Characterization of T-cell epitopes for antigen-specific immunotherapy concepts targeting myeloproliferative neoplasms and multiple myeloma

Charakterisierung von T-Zell-Epitopen für Antigen-spezifische Immuntherapie-Ansätze gegen myeloproliferative Neoplasien und das Multiple Myelom

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
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Tübingen
2020

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	23.10.2020
Stellvertretender Dekan:	Prof. Dr. József Fortágh
1. Berichterstatter:	Prof. Dr. Stefan Stevanović
2. Berichterstatter:	Prof. Dr. Hans-Georg Rammensee

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The antigenic landscape of multiple myeloma: mass spectrometry (re)defines targets for T-cell-based immunotherapy

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Summary

In the past years, cancer-immunotherapy has gained more and more momentum. To this end, several studies provide evidence that immunological control can contribute to remission, long-term control, or even cure of malignant disease. Apart from unspecific strategies including immune checkpoint inhibitors, advanced antigen-specific approaches hold the promise to target cancer cells more specifically. Particularly T-cell-based strategies are on the advance to restore immune responses and thereby improve the outcome in patients. The prerequisite for such approaches is the selection of feasible targets for anti-cancer T-cell responses. Ideally, such targets are tumor-associated peptides that are frequently and exclusively presented by human leukocyte antigen (HLA) on malignant cells. To identify such antigens, our group implemented a mass spectrometry-based approach with subsequent immunological characterization of the identified peptides. This thesis aims at the further characterization of the identified T-cell epitopes for the development of antigen-specific immunotherapy concepts to target myeloproliferative neoplasms (MPNs) and multiple myeloma (MM).

CHAPTER 1 illustrates the comprehensive mapping of the naturally presented immunopeptidome landscape in chronic myeloid leukemia (CML). We detected spontaneous preexisting T-cell responses against novel, frequently presented, highly immunogenic, CML-associated peptides and induced multifunctional and cytotoxic antigen-specific T cells *de novo* in CML samples. Thus, we validated these antigens as prime targets for T-cell-based immunotherapy. In **CHAPTER 2** we identified novel frequently presented MPN-associated peptides in primary samples of BCR-ABL-negative MPN patients. Alignment of MPN- and acute myeloid leukemia (AML)-exclusive antigens revealed a significant proportion of shared proteins. In consequence, we detected *in vitro* multifunctional memory T cells specific for AML-associated T-cell epitopes in MPN patients. **CHAPTER 3** provides a proof-of-concept study for the natural HLA-dependent presentation of peptides derived from intracellular domains of established tumor-associated surface antigens as potential targets for immunotherapy. We identified the HLA-B*18 ligand P(BCMA)$_{B*18}$ derived from the B-cell maturation antigen on primary MM and chronic lymphocytic leukemia (CLL) samples. P(BCMA)$_{B*18}$ is highly immunogenic as demonstrated by spontaneous BCMA-specific T-cell responses in MM samples. Combination of P(BCMA)$_{B*18}$ with *in vitro* immune checkpoint inhibition in MM samples induced multifunctional cytotoxic T cells *de novo*. P(BCMA)$_{B*18}$ is thus a promising target for immunotherapy and immunomonitoring in B-cell malignancies.

Taken together, we provide a novel category of highly immunogenic antigens for tailored off-the-shelf T-cell-based and combinatorial immunotherapy approaches to target hematological malignancies and to circumvent leukemic transformation and progression of disease.

Zusammenfassung

In den vergangenen Jahren hat die Tumor-Immuntherapie zunehmend an Bedeutung gewonnen. Insbesondere Antigen-spezifische Therapien sind ein vielversprechender Ansatz für eine gezielte Bekämpfung von Krebszellen. Zahlreiche Studien zeigen, dass die Aktivierung des Immunsystems zur Reduktion der Tumorlast, Langzeitkontrolle oder Heilung von Tumorerkrankungen beitragen kann. Insbesondere T-Zell-basierte Strategien können hierzu einen wertvollen Beitrag leisten. Die Grundvoraussetzung hierfür ist die Auswahl geeigneter Antigene, die eine gezielte anti-Tumor T-Zell-Antwort ermöglichen. Diese Antigene sind Tumor-assoziierte Peptide, die idealerweise hochfrequent und exklusiv über humanes Leukozytenantigen (HLA) von malignen Zellen präsentiert werden. Zur Identifizierung solcher Antigene hat unsere Gruppe einen Massenspektrometrie-basierten Ansatz mit anschließender immunologischer Charakterisierung entwickelt. Diese Dissertation befasst sich vorrangig mit der Charakterisierung der identifizierten, HLA-präsentierten Peptide zur Entwicklung Antigen-spezifischer Immuntherapie-Konzepte gegen myeloproliferative Neoplasien (MPNs) und das Multiple Myelom (MM).

KAPITEL 1 beschreibt die umfangreiche Analyse der natürlich präsentierten HLA-Liganden in Primärproben der chronischen myeloischen Leukämie (CML). Gegen die identifizierten, hochfrequent präsentierten, CML-assoziierten Antigene konnten wir spontane Gedächtnis-T-Zell-Antworten sowie *de novo* induzierte, multifunktionale und zytotoxische T-Zellen in Proben von CML Patienten nachweisen. Diese Antigene konnten somit als geeignete Zielstrukturen für T-Zell-basierte Immuntherapien bestätigt werden. In **KAPITEL 2** identifizierten wir in Primärproben BCR-ABL-negativer MPN-Patienten hochfrequente MPN-assoziierte Peptide. Der Vergleich von MPN- mit akute myeloische Leukämie (AML)-exklusiven Antigenen zeigte einen beachtlichen Anteil gemeinsamer Proteine. Hierauf basierend konnten wir multifunktionale Gedächtnis-T-Zellen gegen AML-assoziierte T-Zell-Epitope *in vitro* in MPN Patienten detektieren. **KAPITEL 3** beschreibt natürlich präsentierte Peptide aus intrazellulären Domänen bekannter Tumor-assozierter Oberflächenantigene als mögliche Zielstrukturen für Immuntherapien. Hierzu identifizierten wir in Proben von MM Patienten den HLA-B*18 Liganden P(BCMA)$_{B*18}$ aus dem B-Zell-Reifungsantigen. Die Immunogenität von P(BCMA)$_{B*18}$ konnte durch spontane T-Zell-Antworten in MM Patientenproben bestätigt werden. Des Weiteren induzierte P(BCMA)$_{B*18}$ in Kombination mit Immuncheckpoint-Inhibitoren *in vitro* multifunktionale, zytotoxische T-Zellen in MM Proben. P(BCMA)$_{B*18}$ ist daher ein vielversprechendes Zielantigen für Immuntherapien von B-Zell-Erkrankungen.

Zusammenfassend beschreiben wir neue, immunogene Zielstrukturen für T-Zell-basierte Immuntherapien zur gezielten Bekämpfung hämatologischer Neoplasien und deren Vorstufen.

General introduction

The immune system

The human body is constantly challenged with pathogens but despite this continuous exposure an affliction occurs rarely due to our protective immune system. The immune system is our defense against a variety of disease-causing pathogens and alterations. A functional defense system is the key to health and survival. Due to distinct challenges, the immune system of every species is unique. Therefore, it is crucial to study the human immune system and its defense mechanisms in order to fight pathogens and diseases.[1]

The human immune system can be divided into two major categories: the innate and the adaptive component. The innate immunity is native, reacts immediately against a wide range of infectious agents, and is the first line of defense against pathogens such as bacteria, fungi, or parasites. It includes barriers (physical or chemical), the complement system, chemokines and cytokines acting as inflammation mediators, as well as innate lymphoid cells.[2,3]

The adaptive immune system, on the other hand, is acquired, reacts delayed but specific against a broad range of microbial as well as non-microbial pathogens, and involves development of long-lasting memory for a more rapid and intense secondary response in case of a repeated exposure.[4] The key players of the adaptive immune responses are B and T lymphocytes. B cells mature in the bone marrow and are activated by antigens recognized by the B-cell receptor, which leads to proliferation and differentiation into antibody-secreting plasma cells. In contrast, T cells mature in the thymus and recognize antigen-derived peptides *via* their T-cell receptor (TCR).[5] Such peptides are derived from extracellular or cytoplasmic proteins and are presented on the surface of for example infected cells or antigen-presenting cells (APCs) by the major histocompatibility complex (MHC). The human MHC molecule is referred to as human leukocyte antigen (HLA).

HLA molecules are categorized in two groups - HLA class I and HLA class II - exhibiting distinct structural and functional characteristics. Each individual carries three genes of the major HLA class I (encoding for HLA-A, -B, -C) and HLA class II loci (HLA-DP, -DQ, -DR) in addition to minor HLA class I loci (HLA-E, -F, -G). For each of the genes two different HLA molecules can be expressed. Each HLA allotype binds peptides with a specific sequence

motif characterized by anchor amino acids (aa) in defined positions.[6,7] Whereas the sequence motifs for HLA class I-restricted ligands are predominantly well explored, the binding motifs of HLA class II-restricted peptides remain less characterized due to their promiscuity. HLA class I molecules are expressed by every nucleated cell and present endogenous peptides of 8-12 aa derived from intracellular viral or tumor-antigens, which are processed by the proteasome (Figure 1a).[8–10] These HLA ligands are recognized by peptide-specific TCRs and the binding is stabilized by the co-receptor cluster of differentiation (CD)8, which is expressed on the surface of $CD8^+$ cytotoxic T lymphocytes (CTLs).[5]

HLA class II molecules are expressed on professional APCs - dendritic cells (DCs), macrophages, B cells - following phagocytosis of exogenous proteins and subsequent processing by endolysosomal enzymes (Figure 1b). The resulting peptides with a length of 9-25 aa are loaded to the binding groove of HLA class II molecules by displacing the invariant chain. The HLA-peptide complex is transported to the cell surface, where the peptide is recognized by the TCR of helper T cells (Th) and stabilized by the binding of their CD4 co-receptor to the HLA class II molecules.[9]

An effective immune response depends on the activation of DCs. For a functional activation of DCs, three signals are required: loading of peptides onto HLA molecules, expression of co-stimulatory molecules (CD40, CD80, CD86), and cytokines.[11] DCs located in the periphery (e.g. skin) scan their surroundings for antigens to ingest and process. Upon activation they travel to lymphoid tissues and generate immune responses against this antigen by priming naïve $CD4^+$ T cells and natural killer (NK) cells.[12]

Additionally, professional APCs are also able to prime naïve $CD8^+$ T cells - a special feature termed cross-priming.[13] This process requires cross-presentation: the ability to ingest, process, and present extracellular antigens *via* HLA class I to $CD8^+$ CTLs. Therefore, exogenous antigens are imported into the cytosol, processed by the proteasome or re-imported into the phagosome and loaded on HLA class I.[14] CTL stimulation can be enhanced by activated $CD4^+$ T cells that secrete interleukin-2 (IL-2) and license DCs for cross-priming *via* CD40L-CD40 interaction.[15] Licensed DCs, in turn, downregulate expression of inhibitory molecules such as programmed cell death-ligand 1 (PD-L1) and upregulate CD80, CD86, and other co-stimulators. Signaling *via* toll-like receptors (TLRs) promotes endosomal peptide loading onto HLA class I thereby leading to an increased cross-presentation.[13]

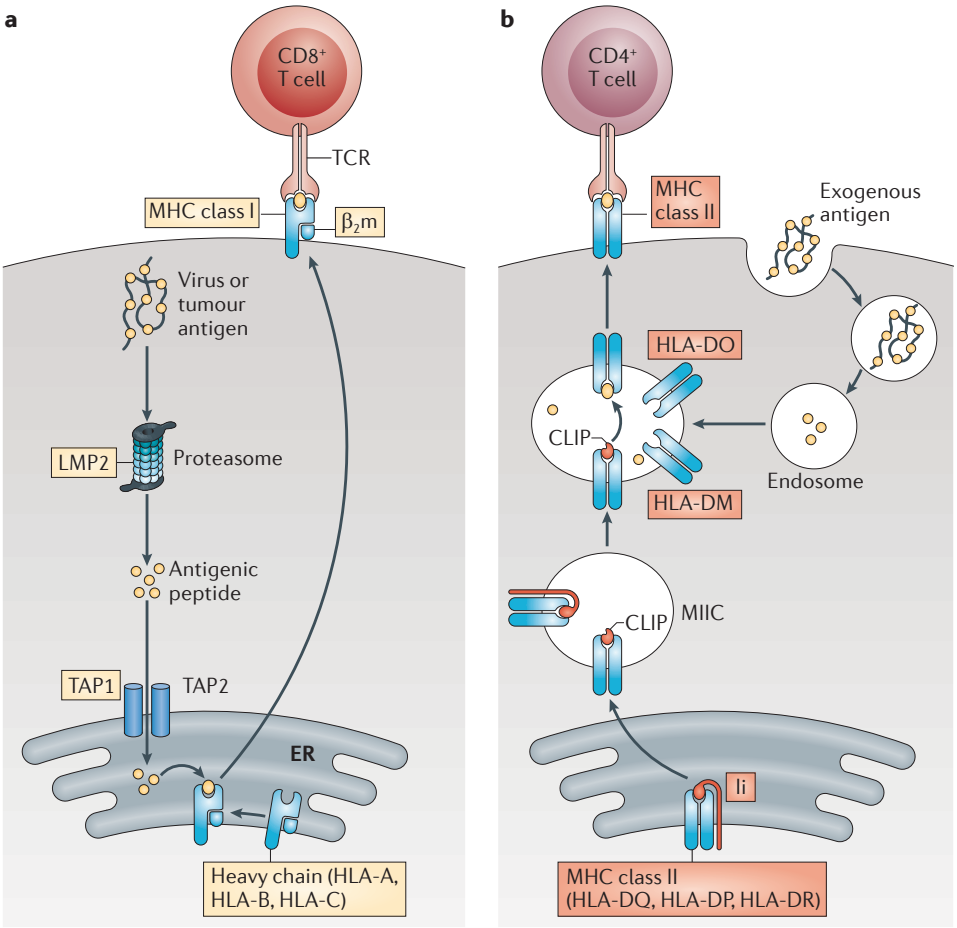

Figure 1: Processing and presentation of HLA class I- and HLA class II-restricted peptides. (a) Intracellular viral or tumor-associated antigens are processed by the proteasome, transported into the endoplasmic reticulum (ER), loaded on HLA class I molecules, and exported to the cell surface. HLA class I-presented peptides are recognized by the TCR of $CD8^+$ T cells. (b) Exogenous antigens are processed by endolysosomal enzymes and the resulting peptides are loaded on HLA class II molecules by displacing the invariant chain. HLA class II-restricted peptides are presented to $CD4^+$ T cells. From[16] under license number 4774811115564.

For an effective priming of naïve T cells the formation of the immunological synapse comprising three main features is required: (i) recognition of an HLA-peptide complex *via* an antigen-specific TCR, (ii) co-stimulation by CD80/CD28 (APC/CTL), and (iii) cytokines directing T-cell differentiation (usually the general T-cell growth factor IL-2, the hematopoietic growth factor IL-7, and IL-12 promoting T-cell growth and activity).[17,18]

Upon activation, T cells proliferate and differentiate into distinct effector phenotypes dedicated to either stimulation, cytotoxicity, or immune regulation. Th cells provide co-stimulatory signals for CTLs, B lymphocytes and NK cells.[19] CTLs kill target cells *via* granzyme B and perforin as well as *via* FasL-induced apoptosis and regulatory T cells (Tregs) are suppressors assigned for immune response control.

Tumor immunology – The immune system against cancer

The first recorded case of cancer is dated approximately 4,600 years back and was documented in the ancient Egypt by the high priest, pharaoh chancellor, and physician Imhotep.[20] Since then, rumors about spontaneous regressions of cancer as well as remissions following acute infections were circulating throughout history.[21,22] In the 19th century the idea to employ and modulate the immune system against neoplastic malignancies has begun to gain momentum when the German physicians Busch and Fehleisen reported their independent observations of tumor regression following bacterial skin infections termed erysipelas. In order to prove the infections being the cause for cancer regress, Wilhelm Busch intentionally infected a cancer patient and observed reduction of tumor mass.[23,24] Fehleisen identified *Streptococcus pyogenes* as causative agent of erysipelas and validated Busch's results.[25]
Almost a decade later William Coley, the "Father of Immunotherapy", reported on the treatment of malignant tumors using heat-inactivated bacteria,[26] known as Coley's toxins, and induced enduring remissions and even cures that were achieved in >1,000 cancer patients suffering mostly from sarcoma.[27] Due to the inconsistency of methods, the irreproducibility of results, and the development of chemo- and radiotherapy, the interest for Coley's toxins faded.[21] Nevertheless, Coley's principles were endorsed by Morales in 1976 who introduced the Bacillus Calmette-Guérin (BCG) for the therapy of superficial bladder tumors.[28] Paul Ehrlich hypothesized from the results that subjects with a defective immune system suffer more frequently from cancer and implied that the immune system is capable of controlling cancer development and progression.[29] Ehrlich's rationale was supported in the 1950s by Macfarlane Burnet and Lewis Thomas and their hypothesis of cancer immunosurveillance.[30-34] The theory suggested that the immune system detects and eliminates the constantly emerging malignant cells that differ from the "self" well before clinical evidence of malignancy occurs.
The cancer immunosurveillance concept was amended by Robert Schreiber and his description of cancer immunoediting.[35,36] Immunoediting describes the shaping of non-eradicated tumor cell immunogenicity by the selection pressure of immunosurveillance and consists of three phases termed elimination, equilibrium, and escape. During the elimination phase the immune system recognizes a malignancy, eliminates the affected cells, and thus returns the tissue to its normal healthy state. If a tumor manages to survive the initial elimination, it re-

mains dormant or enters a dynamical equilibrium phase with the immune system. Thereby the malignancy is experiencing a constant selection pressure from the lymphocytes, which cannot completely obliterate the malignancy but instead give rise to new mutations resulting in resistance and reduced immunogenicity. If the tumor successfully evades the immune control, the malignant cells progress to the escape phase where they can unopposedly proliferate and metastasize. The generally accepted concept of immune evasion represents one important component of Hanahan and Weinberg's updated "Hallmarks of Cancer".[37]

Since the postulations of Busch, Coley, and Ehrlich many efforts have been made unraveling the role of the immune system in the development and progression of cancer. Recent advances include demonstration of profound anti-tumor effects by immune checkpoint blockade,[38] first described by James Allison that is especially effective in malignancies with high mutational burden.[39,40] Other investigations revealed the potential of antigen-specific tumor infiltrating lymphocytes (TILs), which not only keep the tumor growth in check, but also represent a prognostic factor for various cancer types[41–44] and have additionally been associated with response to treatment.[45]

As a result, the past 150 years of research manifest the feasibility for the employment of the immune system against malignant neoplasms and fuel the development of tumor immunotherapy approaches.

Hematological malignancies

In line with Robert Schreiber's concept of immunoediting, most types of hematological malignancies (HMs) are affecting predominantly elderly patients insinuating the decrease of functionality and effectiveness of the ageing immune system along with clonal hematopoiesis.[46]

In contrast to solid tumors, hematological malignancies can be targeted especially effective by immunotherapeutic approaches due to the identical localization of malignant cells and immune cells. Furthermore, the simple acquisition of malignant cells from blood samples makes hematological neoplasms an attractive study object.

Hematological malignancies can affect lymphoid or myeloid cells. Malignancies arising from the lymphoid lineage comprise lymphocytic leukemias (chronic lymphocytic leukemia

(CLL) and acute lymphocytic leukemia (ALL)), lymphomas (Hodgkin's and Non-Hodgkin's lymphoma), and the monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (SMM), and multiple myeloma (MM) sequence.

Disorders affecting the myeloid cell compartment include the myeloid leukemias (chronic myeloid leukemia (CML) and acute myeloid leukemia (AML)), myelodysplastic syndrome (MDS), essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). The myeloproliferative neoplasms (MPNs) can be further classified into Philadelphia chromosome-positive (Ph+) CML and Philadelphia chromosome-negative (Ph-) ET, PV, and PMF. The latter represent precursor conditions that might progress to post-ET-MF (PET-MF) or post-PV-MF (PPV-MF) and further to AML.

Chronic myeloid leukemia

CML is a clonal myeloproliferative disorder of hematopoietic stem cells (HSCs) affecting elderly individuals with a median age of 65 years.[47] CML occurs at an annual incidence rate of 1/100,000 with the accumulation of granulocytes and their precursors. Non-specific symptoms include fatigue or weight loss.[48,49] CML is usually diagnosed in the chronic phase and can progress to an accelerated phase and ultimately a blast crisis, which can present in a myelogenous or lymphocytic form.[50,51]

The main characteristic of CML is the first described tumor-specific chromosomal aberration identified 1960 by Nowell and Hungerford in Philadelphia (USA) and is being referred to as the Philadelphia chromosome (Ph) ever since.[52] It results from a reciprocal translocation of the chromosome 9 encoding the proto-oncogene Abelson leukemia virus (ABL) tyrosine kinase and chromosome 22 containing the breakpoint cluster region (BCR) housekeeping gene.[53] The t(9;22) translocation leads to the juxtaposition of BCR and ABL and consequentially to the BCR-ABL fusion transcript. The encoded tyrosine kinase is constitutively activated and favors clonal expansion of myeloid cells by engaging signaling pathways such as MAPK, STAT5, and MYC.[54] Ph can be ascertained in 95% of CML patients in every hematopoietic cell, even in lymphocytes.[55]

Primary treatment options were based on busulfan and later hydroxyurea (HU). In the 1990s, Interferon-alpha (IFN-α) was introduced as gold standard and persisted for ten years until the introduction of tyrosine kinase inhibitors (TKIs) targeting the BCR-ABL fusion protein. TKIs

revolutionized therapy of CML patients allowing for the achievement of deep molecular remissions with the disease not being detectable at molecular level. In case of TKI resistance and repeated TKI failure, an allogeneic stem cell transplantation (alloSCT) is recommended as a potentially curative option.[56,57] However, the number of patients enduring alloSCT has decreased significantly since the introduction of TKIs.[49]

To date, five TKIs have been approved for the treatment of CML: the 1st generation Imatinib, the 2nd generation Nilotinib, Dasatinib, and Bosutinib, as well as the 3rd generation Ponatinib.[55] TKIs are competitive small molecule inhibitors, which are blocking the binding site for adenosine triphosphate (ATP) on the BCR-ABL tyrosine kinase. ATP is necessary to provide phosphate, which is required for phosphorylation of adapter proteins and thus for the activation of downstream signaling cascades in order to propagate proliferation. Unfortunately, cells bearing the BCR-ABL transcript are susceptible for further cytogenetic aberrations due to their genomic instability.[58] This in turn is promoting TKI resistance either due to clonal evolution of additional genomic aberrations or in a BCR-ABL-dependent manner *via* point mutations in the kinase domain region.[49]

Another downside of TKIs might be the repeatedly postulated and controversially discussed dysregulating effects on immune responses.[59–62] This argument is not settled yet since a variety of publications are claiming to prove a link between autoimmune phenomena and TKI treatment.[63–66] Elimination of Tregs by TKIs, for example, might improve anti-cancer immunity but can also be accompanied by autoimmune adverse effects.[67,68]

Potential negative effects of TKIs are not the only explanation for immunosuppression in CML. The impaired immune system might also be due to a general immunosuppressive state resulting from HLA-G, elevated numbers of myeloid-derived suppressor cells (MDSCs) and Tregs, or increased PD-1 expression.[68,69] The checkpoint molecule HLA-G is a strong ligand for inhibitory receptors on the surface of immune cells and capable of obstructing their effector function.[70,71] HLA-G is thus associated with poor outcome in CML patients.[72] Together with other inhibitory molecules HLA-G can be acquired by both, primarily activated CD4$^+$ and CD8$^+$ T cells from APCs and thus lead to a hyporesponsive immune phenotype with regulatory properties.[73,74] Furthermore, mesenchymal CML stem cells orchestrate activation of MDSCs,[75] which particularly suppress T-cell activation and proliferation.[76] The immunosuppression is further evident from decreased and defective NK-cell and CTL re-

sponses along with enhanced PD-1 expression on T cells of patients under TKI treatment prior to molecular response (MR) as well as at the time of diagnosis.[68,69]

Since TKIs are not restricted to the BCR-ABL kinase, they can also bind other kinases or growth factor receptors.[49,54] The off-target binding can not only result in hematological effects such as anemia, but also in non-hematological adverse effects such as nausea, diarrhea, and vomiting thereby decreasing the quality of life for CML patients.[77] Thus, various TKI stop studies investigate long-term effects of therapy discontinuation.[78] Although many patients desire the cessation of TKIs, the discontinuation is recommended only within controlled trials with the prerequisite of complete molecular remission and very deep MR.[49] An enduring benefit could be observed only in a subset of patients with 40-50% achieving treatment-free remission (TFR) upon TKI discontinuation.[79,80] In order to ensure a durable therapeutical effect along with a good quality of life, new approaches are needed to endorse the positive impact of TKIs. One approach relies upon the evidence of the immune system being capable to control the disease and promote achievement of deep MR in CML patients receiving TKIs.[81–83] In conclusion, reinforcement of anti-cancer immunity might enable long-term TKI-free survival or even cure for CML patients.

Immunotherapy, such as the peptide-based vaccination, represents approaches with few adverse effects and good targeting capability that might allow long-term control of CML. Such strategies require the knowledge of target structures for anti-cancer immune responses. One such target structure might be BCR-ABL: the most abundant property of CML. The junctional regions of BCR-ABL harbor a unique and strictly CML-specific protein sequence,[84,85] which can result in natural presentation of immunogenic peptides *via* HLA.[86–91] Nonetheless, the majority of DNA mutations do not result in naturally presented neoepitopes.[92,93] Further attractive targets for immunotherapy might be antigens that are overrepresented or expressed selectively in CML. Representatives for such antigens are leukemia-associated antigens (LAAs) such as WT1, RHAMM, PR3, hTERT, or Survivin,[94] and cancer/testis antigens (CTAs), for example MAGE, PRAME, and NY-ESO.[95–97]

Philadelphia chromosome-negative myeloproliferative neoplasms

Classical Ph- MPNs include the clonal disorders PV, ET, and PMF arising from pluripotent HSCs and progenitors with a worldwide annual incidence of 0.4-5.9/100,000.[98–102] MPNs

can be classified by their mutational landscape of recurrent somatic alterations. The mutations (Figure 2) can be detected in all mature myeloid cells as well as in HSCs and progenitor cells and typically harbor alterations in either of the three proteins: Janus kinase 2 (JAK2), calreticulin (CALR), or myeloproliferative leukemia (MPL).[103–107]

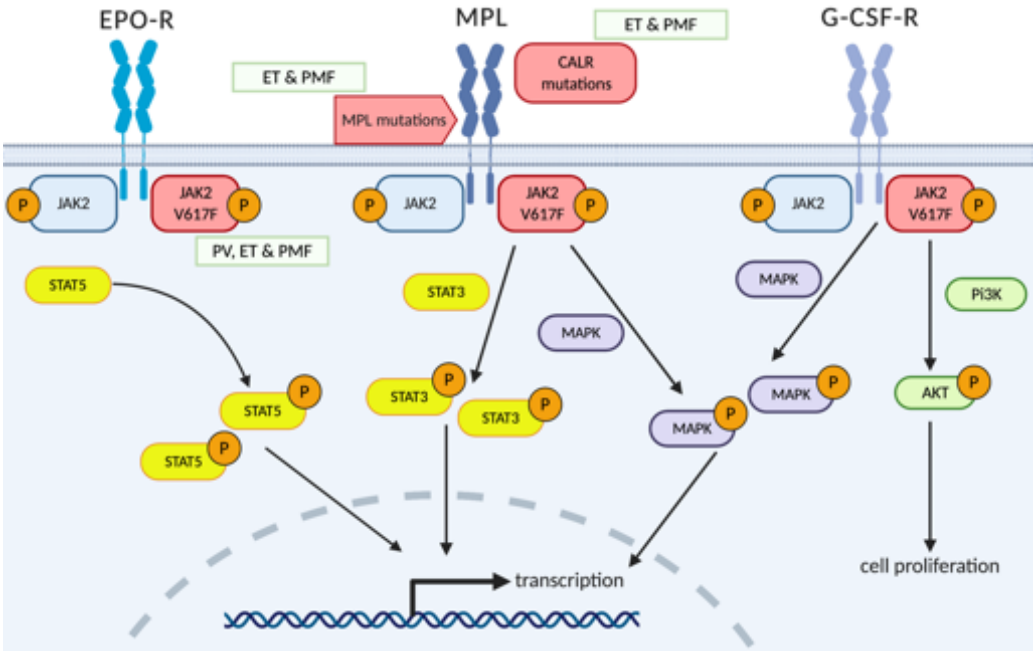

Figure 2: Mutations of CALR, MPL, and JAK2 involved in MPN pathogenesis affecting different pathways. Mutation of JAK2 leads to a constitutive activation of the receptors for erythropoietin (EPO), thrombopoietin (TPO), or granulocyte colony-stimulating factor (G-CSF) thereby potentially triggering genesis of PV, ET, or PMF *via* signal transducer and activator of transcription (STAT) activation and enhanced gene transcription. Mutations of the TPO receptor MPL similarly lead up to activation of JAK-STAT and MAP kinase pathways. Mutant CALR can bind MPL and thereby promote ET or PMF. Created with BioRender and adapted from[108].

