from CoV-2 #1 obtained without stimulation (control, top) or in the presence of N peptides (bottom). Numbers indicate frequencies among CD8⁺ T cells in %. (B) The results for marker⁺ m24⁺ CD8⁺ T cells obtained from SARS-CoV-2 convalescents ($n=3$, CoV-2 #1 to #3) and unexposed healthy donors ($n=4$, UD #1 to #4) are shown as graphs (after subtraction of background assessed in the control test). No significant difference was found using two-tailed Mann–Whitney U -tests. Numbers indicate frequencies among CD8⁺ T cells in %. Gating strategy is as shown in Figure 2A.

We next tested CD4⁺ T cell reactivity in the blood of a healthy volunteer who had been immunized ~6 weeks before with a cocktail of synthetic peptides containing HLA-class II SARS-CoV-2 sequences derived from the N protein (CoV- 2/IGY and CoV-2/ASA) and from the Env protein (CoV- 2/FYV), as well as one recall CMV pp65 peptide (CMV/YQE) [(13 Preprint)]. Virtually, no m24⁺ cells producing CD154 or cytokines were detected when cells were left unstimulated for 6 h (0–0.001%, Figure 9A). The CMV/YQE stimulation caused a strong expression of CD154 (0.418%), TNF (0.312%), IFN- γ (0.382%), and IL-2 (0.253%) within m24⁺ cells (Figure 9B). The three SARS-CoV-2 peptides used for vaccination induced variable responses, with 0.094, 0.060, and 0.012% of m24⁺ CD154⁺ CD4⁺ T cells for SARS-CoV-2 N/IGY, N/ASA, and Env/FYV, respectively, as well as for the production of all three cytokines (Figures 9C–E). These results are coherent with those obtained by IFN- γ ELISpot ~3 weeks after immunization [(13 Preprint)].

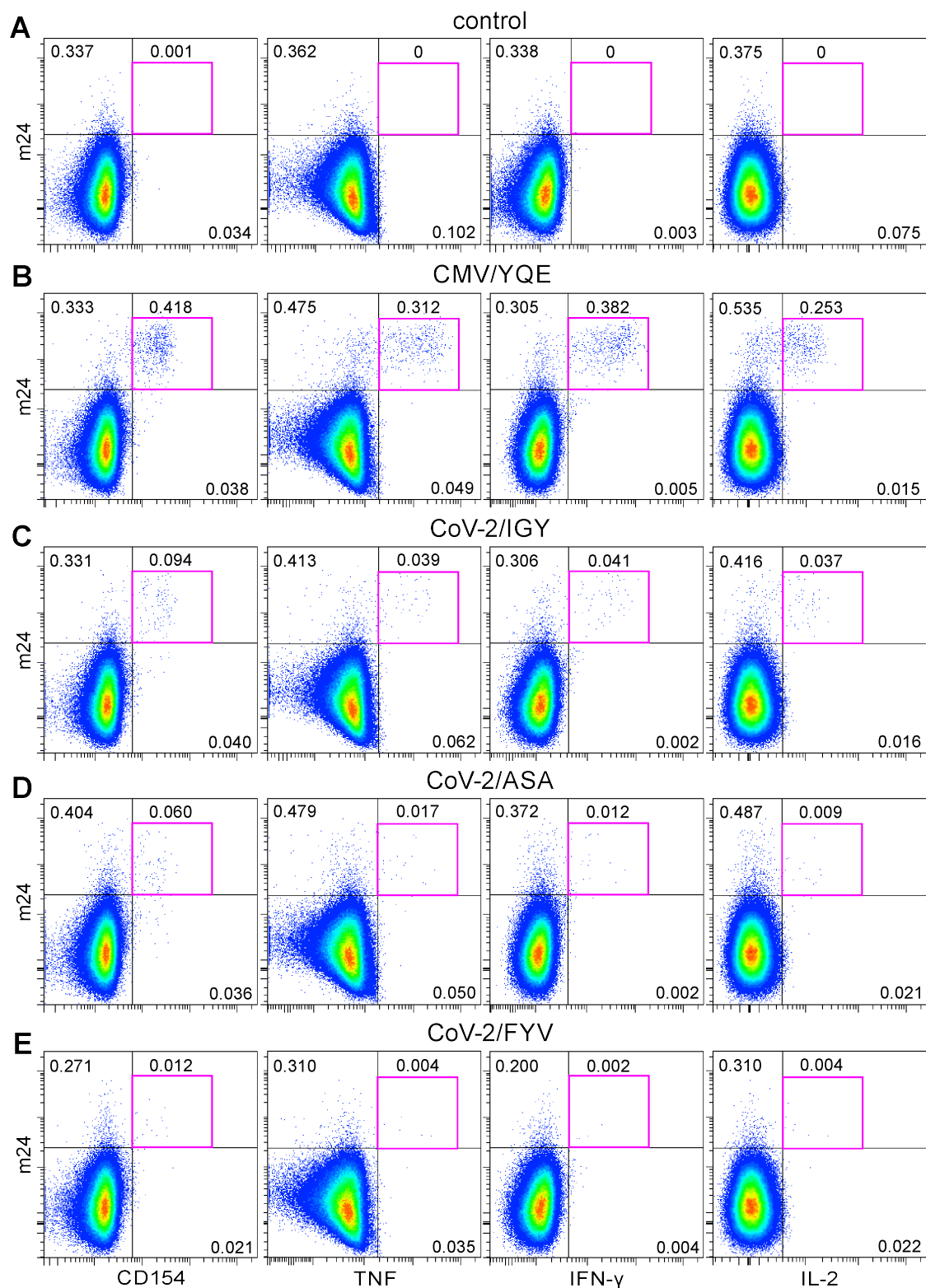


Figure 9. The functional profile of CMV- and SARS-CoV-2-specific CD4⁺ WB T cells after peptide vaccination. (A-E) m24 Ab staining in combination with CD154, TNF, IFN-γ or IL-2 expression obtained from a donor vaccinated with one CMV and three SARS-CoV-2 major histocompatibility complex (MHC)-class II peptides without stimulation (control, A), with CMV/YQE (B), CoV-2/IGY (C), CoV-2/ASA (D) or CoV-2/FYV peptides (E) for 6 h stimulation. Numbers indicate frequencies among CD4⁺ T cells in %. Gating strategy is as shown in Figure 1A.

m24 Ab staining of antigen-specific CD4⁺ and CD8⁺ T cells in frozen/thawed PBMCs

We have also tested our method for the detection of T cells within cryopreserved PBMC samples. First, two donors, with high (~2% of DRB1*11/HPT tetramer⁺) and low (~0.05% CD154⁺ against HBV/Env) frequencies of virus-specific CD4⁺ T cells, were selected (experiments with WB of the same donors are reported in Figures 3, 5, 6). PBMCs were stimulated with the CMV/HPT peptide (first donor), were stimulated with HBV/Env overlapping peptides (second donor), or remained unstimulated for 6h, followed by incubation with m24 Ab plus EDTA treatment and staining for intracellular CD154 and cytokines (TNF, IFN- γ , and IL-2).

For the first donor, β_2 -integrin activation (m24 Ab binding) and functionality (CD154, TNF, IFN- γ , and IL-2 production) were stronger in PBMCs than in WB (1.90 vs. 1.36%, respectively, of m24⁺ CD154⁺ cells; compare Supplementary Figure 5B and Figure 5B). Similar to what we observed with WB (Figure 5A), the upregulation of CD154 and cytokines was exclusively confined to the m24⁺ cell subset and almost all m24⁺ cells (93%) expressed CD154, TNF, or IFN- γ (Supplementary Figure 5B). Moreover, cytokine-producing cells were mainly m24⁺ CD154⁺. Only 17, 12, and 1% of the TNF-, IFN- γ -, or IL-2-producing cells did not express CD154, and virtually none were m24^{neg} (Supplementary Figure 5B bottom). Similar observations were made for the second donor, with a more robust response in PBMCs than in WB (0.058 vs. 0.041% of m24⁺ CD154⁺ cells for PBMCs and WB, respectively; compare Supplementary Figure 5C and Figure 6B). Again, some single-stained m24⁺ or CD154⁺ events were observed in unstimulated cells, but virtually no double-stained m24⁺ CD154⁺ events were detected (Supplementary Figure 5C top, left). Almost none of the TNF-, IFN- γ -, or IL-2-producing CD4⁺ T cells were CD154^{neg}, whereas only 1, 0, and 8% were m24^{neg}, respectively (Supplementary Figure 5C bottom). Thus, m24 Ab staining together with the expression of CD154 allows the reliable identification of low frequency, polyfunctional CD4⁺ T cells in cryopreserved PBMCs.

We finally applied the m24 Ab assay for simultaneous detection of antigen-specific CD4⁺ and CD8⁺ T cells within PBMCs of one prescreened donor (with a known low CD4⁺ T cell reactivity against an HLA-class II peptide mix containing CMV-, EBV-, and Flu-derived epitopes, and a low frequency of CD8⁺ T cells against one HLA-A*02 restricted EBV-derived epitope). Background staining was observed with single m24 or single CD154 Abs on unstimulated CD4⁺ T cells; however, the combination of both markers allowed to readily detect ~0.05% of reactive CD4⁺ T cells (Figure 10A), whereas the combination of m24 Ab with TNF and IFN- γ (but not with CD154 or IL-2) sensitively identified EBV-specific CD8⁺ T cells (Figure 10B).

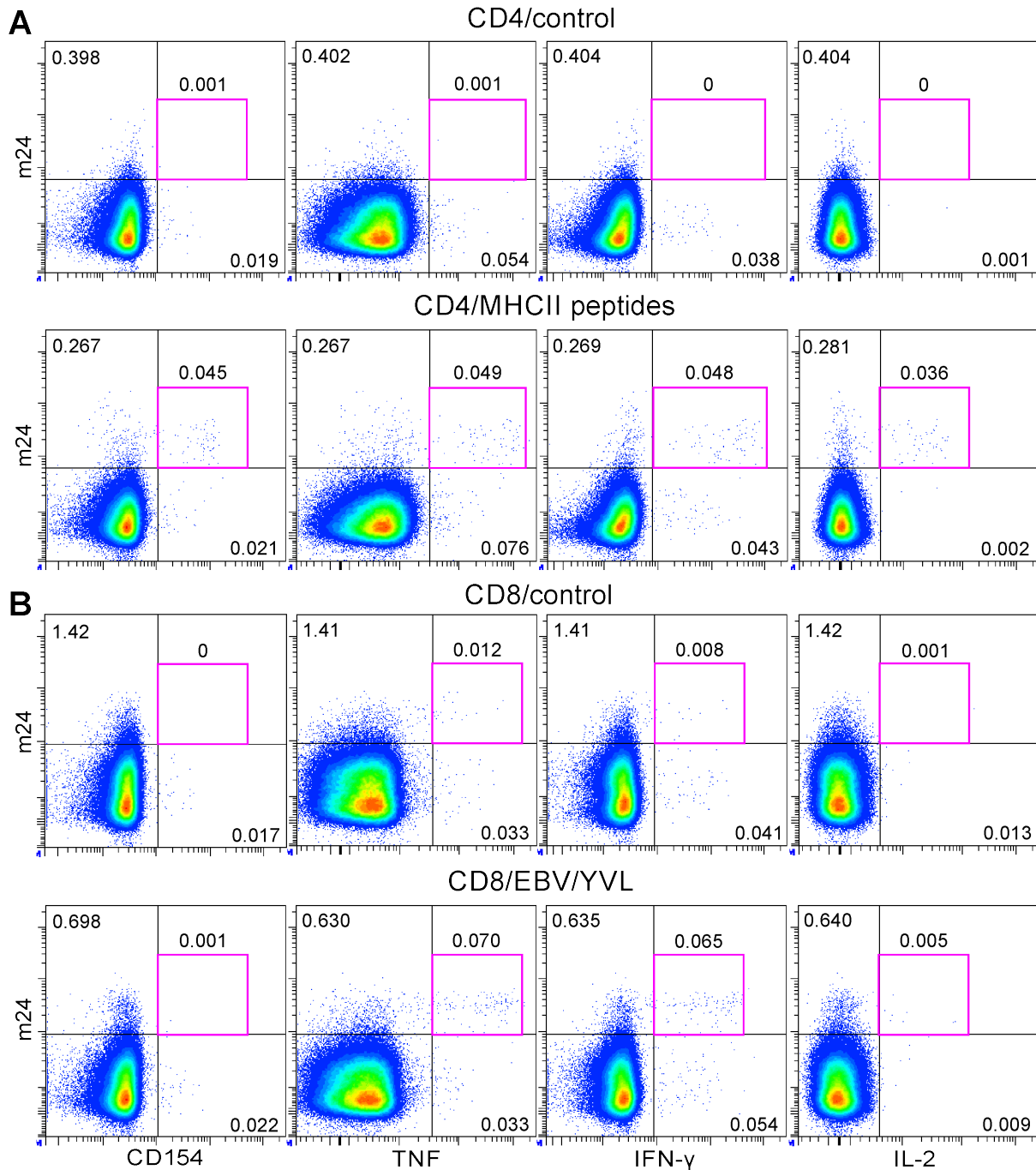


Figure 10. The application of the m24 Ab assay for simultaneous detection of antigen-specific CD4⁺ and CD8⁺ T cells within cryopreserved peripheral blood mononuclear cells (PBMCs). PBMCs of a preselected healthy donor were thawed and rested overnight before a 4 h stimulation with a pool of HLA-class II and HLA-A*02 peptides. (A) m24 Ab staining and CD154, TNF, IFN- γ and IL-2 expressions for unstimulated (control) or major histocompatibility complex (MHC)-class II peptide-stimulated CD4⁺ T cells (mix of $n=9$ virus-derived peptides). (B) The same stainings for CD8⁺ T cells without stimulation (control) or after stimulation with a HLA-A*02 epitope (YVLDHLIVV, EBV/YVL). Gating strategy is similar to that of Figures 1A, 2A, with Zombie Aqua included as a live/dead cell marker. See also Supplementary Figure 5A.

5.2.5 Discussion

We describe a protocol for sensitive assessment of virus-specific CD4⁺ T cells, which, comparable to our prior ICAM-1 multimer assay for flow cytometry-based detection of CD8⁺ T cells, takes advantage of the conformational change of membrane-bound β_2 -integrins after the activation. Instead of multimers of the integrin ligands, the modified method uses a monoclonal Ab (m24 Ab) specific for the open, high-affinity conformation of activated β_2 -integrins. The m24 Ab detects very low numbers of effector CD4⁺ and CD8⁺ T cells in WB or cryopreserved PBMCs specific for a range of viruses (i.e., SARS-CoV-2, CMV, EBV, and HBV). The combination of m24 and CD154 Ab gives a remarkable low background in unstimulated samples ($\leq 0.002\%$ of CD4⁺ T cells), allowing the reliable detection of antigen-specific CD4⁺ T cells at extremely low frequencies (down to 0.004%). This is far below the lower detection limit of the standard flow cytometry assays and in the range of what can be resolved with combinatorial approaches (6, 17-19). A scheme summary of our suggested experimental procedure for detection of activated CD4⁺ and CD8⁺ T cells using m24 Ab is given in Figure 11.

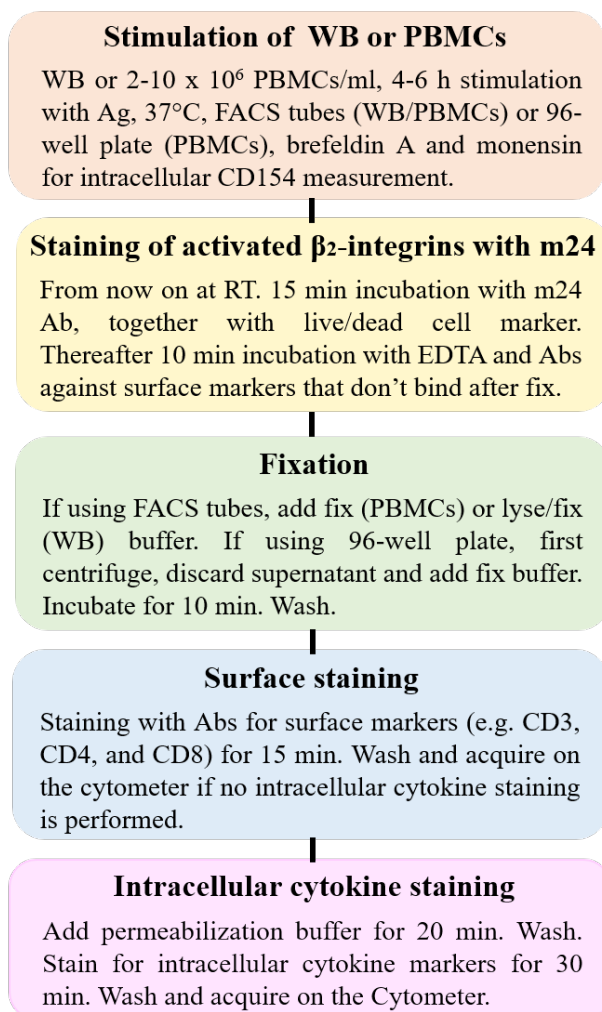


Figure 11. Figure The scheme of experimental procedure for flow cytometry-based detection of activated CD4⁺ and CD8⁺ T cells using m24 Ab. The procedure can be performed on WB or PBMCs, either in fluorescent-activated cell sorting (FACS) tubes (WB and PBMCs) or 96 well plates (PBMCs) and is divided into five main steps: (1) antigen-specific stimulation, (2) staining of activated β_2 -integrins with m24 Ab, followed by EDTA treatment, (3) fixation/erythrocyte lysis of WB or fixation of PBMCs, (4) cell surface marker staining, (5) permeabilization and intracellular staining. Additional modifications can be made for covering different specific questions as indicated in the scheme.

While the activation of β_2 -integrins on CD8⁺ T cells occurs within several minutes of peptide stimulation (5), we show that several hours are needed for CD4⁺ T cells. This difference might reflect specificities in the integrin activation pathways of the two main T lymphocyte subsets (e.g., differences in the expression of adaptor molecules downstream of the TCR signaling pathway). It might also explain their distinct patterns of immunological synapse formation (transient vs. stable), which may be adapted to their ultimate main functions (serial killing of numerous target cells vs. sustained cytokine production) (10). We know that rapid integrin activation can be found on various differentiation stages of memory and effector CD8⁺ T cells (5). Preliminary experiments for CD4⁺ T cells using CD27, CD28 and CCR7 markers indicate that activated β_2 -integrins after 4-6 hours of stimulation mark all CD4⁺ T cells irrespective of their differentiation stages (data not shown). However, it cannot be excluded that certain subsets require even longer time for activation of β_2 -integrins.

Although several hours of stimulation are needed for β_2 -integrin activation on CD4⁺ T cells, this event is still detectable earlier than the conventional flow cytometry markers on CD4⁺ T cells, such as CD154 and cytokines. We observed that functional (i.e. cytokine producing) CD4⁺ T cells were contained exclusively within the m24⁺ fraction, demonstrating that robust activation of β_2 -integrins reveals highly effective, functional antigen-specific CD4⁺ T cells. The intensity of m24 Ab staining on CD4⁺ T cells is relatively dim and has some background staining. However, the combination with other markers, for example, CD154, increases the signal-to-noise ratio, allowing detection of very low frequencies of CD4⁺ antigen-specific T cells. m24 Ab stained surface activated integrins, whereas CD154 was determined intracellularly together with a range of cytokines after stimulation in the presence of brefeldin A and monensin as previously described in Frentsch et al.(7). To simplify the assay, an option might be the cell surface staining of both activated β_2 -integrins and CD154, provided that a CD40-specific Ab is added to the culture to stabilize extracellular CD154 neo- expression (7).

EDTA treatment was required to visualize singlet responding CD4⁺ T cells, suggesting intense cell clustering between T helper and HLA-class II⁺ antigen-presenting cells. Since EDTA reversed the binding of ICAM-1 multimers to the activated β_2 -integrins, we used the m24 Ab whose binding was not affected by subsequent EDTA treatment. Similar to the detection with ICAM-1 multimers(5), staining of the β_2 -integrin activation with m24 Ab on CD8⁺ T cells was maximal after 4–16 min of stimulation with peptides. However, in contrast to the ICAM-1 multimers, m24 Ab binding on antigen-specific CD8⁺ T cells diminished only slightly following prolonged activation (4–6h). Therefore, m24 Ab staining can be used for simultaneous screening of both CD4⁺ and CD8⁺ T cell reactivities. Since the fluorescence

intensity of m24 Ab is generally dimmer than that of ICAM-1 multimers, we however recommend to perform staining after a short-term stimulation with ICAM-1 multimers (without EDTA treatment) when only antigen-specific CD8⁺ T cells are assessed [see Figure 2 and Supplementary Figure 10 from Schöllhorn et al. (20 Preprint)].

To use the assay for clinical samples, it was important to optimize the protocol for the detection of T cells not only in WB but also in cryopreserved PBMCs. We used 2×10^6 PBMCs in 1ml medium per test in FACS tubes, which was suitable for the detection of antigen-specific CD4⁺ and CD8⁺ T cells after a 4–6h stimulation. If needed, upscaling of the cell concentration is possible without major problems. This is in contrast to the short-term stimulation protocol for the detection of antigen-specific CD8⁺ T cells alone. In the short term stimulation, we observed that the PBMC concentration (2×10^6 cells in 1 ml or 0.2 ml medium) influenced detection due to heavy aggregation in the presence of activating peptide when the higher PBMC concentration was used [see Supplementary Figures 6–9 from Schöllhorn et al.(20 Preprint)]. Another observation we made was that frequencies of antigen-specific CD4⁺ T cells were higher within PBMCs than when using WB, possibly reflecting different cell– cell interaction conditions in the two cell sources. We also observed that the method is transferable to 96-well plates, which allows easier handling of many tests simultaneously. By repeated testing of the very same donor and specificity ($\sim 0.2\%$ of the CD4⁺ subset), we also observed excellent intra- (triplicate tests) and inter-experiment (repetition on 3 days) variability, with percentage coefficients of variation ranging from ~ 3 to 13%, which is in the range of other functional T cell assays (data not shown).

As a currently highly relevant research topic, we evaluated CD4⁺ and CD8⁺ antigen-specific T cells in SARS-CoV-2 convalescents in comparison to unexposed healthy donors. CD4⁺ T cells specific for S, N, and M proteins were detected in all three convalescents, whereas CD8⁺ T cells were detected only toward the N protein. In line with other recent reports, we also observed in one unexposed donor very low frequencies of CD4⁺ and CD8⁺ T cells against S and N proteins, respectively, probably due to cross-reactive cells primed against other SARS viruses (21-23). We also used m24 Ab staining to confirm the induction of anti-vaccine CD4⁺ T cells after experimental immunization of one healthy volunteer with synthetic peptides from the S and N proteins (13 Preprint). Importantly, the results matched those of a previous IFN- γ ELISpot, and our method allowed reliable detection of the weakest anti-peptide response (0.012% m24⁺ CD154⁺ CD4⁺ T cells vs. 29 spots/300,000 PBMCs against CoV-2/FYV peptide).

The m24 Ab identifies functional cells (i.e., those that produce cytokines upon stimulation). Interestingly, different CD4⁺ antigen specificities showed unique cytokine profiles, which were also distinct in post infection vs. vaccination conditions (Figure 12).

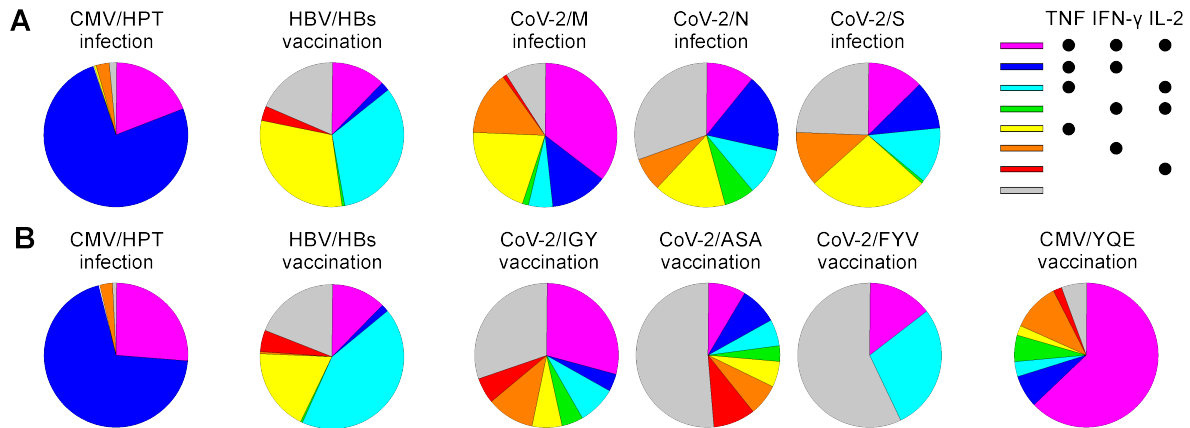


Figure 12. The polyfunctional profile of CD154⁺ m24⁺ CD4⁺ WB T cells for various viruses and vaccination conditions. The pie charts represent the distribution of the cytokine production patterns from the donors and antigens tested in the study after CMV peptide vaccination ($n=1$), CMV infection ($n=1$), CoV-2 infection ($n=3$, mean is represented), CoV-2 peptide vaccination ($n=1$) or HBV vaccination ($n=2$, mean is represented) after 4 h (A) or 6 h (B) of stimulation with the respective peptides. Samples of the CMV infection and HBV vaccination were stimulated for 4 and 6 h for comparative purposes.

Chronically CMV-infected individuals showed a prevalent TNF and IFN- γ production pattern, with some IL-2, while CMV-peptide boost vaccination was predominated by a triple cytokine production with higher IL-2 levels. The HBV vaccination selected for a TNF-dominant memory cell pool that was almost equally distributed in the TNF production alone, together with IL-2, or with both IL-2 and IFN- γ . SARS-CoV-2 convalescents had a more heterogeneous functional profile of either single, double, or triple cytokine-producing cells, possibly reflecting a peculiar immune response to the virus and/or recent infection. Only the response to the M protein was dominated by triple-positive cells. Finally, the vaccination response to one of the three CoV-2 peptides (CoV-2/IGY) was not only the strongest but also characterized by the highest proportion of triple cytokine positive cells. Thus, ex vivo costaining for activated integrins and CD154 on very low frequencies of effector/memory CD4⁺ T cells allows gaining precise information about the polyfunctionality of the immune response. This is of importance since prior findings showed that the magnitude of the vaccine-induced polyfunctionality highly correlates with protection against pathogens (24-26). Although we essentially analyzed Th1 cells, including Abs against further cytokines could be useful to dissect the functional heterogeneity and refine Th profiles of pathogen- or vaccine-specific CD4⁺ T cell responses (27).

In conclusion, we present a cytometry-based assay for the identification of antigen-specific CD4⁺ and CD8⁺ T cells. For CD4⁺ T cells, in particular, costaining of β_2 -integrins with the activation marker CD154 allows the detection of extremely low frequencies of

responding polyfunctional cells. The assay can be used for highly sensitive screening of T cell reactivities, for example, in epitope mapping studies, as they are currently performed for the new SARS-CoV-2 virus or in translational approaches.