The adapter protein JAK2 is responsible for intracellular signal transduction of EPO, TPO, and G-CSF receptors. Originally termed "just another kinase" the group of proteins was eventually renamed after the Roman god of gates and passages *Janus*, who could look in two directions at once.[109] The main occurring JAK exon 9 mutation is the substitution of valine (V) by phenylalanine (F) in position 617, which is the pseudokinase autoinhibitory domain.[110–116] The mutation results in hypersensitivity and activation of the downstream signaling cascades of STAT, MAPK (RAS/RAF/MEK/ERK), and PI3K/AKT/mTOR pathways. The constitutive activation of JAK-STAT pathways is common to all MPNs regardless of the underlying driver mutation.[106,108,117] The STAT proteins are activated differently: STAT3 and STAT5 phosphorylation is increased in PV patients, whereas phosphorylation of STAT3

is increased and that of STAT5 is reduced in ET patients. In PMF, phosphorylation of both STAT3 and STAT5 is reduced.[118] Respectively, the downstream signaling of the three receptors involves different STAT molecules: EPO receptor is signaling mainly *via* STAT5; STAT3 is downstream of MPL and G-CSF receptors; STAT1 is part of MPL signaling.[119,120] Mutations in these receptors and the activation of their downstream signaling leads to EPO-, TPO-, or G-CSF-independent proliferation and differentiation of hematopoietic cells.[121,122] The activation of signaling pathways by different receptors provides an explanation for how three distinct entities can harbor the very same driver mutation.[108]

The TPO receptor MPL is a member of the homodimeric type I cytokine receptor family and requires JAK2 for signaling.[106] The most frequently described mutations for MPL occur in exon 10 at position 515, which is located just between the cytoplasmic and the transmembrane domain. At this junction site the tryptophan (W) in position 515 is part of a motif, that is involved in the negative regulation of MPL.[123] Therefore, substitutions of W515 result in a constitutively activated phenotype and TPO-independent signaling and thus in uncontrolled megakaryopoiesis and platelet production.[124]

CALR is not directly involved in cytokine receptor signaling: it is rather a multifunctional protein required as a calcium-binding chaperone for regulation of calcium homeostasis as well as quality control, accurate protein folding, and oligomeric assembly in the endoplasmic reticulum (ER).[125,126] Overall, more than 50 CALR mutations with a shared 34-amino acid consensus sequence have been identified in MPN patients. The two most frequent mutations occur in 80% of patients with a CALR exon 9 mutation: the prognostically more favorable type 1 (deletion of 52 base pairs, 1092_1143del or L367fs*46) and type 2 (insertion of 5 base pairs, 1154_1155insTTGTC or K385fs*47).[127–130] These mutations are likely an early event as they are present in HSCs.[128] Mutated CALR is missing the ER retention signal KDEL, mislocalizes from the ER, and binds to MPL.[131–133] Binding of CALR to MPL (but not to EPO and only weakly to G-CSF receptors) *via* the positively charged C-terminus results in JAK-STAT activation.[134–136] Physiological CALR is not only an ER chaperone but is also part of the peptide loading complex: CALR associates with HLA class I and recruits it to the peptide loading complex.[137] Mutated CALR affects antigen presentation, inhibits recruitment of HLA class I, and decreases expression of HLA molecules on the cell surface by introducing functional derogation to the peptide loading complex.[137,138]

Polycythemia vera is characterized by the clonal expansion and accumulation of erythrocytes and their precursors in the bone marrow and peripheral blood. In some cases increased erythrocytes are accompanied by elevated numbers of leukocytes and platelets.[101] The annual incidence in the European population is 0.4-2.8/100,000 with the age at diagnosis ranging from 65 to 74 years.[102] Symptoms are mainly constitutional such as pain, fever, fatigue, nightly sweats, or weight loss, but also hypertension, erythromelalgia, and vision disorders which lead to a diminished quality of life.[139] The mean overall survival is approximately 14 years and life expectancy, compared to the common population, is reduced especially in patients younger than 50 years.[140,141]

The characteristic JAK2 V617F mutation occurs in 95% of PV patients (Figure 3).[110–116] In less than 3% of PV patients (but never in ET or PMF) JAK2 is mutated in exon 12.[142] Only in rare PV cases (1-2%) JAK2 mutations are absent.

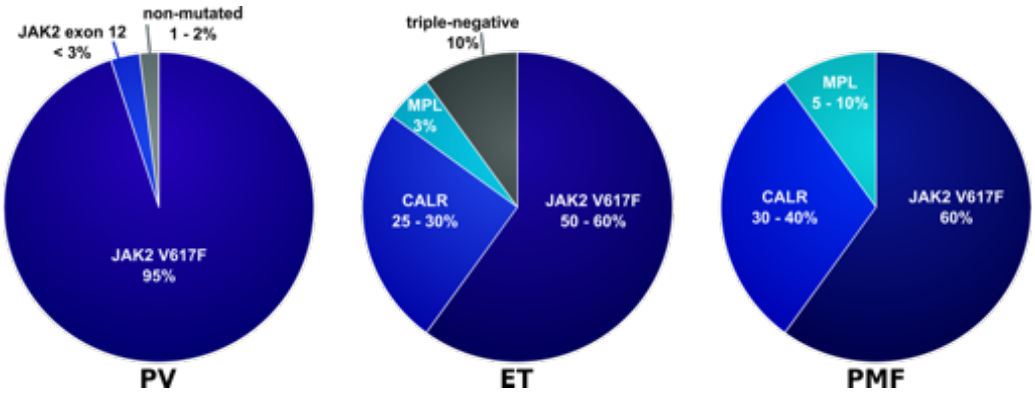

Figure 3: Distribution of the most common driver mutations in MPNs. The three most frequently mutated proteins JAK2, MPL, and CALR and their distribution within the cohorts of PV patients (left), ET patients (middle), or PMF patients (right). Adapted from[106] and[143].

Therapy of PV comprises phlebotomy to reduce the erythrocyte load or cytoreductive measures such as HU and IFN-α. The latter has anti-angiogenic effects, inhibits HSC proliferation and can even reduce V617F allele burden.[144–147] IFN-α-induced molecular remissions as well as hematological responses were observed in a significant proportion of patients.[148,149] Adverse effects are quite severe ranging from flu-like to psychiatric and neurological symptoms. In case of progredient PPV-MF, HU incompatibility or for HU non-responders, Ruxolitinib – a small molecule ATP-mimetic JAK inhibitor – is an approved therapeutic option. Improvement of symptoms and therapeutic efficacy is attributed to the

profound reduction of pro-inflammatory cytokines.[150] The downside of Ruxolitinib is the absent specificity for the V617F point mutation and therefore the unspecific targeting of all JAK proteins. In contrast to alloSCT and IFN-α therapy, the application of Ruxolitinib cannot eliminate the malignant V617F clones. The most common adverse effect is myelosuppression, which is generally manageable by dose adjustment.[151]
Progress to PPV-MF is described as the post-polycythemic phase and occurs in 0.5% of PV patients per year. Progress to the established AML is observed in 2.3% to 5.5% and 7.9% of PV patients over the time of 10 to 15 and 20 years past initial diagnosis, respectively.[152]

Essential thrombocytosis is characterized by an accumulation of platelets in blood and bone marrow and affects elderly people of 64-73 years with an annual incidence of 0.4-1.7/100,000 Europeans.[101,102] The disease is mostly asymptomatic and life expectancy is barely affected in comparison to the general population.[153] In contrast to PV, only 50-60% of ET patients harbor the JAK2 V617F mutation. The remaining 25-30% of ET patients harbor a CALR mutation, approximately 3% have an MPL exon 10 mutation, and 10% of ET patients are triple-negative (Figure 3).[102] Interestingly, CALR-mutated ET patients present with increased platelet and decreased leukocyte counts, whereas JAK2-mutated patients display enhanced leukocyte counts and elevated hemoglobin.[154]
Therapy is indicated for high-risk patients only and comprises HU for older patients, IFN-α, and anagrelide to reduce platelets and inhibit their aggregation. Ruxolitinib is indicated solely for PET-MF patients. Progress to PET-MF occurs at an annual rate of 5% and to AML in 1-2.5% of ET patients.[55]

Myelofibrosis, or osteomyelofibrosis, occurs in its initial phase with a proliferation of megakaryocytes and granulocytes at a European annual incidence rate of 0.1-1/100,000 affecting the elderly with a median age of 69-76 years at diagnosis.[101,102] MF can manifest directly as a primary myelofibrosis or progress from ET or PV as a secondary PET-MF or PPV-MF. MF is characterized by a fibrotic bone marrow (reticulin/collagen) and extra-medullary hematopoiesis in the spleen and liver, resulting in symptomatic anemia and splenomegaly.[102] Other symptoms comprise fatigue, increased peripheral blood blast counts, and teardrop erythrocytes.[55,102] The mutational landscape is covering 60% of MF

patients with JAK2 V617F along with 30-40% of patients harboring CALR mutations (most frequently type 1) and 5-10% with an MPL exon 10 mutation (Figure 3).[108,155]

ET and PV patients can be assigned to a low-risk or a high-risk group according to two criteria: age $\geq$60 years and thrombosis history.[102] The risk group assessment for MF is more intricate according to the criteria of the International Prognostic Scoring System (IPSS),[156] the dynamic IPSS (DIPSS),[157] or the DIPSS plus.[158] The criteria include age ($\geq$65 years), constitutional symptoms, leukocyte and platelet counts, circulating blasts, cytogenetic alterations, and hemoglobin values and enable the classification into low-risk, high-risk, intermediate-1, and -2 groups.

Whereas the median survival for PV and ET patients is 8-10 years and longer,[101] the period for MF patients is considerably shorter. It varies between the respective risk groups: median survival of low-risk patients is 11 years, that of high-risk patients only 2 years.[156]

Therapy of MF is either watch-and-wait for low-risk and intermediate-1 patients or mainly palliative and symptomatic for intermediate-2 and high-risk patients.[159] Palliative therapies comprise HU or prednisone, immunomodulatory drugs (IMiDs) such as lenalidomide and thalidomide, or androgens as response modifiers. Symptomatic therapy includes EPO or prednisone for anemia, HU or IFN-α in combination with ASA can counteract thrombocytosis. For the treatment of splenomegaly HU, IFN-α, splenectomy or Ruxolitinib are an option. Symptom reduction following Ruxolitinib is due to decrease of inflammatory cytokines but the persistent fibrosis suggests the JAK inhibition being insufficient for elimination of the malignant clones.[160] For younger high-risk patients alloSCT is a potentially curative option. MF has the highest risk to progress to AML in 10-20% of patients within 10 years.[152]

Overall, the successful employment and impressive results of unspecific immunotherapy such as alloSCT and IFN-α elucidate the importance of the immune system for an effective therapy directed against premalignant and established hematological neoplasms, which underlines the necessity for more personalized and specific anti-cancer therapy approaches.

Multiple myeloma

Multiple myeloma is a clonal plasma cell disorder that is affecting the lymphoid lineage. The term multiple myeloma was introduced as early as 1873 by J. von Rustizky describing multiple tumorous lesions of the bone marrow (Greek *"muelós"* for marrow).[161] MM is creditable for 10% of hematological cancers and for 1% of all neoplasms.[162] The disease is affecting predominantly elderly people with a median age of 72 years at diagnosis and an annual incidence of 4.5-6/100,000 European individuals.[163] The overall survival for MM patients is 4-10 years.[55] The majority of patients traverse the preliminary stages of MM termed MGUS and asymptomatic (indolent) SMM. The annual progress rate from MGUS to established MM is approximately 1%. The progress rate of the more advanced SMM varies with the time lapsed from initial diagnosis. While in the first 5 years the annual progress averages 10%, the risk drops to 3% for 5-10 years post diagnosis and ultimately to 1.5% thereafter.[164]

Multiple myeloma is characterized by the clonal expansion of plasma cells which are excessively producing monoclonal antibodies (mAbs) or antibody fragments (light chains) leading to an accumulation of monoclonal protein in the blood serum and urine.[162] The aggregation of malignant plasma cells in the bone marrow displaces the resident HSCs and precursors and eventually results in anemia and an impaired immune system. One clinical feature of MM is the destructive osteolytic bone disease in 80% of MM patients that is propagated by suppression of bone-building osteoblasts and stimulation of bone resorbing osteoclasts.[165–167] Bone resorption leads to the release of growth factors, which in turn promote proliferation of myeloma cells.[168]

General standard of care for MM patients comprises chemotherapy, IMiDs, and proteasome inhibitors such as bortezomib or carfilzomib. The only potentially curative option to date is the allogeneic SCT but is available only for a small proportion of patients. For the majority of patients MM remains incurable due to ultimately occurring therapy resistance.

For these patients, immunotherapy might represent an approach that might allow long-term control of MM. Immunotherapy is especially effective in the state of minimal residual disease (MRD) after achievement of remission due to an optimal ratio of T cells *versus* myeloma cells. Moreover, immunotherapy could be a promising approach for patients suffering from early-stage MGUS or SMM since there are currently no beneficial therapeutic options available so far.

The rationale for the employment of immunotherapy in MM is based on the immunogenic potential demonstrated by the graft-*versus*-MM effect after alloSCT.[169] Unspecific immunotherapy is represented by donor lymphocyte infusion (DLI),[170] alloSCT,[171,172] immune checkpoint inhibitors,[173] and IMiDs (Figure 4).[174,175] In addition, antigen-specific immunotherapies such as mono-/bispecific antibodies,[176–179] chimeric antigen receptor (CAR) T cells,[180] TCR-engineered T cells,[181] and DC- or peptide-based vaccines[182–185] are on advance.

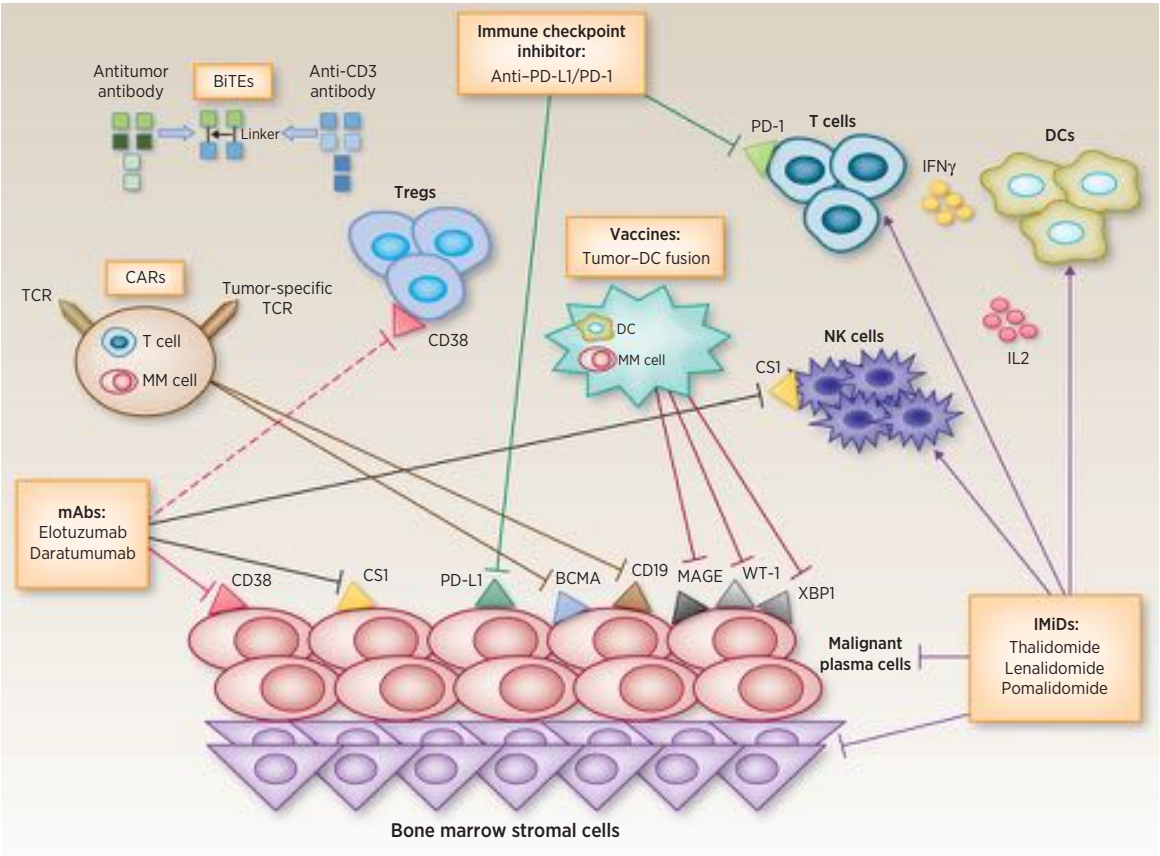

Figure 4: Immunotherapeutic approaches and target antigens in multiple myeloma. Cellular components of the bone marrow microenvironment and the MM patient's immune system together with antigen-specific and -unspecific treatment options including immune checkpoint inhibitors, IMiDs, mAbs, DC-based vaccination, or CAR T cells are illustrated indicating inhibitory or stimulatory effects. Additionally, target antigens for immunotherapy of MM are linked with potential therapeutic options. From[173] under licence number 4774811468272.

Effective antigen-specific immunotherapy requires the knowledge of anti-cancer T-cell target structures. To this end, numerous antigens have been described for MM in the past decades including CD138, CD38, SLAMF7, WT1, DKK1, and many more.[186–189] One particularly promising target is the B-cell maturation antigen (BCMA), which is selectively expressed by cells of the B-lineage including MM cells and therefore is one of the most interesting target structures for immunotherapeutic approaches in MM patients.

Immunotherapeutic approaches against cancer

Since Busch's and Coley's experiments, the field of tumor immunology and immunotherapy has evolved greatly leading to the development of various strategies to utilize the immune system against neoplastic disorders. Immunotherapeutic strategies can be divided in antigen-unspecific and antigen-specific approaches (Figure 5). The latter can be subdivided in HLA-dependent and HLA-independent strategies.

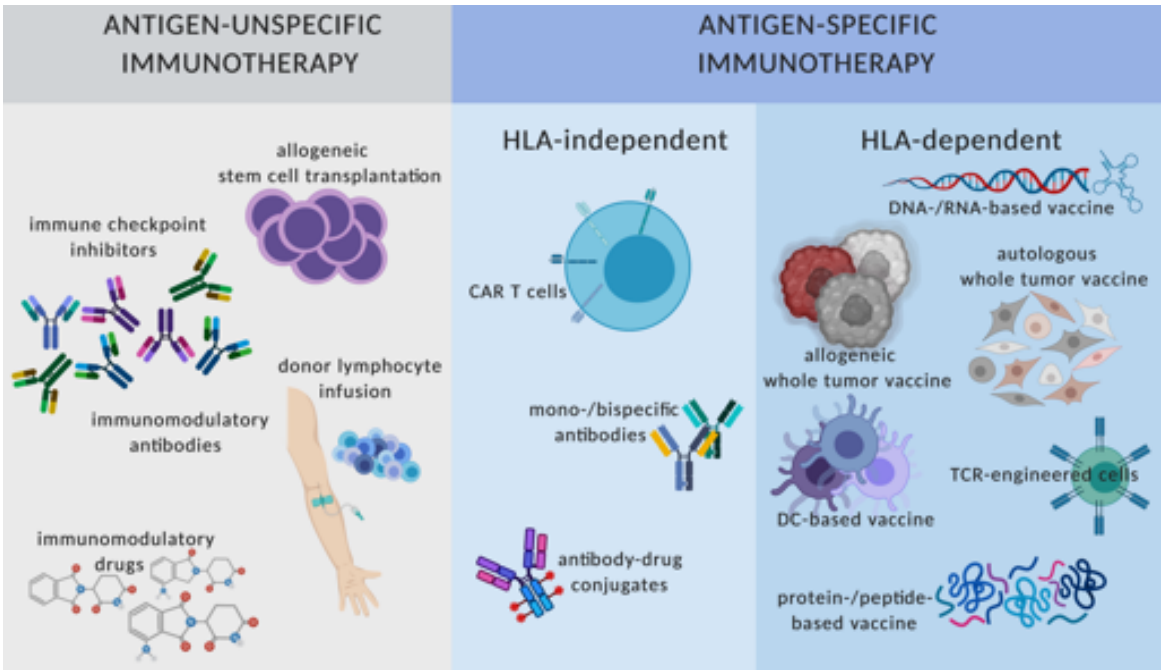

Figure 5: Antigen-specific and -unspecific immunotherapy approaches. Antigen-unspecific immunotherapy includes alloSCT, DLI, IMiDs, immunomodulatory and immune checkpoint blocking antibodies. Mono- and bispecific antibodies, antibody-drug conjugates (ADCs), and CAR T cells represent the antigen-specific HLA-independent strategies. HLA-dependent approaches comprise adoptive transfer of *in vitro* activated, enriched or TCR-transduced T cells, allogeneic and autologous whole tumor vaccines, DC-, DNA-/RNA-, or protein-/peptide-based vaccinations. Created with BioRender.

Unspecific immunotherapy includes alloSCT, DLI, IMiDs, cytokines (IL-2, IFN-α), immune checkpoint blocking and immunomodulatory antibodies, which target either inhibitory and stimulatory co-receptors on the T-cell surface or the corresponding ligands on the surface of APCs.[190–192] The mode of action of alloSCT and DLI is the cytotoxicity of donor lymphocytes against malignant cells in a graft-*versus*-malignancy reaction.[12] On the downside alloSCT is available for few patients only and harbors a substantial risk for morbidity and mortality.[193,194] Properties of IMiDs comprise anti-inflammatory, anti-proliferative, and anti-angiogenic features.[195] Antigen-specific approaches are discussed in more detail in the following sections.

Antigen-specific immunotherapy

Antigen-specific immunotherapy approaches comprise (i) monospecific and bispecific antibodies, (ii) ADCs, (iii) adoptive cell transfer of TCR-engineered T cells, CAR T cells or enriched TILs, or (iv) therapeutic cancer vaccines such as DNA-/RNA- or peptide-based vaccination.[196,197] The targets of such immunotherapy are either tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs). Generally, TAAs are displayed on both, malignant and normal cells and are represented by CTAs or derived from over-expressed antigens and differentiation antigens.[198] In contrast, TSAs are expressed exclusively by malignant cells. An ideal target antigen for immunotherapeutic strategies is one that is expressed frequently and exclusively on malignant cells in a vast majority of patients and that is immunogenic. Furthermore, antigen-specific immunotherapies can be distinguished based on the targeting of antigens either in an HLA-dependent or HLA-independent approach.[199]

HLA-independent immunotherapy includes antibodies, ADCs, and CAR T cells and targets antigens that are expressed preferentially on the surface of malignant cells. In general, antibodies can either block interactions, opsonize the target to mark it for phagocytosis, engage the complement system for cell lysis known as complement-dependent cytotoxicity (CDC), or induce antibody-dependent cellular cytotoxicity (ADCC) *via* binding to Fc receptors located on effector cells.[200] In case of ADCs, conjugated antibodies are internalized by the target cell, where the conjugated drug is activated.[201]

Patient-derived CAR T cells are genetically engineered and display an artificial TCR, which combines the superficial TAA-binding domain of an antibody with intracellular T-cell activating signals.[202] Toxicity and immense production expenses however underline the necessity for improvement of this approach.[203,204]

HLA-dependent immunotherapy includes adoptive transfer of activated or TCR-transduced T cells and DNA-/RNA-, DC-, or peptide-based vaccines. One of the first adoptive cell transfers was conducted using *ex vivo* expanded TILs in combination with IL-2 and revealed responses in approximately 33% of patients suffering from metastatic melanoma.[205] In this context, particularly $CD8^+$ TILs correlated with improved survival.[206] Therefore, adoptive transfer of enriched TILs or TCR-engineered T cells is considered to be one feasible strategy to target malignant cells. Another option to engage the immune system against cancer is the therapeutic vaccination, which is subsequently described in more detail.

Therapeutic anti-cancer vaccines

In contrast to prophylactic vaccines used to protect the healthy individuum, therapeutic cancer vaccines have the purpose to reinforce the immune system of patients to eliminate malignancies.[207] The first types of anti-cancer vaccinations were autologous, patient-derived, irradiated tumor cells from various solid cancer entities combined with immunostimulatory compounds such as BCG.[208] The advantage of autologous whole tumor vaccines lies in the presentation of the complete range of TAAs but is limited to distinct entities due to a minimal required amount of tumor mass for production. Additionally, the whole tumor vaccine can be modified to obtain immunostimulatory properties or to block inhibitory molecules.[209]

Another strategy is the allogeneic whole tumor vaccination using human tumor cell lines. The production is highly standardized without being limited by lack of tumor mass. This strategy was dismissed after failure to achieve efficacy in phase III clinical trials.[210,211]

The generation of TAA-loaded autologous DCs from patient-derived hematopoietic progenitor cells (HPCs) or monocytes from peripheral blood represents one further approach to harness the immune system against neoplastic disorders. Such DC-based vaccines are *ex vivo* maturated cells loaded with antigen and subsequently re-administered together with adjuvants to the patient.[212] The first therapeutic vaccination to gain Food and Drug Administration (FDA) approval was Sipuleucel-T (Provenge) for treatment of prostate cancer patients. The DC-based Sipuleucel-T is produced using prostate-specific antigen-stimulated peripheral blood mononuclear cells (PBMCs) in addition to GM-CSF.[213] Nevertheless, its efficacy remains moderate calling for more optimized and improved vaccines.

One approach for administration of multiple antigens and for simultaneous stimulation of different immune system components is the DNA-/RNA-based vaccination.[214] Here, DNA delivers the genetic blueprint for numerous TAAs and simultaneously acts as a TLR agonist. Therefore, DNA-based vaccines can elicit humoral and cellular immune responses, but on the downside struggled to translate their efficacy into the clinical setting.[215] Autologous messenger RNA derived from a patient's malignant tissues can elicit antigen-specific cytotoxic immune responses with the advantage of instability: due to the rapid clearance of RNA such vaccines are not prone to invoke autoimmune or adverse effects.[216,217]

Since this thesis is focused predominantly on peptide-based immunotherapy, this approach is discussed in more detail in the following section.

Peptide-based vaccination

Peptide-based immunotherapy approaches have gained momentum in the past decades. This is due to many beneficial features. Synthetic peptides - in contrast to recombinant proteins - are free of bacterial or viral contaminants, allow for rapid adjustments, and have no oncogenic properties.[185] Peptide-based vaccination displayed only minimal toxicity if any, revealed little autoimmune and no adverse effects, and did not induce any secondary disorders.[207] This strategy employs the body's very own immune system to target TAAs and TSAs. Since peptides are endogenously present in the human immune system, no foreign substances or chemicals need to be introduced apart from the required adjuvants. Due to the HLA-dependent nature of peptide-based vaccination the targeting of TAAs and TSAs is not limited to surface antigens. In consequence, peptide-based approaches provide more possibilities to target TAAs and broaden the range and variety of antigen targets.[199]

An additional benefit is the "epitope spreading", which is triggered by vaccine-induced tumor-specific CTLs and the subsequent effector-mediated cancer cell lysis that ultimately results in the exposition of a variety of novel antigenic epitopes. These in turn can be ingested, processed, and presented by local APCs and ultimately prime and generate T cells with a broad novel cancer-specific T-cell repertoire.[218,219]

In order to generate an effective and long-lasting anti-cancer immune response, the activation of both, $CD4^+$ Th cells and $CD8^+$ CTLs, is essential.[220–224] Exclusive application of short peptides might result in exogenous loading of these epitopes onto HLA class I molecules that are displayed on every nucleated cell including T cells. Such T cells loaded with short peptides in absence of immunostimulatory adjuvants can migrate to lymph nodes and induce immunological tolerance due to the absence of a co-stimulus.[225–228] Combination of short and long peptides representing $CD8^+$ and $CD4^+$ T-cell epitopes can overcome such tolerance mechanisms. Moreover, conjugation to a single linear hybrid peptide that is presenting both kinds of epitopes might further enhance the efficiency.[228,229]

Previously conducted investigations revealed increased frequencies of tumor-specific T cells directed predominantly against HLA class II-restricted epitopes.[230,231] Thus, $CD4^+$ T cells are considered to be highly important and efficient in the rejection of neoplasms.[19] In fact, T-cell responses assessed by immunomonitoring following peptide-based vaccination revealed strong and early induction of $CD4^+$ peptide-specific cells along with delayed but

weak induction of $CD8^+$ CTLs.[232–236] Additionally, cytotoxic properties of $CD4^+$ T cells are further emphasizing the importance of helper T-cell epitopes in immunotherapy.[237,238]

One of the challenges for peptide-based immunotherapy is immunoediting, which can contribute to tumor progression and relapse.[239] Combination of immunotherapy concepts or simultaneous targeting of diverse antigens might be a solution to overcome loss-of-antigen.[199] Particularly if multiple antigens are employed, the loss-of-antigen might represent a prediction marker for complete regression of immune-responsive tumors, in contrast to an unaffected TAA expression in therapy-resistant lesions.[240,241] Additionally, one has to bear in mind that even if substantial tumor burden reduction or extension of relapse-free survival remain absent, a beneficial effect of anti-cancer vaccinations might still be present in terms of decelerated tumor growth or an overall improved survival.[242]

Strategies to improve peptide-based approaches comprise various combinations with adjuvants, immunomodulators, or immune checkpoint blockade. Adjuvants help the innate compartment required for adaptive immune reactions. Innate immunity is best triggered by TLR agonists, which ultimately are crucial for APC activation.[243] Examples for frequently used TLR agonists are BCG that activates TLR2 and TLR4, the natural TLR4 ligand LPS (bacterial lipopolysaccharide), the TLR7 agonist Imiquimod, the TLR9 targeting Resiquimod, and the novel TLR1/TLR2 agonist XS15.[207,244] Encouraging examples for combinations are provided by clinical trials including a phase III study investigating a peptide-based vaccine combined with IL-2 in patients with advanced melanoma or anti-cancer vaccines in addition to IFN-α therapy.[245–247] Combination of vaccines with checkpoint blocking antibodies can enhance T-cell avidity and thus anti-tumor effects.[248–252] The combination of peptide-vaccines with anti-PD1 for example can amplify T-cell expansion and prolong the impact of the vaccination.[253–255] In this context, Wang *et al.* suggested the “one activation and two inhibitions” concept where one activation (e.g. by antigen) is combined with two inhibitions of immune checkpoints or immunosuppressive cells.[196]

Another adjustment to consider is the ideal timing for an employment of immunotherapy. Due to an optimal ratio of T cells *versus* malignant cells an immunotherapeutic approach is likely most effective against a low tumor burden in the state of MRD.[256]

One major prerequisite for effective immunotherapy is the identification of suitable target antigens. For entities with a low mutational burden a peptide-based approach targeting

(i) non-mutated, (ii) frequently and (iii) naturally presented, (iv) immunogenic, (v) tumor-specific epitopes is considered being most eligible.[257–259] Peptides derived from mutated proteins – so called neoepitopes – are recommended for high mutational burden malignancies and are especially attractive due to their cancer-exclusivity. On the downside, neoepitopes derived from driver mutations are presented with a low abundance on HLA molecules of malignant cells, which seems to be part of immune escape tactics. This is emphasized by different publications stating that from 1,000 mutations only about one neoepitope results in HLA presentation.[92 93 232 260–262]

For the identification of neoepitopes or naturally processed tumor-associated peptides it has to be taken into account that the immunopeptidome is the result of very complex and highly dynamic processes. Moreover, the HLA ligandome does neither mirror the proteome nor the transcriptome, which is emphasized by the absent correlation of HLA-dependent antigen presentation with the gene expression.[263–266] This distorted correlation is likely due to the complexity of immunopeptidome genesis and to the differential protein processing frequently altered in malignant cells.[267–269] The altered antigen processing leads to a significant modification of the displayed peptide repertoire and can ultimately result in reduced presentation of TAA- and TSA-derived epitopes and thus promote immune evasion and tumor progress.[270] Regardless of which kind of peptide is chosen – non-mutated peptides or neoepitopes – the identification and selection of eligible T-cell epitopes is more and more directed towards personalized approaches.[236 259 271 272]

To sum up, T-cell epitopes should be cautiously selected in an entity-specific manner from the naturally presented tumor-associated or -specific repertoire of HLA ligands of the respective tumor. Multiple immunogenic epitopes with HLA class I and class II restriction should be combined with the best suitable adjuvant. Combinations with other immunotherapeutic agents such as immune checkpoint inhibitors, IFN-α, or IMiDs should be considered thoroughly. The vaccination should be administered in the state of MRD due to the optimal ratio of T cells *versus* malignant cells. Non-mutated peptides are potentially more feasible for low mutational burden entities in contrast to neoepitopes, that are suitable for high mutational burden malignancies but need improvement regarding the identification pipelines.