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5.3 Part III

Staining of activated β_2 -integrins in combination with CD137 and CD154 for sensitive identification of functional antigen-specific CD4⁺ and CD8⁺ T cells

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Supplementary data of part III see pages 195 to 202.

The author of the present thesis was involved in the conception of the entire study. The author planned, performed, and analyzed all experiments that are presented in the manuscript and prepared all figures. The author gave intellectual input and was involved in the interpretation of the data and in writing and revising the manuscript.

5.3.1 Abstract

Common flow cytometry-based methods used for functional assessment of antigen-specific T cells rely on *de novo* expression of intracellular cytokines or cell surface activation induced markers. They come with some limitations such as complex experimental setting, loss of cell viability and often high unspecific background which impairs assay sensitivity. We have previously shown that staining of activated β_2 -integrins either with multimers of their ligand ICAM-1 or with a monoclonal antibody can serve as a functional marker detectable on T cells after minutes (CD8⁺) or few hours (CD4⁺) of activation. Here, we present a simple method for detection of activated β_2 -integrins in combination with established cell surface activation induced markers. We observed that activated β_2 -integrins were still detectable after 14 hours of stimulation, allowing their detection together with CD137 and CD154. Combinatorial gating of cells expressing activated β_2 -integrins and CD137 or CD154 reduced background in unstimulated samples, increasing the signal-to-noise ratio and allowing improved assessment of low-frequency T cell responses. Extracellular staining of these markers highly correlated with production of intracellular cytokines IL-2, TNF or IFN- γ in CD4⁺ and CD8⁺ T cells. As an exemplary application, SARS-CoV-2 spike-specific T cell responses were assessed in individuals after COVID-19 vaccination. This method should be useful for epitope discovery projects and for the simultaneous monitoring of low-frequency antigen-specific CD4⁺ and CD8⁺ T cell responses in various physiological situations.

5.3.2 Introduction

CD4⁺ and CD8⁺ T cells play an essential role in immunity against infectious diseases and cancer. For both basic and translational research (e.g. epitope mapping or monitoring of clinical specimens), testing of antigen-specific T cells should be sensitive enough to detect and quantify low-frequency antigen-specific T cells, robust and reproducible, as well as applicable to high numbers of tests and limited amount of material. Flow cytometry is commonly used to assess antigen-specific T cells, as it presents the unique advantage to deliver multiparametric, phenotypic and functional information at a single cell level while allowing to routinely analyze millions of cells in a short time (1, 2). A common method for assessment of T cell functionality is intracellular cytokine staining (ICS) for detection of e.g. IFN γ , TNF and IL-2, which can be completed by cell surface or intracellular assessment of activation markers expressed after T cell stimulation (e.g. CD154, CD107a). However, ICS is per se limited by the choice of the cytokines to be detected. The method is also intrinsically complex, requiring cell culture over several hours in the presence of protein transport inhibitors and time-consuming staining procedures including permeabilization/ fixation

steps which do not preserve cell viability. In addition, ICS has been shown to be sensitive to small variations in protocols and cell handling (3-5).

Cell surface activation induced markers (AIMs) identify reactive antigen-specific T cells independently of their cytokine production pattern, and are not restricted to certain T cell subsets or differentiation states (6). As an example, CD154 (CD40 ligand), a costimulatory molecule expressed on activated Th cells, is routinely used intracellularly or extracellularly for assessing antigen-specific CD4⁺ T cells (7-9). Further AIMs can be implemented for detection of antigen-specific CD4⁺ T cells, like combinations of CD69/CD154 or OX40/CD25, and more recently OX40/PD-L1 or OX40/CD137 (4-1BB), with stimulation times between 9 and 20 hours (h), depending on the marker combination (10-12). Since CD154 is not broadly expressed on CD8⁺ T cells, it can be replaced by CD137, OX40 or CD25 for assessing this subset (12-14). In a recent screening study of SARS-CoV-2-reactive T cells in convalescent donors, the AIM combinations CD137/OX40 and CD137/CD69 were analyzed separately for CD4⁺ and CD8⁺ T cells, respectively, after 24 h of stimulation (15). Although it would be material- (clinical samples are available in limited amount) and time-advantageous, simultaneous detection of CD4⁺ and CD8⁺ T cells with the very same AIM combination is rarely performed. Moreover, AIM expression on the cell surface of unstimulated cells is not negligible, which impairs the sensitive detection of low-frequency T cells.

β_2 -integrins (e.g. LFA-1) are already expressed on the cell surface of resting antigen-experienced T cells in a steady-state, inactivated conformation. TCR-mediated activation induces a conformational change to an open high-affinity form and clustering of β_2 -integrins, leading to an increase in affinity and valency which allows interaction with its ligand intercellular adhesion molecule (ICAM)-1 (16, 17). We have previously used activated β_2 -integrins as an early cell surface activation marker that is detectable after minutes (CD8⁺ T cells) or few hours (CD4⁺ T cells) of antigen-specific stimulation. Activated β_2 -integrins were specifically stained with fluorescent multimeric ICAM-1 (on CD8⁺ T cells) or with a conformational-specific monoclonal antibody (clone m24, on CD8⁺ and CD4⁺ T cells) (18, 19). Moreover, combination of activated β_2 -integrin staining and intracellular cytokine staining (m24+ICS) demonstrated that m24 antibody (Ab) staining is a surrogate marker for cytokine production and allowed identification of very low frequency functional antigen-specific T cells after short *in vitro* stimulation (19).

We now introduce activated β_2 -integrins as a cell surface activation marker that can be combined to already established AIMs for simultaneous detection of functional antigen-specific CD4⁺ and CD8⁺ T cells. First, several AIMs were tested for combination with m24 Ab staining: CD25, CD69, CD137 and CD154. Based on the following considerations (i) the expression kinetics of the different markers, ii) the feasibility of detecting simultaneously

CD4⁺ and CD8⁺ T cells, and iii) the need to reduce background staining in unstimulated cells for improved detection of rare events, we propose to combine cell surface staining of β_2 -integrins with CD137 and CD154 (m24+AIM). Detection of these markers together decreased background and correlated with intracellular cytokine staining, with higher frequencies of antigen-specific T cells being detected with m24+AIM than with m24+ICS. As an example of the application of the assay to clinical samples, we show that SARS-CoV-2 spike-specific CD4⁺ and CD8⁺ T cells can be readily detected in healthy donors after SARS-CoV-2 vaccination.

5.3.3 Materials and methods

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from mononuclear cell blood concentrates (cones) or Na-heparinized blood by cell density centrifugation (Lymphoprep, Biowest). PBMCs were washed twice with PBS (w/o Ca²⁺/Mg²⁺) and counted with an automated cell counter (NucleoCounter, Chemometec) using Solution 18 (AO/DAPI, Chemometec). Aliquots of ~20 Mio cells/ml were frozen in 90 % heat-inactivated fetal bovine serum (FBS; Capricorn Scientific) containing 10 % DMSO (Merck) at -80°C and stored in liquid nitrogen until usage.

Study subjects and experimental settings

PBMCs from altogether 21 healthy donors (HD) were used (Supplementary Table 1). Expression kinetics were performed with two of them (Supplementary Fig 1A, HD2 and HD21). Three HLA-A*02⁺ donors with detectable Flu-, CMV- or EBV-specific CD4⁺ and CD8⁺ T cell responses in pre-screening experiments (performed with multimer stainings for CD8⁺ T cells and ICS after synthetic peptide stimulation for CD4⁺ T cells) were used for further AIM selection (Supplementary Fig 1B,C, HD1, 3 and 4). Three HLA-A*02⁺ and two HLA-A*02⁻ donors (HD4, HD11-14) were tested with HIV-derived peptides. Three donors were used to compare the staining procedure of m24+AIM with and without cell fixation (Supplementary Figure 4, HD11, HD19 and HD20). For the comparison of m24+AIM and m24+ICS assays, we collected PBMCs from six healthy donors between three to five weeks after the third SARS-CoV-2 vaccination and screened them for SARS-CoV-2-specific, as well as for Flu-specific T cell responses (HD5-10, Figures 1B, C, 2A, B, 3A, 4A, B, 5A). Pre-pandemic samples from five individuals collected between September 2019 and January 2020 were also tested for SARS-CoV-2-specific T cell responses (HD7, HD15-18, Figure 5B), one of these (HD7) was also tested after SARS-CoV-2 vaccination.

Blood collection and study were approved by the Ethics Committee of the University of Tübingen (approvals 156/2012B01 and 713/2018B02), and all participants gave written informed consent.

Peptides and stimuli

For simultaneous antigen-specific stimulation of CD8⁺ and CD4⁺ T cells, synthetic HLA-class I and -class II peptides either produced in-house or purchased as 15-mer overlapping peptide pools were used (Supplementary Table 1).

A HLA-class II peptide mix of nine described epitopes derived from various proteins of CMV (n = 2), EBV (n = 6), and Flu (n = 1) viruses (Mix-II) together with the HLA-A*02 binding GLCTLVAML epitope from EBV BMLF1 protein, aa 259-267 (EBV/GLC) was used. For screening HIV-specificities, the epitopes ILKEPVHGV (HIV RT, aa 476-484, HLA-A*02 binder) and YVDRFYKTLRAEQASQEV (HIV Gag, aa 164-181, HLA-DRB1 binder) were pooled (HIV I+II). All in-house synthesized peptides were dissolved in 10 % DMSO/H₂O and stored at -80°C at a concentration of 1 mg/ml. Pools of 15-mer peptides overlapping by eleven amino acids and spanning the entire CMV/pp65 (CMV/pp65 peptides) and Flu/matrix proteins (Flu/M peptides) were purchased from JPT Peptide Technologies, those spanning the entire SARS-CoV-2/spike (CoV-2/S peptides) from Miltenyi Biotec. Peptide pools from JPT were dissolved to a concentration of 500 µg/ml in 100 % DMSO, while the spike peptide pool was dissolved to a concentration of 100 µg/ml in 20 % DMSO/H₂O, aliquoted and stored at -80°C. As positive control, cells were stimulated with Staphylococcal enterotoxin B (SEB; Sigma-Aldrich). SEB was dissolved in PBS w/o Ca²⁺/Mg²⁺ and stored at -20°C in 1 mg/ml aliquots. Unstimulated control samples were supplemented with a final concentration of DMSO identical to that used in the stimulated tests.

Thawing and resting of PBMCs

Cryopreserved PBMCs were thawed in IMDM (Gibco) containing 2.5 % heat-inactivated (hi) human serum (HS; Capricorn Scientific), 100 U/ml penicillin/ 100 µg/ml streptomycin (1X P/S, Sigma-Aldrich) and 50 µM β-mercaptoethanol (β-ME; Merck). After washing once (300 x g, 5 min, room temperature (RT)) with serum free IMDM medium (IMDM, P/S, 50 µM β-ME), cells were counted and rested for 4 to 6 h in T cell medium (TCM; IMDM, 10 % HS hi, 1X P/S, 50 µM β-ME) containing 1 µg/ml DNase I (Sigma-Aldrich) at a concentration of 2-3 Mio cells/ml and not more than 20 Mio cells per 50 ml Falcon tube at 37°C and 7.5 % CO₂.

Kinetics for detection of cell surface AIMs CD25, CD69, CD137, OX40 and CD154

After resting, cells were washed (300 x g, 5min, RT) with serum free IMDM medium, counted, and resuspended in the appropriate volume of TCM for stimulation. For cell surface staining of CD25, CD69 and CD137, 2 Mio PBMCs were incubated with 10 µg/mL SEB or left unstimulated in a final volume of 150 µl in sterile 96 well U-bottom culture plates (Greiner Bio-One) for 4, 6, 14 and 24 h at 37°C and 7.5 % CO₂. After stimulation, 50 µl FACS buffer (PBS, 2 % FBS hi, 2mM EDTA and 0.02 % sodium azide) was added to the wells,

samples were centrifuged (380 x g, 5 min, RT), washed with 150 µl FACS buffer and stained with a mix of live/dead dye (Zombie aqua fixable dye, Biolegend), CD4-FITC (in-house production), CD8-AF700, CD25-PE-Cy7, CD69-BV421, CD137-PE-Dazzle594 and OX40-APC antibodies (Abs) in 50 µl FACS buffer. For extracellular detection of CD154, cells must be stimulated in the presence of a purified CD40 Ab to prevent cell surface downregulation and internalization of CD154 upon ligand binding (6, 8, 20). 2 Mio PBMCs were incubated with 10 µg/mL SEB or left unstimulated in the presence of 2 µg/ml CD40 Ab (pure-functional grade, clone HB14, Miltenyi Biotec) in a final volume of 150 µl in sterile 96 well U-bottom culture plates for 4, 6, 14 and 24 h at 37°C and 7.5 % CO₂. After stimulation, 50 µl FACS buffer was added, samples were centrifuged, washed with 150 µl FACS buffer and stained with a mix of Zombie aqua, CD4-FITC, CD8-AF700 and CD154-APC Abs in 50 µl FACS buffer. Stainings were performed for 20 min at 4°C in the dark. Cells were washed twice with 150 µl FACS buffer, resuspended in FACS buffer and analyzed on the same day. All Abs were purchased from Biolegend, if not stated differently, and were used at recommended or pre-tested dilutions.

Stimulation of antigen-specific CD4⁺ and CD8⁺ T cells

After resting, cells were washed twice (300 x g, 5min, RT) with CTS medium (CTS OpTmizer T-Cell Expansion SFM containing supplement (Gibco), 2 mM L-Glutamine (Sigma-Aldrich) and 1X P/S) and counted before resuspending in CTS medium + 2 µg/ml DNase I for stimulation (optimized medium conditions for T cell recovery and m24 Ab staining). 2 Mio PBMCs were stimulated in a final volume of 200 µl in sterile 96 well U-bottom culture plates (Greiner Bio-One) for 14 h at 37°C and 7.5 % CO₂ in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich) and Golgi Stop (1:1500, BD Biosciences) for intracellular marker staining (m24+ICS), or in the presence of 2 µg/ml CD40 Ab (pure-functional grade, clone HB14, Miltenyi Biotec) for staining of cell surface activation induced marker (m24+AIM). Purchased overlapping peptide pools or in-house synthesized peptides were used at a final concentration of 2 µg/ml or 4 µg/ml per peptide, respectively.

m24 Ab staining combined to intracellular marker staining (m24+ICS)

After stimulation, samples were incubated for 4 min at RT while removing 100 µl of the supernatant thus leaving 100 µl of the stimulation volume without disturbing the cell pellet. Activated β_2 -integrins were stained with a PE-labeled monoclonal Ab (m24 Ab clone, 1:100) and live/dead dye Zombie aqua for 15 min. 100 mM EDTA diluted in PBS was added to a final concentration of 4 mM EDTA and incubated for 10 min. Samples were centrifuged (380 x g, 5 min), fixed with 200 µl 1X FACS-Lysing solution (BD Biosciences) for 10 min, and washed with PBS/ 0.5 % BSA/ 0.1 % sodium azide (500 x g, 5 min). All following centrifugation steps were performed at 500 x g (5 min) and all washing steps, as well as Ab

dilutions, were in PBS/ 0.5 % BSA/ 0.1 % sodium azide. CD3-PerCP-Cy5.5, CD4-BV421, and CD8-APC-Cy7 Abs were added in 50 µl volume for 15 min. Cells were washed by adding 150 µl wash buffer, followed by permeabilization for 20 min with 200 µL 1X FACS-Permeabilizing solution 2 (BD Biosciences). Samples were centrifuged and stained for intracellular markers with TNF-BV605, IFN γ -FITC, IL-2-PE-Cy7 and CD154-APC Abs in 50 µl volume for 30 min. After two more washing steps, cells were resuspended in FACS buffer and analyzed on the same day. All Abs were purchased from Biolegend and pre-titrated. Incubation, staining and centrifugation steps were performed at RT in the dark.

m24 Ab staining combined to cell surface activation marker staining (m24+AIM)

After stimulation, samples were incubated for 4 min at RT while removing 100 µl of the supernatant thus leaving 100 µl of the stimulation volume without disturbing the cell pellet. Activated β_2 -integrins were stained with PE-labeled m24 Ab (1:100) for 15 min. A mix of live/dead dye Zombie aqua, CD3-PerCP-Cy5.5, CD4-BV421, CD8-APC-Cy7, CD154-APC, CD137-PE-Dazzle594 Abs diluted in PBS/ 0.5 % BSA/ 0.1 % sodium azide supplemented with 4 mM EDTA was added directly into the remaining stimulation volume (final volume 125 µl) and incubated for 15 min. When CD69 was stained (Supplementary Figure 1C, HD4), Zombie aqua, CD3-PerCP-Cy5.5, CD4-APC-Cy7 (BD Biosciences), CD8-AF700, CD154-APC, CD137-PE-Dazzle594 and CD69-BV421 Abs were used. Afterwards, samples were centrifuged (380 x g, 5 min), fixed with 200 µl 1X FACS-Lysing solution (BD Biosciences) for 10 min and washed with PBS/ 0.5 % BSA/ 0.1 % sodium azide (500 x g, 5 min). Cells were resuspended in FACS buffer and analyzed on the same day. All Abs were purchased from Biolegend, if not stated differently, and pre-titrated. Incubation, staining and centrifugation steps were performed at RT in the dark.

Flow cytometry analysis

Samples were acquired on a BD LSRFortessa with the Diva software V.6 (between 450,000 and 1,000,000 events were recorded) and analyzed with Flow Jo 10.6.2 (BD Biosciences). Gating strategy is shown in Supplementary Figure 2A. Results are presented as percent of cells within the parent CD4⁺ or CD8⁺ living CD3⁺ lymphocyte population. To compare the combination of m24 Ab and cell surface AIM staining (m24+AIM) with the combination of m24 Ab and intracellular activation marker staining (m24+ICS), frequencies of m24⁺ and at least one additional marker⁺ T cells after Boolean gating (21) were summed up (Supplementary Figure 1D, Figures 3A, B, 4A, 5A, B). Depending on the cell type (CD4⁺ or CD8⁺) and the staining procedure (m24+AIM or m24+ICS), different marker combinations were considered: m24 Ab, CD154 and CD137 or m24 Ab, TNF, IFN γ , IL-2 and CD154 for CD4⁺ T cells; m24 Ab and CD137 or m24 Ab, TNF and IFN γ for CD8⁺ T cells. For assessing antigen-specific T cells, frequencies of marker⁺ cells detected in the unstimulated

control samples were subtracted from the frequencies of marker⁺ cells in the stimulated samples (background subtracted). Stimulated:control ratios were calculated by dividing the mean frequency of antigen-specific T cells by the mean frequency of the corresponding unstimulated control samples (Figures 1C, 3A, 5A, B).

Graphs and statistical analysis

Graphs and statistical analyses were prepared with Prism (GraphPad, version 9.4.0). For comparison of m24+AIM and m24+ICS datapoints, a Wilcoxon matched pairs test was performed (significant if $p < 0.05$, Figures 3A, B, 5A, B). Spearman correlation analyses and simple linear regressions were used to determine Spearman r and x-intercepts (Figures 4A, B, Supplementary Figure 3), respectively. x-intercept gives information on the closeness of the results between the two parameters or assays compared. If data was plotted on a logarithmic scale, values that were 0 were set to 0.0001 (Figures 1C, 3A, 4A, 5A and Supplementary Figure 3).

5.3.4 Results

Combination of cell surface staining of activated β_2 -integrins and AIMs CD137 and CD154 is optimal for detection of antigen-specific CD4⁺ and CD8⁺ T cells

To determine the optimal conditions for co-staining activated β_2 -integrins with other established AIMs, we first examined which markers can be detected simultaneously. The expression kinetics of various AIMs, namely CD25, CD69, CD137, OX40 and CD154, were assessed in PBMCs from two healthy donors (HD PBMCs) stimulated with SEB for 4, 6, 14 and 24 h. CD25 expression was almost not detectable at 4 and 6 h and reached maximum at 24 h on both CD4⁺ and CD8⁺ T cells. OX40 shows very low expression on CD8⁺ T cells and low expression on CD4⁺ T cells after 4 and 6 h of stimulation, with maximum also reached at 24 h. Both markers are therefore not favorable for a fast read out (Supplementary Figure 1A). CD69 and CD137 were upregulated on both CD4⁺ and CD8⁺ T cells; CD69 expression was evident already at 4 h of stimulation and remained stable up to 24 h, while CD137 expression peaked between 6 and 14 h of stimulation (Supplementary Figure 1A). In accordance with previous reports (8, 9), CD154 was detectable on CD4⁺ T cells, with maximum expression reached at 4 h and maintained up to 14 h after stimulation (Supplementary Figure 1A), but was poorly expressed on CD8⁺ T cells (Supplementary Figures 1A, C, D). Hence, expression of CD69, CD137 and CD154 can be optimally detected after overnight (14 h) T cell activation.

We previously showed that β_2 -integrin activation is an early event (approx. 5-15 min and 4-6 h for CD8⁺ and CD4⁺ T cells, respectively) following antigen-specific *in vitro* stimulation (18, 19). To combine this marker with further AIMs, we verified that β_2 -integrin activation was detectable after 14 h of stimulation (Supplementary Figure 1B). For CD4⁺ T cells, frequency of m24⁺ cells increased between 4 and 14 h of stimulation (e.g. 0.58% to 0.70% for HD1), with minor changes in the expression within the unstimulated control (e.g. 0.063% to 0.075% for HD1, Supplementary Fig 1B, top). For CD8⁺ T cells, frequency of m24⁺ cells decreased between 4 and 14 h of stimulation, however frequency in the unstimulated control also decreased, which led to an increased signal-to-noise (stimulated:control) ratio (Supplementary Figure 1B, e.g. for HD1 stimulated:control ratios 5.8 (4 h) and 8.3 (14 h)).

Combination of m24 Ab staining with detection of CD69, CD137 and CD154 after 14 h of stimulation in the presence of CD40 Ab (to prevent downregulation of CD154 upon ligand binding) was tested in four donors that were previously screened for antigen reactivities, one representative donor is presented in Supplementary Figure 1C. Staining of CD69 showed very high background in unstimulated cells, and a distinct population in the antigen-stimulated sample was difficult to identify (Supplementary Figure 1A, C left panel), hence, this marker was not kept for further testing. In contrast, combination of m24 Ab

staining with detection of CD137 (for CD8⁺ and CD4⁺ T cells) and CD154 (for CD4⁺ T cells) allowed clear identification of antigen-reacting T cells (Supplementary Figure 1C, middle and right panels). Based on these results our final protocol combines m24 Ab staining with AIMs CD137 for CD8⁺ T cells or CD137 and CD154 for CD4⁺ T cells (m24+AIM; Figure 1A for schematic overview). The gating strategy adopted for the analysis is presented in Supplementary Figure 2A and a representative dot plot is shown in Figure 1B.

While analysis of single markers showed notable background staining in unstimulated control cells, the combination of CD137 or CD154 detection with m24 Ab staining was sufficient to decrease the background (to <0.01% cells and 0.02% in mean for CD4⁺ and CD8⁺ T cells, respectively; Figures 1B, C). In the stimulated samples, the combination of markers also showed a decline in the frequencies of CD4⁺ responding T cells compared to single marker stainings, but not as consequent as for the controls (Fig 1C). Importantly, the stimulated:control ratio was greatly improved when combining m24 Ab with CD137 or CD154 staining for CD4⁺ T cells, or m24 Ab and CD137 for CD8⁺ T cells (Figure 1C, bottom tables). Hence, combination of m24 Ab staining with AIMs CD137 and CD154 improved the signal-to-noise ratio and thereby gives the possibility to detect very low frequencies of antigen-specific CD4⁺ and CD8⁺ T cells.

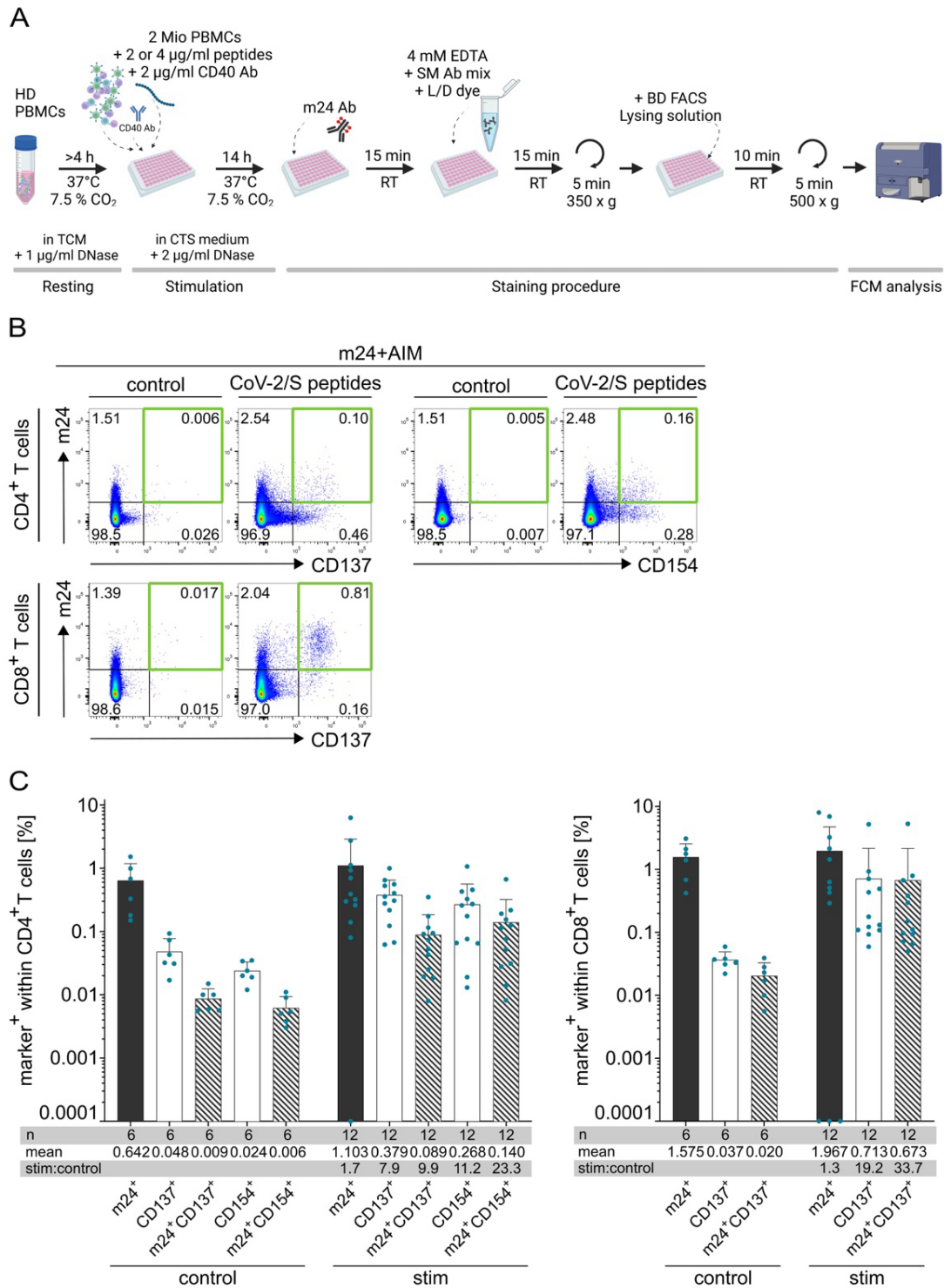
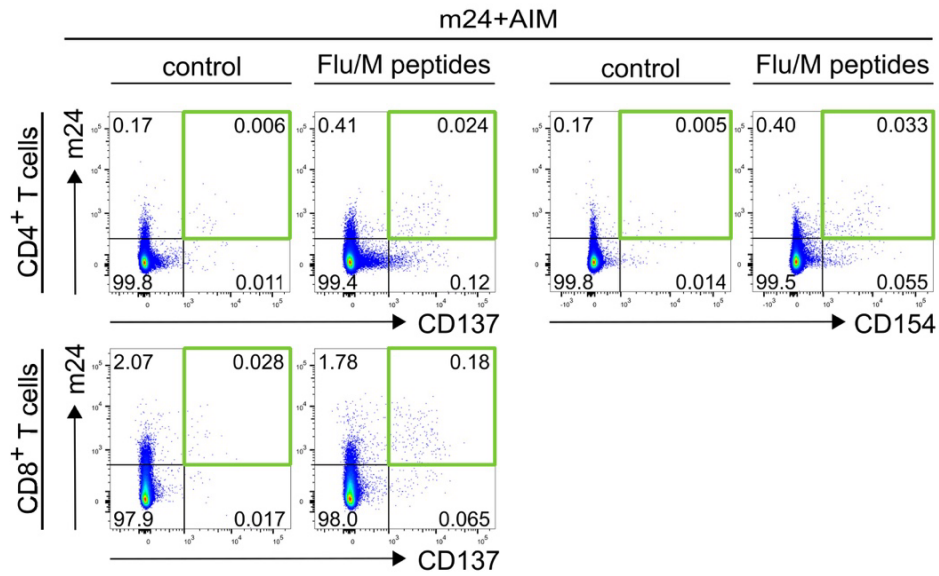


Figure 1. Co-staining of cell surface activated β 2-integrins with CD137 and CD154 (m24+AIM) allows identification of antigen-specific T cells. (A) Scheme of the m24+AIM protocol (created with BioRender.com). PBMCs from healthy donors (HD) are stimulated with antigenic peptides or left unstimulated for 14 h in CTS medium + DNase in the presence of CD40 Ab. Activated β 2-integrins are then stained with m24 Ab, followed by incubation with EDTA, surface markers (SM) and live/dead dye (L/D) before fixation and analysis by flow cytometry. (B) Exemplary dot plots of PBMCs from donor HD5 incubated with SARS-CoV-2/S overlapping peptides or unstimulated control. m24 Ab staining in combination with CD137 or CD154 expression on CD4⁺ T cells (top) or m24 Ab staining and CD137 expression on CD8⁺ T cells (bottom). Numbers represent frequencies within CD4⁺ or CD8⁺ T cells, the green squares mark the subsets of interest. (C) Mean + SD of the frequencies of cells expressing individual activation markers (unicolored bars) and combination of markers (striped bars) within CD4⁺ (left) and CD8⁺ (right) T cells. Number of donor/antigen combinations tested (n), mean and stimulated:control ratios (stim:control) are indicated. Frequencies of stimulated samples are background subtracted. Values that were 0 were set to 0.0001.