Implications for peptide-based immunotherapy in MPNs

Although TKIs have revolutionized the therapy of CML and greatly improved the clinical outcome of patients, novel strategies might allow enduring long-term control of CML with less adverse effects and better quality of life for a greater proportion of CML patients.
CML is a paragon for neoepitope-based therapy, due to the BCR-ABL driver mutation. Therefore, various efforts have been made to identify and employ BCR-ABL-derived neoepitopes for immunotherapeutic approaches. Several predicted BCR-ABL- and ABL-BCR-derived peptides revealed immune responses *in vitro* as well as clinical responses *in vivo* in clinical trials.[273–275] Possible HLA restrictions of peptides derived from the BCR-ABL junction site suggested protective properties of HLA-A*03, -B*08, and especially their combined expression.[276] Additional studies employing multi-peptide vaccinations reported induction of immune responses, improvement of clinical parameters, as well as MRs and cytogenetic remissions in CML patients. The responses to peptide-based vaccines are even more improved in combination with IFN, TKIs, and IMiDs.[277,278] These results seem to contradict the argument of BCR-ABL being non-immunodominant.[279] In fact, one must consider, that natural processing and presentation on HLA molecules of such driver mutations occurs rarely. Nevertheless, the kinase activity of BCR-ABL can enhance expression of other TAAs and might thereby – despite the lack of HLA-presented BCR-ABL neoepitopes – contribute to a success of vaccination-based approaches.[280,281]

Chronic Ph- neoplasms generally display a driver mutation in either JAK2, MPL, or CALR. Therapeutic options are being investigated including agents inhibiting the telomerase, PI3 kinase, or histone deacetylase as well as anti-fibrotic and hypomethylating agents, however all these approaches are limited by toxicity along with moderate clinical responses.[108,282–284] Novel therapeutic approaches such as peptide vaccination with few side effects and efficient targeting are representing a sought option. The rationale is the immunogenicity of MPNs demonstrated for example by the graft-*versus*-MF effect observed following DLI.[285]
Various peptides have been predicted from JAK2 V617F as well as CALR exon 9 mutations, thereby identifying potential T-cell epitopes.[233,234,286] Only weak spontaneous responses were detected in MPN patients directed against JAK2 V617F epitopes and some endeavors to identify binding peptides from this driver mutation even failed completely.[286–288] These

data suggest a low abundancy of naturally presented JAK2 V617F-derived neoepitopes.

In contrast to JAK2, mutations in MPL and CALR represent a rich source of neoepitopes that are suitable for various HLA molecules. Indeed, highly immunogenic CALR-derived neoepitopes revealed spontaneous T-cell responses in samples of CALR-mutated MPN patients.[233,234,286,287] These immune responses were mainly due to strong activation of $CD4^+$ T cells directed against HLA class II-restricted peptides. Additionally, immune responses directed against some of the tested HLA class I-restricted CALR-neoepitopes were attributed to weak $CD8^+$ T-cell activation. Interestingly, the most frequent T-cell responses were observed in samples of ET patients, less frequently in PV samples, with a lower frequency in pre-MF samples, and finally with the lowest frequency in samples of PMF patients.[233,289] This correlation of spontaneous T-cell responses with disease stage and clinical response underlines the profound immune disruption that is promoted with disease progression. In consequence, this emphasizes the importance of immunotherapy for patients in early disease states instead of the watch-and-wait strategy.

Notably, preexisting spontaneous CALR-specific T-cell responses have been observed in healthy volunteers (HVs) and MPN patients without detectable CALR mutations.[235] The CALR-directed responses were more frequent and stronger in HVs and specific T cells displayed a $CD4^+$ memory phenotype with an increased CD107a expression upon stimulation. The hypothesis is that HVs acquire the driver mutation and their functional immune system clears the altered cells and generates CALR-specific memory cells. This in turn seems to be an evidence for immune surveillance. Apart from the classical driver mutations, other TAA- and CTA-derived peptides have been searched for, but without success.[290,291]

The immunogenicity of the analyzed neoantigens holds promise for the successful development of effective antigen-specific immunotherapies.[292] One potential obstacle for immunotherapy is the immunosuppressive environment (enhanced PD-1/PD-L1 expression) and hyperinflammatory state (increased numbers of Tregs and MDSCs).[293–295] A multipeptide vaccine containing PD-L1-derived peptides or combination with checkpoint blockade can restore and enhance T-cell responses *in vitro* and *in vivo*.[289,296–299] However, in case of CALR-mutated malignancies, extracellular CALR is shown to inhibit phagocytosis of malignant cells by DCs, which might suppress the efficacy of checkpoint blockade.[133,138]

Besides the PD-1/PD-L1 axis, the immune disruption can be targeted by JAK inhibition. Rux-

olitinib can normalize the cytokine profile, suppress differentiation of pro-inflammatory Th17 cells, and deplete suppressive Tregs *in vitro* and *in vivo* in a dose-dependent manner.[300–303] Despite these benefits, one has to bear in mind the myelosuppressive effects that have been described for Ruxolitinib and therefore deploy Ruxolitib following immunotherapy.[294,304,305]
Another option is to enhance HLA molecule expression, since particularly the compromised CALR chaperone function leads to decreased peptide loading and impairs HLA surface expression.[137,306,307] Aside from decreasing the allele burden,[308] IFN-α can induce expression of HLA-related genes and activate lymphocytes.[309–312] These anti-proliferative and immunomodulatory effects qualify IFN-α as companion for peptide-based approaches.[246,247,313]
The group of IMiDs can also alter the cytokine milieu,[314] hamper Treg functionality,[315] activate NKT cells,[316] and reinstate immunological synapse formation.[317]
Taken together, targeting both, driver mutations and immunoregulation by combining peptide-based strategies with synergistic approaches might allow progression-free long-term control for patients suffering from myeloproliferative neoplasms.[318,319]

Peptide-based immunotherapy in MM

Apart from alloSCT and IMiDs, various modern immunotherapeutic approaches such as CAR T cells, mAbs, and peptide-based vaccines are on advance for MM. Multi-peptide vaccinations can counteract tumor escape strategies such as mutations, loss-of-antigen, or HLA molecule down-regulation.[186] Vaccination studies with WT1-derived peptides emphasize the importance of timing, which is best in the MRD state.[320,321] Moreover, the prevailing immunosuppressive state of the myeloma microenvironment needs to be addressed for example by studying combinations with immune checkpoint blockade.[322–324] Vaccination studies targeting MM-associated TAAs and CTAs demonstrate their immunogenicity, multifunctionality of induced T cells (CD107a, IFN-γ, tumor necrosis factor (TNF), IL-2 production), and even clinical responses by targeting RHAMM, MAGE-C1, NY-ESO, SLAMF7, hTERT, and CD138 amongst others.[325–330] One of the most promising antigens is BCMA, which is studied extensively. First encouraging results are demonstrated using antibodies, ADCs, and CAR T cells.[331,332] Nevertheless, particularly CAR T cells are cost- and labor-intensive.[333–336] Besides adverse effects, such approaches have the disadvantage to exclusively target surface domains and thus bear a substantial risk for immune evasion *via* antigen loss.[337–339]

In consequence, peptide-based immunotherapy appears to be more attractive as demonstrated by Bae *et al.* using peptide vaccines and peptide-encapsulated nanoparticles.[340,341] The identified BCMA-derived peptides – despite their extracellular domain origin – induced multifunctional memory CTLs for an effective long-lasting immunity. Combination with immune agonists, checkpoint inhibitors, or IMiDs further enhanced immune response and functionality. Since MM is counted among the intermediate mutational burden entities, neoantigen-based approaches could provide therapeutical benefits. Perumal *et al.* recently reported on *in vitro* anti-cancer activity in correlation with *in vivo* clinical response to immunogenic neoantigens in MM patients.[342] Taken together, the potent peptide-based vaccines as off-the-shelf products for immunotherapy represent a promising strategy for more efficient therapy and a broad range of MM patients.

Aim of thesis

The immune system and immunological control are of great importance for the surveillance and restriction of neoplastic malignancies and can influence their genesis and progress. Particularly T-cell-based immunotherapy approaches are on the advance to harness the immune system against cancer and to reinstate the anti-cancer immune response. Such approaches are represented for example by tailored peptide-based strategies with few adverse effects and a good targeting capability. This thesis aims to characterize a novel category of immunogenic antigen targets for such off-the-shelf T-cell-based and combinatorial immunotherapeutic approaches to target hematological malignancies and to circumvent leukemic transformation and progression of disease. Therefore, using mass spectrometry (MS) we aimed to identify novel, highly immunogenic, CML-associated T-cell epitopes and took a first step towards the identification of ET-, PV-, and PMF-associated target antigens derived from mutated and non-mutated proteins for immunotherapy and subsequent immunomonitoring. In addition, we provided a proof-of-concept study for the natural HLA-dependent presentation of peptides derived from intracellular domains of established tumor-associated surface antigens as potential targets for immunotherapy by characterizing P(BCMA)$_{B*18}$ as a highly immunogenic T-cell epitope in B-cell malignancies. The main focus of this thesis is set on the immunological characterization of the identified antigens using T-cell-based experiments.

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CHAPTER 1

The HLA ligandome landscape of chronic myeloid leukemia delineates novel T-cell epitopes for immunotherapy

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Blood. 2019 Feb 7;133(6):550-565 PMID: 30530751 doi: 10.1182/blood-2018-07-866830

Disclosure of authorship contributions

TB and AN contributed equally to this publication. TB was involved in the study design. TB planned and performed all *in vitro* T-cell experiments and analyzed the respective data. TB designed and created all figures and tables involving T-cell-based assays: Figure 5-7, supplemental Table S1 and S3, and supplemental Figure S10-S13. TB drafted the manuscript together with JSW and AN. For further information see the declaration provided in the "Authorship Contribution" section at the end of this chapter/manuscript.

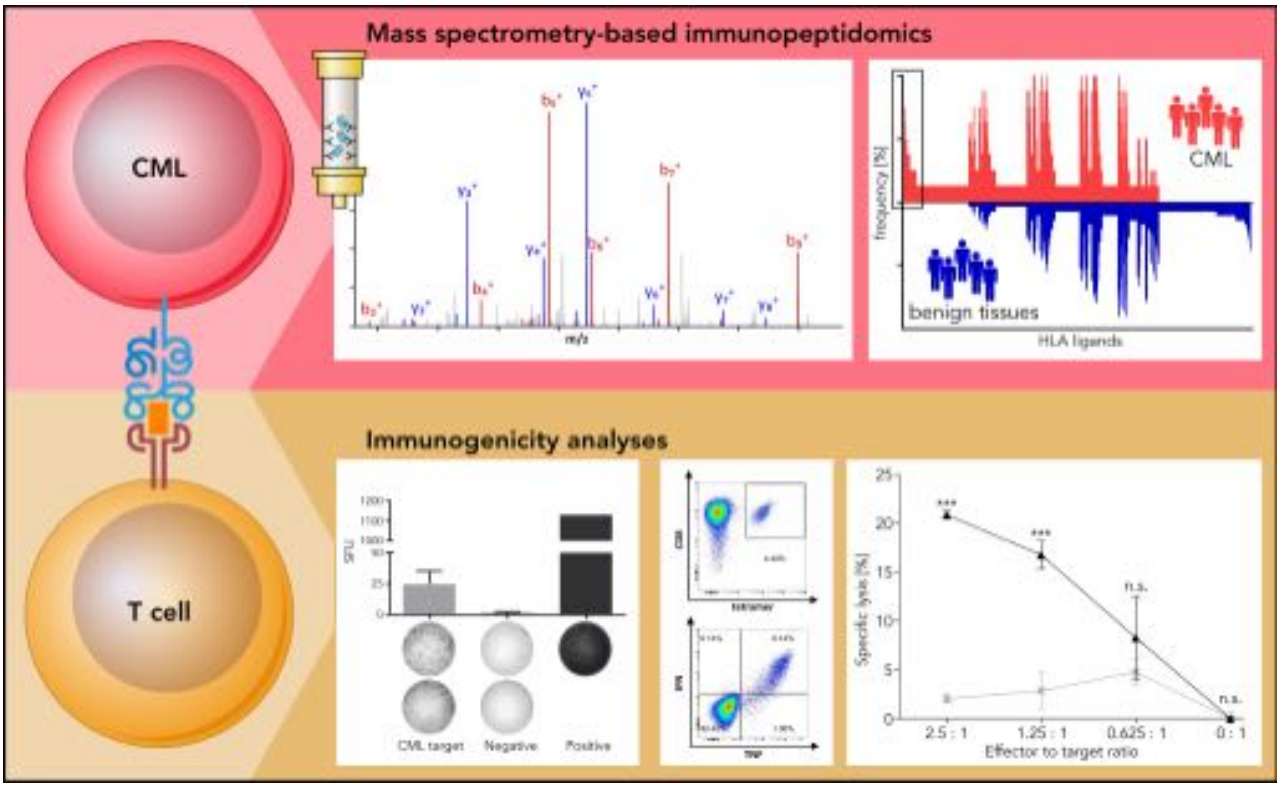
Abstract

Anti-leukemia immunity plays an important role in disease control and maintenance of TKI-free remission in CML. Thus, antigen-specific immunotherapy holds promise for strengthening immune control in CML but requires the identification of CML-associated targets. In this study, we used a mass spectrometry-based approach to identify naturally presented HLA class I- and class II-restricted peptides in primary CML samples. Comparative HLA ligandome profiling using a comprehensive dataset of different hematological benign specimens and samples from CML patients in deep molecular remission delineated a panel of novel frequently presented CML-exclusive peptides. These non-mutated target antigens are of particular relevance because our extensive data-mining approach suggests the absence of naturally presented BCR-ABL- and ABL-BCR-derived HLA-restricted peptides and the lack of frequent tumor-exclusive presentation of known cancer/testis and leukemia-associated antigens. Functional characterization revealed spontaneous T-cell responses against the newly identified CML-associated peptides in CML patient samples and their ability to induce multifunctional and cytotoxic antigen-specific T cells *de novo* in samples from healthy volunteers and CML patients. Thus, these antigens are prime candidates for T-cell-based immunotherapeutic approaches that may prolong TKI-free survival and even mediate cure of CML patients.

Key Points

- Mass spectrometry-based HLA ligandome analysis of primary CML patient samples revealed a panel of novel CML-associated target antigens.
- These antigens induced multifunctional T-cell responses and may be used as targets for T-cell-based immunotherapeutic approaches.

Visual Abstract



Introduction

CML is characterized by the translocation t(9;22) that leads to the formation of the BCR-ABL fusion transcript.[1,2] To inhibit the resulting fusion protein, which mediates constitutive tyrosine kinase activity, 5 approved TKIs are available that have led to an impressive improvement in the prognosis of CML patients.[3-7] Currently, the main treatment goal in CML is the achievement of a so-called "deep molecular response", in which discontinuation of TKI therapy can be considered. However, only few patients are able to permanently stop TKI therapy without suffering from molecular relapse.[8,9] Thus, lifelong TKI therapy is the standard of care for most CML patients, but it can be associated with significant side effects and the risk of developing resistance to TKIs.[10,11] Several studies provided evidence that immunological control may contribute to and even represent a marker for the achievement of deep MR in CML patients under TKI treatment (CML_{TKI} patients) and TFR. The restoration of immune responses is characterized by increased NK-cell and T-cell responses,[12] reduced PD-1 expression on T cells,[12] the correlation of CD62L expression on T cells[13] in patients with MR, and the association between increased NK-cell count[14] and $CD86^+$ plasmacytoid dendritic cell count and function[15] with TFR.

In turn, reinforcing CML-specific immune responses by T-cell-based immunotherapy may serve to enlarge the fraction of patients achieving long-term TFR or even cure. It has been shown that "nonspecific" immunotherapy approaches, such as allogeneic stem cell transplantation or IFN-α therapy, enable long-lasting remissions in CML patients after discontinuation of TKI therapy.[16-20] Immune checkpoint inhibitors, which have revolutionized the treatment of many solid tumors in recent years,[21-23] are currently being evaluated in CML therapy.[24] More advanced strategies to treat CML patients comprise agents inducing an immune response specifically directed against the leukemic cells, such as vaccines,[25-27] T-cell receptor mimic antibodies,[28-30] or engineered T cells.[31,32] The prerequisite for such T-cell-based immunotherapeutic approaches is the identification of targets for CML-specific T-cell responses, which, in general, are represented by tumor-associated HLA-presented peptides on malignant cells.[33,34] Several studies have suggested neoepitopes arising from tumor-specific mutations as central specificities of checkpoint inhibitor-induced T-cell responses in solid tumors with high mutational burden.[33,35] However, the role of neoantigens for T-cell responses in cancer entities with low mutational burden, including CML, remains

unclear. In addition to neoantigens, we and other investigators identified non-mutated tumor-associated HLA peptides that are able to induce peptide-specific T-cell responses and can serve as targets for T-cell-based immunotherapy approaches.[36-39] In recent years, we implemented the characterization of such tumor-associated antigens in hematological malignancies (HMs) based on the direct isolation of naturally presented HLA ligands from leukemia cells and their subsequent identification by MS. Thus far, for AML, CLL, and MM, we identified >100 tumor-exclusive highly frequent antigens that were validated as immunogenic targets for T-cell-based immunotherapy approaches.[38,40,41] An extensive meta-analysis of our HM immunopeptidome data revealed only a small set of entity-spanning antigens that was predominantly characterized by low presentation frequencies within the different patient cohorts,[42] indicating that T-cell-based immunotherapies for HMs should be designed in an entity-specific manner. For CML, very few non-mutated tumor-associated antigens[43-46] or peptides derived from the BCR-ABL fusion region[47-49] have been described and validated as immunogenic targets of anticancer T-cell responses.[50-52] Here, we comprehensively mapped the landscape of naturally presented HLA class I and II peptides in primary CML samples to identify novel CML-associated antigens covering a broad range of HLA allotypes. These antigens were further validated for their potential to induce T-cell responses, particularly in the context of immunomodulatory effects induced by TKI treatment in CML patients.[53-56]

Methods

A detailed description of the methods used can be found in Supplemental Methods (available on the Blood web site).

Patients and blood samples

PBMCs from CML patients were collected at the Departments of Hematology and Oncology in Tübingen, Leipzig, and Aachen, Germany. Informed consent was obtained in accordance with the Declaration of Helsinki protocol. The study was performed according to the guidelines of the local ethics committees. Patient characteristics are provided in supplemental Table 1.

HLA surface molecule quantification

HLA surface expression was determined using a QIFIKIT quantification flow cytometric assay (Dako).[40,57] Cells were stained with the pan-HLA class I-specific W6/32, HLA-DR-specific L243 mAbs, or isotype control. Surface marker staining was performed with fluorescence-conjugated antibodies against CD33, CD13, CD117, and CD34.

Isolation of HLA ligands

HLA molecules were isolated by standard immunoaffinity purification[40,58] using the mAbs W6/32, Tü-39, and L243.

Analysis of HLA ligands by liquid chromatography-tandem MS

HLA ligand extracts were analyzed as described previously.[38] Peptides were separated by nanoflow high-performance liquid chromatography. Eluted peptides were analyzed in an online-coupled LTQ Orbitrap XL mass spectrometer. Furthermore, parallel reaction monitoring targeting BCR-ABL- and ABL-BCR-derived peptides (supplemental Table 2) was performed on an Orbitrap Fusion Lumos mass spectrometer.

Data processing

Data processing was performed as described previously.[38,57] Proteome Discoverer (v1.3, Thermo Fisher Scientific) was used to integrate the search results of the Mascot search engine (v2.2.04, Matrix Science) against the human proteome (Swiss-Prot database). For the search of BCR-ABL- and ABL-BCR-derived neoantigens, the human proteome was extended by BCR-ABL sequences from the TrEMBL database and by published ABL-BCR sequences.[59,60] The false discovery rate (FDR; estimated by the Percolator algorithm 2.04[61]) was limited to 5% for HLA class I and 1% for HLA class II. HLA class I annotation was performed using SYFPEITHI 1.0[62] and NetMHCpan 3.0.[63,64] The lists of HLA class I and II peptides identified on CML, CML_{MR}, and hematological benign tissue samples are provided in supplemental Data Set 1.

Peptide synthesis

Peptides were produced with the Liberty Blue Automated Peptide Synthesizer (CEM) using the 9-fluorenylmethyl-oxycarbonyl/tert-butyl strategy.[65]

Amplification of peptide-specific T cells and IFN-γ ELISPOT assay

PBMCs from CML patients and HVs were pulsed with 1 mg/mL (class I) or 5 mg/mL (class II) per peptide and cultured for 12 days.[38,40] Peptide-stimulated PBMCs were analyzed by enzyme-linked immunospot (ELISPOT) assay.[41,66]

aAPC priming of naïve CD8$^+$ T cells

Priming of peptide-specific cytotoxic T lymphocytes was conducted using artificial antigen-presenting cells (aAPCs).[37,67] MACS-sorted CD8$^+$ T cells were cultured with IL-2 and IL-7. Weekly stimulation with peptide-loaded aAPCs and IL-12 was performed four times.

Cytokine and tetramer staining

The functionality of peptide-specific CD8$^+$ T cells was analyzed by intracellular cytokine staining (ICS).[66,68] Cells were pulsed with peptide, brefeldin A, and GolgiStop. Staining was performed using mAbs against CD8, TNF, IFN-γ, and CD107a. The frequency of peptide-specific CD8$^+$ T cells was determined by anti-CD8 and tetramer staining.[69]

Cytotoxicity assay

The cytolytic capacity of peptide-specific CD8$^+$ T cells was analyzed using the flow cytometry-based VITAL assay.[70,71] Autologous target cells were loaded with test peptides or irrelevant control peptides and labeled with CFSE or FarRed, respectively. Effector cells were added at the indicated effector-to-target ratios. Specific lysis of peptide-loaded target cells was calculated relative to control targets.

Data availability

The MS data have been submitted to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the PRIDE[72] partner repository (dataset identifier PXD010450).

Results

Myeloid and precursor cells of primary CML samples express high levels of HLA molecules

T-cell-based immunotherapy requires sufficient HLA expression on target cells, which, in the case of CML, consist of myeloid cells and myeloid precursor cells. Thus, as a first step, we quantified HLA surface expression on CD33⁺ and CD13⁺ myeloid cells, as well as on CD117⁺CD34⁺ precursor cells, using PBMCs from CML patients (n = 7; supplemental Table 1). HLA class I surface levels showed substantial heterogeneity, with molecule counts per cell of 78,600 to 202,100 (mean 153,600) for CD33⁺ cells and 69,500 to 322,000 (mean 184,600) for CD13⁺ cells (Figure 1A). HLA class II expression ranged from 37,700 to 230,000 (mean 101,200) molecules per cell for DR⁺CD33⁺ cells and from 39,000 to 286,300 (mean 158,900) molecules per cell for DR⁺CD13⁺ cells (Figure 1B). Notably, the highest HLA surface levels were detected on precursor cells, with 112,000 to 316,500 (mean 221,100) and 99,700 to 487,200 (mean 228,300) molecules per cell for HLA class I and II, respectively.

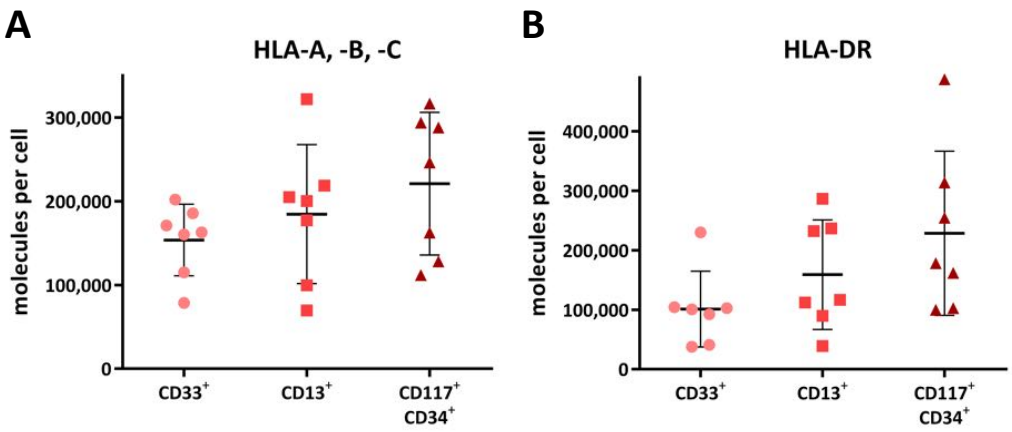


Figure 1: HLA surface expression of primary CML cells. HLA class I (A) and HLA-DR (B) expression was determined by flow cytometry for $CD33^+$ and $CD13^+$ myeloid cells, as well as for $CD117^+CD34^+$ precursor cells, from the peripheral blood of CML patients (n = 7) at the time of diagnosis. Data points represent individual samples. Horizontal lines indicate mean values ± standard deviation.

MS identifies naturally presented CML-associated HLA class I ligands in CML patient samples

MS analysis of 21 primary CML samples revealed a total of 11,945 unique HLA class I ligands (range 535-2,107; mean 1,080 per sample) from 5,478 source proteins (supplemental Figure 3A; supplemental Data Set 1), obtaining 82% of the estimated maximum attainable coverage in HLA ligand source proteins (Figure 2A). For the identification of CML-associated antigens, we established a comparative cohort of hematological benign tissues (n = 108), including PBMCs (n = 63), granulocytes (n = 14), $CD19^+$ B cells (n = 5), bone marrow (n = 18), and $CD34^+$ hematopoietic progenitor cells (HPCs; n = 8). A total of 51,232 naturally presented HLA class I ligands (range 101-7,587; mean 1,404 per sample) from 11,437 source proteins (supplemental Data Set 1), obtaining 95% of maximum attainable coverage (supplemental Figure 4A), were identified. Furthermore, we created an additional comparative benign ligandome dataset of PBMCs from CML patients in deep molecular remission (CML_{MR}, n = 15) comprising a total of 5,907 unique HLA class I ligands (range 311-1,145; mean 655 per sample; supplemental Data Set 1).

The CML cohort included a total of 31 different HLA class I allotypes, with the most frequent being HLA-C*07 (n = 11), HLA-A*02 (n = 10), HLA-A*03 (n = 8), HLA-B*07 (n = 7), and HLA-B*35 (n = 6; supplemental Figure 5A). Among the world's population, 99.3% of individuals carry $\geq$1 HLA class I allotype that is represented within this cohort[73,74] (supplemental Figure 6A). The comparative hematological benign cohort showed an HLA allotype population coverage of 99.9% (supplemental Figure 6B) and matched 89% of HLA-A allotypes, 100% of HLA-B allotypes, and 88% of HLA-C allotypes within the CML cohort (supplemental Figure 5B).