Combined cell surface staining of activated β_2 -integrins with CD137 and CD154 is more sensitive than intracellular marker staining to identify functional antigen-specific T cells

Activated β_2 -integrins are expressed by the majority of cytokine-expressing T cells after antigen-specific stimulation (18, 19). We therefore compared the frequencies of antigen-specific T cells detected by combination of activated β_2 -integrins and cell surface activation marker staining (m24+AIM) with that detected by combination of activated β_2 -integrin and intracellular activation marker staining (m24+ICS), which is a broadly accepted read out to detect functional T cells. Since cell surface expression of activation markers is inhibited in the presence of brefeldin A and monensin [(7) and data not shown], all markers could not be assessed simultaneously, and two independent tests were performed in parallel (m24+AIM vs m24+ICS). For CD4⁺ T cells, the combinations of m24 Ab with CD154 or CD137 vs m24 Ab with TNF, IFN γ , IL-2 or intracellular CD154 were assessed. For CD8⁺ T cells, the combination of m24 Ab and CD137 was compared to that of m24 Ab and TNF or IFN γ . PBMCs from six donors (HD5-HD10) were tested for SARS-CoV-2/S- and Flu/M-reactive T cell responses (n=12 donor/antigen combinations). Exemplary dot plots of m24+AIM and m24+ICS stainings of CD4⁺ and CD8⁺ T cells stimulated with Flu/M overlapping peptides are shown in Figure 2A, B for donor HD6. An additional example for m24+AIM and m24+ICS stimulated with SARS-CoV-2/S peptides is presented for donor HD5 in Figure 1B and Supplementary Figure 2B, respectively.

A



B

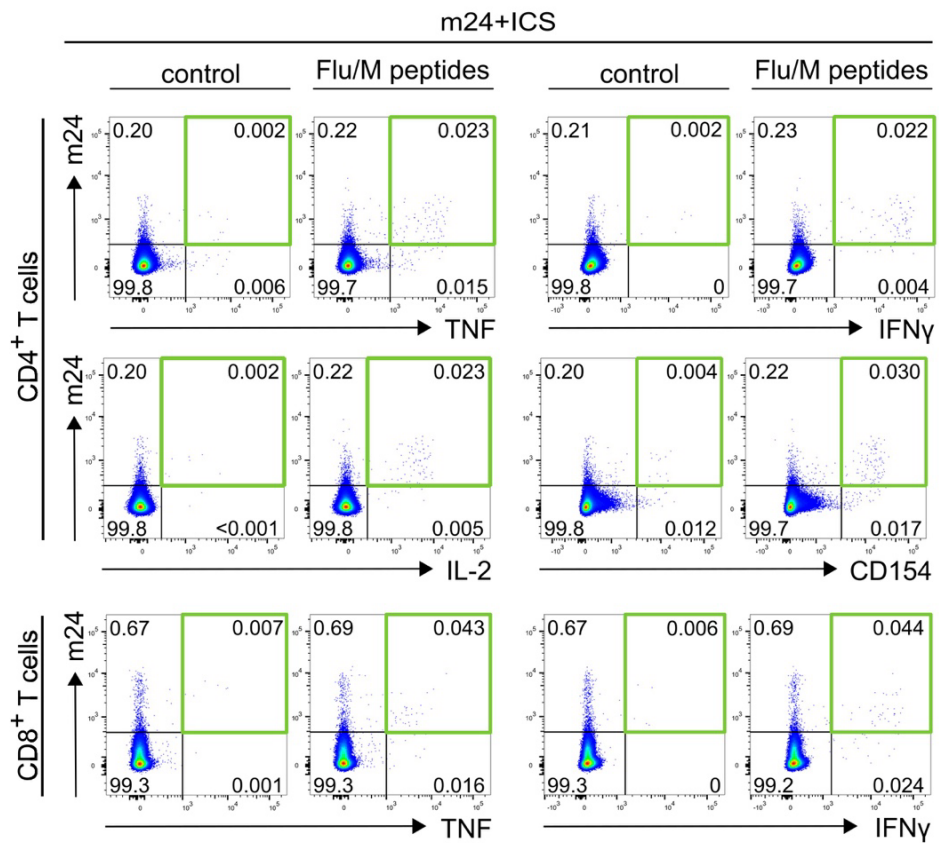


Figure 2. Detection of antigen-specific T cells using m24+AIM (A) or m24+ICS (B). Exemplary dot plots of PBMCs from donor HD6 incubated with Flu/M overlapping peptides or left unstimulated for 14 h in the presence of CD40 Ab (m24+AIM) or brefeldin A and monensin (m24+ICS). (A) m24+AIM: m24 Ab staining in combination with CD137 or CD154 expression on CD4⁺ T cells, or m24 Ab staining and CD137 expression on CD8⁺ T cells (B) m24+ICS: m24 Ab staining in combination with intracellular TNF, IFN γ , IL-2 and CD154 expression for CD4⁺ T cells, or intracellular TNF and IFN γ expression for CD8⁺ T cells. Numbers represent frequencies within CD4⁺ or CD8⁺ T cells. The green squares in (A, B) mark the populations of interest co-expressing two markers.

The summed frequencies of m24⁺ and at least one additional marker⁺ T cells after Boolean gating were calculated for each donor/antigen combination (n=12) detected with either m24+AIM or m24+ICS. Figure 3A displays the results obtained for unstimulated controls and antigen-specific stimulations of CD4⁺ (left graph) and CD8⁺ (right graph) T cells. The frequencies of reactive T cells in unstimulated control samples were slightly higher in both T cell subsets for m24+AIM compared to m24+ICS, but these differences were not significant (n=6, Figure 3A). The frequency of antigen-specific T cell responses was however significantly increased in CD4⁺ T cells (p=0.001) and even more pronounced in CD8⁺ T cells (p<0.001) for m24+AIM compared to m24+ICS (n=12, Figure 3A).

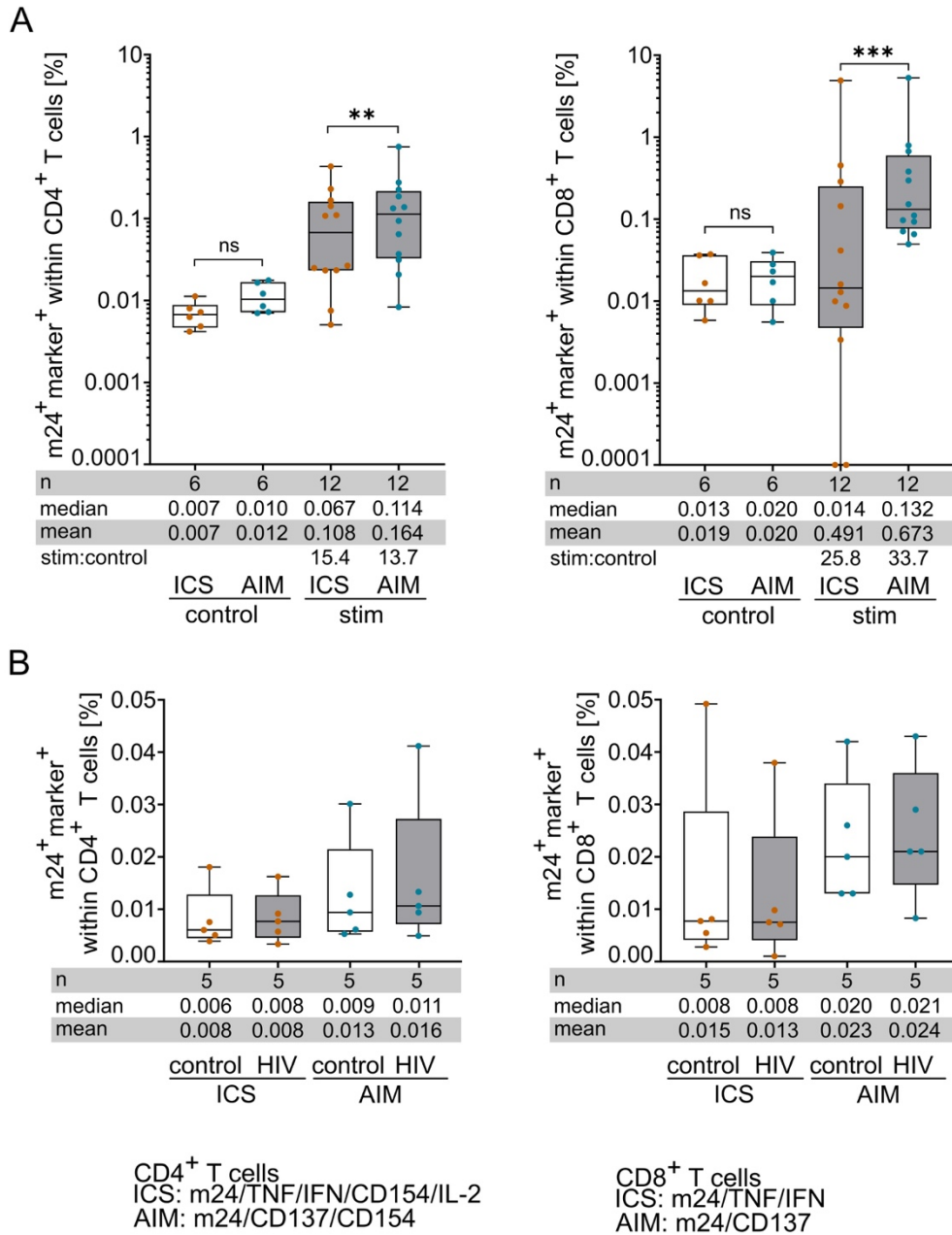


Figure 3. m24+AIM staining detects antigen-specific T cells with increased sensitivity compared to m24+ICS. PBMCs were incubated with antigenic peptides or left unstimulated for 14 h in the presence of CD40 Ab (m24+AIM) or brefeldin A and monensin (m24+ICS). Summed frequencies of m24⁺ and at least one additional marker⁺ T cells after Boolean gating are presented as box and whisker plots showing median, interquartile range, minimum and maximum for control and peptide-stimulated CD4⁺ (left graphs) and CD8⁺ (right graphs) T cells detected with m24+ICS or m24+AIM. Number of samples (n), median, mean and stimulated:control ratios (stim:control) are indicated. (A) Comparison of CD4⁺ (left) and CD8⁺ (right) T cell frequencies detected with m24+ICS or m24+AIM in unstimulated control and peptide-stimulated tests (n=6 HD, 12 donor/antigen combinations). Frequencies of stimulated samples are background subtracted. Values that were 0 were set to 0.0001. (B) Comparison of CD4⁺ (left) and CD8⁺ (right) T cell frequencies detected with m24+ICS or m24+AIM in control or HIV peptide-supplemented samples (n=5 HD). To compare both conditions, HIV tests were not background subtracted. Wilcoxon matched pairs, p<0.05 (** 0.002, *** < 0.001).

To rule out that non-antigen specific T cells can express activated β_2 -integrins and AIMs in our experimental setting (which could explain the difference in the observed frequencies after m24+AIM vs m24+ICS staining), we incubated HD PBMCs (n=5, n=2 HLA-A*02⁻ and n=3 HLA-A*02⁺ donors) with a mix of two known HIV-derived epitopes, one for HLA-class I

(HLA-A*02) and one for HLA-class II (HLA-DRB1), against which we did not expect to observe any reactive T cells. Again, the summed frequencies of m24⁺ and at least one additional marker⁺ T cells after Boolean gating were calculated for each donor. These frequencies were neither significantly different between the HIV- and the corresponding control stimulations nor between the m24+AIM vs the m24+ICS assays, both for the CD4⁺ (left graph) and CD8⁺ (right graph) T cell subsets (Figure 3B). Altogether, these results suggest that T cells detected with the m24+AIM assay are not activated by our experimental conditions but are indeed antigen-specific, and that the assay is able to detect higher frequencies of antigen-reactive T cells as compared to m24+ICS staining.

For in depth comparison of both assays (m24+AIM vs m24+ICS), we additionally performed correlation analyses and calculated x-intercepts (n=12 donor/antigen combinations from Figure 3A). Combination of m24 Ab, CD137 and CD154 highly correlated with combination of m24 Ab, IFN γ , TNF, IL-2 and CD154 for CD4⁺ T cells with a Spearman r of 0.90 (p=0.0002, Figure 4A left graph). The two assays (m24 Ab and CD137 vs m24 Ab, IFN γ and TNF) also correlated for CD8⁺ T cells, but with a lower Spearman r of 0.68 (p=0.0189, Figure 4A middle graph). The only marker that could be directly compared in intracellular (m24+ICS) vs extracellular (m24+AIM) staining was CD154 on CD4⁺ T cells, with a notable Spearman r of 0.92 (p<0.0001, Figure 4A right graph). For CD4⁺ T cells, x-intercepts were -0.019 (m24+AIM vs m24+ICS) and -0.017 (extracellular vs intracellular CD154 stainings) (Figure 4A). For CD8⁺ T cells, a higher x-intercept value was observed (0.154, Figure 4A), reflecting the increased frequencies of antigen-specific T cells detected with m24+AIM compared to m24+ICS (Figure 3A).

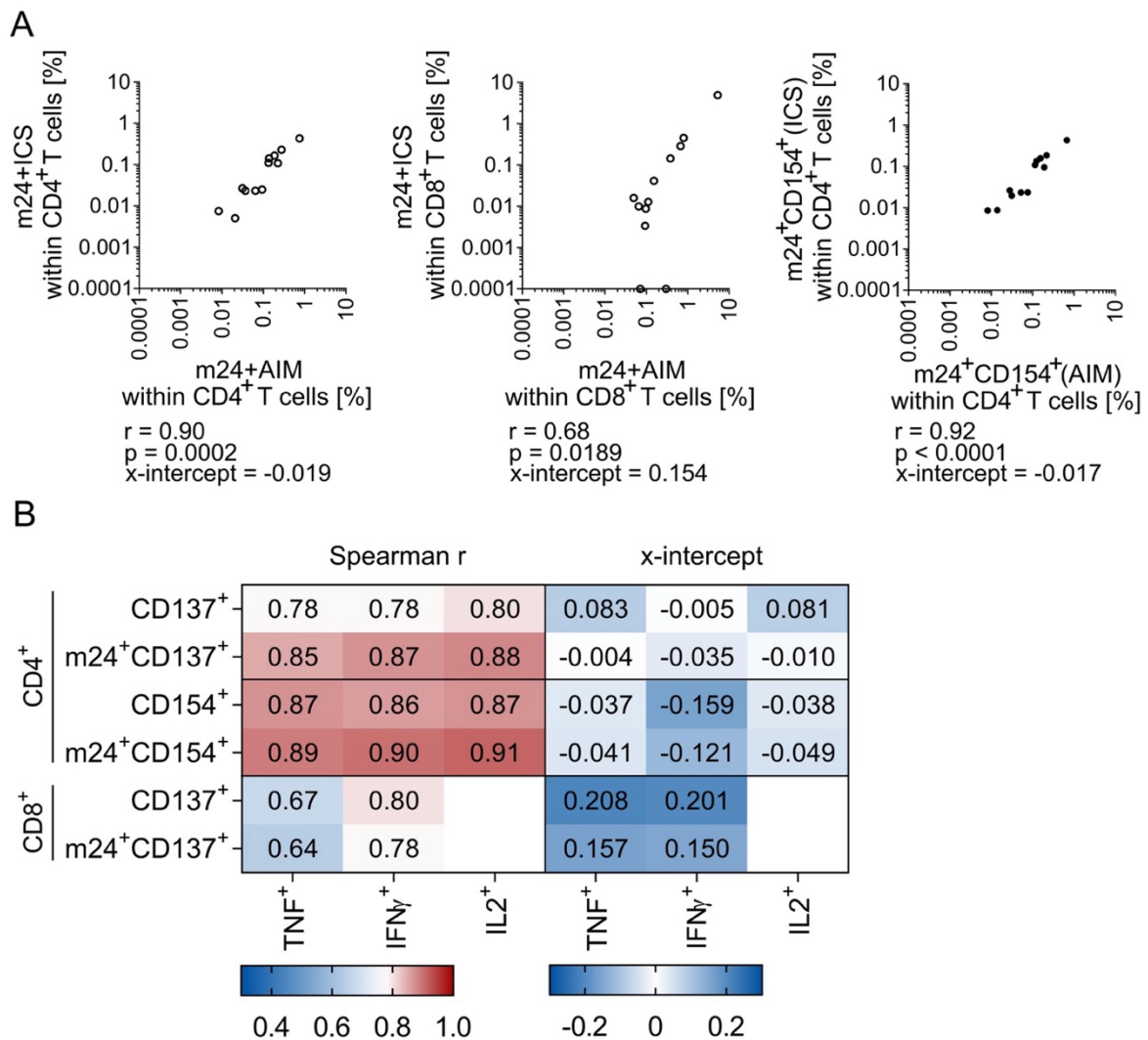


Figure 4. Frequencies of antigen-specific T cells assessed by m24+AIM correlate with those detected with m24+ICS. Correlation between antigen-specific T cell frequencies assessed by AIM or ICS in HD PBMCs (n=12 donor/antigen combinations). Spearman r ($p < 0.05$) and x-intercepts are indicated. Frequencies of stimulated samples are background subtracted. Values that were 0 were set to 0.0001. (A) Comparison of frequencies detected with m24+AIM vs m24+ICS (summed frequencies of m24⁺ and at least one additional marker⁺ after Boolean gating) for CD4⁺ (left) and CD8⁺ (middle) T cells. Comparison of extracellular vs intracellular CD154 staining for CD4⁺ T cells (right). (B) Heat map of Spearman r (left) and x-intercept (right). Frequencies of antigen-specific CD4⁺ (top) and CD8⁺ (bottom) T cells detected by single AIMS or in combination with m24 Ab staining were compared to single cytokine expression. Corresponding correlation graphs are shown in Supplementary Figure 3.

To verify the utility of combining AIM markers with m24 Ab staining, we finally performed correlations with single cytokine expression (frequencies of cells detected by CD154 or CD137 expression alone or combined to m24 Ab staining vs TNF, IFN γ or IL-2 production, see Figure 4B and corresponding graphs Supplementary Figure 3). For CD4⁺ T cells, CD137 expression alone showed a weaker correlation with cytokine production as compared to CD137 combined to m24 Ab staining, CD154 expression alone and CD154 combined to m24 Ab staining (Spearman r , Figure 4B left panel). For example, Spearman r were 0.78 vs 0.85 for TNF detection with CD137 alone vs CD137 combined to m24 Ab staining, respectively. For CD8⁺ T cells, CD137 alone or in combination with m24 Ab showed

similar correlations with both TNF and IFN γ (Spearman r 0.64-0.80, Fig 4B left panel), however, x-intercepts were improved for CD137 expression combined to m24 Ab staining vs solely CD137 (Figure 4B right panel). Finally, detection of CD154 alone or together with m24 Ab staining appears equally good for identifying antigen-specific CD4 $^{+}$ T cells (Spearman r and x-intercepts). Altogether, m24+AIM correlates with m24+ICS and the combination of m24 Ab staining with detection of CD154 or CD137 showed improved correlations with cytokines as compared to CD137 alone.

In conclusion, m24+AIM surface staining highly correlates with intracellular cytokine production, while detecting functional antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T cells with an increased sensitivity.

Sensitive detection of SARS-CoV-2 spike-specific T cell responses in SARS-CoV-2 vaccinated and pre-pandemic healthy donors

Finally, we compared the two assays m24+AIM and m24+ICS for their ability to detect antigen-specific T cell responses in a clinically relevant setting. For this, the T cell responses obtained for SARS-CoV-2 vaccinated donors after stimulation with overlapping spike peptides (data already included in previous sections together with other antigen stimulations, i.e. in Figures 1, 3, 4) were compared to those detected in PBMCs isolated before the SARS-CoV-2 outbreak (Figures 5A, B; CD4 $^{+}$ and CD8 $^{+}$ T cell responses on left and right graphs, respectively). We detected spike-specific CD4 $^{+}$ T cell responses with both assays in 6 of 6 vaccinees, while for CD8 $^{+}$ T cells, T cell reactivity was observed in 5 of 6 donors with m24+ICS, and in 6 of 6 donors with m24+AIM (Figure 5A). In line with published results (15, 22, 23), spike-specific CD4 $^{+}$ and/or CD8 $^{+}$ T cell responses could also be detected in 4 of 5 pre-pandemic donors, suggesting T cell cross-reactivity between CoV-2 and other viruses from the SARS family. Altogether, the mean and median frequencies of specific T cells detected with the m24+AIM assay were equivalent or even higher (Figure 5A, upper right panel for CD8 $^{+}$ T cell responses in vaccinees) than those detected with the m24+ICS combination. In addition, the stimulated:control ratio was elevated in 3 out of 4 cases, confirming our previous results.

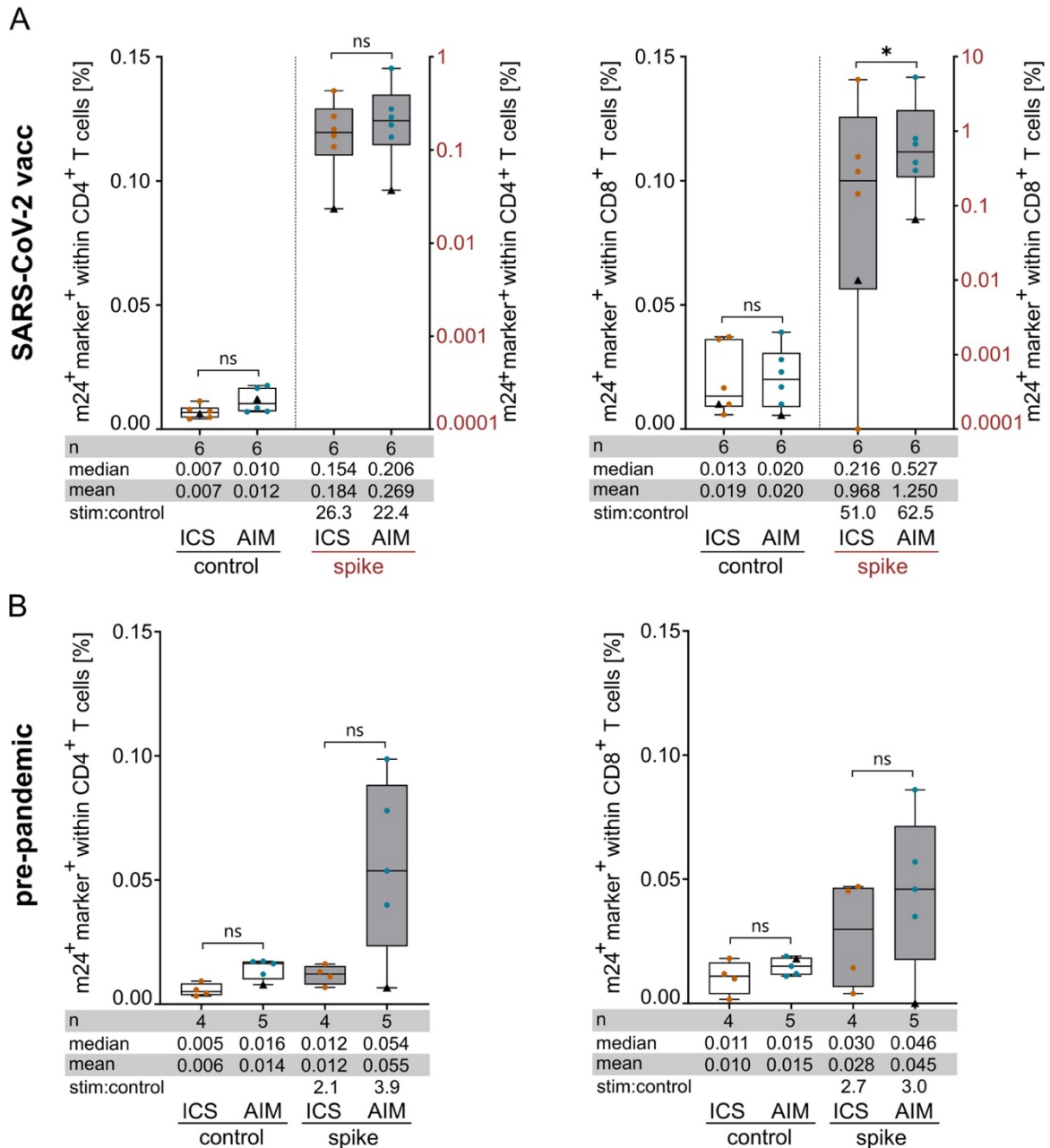


Figure 5. Assessment of spike-specific T cell responses with m24+ICS and m24+AIM in SARS-CoV-2 vaccinated (A) and pre-pandemic (B) healthy donors. PBMCs were incubated with SARS-CoV-2/S overlapping peptides or left unstimulated for 14 h in the presence of CD40 Ab (m24+AIM) or brefeldin A and monensin (m24+ICS). Summed frequencies of m24⁺ and at least one additional marker⁺ T cells after Boolean gating are presented as box and whisker plots showing median, interquartile range, minimum and maximum for CD4⁺ (left graphs) and CD8⁺ (right graphs) T cells. Number of donors (n), median, mean and stimulated:control ratios (stim:control) are indicated. For donor HD7 (black triangle), pre-pandemic and post-vaccination samples are shown. Frequencies of stimulated samples are background subtracted. (A) T cell responses in SARS-CoV-2 vaccinated donors (n=6 HD). Frequencies of marker⁺ cells in unstimulated controls (left y-axis) and after spike-peptide stimulation (right y-axis) are plotted for CD4⁺ and CD8⁺ T cells. This dataset is also part of Figures 1, 3 and 4. Values that were 0 were set to 0.0001. (B) Frequencies of marker⁺ cells in pre-pandemic PBMCs tested with m24+AIM (n=5 HD) and m24+ICS (n=4 HD, donor HD7 symbolized by a black triangle was tested only with m24+AIM). p<0.05 (* 0.03). ns, not significant.

5.3.5 Discussion

We describe a protocol for simultaneous and sensitive assessment of functional antigen-specific CD4⁺ and CD8⁺ T cells using a combination of solely cell surface activation markers, namely activated β_2 -integrins, CD137 and CD154. Several features of activated β_2 -integrins make them an attractive new activation induced marker to detect antigen-specific T cells, i.e. their expression on T cells at various differentiation stages, high correlation with cytokine production, and the possibility to preserve cell viability during labelling (18, 19). We have previously observed that detection of activated β_2 -integrins showed some background staining (19), which is also the case for other established cell surface AIMs. Hence, we reasoned that the combination of these cell surface activation markers will decrease unspecific signal and lower the threshold of detection, as we demonstrated for the combination of activated β_2 -integrins with intracellular cytokines (19).

We found that m24 Ab staining could be combined with AIMs CD137 and CD154 after a relatively short stimulation time of 14 hours (i.e. overnight). This time was compatible with the expression kinetics of these two receptors (Supplementary Figure 1) and in accordance with previous reports from others (6). Other described AIMs require longer stimulation times of 18 to 20 h for peak expression [e.g. OX-40, CD25 (11, 12)], or show a very high background staining in unstimulated cells [e.g. CD69, Supplementary Figure 1 and (6)]. Overall, combined staining of activated β_2 -integrins (using m24 Ab) and CD137 decreased background of unstimulated controls and improved signal-to-noise ratio for CD8⁺ T cells. The same was seen for CD4⁺ T cells with combination of m24 Ab staining and detection of CD137 or CD154.

Interestingly, not all AIM⁺ T cells co-stained for activated β_2 -integrins. This was especially true for the CD4⁺ subset, for which we repeatedly detected CD137- or CD154-expressing CD4⁺ T cells that were not stained with m24 Ab (Figure 1C). For the CD8⁺ subset however, the vast majority of antigen-specific CD137-expressing cells stained positive for activated β_2 -integrins (>80%, Figure 1C). Additionally, the majority of m24⁺CD137⁺ CD4⁺ T cells also expressed CD154 (74%) and inversely (66%, data not shown). Hence, the three AIMs identify overlapping, but not fully identical CD4⁺ T cell subsets, as previously reported using AIM assays combining CD69/CD154 and OX40/CD25 (11).

To investigate the association between cell surface activation marker expression (m24+AIM) and cytokine production (m24+ICS), we performed both tests in parallel after peptide stimulation. A first finding was that the overall frequency of antigen-specific T cells detected with m24+AIM was significantly higher than that detected with m24+ICS (in mean 1.5-fold for CD4⁺ and 1.4-fold for CD8⁺ T cells, Figure 3A). In some cases, we could even reveal T cell responses with m24+AIM that were not detectable with m24+ICS (Figures 3A,

5A, B). In contrast, addition of HIV peptides during the culture did not lead to an increase in the frequencies of responding T cells in the m24+AIM vs m24+ICS assays (Figure 3B). This suggests that cells revealed by cell surface staining are antigen-specific, but do not produce detectable amount of the cytokines (IFN γ , TNF and IL-2) tested. In line with these findings, AIM assays have previously been reported to identify higher frequencies of antigen-specific T cells compared to cytokine-based assays (12, 24). It is important to note that, although bystander activation is unlikely (11), it cannot be ruled out in our system. A second observation made by comparing m24+AIM and m24+ICS was an excellent correlation (Spearman r approx. 0.9) of cell surface staining with cytokine production for CD4 $^{+}$ T cells. Notably, the combination of m24 Ab staining together with CD137 or CD154 showed improved correlations compared to the detection of CD137 alone (but not CD154 alone). For CD8 $^{+}$ T cells, the correlation was weaker (Spearman r approx. 0.7) than for CD4 $^{+}$ T cells, however x-intercept was improved when m24 Ab and CD137 were combined compared to CD137 alone.

Considering these results together, i.e. the frequencies of detected cells, the signal-to-noise ratio and the correlation with cytokine production, staining of activated β_2 -integrins, CD137 and CD154 allows optimized and simultaneous detection of functional antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T cells. With this setting, frequencies of antigen-specific cells below 0.01% and 0.05% were detected for CD4 $^{+}$ and CD8 $^{+}$ T cells, respectively. Hence, the assay is highly sensitive, although spiking experiments will be needed to determine the lowest limits of quantification more precisely. Moreover, the assay is simpler than the ICS method, since it doesn't require permeabilization, resulting in less washing and centrifugation steps. As an exemplary application, we used our new method for detection of antigen-specific T cells in a cohort of SARS-CoV-2 vaccinated and unexposed donors. Spike-specific CD4 $^{+}$ and CD8 $^{+}$ T cells were detected in all SARS-CoV-2 vaccinated individuals with m24+AIM, including one CD8 $^{+}$ T cell response that was not detectable with m24+ICS (Figure 5A). Additionally, we observed spike-specific T cell responses in pre-pandemic donors, and again, one of the CD8 $^{+}$ T cell responses was detected with m24+AIM and not with m24+ICS (Figure 5B). This underlines that not all antigen-specific T cells are necessarily expressing cytokines. Hence, cell surface staining should allow for a fast and easy detection of SARS-CoV-2-specific T cell responses in convalescent and/or vaccinated individuals.

Compared to our previously published work (19), we have further optimized the protocol of activated β_2 -integrin staining using m24 Ab for cryopreserved PBMC samples and for usage of 96 well plates, which is critical for high-throughput screening of clinical samples. Additionally, the use of serum-free CTS medium containing DNase I for stimulation was superior to a human serum-supplemented medium by reducing the

background signal within unstimulated controls (comparison not shown). The protocol described in this work makes usage of FACS-Lysing solution (BD) to fix the cells after staining which preserves the activated conformation of β_2 -integrins. However, comparison experiments show that the assay can also be performed without fixation (Supplementary Figure 4), which would allow to maintain cell viability for downstream analysis, similarly to what we previously showed using mICAM multimers for CD8⁺ T cells (18).

In conclusion, we introduce activated β_2 -integrins as an additional early detectable cell surface AIM applicable for detection of CD4⁺ and CD8⁺ T cells in combination with already established AIMs CD137 and CD154. Our new assay allows assessment of functional antigen-specific CD4⁺ and CD8⁺ T cells with an increased sensitivity compared to intracellular cytokine detection. It could therefore be applied for T cell epitope screening projects and for the monitoring of low frequency antigen-specific T cell responses in the context of pathogen infection, autoimmunity and cancer.

5.3.6 References

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6 General discussion and perspectives

The breakthrough of checkpoint inhibitors in the treatment of many types of cancer has initiated in a new era of immunotherapy (1, 2). Besides checkpoint inhibition different T cell-based immunotherapeutic approaches including other antibody-based therapies like T cell engaging antibodies (3, 4), cell-based therapies, such as tumor-specific T cells expanded from tumor infiltrating lymphocytes (TILs) or T cells with genetically modified TCRs (5, 6), or cancer vaccines like the autologous PBMC-derived GM-CSF stimulated vaccine Sipuleucel-T (7) were shown to improve the clinical outcome of some patients in different cancer types.

Besides promising results in preclinical studies and early clinical trials of immunotherapy-based approaches in SCLC (8, 9), anti-PD-L1 checkpoint inhibitors combined to standard of care treatment for extensive stage (ES)-SCLC remain the only approved immunotherapeutic treatment in SCLC (10). However, SCLC patients are still confronted with a very poor prognosis.

A critical factor for the development of effective T cell-based immunotherapies is the identification and selection of target antigens. T cell targets should be present on malignant cells but absent on healthy tissue to avoid on target/off tumor toxicity. Mutated neoantigens that are tumor-specific by definition are suggested to be ideal tumor antigens. They were found to be targeted under checkpoint inhibition in animal models (11) and were shown to mediate anti-tumor T cell responses and tumor regression in melanoma (12, 13). A high tumor mutational burden (TMB) is therefore assumed to be indicative for effective immune checkpoint inhibition (14). SCLC is associated with a high TMB (15-17), which was reported to be a predictive marker for SCLC therapy response to checkpoint inhibitors in one study (18) but lack a predictive potential in other studies (10). Still, only a fraction of SCLC patients is benefiting from immune checkpoint inhibition (10, 18). We identified and validated only one naturally presented mutated neoantigen in our SCLC cohort. This is supported by studies showing that only a limited number (approx. 0.1% (19)) of genomic mutations end up being presented on HLA in malignant cells (19-22). We were therefore concentrating on the identification of tumor-associated self-antigens that were found to be naturally presented on SCLC using MS-based immunopeptidomics. Highly valuable datasets of >2 million naturally presented HLA class I and II peptides that derived from different benign tissue (lung, brain, kidney, spleen, etc.) and cell types (B cells, granulocytes, etc.) were published by our institute (23-26). A comparative analysis with these datasets enables the identification of tumor-exclusive antigens, which are also referred to as tumor-specific, as they are absent in healthy tissue. These identified targets could be exploited in the future by different T cell-based immunotherapies, including

adoptive T cell transfer of TCR engineered or amplified tumor-specific cells, or different vaccine approaches. Our focus is the design of peptide-based vaccines that can be combined with standard of care therapy. Peptide vaccines using mutated neoantigens (12) or self-antigens (27, 28) successfully induced T cell responses in cancer patients. Peptide vaccination with tumor-associated self-antigens, however, needs to overcome self-tolerance of T cells, which can be achieved with appropriate adjuvants. The TLR1/2 agonist XS15 was shown to be an efficient adjuvant for peptide vaccination (29). One advantage of peptide vaccination is that different HLA class I and II peptides can be mixed in a vaccine cocktail targeting multiple epitopes of a tumor. This is favorable to induce a broad CD8⁺ and CD4⁺ T cell response against different antigens to fight malignant cells.

A T cell target should not only be presented by HLA molecules, it should also be able to induce a T cell response in patients, thus be immunogenic (30). Not all antigens are immunogenic, e.g. due to central tolerance of T cells against this antigen that was generated in the thymus during T cell development or due to intrinsic properties of peptides (31, 32). Hydrophobic peptides, for example, were found to be more immunogenic than less hydrophobic peptides (32). Immunogenicity can be tested by recall experiments of memory T cells in PBMCs of cancer patients or priming experiments using aAPCs in PBMCs of healthy donors. A T cell response can be assessed by different read outs, including flow cytometry-based methods. T cell assays are not only required for immunogenicity screening of target antigens, but also for monitoring vaccine induced T cell responses in cancer patients after e.g. peptide-based vaccination. Standard methods that are routinely used for identification of functional T cells with their advantages and limitations are reviewed in (33). Some assays cannot discriminate between CD8⁺ and CD4⁺ T cell responses, can only be used for identification of CD8⁺ T cells, or require the addition of protein transport inhibitors and permeabilization. A novel flow cytometry-based T cell assay that is based on the detection of activated β_2 -integrins, such as LFA-1, with fluorescent multimers of their ligand ICAM-1 (mICAM-1) was developed by our group and colleagues (34). CD8⁺ T cell activation was detected within minutes after antigen stimulation and surface staining of activated integrins allowed sorting of viable CD8⁺ T cells (34). To that point, the assay was limited to the detection of CD8⁺ T cells and could not be applied for CD4⁺ T cells due to heavy aggregation of CD4⁺ T cells with monocytes during the procedure. In cancer immunology, it is however important to not only detect CD8⁺ T cells, but also CD4⁺ T cells. Throughout this thesis, we have optimized and further developed this assay. In the optimized assay, mICAM-1 is replaced by a fluorescent antibody, clone m24 (m24 Ab), that is specifically binding to the open high affinity form of LFA-1. After binding of the m24 Ab to activated β_2 -integrins, the activation is not reversed by the addition of

EDTA, allowing the detection of both CD4⁺ and CD8⁺ T cells. Additional adaptation steps included the combination with staining of intracellular markers and the usage of cryopreserved PBMCs instead of whole blood. The protocol was published in *Frontiers in Immunology* (35). A special application for $\gamma\delta$ T cells was published by colleagues (36). In a next step, we were interested in avoiding intracellular detection of activation markers, which requires permeabilization of the cells, thereby killing the cells, and requires multiple incubation and washing steps that prolong the staining time and potentially lead to loss of cells during the procedure. Instead, we used activation-induced markers (AIMs) that are detectable on the cell surface, avoiding the need for permeabilization and providing the opportunity to maintain cell viability, as well as reducing washing steps and processing time. The detection of activated β_2 -integrins using the m24 Ab in combination with detection of CD137 (CD8⁺ T cells) or CD137 and CD154 (CD4⁺ T cells) correlated with intracellular marker staining and allowed sensitive detection of functional T cells. Additional adaptation steps included the switch from FACS tube-based staining to 96 well plate staining increasing the throughput and implementation of optimized culture medium conditions to decrease unspecific activation of β_2 -integrins. The protocol was published in the special section methods in T cell biology in *Frontiers in Immunology* (37). Methods were optimized using viral model antigens, but they can also be applied for detection of cancer-specific T cells and will be used for immunogenicity testing of selected peptides in SCLC patient samples.

With the immunopeptidomics dataset that was acquired for SCLC in this thesis, several other aspects could be addressed in future studies. These include the analysis of other antigens such as cryptic peptides (38), microbe-derived peptides (39, 40) or posttranslational modified peptides (41) that gained interest as potential tumor-specific antigens. In addition, the relevance of soluble HLA of plasma from patients could be addressed in more depth.

So far, SCLC is treated as one homogenous disease despite growing evidence that there is inter- and intratumoral heterogeneity in SCLC exhibiting different vulnerabilities (42, 43). The multi-epitope approach combining different peptides in one vaccination cocktail allows to target different tumor clones of SCLC at the same time and the usage of frequently shared tumor antigens allows simple production and broad applicability. Future clinical phase I trials in SCLC testing a multi-peptide vaccine, e.g. with antigens that were identified throughout this study, should be tested in combination with the new adjuvant XS15 (TLR1/2 agonist). XS15 was shown to be safe (29) and first clinical trials using XS15 plus Montanide ISA™51 in combination with a multi-peptide vaccine in the context of SARS-CoV-2 infection showed the induction of long lasting vaccine-specific multifunctional CD4⁺

and CD8⁺ T cell responses (44). XS15 and Montanide ISA™51 in combination with peptide vaccines for cancer treatment are also tested in ongoing clinical trials for glioblastoma (NCT04842513), fibrolamellar hepatocellular carcinoma (NCT05937295), chronic lymphocytic leukemia (NCT04688385) and sarcoma patients (NCT06094101). Future immuno-peptidomics studies in SCLC should maybe additionally segregate the different subtypes of SCLC to identify distinct but also overlapping targets on the ligandome level and include the selective analysis of relapsed and therapy resistant SCLC tumors to combat rare tumor clones that get enriched and are potentially involved in relapse.

All in all, this study paved the way for first identification of SCLC-associated T cell targets that could be applied in T cell-based immunotherapy presenting alternative treatment options. Such T cell-based immunotherapies can be combined to already established or other novel therapies for SCLC patients that are still faced with a poor prognosis and limited treatment options.

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7 Index of Abbreviations

aa	Amino acid	ER	Endoplasmatic reticulum
aAPC	Artificial antigen presenting cell	ES	Extensive stage
Ab	Antibody	EZH2	Enhancer of zeste homolog 2
ACN	Acetonitrile	FBS	Fetal bovine serum
AIM	Activation induced marker	FDA	Food and drug administration
APC	Antigen presenting cell	FoxP3	Forkhead box P3
ASCL1	Achaete-scute homolog 1	GM-CSF	granulocyte-macrophage colony-stimulating factor
BCG	Bacillus Calmette-Guerin	HBV	Hepatitis B virus
BCL-2	B-cell lymphoma 2	HCD	Higher-energy collisional dissociation
BCMA	B cell maturation antigen	HD	Healthy donorm/
BCR	B cell receptor	HER-2	human epidermal growth factor receptor 2
BiTE	Bispecific T cell engager	HLA	Human leukocyte antigen
BTK	Bruton's tyrosine kinase	HPV	Human papillomavirus
CAF	Cancer-associated fibroblasts	ICAM	Intercellular adhesion molecule 1
CAR	Chimeric antigen receptor	ICI	Immune checkpoint inhibitors/inhibition
CCR7	CC-chemokine receptor 7	ICOS	Inducible costimulator
CD	Cluster of differentiation	ICS	Intracellular marker staining
CDCA1	Cell division cycle-associated protein 1	IFN- γ	Interferon gamma
CDR	Complementarity-determining region	Ig	Immunoglobulin
CDX	Circulating tumor cell derived xenograft	IL	Interleukin
CHGA	Chromogranin A	INSM1	insulinoma-associated protein 1
CHK1	Checkpoint kinase 1	KIF20A	Kinesin family member 20A
CID	Collision-induced dissociation	KIR	Killer immunoglobulin-like receptors
CLIP	Class II-associated invariant chain peptide	LAG-3	Lymphocyte-activation gene 3
CMV	Cytomegalovirus	LAMP-1	Lysosomal Associated Membrane Protein 1
COL6A3	Collagen type VI a-3	LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
CTC	Circulating tumor cell	LCNEC	Large cell neuroendocrine carcinoma
CTL	Cytotoxic T cell	LFA-1	Lymphocyte function-associated antigen 1
CTLA-4	Cytotoxic T lymphocyte antigen 4	LS	Limited stage
DC	Dendritic cell	Lumos	Orbitrap Fusion Lumos
DDA	Data-dependent acquisition	m/z	Mass to charge ratio
DLL3	Delta-like 3	MDSC	Myeloid derived suppressor cells
DMSO	Dimethyl sulfoxide	MHC	Major histocompatibility complex
DNA	Deoxyribonucleic acid	mRNA	Messenger ribonucleic acid
EBV	Epstein-Barr virus	MS	Mass spectrometry
EDTA	Ethylenediaminetetraacetic acid	NCAM1	Neural cell adhesion molecule 1
ELISA	Enzyme-linked immunosorbent assay	NE	Neuroendocrine
ELISpot	Enzyme linked immune spot	NEUROD1	Neurogenic differentiation 1
EMA	European medicine agency		
EMT	Epithelial to mesenchymal transition		

NGS	Next generation sequencing	T _{CM}	Central memory T cell
NK cells	Natural killer cell	TCM	T cell medium
NKG2	Natural killer group 2	TCR	T cell receptor
NSCLC	Non small cell lung cancer	T _{EM}	Effector memory T cell
PAMP	Pathogen associated molecular pattern	T _{EMRA}	Effector memory T cell re-expressing CD45RA
PAP	Prostatic acid phosphatase	TERT	telomerase reverse transcriptase
PARP1	Poly [ADP-ribose] polymerase 1	TFA	Trifluoroacetic acid
PASEF	Parallel accumulation-serial fragmentation	Tfh	Follicular helper T cell
PBS	Phosphate buffered saline	TGF- β	Transforming growth factor beta
PBMCs	Peripheral blood mononuclear cells	Th	T helper cell
PD-1	Programmed cell death protein 1	TIGIT	T cell immunoreceptor with Ig and ITIM domains
PD-L1	Programmed cell death ligand 1	TIL	tumor infiltrating lymphocyte
PDX	Patient (biopsy) derived xenograft	TIM-3	T-cell immunoglobulin and mucin-domain containing-3
Pen/Strep	Penicillin-Streptomycin	timsTOF	Trapped ion mobility spectrometry coupled to time of flight
pHLA	Peptide-Human leukocyte antigen	TLR	Toll-like receptor
POU2F3	POU domain class 2 transcription factor 3	TLS	Tertiary lymphoid structures
PRR	Pattern recognition receptor	TMB	Tumor mutational burden
PTM	Posttranslational modification	TME	Tumor microenvironment
QE	Q Exactive HF	T _N	Naive T cell
RB1	Retinoblastoma protein 1	TNF	Tumor necrosis factor
RNA	Ribonucleic acid	TNM	Tumor Node Metastasis
RT	Room temperature	TRAIL	TNF-related apoptosis-inducing ligand
SARS-CoV-2	Severe acute respiratory syndrome-related coronavirus 2	Treg	Regulatory T cell
SCLC	Small cell lung cancer	Trop-2	Trophoblast cell-surface antigen
SEB	Staphylococcus enterotoxin B	TSA	Tumor-specific antigen
SMAC	Supramolecular activation cluster	T _{SCM}	Stem like memory T cell
STING	Stimulator of interferon genes	UICC	Union for International Cancer Control
SOX2	SRY-Box transcription factor 2	VALSG	Veterans Administration Lung Cancer Study Group
sHLA	Soluble human leukocyte antigen	WB	Whole blood
TAA	Tumor-associated antigen	WHO	World health organization
TAM	Tumor-associated macrophage	YAP1	Yes-associated protein 1
TAP	Transporter associated with antigen processing		

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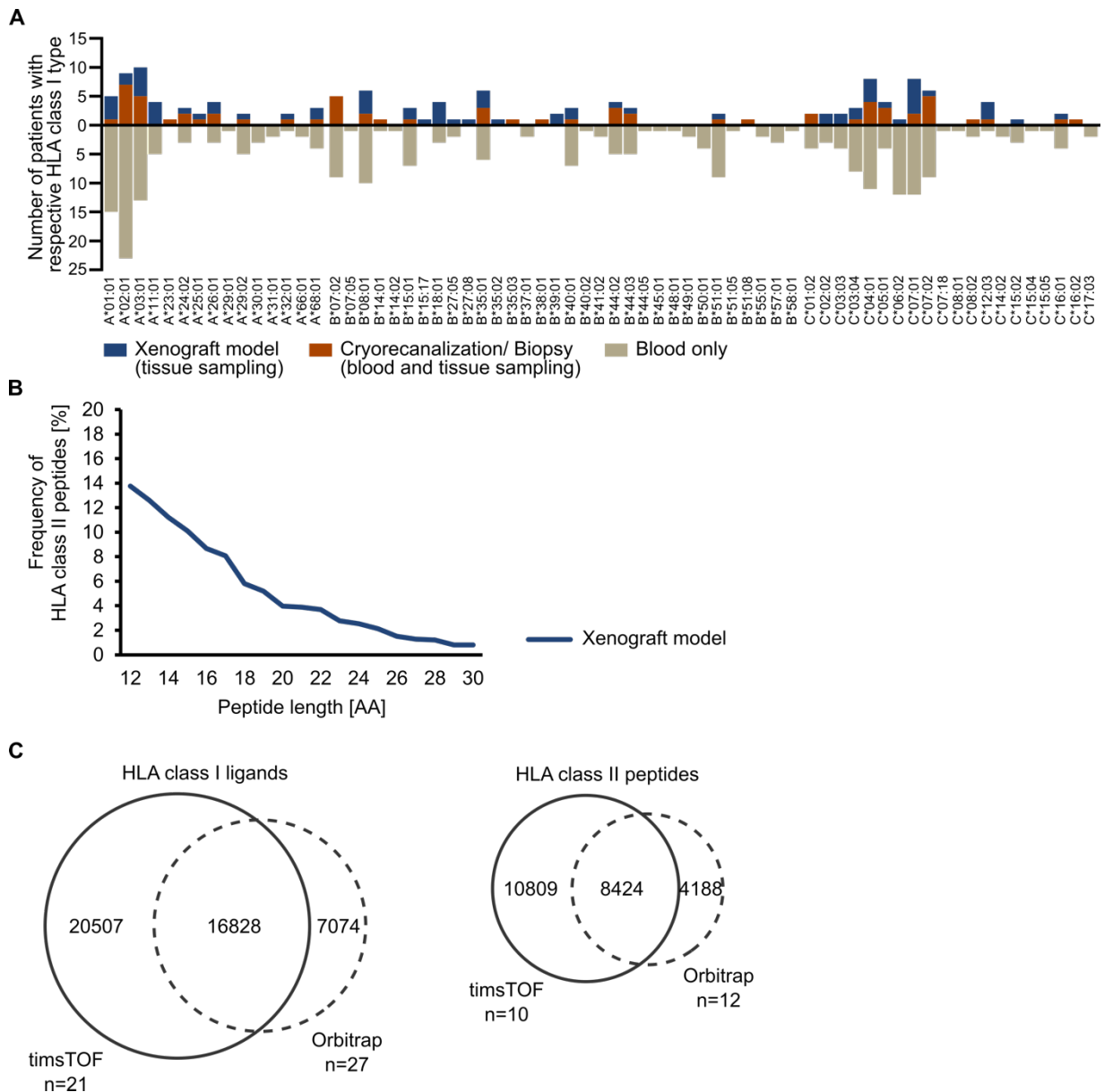
Additionally, I want to thank everyone who was keeping the daily business running: Beate, Claudia, Franziska, Adriana, Carmen, Gerhard, Mathias, and everyone I may have forgotten.

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9 Supplementary data

9.1 Supplementary data Part I



Supplementary Figure 1. A) Number of SCLC patients with respective HLA class I type from all SCLC samples. Top panel: patients with tissue sampling for immunopeptidomics (orange: cryorecanalization/biopsy and blue: xenograft models, n= 24). Blood samples were additionally collected for the cryorecanalization/ biopsy samples (except for S02243). Lower panel: patients with blood samples only (PBMCs and plasma, gold, n= 45). B) Length distribution of HLA class II peptides eluted with Tü39/L243 from samples of xenograft models. C) Venn diagram of sequences identified with timsTOF and/or Orbitrap-based MS devices for HLA class I ligands (left) and HLA class II peptides (right).