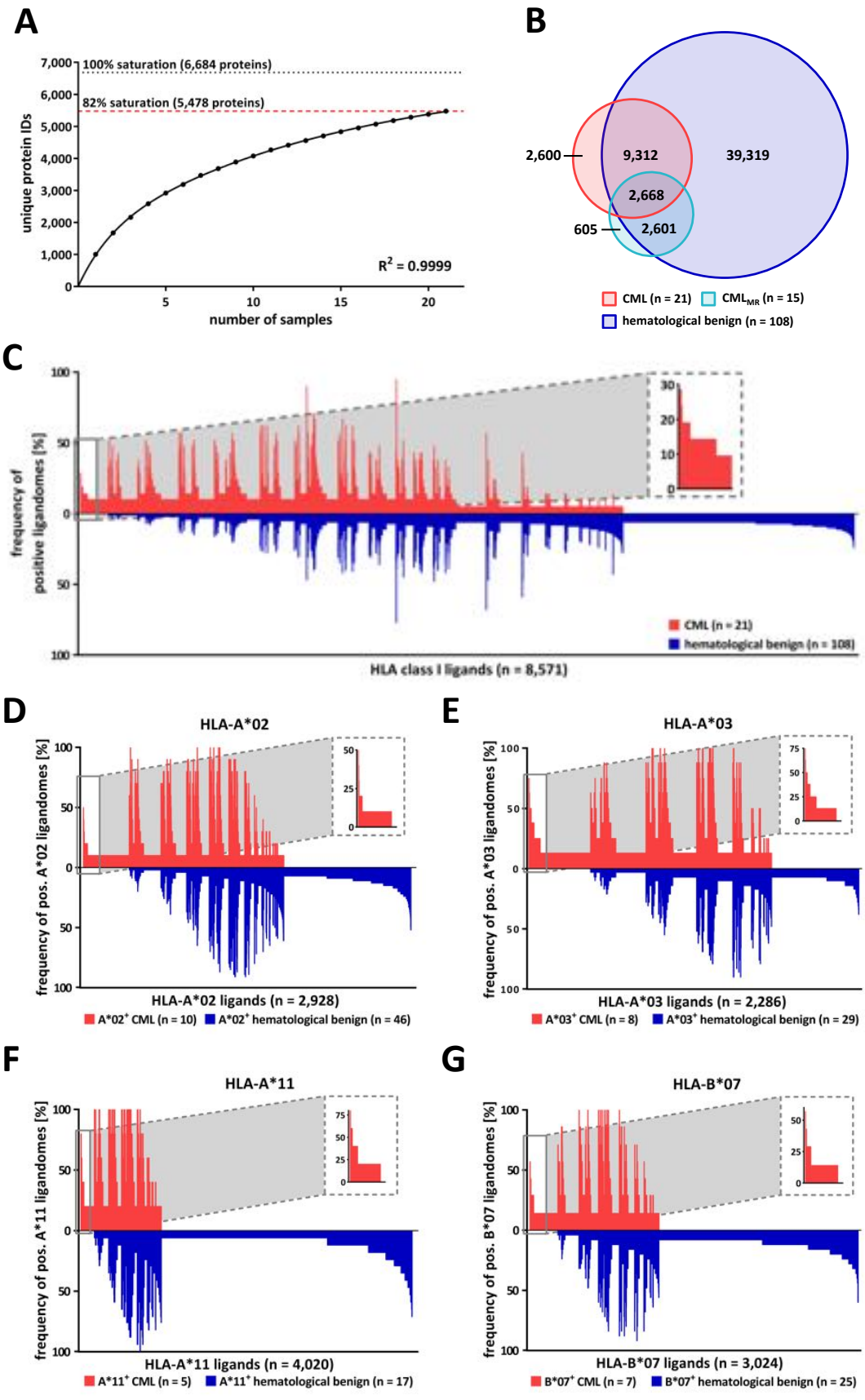

A
100% saturation (6,684 proteins)
82% saturation (5,478 proteins)
unique protein IDs
number of samples
R² = 0.9999
B
2,600
9,312
39,319
2,668
605
2,601
CML (n = 21)
CML_MR (n = 15)
hematological benign (n = 108)
C
frequency of positive ligandomes [%]
HLA class I ligands (n = 8,571)
CML (n = 21)
hematological benign (n = 108)
D
HLA-A*02
frequency of pos. A*02 ligandomes [%]
HLA-A*02 ligands (n = 2,928)
A*02+ CML (n = 10)
A*02+ hematological benign (n = 46)
E
HLA-A*03
frequency of pos. A*03 ligandomes [%]
HLA-A*03 ligands (n = 2,286)
A*03+ CML (n = 8)
A*03+ hematological benign (n = 29)
F
HLA-A*11
frequency of pos. A*11 ligandomes [%]
HLA-A*11 ligands (n = 4,020)
A*11+ CML (n = 5)
A*11+ hematological benign (n = 17)
G
HLA-B*07
frequency of pos. B*07 ligandomes [%]
HLA-B*07 ligands (n = 3,024)
B*07+ CML (n = 7)
B*07+ hematological benign (n = 25)

Figure 2: Comparative HLA class I ligandome profiling and identification of CML-associated antigens. (A) Saturation analysis of HLA class I ligand source proteins of the CML patient cohort. Number of unique HLA ligand source protein identifications are shown as a function of cumulative HLA ligandome analysis of CML samples (n = 21). Exponential regression allowed for the robust calculation ($R^2 = 0.9999$) of the maximum attainable number of different source protein identifications (dotted line). The dashed red line depicts the source proteome coverage achieved in our CML patient cohort. (B) Overlap analysis of HLA class I ligand identifications of primary CML samples (n = 21), CML_{MR} samples (n = 15), and hematological benign samples (n = 108), including PBMCs (n = 63), granulocytes (n = 14), $CD19^+$ B cells (n = 5), bone marrow (n = 18), and $CD34^+$ HPCs (n = 8). (C) Comparative profiling of HLA class I ligands based on the frequency of HLA-restricted presentation in CML and hematological benign ligandomes. Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. To allow for better readability, HLA ligands identified on <5% of the samples within the respective cohort were not depicted in this plot. The box on the left and its magnification highlight the subset of CML-associated antigens showing CML-exclusive high frequent presentation. Allotype-specific comparative profiling of HLA-A*02-positive (D), HLA-A*03-positive (E), HLA-A*11-positive (F), and HLA-B*07-positive (G) samples, as described above. Abbreviations: ID, identifications; pos., positive.

To identify CML-associated antigens, we performed comparative HLA class I ligandome profiling of the CML cohort with the hematological benign and CML_{MR} cohorts. Overlap analysis revealed that 2,600 HLA class I ligands were presented exclusively on CML samples (Figure 2B) and never detected on hematological benign or CML_{MR} samples. For the identification of broadly applicable CML-associated antigens, we aimed for the selection of target antigens that not only fulfill the criterion of CML exclusivity, but also exhibit high prevalence within the CML cohort. At a target-definition FDR <5% (<1%) a total of 23 (5) HLA class I ligands with a representation frequency $\geq$19% ($\geq$24%) were identified (Figure 2C; supplemental Figure 7A; supplemental Table 4). The most common HLA allotype restrictions of these HLA ligands included HLA-A*02, HLA-A*03, HLA-A*11, and HLA-B*07. To identify CML-associated targets with even higher representation frequencies, we subsequently performed HLA allotype-specific immunopeptidome profiling. Setting the target FDR <5% (<1%), we identified 4 (1) HLA-A*02-, 35 (15) HLA-A*03-, 3 (0) HLA-A*11-, and 8 (2) HLA-B*07-restricted ligands with representation frequencies of $\geq$40% ($\geq$50%), $\geq$38% ($\geq$50%), $\geq$80% ($\geq$80%), and $\geq$43% ($\geq$57%), respectively (Figure 2D-G; supplemental Figure 7B-E; supplemental Table 4). To further validate these CML-associated targets, we compared them with an additional benign dataset comprising 28 different non-hematological tissue entities (n = 166; e.g. liver, lung, brain, skin) with a total of 128,590 unique HLA class I peptides from 16,405 source proteins. Thus, we selected a panel of 8 CML-exclusive target antigens, including 2 HLA-A*02-restricted, 3 HLA-A*03-restricted, 1 HLA-A*11-restricted, and 2 HLA-B*07-restricted ligands, for further immunological characterization.

HLA class II ligandome profiling delineates 3 novel groups of CML-associated antigens

Mapping the HLA class II ligandomes of 20 primary CML samples, we identified 5,991 different HLA class II-restricted peptides (range 172-1,162; mean 641 per sample) derived from 1,302 source proteins (supplemental Figure 3B; supplemental Data Set 1), achieving 72% of maximum attainable coverage (Figure 3A). Our HLA class II hematological benign tissue cohort (n = 88; PBMCs, n = 38; granulocytes, n = 18; $CD19^+$ B cells, n = 9; bone marrow, n = 15; $CD34^+$ HPCs, n = 8) contained 42,753 unique peptides (range 111-6,267; mean 1,197 per sample) from 4,877 source proteins (supplemental Data Set 1), obtaining 84% of maximum attainable coverage (supplemental Figure 4B). The benign CML_{MR} ligandome dataset (n = 15) included a total of 1,529 HLA class II peptides (range 74-281; mean 164 per sample; supplemental Data Set 1).

For the identification of HLA class II-restricted CML-associated antigens, we established an innovative HLA class II ligandome-profiling platform that delineated 3 groups of antigens: peptide targets, protein targets, and hotspot targets. First, we performed comparative ligandome profiling at the peptide level. Overlap analysis revealed that 1,949 peptides were exclusively presented on CML (Figure 3B) and were never detected on hematological benign or CML_{MR} samples. Of these, 36 peptides were identified with a representation frequency $\geq$20% based on an FDR <1%; however, 30 of 36 peptide targets showed length variants (>50% overlap) presented on benign hematological samples and, therefore, were excluded (peptide targets; Figure 3C; supplemental Figure 8A; supplemental Table 5). Further ligandome profiling was performed at the HLA class II source protein level. Based on an FDR <5% (<1%), a total of 4 (2) source proteins were identified with a frequency $\geq$20% ($\geq$25%) representing 10 (4) unique HLA class II peptides (protein targets; Figure 3D; supplemental Figure 8B; supplemental Table 5). As a third group of CML-associated antigens, we analyzed CML-exclusive hotspots by peptide clustering, which validated the previously described targets and identified 1 additional CML-associated hotspot with a representation frequency of 20% comprising 3 unique HLA class II peptides (hotspot targets; Figure 3E; supplemental Table 5). Subsequent validation of these targets using our non-hematological

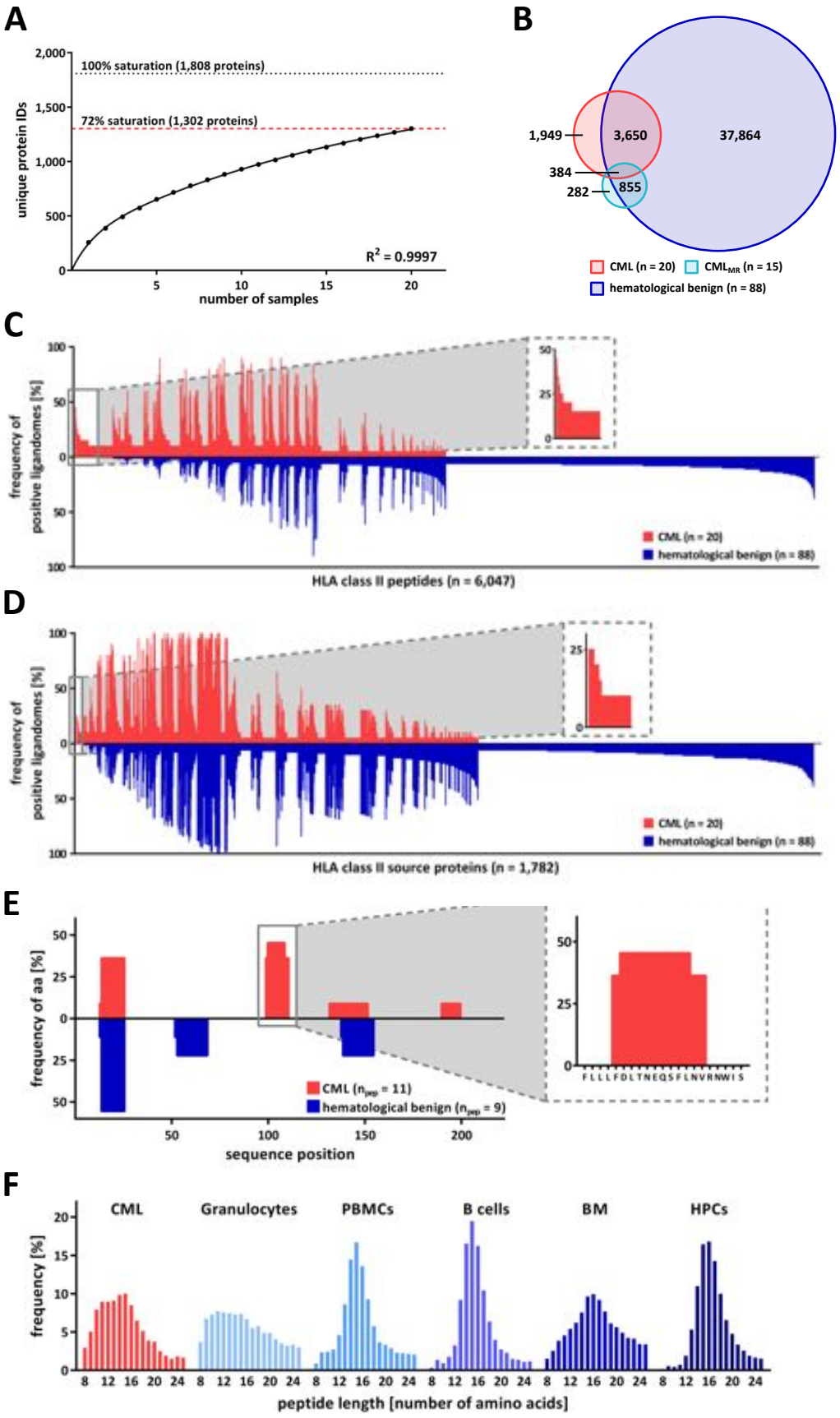

A
unique protein IDs
100% saturation (1,808 proteins)
72% saturation (1,302 proteins)
R² = 0.9997
number of samples
B
1,949
3,650
37,864
384
282
855
CML (n = 20)
CML_MR (n = 15)
hematological benign (n = 88)
C
frequency of positive ligandomes [%]
CML (n = 20)
hematological benign (n = 88)
HLA class II peptides (n = 6,047)
D
frequency of positive ligandomes [%]
CML (n = 20)
hematological benign (n = 88)
HLA class II source proteins (n = 1,782)
E
frequency of aa [%]
CML (n_pep = 11)
hematological benign (n_pep = 9)
sequence position
F L L L F D L T N E Q S F L N V R N W I S
F
frequency [%]
CML
Granulocytes
PBMCs
B cells
BM
HPCs
peptide length [number of amino acids]

Figure 3: Comparative HLA class II ligandome profiling and identification of CML-associated antigens. (A) Saturation analysis of HLA class II peptide source proteins of the CML patient cohort. Number of unique HLA peptide source protein identifications as a function of cumulative HLA ligandome analysis of CML samples (n = 20). Exponential regression allowed for the robust calculation ($R^2 = 0.9997$) of the maximum attainable number of different source protein identifications (dotted line). The dashed red line depicts the source proteome coverage achieved in our CML patient cohort. (B) Overlap analysis of HLA class II peptides of primary CML samples (n = 20), CML_{MR} samples (n = 15), and hematological benign samples (n = 88), including PBMCs (n = 38), granulocytes (n = 18), $CD19^+$ B cells (n = 9), bone marrow (n = 15), and $CD34^+$ HPCs (n = 8). Comparative profiling of HLA class II peptides (C) and HLA class II source proteins (D) based on the frequency of HLA-restricted presentation in CML and hematological benign ligandomes. The frequencies of positive immunopeptidomes for the respective HLA peptides or source proteins (x-axis) are indicated on the y-axis. To allow for better readability, HLA peptides or source proteins identified on <5% of the samples within the respective cohort are not depicted. The boxes on the left and their magnifications highlight the subset of CML-associated antigens showing CML-exclusive high frequent presentation in CML samples. (E) Hotspot analysis of the protein RB27A by peptide clustering. Identified peptides were mapped to their amino acid positions within the source protein. Representation frequencies of amino acid counts within each cohort for the respective amino acid position (x-axis) were calculated and are indicated on the y-axis. The box on the left and its magnification highlight the identified hotspot with the respective amino acids on the x-axis. (F) Tissue-specific HLA class II peptide length distribution (number of amino acids) of all identified peptides on primary CML samples (n = 20), granulocytes (n = 18), PBMCs (n = 38), $CD19^+$ B cells (n = 9), bone marrow (n = 15), and $CD34^+$ HPCs (n = 8). Abbreviations: aa, amino acids; IDs, identifications; n_{pep}, number of peptides.

benign tissue dataset (n = 166, 28 tissues, 143,652 HLA class II peptides, 13,410 source proteins) delineated a panel of 6 strongly CML-associated target antigens for immunological characterization. Notably, most of the identified targets showed unusual short peptide lengths for HLA class II-restricted peptides (mean 12 amino acids), which are reflected by a general length distribution shift in myeloid cell-containing samples representing shorter HLA class II-restricted peptides (Figure 3F; supplemental Figure 9).

The roles of CTAs, LAAs, and BCR-ABL-derived neoantigens in the immunopeptidome of CML

In addition to the definition of novel CML-associated antigens, we focused on the identification and ranking of established CTAs[75,76] and LAAs[43,77] in our dataset of naturally presented HLA peptides. We identified 170 different HLA class I peptides and 382 HLA class II peptides from 39 and 18 CTAs, respectively, as well as 1,429 HLA class I peptides and 3,428 HLA class II peptides from 131 and 77 LAAs, respectively (supplemental Tables 6-9). Notably, these antigens were represented in CML immunopeptidomes, as well as on hematological benign samples (Figure 4). Hence, this analysis delineated only a small panel of 7 (4% of total) CML-exclusive, but infrequent, CTAs and LAAs that represent suitable candidates for T-cell-based immunotherapy in selected CML patients.

Because the characteristic BCR-ABL translocation may result in the presentation of BCR-ABL or ABL-BCR neoepitopes, we further screened our CML cohort for naturally presented BCR-ABL-derived and ABL-BCR-derived peptides by data-dependent acquisition of all CML samples, as well as by targeted parallel reaction monitoring of 4 CML samples (supplemental Table 2). Despite the fact that the BCR-ABL and ABL-BCR fusion sites potentially provide HLA-binding motifs for several HLA allotypes, no naturally presented HLA peptides were identified.

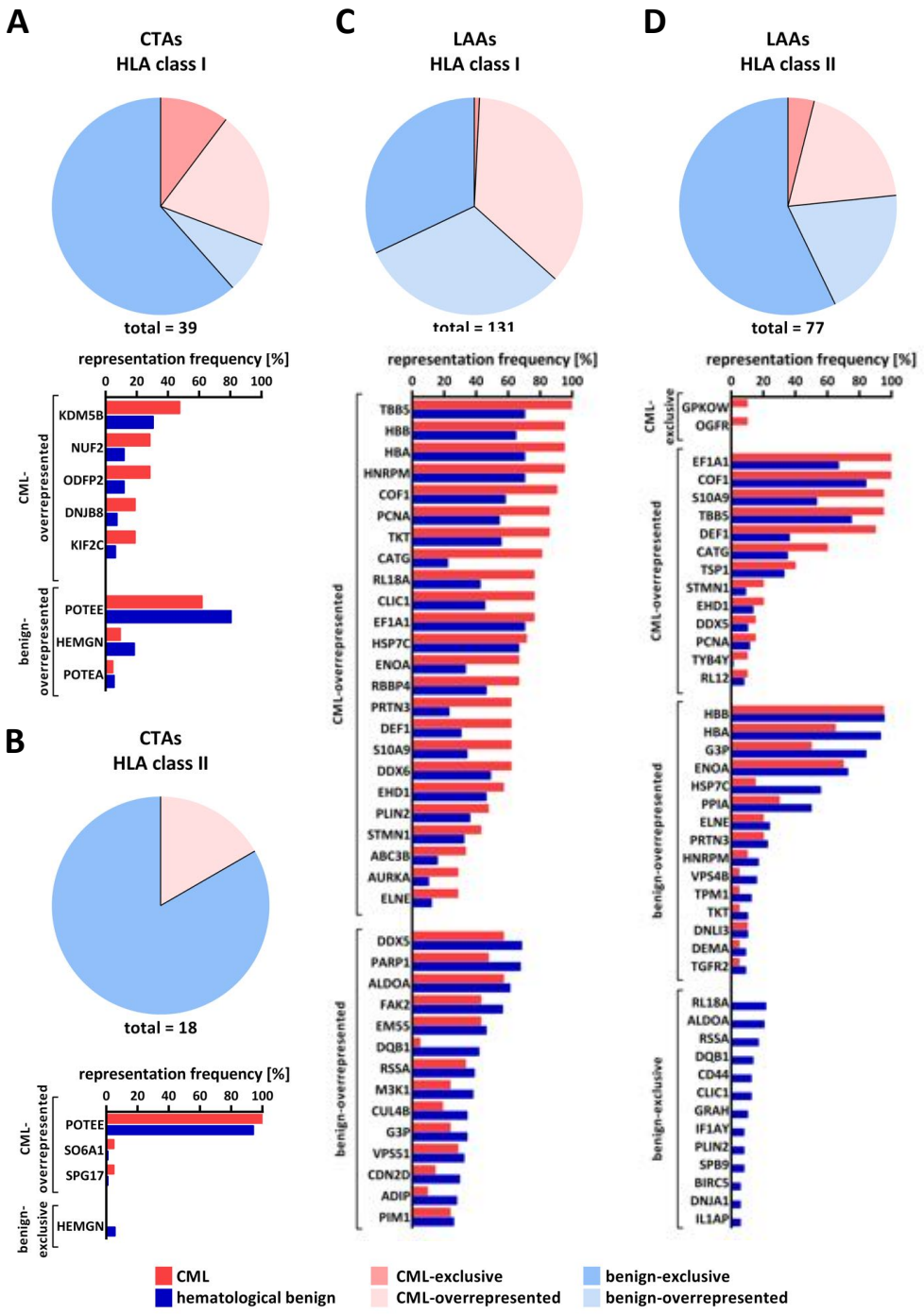


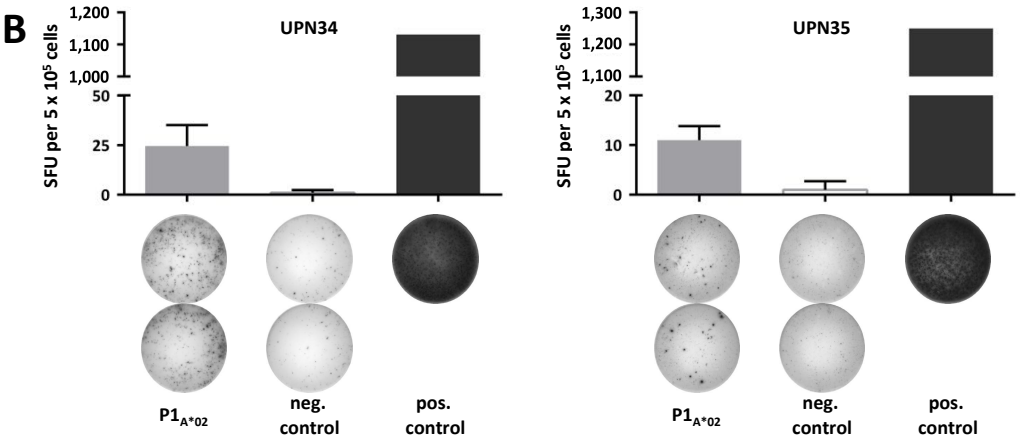
Figure 4: Representation of published CTAs and LAAs in CML and hematological benign HLA ligandomes. Representation frequencies of published CTAs in HLA class I (A) and II (B) ligandomes, as well as published LAAs in HLA class I (C) and class II (D) ligandomes in CML patient and hematological benign samples. Pie charts represent the total amount of identified CTAs and LAAs assigned to their degree of CML association (i.e., CML-exclusive, CML-overrepresented, benign-overrepresented, benign-exclusive). Bar diagrams depict the relative representation (%) of the respective antigens on CML and hematological benign samples allocated to their CML association. Only antigens with representation frequencies >5% (A-B,D) or >25% (C) in the respective cohort are shown.

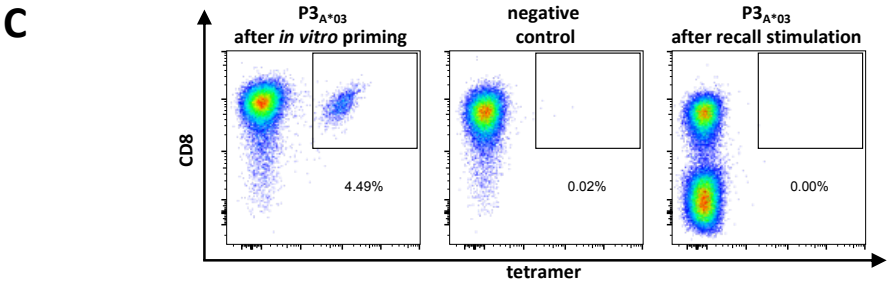
HLA class I-restricted CML-associated antigens induce functional peptide-specific T cells in samples from HVs and CML patients

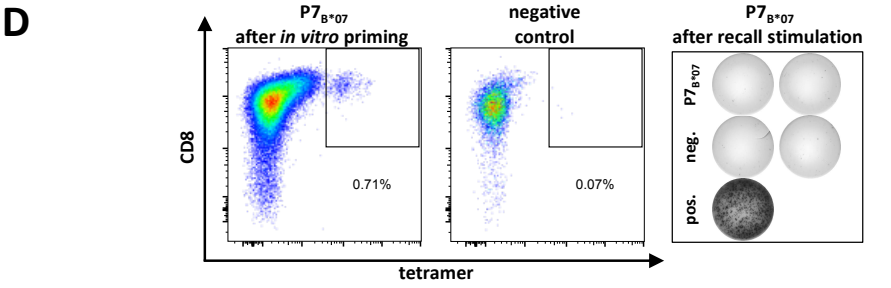
To confirm immunogenicity and detect preexisting memory T-cell responses against the identified CML-associated antigens (Figure 5A), we performed IFN-γ ELISPOT and tetramer staining assays using HLA-matched PBMCs from CML_{TKI} patients and HVs. We observed IFN-γ secretion for 1 of 8 CML-associated ligands in 2 of 17 (12%) analyzed CML_{TKI} patients (Figure 5B), as well as for 2 peptides in 1 HV, respectively (supplemental Figure 10). It appears to be unlikely that cross-reacting microorganism-specific or virus-specific T cells are the reason for the observed T-cell responses in single HVs, because no sequence similarity was found between the CML-associated antigens and the proteins from microorganisms and viruses. In addition, low frequent peptide-specific $CD8^+$ T cells were detected by tetramer staining for 4 of 8 peptides in 3 of 18 CML_{TKI} patient samples after a 12-day stimulation without any detectable preexisting peptide-specific T cells *ex vivo* prior to stimulation (supplemental Figure 11). To assess the immunogenicity of the remaining HLA class I-restricted ligands, we performed *in vitro* aAPC-based priming experiments using $CD8^+$ T cells from HVs and CML patients. Effective priming and expansion of antigen-specific T cells were observed for all 8 CML-associated peptides in $\geq$70% of analyzed HVs, with frequencies of peptide-specific T cells ranging from 0.1% to 33.9% (mean 2.2%) within the $CD8^+$ T-cell population (Figure 5A,C; supplemental Figure 12). Furthermore, all analyzed CML-associated peptides induced peptide-specific T cells using CML patient samples with frequencies of 0.1% to 2.2% (mean 0.4%) within the $CD8^+$ T-cell population (Figure 5A,D). Notably, peptide-specific immune responses were even induced in CML_{TKI} patient samples that had not shown preexisting immune responses. Priming experiments with control peptides frequently presented by HLA-A*02 and HLA-A*03 on tumor and benign tissues (peptide presentation >90% in HLA-matched sources) confirmed the CML specificity of the induced T-cell responses (Figure 5E). Furthermore, multifunctionality of peptide-specific T cells was shown for 6 of 8 CML-associated peptides by IFN-γ and TNF production and upregulation of the degranulation marker CD107a (Figure 6A-B). Finally, cytotoxicity assays with polyclonal peptide-specific effector T cells revealed the capacity to induce antigen-specific lysis for 3 of 4 analyzed peptides (Figure 6A,C-E; supplemental Figure 13).

A

Peptide ID	Peptide sequence	Source protein	CD8⁺ T-cell response in CML	CD8⁺ T-cell response in HVs	*in vitro* CD8⁺ T-cell priming in HVs	*in vitro* CD8⁺ T-cell priming in CML
$P1_{A*02}$	ALHPLVILNI	CSN6	2/17 (12%)	0/12 (0%)	5/6 (83%)	1/3 (33%)
$P2_{A*02}$	KQGFEPPSFV	GELS	0/17 (0%)	0/12 (0%)	6/7 (86%)	1/5 (20%)
$P3_{A*03}$	RTAGHPLTR	PTN7	0/10 (0%)	0/25 (0%)	9/10 (90%)	1/4 (25%)
$P4_{A*03}$	HLLEQVAPK	PLSL	0/7 (0%)	1/24 (4%)	7/10 (70%)	2/2 (100%)
$P5_{A*03}$	KIFWIPLSH	DHRS9	0/7 (0%)	0/12 (0%)	5/5 (100%)	n.t.
$P6_{A*11}$	AVNPGVVVR	BPI	0/3 (0%)	0/15 (0%)	3/3 (100%)	n.t.
$P7_{B*07}$	RAMVARLGL	CD24	0/5 (0%)	0/12 (0%)	6/6 (100%)	1/1 (100%)
$P8_{B*07}$	APGQPLRVL	CEBPE	0/5 (0%)	1/12 (8%)	6/6 (100%)	n.t.