Supplementary Table 1: Gene names and UniProt ID of genes or proteins that were found to be associated with SCLC and main alteration or effects on SCLC tumorigenesis. The reference numbers refer to the bibliography list below the table.

	Gene name	UniProt ID	Aberration/SCLC association	Reference
Cell cycle and apoptosis				
	<i>TP53</i>	P04637	Inactivating mutation, loss of function	(1-6)
	<i>RB1</i>	P06400	Inactivating mutation, loss of function	(1-6)
	<i>RBL1</i>	P28749	inactivating mutation	(3)
	<i>RBL2</i>	Q08999	inactivating mutation	(3)
	<i>CCND1</i>	P24385	activating mutations, overexpression in WT RB1 tumors	(3)
	<i>ATR</i>	Q13535	vulnerable to ATR inhibition	(7)
	<i>CHK1</i>	O14757	overexpression, vulnerable to CHK1 inhibition	(7, 8)
	<i>TP73</i>	O15350	activating alteration, rearrangement to oncogenic version TP73 Δ ex2/3	(3)
	<i>BCL-2</i>	P10415	overexpression	(9)
	<i>CASP9</i>	P55211	cancer promoting	SFB1399 (A08)
Epigenetic regulators				
	<i>CREBBP</i>	Q92793	inactivating mutation, mutations clustered around HAT domain	(1, 3)
	<i>EP300</i>	Q09472	inactivating mutation, mutations clustered around HAT domain	(1-3)
	<i>KMT2A</i>	Q03164	mutated	(1, 6)
	<i>KMT2C</i>	Q8NEZ4	mutated	(6)
	<i>KMT2D</i>	O14686	inactivating mutation	(2, 6)
	<i>EZH2</i>	Q15910	high expression	(10, 11)
	<i>EZH1</i>	Q92800	expression	SFB1399 (C04)
	<i>KMT9</i>	Q9Y5N5/ Q9UI30	high levels correlate with poor survival	(12)
	<i>EHMT2</i>	Q96KQ7	suppression of chemokine expression	SFB1399 (C04)
	<i>EHMT1</i>	Q9H9B1	suppression of chemokine expression	SFB1399 (C04)
	<i>KDM6A</i>	O15550	mutated	(6)
	<i>SETD2</i>	Q9BYW2	mutated	(6)
	<i>PBRM1</i>	Q86U86	mutated	(6)
	<i>ARID1A</i>	O14497	mutated	(6)
	<i>ARID1B</i>	Q8NFD5	mutated	(6)
	<i>CHD7</i>	Q9P2D1	mutated	(6)
	<i>PIF1</i>	Q9H611	upregulated	SFB1399 (B06)
	<i>DHX36</i>	Q9H2U1	upregulated	SFB1399 (B06)
	<i>Parp1</i>	P09874	high expression	(13, 14)
Kinase signaling				
	<i>PTEN</i>	P60484	inactivating mutation	(1-3)
	<i>PIK3CA</i>	P42336	activating mutation	(2, 3, 5, 6, 15)
	<i>BRAF</i>	P15056	mutated	(3)
	<i>EPHA7</i>	Q15375	mutated	(1)
	<i>FGFR1</i>	P11362	amplification	(1-3, 13, 16)
	<i>aurora kinase A, B, C</i>	O14965, Q96GD4, Q9UQB9	activation	(13)
	<i>KIT</i>	P10721	activating alteration, overexpression	(3, 17)

<i>YES1</i>	P07947	overexpression, amplification	(18)
Cytoskeleton and cell adhesion			
<i>SLIT2</i>	O94813	mutated	(1)
<i>COBL</i>	O75128	mutated	(1)
<i>NFIB</i>	O00712	overexpression	(19, 20)
<i>FMN2</i>	Q9NZ56	inactivating mutation	(3)
<i>KIAA1211</i>	Q6ZU35	mutated	(3)
<i>COL22A1</i>	Q8NFW1	inactivating mutation	(3)
<i>ASPM</i>	Q8IZT6	mutated	(3)
<i>ALMS1</i>	Q8TCU4	mutated	(3)
<i>PDE4DIP</i>	Q5VU43	mutated	(3)
<i>CSMD3</i>	Q7Z407	mutated	(4)
<i>ITGA2, (CD49b)</i>	P17301	high expression	(21)
Transcription factors			
<i>SOX2</i>	P48431	amplification	(2, 22)
<i>MYC</i>	P01106	amplification, promotes gradual non-NE differentiation upregulates YAP-1	(2, 3, 23, 24)
<i>MYCL</i>	P12524	amplification and aberrant expression, recurrent RLF-MYCL1 fusion	(1-3, 24, 25)
<i>MYCN</i>	P04198	amplification and aberrant expression	(1, 3, 25, 26)
<i>REST1</i>	Q13127	higher expressed in non-NE; suppresses NE differentiation	(27)
<i>ASCL4</i>	Q6XD76	higher expressed in non-NE	(27)
SCLC subtype associated transcription factors			
<i>ASCL1</i>	P50553	high expression, SCLC-A	(28)
<i>HES1</i>	Q14469	expression, SCLC-A2	(10, 29)
<i>NEUROD1</i>	Q13562	high expression, SCLC-N	(28)
<i>POU2F3</i>	Q9UKI9	high expression, SCLC-P	(28)
<i>YAP1</i>	P46937	high expression, SCLC-Y	(28)
<i>ATOH1</i>	Q92858	expression	(10, 26)
Neuroendocrine markers			
<i>CHGA</i>	P10645	high expression	(3, 30)
<i>GRP</i>	P07492	high expression	(3)
<i>DLK1</i>	P80370	high expression	(3)
<i>SYP</i>	P08247	high expression	(3, 30)
<i>NCAM1</i>	P13591	high expression	(3, 30)
<i>DLL3</i>	Q9NYJ7	high expression	(13, 25, 31)
<i>INSM1</i>	Q01101	marker in immunohistochemistry	(30)
Ferroptosis associated			
<i>SLC7A11</i>	Q9UPY5	upregulated	(27)
<i>GPX4</i>	P36969	expressed	(27)
<i>TFRC (CD71)</i>	P02786	elevated expression in SCLC cell lines	(27)
<i>LPCAT3</i>	Q6P1A2	higher expressed in non-NE SCLC	(27)
NF-kappa B signaling			
<i>NEMO</i>	Q9Y6K9	deletion delayed SCLC onset (mouse models)	(32), SFB1399 (A04)
<i>RELA</i>	Q04206	deletion delayed SCLC onset (mouse models)	(32), SFB1399 (A04)
<i>ADAR1</i>	P55265	regulates ZBP1	SFB1399 (A04)
<i>ZBP1</i>	Q9H171	mediates cell death and type IFN responses	SFB1399 (A04)

	<i>NFKB1</i>	P19838	NF- κ B subunit	SFB1399 (A04)
	<i>NFKB2</i>	Q00653	NF- κ B subunit	SFB1399 (A04)
	<i>IKKA</i>	O15111	catalytic subunit promoting NF- κ B signaling	(32), SFB1399 (A04)
	<i>IKKB</i>	O14920	catalytic subunit promoting NF- κ B signaling	(32), SFB1399 (A04)
Glutamate receptor associated				
	<i>GRM8</i>	O00222	expression in SCLC-A and SCLC-N	(33), SFB1399 (A01)
	<i>GRID1/ KIAA1220</i>	Q9ULK0	mutated	(2, 33)
	<i>GRM1</i>	Q13255	mutated	(33)
	<i>GRM2</i>	Q14416	expression in SCLC	(33)
	<i>GRM3</i>	Q14832	mutated	(33)
	<i>GRM4</i>	Q14833	expression in SCLC	(33)
	<i>GRM5</i>	P41594	mutated	(33)
	<i>GRIA1</i>	P42261	mutated	(33)
	<i>GRIA2</i>	P42262	expression in SCLC	(33)
	<i>GRIA3</i>	P42263	mutated	(33)
	<i>GRIA4</i>	P48058	mutated	(33)
	<i>GRIK2</i>	Q13002	mutated	(33)
	<i>GRIK3</i>	Q13003	expression in SCLC	(33)
	<i>GRIK4</i>	Q16099	mutated	(33)
	<i>GRIK5</i>	Q16478	expression in SCLC	(33)
	<i>MAP2</i>	P50579	neuron marker	SFB1399 (A01)
Others				
	<i>NOTCH Notch1,2,3,4</i>	P46531, Q04721, Q9UM47, Q99466	inactivating mutation, significantly damaged, active in non-NE	(3, 4, 6, 13, 27)
	<i>IRS2</i>	Q9Y4H2	amplification	(3)
	<i>PD-L1 (CD274)</i>	Q9NZQ7	target of checkpoint inhibitors, amplification in subsets of patients	(34, 35)
	<i>SRSF1</i>	Q07955	overexpression	(4)
	<i>RGS7</i>	P49802	mutated	(3)
	<i>FPR1</i>	P21462	mutated	(3)
	<i>XRN1</i>	Q8IZH2	mutated	(3)
	<i>PTGFRN</i>	Q9P2B2	mutated	(3)
	<i>ZMYM3</i>	Q14202	promotes cell proliferation	(36)
	<i>FASLG</i>	P48023	therapeutic potential in mice by blocking	SFB1399 (C06)
	<i>TRAIL</i>	P50591	therapeutic potential in mice by blocking	SFB1399 (C06)
	<i>TERT</i>	O14746	chromosome 5 amplification, elevated expression	(10), SFB1399 (A07, A03)
	<i>GSDME</i>	O60443	downregulation	SFB1399 (A09)
	<i>PLCG2</i>	P16885	expression defines a SCLC subpopulation	(37)
	<i>NKX2-1</i>	P43699	neuronal differentiation	(38)
Chromosome level copy number alterations in SCLC				
	<i>MYCL</i>	P12524	amplification chromosome 1	(10)
	<i>RRAGC</i>	Q9HB90	amplification chromosome 1	(10)
	<i>TERT</i>	O14746	amplification chromosome 5	(10)

<i>DROSHA</i>	Q9NRR4	amplification chromosome 5	(10)
<i>RICTOR</i>	Q6R327	amplification chromosome 5	(10)

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Supplementary Table 2: SCLC patient characteristics.

	Patient ID	Site	Sex	Age	HLA type*	Clinical histology	TNM classification	Cancer stage UICC	Pre-treatment (at sampling)	Smoking	Sample collection			Immunopeptidomic	
											Tissue	PBMC # of samples	Plasma	Tissue	Plasma
SCLC patient cohort 1	S02496	Cologne	m	71	A*03:01;A*26:01;B*18:01;B*39:01;C*05:01;C*02:02	SCLC	T2c N3c M1	IV	yes	yes	CDX			yes	
	S02521		m	61	A*01:01;A*02:01;B*08:01;C*07:01	SCLC	T1 N2 M1	IV	no	yes	CDX			yes	
	S02544		f	62	A*03:01;A*11:01;B*15:17;B*44:03;C*07:01;C*16:01	SCLC	N/A	IV	yes	yes	CDX			yes	
	S02714		f	71	A*02:01;A*26:01;B*18:01;B*44:02;C*12:03;C*07:02	SCLC	T3 N2 M1b	IV	yes	N/A	CDX			yes	
	S02513		f	88	A*03:01;A*29:02;B*15:01;B*39:01;C*03:03;C*12:03	SCLC	T4 N2 M1b	IV	yes (1 cycle)	yes	CDX			yes	
	S02658		m	77	A*03:01;A*32:01;B*27:08;B*35:01;C*04:01;C*06:02	SCLC	T4 N3 M1b	IV	no	yes	CDX			yes	
	S02730		f	64	A*11:01;A*68:01;B*18:01;B*35:01;C*04:01;C*07:01	SCLC	T4 N2 M1c	IVb	no	yes	2x CDX (_03/_10)			yes	
	S02895		m	48	A*24:02;B*15:01;B*40:01;C*03:03;C*03:04	SCLC + LCNEC	T2 N1 M1b	IV	no	yes	CDX			yes	
	S02841		m	57	A*03:01;A*24:02;B*27:05;B*40:01;C*02:02;C*03:04	SCLC	T2 N3 M1	IV	no	yes	CDX			yes	
	S03381		m	61	A*01:01;A*11:01;B*08:01;B*35:02;C*07:01;C*04:01	SCLC - Brain met	T4 N2 M0	IIIB	no	yes	PDX and cells			yes	
	S03387		m	64	A*01:01;A*25:01;B*08:01;B*18:01;C*07:01;C*12:03	SCLC + LCNEC	T4 N3 M1	IV	no	no	PDX			yes	
	S03505		m	64	A*11:01;B*35:01;C*04:01	SCLC	T4 N3 M1	IVb	no	yes	PDX			yes	
	S03536		f	59	A*01:01;A*68:01;B*08:01;B*51:01;C*07:01;C*15:02	SCLC	T3 N0 M0	IIB	no	N/A	PDX			yes	
Cryorecanitization/ Biopsy (blood + tissue sampling)	S02243		m	64	A*03:01;A*26:01;B*07:02;B*38:01;C*07:02;C*12:03	SCLC	T1a N0 M0	I	no	yes	biopsy			yes	
	SCLC 1.02		m	66	A*02:01;A*03:01;B*07:02;B*51:08;C*07:02;C*16:02	SCLC	T3c N2c M0c	IIIB	yes	yes	cryorec	2		yes	
	SCLC 1.03		f	57	A*03:01;A*32:01;B*07:02;B*14:01;C*07:02;C*08:02	SCLC	T4c N3c M1c	IV	yes	yes	cryorec	1		yes	
	SCLC 1.05		f	59	A*02:01;A*24:02;B*35:01;B*40:01;C*03:04;C*04:01	SCLC	T4c N2c M1c	IV	yes	yes	cryorec	2	1	yes	yes
	SCLC 1.06		f	62	A*01:01;A*02:01;B*08:01;B*35:03;C*04:01;C*07:01	SCLC	T2b N2 M1	IV	yes	yes	cryorec	2	2	yes	yes
	SCLC 1.07		f	75	A*02:01;A*24:02;B*07:02;B*15:01;C*07:02;C*01:02	SCLC	T4c N3c M1c	IV	no	yes	cryorec	1	1	yes	yes
	SCLC 1.08		f	85	A*02:01;A*68:01;B*44:02;B*51:01;C*05:01;C*01:02	SCLC	T4 N3 M1c	IV	no	yes	cryorec	1	1	yes	yes
	SCLC 1.09		f	61	A*03:01;A*23:01;B*35:01;B*44:03;C*04:01	SCLC	T4 N3 M1a	IV	no	yes	cryorec	2	2	yes	yes
	SCLC 1.10		m	58	A*03:01;A*26:01;B*07:02;B*35:01;C*07:02;C*04:01	SCLC	T4N0M0	IIIA	no	yes	cryorec	2	2	yes	yes
	SCLC 1.11		m	66	A*02:01;A*25:01;B*08:01;B*44:02;C*07:01;C*05:01	SCLC	T4N3M0	IIIC	no	yes	cryorec	2	2	yes	yes
	SCLC 2.20		f	67	A*02:01;A*29:02;B*44:02;B*44:03;C*05:01;C*16:01	SCLC	T4 N3 M1	IV	yes	yes	biopsy (T1)+ cryorec (T2)	4	4	yes	yes
	SCLC 2.01		m	64	N/A	SCLC	T4 NX M1	IV	yes	yes		1			
	SCLC 2.03		f	76	A*01:01;A*23:01;B*08:01;B*14:01;C*07:01;C*08:02	SCLC	T4 N3 M1	IV	yes	yes		2	1		
SCLC 2.04		m	52	A*03:01;A*29:02;B*07:02;B*44:03;C*07:02;C*16:01	SCLC	T3 N3 M0	IV	yes	yes		2	2	yes (t1)		
SCLC 2.05		f	67	A*01:01;A*25:01;B*27:05;B*35:01;C*02:02;C*04:01	SCLC	T4 N2 M1c	IV	no	yes		1	1			
SCLC 2.06		f	72	A*02:01;A*29:02;B*44:03;B*58:01;C*16:01;C*07:18	SCLC	T4 N2 M0	IIIB	yes	yes		1	1			
SCLC 2.07		m	80	A*01:01;A*03:01;B*07:02;B*37:01;C*07:02;C*06:02	SCLC	T3 N3 M1c	IV	yes	yes		1	1			
SCLC 2.08		f	66	A*02:01;A*25:01;B*18:01;B*50:01;C*12:03;C*06:02	SCLC	T2 N2 M0	IIIA	yes	yes		2	2			
SCLC 2.09		m	81	A*03:01;A*68:01;B*35:01;B*44:02;C*04:01;C*07:04	SCLC	T4 N3 M1c	IVb	yes	yes		1	1			
SCLC 2.10		m	77	A*03:01;A*30:01;B*07:02;B*44:03;C*07:02;C*04:01	SCLC	T4 N3 M1c	IVb	yes	N/A		2	2			
SCLC 2.11		f	46	A*01:01;A*03:01;B*07:02;B*51:01;C*07:02;C*15:02	SCLC	T3 N2 M1	IV	yes	yes		2	2		yes	

Supplementary Table 2: SCLC patient characteristics (continued).

SCLC patient cohort 2	Tübingen	f	67	A*02:01;B*15:01;B*44:02; C*03:03;C*05:01	SCLC	Tx Nx M1	IV	yes	yes		2	2		
		m	71	A*30:01;A*68:01;B*13:02; B*51:01;C*06:02;C*15:04	SCLC	T4 N3 M1	IV	yes	yes		2	2		
		m	59	A*29:02;A*31:01;B*44:03; B*51:01;C*16:01;C*15:02	SCLC	T4 N3 M0	IIIC	yes	yes		1	1		
		m	67	A*02:01;A*11:01;B*15:01; B*55:01;C*03:04;C*03:03	SCLC	T2b N0 M1c	IV	yes	yes		2	2		
		f	60	A*01:01;A*02:01;B*08:01; B*40:01;C*07:01;C*03:04	SCLC	Tx N2 M1c	IV	yes	yes		2	2		yes
		m	62	A*01:01;A*11:01;B*08:01; B*35:01;C*07:01;C*04:01	SCLC	T4 N2 M0	IIIB	yes	yes		2	2		
		m	64	A*01:01;A*03:01;B*08:01; B*40:01;C*07:01;C*03:04	SCLC	T1 N2 M1	IV	yes	yes		2	2		
		f	64	A*02:01;A*03:01;B*40:01; B*51:01;C*03:04;C*14:02	SCLC	T4 N0 M0	IIIA	yes	yes		2	2		
		f	53	A*02:01;B*48:01;B*50:01; C*06:02;C*08:01	SCLC	T4 N1 M1	IV	yes	yes		2	2		
		m	74	A*03:01;A*29:01;B*07:05; B*51:01;C*15:05;C*01:02	SCLC	T4 N3 M1	IV	yes (1 cycle)	yes		1	1		yes
		m	68	A*01:01;A*31:01;B*08:01; B*51:01;C*07:01;C*15:02	SCLC	T3 N1 M1	IV	yes (1 cycle)	N/A		1	1		yes
		f	82	A*02:01;A*66:01;B*15:01; B*41:02;C*01:02;C*17:03	SCLC	T4 N1 M0	IIIA	yes	yes		1	1		
		f	73	A*01:01;A*11:01;B*15:01; B*57:01;C*04:01;C*06:02	SCLC	T1 N3 M0	IIIB	yes	yes		1	1		
		m	72	A*02:01;A*11:01;B*15:01; B*37:01;C*04:01;C*06:02	SCLC	T4 N3 M1	IV	yes (1 cycle)	yes		2	2		yes
		m	55	A*01:01;A*02:01;B*08:01; B*40:01;C*07:01;C*03:04	SCLC	T4 N0 M1	IV	yes	yes		2	2		
		f	52	A*26:01;A*29:02;B*49:01; B*50:01;C*07:01;C*06:02	SCLC	T2b N1 M1c	IV	yes (1 cycle)	yes		2	2		
		m	61	A*32:01;A*68:01;B*40:02; B*51:01;C*02:02;C*07:04	SCLC	T4 N3 M1c	IV	yes (1 cycle)	yes		2	2		
		f	65	A*01:01;A*03:01;B*07:02; B*08:01;C*07:02;C*07:01	SCLC	T3 N2 M0	IIIB	yes (1 cycle)	yes		1	1		
		m	57	A*03:01;A*24:02;B*14:02; B*35:01;C*08:02;C*04:01	SCLC	T4 N3 M1b	IV	yes (1 cycle)	yes		2	2		
		f	56	A*01:01;A*02:01;B*08:01; B*27:05;C*07:01;C*01:02	SCLC	T4 N1 M1c	IV	no	yes		2	2		yes
		f	61	A*02:01;A*23:01;B*07:02; B*40:01;C*07:02;C*03:04	SCLC	T4 N3 M0	IIIC	no	yes		3	2		yes
		m	46	A*02:01;A*66:01;B*41:02; B*44:02;C*17:03;C*05:01	SCLC	T4 N3 M1c	IV	yes (1 cycle)	yes		2	2		
		f	62	A*02:01;A*24:02;B*08:01; B*18:01;C*07:01	SCLC	T4 N0 M1b	IV	no	yes		1	1		yes
		m	75	A*02:01;A*03:01;B*49:01; B*55:01;C*07:01;C*03:03	SCLC	Tx Nx M1c	IV	yes	yes		2	2		
		m	79	A*02:01;B*07:02;B*40:01; C*07:02;C*03:04	SCLC	T3 N0 M0	IIIB	yes (1 cycle)	yes		2	2		
		f	65	A*02:01;A*26:01;B*07:02; C*07:02	SCLC	Tx N3 M1	IV	no	yes		2	2		
		m	66	A*01:01;A*02:01;B*08:01; B*15:01;C*07:01;C*03:03	SCLC	T1a N1 M0	IV	yes	yes		2	2		
		f	59	A*02:01;A*30:01;B*44:02; B*45:01;C*05:01;C*06:02	SCLC	Tx Nx M1c	IV	no	yes		2	2		
		f	73	A*02:01;B*35:01;B*57:01; C*04:01;C*06:02	SCLC	T4 N3 M1c	IV	no	yes		2	2		yes
		f	74	A*01:01;A*68:01;B*44:05; B*57:01;C*02:02;C*06:02	SCLC	T4 Nx M1b	IV	no	yes		2	2		
		m	64	A*11:01;A*24:02;B*07:02; B*15:01;C*07:02;C*04:01	SCLC	T4 N1 M1b	IV	no	yes		2	2		
		f	72	A*02:01;A*03:01;B*51:01; B*51:05;C*14:02;C*04:01	SCLC	T3 N3 M0	IIIC	no	yes		2	2		
		m	69	A*01:01;A*03:01;B*40:01; B*51:01;C*03:04;C*01:02	SCLC	Tx Nx M1b	IV	no	yes		1	1		yes
		m	82	A*02:05;A*26:01;B*35:01; B*50:01;C*04:01;C*06:02	SCLC	T4 N3 M0	IIIC	no	yes		1	1		
		f	58	A*02:01;B*13:02;B*18:01; C*06:02;C*07:01	SCLC	T2 N2 M0	IIIA	no	yes		2	2		

For three patients (S02730_03/_10, S03381_T/_cells and SCLC2.20_T1/_T2) two different tumor samples were collected (see also Supplementary Tables 2 and 3). In SCLC patient cohort 2 patients are labeled in bold, if immunopeptidomics of plasma samples was performed. t1 refers to the first timepoint of plasma collection.

Abbreviations: m, male; f, female; N/A, not available; LCNEC, large cell neuroendocrine tumor; T, tumor; N, (lymph) node; M, metastasis; UICC, Union for International Cancer Control; CDX, circulating tumor cell derived xenograft; PDX, patient (biopsy) derived xenograft; Cryorec, cryorecanalization

Supplementary Table 3: Sample and MS specifications of SCLC samples from xenograft models.

Sample specifics						Orbitrap-based MS										Orbitrap/timsTOF combined	
Sample ID	Batch #	Sample type	Tissue mass [mg]	FASTA	# of mutations included in FASTA	Zip-Tip	MS specifics (HLA I)	HLA I peptides (h h/m m)	HLA I peptides (h + h/m)	HLA I ligands (h + h/m)	Purity [%] (h + h/m)	HLA I peptides (h h/m m)	HLA I peptides (h + h/m)	HLA I ligands (h + h/m)	Purity [%] (h + h/m)	HLA I ligands (h + h/m)	HLA I ligands (h)
S02496	1	CDX	35.8	Human 201014 + Mouse 210203	367 (13 fs; 354 SNM)	Lumos DE: 7s MaxIT: 150 ms		1309 (429 808 72)	1237	1149	92.9					1149	405
S02521	1	CDX	87	Human 201014 + Mouse 210203 + mutations				683 (136 385 162)	521	341	65.5					341	91
S02544	1	CDX	122	Human 201014 + Mouse 210203				234 (39 138 57)	177	92	52.0					92	9
S02714	1	CDX	78.1	Human 201014 + Mouse 210203				176 (56 98 22)	154	58	37.7					58	18
S02513	2	CDX	34.1	Human 201014 + Mouse 210203 + mutations	141 (5 fs; 136 SNM)	ZT	QE DE 1s MaxIT: 200 ms	992 (373 572 47)	945	635	67.2	2183 (822 1247 114)	2069	1312	63.4	1496	526
S02658	2	CDX	23.6	Human 201014 + Mouse 210203 + mutations	140 (6 fs; 134 SNM)			2332 (859 1434 39)	2293	570	24.9	6056 (2187 3738 131)	5925	1472	24.8	1616	551
S02730_03	2	CDX (Tissue Tek)	171.3	Human 201014 + Mouse 210203 + mutations	360 (21 fs; 339 SNM)			2517 (838 1616 63)	2454	2340	95.4	4248 (1483 2609 156)	4092	3883	94.9	4383	1600
S02730_10	2	CDX	23.7	Human 201014 + Mouse 210203 + mutations	25 (25 SNM)			2265 (926 1290 49)	2216	2087	94.2	3823 (1518 2214 91)	3732	3470	93.0	4012	1647
S02895	2	CDX	75.4	Human 201014 + Mouse 210203 + mutations				1061 (370 616 75)	986	766	77.7	2280 (823 1291 166)	2114	1600	75.7	1788	619
S02841	2	CDX	43.2	Human 201014 + Mouse 210203 + mutations	379 (379 SNM)			886 (295 558 33)	853	763	86.1	1994 (734 1175 85)	1909	1658	86.9	1852	661
S03381_cell	3	PDX	80 Mio	Human 201014 + Mouse 210203 + mutations	143 (7 fs; 3 delins; 133 SNM)			4190 (1770 2344 76)	4114	3763	89.8	6427 (2854 3452 121)	6306	5557	88.1	6544	2831
S03381_T	3	PDX	36	Human 201014 + Mouse 210203 + mutations				217 (95 90 32)	185	28	12.9	679 (354 268 57)	622	68	10.9	79	25
S03387	3	PDX	24	Human 201014 + Mouse 210203 + mutations	64 (1 fs; 1 ins; 62 SNM)			481 (349 99 33)	448	73	15.2	1085 (707 307 71)	1014	170	16.8	188	112
S03505	3	PDX	9	Human 201014 + Mouse 210203 + mutations	216 (16 fs; 1 delins; 199 SNM)			356 (162 102 92)	264	32	9.0	623 (285 198 140)	483	50	10.4	61	182
S03536	3	PDX	21	Human 201014 + Mouse 210203 + mutations	321 (16 fs; 1 delins; 304 SNM)			441 (222 185 34)	407	157	35.6	680 (369 258 53)	627	198	31.6	256	100

The description in brackets (h | h/m | m), gives information whether the peptide sequences derived from human only (h), human/mouse (h/m) or mouse only (m) proteins.

Abbreviations: T, tumor; CDX, circulating tumor cell derived xenograft; PDX, patient (biopsy) derived xenograft; fs, frameshift; SNM, single nucleotide mutation; ins, insertion; del, deletion; DE, dynamic exclusion; MaxIT, maximal injection time; h, human; m, mouse

Supplementary Table 4: Sample and MS specifications of SCLC samples from cryorecanalization or biopsy.

Sample specifics						Orbitrap-based MS					timsTOF mS				Orbitrap/ timsTOF combined	
Sample ID	Sample type	Tissue mass [mg]	FASTA	# of mutations included in FASTA	Zip-Tip	MS specifics (HLA I/ II)	HLA I peptides	HLA I ligands	Purity [%]	HLA II peptides	HLA I peptides	HLA I ligands	Purity [%]	HLA II peptides	HLA I ligands	HLA II peptides
S02243	Biopsy (TissueTek)	245	Human 201014 + mutations	204 (4 delins; 200 SNM)	ZT	Lumos DE: 7s/10s	1913	1401	73.2	648					1401	648
SCLC1.02_Prep1	Cryorec	641	Human 201014			MaxIT: 150 ms				2052						2052
SCLC1.02_Prep2	Cryorec	327	Human 201014				3012	2822	93.7	2675					2822	2675
SCLC1.03	Cryorec	285	Human 201014				3674	3520	95.8	2977	5249	4761	90.7	3124	6361	4839
SCLC1.05	Cryorec	298	Human 201014				5241	5080	96.9	3245	10206	9631	94.4	5619	11274	6603
SCLC1.06	Cryorec	36	Human 201014				1429	1179	82.5	1560	3824	3063	80.1	1251	3381	2400
SCLC1.07	Cryorec	150	Human 201014			QE	506	388	76.7	351	3082	2238	72.6	2047	2292	2091
SCLC1.08	Cryorec	28	Human 201014			DE: 7s/ 10s MaxIT: 150 ms	803	598	74.5	379	1564	1105	70.7	418	1256	562
SCLC1.09	Cryorec	50	Human 201014				2039	1930	94.7	1223	6190	5711	92.3	2464	5922	2654
SCLC1.10	Cryorec	31	Human 201014				529	415	78.4	256	1352	1017	75.2	556	1099	619
SCLC1.11	Cryorec	21	Human 201014				2823	2651	93.9	1887	5950	5522	92.8	4077	6075	4395
SCLC2.20_T1	Biopsy	15	Human 201014				4316	1201	27.8	1124	10428	2686	25.8	1875	2895	2111
SCLC2.20_T2	Cryorec	281	Human 201014				4285	4120	96.1	2465	7563	7045	93.2	6002	8039	6308

For SCLC1.02, two HLA immunoprecipitations (Prep 1 and 2) were performed from the same tumor, for SCLC 2.20, tumor tissue was sampled twice at different timepoints (T1 and 2).

Abbreviations: Prep, HLA immunoprecipitation; T, tumor; Cryorec, cryorecanalization; del, deletion; ins, insertion; SNM, single nucleotide mutation; DE, dynamic exclusion; MaxIT, maximal injection time

Supplementary Table 5: Sample and MS specifications of plasma samples from SCLC patients.

Sample specifics					Orbitrap-based MS					timsTOF MS				Orbitrap/ timsTOF combined	
Sample ID	Sample type	Volume plasma [ml]	FASTA	Zip-Tip	MS specifics (HLA I/ II)	HLA I peptides	HLA I ligands	Purity [%]	HLA II peptides	HLA I peptides	HLA I ligands	Purity [%]	HLA II peptides	HLA I ligands	HLA II peptides
SCLC1.05_002_sHLA	Plasma	20.2	Human 201014	ZT	QE DE: 7s/ 10s MaxIT: 150 ms	3654	3540	96.9	426	9151	8635	94.4	1706	9122	1789
SCLC1.06_001_sHLA	Plasma	26	Human 201014	ZT		3579	3431	95.9	779	8260	7720	93.5	1840	8276	2001
SCLC1.06_002_sHLA	Plasma	24	Human 201014	ZT		2097	2036	97.1	233	5701	5389	94.5	675	5621	720
SCLC1.07_001_sHLA	Plasma	23.2	Human 201014	ZT		2226	2193	98.5	762	6433	6222	96.7	2137	6433	2234
SCLC1.08_001_sHLA	Plasma	15	Human 201014	ZT		1289	1166	90.5	34	2099	1918	91.4	130	2173	132
SCLC1.09_001_sHLA	Plasma	18.5	Human 201014	ZT		1740	1703	97.9	721	2807	2741	97.6	1167	3099	1358
SCLC1.09_002_sHLA	Plasma	27	Human 201014	ZT		3579	3521	98.4	335	7143	6964	97.5	762	7535	810
SCLC1.10_001_sHLA	Plasma	22	Human 201014	ZT		645	618	95.8	49	1507	1445	95.9	110	1513	118
SCLC1.10_002_sHLA	Plasma	19	Human 201014	ZT		39	34	87.2	14	661	627	94.9	53	627	58
SCLC1.11_001_sHLA	Plasma	19.4	Human 201014	ZT		1511	1383	91.5	829	3576	3242	90.7	1444	3480	1604
SCLC1.11_002_sHLA	Plasma	25.4	Human 201014	ZT		2366	2130	90.0	759	3610	3267	90.5	896	3818	1118
SCLC2.20_001_sHLA	Plasma	24	Human 201014	ZT		3030	2920	96.4	405	4160	3976	95.6	683	4733	814
SCLC2.20_002_sHLA	Plasma	22	Human 201014	ZT		1555	1466	94.3	95	2528	2338	92.5	189	2674	202
SCLC2.20_003_sHLA	Plasma	19	Human 201014	ZT		503	479	95.2	191	1243	1172	94.3	458	1217	505
SCLC2.20_004_sHLA	Plasma	21	Human 201014	ZT		273	258	94.5	23	540	517	95.7	27	567	32
SCLC2.04_001_sHLA	Plasma	15	Human 201014	ZT		403	363	90.1	782	1618	1381	85.4	1549	1405	1725
SCLC2.11_001_sHLA	Plasma	25	Human 201014	ZT		2659	2535	95.3	745	7197	6680	92.8	1495	7073	1666
SCLC2.11_002_sHLA	Plasma	16	Human 201014	ZT		369	248	67.2	702	2286	1416	61.9	2005	1441	2175
SCLC2.16_001_sHLA	Plasma	16.2	Human 201014	ZT		2559	2472	96.6	468	5355	5003	93.4	2072	5503	2153
SCLC2.16_002_sHLA	Plasma	18	Human 201014	ZT		2417	2334	96.6	892	5003	4672	93.4	1477	5148	1705
SCLC2.23_001_sHLA	Plasma	20	Human 201014	ZT		671	530	79.0	340	1435	1122	78.2	374	1254	475
SCLC2.24_001_sHLA	Plasma	24	Human 201014	ZT		649	614	94.6	85	1363	1268	93.0	172	1369	187
SCLC2.27_001_sHLA	Plasma	25.5	Human 201014	ZT		2416	2273	94.1	1269	4738	4377	92.4	1539	4796	1982
SCLC2.27_002_sHLA	Plasma	20.5	Human 201014	ZT		2393	1890	79.0	1556	3796	2779	73.2	1796	3296	2319
SCLC2.33_001_sHLA	Plasma	24	Human 201014	ZT		879	830	94.4	89	1621	1468	90.6	129	1610	159
SCLC2.33_002_sHLA	Plasma	17	Human 201014	ZT		689	623	90.4	94	1041	914	87.8	110	1037	138
SCLC2.34_001_sHLA	Plasma	16	Human 201014	ZT		3753	3651	97.3	462	6387	6141	96.1	776	6936	854
SCLC2.34_003_sHLA	Plasma	21.5	Human 201014	ZT		5157	5060	98.1	220	8781	8502	96.8	489	9754	514
SCLC2.36_001_sHLA	Plasma	21.5	Human 201014	ZT		1171	1049	89.6	78	1774	1505	84.8	109	1683	135
SCLC2.42_001_sHLA	Plasma	22.3	Human 201014	ZT		2995	2860	95.5	198	6808	6297	92.5	402	6814	446
SCLC2.42_002_sHLA	Plasma	27	Human 201014	ZT	2934	2831	96.5	1694	3908	3720	95.2	2433	4714	2843	
SCLC2.46_001_sHLA	Plasma	22.4	Human 201014	ZT	3299	3136	95.1	777	5166	4953	95.9	936	5786	1118	

For some patients, plasma was collected at different timepoints indicated with the number in the sample ID (e.g. 001).

Abbreviations: Prep, HLA immunoprecipitation; T, tumor; QE, Q Exactive HF; timsTOF, trapped ion mobility spectrometry coupled to time of flight; DE, dynamic exclusion; MaxIT, maximal injection time

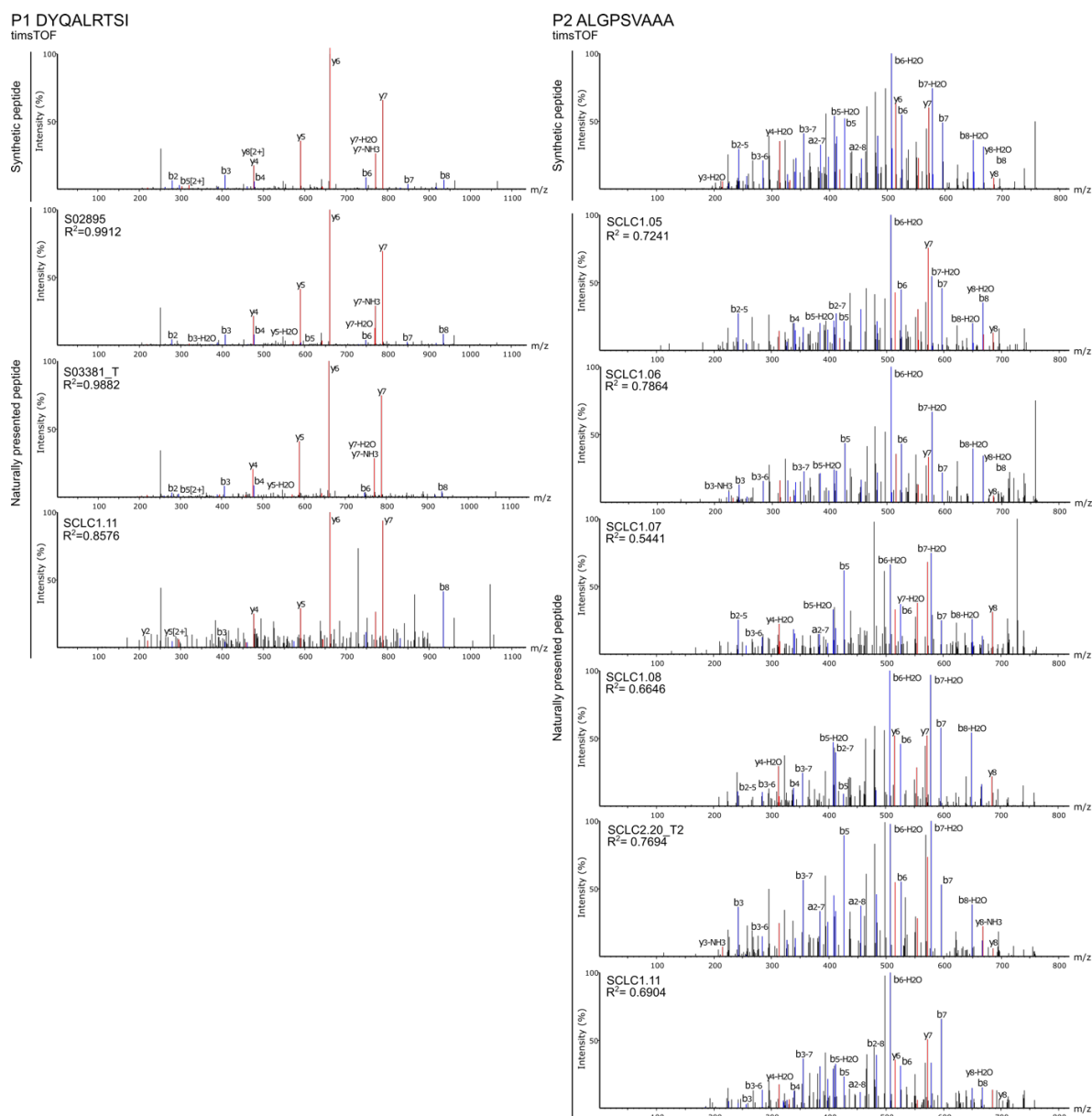
Supplementary Table 6: Synthetic peptide validation of frequently shared peptide sequences

Peptide ID	Peptide Sequence	Synthetic peptide validation					
		MS device	timsTOF				Orbitrap (ongoing)
			Sample	R ²	RT	Precursor ID	Sample
Frequently shared peptide sequences							
P1 B*08	DYQALRTSI	TT/QE	Synpep (z=2) S02895 (z=2) S03381_T (z=2) SCLC1.11 (z=2)	- 0.9912 0.9882 0.8576		43410 29909 29487 34638	Synpep S02895 S03536 S03381_T S03387 SCLC1.11 (S02521)
P2 A*02	ALGPSVAAA	TT	Synpep (z=1) SCLC1.05 (z=1) SCLC1.06 (z=1) SCLC1.07 (z=1) SCLC1.08 (z=1) SCLC2.20_T2 (z=1) SCLC1.11 (z=1)	- 0.7241 0.7864 0.5541 0.6646 0.7694 0.6904	20.01 18.77 18.75 18.75 21.74 21.75 21.63	34127 28469 22913 22786 19945 27724 29770	
P3 A*03	SINKPTSER	QE					Synpep S02496 S02513 S02658 S02841 SCLC1.02 SCLC1.03
P4 mix	SGALHVYSL	TT/QE	Synpep (z=2) S02895 (z=2) S02513 (z=2) S02730_03 (z=2) S02730_10 (z=2) S02841 (z=2)	- 0.8729 0.9197 0.8638 0.865 0.8926		53395 33866 33085 24108 33616 28087	(S02521)
P5 A*02	VLSGPPAVA	TT	Synpep (z=1) SCLC1.05 (z=1) SCLC1.06 (z=1) SCLC1.07 (z=1) SCLC1.08 (z=1) SCLC2.20_T2 (z=1)	- 0.9536 0.9216 0.953 0.973 0.9678		36299 32376 26482 25476 25891 31331	
P6 A*03	KSKEYFSKQK	QE					Synpep S02658 S02841 SCLC1.02 (S02496) (S02544)
P7 mix	EVFGRSEQY	TT/QE	Synpep (z=2) SCLC1.10 (z=2) SCLC1.11 (z=2)	- 0.9263 0.9383		31112 20386 25060	Synpep SCLC1.10 SCLC1.11 (S02243) (S02496) (S02714)
P8 mix	VPSEIDAAF	TT/QE	Synpep (z=1) S03381_cells (z=1) SCLC1.05 (z=1) SCLC1.06 (z=1) SCLC1.09 (z=1) SCLC1.10 (z=1)	- 0.9678 0.9854 0.9498 0.9708 0.9282		53622 47602 49173 37264 44116 32250	Synpep SCLC1.05 SCLC1.09
P9 mix	MYDGPVYEV	TT/QE	Synpep (z=1) S03381_cells (z=1) S02730_03 (z=1) S02730_10 (z=1) SCLC1.05 (z=1) SCLC1.09 (z=1)	- 0.9671 0.886 0.897 0.8093 0.7463	24.33 25.45 25.42 25.36 22.83 25.37	48431 41450 25212 31851 41948 39332	Synpep S02730 S03381_cells
P10 mix	TIIDKSKSV	TT/QE	Synpep (z=2) S02895 (z=2) S02513 (z=2) S02841 (z=2) SCLC1.05 (z=2)	- 0.9826 0.9897 0.9949 0.9697		33172 16529 16108 14648 12295	Synpep S02513 S02895 S02841 SCLC1.05
P11 A*01	EVDPIGHLY	TT/QE	Synpep (z=1) Synpep (z=2) S03381_cells (z=1) S03536 (z=2)	- - 0.9773 0.9714		52255 52276 42216 31112	Synpep S03381_cells S03536
P12 A*02	ILLDVKTRL	TT	Synpep (z=2) SCLC1.05 (z=2) SCLC1.06 (z=2) SCLC2.20_T2 (z=2) SCLC1.11 (z=2)	- 0.8842 0.9691 0.8176 0.9773		84315 36408 28575 36974 37999	

P13 A*02	LLPAVPVGA	TT/QE	Synpep (z=1) SCLC1.05 (z=1) SCLC2.20 (z=1) SCLC1.11 (z=1)	- 0.9801 0.9478 0.8217		73280 68423 56340 58080	Synpep SCLC1.02 SCLC1.05
P14 A*02	GLAEEVQRL	TT/QE	Synpep (z=2) SCLC1.05 (z=2) SCLC1.11 (z=2)	- 0.9816 0.9907		50454 36674 35963	Synpep SCLC1.02 SCLC1.05 SCLC1.11 (S02521)
P15 A*03	ALNSGVSQK	QE					Synpep S02513 S02658 S02841
P16 A*03	VLSGPGTGK	TT/QE	Synpep (z=2) S02513 (z=2) S02841 (z=2)	- 0.9950 0.9947		20891 12988 10026	Synpep S02513 S02841 SCLC1.02 SCLC1.03
P17 A*03/A*11	KSSDVFSK	QE					Synpep S02513 S02658 S02730_10 S02841
P18 A*03/A*11	STAKHVFKEK	QE					S02513 S02730_10 S03381 (S02496)
P19 A*11	ATAQFKINKK	TT	Synpep (z=2) S03381_cells (z=2) S02730_03 (z=3) S02730_10 (z=3)	- 0.865 0.4132 0.4652	29.68 14.8 14.7 14.71	67089 13063 8760 13582	
P20 A*11/A*03	GLAPNTPGK	TT/QE	Synpep (z=2) S02513 (z=2) S02730_03 (z=2) S03381_cells (z=2)	- 0.9685 0.9893 0.9904		22505 15872 14564 15776	Synpep S03381_cells
P21 A*24/A*23	EYLDDRNTF	TT/QE	Synpep (z=2) SCLC1.07 (z=2) SCLC1.09 (z=2) S02841 (z=2) S02895 (z=2)	- 0.9851 0.9868 0.9291 0.9663		40388 23731 31536 23089 40388	Synpep S02841 S02895 SCLC1.09
P22 B*35/B*07	SPLDTSSVL	TT	Synpep (z=1) SCLC1.03 (z=1) SCLC1.05 (z=1) SCLC1.06 (z=1) SCLC1.07 (z=1)	- 0.9366 0.8493 0.9422 0.9078		55165 44508 47977 37209 34543	
P23 B*07	VPLPAGGGTV	TT/QE	Synpep (z=1) SCLC1.03 (z=1)	- 0.9812		39716 33999	Synpep SCLC1.02
P24 B*51/B*07	YPRISVNNV	TT/QE	Synpep (z=2) SCLC1.03 (z=2)	- 0.8401		47906 30125	Synpep SCLC1.02 SCLC1.03
P25 B*51/B*08	DGKKKISTV	QE					Synpep S03381_cells SCLC1.02 SCLC1.06

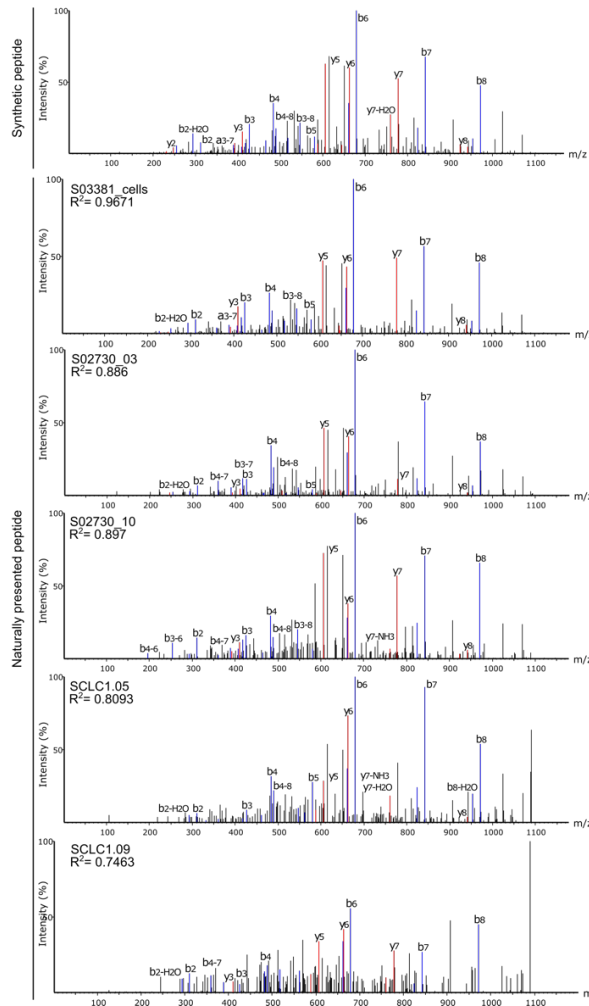
Synthetic peptides are analyzed with the same MS instrument than the naturally presented peptide sequence, indicated in the column MS device. Validation will only be performed with timsTOF and the QE. R² values > 0.8 are indicated in green, R² values > 0.6 in yellow and R² values < 0.6 in red. In the column Orbitrap (ongoing) samples are listed for which synthetic peptide validation is ongoing but will not be included in this thesis. Samples that are listed in brackets were analyzed with the Lumos and will not be confirmed separately.

Abbreviations: MS, mass spectrometry; TT, timsTOF, trapped ion mobility spectrometry coupled to time of flight; QE, Q Exactive HF; RT, retention time; Precursor ID, identification number of the precursor ion that was used for spectral validation

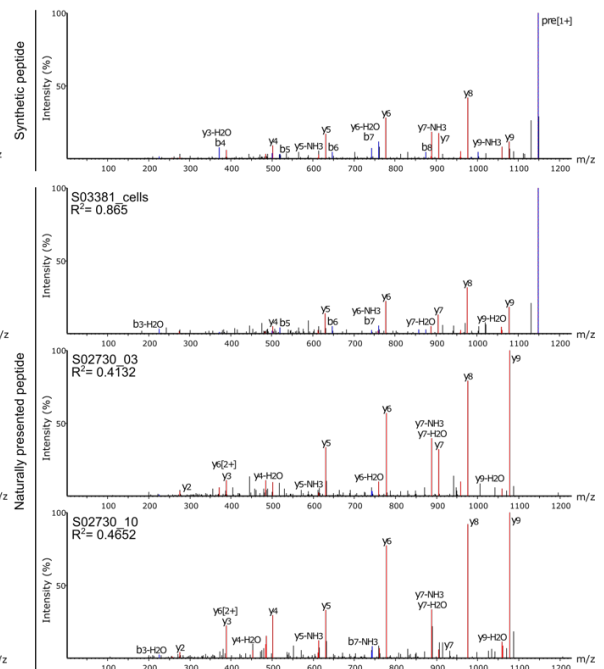


Supplementary Figure 2. Synthetic peptide validation of P1 DYQALRTSI and P2 ALGPSVAAA with timsTOF MS. On top the spectrum of the synthetic peptide and below the spectra of all naturally presented peptides. Intensity [%] is plotted against the m/z ratio. Annotated b- and y-ions are indicated. The similarity between the synthetic and experimental spectrum was calculated (R^2). Y-ions are indicated in red and b-ions in blue.

P9 MYDGPVYEV
timsTOF



P19 ATAQFKINKK
timsTOF



Supplementary Figure 3. Synthetic peptide validation of P9 MYDGPVYEV and P19 ATAQFKINKK with timsTOF MS. On top the spectrum of the synthetic peptide and below the spectra of all naturally presented peptides. Intensity [%] is plotted against the m/z ratio. Annotated b- and y-ions are indicated. The similarity between the synthetic and experimental spectrum was calculated (R^2). Y-ions are indicated in red and b-ions in blue.