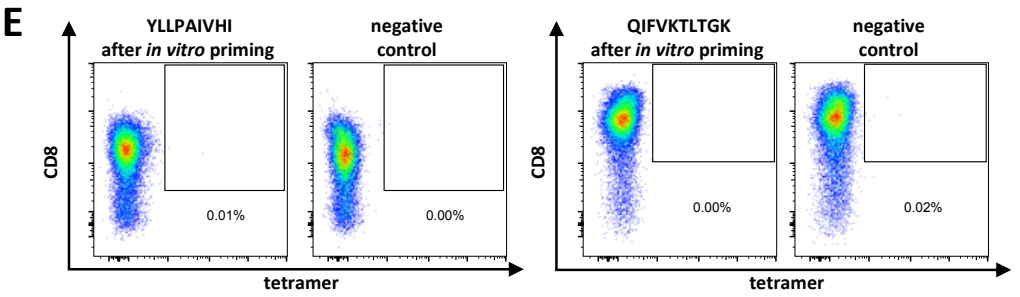
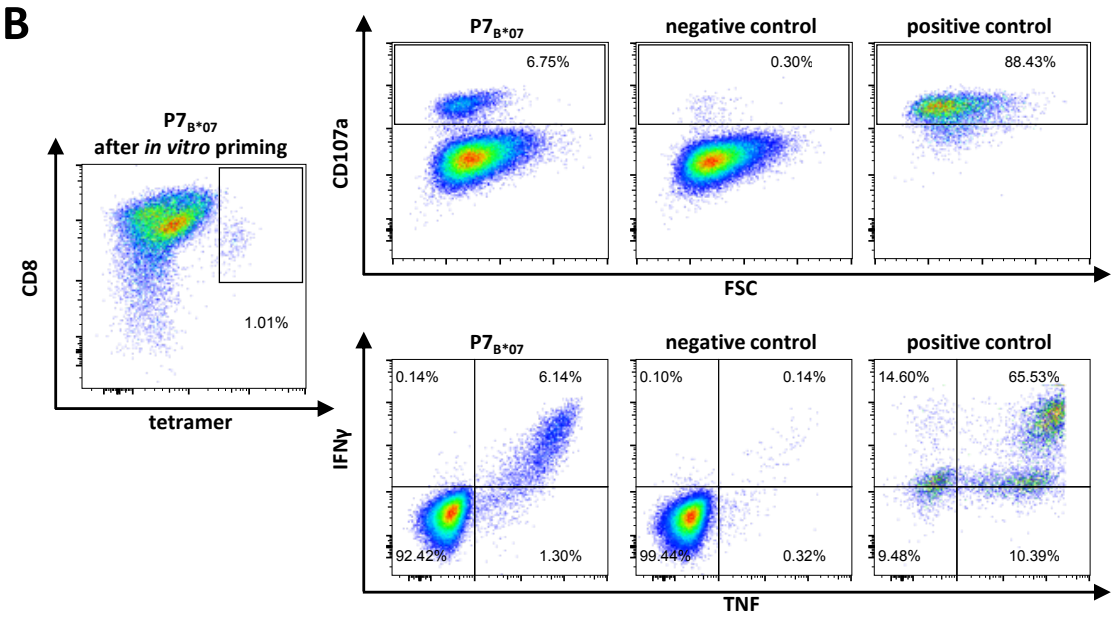
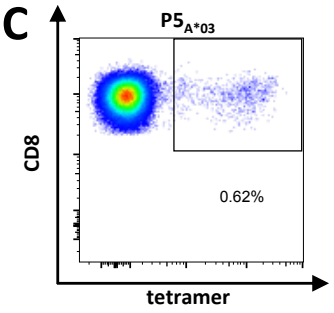


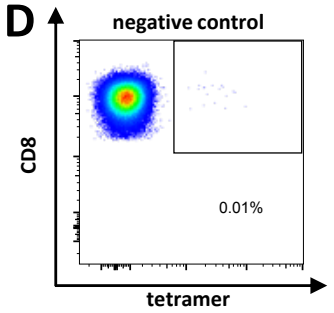
Figure 5: Immunogenicity of HLA class I-restricted CML-associated antigens. (A) Immunogenicity analysis results for the 8 HLA class I-restricted CML-associated peptides with their respective frequencies of preexisting immune recognition by PBMCs from CML patients or HVs in IFN-γ ELISPOT assays ($CD8^+$ T-cell response in CML/HVs), as well as the frequencies of peptide-specific $CD8^+$ T cells detected after *in vitro* aAPC-based priming experiments with naïve $CD8^+$ T cells from HVs and CML patients. (B) Examples of CML-associated ligands evaluated in IFN-γ ELISPOT assays after a 12-day stimulation using PBMCs from CML patients. Results are shown for immunoreactive peptides only. Phytohemagglutinin (PHA) was used as positive control and the HLA-A*02-restricted DDX5_HUMAN$_{148-156}$ peptide YLLPAIVHI served as negative control. Data are expressed as mean $\pm$ standard deviation of 2 independent replicates. Naïve $CD8^+$ T cells from (C) HVs and (D) CML patients were primed *in vitro* using aAPCs. Graphs show single viable cells stained for CD8 and PE-conjugated multimers of indicated specificity. Tetramer staining was performed after 4 stimulation cycles with peptide-loaded aAPCs. The left panels show (C) P3$_{A*03}$-tetramer or (D) P7$_{B*07}$-tetramer staining. The middle panels (negative control) depict (C) P3$_{A*03}$-tetramer or (D) P7$_{B*07}$-tetramer staining of respective T cells primed with an irrelevant peptide. The right panels show T cells from the same donor that were tested for the absence of preexisting memory T cells after (C) a 12-day recall stimulation by tetramer staining or (D) IFN-γ ELISPOT assay. (E) Tetramer staining after 4 stimulation cycles with negative control peptide-loaded aAPCs (HLA-A*02, YLLPAIVHI, DDX5_HUMAN$_{148-156}$ and HLA-A*03, QIFVKTLTGK, UBC_HUMAN$_{2-11}$). Abbreviations: ID, identification; neg., negative; n.t., not tested; pos., positive; SFU, spot-forming unit; UPN, uniform patient number.

A

Peptide ID	Peptide sequence	Source protein	ICS of peptide-specific CD8⁺ T cells	VITAL assay
$P1_{A*02}$	ALHPLVILNI	CSN6	negative	positive
$P2_{A*02}$	KQGFEPPSFV	GELS	TNF⁺ / IFNγ⁺	n.t.
$P3_{A*03}$	RTAGHPLTR	PTN7	TNF⁺ / IFNγ⁺ / CD107a⁺	n.t.
$P4_{A*03}$	HLLEQVAPK	PLSL	TNF⁺ / IFNγ⁺ / CD107a⁺	negative
$P5_{A*03}$	KIFWIPLSH	DHRS9	TNF⁺ / IFNγ⁺ / CD107a⁺	positive
$P6_{A*11}$	AVNPGVVVR	BPI	TNF⁺ / IFNγ⁺ / CD107a⁺	n.t.
$P7_{B*07}$	RAMVARLGL	CD24	TNF⁺ / IFNγ⁺ / CD107a⁺	positive
$P8_{B*07}$	APGQPLRVL	CEBPE	negative	n.t.







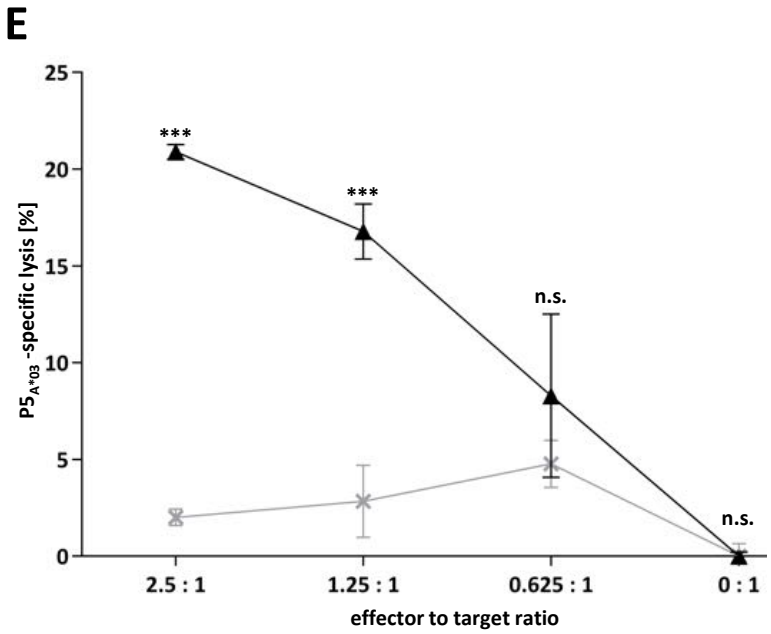
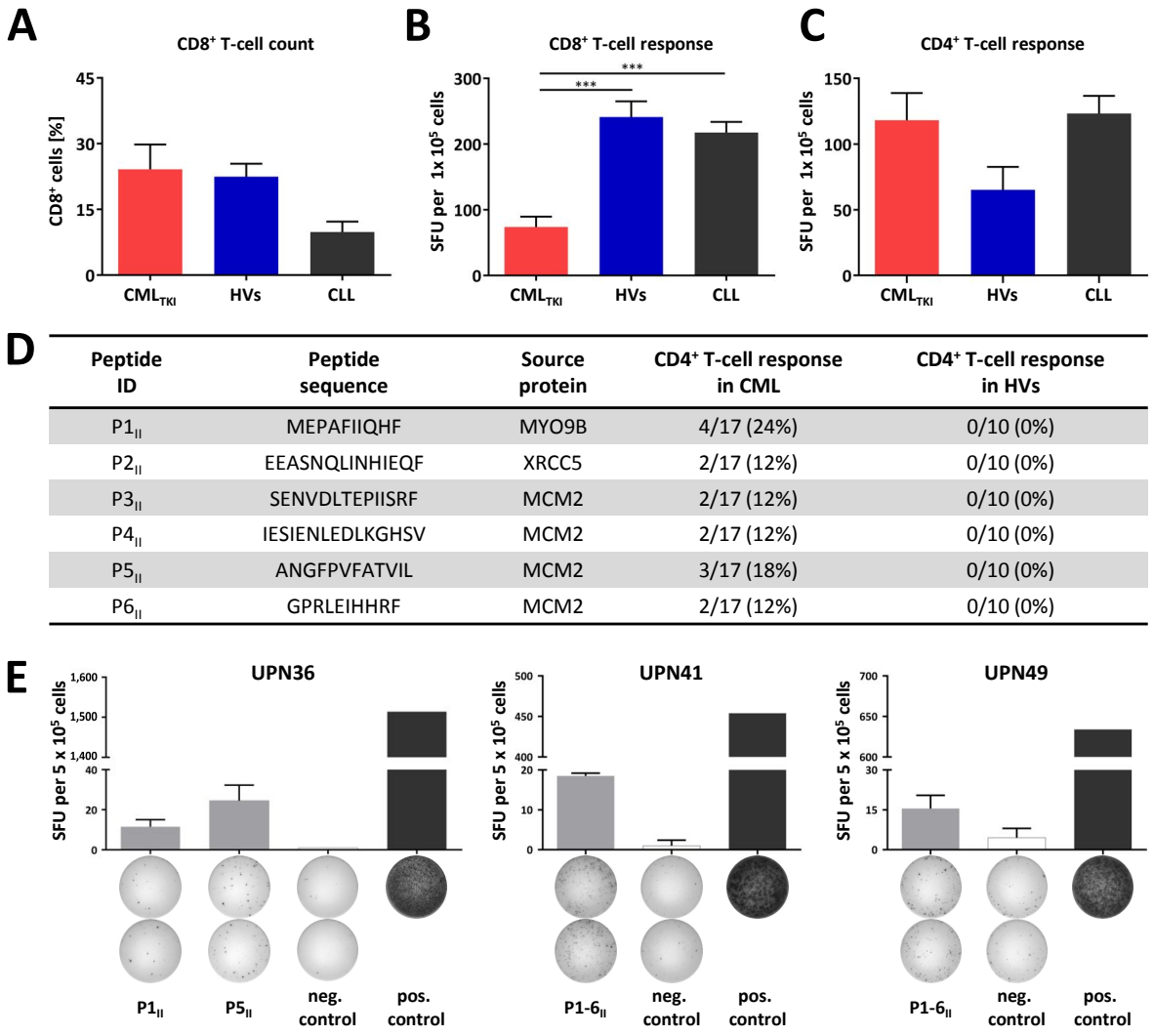


Figure 6: Functional characterization of CML-associated antigen-specific CD8$^+$ T cells. (A) Functional characterization of CML-associated antigen-specific CD8$^+$ T cells, including their CD107a and cytokine expression profile detected by ICS following aAPC-based priming experiments and their cytotoxic capability (VITAL assay). (B) Representative example of increased IFN-γ and TNF production, as well as CD107a expression, after stimulation with the respective P7$_{B*07}$-peptide used for the stimulation with aAPCs compared with the corresponding negative control peptide (HLA-B*07, TPGPGVRYPL, NEF_HV1BR$_{128-137}$). Phorbol myristate acetate (PMA) and ionomycin served as positive control. The P7$_{B*07}$-specific CD8$^+$ T-cell population showed a frequency of 1.01%, as detected by tetramer staining (far left panel). (C-E) Selective cytotoxicity of P5$_{A*03}$-specific effector T cells analyzed in a VITAL cytotoxicity assay with *in vitro* primed CD8$^+$ T cells from an HV. Tetramer staining of polyclonal effector cells before performance of the VITAL assay determined the amount of P5$_{A*03}$-specific effector cells in the population of successfully P5$_{A*03}$-primed CD8$^+$ T cells (C) and in the population of control cells (D) from the same donor primed with an HLA-matched irrelevant peptide. (E) At an effector-to-target ratio of 2.5:1, P5$_{A*03}$-specific effectors (red) exerted 20.9% ($\pm$0.4%) P5$_{A*03}$-specific and significant higher lysis of P5$_{A*03}$-loaded autologous target cells in comparison with control peptide-loaded target cells (HLA-A*03, RLRPGGKKK, GAG_HV1BR$_{20-28}$). P5$_{A*03}$-unspecific effectors (blue) only showed 2.0% ($\pm$0.4%) unspecific lysis of the same targets. Results are shown as mean $\pm$ standard error of the mean for 3 independent replicates. Abbreviations: ***, P $<$ .001. FSC, forward scatter; ID, identification; n.s., not significant; n.t., not tested.

Reduced functionality of CD8$^+$ T cells in CML$_{TKI}$ patients

Subsequently, we reasoned that weak preexisting immune responses against the CML-associated HLA class I-restricted peptides in our IFN-γ ELISPOT assays could have been caused by an impairment of CD8$^+$ T-cell functionality that reportedly occurs upon TKI treatment.[53-56] Therefore, we compared T-cell responses against viral epitopes of CML$_{TKI}$ patients, HVs, and CLL patients[38] in IFN-γ ELISPOT assays. Although CD8$^+$ T-cell counts themselves were not reduced in CML$_{TKI}$ patients (Figure 7A), we observed significantly reduced IFN-γ release by T cells compared with HVs and CLL patients (P $<$ 0.001, Figure 7B). In contrast, no significantly reduced IFN-γ production was observed upon stimulation with HLA class II-restricted viral epitopes (Figure 7C). These results were confirmed by the functional characterization of 6 HLA class II-restricted CML-associated peptides in IFN-γ ELISPOT assays (Figure 7D-E). Frequencies of CD4$^+$ T-cell responses reached up to 24% (4/17) of analyzed CML patient samples; however, some peptides were only analyzed in pooled read-outs because of low cell numbers.

Taken together, we characterized a panel of novel CML-associated HLA class I and II antigens that, even in the context of the immunosuppressive effects induced by TKI treatment, were able to induce multifunctional T-cell responses and, therefore, could serve as prime targets for the development of antigen-specific immunotherapies in CML.



D

Peptide ID	Peptide sequence	Source protein	CD4⁺ T-cell response in CML	CD4⁺ T-cell response in HVs
$P1_{II}$	MEPAFIIQHF	MYO9B	4/17 (24%)	0/10 (0%)
$P2_{II}$	EEASNQLINHIEQF	XRCC5	2/17 (12%)	0/10 (0%)
$P3_{II}$	SENVDLTEPIISRF	MCM2	2/17 (12%)	0/10 (0%)
$P4_{II}$	IESIENLEDLKGHSV	MCM2	2/17 (12%)	0/10 (0%)
$P5_{II}$	ANGFPVFATVIL	MCM2	3/17 (18%)	0/10 (0%)
$P6_{II}$	GPRLEIHHRF	MCM2	2/17 (12%)	0/10 (0%)

Figure 7: General functionality of T cells in CML_{TKI} patients and immunogenicity of HLA class II-restricted CML-associated antigens. (A) $CD8^+$ T-cell counts for CML patients under TKI treatment (CML_{TKI} patients, n = 7) compared with HVs (n = 10) and CLL patients (n 5 10). Retrospective analysis of preexisting immune responses directed against HLA class I-restricted (B) and HLA class II-restricted (C) viral T-cell epitopes (supplemental Table 3) analyzed in IFN-γ ELISPOT assays after a 12-day recall stimulation of PBMCs from CML_{TKI} patients (HLA class I, n = 10; HLA class II, n = 12), HVs (HLA class I, n = 14; HLA class II, n = 6), and CLL patients (HLA class I, n = 31; HLA class II, n = 24). (D) HLA class II-restricted CML-associated peptides with their corresponding source proteins and frequencies of preexisting immune recognition by $CD4^+$ T cells from CML patients or HVs in IFN-γ ELISPOT assays after a 12-day stimulation. (E) Examples of CML-associated HLA class II-restricted peptides evaluated in IFN-γ ELISPOT assays using PBMCs from CML patients. Results are shown for immunoreactive peptides only. PHA was used as positive control and the HLA class II-restricted FLNA_HUMAN$_{1669-1683}$ peptide ETVITVDTKAAGKGK served as negative control. Because of low cell numbers, the results for UPN41 and UPN49 are shown as pool read-outs of all 6 HLA class II-restricted CML-associated peptides. Data are expressed as mean $\pm$ standard deviation of 2 independent replicates. Abbreviations: ***, $P < .001$. ID, identification; neg., negative; pos., positive; SFU, spot-forming unit; UPN, uniform patient number.

Discussion

Several studies have shown that immunological control plays a major role in the course of disease and for treatment success in CML.[12,14,16] Therefore, various immunotherapeutic approaches are currently being evaluated,[17-20,24,29] with the main goal to achieve deep remissions that enable long-term TKI-free survival or even cure of CML patients. An attractive approach is the further development of tailored peptide-based immunotherapy, which enables specific targeting of CML cells with minor side effects. Therefore, the identification of novel naturally presented and highly frequent CML-associated target antigens is required. In this study, we present a large-scale immunopeptidomics-based approach to identify and functionally characterize such CML-associated HLA class I- and class II-restricted peptides. We confirmed strong HLA surface expression on myeloid cells, as well as on hematopoietic precursor cells, from CML patients in a range that is comparable to different healthy hematological cell types,[40,41] other HMs,[38,40,41] and solid tumors[37]; this constitutes a major prerequisite for immunotherapeutic approaches. The comprehensive comparison of HLA ligandomes from CML samples with benign tissues and PBMC samples from CML_{MR} revealed a total of 50 CML-associated HLA class I ligands for 4 of the most common HLA allotypes.[78] The allele-specific prevalence of these CML-associated targets reached up to 80%. This enables the creation of personalized multipeptide vaccine cocktails, as well as the broadly applicable off-the-shelf development of single-peptide-based immunotherapeutic approaches, such as adoptive T-cell transfer or T-cell receptor therapies.

In addition to cytotoxic $CD8^+$ T cells, $CD4^+$ T cells play important direct and indirect roles in anticancer immunity.[79-85] Thus, we expanded our profiling approach to the HLA class II peptidome identifying 19 additional CML-associated peptides. Interestingly, length distribution of HLA class II-restricted peptides could be correlated with specific cell types and lineages, because, in general, myeloid cell-derived peptides are represented by shorter peptide sequences. This is in line with the previous observations that the immunopeptidome directly mirrors cell type biology and specificity, which is reflected by the general peptide composition,[42] as well as by the length distribution of HLA-presented peptides, as demonstrated by our data.

Because spontaneous pathophysiologically relevant T-cell responses against non-mutated LAAs were described for other HMs,[38,86,87] we analyzed our CML patient cohort for pre-

existing T-cell responses against our newly defined targets. Of note, although preexisting T-cell responses against HLA class II peptides were identified with comparable frequencies as previously described for CLL,[38] acute myeloid leukemia,[40] and multiple myeloma,[41] functional T cells targeting HLA class I antigens were only of low frequency in CML_{TKI} patient samples. In line with previous studies reporting a negative[53-56] or dysregulating[88] impact of TKI treatment on immune responses, $CD8^+$ T-cell functionality in our CML_{TKI} patient cohort was impaired, potentially explaining the reduced frequencies of preexisting memory T-cell responses to CML-associated HLA class I ligands. Of note, because no CML patients without TKI treatment were included in the immunogenicity analyses, the reduced T-cell functionality could not be directly correlated with TKI treatment; it might also be linked to a general immunosuppressive state in CML disease caused, for example, by HLA-G,[89] elevated myeloid-derived suppressor cells,[12] and regulatory T cells,[12,90] as well as by increased PD-1 expression on immune cells.[12] However, the immunogenicity of all of our CML-associated HLA class I antigens was proven by *in vitro* induction of multifunctional and cytotoxic T cells from HVs. Strikingly, CML-specific T cells could also be induced *de novo* using PBMCs from CML_{TKI} patients, which qualifies the identified targets as promising candidates for peptide-based immunotherapy approaches in CML patients after termination of TKI therapy, as well as for tailored combinations with TKI treatment. Furthermore, several studies showed the pathophysiological relevance of preexisting peptide-specific T-cell responses to clinical outcomes in cancer patients,[38,86,87] suggesting that such a T-cell response, induced or boosted by peptide-based immunotherapies, might result in clinical effectiveness.

Mutated neoantigens have been described as the main specificities of anticancer T-cell responses induced by immune checkpoint inhibitors in solid tumors with high mutational burden.[91] However, only a very small fraction of mutations at the DNA sequence level results in peptides naturally presented in the HLA ligandome.[92-94] This raises the question about the relevance of mutated neoepitopes for T-cell-based immunotherapy, in particular for malignancies with low mutational burden, including CML. Despite an extensive search for naturally presented BCR-ABL- and ABL-BCR-derived peptides, none could be validated in our CML cohort by MS. However, we have to emphasize that the absence of evidence does not mean that there is evidence of absence; the sensitivity of shotgun mass spectrometric discovery approaches, even in the context of immense technical improvements in the last decades,[95] is

limited because the HLA immunopeptidome is a highly dynamic, rich, and complex assembly of peptides. Therefore, we cannot exclude low-level presentation of mutation-derived peptides in our CML patient cohort. Nevertheless, MS-based immunopeptidomics is the only unbiased methodology that can identify all naturally processed and presented HLA peptides in primary tissue samples[96]; this enables us to identify and characterize target antigens in low mutational-burden cancer entities that are non-mutated, naturally presented, highly frequent, and tumor-specific.

This is further emphasized, because the extensive screening of our CML and hematological benign cohorts for HLA-presented peptides derived from previously described CTAs[75,76] and LAAs[43,77] did not reveal any highly frequent tumor-exclusive presentation. Together with previous findings showing a distorted correlation between gene expression and HLA-restricted antigen presentation meaning that the immunopeptidome that does not mirror neither the transcriptome nor the proteome,[40,93,97–100] this precludes, in our view, these antigens as optimal candidates for T-cell-based immunotherapy. Nevertheless, tumor exclusivity can be determined at the level of HLA ligands or at the level of entire antigens. In this study, CTA and LAA analyses were performed at the level of entire antigens and do not consider presentation of CTA- and LAA-derived single HLA ligands, as they might potentially be tumor-exclusive as a result of differential antigen processing in cancer cells.

In conclusion, the cell biology-specific character of the immunopeptidome[42] calls for entity-centered identification of tumor-associated targets. Therefore, our study provides profound insights into the naturally presented immunopeptidome of CML, delineating a panel of novel, immunogenic, non-mutated, and CML-associated T-cell epitopes. These antigens aid in the development of antigen-specific therapeutic approaches that may provide options to enable achievement of deep remission, long-term TKI-free survival, or even cure for CML patients.

Acknowledgments

The authors thank Ulrike Schmidt, Claudia Falkenburger, Patricia Hrstić, Nicole Bauer, Beate Pömmerl, and Ulrich Wulle for excellent technical support.

This work was supported by the German Cancer Consortium, the Deutsche Forschungsgemeinschaft (STI 704/1-1 and SFB 685), the Wilhelm Sander Stiftung (2016.177.1), the Bundesministerium für Bildung und Forschung (031A535A), and the European Union (ERC AdG339842 MUTAEDITING).

Authorship Contribution

T.B., A.N., H.-G.R., S.S., and J.S.W. designed the study; A.N., D.J.K., H.S., A.M., J.B., and M.M. performed HLA ligandome experiments; T.B., M.L., J.R., and J.K.P. conducted *in vitro* T-cell experiments; L.B., C.-C.T., M.C.N., M.S., T.H.B., V.V., D.N., O.K., and J.S.W. provided new reagents/analytic tools/samples; H.R.S., M.C.N., M.S., T.H.B., V.V., D.N., M.R., R.K., L.K., and J.S.W. collected patient data and performed medical evaluations; T.B., A.N., L.B., D.J.K., H.S., S.S., and J.S.W. analyzed data; T.B., A.N., H.R.S., L.K., H.-G.R., S.S., and J.S.W. drafted the manuscript; and H.-G.R., S.S., and J.S.W. supervised the study.

Conflict-of-interest disclosure

D.J.K. and H.S. are employees of Immatics Biotechnologies. C.-C.T. is an employee of Immatics US. H.-G.R. is a shareholder of Immatics Biotechnologies and Curevac. The remaining authors declare no competing financial interests.

Footnotes

Submitted 31 July 2018; accepted 1 December 2018. Prepublished online as Blood First Edition paper, 10 December 2018; DOI 10.1182/blood-2018-07-866830.

*T.B. and A.N. contributed equally to this work.

The MS data reported in this article have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the PRIDE partner repository (dataset identifier PXD010450).

The online version of this article contains a data supplement.

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CHAPTER 2

Immunopeptidome-based identification of T-cell epitopes for antigen-specific immunotherapy of Philadelphia chromosome-negative myeloproliferative neoplasms

Disclosure of authorship contributions

TB was involved in all parts of this study. TB planned all experiments, analyzed and evaluated the MS-acquired data and performed *in vitro* T-cell experiments. TB drafted this chapter and designed and created all presented figures and tables.

Abstract

Myeloproliferative neoplasms are heterogeneous diseases that are characterized by a clonal proliferation of hematopoiesis and bear a substantial risk for leukemic transformation. Philadelphia chromosome-negative MPNs include PV, ET, and PMF and usually harbor one of three driver mutations in either JAK2, MPL, or CALR leading to a constitutive activation of myeloid cytokine receptors. Unspecific immunotherapy such as alloSCT and IFN-α therapy demonstrate positive effects in MPN patients, providing a rationale for the further development of more specific immunotherapy strategies including peptide-based approaches. Effective antigen-specific immunotherapy requires the knowledge of tumor-associated HLA-presented T-cell epitopes. Thus, using a mass spectrometry-based approach we here chraracterized the immunopeptidome of primary samples from Ph- MPN patients (ET, PV, PMF) to identify and characterize naturally presented tumor-associated antigens.

Comparative immunopeptidome analysis of the MPN immunopeptidomes and an extensive dataset of different hematological and non-hematological benign specimens delineated novel highly abundant non-mutated peptides with tumor-exclusive presentation. Such target antigens are especially relevant since our observations suggest the absence of naturally presented driver mutation-derived neoepitopes.

As particularly myelofibrosis bears the risk for leukemic transformation we further analyzed immunopeptidome similarities of MPN- and AML-exclusive antigens and identified a significant proportion of shared tumor-exclusive HLA class I source proteins between PMF and AML. In consequence, IFN-γ ELISPOT assays revealed preexisting spontaneous memory T-cell responses against AML-associated T-cell epitopes in PBMCs of ET and PMF patients. Observed immune responses were mediated by polyfunctional $CD4^+$ and $CD8^+$ T cells.

Taken together, these results represent a first step towards the identification and characterization of novel T-cell epitopes for the development of antigen-specific immunotherapy approaches for MPN patients.

Introduction

In recent years T-cell-based immunotherapy approaches, such as immune checkpoint inhibitors, CAR T cells, adoptive T-cell transfer, or vaccination strategies have entered the treatment landscape of hematological malignancies. In contrast to the established disease, where profound immune defects can impede T-cell-based approaches, premalignant states have the advantage of an optimal ratio of functional T cells *versus* malignant cells.[1] This can reinforce immunotherapy approaches early in the premalignant lesions to allow for the achievement of an effective immunological control and thereby impede progress to the established disease. The Ph- myeloproliferative neoplasms essential thrombocythemia, polycythemia vera, and myelofibrosis represent such premalignant lesions that bear a substantial risk for leukemic transformation to the established AML.[2,3]

Several studies suggest that a functional immune system and particularly the T-cell-mediated immune response are of great importance to decelerate or even prevent leukemic transformation. This is endorsed by the profound immune dysregulation reflected by the deregulated cytokine milieu and immune phenotype in MPN patients and thus the defective immune surveillance, which is believed to contribute to disease evolution and progress.[4–7] Reduced circulating DCs, impaired monocyte differentiation, and a reduced Th1 compartment have been observed in advanced high-risk myelofibrosis patients.[8] The general hyperinflammatory state contributes to an enhanced PD-1/PD-L1 expression and thus to suppression of T-cell responses.[9–11] In addition, increased numbers of immunosuppressive cells such as MDSCs - that are even more T-cell suppressive than the MDSCs in HVs - can further hamper immune responses.[12] Evasion of immune surveillance can furthermore be reinforced by down-regulation of HLA class I and HLA class II expression.[13,14]

Unspecific immunotherapy such as alloSCT, DLI, and IFN-α achieved promising results and revealed beneficial therapeutic effects, including the normalization of the cytokine milieu and depletion of malignant clones.[15–21] Such approaches build the foundation for more advanced strategies targeting malignant cells more specifically. These strategies are represented for example by HLA-dependent peptide-based approaches such as the therapeutic cancer vaccination.[22] Employment of peptide-based vaccines is especially attractive due to the low side effect profile of this therapeutic strategy. In order to generate an effective antigen-specific therapy, the knowledge of suitable immunogenic tumor-associated target antigens is indis-

pensable. The rationale for the use of such immunogenic antigens has been provided for example by Holmström *et al.* using predicted peptides derived from JAK2 and CALR driver mutations. These neoepitopes were recognized by specific T cells of MPN patients and even HVs, demonstrating frequent spontaneous cellular immune responses and thus the high immunogenicity of these antigens.[23,24] However, it has to be kept in mind that only few mutations result in peptides that are naturally presented in the HLA ligandome.[25–27] Therefore, the relevance of neoepitopes for low mutational burden malignancies such as MPNs in the context of T-cell-based immunotherapy remains inconclusive.

We here aimed for the MS-based identification of naturally presented antigens using primary samples of MPN patients. We identified novel, non-mutated, frequently and naturally presented MPN-associated antigens, which are especially relevant since our observations suggest the absence of naturally presented driver mutation-derived neoepitopes. As particularly myelofibrosis bears the risk for leukemic transformation we detected shared tumor-exclusive antigens between PMF and AML and furthermore assessed preexisting spontaneous memory T-cell responses against AML-associated T-cell epitopes in MPN patients.