Supplementary Table 7: Synthetic peptide validation of individually interesting peptide sequences

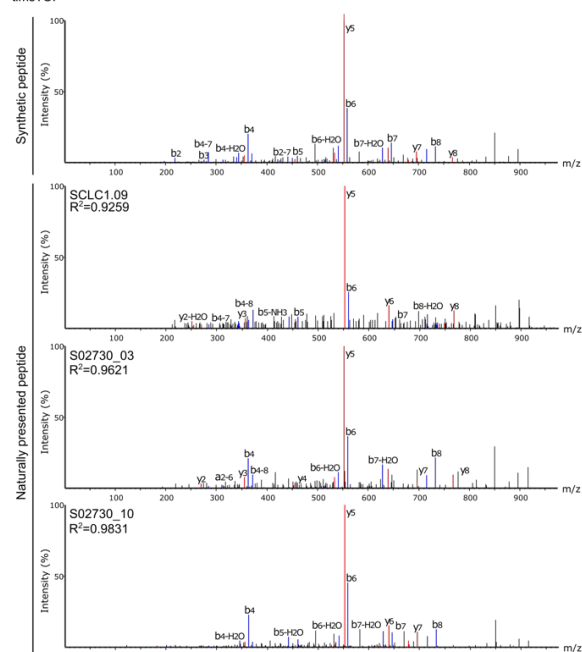
Peptide ID	Peptide Sequence	Synthetic peptide validation					
		MS device	timsTOF			Orbitrap (ongoing)	
			Sample	R ²	RT		Precursor ID
Individually interesting selected peptides							
P26 _{SCLC1.09}	MAGSPVSSY	TT	Synpep (z=1) SCLC1.09 (z=1) S02730_03 (z=1) S02730_10 (z=1)	- 0.9259 0.9621 0.9831		25156 23399 12679 21383	
P27 _{1.05,1.06,1.09}	AFDSSLVFI	TT/QE	Synpep (z=1) SCLC1.05 (z=1) SCLC1.06 (z=1) SCLC1.09 (z=1)	- 0.9243 0.8721 0.9172		84212 76723 58972 61949	Synpep SCLC1.05
P28 _{SCLC1.05, 1.09}	TFDETVSTY	TT/QE	Synpep (z=1) SCLC1.05 (z=1) SCLC1.09 (z=1) S03381_cells (z=1)	- 0.9249 0.9019 0.8413		42413 36341 33420 35864	Synpep S03381_cells SCLC1.05
P29 _{1.05,1.06,1.09}	NFDFQVQKF	TT/QE	Synpep (z=2) SCLC1.05 (z=2) SCLC1.06 (z=2) SCLC1.09 (z=2)	- 0.8833 0.9198 0.7681	26.88 24.53 24.53 28.26	57430 47895 36360 45196	Synpep SCLC1.09
P30 _{SCLC1.05, 1.06}	VLLDKIKNL	TT	Synpep (z=2) SCLC1.05 (z=2) SCLC1.06 (z=2)	- 0.79 0.0102	36.45 21.53 21.55	87231 37326 29903	
P31 _{SCLC1.09, 1.10}	KPLDGSALY	TT/QE	Synpep (z=2) SCLC1.09 (z=2) SCLC1.10 (z=2)	- 0.9898 0.9602		43470 30904 24386	Synpep SCLC1.09
P32 _{SCLC1.05}	ILPPAPSSYF	TT	Synpep (z=1) SCLC1.05 (z=1) S02895 (z=1)	- 0.9846 0.9992		63110 59955 49763	
P33 _{SCLC1.03, 1.09}	RSAPVGLNK	TT/QE	Synpep (z=2) SCLC1.03 (z=2) SCLC1.09 (z=2)	- 0.8885 0.9757		38633 10690 19033	Synpep SCLC1.03 SCLC1.09
P34 _{SCLC1.05, 2.20}	ALATGLVGG	TT	Synpep (z=1) SCLC1.05 (z=1) SCLC2.20_T2 (z=1)	- 0.9786 0.8742		43036 37452 33957	
P35 _{SCLC1.02}	AARVPGPSSSL	QE					Synpep SCLC1.02
P36 _{SCLC1.03}	STYYWPRPR	QE					Synpep SCLC1.03
P37 _{SCLC1.03}	KISGGPRISY	TT	Synpep (z=2) SCLC1.03 (z=2)	- 0.9983		63042 16417	
P38 _{SCLC1.03}	LPQKTFQSL	QE					Synpep SCLC1.03
P39 _{SCLC1.05}	SPNNFLSY	TT	Synpep (z=1) SCLC1.05 (z=1)	- 0.9116		60990 56281	
P40 _{SCLC1.05}	SLEAGLPLQV	TT	Synpep (z=1) SCLC1.05 (z=1)	- 0.9344		68282 63499	
P41 _{SCLC1.05}	DAWPGTFSF	TT	Synpep (z=1) SCLC1.05 (z=1)	- 0.9979		81288 74457	
P42 _{SCLC1.05}	FEDHNPPQL	QE					Synpep SCLC1.05
P43 _{SCLC1.05}	DPFLNSGT	TT	Synpep (z=1) SCLC1.05 (z=1)	- 0.6997	26.57 24.89	56270 49181	
P44 _{SCLC1.05}	GEEDSPSHI	QE					Synpep SCLC1.05
P45 _{SCLC1.06}	IPLESTDGERL	TT	Synpep (z=2) SCLC1.06 (z=2)	- 0.9853		41903 25681	
P46 _{SCLC1.06}	DLVKLKY	TT	Synpep (z=2) SCLC1.06 (z=2)	- 0.7745	37.66 21.05	90011 28650	
P47 _{SCLC1.10}	LKPGAPLPAL	TT	Synpep (z=2) SCLC1.10 (z=2)	- 0.0664	27.77 26.78	60534 31069	
P48 _{SCLC1.11}	EALLRVLLL	TT	Synpep (z=2) SCLC1.11 (z=2)	- 0.5457	44.91 36.61	104722 60196	
P49 _{SCLC1.11}	GVFELQIHSF	TT/QE	Synpep (z=2) SCLC1.11 (z=2)	- 0.9787		73347 52361	Synpep SCLC1.11
P50 _{SCLC1.11}	DVSSSVYSTW	TT	Synpep (z=1) SCLC1.11 (z=1)	- 0.6865	27.82 27.35	60669 43753	
P51 _{SCLC2.20_T1/2}	FMKAITDRY	TT	Synpep (z=2) SCLC2.20_T1 (z=2) SCLC2.20_T2 (z=2)	- 0.9318 0.8531		51182 24646 18163	
P52 _{SCLC2.20}	GEELFFDYRY	TT	Synpep (z=2) SCLC2.20_T2 (z=2)	- 0.723	38.45 30.6	91857 51433	
P53 _{SCLC2.20}	QETLSVLKF	QE					Synpep SCLC2.20_T2
P54 _{SCLC2.20}	SEVIEPPSL	TT	Synpep (z=1) SCLC2.20_T2 (z=1)	- 0.9817		54395 47912	

P55 _{SCLC2.20}	EEYDSIIVFY	QE					Synpep SCLC2.20_T2
P56 _{SCLC2.20}	NEAGLVHGY	QE					Synpep SCLC2.20_T2
P57 _{SCLC2.20}	SEVGSPPSY	TT	Synpep (z=1) SCLC2.20_T2 (z=1)	- 0.9808		32781 23556	
P58 _{FLLDGSANV}	FLLDGSANV	TT/QE	Synpep (z=1) SCLC1.05 (z=1) SCLC1.06 (z=1) SCLC1.11 (z=1)	- 0.7017 0.6528 0.5014	30.06 27.01 27.01 29.95	68375 56284 43250 47585	Synpep SCLC1.05
P59 _{mut_R4M}	KTMGFSFPR	TT	Synpep (z=2) S02730_03 (z=2)	- 0.8318		74193 17763	

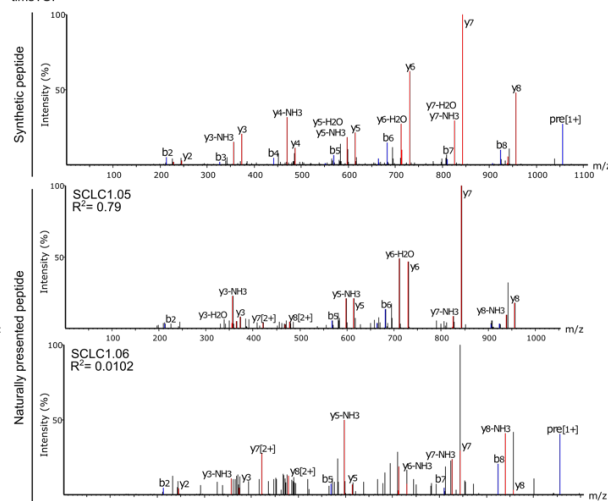
Synthetic peptides are analyzed with the same MS instrument than the experimentally identified peptide sequence, indicated in the column MS device. Validation will only be performed with timsTOF and QE. R2 values > 0.8 are indicated in green, R2 values > 0.6 in yellow and R2 values < 0.6 in red. In the column Orbitrap (ongoing) samples are listed for which synthetic peptide validation is ongoing but will not be included in this thesis.

Abbreviations: MS, mass spectrometry; TT, timsTOF, trapped ion mobility spectrometry coupled to time of flight; QE, Q Exactive HF; RT, retention time; Precursor ID, identification number of the precursor ion that was used for spectral validation.

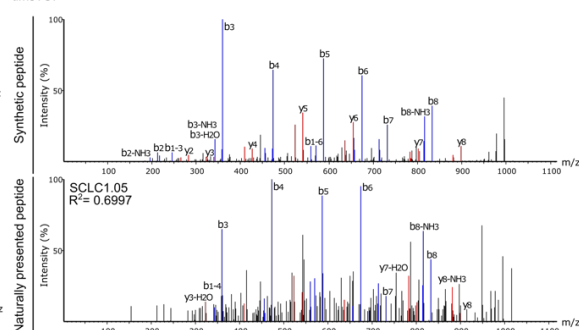
P26 MAGSPVSSY_ASCL1
timsTOF



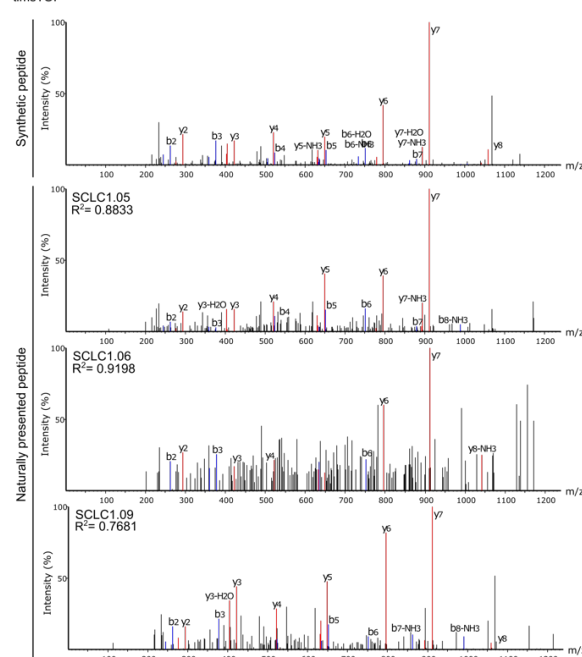
P30 VLLDKIKNL_COL6A3
timsTOF



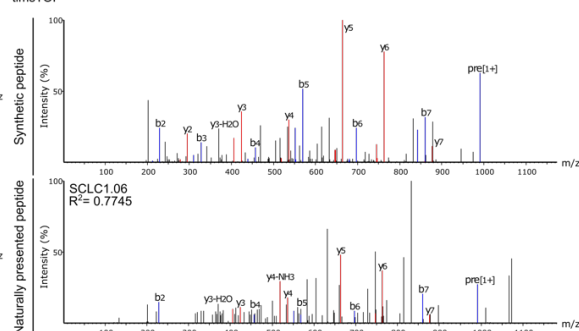
P43 DPFLNSGT_YAP1
timsTOF



P29 NFDFQVQKF_Protein not on benign
timsTOF

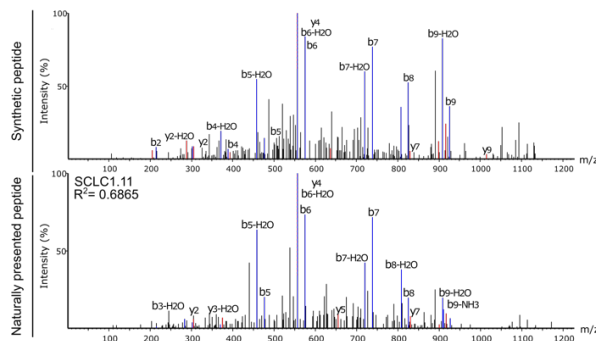


P46 DLVKKLYL_MAGEB3
timsTOF

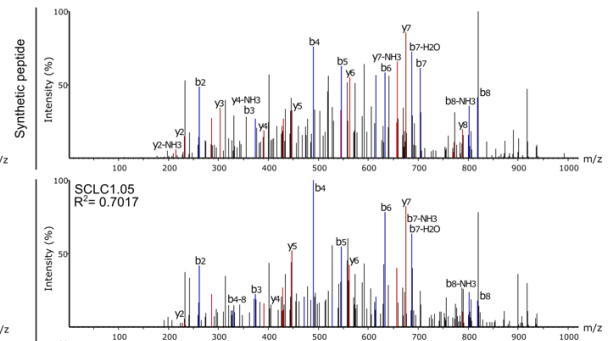


Supplementary Figure 4. Synthetic peptide validation of P26 MAGSVSSY, P29 NFDFQVQKF, P30 VLLDKIKNL, P43 DPFLNSGT and P46 DLVKKLYL with timsTOF MS. On top the spectrum of the synthetic peptide and below the spectra of all naturally presented peptides. Intensity [%] is plotted against the m/z ratio. Annotated b- and y-ions are indicated. The similarity between the synthetic and experimental spectrum was calculated (R^2). Y-ions are indicated in red and b-ions in blue.

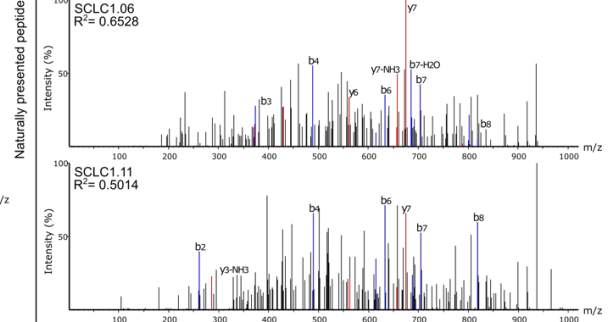
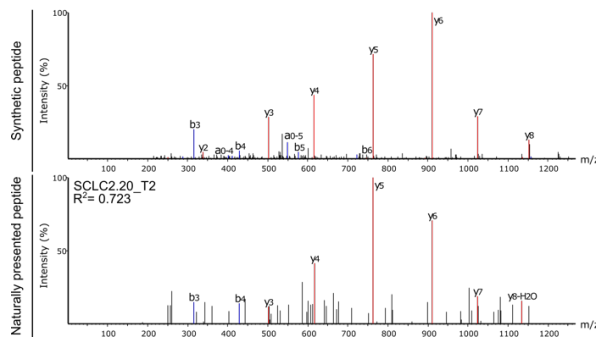
P50 DVSSSVYSTW_KIT
timsTOF



P58 FLLDGSANV_COL6A3
timsTOF

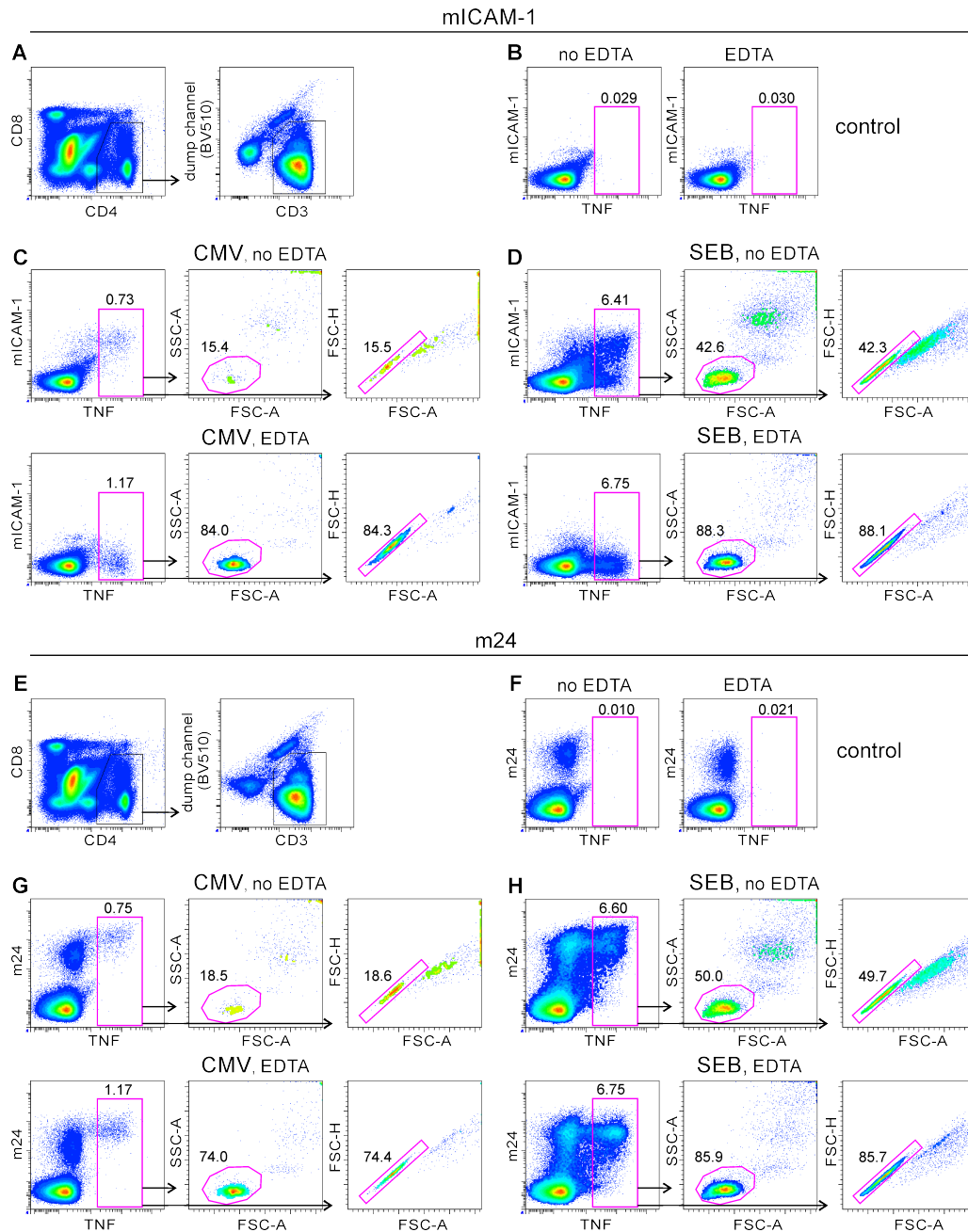


P52 GEELFFDYRY_EZH2
timsTOF

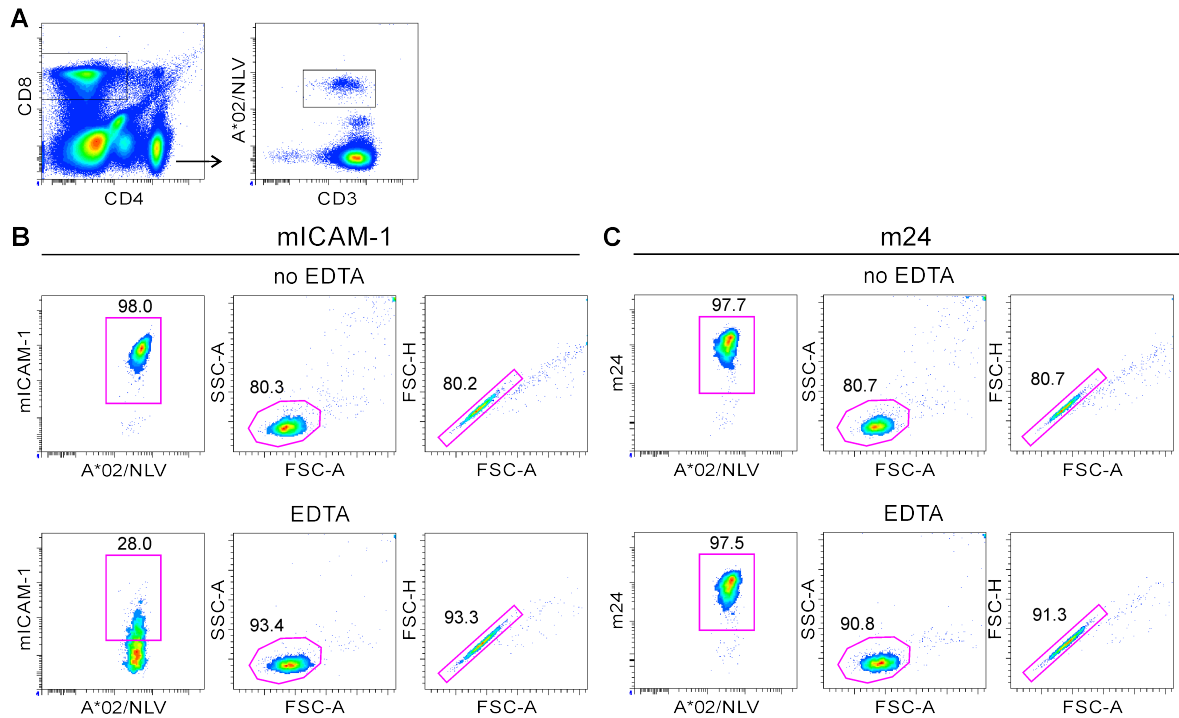


Supplementary Figure 5. Synthetic peptide validation of P50 DVSSSVYSTW, P52 GEELFFDYRD and P58 FLLDGSANV with timsTOF MS. On top the spectrum of the synthetic peptide and below the spectra of all naturally presented peptides. Intensity [%] is plotted against the m/z ratio. Annotated b- and y-ions are indicated. The similarity between the synthetic and experimental spectrum was calculated (R^2). Y-ions are indicated in red and b-ions in blue.

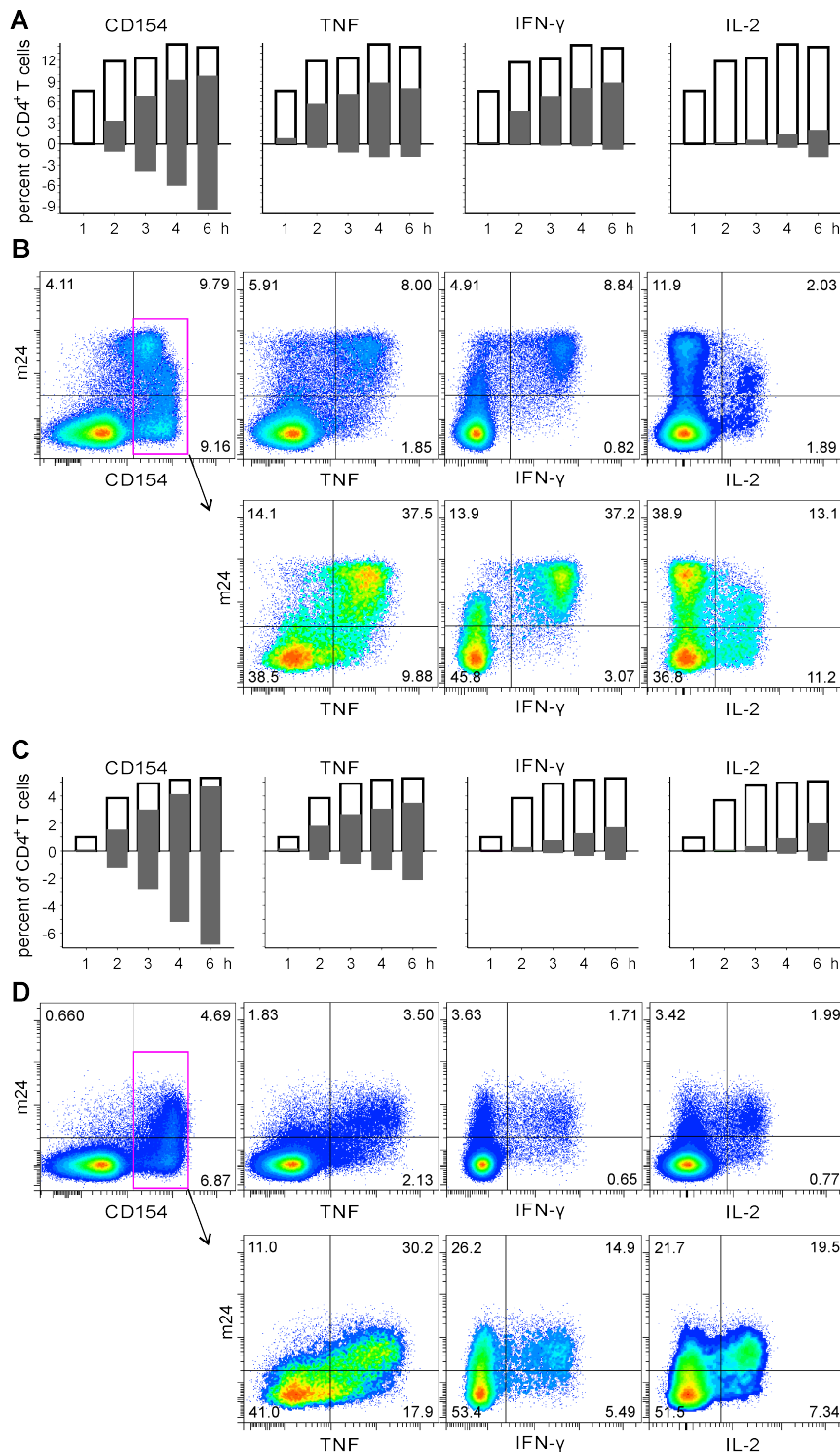
9.2 Supplementary data Part II



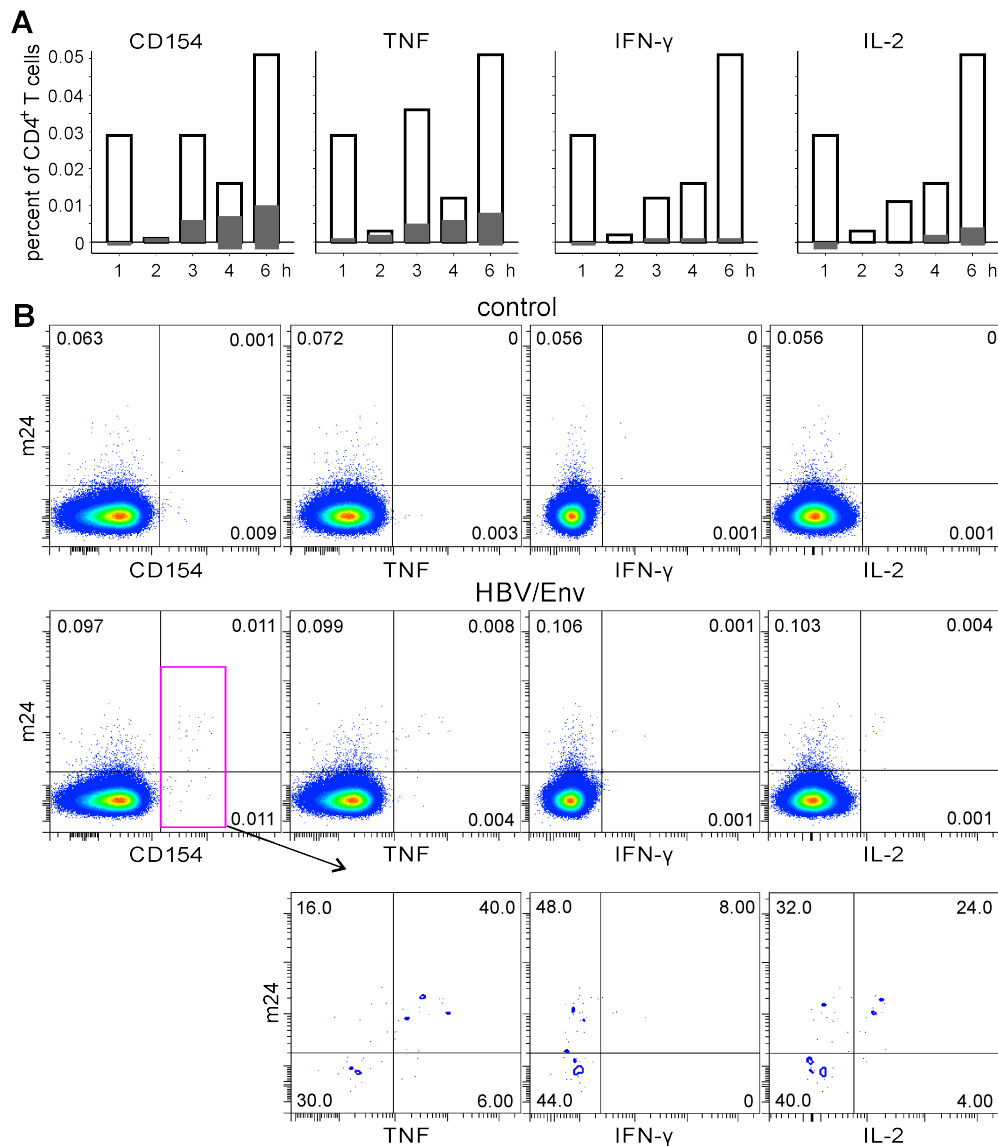
Supplementary Figure 1: Detection of TNF⁺ CD3⁺ CD4⁺ WB T cells within the lymphocyte and singlet gates in relation to EDTA treatment. The figure shows results obtained for mICAM-1/m24 Ab stained samples and analysis of basic parameters FSC and SSC. (A and E) Gating strategy. From left to right, the gating of CD4⁺ events and of CD3⁺ T cells. (B-D and F-H) WB cells were cultured without stimulus (B, F), with the CMV/HPT peptide (C, G) or with SEB (D, H). TNF⁺ cells within the CD4⁺ T cells were defined (left, pink frame, frequencies are indicated) and their distribution on FSC-A/SSC-A plots (middle) or FSC-A/FSC-H plots (right) without (top) or with EDTA treatment (bottom) after the staining with mICAM-1 (B-D) or m24 Ab (F-H) was assessed. Numbers on the FSC-A/SSC-A and FSC-A/FSC-H plots indicate the percentages of TNF⁺ CD4⁺ T cells falling within the gates (pink). Note that results are partially shown in Fig.1, but here the FSC-A/SSC-A lymphocyte gate and the FSC-A/FSC-H duplet exclusion are not included in the gating strategy. Without lymphocyte pregating, a population of m24⁺ TNF^{neg} cells is visible also in the unstimulated control. These are very large events containing probably aggregated T cells and monocytes (the latter binding to m24 Ab after stimulation).