Results

HLA expression on myeloid and precursor cells of primary samples

T-cell-based immunotherapy requires sufficient HLA expression on target cells, which are represented by myeloid cells and myeloid precursor cells in MPNs. As a first step, we quantified HLA surface expression on CD33⁺ and CD13⁺ myeloid cells using isolated PBMCs and granulocytes (Figure 1A, supplemental Table S1). Additionally, we quantified the HLA expression on CD34⁺ precursor cells within primary PBMC samples of PMF patients only (Figure 1B), since CD34⁺ are most abundant in the peripheral blood of PMF patients in comparison to ET or PV patients.[28,29]

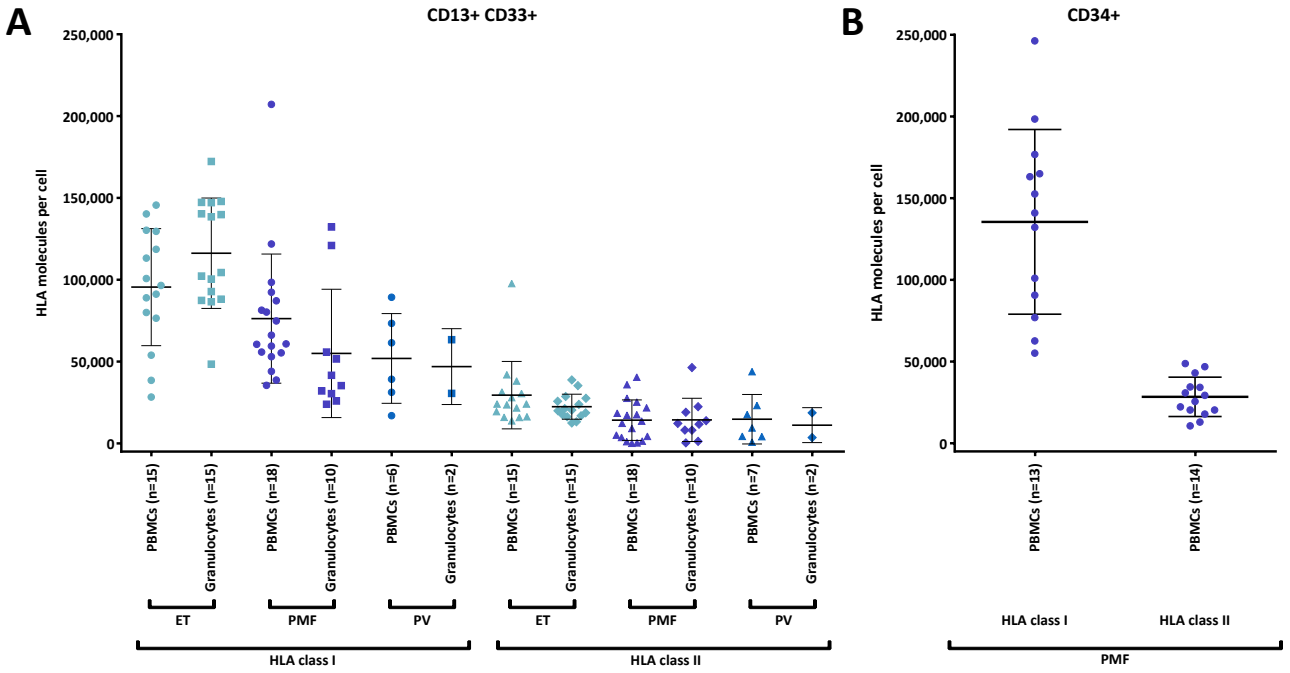

Figure 1: HLA surface expression on primary cells from ET, PMF, and PV patients. HLA class I and HLA-DR expression determined for (A) CD33$^+$ and CD13$^+$ myeloid cells within the isolated PBMCs or granulocytes from peripheral blood of ET, PMF, and PV patients. (B) HLA class I and HLA-DR expression on CD34$^+$ precursor cells within the PBMCs of PMF patients. Data points represent individual samples. Horizontal lines indicate mean values ± standard deviation.

HLA class I and II surface levels showed substantial heterogeneity with a generally decreased HLA class II molecule expression throughout the cohorts. Within the CD33⁺ and CD13⁺ myeloid cell compartment the HLA class I surface levels on PBMCs from ET patients (n = 15) averaged 95,507 HLA molecules/cell and on granulocytes (n = 15) 116,248 HLA molecules/cell. Myeloid cells of PMF patients displayed 76,288 HLA class I molecules/cell within the PBMCs (n = 18) and 54,998 molecules/cell within the granulocytes (n = 10). HLA

class I molecule expression on myeloid cells of PV patients averaged 51,936 molecules/cell within the PBMCs (n = 6) and 46,965 molecules/cell within the granulocytes (n = 2).
HLA class II expression on myeloid cells within the PBMCs of ET patients (n = 15) added up to 29,517 and within the granulocytes (n = 15) to 22,460 molecules/cell. Myeloid cells of PMF patients displayed 14,200 HLA class II molecules/cell within the PBMCs (n = 18) and 14,364 molecules/cell within the granulocytes (n = 10). HLA class II molecule expression on myeloid cells of PV patients averaged 14,791 molecules/cell within the PBMCs (n = 7) and 11,190 molecules/cell within the granulocytes (n = 2). The $CD34^+$ progenitor cells of PMF patients expressed an average of 135,532 HLA class I molecules/cell (n = 13) as well as 28,409 HLA class II molecules/cell (n = 14). Together, these results prove sufficient HLA expression for immunopeptidome analyses of Ph- MPNs.

MS-based identification of naturally presented HLA class I ligands

MS-based HLA class I ligandome analysis of primary samples obtained from 7 ET patients identified a total of 6,347 unique HLA ligands (mean 1,290 per sample) derived from 3,844 unique source proteins (mean 1,248 per sample), obtaining 73% of the maximum attainable protein coverage (Table 1, supplemental Table S2, supplemental Figures S1A, S2A). HLA class I ligandome analysis using primary samples of 13 PV patients revealed a total of 14,604 unique HLA ligands (mean 1,424) from 6,422 unique source proteins (mean 1,346), obtaining 77% of the maximum attainable protein coverage (Table 1, supplemental Figures S1C, S3A) Immunopeptidome analysis of primary samples from 21 PMF patients identified a total of 30,863 unique HLA ligands (mean 2,283) from 8,517 unique source proteins (mean 1,798), obtaining 89% of the maximum attainable protein coverage (Table 1, Figure 2A, supplemental Figure S1E). Combined analysis of primary samples obtained from ET, PV, and PMF patients (MPN, n = 41) revealed a total of 37,384 unique HLA ligands (mean 1,663) from 9,516 unique source proteins (mean 1,727), obtaining 92% of the maximum attainable protein coverage (Table 1, supplemental Figure S4A). Due to the ongoing sample acquisition and the maximum attainable source protein coverage of $\leq$80% within the ET and PV datasets, further extensive evaluations are performed solely for the PMF dataset in a separate results section.
For the identification of MPN-associated antigens, we established a comparative cohort

of hematological benign tissues (n = 130), including PBMCs (n = 63), granulocytes (n = 32), T cells (n = 1), CD19⁺ B cells (n = 6), bone marrow (n = 20), and CD34⁺ HPCs (n = 8). A total of 54,419 (mean 1,251) naturally presented HLA class I ligands from 11,445 (mean 1,091) source proteins were identified. To achieve a more stringent selection of MPN-associated targets, we compared the HLA ligandomes of the MPN cohorts with the immunopeptidome of our hematological benign cohort in addition to an extensive dataset comprising 30 different benign non-hematological tissue entities (n = 221) including liver, lung, brain, and skin. The non-hematological benign dataset includes a total of 66,457 (mean 1,570) unique HLA class I ligands from 13,669 (mean 1,380) unique source proteins. The source proteins of both - hematological and non-hematological benign tissues - combined obtained 99% of the maximum attainable protein coverage (supplemental Figure S5A).

Dataset	HLA class I							HLA class II						
	unique ligands	unique proteins	samples	patients	tissue types	tumor-exclusive		unique peptides	unique proteins	samples	patients	tissue types	tumor-exclusive	
						ligands	proteins						peptides	proteins
PMF	30,863 (mean 2,283)	8,517 (mean 1,798)	29	21	2	7,956	149	17,989 (mean 1,779)	2,602 (mean 527)	28	20	2	6,108	174
PV	14,604 (mean 1,424)	6,422 (mean 1,346)	15	13	2	2,556	105	8,041 (mean 1,495)	1,511 (mean 481)	10	6	2	1,873	47
ET	6,347 (mean 1,290)	3,844 (mean 1,248)	8	7	2	584	14	5,335 (mean 1,285)	1.244 (mean 483)	8	7	2	868	29
MPN	37,384 (mean 1,663)	9,516 (mean 1,727)	52	41	2	10,305	257	21,837 (mean 1,500)	2,938 (mean 601)	46	33	2	7,706	222
hematological benign	54,419 (mean 1,251)	11,445 (mean 1,091)	130	130	6	-	-	43,477 (mean 1,232)	4,893 (mean 432)	89	89	6	-	-
benign tissues	66,457 (mean 1,570)	13,669 (mean 1,380)	221	14	30	-	-	152,096 (mean 2,466)	9,883 (mean 729)	223	17	29	-	-

Table 1: Overview of HLA peptide and source protein yields per cohort. Overview of HLA peptide and source protein yields identified in the ET, PV, PMF, MPN, hematological benign, and non-hematological benign tissue cohorts together with the number of samples, patients, and tissue types. Comparative analysis of the MPN immunopeptidomes with the total benign immunopeptidome dataset revealed various amounts of tumor-exclusive peptides and source proteins.

Comparative HLA class I ligandome profiling using our hematological benign dataset revealed 974 (15%) ET-, 3,508 (24%) PV-, 10,550 PMF- (34%), and 13,660 (37%) MPN-exclusive unique HLA class I ligands (Figure 2B-C, supplemental Figures S2C-D, S3C-D, S4C-D). Using our total dataset of benign tissue samples (n = 351, 120,876 ligands from 25,114 source proteins) we identified 584 (9%) ET-, 2,556 (18%) PV-, 7,956 (26%) PMF-, and 10,305 (28%) MPN-exclusive HLA class I ligands, so far (Table 1). An evaluation on the protein level using the hematological benign dataset revealed 48 (1%) ET-, 408 (6%) PV-, 552 (6%) PMF-, and 896 (9%) MPN-exclusive unique source proteins (supplemental Figure S6). Subsequent comparison with the total benign tissues dataset identified 14 (0.4%) ET-, 105 (2%) PV-, 149 (2%) PMF-, and 257 (3%) MPN-exclusive source proteins (Table 1).

Identification of PMF-associated HLA class I ligands

The PMF cohort included a total of 38 different HLA class I allotypes, with the most frequent being HLA-A*02 (n = 12), HLA-C*03 (n = 8), HLA-B*51 (n = 7), HLA-C*07 (n = 7), HLA-A*24 (n = 6), HLA-B*15 (n = 6), HLA-C*04 (n = 6; supplemental Figure S7A). Among the world population, 99.9% of individuals carry ≥1 HLA class I allotype that is represented within this PMF cohort (supplemental Figure S8A).[30,31] The comparative hematological benign cohort also showed an HLA allotype population coverage of 99.9% (supplemental Figure S8B) and matched the allotypes within the PMF cohort (supplemental Figure S7B).

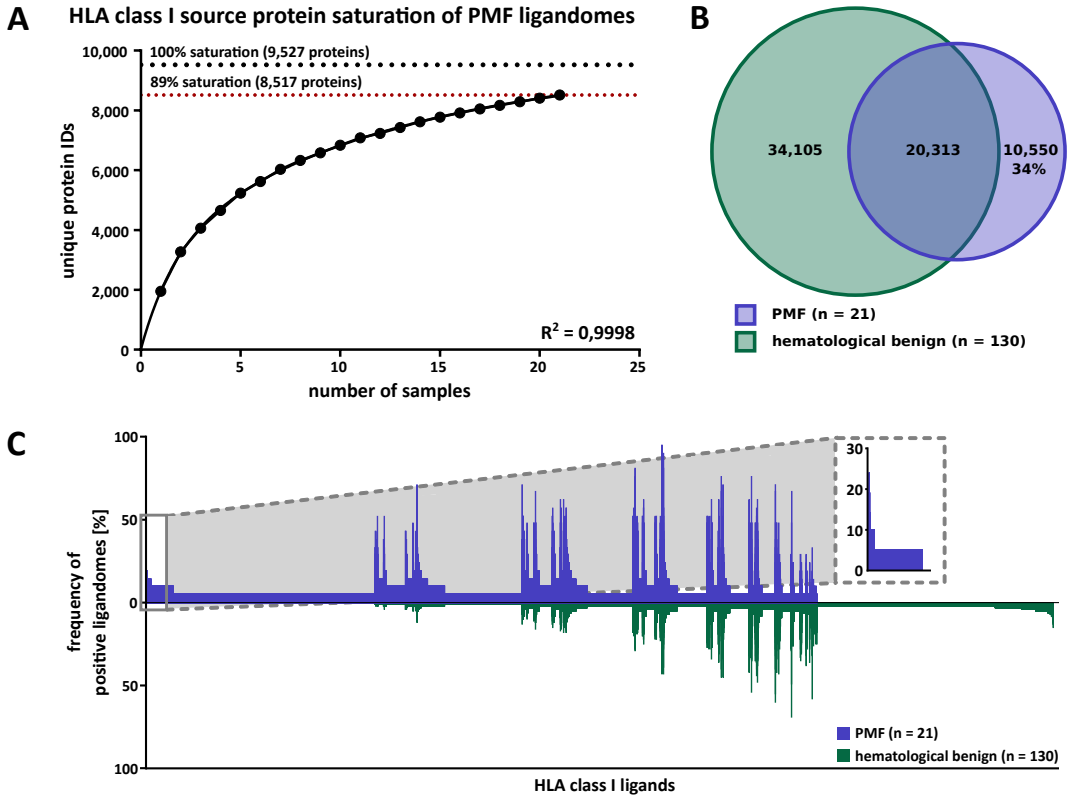

Figure 2: Comparative HLA class I ligandome profiling for PMF. (A) Saturation analysis of HLA class I source proteins within the PMF patient cohort. Unique source protein identifications are shown as a function of cumulative HLA ligandome analyses. Exponential regression allowed for the robust calculation ($R^2 = 0.9998$) of the maximum attainable source protein coverage (dotted black line). The dashed red line depicts the source proteome coverage achieved in our PMF cohort. (B) Overlap analysis of HLA class I ligand identifications in primary PMF samples (n = 21) and hematological benign samples (n = 130). Created using BioVenn.[32] (C) Comparative profiling of HLA class I ligands based on the frequency of HLA-restricted presentation in PMF and hematological benign immunopeptidomes. Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. To allow for better readability, HLA ligands identified on ≤2% of the hematological benign samples are not depicted in this plot. The box on the left and its magnification highlight PMF-exclusive antigens. Abbreviation: IDs, identifications.

To identify PMF-associated antigens, we performed comparative HLA class I ligandome profiling of the PMF cohort with the hematological and non-hematological benign dataset. Overlap analysis revealed exclusive presentation of 7,956 HLA class I ligands on PMF samples (Table 1), that were never detected on hematological or non-hematological benign tissue samples. For the identification of broadly applicable PMF-associated antigens, we aimed for the selection of target antigens that are not only PMF-exclusive, but also highly abundant within the PMF cohort.

Setting the target-definition FDR to <5% (<1%) we identified a total of 22 (3) HLA class I ligands with a representation frequency of $\geq$19% ($\geq$24%), which are listed in Table 2 (supplemental Figure S9A). The most common HLA allotype restriction of these highly frequent PMF-associated peptides was HLA-B*51.

An evaluation on source protein level using the hematological benign dataset revealed 552 PMF-exclusive unique source proteins (supplemental Figure S6). Subsequent comparison with the total benign tissues dataset identified 149 PMF-exclusive proteins. However, at a target-definition FDR of <5% or <1% none of the PMF-exclusive source protein targets was represented with a high frequency within the PMF cohort (supplemental Figure S9B).

To identify PMF-associated targets on peptide level with even higher representation frequencies, we subsequently performed HLA allotype-adjusted comparative immunopeptidome profiling with the respectively HLA allotype-adjusted immunopeptidome of our PMF cohort and the total dataset of hematological and non-hematological benign tissues. In preliminary analyses for selected HLA allotypes we identified 2 (8) HLA-A*02-restricted ligands with representation frequencies of $\geq$33% ($\geq$25%), respectively (supplemental Table S3, supplemental Figure S10A-B). In addition, we identified 1, 7, and 20 HLA-B*51-restricted ligands with representation frequencies of $\geq$71%, $\geq$57%, and $\geq$43%, respectively (supplemental Table S3, supplemental Figure S10C-D).

Source protein	Peptide sequence	Peptide length	HLA restriction	Representation frequency in PMF cohort	Representation frequency in HLA-matched PMF samples	Length variants on benign tissues
S23IP	DSFLGQTSI	9	B*51	24%	71%	-
PDC6I	HAAELIKTV	9	B*51, C*03, C*12	24%	n.a.	-
MYD88	SSVPRTAEL	9	B*15, C*03	24%	n.a.	-
TKT	AISESNINL	9	A*02	19%	33%	-
C3AR	DAFLSTHL	8	B*51	19%	57%	-
MED24	DALLEQAMI	9	B*51	19%	57%	-
*	DFPVAMQI	8	B*51	19%	57%	hematological
TLK1	DGFAFQNLV	9	B*51	19%	57%	-
**	EALGRLLVV	9	B*51	19%	57%	non-hematological
SRP54	GQFTLRDMY	9	B*15	19%	67%	-
FUS	GSYGSSSQSSSY	12	A*01, B*15	19%	n.a.	-
XPO2	KLWTPLLQSL	10	A*02, A*32	19%	n.a.	-
***	KQVTVLELF	9	B*15	19%	67%	-
MCMBP	KVNDILELY	9	A*01, B*15	19%	n.a.	-
OR9K2	LLGNVGMMTI	10	A*02	19%	33%	-
MYD88	QADPTGRLL	9	C*03	19%	50%	-
PO210	QALELPLRI	9	B*13, B*15, B*51	19%	n.a.	both
HBM	SPLADLHAL	9	B*07, B*35	19%	n.a.	-
FUS	TIESVADYF	9	A*01, A*25, A*29, C*05	19%	n.a.	non-hematological
CTR9	VPPEILNNV	9	B*51	19%	57%	-
MOT4	VPPVFVVSY	9	B*35	19%	80%	-
****	YLAAVLEYL	9	A*02, B*15	19%	n.a.	-

Table 2: Identified PMF-associated HLA class I ligands. Panel of naturally presented, PMF-associated, HLA class I-restricted ligands identified by HLA class I ligandome profiling of primary PMF samples (n = 21) and a hematological and non-hematological benign tissues dataset (n = 351). Peptides mapping in multiple proteins are marked with asterisks: *CLH1, CLH2; **HBB, HBD, HBE; ***GTR3, GTR14; ****H2A1A, H2A1B, H2A1C, H2A1D, H2A1H, H2A1J, H2A1, H2A1B, H2A3, H2AJ, H2AX, H2AB1, H2AB2. Abbreviation: n.a., not applicable.

Comparative HLA class II ligandome profiling delineates additional MPN-associated antigens

Mapping the HLA class II ligandomes of primary ET samples obtained from 7 patients, we identified a total of 5,335 unique peptides (mean 1,285 per sample) derived from 1,244 unique source proteins (mean 483 per sample), obtaining 77% of the maximum attainable protein coverage (Table 1, supplemental Table S2, supplemental Figures S1B, S2B). HLA class II ligandome analysis of primary PV samples derived from 6 patients revealed a total of 8,041 unique peptides (mean 1,495) from 1,511 unique source proteins (mean 481), obtaining 76% of the maximum attainable protein coverage (Table 1, supplemental Figures S1D, S3B). Immunopeptidome analysis of primary samples from 20 PMF patients identified a total of 17,989 unique peptides (mean 1,779) from 2,602 unique source proteins (mean

527), obtaining 81% of the maximum attainable protein coverage (Table 1, Figure 3A, supplemental Figure S1F). Combined analysis of primary samples from ET, PV, and PMF patients (MPN, n = 33) revealed a total of 21,837 unique peptides (mean 1,500) from 2,938 unique source proteins (mean 601), obtaining 84% of the maximum attainable protein coverage (Table 1, supplemental Figure S4B). Due to the ongoing sample acquisition and the maximum attainable source protein coverage of $\leq$80% within the ET and PV datasets, extensive evaluations are performed solely for the PMF dataset in a separate results section.

For the identification of MPN-associated antigens, we established a comparative cohort of hematological benign tissues (n = 89), including PBMCs (n = 36), granulocytes (n = 20), $CD19^+$ B cells (n = 10), bone marrow (n = 15), and $CD34^+$ HPCs (n = 8). A total of 43,477 (mean 1,232) naturally presented HLA class II peptides from 4,893 (mean 432) source proteins was identified. To achieve a more stringent selection of MPN-associated targets, we compared the HLA ligandomes of the MPN cohorts with the immunopeptidome of our hematological benign cohort in addition to an extensive dataset comprising 29 different benign non-hematological tissue entities (n = 223) including liver, lung, brain, and skin. The non-hematological benign dataset includes a total of 152,096 (mean 2,466) unique peptides from 9,883 (mean 729) unique source proteins. The source proteins of both - hematological and non-hematological benign tissues - combined obtained 91% of the maximum attainable protein coverage (supplemental Figure S5B).

Comparative HLA class II ligandome profiling using our hematological benign dataset revealed 1,262 (24%) ET-exclusive, 2,625 (33%) PV-exclusive, 7,972 PMF-exclusive (44%), and 10,241 (47%) MPN-exclusive unique HLA class I ligands (Figure 3B-C, supplemental Figures S2E-F, S3E-F, S4E-F). Using our total dataset of benign tissue samples (n = 116, 195,573 peptides from 14,777 source proteins) we identified 868 (16%) ET-, 1,873 (23%) PV-, 6,108 (34%) PMF-, and 7,706 (35%) MPN-exclusive peptides, so far (Table 1).

An evaluation on the source protein level using the hematological benign dataset revealed 96 (8%) ET-, 154 (10%) PV-, 516 (20%) PMF-, and 648 (22%) MPN-exclusive unique source proteins (Figure 3D, supplemental Figure S2G-H, S3G-H, S4G-H). Subsequent comparison with the total benign tissues dataset identified 29 (2%) ET-, 47 (3%) PV-, 174 (7%) PMF-, and 222 (8%) MPN-exclusive source proteins (Table 1).

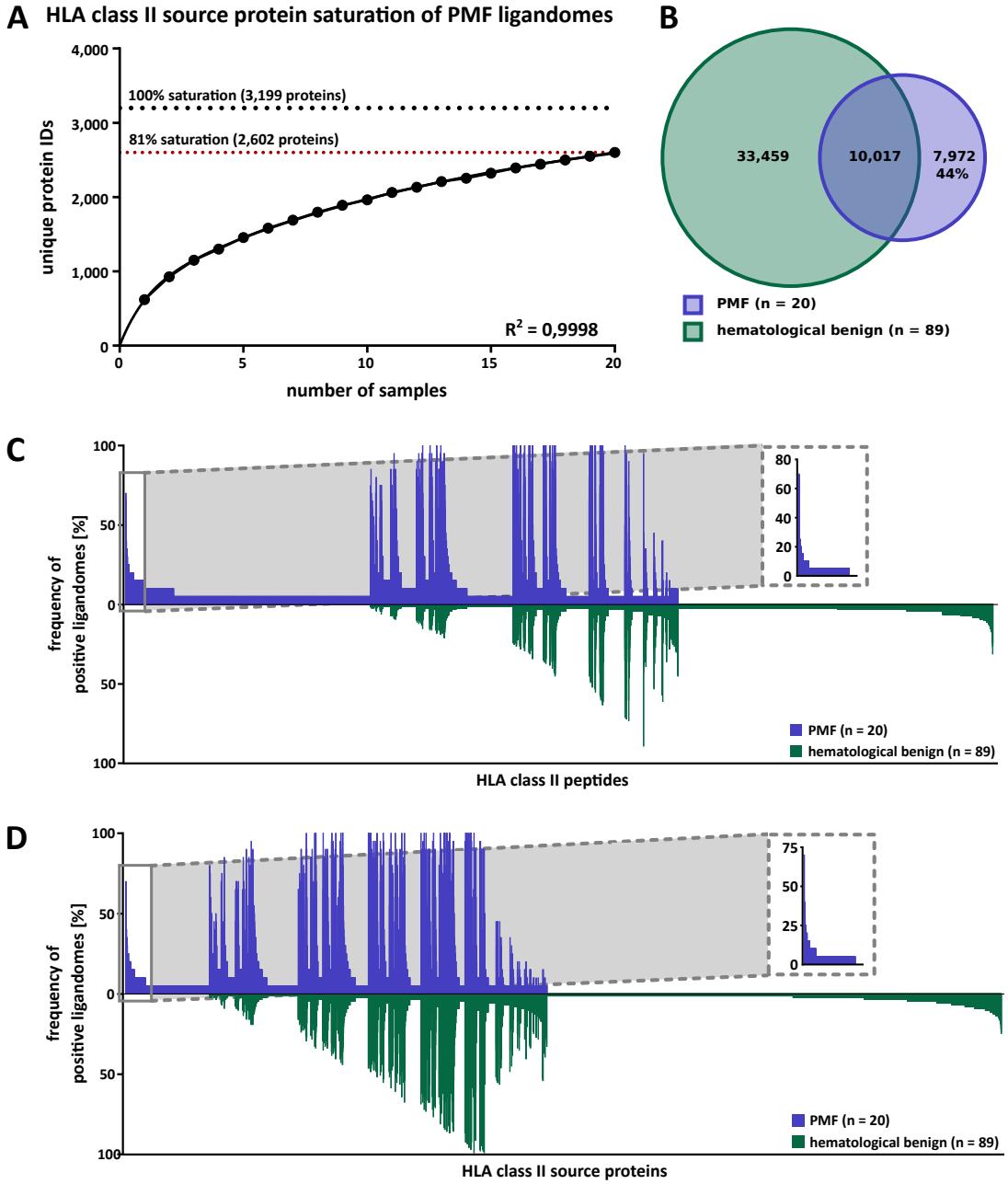

Figure 3: Comparative HLA class II ligandome profiling for PMF. (A) Saturation analysis of HLA class II source proteins within the PMF patient cohort. Unique source protein identifications are shown as a function of cumulative HLA ligandome analyses. Exponential regression allowed for the robust calculation ($R^2 = 0.9998$) of the maximum attainable source protein coverage (dotted black line). The dashed red line depicts the source proteome coverage achieved in our PMF cohort. (B) Overlap analysis of HLA class II peptide identifications in primary samples from 20 PMF patients and 89 different hematological benign samples. Created using BioVenn.[32] Comparative profiling of (C) HLA class II peptides and (D) source proteins based on the frequency of HLA-restricted presentation in PMF and hematological benign immunopeptidomes. Frequencies of positive immunopeptidomes for the respective peptides or proteins (x-axis) are indicated on the y-axis. The boxes on the left and their magnifications highlight PMF-exclusive antigens. Abbreviation: IDs, identifications.

Identification of novel HLA class II-restricted PMF-associated antigens

For the identification of HLA class II-restricted PMF-associated antigens, we previously established an HLA class II ligandome profiling platform to investigate three groups of antigens: peptide targets, protein targets, and hotspot targets.[33] To identify peptide targets we performed comparative immunopeptidome profiling on HLA class II peptide level. Overlap analysis using our hematological benign dataset revealed 7,972 peptides that were presented exclusively on PMF samples (Figure 3B-C) and were never detected on hematological benign samples. Comparative analysis with the immunopeptidome dataset of all hematological benign and non-hematological benign tissue samples revealed 6,108 PMF-exclusive peptides (Table 1). Of these, 34 peptides were identified with a representation frequency of $\geq$30% and a target-definition FDR of <1%. However, 28 of these 34 peptide targets showed length variants (>50% overlap) presented on benign samples and thus were excluded from further analyses (peptide targets, Table 3, supplemental Figure S9C).

For the identification of protein targets, ligandome profiling was performed at the HLA class II source protein level. Overlap analysis using our hematological benign dataset revealed 516 PMF-exclusive source proteins (Figure 3D). Comparative analysis with the immunopeptidome dataset of both hematological and non-hematological benign tissue samples revealed 176 PMF-exclusive proteins (Table 1). Based on a target-definition FDR of <5% (<1%), a total of 9 (8) source proteins were identified with a frequency of $\geq$15% ($\geq$20%) representing 24 (21) unique HLA class II peptides (protein targets, Table 3, supplemental Figure S9D).

The third group of hotspot targets was analyzed by peptide clustering: identified peptides were mapped to their amino acid positions within their source protein. Representation frequencies of aa counts within each cohort for the respective aa position were calculated. The hotspot analysis validated the peptide and protein targets and revealed 7 additional PMF-associated hotspots with representation frequencies of $\geq$30% comprising 24 unique HLA class II peptides (hotspot targets, Table 3, supplemental Figure S11).