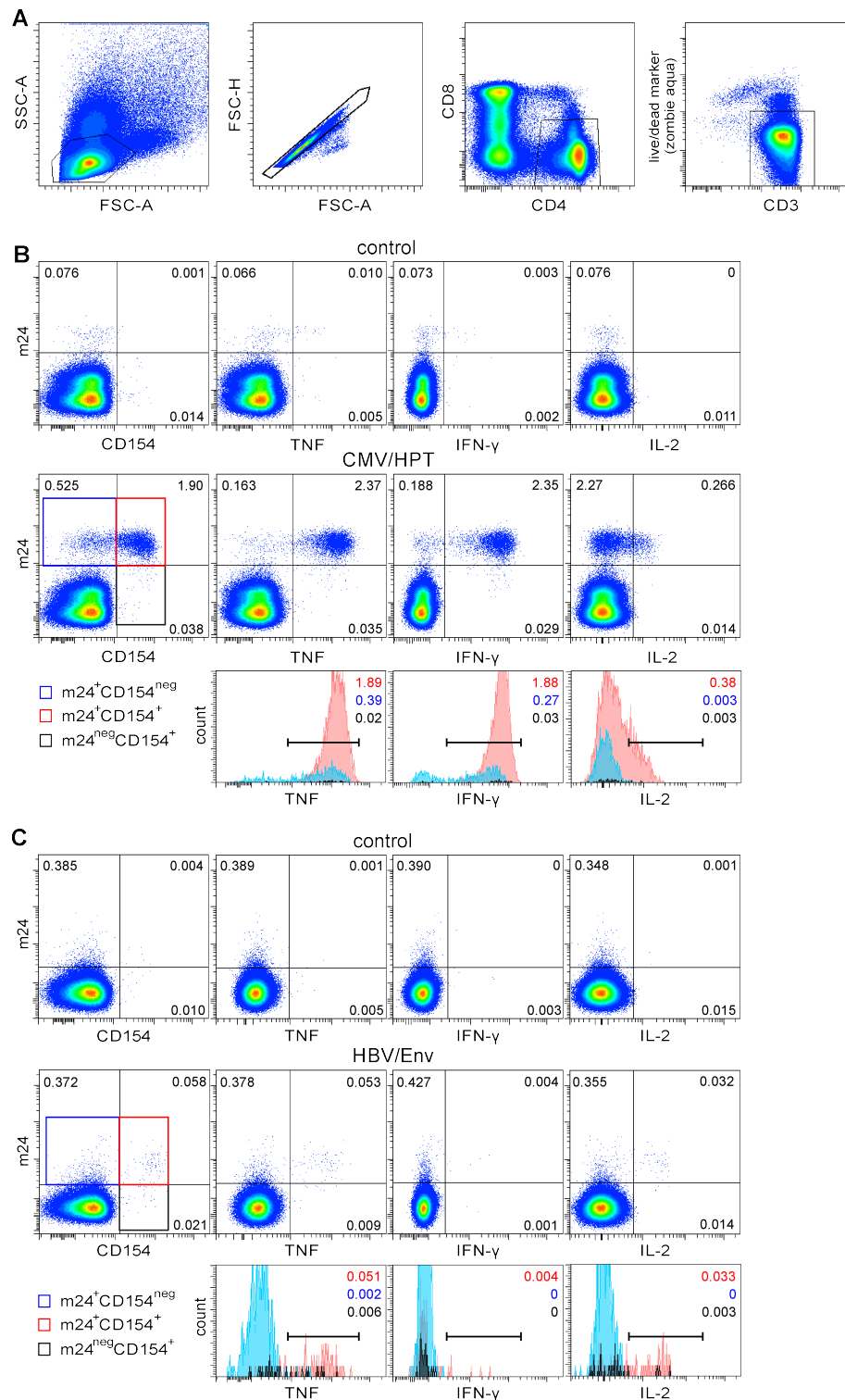
Supplementary Figure 2: Detection of HLA-A*02 restricted, CMV/NLV-specific CD8⁺ WB T cells (costained with CD3, CD4, CD8 Abs, and mICAM-1 or m24 Ab) within the lymphocyte singlet cell gate does not depend on the EDTA treatment. (A) Gating strategy. From left to right: the gating of CD8⁺ events and tetramer A*02/NLV⁺ CD3⁺ T cells. (B, C) β_2 -integrin activation on A*02/NLV⁺ cells (left) and distribution within FSC-A/SSC-A lymphocyte plots (middle) or FSC-A/FSC-H singlet plots (right), without (top) or with EDTA treatment (bottom) after the staining with mICAM-1 (B) or m24 Ab (C). Numbers indicate the percentages of A*02/NLV⁺ CD8⁺ T cells falling within the gates (pink). Note that results are partially shown in Fig.2, but here the FSC-A/SSC-A lymphocyte gate and the FSC-A/FSC-H duplet exclusion are not included in the gating strategy.



Supplementary Figure 3: Functional profile of SEB-stimulated m24⁺ CD4⁺ WB T cells. m24 Ab staining for the two donors DRB1*11⁺ and HBV-vaccinee shown in Figs. 5 and 6, respectively. (A, C) The results of the SEB stimulations are shown as graphs (at the indicated times and after subtraction of the background assessed in the control test; frequencies within CD4⁺ T cells are shown); empty bars represent total m24⁺ cells, positive gray bars represent marker⁺ (CD154, TNF, IFN- γ , or IL-2) m24⁺ CD4⁺ T cells, while negative gray bars marker⁺ m24^{neg} CD4⁺ T cells. (B, D) m24 Ab staining after 6 h stimulation (top panels). CD154⁺ m24^{neg} and m24⁺ subsets (pink frame) are further displayed according to TNF, IFN- γ , and IL-2 expression (bottom). Numbers on density plots indicate frequencies among total or CD154⁺ CD4⁺ T cells. Gating strategy is as in Fig. 1A, and control stains are shown in Figs. 5 and 6.



Supplementary Figure 4: Functional profile of HBV-specific, m24⁺CD4⁺ WB T cells from a second vaccinee. Cell stimulation was performed with HBV/Env overlapping peptides. (A) Results are shown as graphs (at the indicated times and after subtraction of the background assessed in the control test; frequencies within CD4⁺ T cells are shown); empty bars represent total m24⁺ cells, positive gray bars represent marker⁺ (CD154, TNF, IFN- γ , or IL-2) m24⁺ CD4⁺ T cells, while negative gray bars marker⁺ m24^{neg} CD4⁺ T cells. (B) m24 Ab staining after 6 h without stimulation (top) or in the presence of HBV/Env peptides (middle). CD154⁺ m24^{neg} and m24⁺ subsets (pink frame) are further displayed according to TNF, IFN- γ , and IL-2 expression (bottom). Numbers on the density plots indicate frequencies among total or CD154⁺ CD4⁺ T cells. Gating strategy was as in Fig. 1A.



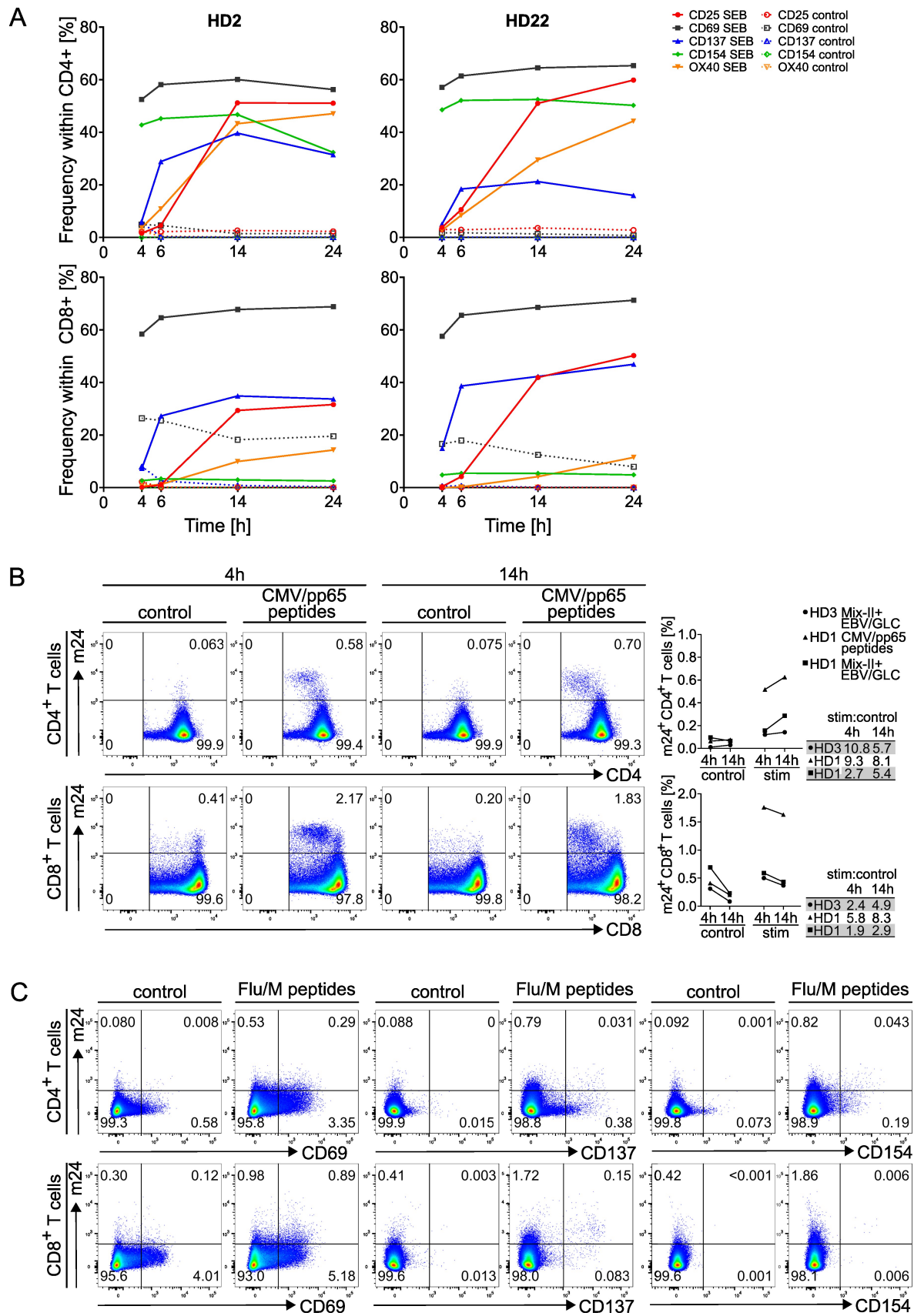
Supplementary Figure 5: Assessment of CMV- and HBV-specific CD4⁺ T cells in frozen/thawed PBMCs. Donors are the same as in Figs. 5 and 6, respectively. (A) Gating strategy. From left to right, the lymphocyte gate, the FSC-A/H duplet exclusion, the gating of CD4⁺, and of living CD3⁺ T cells. (B, C) m24 Ab staining in combination with CD154, TNF, IFN- γ or IL-2 expression (from left to right) after 6 h stimulation of 2×10^6 PBMCs (in 1 ml TCM) without (control), with CMV/HPT (B) or with HBV/Env peptide pool (C). m24⁺CD154^{neg}, m24⁺CD154⁺ and m24^{neg}CD154⁺ subsets were gated (blue, red and black frames, respectively) and further displayed as histograms according to TNF, IFN- γ and IL-2 expression. Frequencies among CD4⁺ T cells are indicated and color-coded on the histograms (markers are shown).

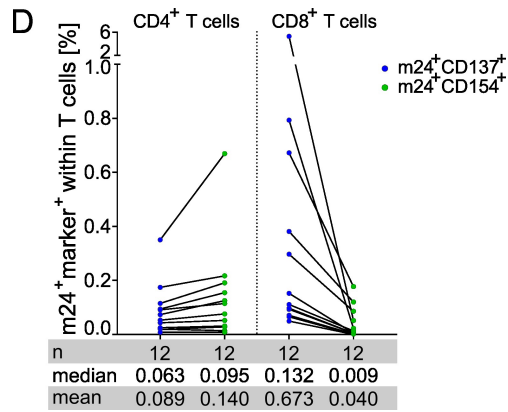
9.3 Supplementary data Part III

Supplementary Table 1 Donor ID, sex and age, collection timepoint, stimulus and corresponding figures in which the donors are presented.

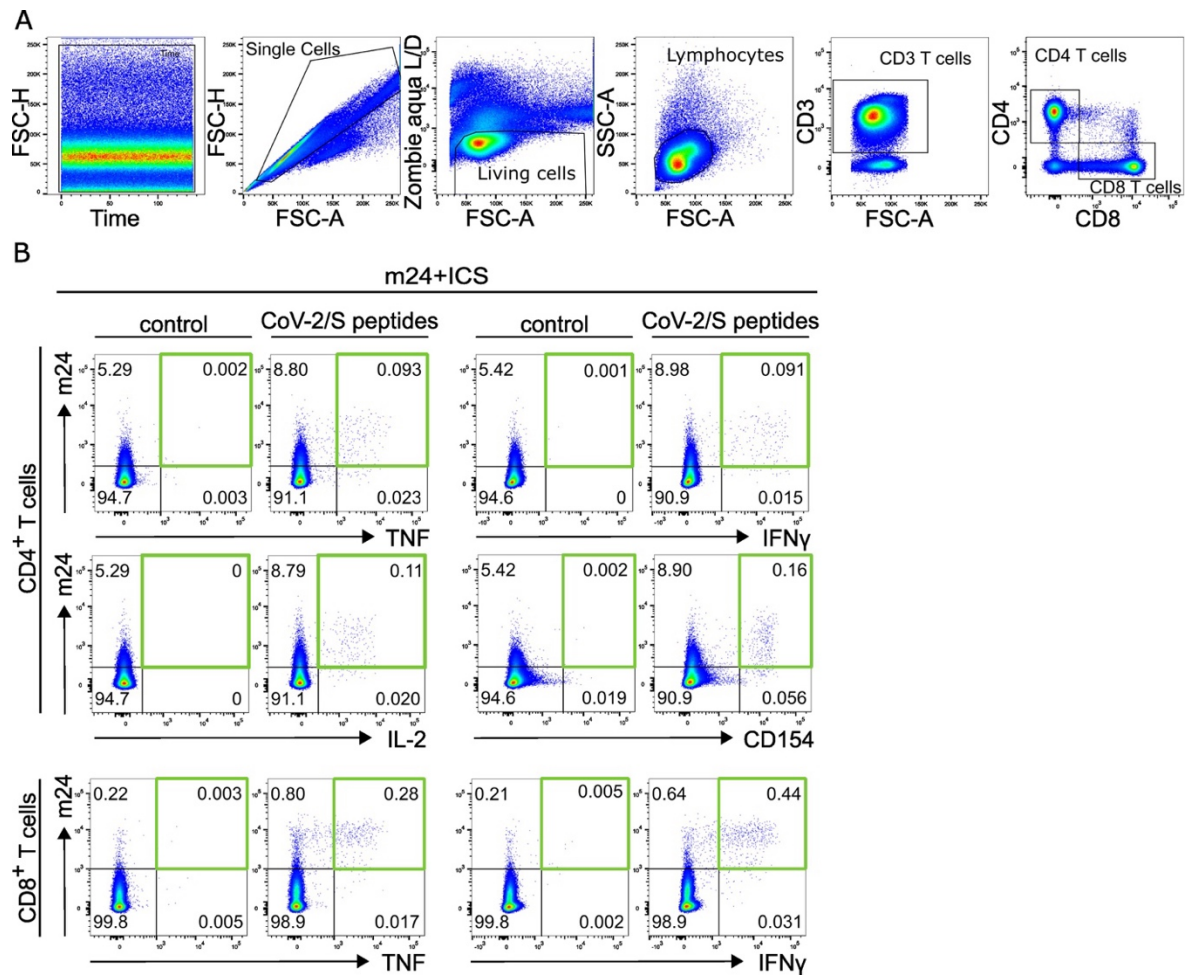
Donor	Sex/ Age	Collection timepoint	Stimulus	Corresponding figures
HD1 (A*02 ⁺)	m/45	Apr 2021	Mix-II+EBV/GLC, CMV/pp65 peptides	S1B
HD2 (A*02 ⁺)	m/55	Sep 2022	SEB	S1A
HD3 (A*02 ⁺)	m/47	Oct 2020	Mix-II+EBV/GLC	S1B
HD4 (A*02 ⁺)	m/34	Sep 2021	Flu/M peptides, HIV I+II	S1C, 3B
HD5	f/29	Jan 2022	CoV-2/S peptides, Flu/M peptides	S1D, 1B, 1C, 3A, 4A, 4B, S2A, S2B, S3, 5A
HD6	f/29	Jan 2022	CoV-2/S peptides, Flu/M peptides	S1D, 1C, 2A, 2B, 3A, 4A, 4B, S3, 5A
HD7	f/54	Dec 2021	CoV-2/S peptides, Flu/M peptides	S1D, 1C, 3A, 4A, 4B, S3, 5A
HD8	f/27	Dec 2021	CoV-2/S peptides, Flu/M peptides	S1D, 1C, 3A, 4A, 4B, S3, 5A
HD9	f/26	Jan 2022	CoV-2/S peptides, Flu/M peptides	S1D, 1C, 3A, 4A, 4B, S3, 5A
HD10	f/22	Jan 2022	CoV-2/S peptides, Flu/M peptides	S1D, 1C, 3A, 4A, 4B, S3, 5A
HD11 (A*02 ⁺)	m/65	Nov 2021	HIV I+II, CMV/pp65 peptides	3B, S4
HD12 (A*02 ⁺)	m/57	Jul 2020	HIV I+II	3B
HD13 (A*02 ⁻)	m/50	Jul 2020	HIV I+II	3B
HD14 (A*02 ⁻)	m/54	Jul 2020	HIV I+II	3B
HD7	f/54	Jan 2020	CoV-2/S peptides	5B
HD15 (A*02 ⁺)	m/29	Sep 2019	CoV-2/S peptides	5B
HD16 (A*02 ⁺)	m/52	Oct 2019	CoV-2/S peptides	5B
HD17 (A*02 ⁺)	m/49	Oct 2019	CoV-2/S peptides	5B
HD18 (A*02 ⁺)	m/28	Oct 2019	CoV-2/S peptides	5B
HD19 (A*02 ⁺)	unknown	Jan 2021	CMV/pp65 peptides	S4
HD20 (A*02 ⁺)	f/25	May 2021	CMV/pp65 peptides	S4
HD21 (A*02 ⁻)	m/38	Sep 2022	SEB	S1A

HD: healthy donor; m/f: male/female; SEB: Staphylococcus enterotoxin B; Mix-II+EBV/GLC: HLA-class II epitopes and GLCTLVAML HLA-A*0201 epitope from EBV BMLF1; CMV/pp65: overlapping peptides from the CMV pp65 protein; Flu/M: overlapping peptides from the Influenza matrix protein; CoV-2/S: overlapping peptides from the SARS-CoV-2 spike protein. HIV I+II: mix of ILKEPVHGV HLA-A*0201 epitope from the HIV reverse transcriptase (RT) protein and YVDRFYKTLRAEQSQEV HLA-class II epitope from HIV group-specific antigen (Gag) protein.



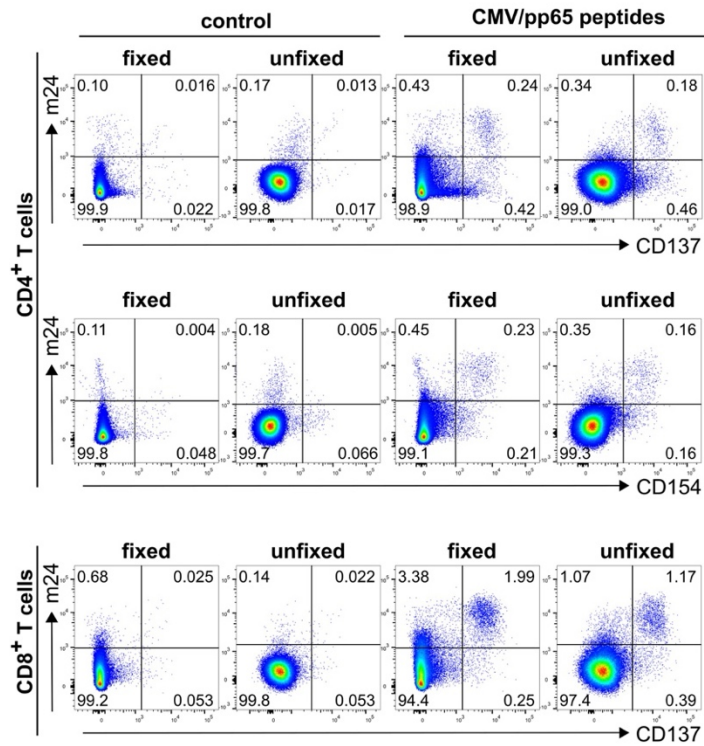


Supplementary Figure 1. Activated β_2 -integrins are detectable after 14 h of stimulation allowing co-staining with cell surface activation induced markers (AIMs) CD137 and CD154. (A) Time course of CD25 (red), CD69 (black), CD137 (blue), CD154 (green) and OX40 (orange) expression on CD4⁺ (top) and CD8⁺ (bottom) T cells after 4, 6, 14 and 24 h of incubation with SEB (solid lines) and on unstimulated PBMCs (dotted line) for two donors (HD2 and HD21). (B) Detection of activated β_2 -integrins with m24 Ab staining after 4 vs 14 h of peptide stimulation on CD4⁺ (top) and CD8⁺ (bottom) T cells. Exemplary dot plots of donor HD1 after incubation with control or CMV/pp65 overlapping peptides for 4 h (left) vs 14 h (middle). Frequencies of marker⁺ cells and stimulated:control (stim:control) ratios for n=3 donor/antigen combinations are shown (right). (C) Dot plots of a representative donor (HD4) for combination of m24 Ab staining with detection of CD69 (left), CD137 (middle) and CD154 (right) for CD4⁺ (top) and CD8⁺ (bottom) T cells after 14 h of incubation with control or Flu/M overlapping peptides. (D) Comparison of m24⁺CD137⁺ and m24⁺CD154⁺ detection on CD4⁺ (left) and CD8⁺ (right) T cells after 14 h of peptide stimulation with mean and median of detected frequencies. n=12 donor/antigen combinations are included. Frequencies of stimulated samples are background subtracted (A, B and D).

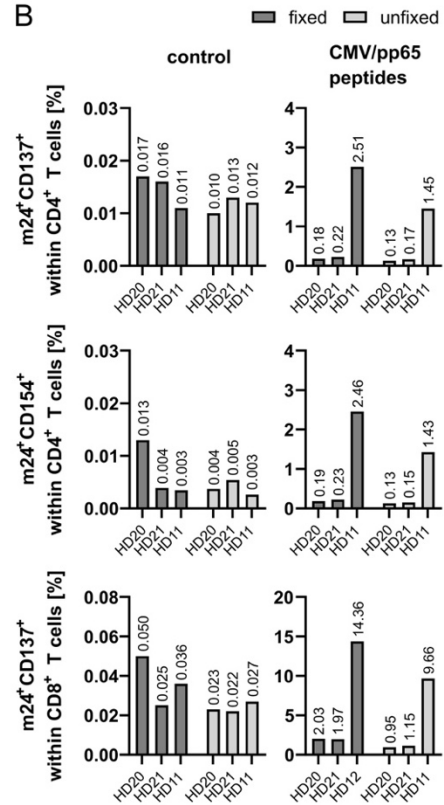


Supplementary Figure 2. (A) Main gating strategy. From left to right: time gate, FSC-A/FSC-H duplet exclusion (single cell gate), Zombie aqua/ FSC-A dead cell exclusion (living cell gate), SSC-A/FSC-A lymphocytes gate, CD3⁺ T cells, followed by gating for CD4⁺ and CD8⁺ T cells. (B) Combined gating of m24 Ab staining with intracellular TNF, IFN γ , IL-2 and CD154 expression (m24+ICS) for CD4⁺ (top), and intracellular TNF and IFN γ expression for CD8⁺ (bottom) T cells of donor HD5 stimulated with SARS-CoV-2/S overlapping peptides or left unstimulated. Numbers represent frequencies within CD4⁺ or CD8⁺ T cells. The green squares mark the populations of interest. The corresponding m24+AIM staining of donor HD5 is shown in Figure 1B.

A



B



Supplementary Figure 4. Staining of activated β_2 -integrins in combination with CD137 or CD154 detection (m24+AIM) is feasible without cell fixation. (A) Dot plots of a representative donor (HD20) for combination of m24 Ab with CD137 and CD154 for CD4⁺ (top) or m24 Ab with CD137 for CD8⁺ (bottom) T cells after 14h incubation with control or CMV/pp65 overlapping peptides. Cells were either fixed with FACS-Lysing solution or left unfixed after the staining procedure. Without fixation, cells were centrifuged once after staining, resuspended in FACS buffer and immediately acquired at the flow cytometer. Fixation was performed as described in the section 2.8. (B) Frequencies of m24⁺CD137⁺ and m24⁺CD154⁺ within CD4⁺ T cells (top and middle) and m24⁺CD137⁺ within CD8⁺ T cells (bottom) of unstimulated control samples (left) or after stimulation with CMV/pp65 overlapping peptides (right; frequencies of stimulated samples are background subtracted) are plotted for n=3 donors.