Interestingly, the hotspot of Ras-related protein Rab-27A (RB27A) was also identified as a CML-associated hotspot target and included 3 of the 7 PMF-associated peptides although with lower frequencies in the CML cohort.[33] The peptide and hotspot target tyrosine-protein kinase SYK is reported to be expressed in HPCs and neutrophils.[34,35] The ETS-related transcription factor Elf-4 is expressed abundantly in cell lines of myeloid leukemia and in the

placenta.[36,37] The Cas scaffolding protein family member 4 is expressed with a high abundance in leukemia cell lines.[38] Finally, from the peptides comprised within these three groups of peptide, protein, and hotspot targets we will select a panel for further immunological characterization, as described in **Chapter 1**.[33]

Protein	Sequence	Peptide length	Representation frequency	Peptide target	Protein target	Hotspot target
Peptide targets						
*	**ETS-related transcription factor Elf-1/-2/-4**					
	GRALRYYYQRGILAK	15	40%	x	x	x
KSYK	**Tyrosine-protein kinase SYK**					
	SFPKPGHRKSSPAQGNRQESTV	22	35%	x		x
RB27A	**Ras-related protein Rab-27A**					
	FDLTNEQSFLNV	12	30%	x		x
VRK1	**Serine/threonine-protein kinase VRK1**					
	KFYQRAAKPEQIQKWIRTRKLKY	23	30%	x		x
PKN1	**Serine/threonine-protein kinase N1**					
	RNPVIERIPRLRRQKKIF	18	30%	x		x
DHX8	**ATP-dependent RNA helicase DHX8**					
	YNRYEEPNAWRISRAFRRR	19	30%	x		x
Protein targets						
*	**ETS-related transcription factor Elf-1/-2/-4**		**40%**			
	GRALRYYYQRGILAK	15	40%	x	x	x
TBD2A	**TBC1 domain family member 2A**		**40%**			
	KFGGKGPIRGWKSRWFF	17	5%		x	x
	FGGKGPIRGWKSRWF	15	5%		x	x
	GGKGPIRGWKSRWF	14	25%		x	x
	GGKGPIRGWKSRWFF	15	15%		x	x
	GPIRGWKSRWF	11	5%		x	x
	RAGVPREHRPRVWRWL	16	5%		x	x
FGD3	**FYVE, RhoGEF and PH domain-containing protein 3**		**30%**			
	RLTDAGIPPEVIMGIFS	17	5%		x	x
	VWKLQWAKQSWY	12	5%		x	x
	DSGHVWKLQWAKQSWY	16	20%		x	x
	DSGHVWKLQWAKQSWYL	17	5%		x	x
LPIN2	**Phosphatidate phosphatase LPIN2**		**25%**			
	KSGRWWFWRK	10	5%		x	x
	SGRWWFWRK	9	20%		x	x
	GRWWFWRK	8	20%		x	x
	IFTPSSVKKKKRRRKKY	17	15%		x	x
MP2K3	**Dual specificity mitogen-activated protein kinase kinase 3**		**20%**			
	KQVVEEPSPQLPADRFSPEFV	21	5%		x	
	MSKPPAPNPTPPRNLDSRTFI	21	5%		x	
	ILRFPYESWGTPFQQLKQV	19	5%		x	
	TIGDRNFEVEADDLVTI	17	5%		x	
	ILRFPYESWGTPF	13	5%		x	
CASS4	**Cas scaffolding protein family member 4**		**20%**			
	RLYFGALFKAISA	13	20%		x	
ASCL2	**Achaete-scute homolog 2**		**15%**			
	AVEYIRALQRLLAEH	15	10%		x	
	VEYIRALQRLLAE	13	5%		x	
	SAVEYIRALQRLLAEHDAV	19	5%		x	

Protein	Sequence	Peptide length	Representation frequency	Peptide target	Protein target	Hotspot target
Hotspot targets						
RB27A	**Ras-related protein Rab-27A**		**30%**			
	AFFRDAMGFLLL	12	5%			x
	LLLFDLTNEQSFLNV	15	5%			x
	LLFDLTNEQSFLNV	14	5%			x
	LFDLTNEQSFLNV	13	5%			x
	FDLTNEQSFLNV	12	30%	x		x
	FDLTNEQSFL	10	5%			x
	DLTNEQSFLNV	11	5%			x
SYLC	**Leucine--tRNA ligase, cytoplasmic**		**40%**			
	TLRERNKIKFGKRY	14	25%			x
	TLRERNKIKFGKRYT	15	5%			x
	TLRERNKIKFGKRYTIYSPK	20	5%			x
	YDSFVRWQFL	10	5%			x
	YDSFVRWQFLTLRERNKIKFGKRY	24	5%			x
	YDSFVRWQFLTLRERNKIKFGKRYT	25	10%			x
FLII	**Protein flightless-1 homolog**		**35%**			
	WSDVFIWLGRKSPRLV	16	15%			x
	WSDVFIWLGRKSPRLVRAA	19	20%			x
	WSDVFIWLGRKSPRLVRAAAL	21	5%			x
IMB1	**Importin subunit beta-1**		**30%**			
	MLVQPRVEFILSF	13	10%			x
	VQPRVEFILSF	11	25%			x
PKN1	**Serine/threonine-protein kinase N1**		**35%**			
	TFRNPVIERIPRLRRQKKIF	20	15%			x
	RNPVIERIPRLRRQKKIF	18	30%	x		x
VRK1	**Serine/threonine-protein kinase VRK1**		**35%**			
	KFYQRAAKPEQIQKWIRTRKLKY	23	30%	x		x
	KFYQRAAKPEQIQKWIRTR	19	10%			x
	KFYQRAAKPEQIQKWIRTRKL	21	10%			x
	FYQRAAKPEQIQKWIRTRKLKY	22	5%			x
	YQRAAKPEQIQKWIRTRKLKY	21	20%			x
	YQRAAKPEQIQKWIRTRKL	19	5%			x
KSYK	**Tyrosine-protein kinase SYK**		**35%**			
	SFPKPGHRKSSPAQGNRQES	20	5%			x
	SFPKPGHRKSSPAQGNRQESTV	22	35%	x		x

Table 3: Identified PMF-associated HLA class II antigens. Naturally presented, PMF-associated HLA class II antigens identified by comparative ligandome profiling of primary samples from 20 PMF patients and our hematological as well as non-hematological benign tissues dataset (n = 312). Peptides are assigned to the respective group of target antigens (peptide targets, protein targets, hotspot targets). Peptides mapping in multiple proteins are marked with asterisks: *ELF1, ELF2, ELF4.

Identification of naturally presented CALR-, JAK2-, and MPL-derived peptides in primary samples from ET, PV, and PMF patients

HLA class I immunopeptidome analysis of primary samples obtained from ET, PV, and PMF patients revealed 26 CALR-, 33 JAK2-, and 14 MPL-derived naturally presented HLA ligands. Of these, 6 JAK2- and 1 MPL-derived peptides showed MPN-exclusive but low frequent presentation. Analysis of the HLA class II-restricted peptides identified 350 CALR-

derived peptides containing a variety of length variants as well as 6 JAK2- and 1 MPL-derived peptide. Of these, 2 CALR- and 1 JAK2-derived peptides were presented exclusively on samples from MPN patients.

To allow for a better overview, the percentage of samples presenting at least one CALR-, JAK2-, or MPL-derived peptide within the cohorts of ET, PV, PMF, and all MPN patients as well as within the hematological, non-hematological, and total benign tissues dataset is illustrated in Figure 4A-B. HLA class I ligands derived from JAK2 are detected on a higher proportion of MPN samples compared to benign tissues. Notably, HLA class I ligands derived from the thrombopoietin receptor MPL could not be identified on any non-hematological benign tissue (Figure 4A). Only few naturally presented JAK2- and MPL-derived peptides with HLA class II restriction were identified in our cohorts (Figure 4B). CALR-derived HLA class I and II peptides were represented equally within the analyzed cohorts.

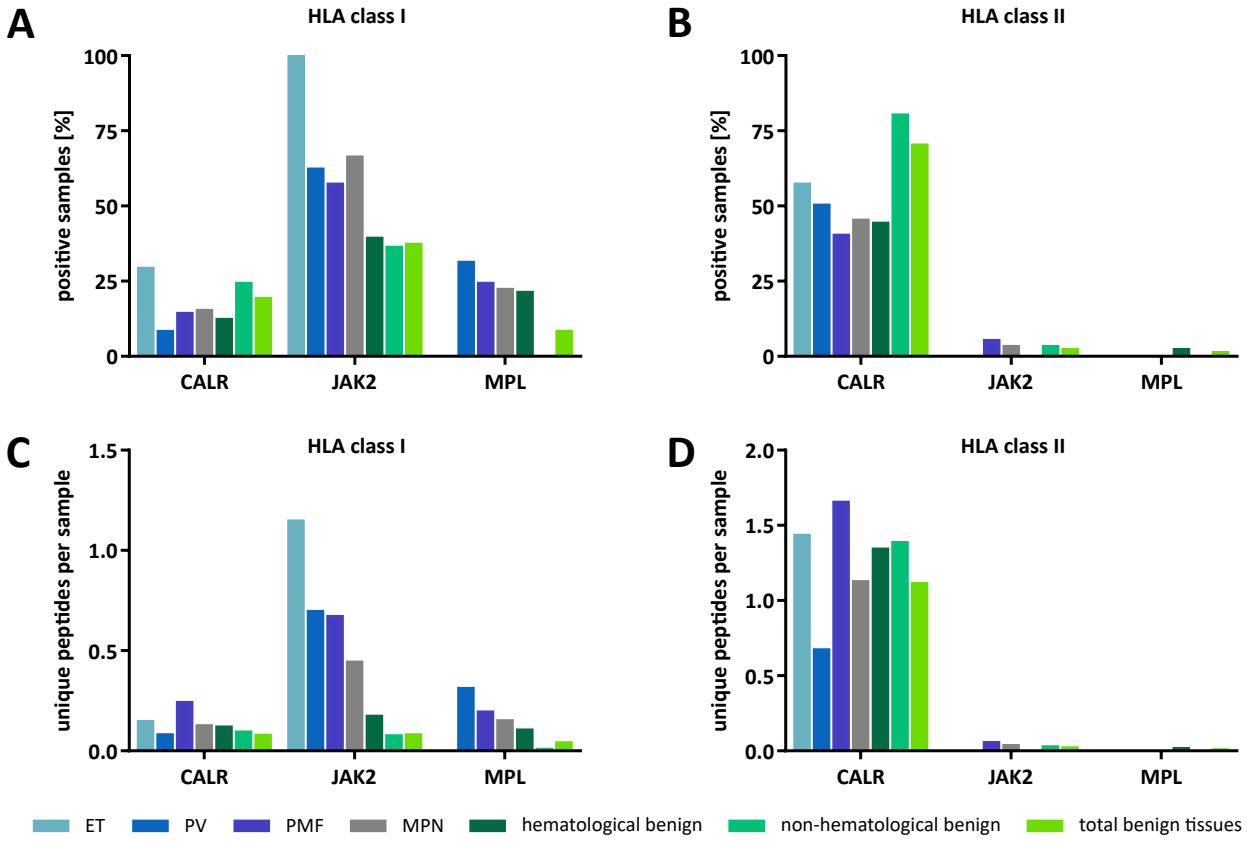

Figure 4: Analysis of CALR-, JAK2-, and MPL-derived peptides. (A) Proportion of samples presenting at least one CALR-, JAK2-, or MPL-derived (A) HLA class I- or (B) class II-restricted peptide within the cohorts of ET, PV, PMF, and combined MPN patients as well as within the hematological benign, non-hematological benign tissues and the total benign tissues dataset. Number of unique (C) HLA class I- or (D) class II-restricted peptides per sample that were detected within each cohort.

The number of unique HLA class I ligands per sample that were detected within each cohort (Figure 4C) is equally distributed for CALR-derived peptides but shows a tendency towards an enhanced representation within the MPN patient cohorts for JAK2- and MPL-derived ligands. Regarding the number of unique HLA class II-restricted peptides per sample only few if any JAK2- and MPL-derived peptides were identified within our datasets (Figure 4D). For CALR-derived HLA class II peptides the amount of unique peptides per sample was distributed equally throughout the cohorts.

Further analyses using peptide clustering confirmed the absence of highly abundant MPN-associated peptides and hotspots (Figure 5) within CALR, JAK2, and MPL.

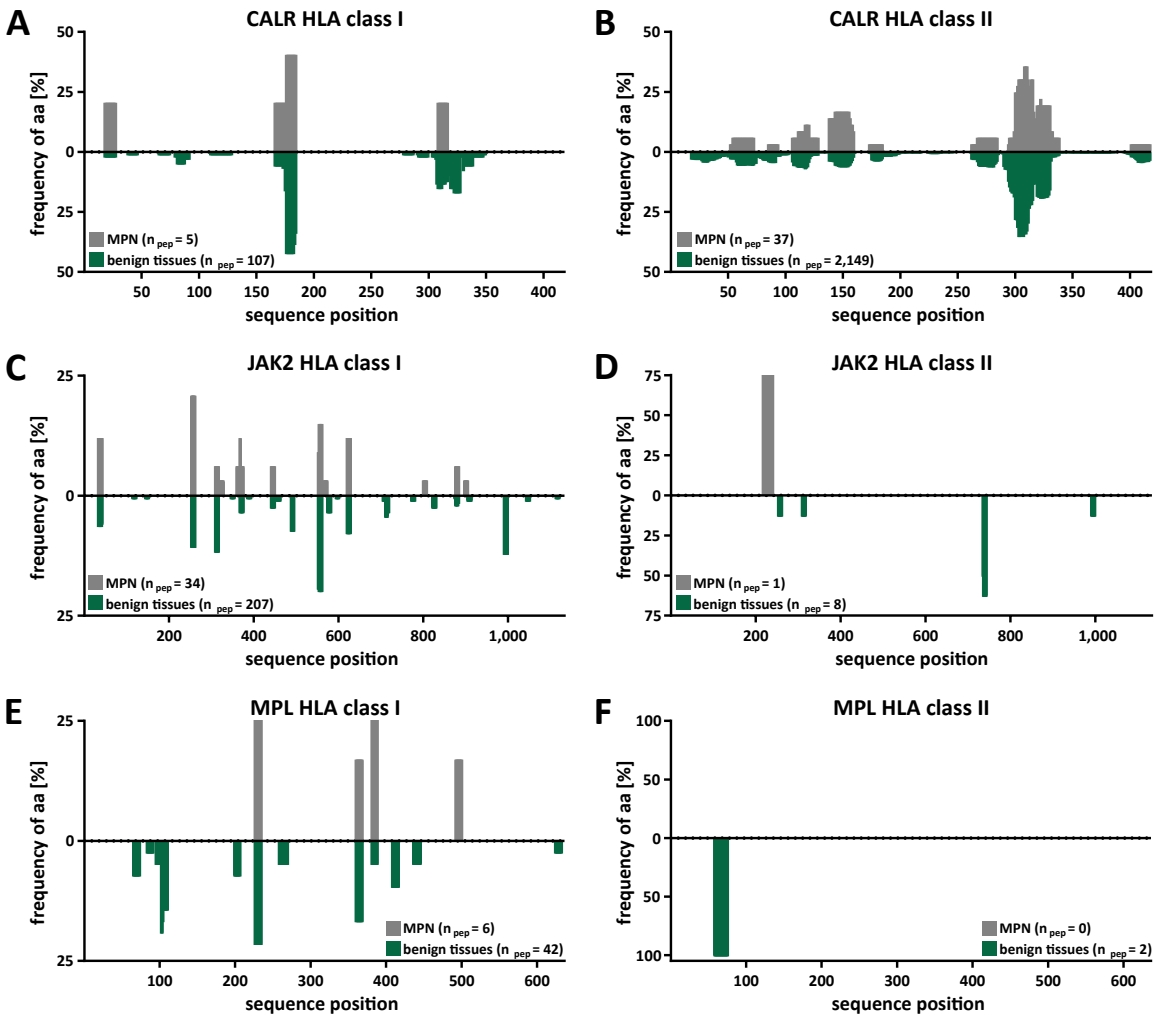

Figure 5: Hotspot analysis of CALR, JAK2, and MPL by peptide clustering. Identified peptides were mapped to their amino acid positions within the respective source protein. Representation frequencies of amino acid counts within each cohort for the respective amino acid position (x-axis) were calculated and are indicated on the y-axis. Abbreviations: aa, amino acid; n_{pep}, number of peptides.

Prediction of potential neoepitopes from the most common CALR, JAK2, and MPL mutations

To date, more than 50 CALR mutations with a shared consensus sequence of 34 amino acids have been identified in MPN patients. The two most frequent mutations type 1 (deletion of 52 base pairs, 1092_1143del or L367fs*46) and type 2 (insertion of 5 base pairs, 1154_1155insTTGTC or K385fs*47) occur in 80% of patients with a CALR exon 9 mutation.[39–42] The most frequent point mutation in JAK2 is the substitution V617F. Other mutations of JAK2 comprise V617I, K607N, K539L, FHK537-539L, and HK538-539QL.[43–45] For MPL the most frequent mutations reported are the substitutions P106L, S505N, W515A, W515K, and W515L.[46–52] For the prediction of possible neoepitopes we used the protein sequences comprising the mutations described above and segmented these sequences in peptides of 8-12 amino acids length harboring the mutated area. Using this peptide list, we performed an HLA class I annotation using SYFPEITHI 1.0[53] and NetMHC 4.0.[54] The cut-off for ligands was set to a NetMHC rank value of $\leq$2.0 and a SYFPEITHI score of $\geq$60%. The predicted potential neoepitopes are plotted together with their annotated HLA class I-restriction in the supplemental Figure S12.

Notably, for the mutations of JAK2 only few neoepitopes could be predicted, which comprised an HLA class I motif compared to the MPL or CALR mutations.

Identification of naturally presented neoepitopes

The top 100 recurrent AML-associated missense mutations in addition to the most common NPM1 and FLT3-ITD mutations (rendered by Annika Nelde) were supplemented with JAK2, MPL, and CALR mutations (supplemental Table S4) and subsequently used for the identification of neoepitopes within the MPN immunopeptidome datasets. To minimize false positive identifications, we applied stringent filter criteria with 5% FDR for HLA class I and 1% for HLA class II, XCorr $\geq$1, and ΔScore $\geq$0.2, which is a measure of the difference between the XCorr for the two best peptide sequences annotated to a distinct spectrum.

We could not detect any neoepitopes derived from the common driver mutations in CALR, JAK2, or MPL within the immunopeptidomes of our MPN patients.

Similarity assessment of the immunopeptidomes in the ET, PV, PMF, and AML cohorts

The myeloproliferative neoplasms ET and PV represent precursor conditions that can progress to the premalignant post-ET-MF or post-PV-MF and further to the established AML. Here, immunopeptidome data of ET, PV, and PMF were compared with each other and with the immunopeptidome data of AML, which were generated and evaluated by Annika Nelde.[55] Overlap analysis of tumor-exclusive ligands (supplemental Figure S13A) and peptides (supplemental Figure S13B) revealed few similarities in the group of ET, PV, and PMF. Similar distributions were observed with the AML dataset on peptide level (data not shown), which is likely due to variant HLA allotype distributions within each cohort. Overlap analysis of tumor-exclusive HLA class I and II source proteins revealed even less analogies between the MPNs (supplemental Figure S13C-D). In contrast to the ET- and PV-exclusive HLA class I source proteins, about 50% of the PMF-exclusive proteins were also found to be AML-exclusive (Figure 6A), which underlines the biological proximity of PMF and AML. Comparison of MPN-exclusive HLA class II source proteins with the AML-exclusive proteins in turn revealed equally distributed resemblance between the MPN entities (Figure 6B).

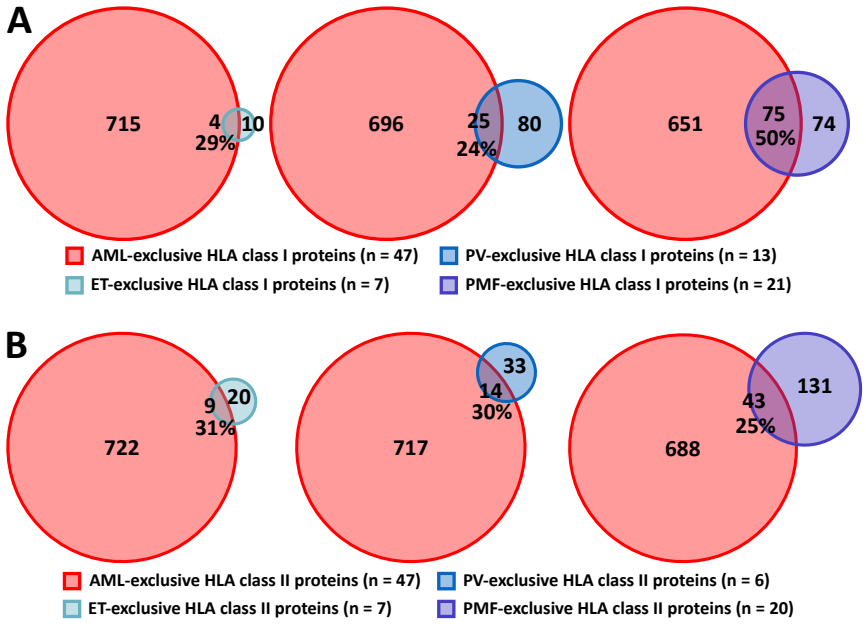

Figure 6: Overlap analysis of ET-, PV-, PMF-, and AML-exclusive source proteins. (A) Overlap analysis of tumor-exclusive HLA class I source proteins identified in primary samples of 7 ET, 13 PV, 21 PMF, and 47 AML patients. (B) Overlap of tumor-exclusive HLA class II source proteins identified in samples of 7 ET, 6 PV, 20 PMF, and 47 AML patients. Created using BioVenn.[32]

T-cell-based assays reveal preexisting immune responses directed against AML-associated antigens in ET and PMF patients *in vitro*

Based on the biological proximity and the detected immunopeptidome overlap of PMF and AML, we conducted T-cell-based experiments using AML-associated peptides (identified and characterized by Annika Nelde[55]) in order to assess preexisting memory T-cell responses against these AML targets. Therefore, we performed IFN-γ ELISPOT assays following a 12-day *in vitro* stimulation using PBMCs from ET and PMF patients. Using a panel of 12 HLA-A*01-, -A*02-, -B*07-, and -C*07-restricted peptides we detected preexisting T-cell responses directed against all three HLA-A*01-restricted AML targets in 2/4 (50%) PMF patients (Figure 7A-B, supplemental Figure S14). The observed immune response was mediated by multifunctional $CD8^+$ peptide-specific T cells showing increased IFN-γ and TNF production and upregulation of the degranulation marker CD107a upon stimulation with the respective test peptide (Figure 7C). We could not detect any preexisting HLA class I-directed memory T-cell response using PBMCs of ET patients, so far.

Using HLA class II-restricted AML-associated peptides we detected multifunctional predominantly $CD4^+$ T cells in 2/12 (17%) ET patients and 5/11 (45%) PMF patients (Figure 8, supplemental Figure S14).

Notably, upon 12-day *in vitro* stimulation of PBMCs obtained from an ET patient (UPN01) we detected preexisting immune responses directed against 13/15 (87%) AML-associated T-cell epitopes (Figure 9). The peptide-specific T cells were predominantly $CD4^+$ and multifunctional (TNF^+, IFN-γ^+, $CD107a^+$, IL-2^+, $CD154^+$), as detected in ICS.

A

Peptide ID	Target type	Peptide sequence	Source protein	T-cell response in ET	ICS in ET	T-cell response in PMF	ICS in PMF	T-cell response in HVs
$P1_{A*01}$	peptide target	DIDTRSEFY	ARP2	n.t.	n.a.	1/4 (25%)	n.a.	0/10 (0%)
$P2_{A*01}$	peptide target	FSEYFGAIY	MYNN	n.t.	n.a.	1/4 (25%)	n.a.	0/10 (0%)
$P3_{A*01}$	peptide target	YLDGRLEPLY	CEBPA	n.t.	n.a.	2/4 (50%)	$CD8^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$	0/10 (0%)
$P4_{A*02}$	peptide target	SLLEADPFL	CCNA1	0/6 (0%)	n.a.	0/6 (0%)	n.a.	0/10 (0%)
$P5_{A*02}$	peptide target	IILDALPQL	DOCK8	0/6 (0%)	n.a.	0/6 (0%)	n.a.	0/10 (0%)
$P6_{A*02}$	peptide target	ILNDVAMFL	RUS1	0/6 (0%)	n.a.	0/6 (0%)	n.a.	0/10 (0%)
$P7_{B*07}$	peptide target	APESKHKSSL	STT3B	0/1 (0%)	n.a.	0/3 (0%)	n.a.	0/10 (0%)
$P8_{B*07}$	peptide target	APGLHLEL	IRF7	0/1 (0%)	n.a.	0/3 (0%)	n.a.	0/10 (0%)
$P9_{B*07}$	peptide target	APTIVGKSSL	OST48	0/1 (0%)	n.a.	0/3 (0%)	n.a.	0/10 (0%)
$P10_{C*07}$	peptide target	SRPPLLGF	UBE3B, UBE3C	0/2 (0%)	n.a.	0/4 (0%)	n.a.	0/8 (0%)
$P11_{C*07}$	peptide target	AYHELAQVY	CSN3	0/2 (0%)	n.a.	0/4 (0%)	n.a.	0/8 (0%)
$P12_{C*07}$	peptide target	IYSGYIFDY	RALY	0/2 (0%)	n.a.	0/4 (0%)	n.a.	2/8 (25%)

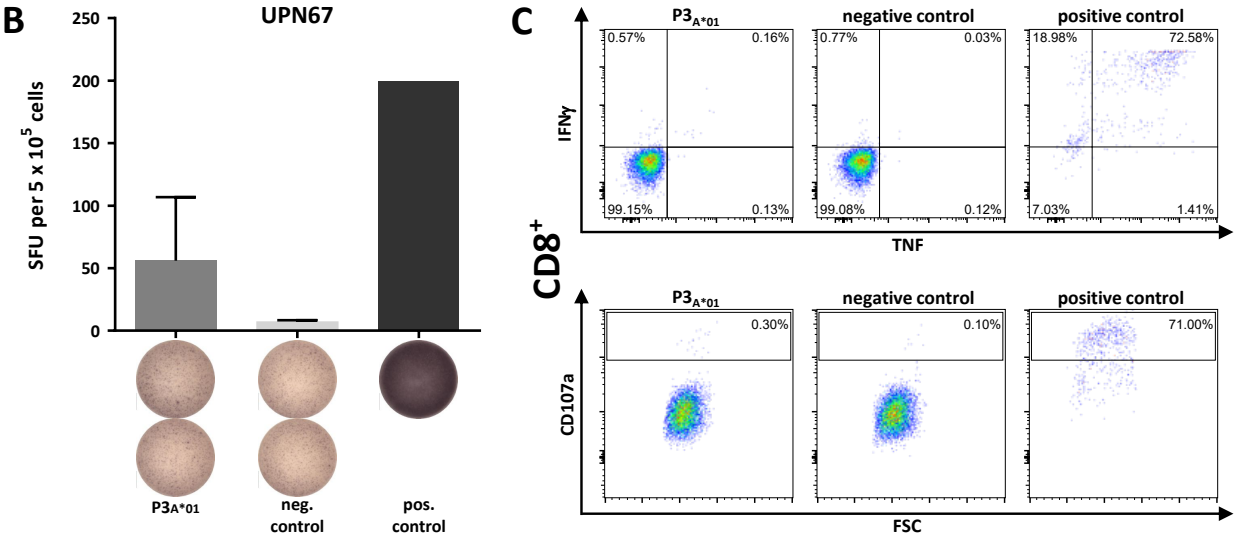

Figure 7: Preexisting HLA class I-directed T-cell responses in ET and PMF patients. (A) Assessment of preexisting memory T-cell responses using HLA class I-restricted AML-associated peptides with respective frequencies of immune recognition by PBMCs from ET and PMF patients or HVs in IFN-γ ELISPOT assays (T-cell response in ET/PMF/HVs) together with the subsequent functional characterization of peptide-specific T cells detected by ICS. T-cell responses in HVs were assessed by Annika Nelde.[55] (B) Results of the IFN-γ ELISPOT assay after 12-day stimulation using PBMCs of a PMF patient. Results are shown for the immunoreactive peptide only. PHA was used as positive control and the HLA-A*03-restricted GAG_HV1BR$_{20-28}$ peptide RLRPGGKKK served as negative control. Data are expressed as mean $\pm$SD of two independent replicates. (C) Cytokine expression profile of peptide-specific T cells from UPN67 showing increased IFN-γ and TNF production, as well as CD107a expression upon stimulation with the respective $P3_{A*01}$-peptide compared to the HLA-A*03-restricted negative control. PMA and ionomycin served as positive control. Abbreviations: ID, identification; n.t., not tested; n.a., not available; UPN, uniform patient number; SFU, spot forming unit; neg., negative; pos., positive; FSC, forward scatter.

A

Peptide ID	Target type	Peptide sequence	Source protein	T-cell response in ET	ICS results in ET	T-cell response in PMF	ICS results in PMF	T-cell response in HVs	ICS results in HVs
$P1_{II}$	peptide target	GNQLFRINEANQLMQ	GALNT7	1/12 (8%)	$CD4^+$ $CD154^+$	2/11 (18%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$	3/15 (20%)	$CD4^+$ $IFN\gamma^+$ TNF^+
$P2_{II}$	peptide target	DRQQMEALTRYLRAAL	CLC11	1/12 (8%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$ $IL\text{-}2^+$ $CD154^+$	1/11 (9%)	negative	1/15 (7%)	n.t.
$P3_{II}$	hotspot target	VVGYALDYNEYFRDL	HPRT	1/12 (8%)	$CD4^+$ $CD154^+$	0/11 (0%)	-	1/14 (7%)	n.t.
$P4_{II}$	LPC-associated protein target	DRVKLGTDYRLHLSPV	TACT (CD96)	1/12 (8%)	negative	0/11 (0%)	-	2/14 (14%)	n.t.
$P5_{II}$	neoepitope	KLKKMWKSPNGTIQNILGGTVF	IDH2 R140Q	1/12 (8%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $IL\text{-}2^+$ $CD154^+$	2/11 (18%)	negative	5/15 (33%)	$CD4^+$ $IFN\gamma^+$ TNF^+
$P6_{II}$	peptide target	LGQEVALNANTKNQKIR	APOB	1/12 (8%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $IL\text{-}2^+$ $CD154^+$	0/11 (0%)	-	1/15 (7%)	$CD4^+$ $IFN\gamma^+$ TNF^+
$P7_{II}$	peptide target	NGRTFHLTRTLTVK	IL1AP	1/12 (8%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $IL\text{-}2^+$ $CD154^+$	1/11 (9%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$	5/15 (33%)	$CD4^+$ $IFN\gamma^+$ TNF^+
$P8_{II}$	protein target	SKPGVIFLTKKGRRF	CCL23	1/12 (8%)	negative	0/11 (0%)	-	4/15 (27%)	$CD4^+$ $IFN\gamma^+$ TNF^+
$P9_{II}$	hotspot target	SPGPFPFIQDNISFYA	FLT3	2/12 (17%)	$CD8^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$	0/11 (0%)	-	1/14 (7%)	negative
$P10_{II}$	hotspot target	LDTMRQIQVFEDEPAR	IL1AP	1/12 (8%)	negative	1/11 (9%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$	0/14 (0%)	-
$P11_{II}$	hotspot target	IGSYIERDVTPAIM	KIT	1/12 (8%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$ $CD154^+$	1/11 (9%)	negative	0/14 (0%)	-
$P12_{II}$	LPC-exclusive peptide target	PHRKKKPFIEKKKAVSFHLVHR	LTV1	1/12 (8%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $IL\text{-}2^+$ $CD154^+$	0/11 (0%)	-	2/14 (14%)	$CD4^+$ $IFN\gamma^+$ TNF^+
$P13_{II}$	LPC-associated peptide target	KHLHYWFVESQKDPEN	PPGB	1/12 (8%)	negative	0/11 (0%)	-	0/14 (0%)	-
$P14_{II}$	LPC-associated peptide target	ETLHKFASKPASEFVK	ITAL	0/12 (0%)	-	0/11 (0%)	-	0/14 (0%)	-
$P15_{II}$	LPC-associated protein target	ERPEWIHVDSRPF	G6PC3	0/12 (0%)	$CD4^+$ $IFN\gamma^+$ TNF^+ $CD107a^+$ $CD154^+$	0/11 (0%)	-	0/14 (0%)	-

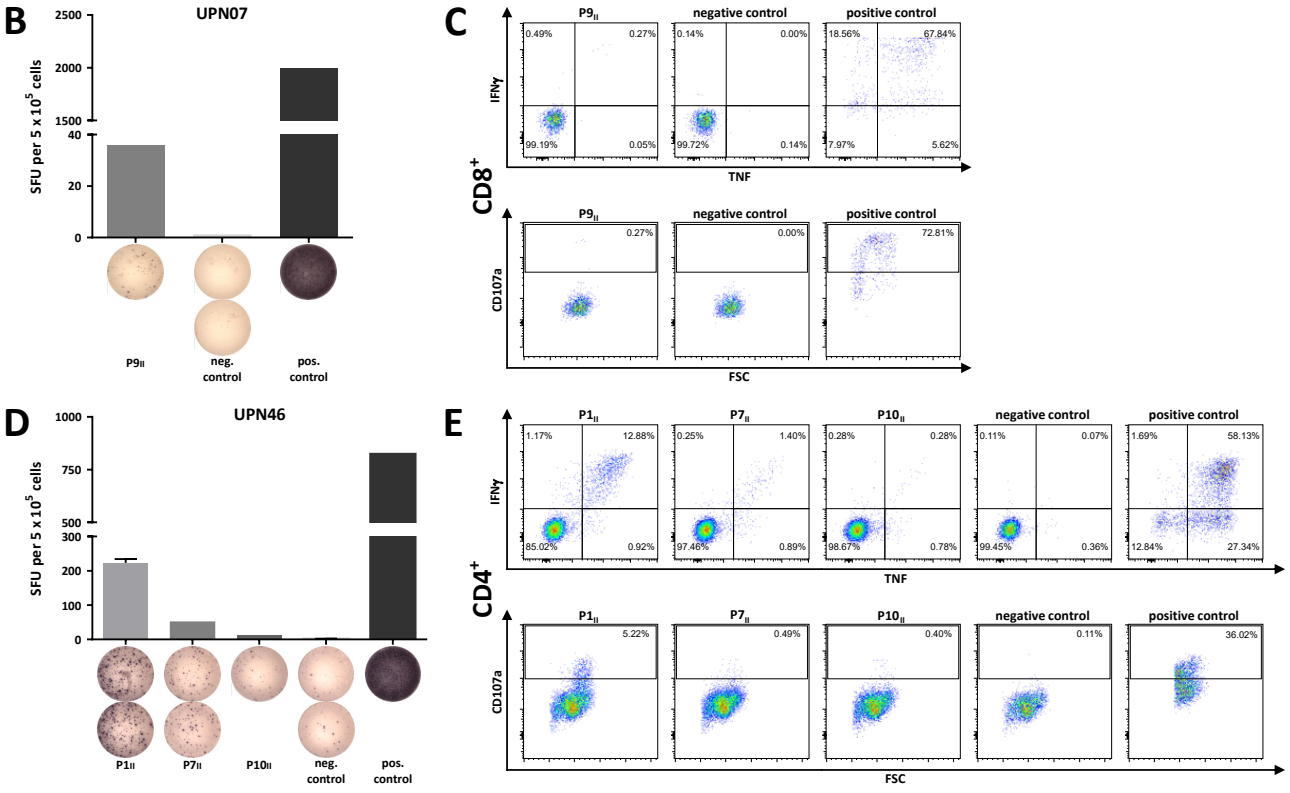

Figure 8: Preexisting HLA class II-directed T-cell responses in ET and PMF patients. (A) Results of analyses for preexisting memory T-cell responses using HLA class II-restricted AML-associated peptides. Respective frequencies of preexisting immune recognition by PBMCs from ET and PMF patients or HVs in IFN-γ ELISPOT assays (T-cell response in ET/PMF/HVs) together with the subsequent functional characterization of peptide-specific T cells, including their CD107a and cytokine expression profile detected by ICS. T-cell responses and functional characterization in HVs were assessed by Annika Nelde.[55] (B-E) Representative results of the IFN-γ ELISPOT assay after 12-day stimulation and subsequent ICS using PBMCs of (B) an ET patient (UPN07) and (D) a PMF patient (UPN46). Results are shown for immunoreactive peptides only. PHA was used as positive control and the HLA class II-restricted FLNA_HUMAN$_{1669-1683}$ peptide ETVITVDTKAAGKGK served as negative control. Data are expressed as mean $\pm$SD of two independent replicates. ICS of peptide-specific T cells from (C) UPN07 and (E) UPN46 showing increased IFN-γ and TNF production, as well as CD107a expression upon stimulation with the indicated peptide compared to the HLA class II-restricted negative control and PMA/ionomycin as positive control. Abbreviations: ID, identification; LPC, leukemic progenitor cell; n.t., not tested; UPN, uniform patient number; SFU, spot forming unit; neg., negative; pos., positive; FSC, forward scatter.

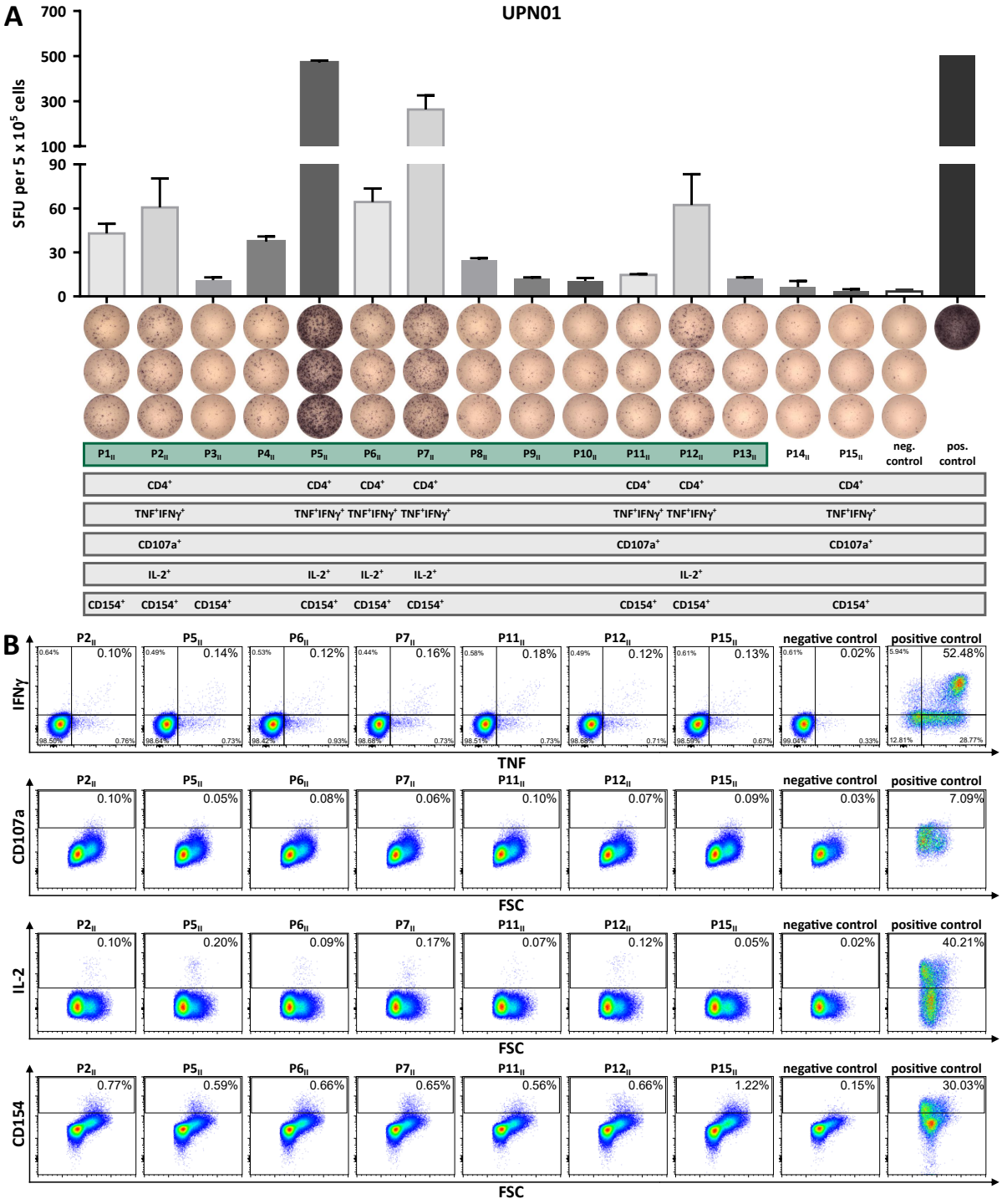

Figure 9: Representative results of preexisting HLA class II-directed T-cell responses in an ET patient. (A) IFN-γ ELISPOT results using HLA class II-restricted AML-associated peptides in an ET patient. PHA was used as positive control and the HLA class II-restricted FLNA_HUMAN$_{1669-1683}$ peptide ETVITVDTKAAGKGK served as negative control. Data are expressed as mean $\pm$SD of three independent replicates. Immunogenic peptides detected by ELISPOT are marked in green. The cytokine profile for each peptide is indicated within the grey bars below each peptide. (B) Subsequent ICS for functional characterization of CD4$^+$ peptide-specific T cells. The IFN-γ, TNF, IL-2, and CD154 production, as well as CD107a expression upon stimulation with the indicated peptide compared to the negative control or PMA/ionomycin positive control. Abbreviations: UPN, uniform patient number; SFU, spot forming unit; neg., negative; pos., positive; FSC, forward scatter.

Discussion

The crucial role of the immune system and particularly the immunological control regarding the genesis and progression of malignancies has become broadly accepted.[56,57] Various strategies harnessing the immune system to target cancer cells are on advance. Particularly antigen-specific immunotherapy, such as adoptive transfer of TCR-engineered cells or peptide-based vaccination, represent promising approaches that enable effective targeting of malignant cells. Tailored and patient-individualized peptide-based vaccination is an attractive strategy to reinstate immune responses and to direct cellular immunity against tumor-specific antigens. Especially for premalignant entities such as Ph- MPNs, peptide-based low side effect approaches represent a particularly promising therapeutic option supported by the generally competent constitution of the immune system along with the optimal T cell-*versus*-malignant cell ratio. The indispensable prerequisite for a clinically effective peptide-based cancer therapy is the identification and selection of valid frequently and naturally presented tumor-associated target antigens. Aside from classical target antigens such as cancer/testis or leukemia-associated antigens, so called neoantigens derived from mutations represent potential targets for anti-cancer immunotherapy. To this end, we implemented an MS-based approach to comprehensively map the immunopeptidome of Ph- MPNs and thus to identify and characterize novel MPN-associated HLA class I- and II-restricted target antigens.

We confirmed sufficient surface expression of HLA molecules on myeloid cells and HPCs from MPN patients. The observed HLA expression was comparable to that of other hematological neoplasms[58–60] as well as healthy peripheral blood cells.[59,60] The comprehensive HLA ligandome mapping of primary samples from ET and PV patients is ongoing, therefore only immunopeptidome data acquired from primary PMF samples was taken into account for an extensive evaluation. Comparative immunopeptidome profiling of PBMCs and granulocytes from PMF patients and our extensive benign tissue dataset revealed 22 PMF-associated HLA class I ligands with representation frequencies of $\geq$19% mostly with HLA-B*51 restriction reaching an allotype-adjusted prevalence of 71%. Of note, these numbers should be handled and interpreted with caution since HLA-B*51$^+$ samples were underrepresented in our benign tissues cohort. Nevertheless, these antigens can serve as targets for the development of single peptide-based immunotherapies such as

the adoptive transfer of T cells or can be included in personalized multi-peptide vaccine cocktails. For a clinically effective and long-lasting anti-cancer immune response it is essential to activate both, cytotoxic $CD8^+$ and $CD4^+$ helper T cells.[61–65] To this end, we applied a previously established profiling platform to additionally characterize three groups of HLA class II-restricted antigens: peptide targets, protein targets, and hotspot targets.[33] We identified a total of 54 unique frequently presented PMF-exclusive HLA class II peptides including 6 PMF-associated peptide targets with representation frequencies of $\geq$30% along with 7 protein targets represented by 24 peptides. These target antigens were verified and complemented by 7 hotspot targets comprising 28 peptides. Notably, the PMF-associated hotspot of RB27A represents also a CML-associated hotspot including 3 identical peptides albeit with lower frequencies in CML samples.[33] This is in line with previous findings stating the cellular biology being directly mirrored by the immunopeptidome regarding for example malignancies arising from cells of the same lineage.[66]

Several studies have revealed neoepitopes derived from tumor-specific mutations as the main anti-cancer T-cell specificities induced by immune checkpoint blockade in high mutational burden cancer entities.[25,67,68] However, only a small amount of such mutations on DNA level is detectable in the immunopeptidome in terms of naturally presented neoantigens.[26,27,69] In consequence, the relevance of such neoepitopes remains ambiguous in the context of broadly applicable T-cell-based immunotherapy, particularly for cancers with low mutational burden such as hematological malignancies. Especially for such entities several non-mutated HLA ligands are indicated as pathophysiologically relevant targets for T-cell-based immunotherapy.[33,58–60,70,71] We analyzed the immunopeptidomes of our MPN patient cohort for naturally presented peptides derived from the MPN-relevant proteins JAK2, CALR, and the thrombopoietin receptor MPL. Whereas most peptides derived from JAK2 and few from MPL were identified in the HLA class I-restricted immunopeptidome and seldom with HLA class II restriction, CALR-derived peptides were generally HLA class II-restricted. This is in line with the general knowledge of an HLA class I-restricted presentation of peptides derived from intracellular proteins (represented by JAK2) and mainly HLA class II-restricted presentation of processed extracellular proteins (represented by CALR).[72,73] Of note, the proportion of MPN samples presenting JAK2- and MPL-derived peptides as well as the amount of unique JAK2- and MPL-peptides identified per MPN-

sample was considerably higher compared to benign tissue samples. However, a hotspot analysis could not reveal any frequently presented tumor-associated JAK2-, CALR-, or MPL-derived peptides.

Screening the immunopeptidomes of our MPN patient cohort for naturally presented neoantigens derived from most abundant mutations in AML patients as well as from common driver mutations in MPNs could not reveal HLA-presented neoepitopes. This could be due to the distorted correlation between gene expression and HLA-restricted antigen presentation since the immunopeptidome does neither mirror the transcriptome nor the proteome.[27,59,74–77] Differential protein processing and the altered immunopeptidome genesis in malignant cells might contribute to this lack of correlation.[78,79] The absence of evidence, however, does not mean that there is evidence of absence.[80] Therefore, we cannot completely preclude low abundant HLA-dependent neoepitope presentation in our MPN patient cohort.

Regardless, MS-based immunopeptidomics enable the identification of valid non-mutated, frequently and naturally presented, tumor-specific target antigens in cancers with low mutational load. Such peptides are promising candidates for single peptide vaccinations,[58,71] antibody-based approaches,[81] transfer of engineered T cells,[82] or combinations of HLA-dependent and -independent strategies.[83] These antigens can also be relevant for immunomonitoring upon employment of different immunotherapy strategies. One major obstacle for broadly applicable off-the-shelf immunotherapy approaches is the HLA allotype restriction. Employment of multi-peptide cocktails combining different HLA allotype restrictions or combination of antigen-specific with -unspecific approaches can circumvent these limitations. Doing so, it is crucial to select the optimal combination partners. Combinations of IMiDs, IFNs, mAbs, or checkpoint inhibitors with other targeted therapies might reveal the true capacity of immunotherapy.[84,85] This has already been proven for the combination of antigen-specific immunotherapy with immune checkpoint blockade, which can potently trigger anti-cancer T-cell responses.[86–91] Peptide-based approaches can also benefit from a direct combination with IFN-α,[92,93] which can normalize the cytokine milieu, immune cell subsets, and immune phenotype, reduce tumor burden, and enhance HLA expression and expression of antigen processing- and presentation-related genes.[94–101] IMiDs normalize cytokine production,[102] activate NKT cells,[103] regulate co-stimulation of T cells, hamper

Tregs,[104] and reinstate the formation of the immunological synapse.[105] In contrast, a direct combination with JAK-inhibitors should be avoided due to observed myelosuppressive effects of Ruxolitinib such as impaired activation, differentiation, and proliferation of DCs and T cells.[106–108] Moreover, preliminary analyses of PBMCs from untreated PMF patients compared to respective PBMCs acquired following *in vivo* Ruxolitinib therapy suggest a profound downregulation of HLA expression.

PMF has the highest risk among the Ph- MPNs for progression to AML. In line with this we observed an overlap of HLA-presented tumor-exclusive antigens within our PMF and AML immunopeptidome datasets.[55] We further observed a low frequent HLA class I-restricted T-cell response directed by multifunctional $CD8^+$ T cells of a PMF patient. In *in vitro* T-cell-based experiments using HLA class II-restricted AML-associated peptides we detected multifunctional $CD4^+$ memory T cells in 2/11 ET patients and 5/12 PMF patients. The pathophysiological relevance of such preexisting peptide-specific T-cell responses for the efficacy of immunotherapy as well as the outcome of cancer patients has been suggested by several studies.[58,109–111] This, in turn, is emphasizing a clinical efficacy of such T-cell responses, that can be induced or boosted by peptide-based strategies.

Taken together, we took a first step to assess the biological and cell type-specific character of the HLA ligandome of Ph- MPNs. Further analyses will include complementary data from our ET and PV patient cohorts that will be analyzed for immunopeptidome alterations in correlation with disease progression, clinical data, as well as presence and functionality of MPN-associated peptide-specific T cells. Thereby we aim to provide a novel category of highly immunogenic antigen targets for tailored T-cell-based and combinatorial immunotherapeutic approaches to target MPNs and to circumvent their leukemic transformation.

Methods

Patients and blood samples

For HLA ligandome analysis, PBMCs and granulocytes from ET, PV, and PMF patients (Departments of Hematology and Oncology in Tübingen and Aachen, Germany) were isolated by density gradient centrifugation (Biocoll, Biochrom) and erythrocyte lysis (EL buffer, Qiagen). For T-cell-based assays PBMCs from ET, PV, and PMF patients were isolated by density gradient centrifugation. HLA typing was carried out by the Department of Hematology and Oncology, Tübingen, Germany. Informed consent was obtained in accordance with the Declaration of Helsinki protocol. The study was performed according to the guidelines of the local ethics committees. Patient characteristics are provided in supplemental Table S1.

HLA surface molecule quantification

HLA surface expression on ET, PV, and PMF patient samples was determined using the QIFIKIT bead-based quantification flow cytometric assay (Dako) according to the manufacturer's instructions.[33] Granulocytes and PBMCs were stained with either pan-HLA class I-specific W6/32 mAb, HLA-DR-specific L243 mAb (both produced in-house), or the respective IgG2a isotype control (BioLegend). Additional surface marker staining was carried out with directly labeled PE/Cy7 anti-human CD33, BV421 anti-human CD13, and APC anti-human CD34 (BD) antibodies. Aqua fluorescent reactive dye (Invitrogen) was used as viability marker. Analysis was performed on a FACS Canto II cytometer (BD).

Isolation of HLA ligands

HLA class I and II molecules were isolated by standard immunoaffinity purification using the pan-HLA class I-specific W6/32, the pan-HLA class II-specific Tü-39, and the HLA-DR-specific L243 mAbs (produced in-house).[33,59,112] For the immunoprecipitation of HLA class II-peptide complexes, we used a mixture of equal amounts of the L243 and Tü-39 mAbs. HLA-DR is known to be expressed at higher levels than the other HLA class II allotypes. Therefore, the HLA-DR-specific L243 mAb was used at the given ratio. The Tü-39 mAb was utilized complementarily to capture the remaining HLA class II complexes.

Analysis of HLA ligands by liquid chromatography-coupled tandem MS

HLA ligand extracts were analyzed as described previously.[58] Peptides were separated by nanoflow high-performance liquid chromatography. Eluted peptides were analyzed using an Orbitrap Fusion Lumos mass spectrometer and for indicated PV samples in an online-coupled LTQ Orbitrap XL mass spectrometer. Isolation of HLA ligands and subsequent MS analysis of the hematological benign dataset were performed by Annika Nelde and the non-hematological benign database was generated by Ana Marcu.[113]

Data processing

Data processing was performed as described previously.[58,114] Proteome Discoverer (v1.3, Thermo Fisher Scientific[115]) was used to integrate the search results of the Mascot search engine (v2.2.04, Matrix Science) for samples acquired with the LTQ Orbitrap XL as well as SequestHT search engine (University of Washington, Seattle, WA, USA) for the samples measured on the Orbitrap Fusion Lumos against the human proteome (Swiss-Prot database[116]) without enzymatic restriction.
Precursor mass tolerance was set to 5 ppm and fragment mass tolerance to 0.5 Da. Oxidized methionine was allowed as dynamic modification. The FDR (estimated by the Percolator algorithm 2.04[117]) was limited to 5% for HLA class I and 1% for HLA class II. Peptide lengths were set to 8-12 or 8-25 amino acids for HLA class I and HLA class II, respectively. HLA class I annotation was performed using SYFPEITHI 1.0[53] and NetMHCpan 4.0.[118]

Identification of ET-, PV-, PMF-, and AML-specific neoepitopes

The non-patient individual FASTA including the top 100 recurrent AML-associated missense mutations specified in the COSMIC database supplemented with the most common NPM1 and FLT3-ITD mutations rendered by Annika Nelde[55] was complemented with sequences of JAK2, MPL and CALR mutations (supplemental Table S4) and used for the identification of neoepitopes within the MPN datasets. Data processing of the HLA immunopeptidome with this FASTA was performed as described above.

Amplification of peptide-specific T cells and IFN-γ ELISPOT assay

PBMCs from ET and PMF patients were pulsed with 1 μg/mL (class I) or 5 μg/mL (class II) per peptide and cultured for 12 days adding 20 U/mL IL-2 (Novartis) on days 3, 5, and 7. Peptide-stimulated PBMCs were analyzed by IFN-γ ELISPOT assay on day 12. Spots were counted using an ImmunoSpot S5 analyzer (CTL) and T-cell responses were considered positive when $\geq$10 spots/well were counted and the mean spot count was at least three-fold higher than the mean spot count of the negative control according to the harmonization guidelines of the international Cancer Vaccine Consortium proficiency panel.[119]

Intracellular cytokine staining

The functionality of peptide-specific T cells was analyzed by ICS as described previously.[120,121] Cells were pulsed with 10 μg/mL of individual peptide and incubated with 10 μg/mL Brefeldin A (Sigma-Aldrich) and 10 μg/mL GolgiStop (BD) for 12-16 h. Staining was performed using Cytofix/Cytoperm solution (BD), APC/Cy7 anti-human CD4 (BD), PE/Cy7 anti-human CD8 (Beckman Coulter), PacificBlue anti-human TNF, FITC anti-human CD107a, and PE anti-human IFN-γ antibodies (BioLegend). PMA and ionomycin (Sigma-Aldrich) served as positive control. For UPN01 an additional ICS was conducted using APC anti-human CD154 and PE/Cy7 anti-human IL-2 (BioLegend).

The response was considered to be CD4$^+$- or CD8$^+$-directed if the frequency of TNF$^+$, IFN-γ^+, CD107a$^+$, and CD4$^+$ or CD8$^+$ T cells respectively exceeded 0.1% and was at least three-fold higher than the frequency of the respective population in the negative control. These criteria are in compliance with the harmonization guidelines for HLA-peptide multimer assays of the international Cancer Vaccine Consortium proficiency panel.[122] All samples were analyzed on a FACS Canto II cytometer (BD).

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CHAPTER 3

Mass spectrometry-based identification of a B-cell maturation antigen-derived T-cell epitope for antigen-specific immunotherapy of multiple myeloma

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Blood Cancer J. 2020 Feb 28;10(2):24 *PMID: 32111817* *doi: 10.1038/s41408-020-0288-3*

Disclosure of authorship contributions

TB was involved in the study design and reevaluated the previously acquired mass spectrometry data. TB performed all immunogenicity analyses and *in vitro* T-cell experiments and analyzed the respective data. TB designed and created all figures and tables. TB wrote the manuscript together with JSW. For further information see the declaration provided in the "Authors' contributions" section at the end of this chapter/manuscript.

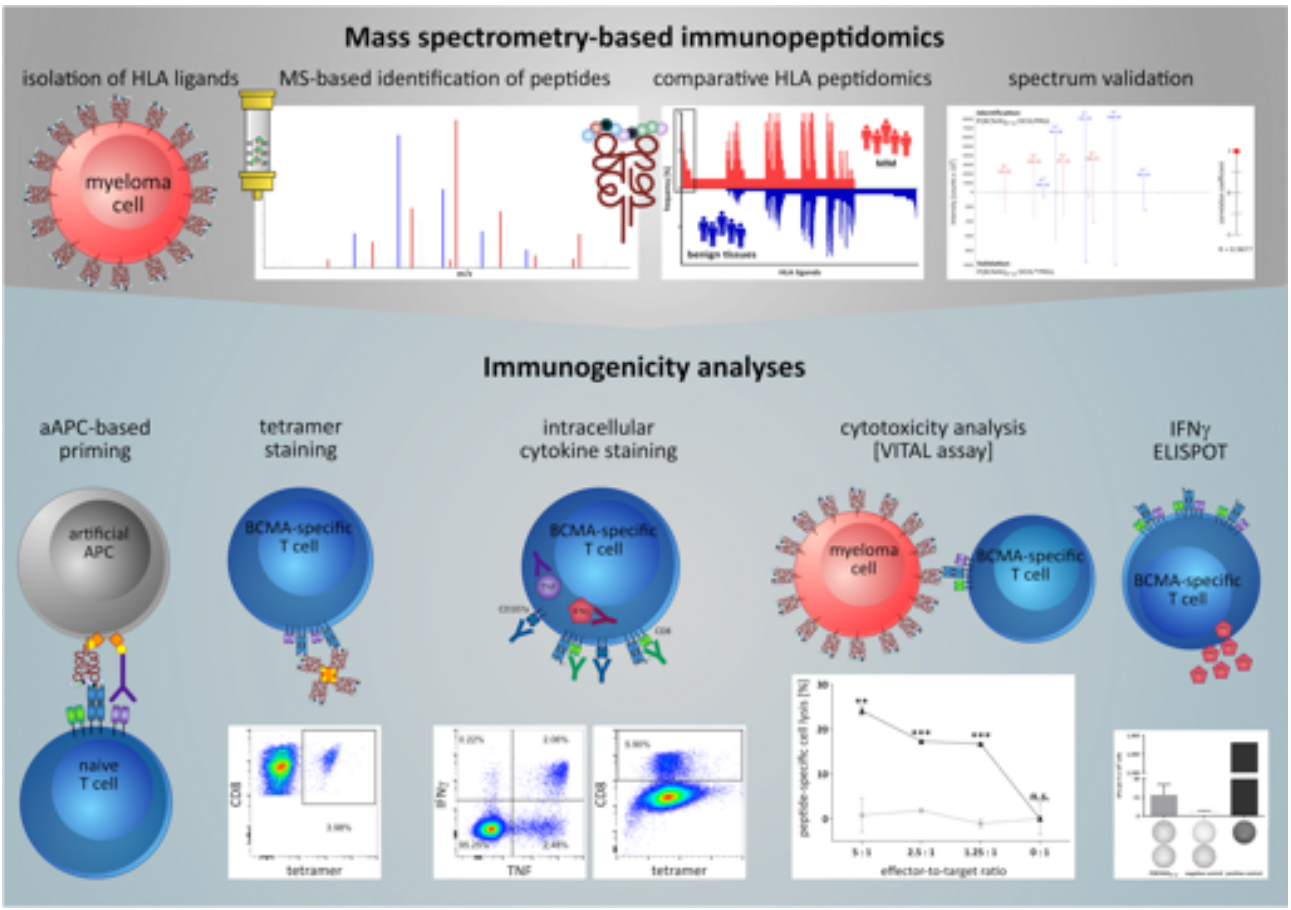
Abstract

The B-cell maturation antigen is currently being evaluated as promising tumor-associated surface antigen for T-cell-based immunotherapy approaches, such as CAR T cells and bispecific antibodies, in MM. Cytotoxic T cells bearing BCMA-specific T-cell receptors might further allow targeting HLA-presented antigens derived from the intracellular domain of BCMA. By analyzing a mass spectrometry-acquired immunopeptidome dataset of primary MM samples and MM cell lines for BCMA-derived HLA ligands, we identified the naturally presented HLA-B*18-restricted ligand P(BCMA)$_{B*18}$. Additionally, P(BCMA)$_{B*18}$ was identified on primary CLL samples, thereby expanding the range for possible applications. P(BCMA)$_{B*18}$ induced multifunctional BCMA-specific cells *de novo* from naïve CD8$^+$ T cells of healthy volunteers. These T cells exhibited antigen-specific lysis of autologous peptide-loaded cells. Even in the immunosuppressive context of MM, we detected spontaneous memory T-cell responses against P(BCMA)$_{B*18}$ in patients. By applying CTLA-4 and PD-1 inhibition *in vitro* we induced multifunctional P(BCMA)$_{B*18}$-specific CD8$^+$ T cells in MM patients lacking preexisting BCMA-directed immune responses. Finally, we could show antigen-specific lysis of autologous peptide-loaded target cells and even MM.1S cells naturally presenting P(BCMA)$_{B*18}$ using patient-derived P(BCMA)$_{B*18}$-specific T cells. Hence, this BCMA-derived T-cell epitope represents a promising target for T-cell-based immunotherapy and monitoring following immunotherapy in B-cell malignancy patients.

Key words

multiple myeloma, immunotherapy, CD8⁺ T cell, T-cell epitope, HLA ligand, BCMA, TNFRSF17

Visual Abstract



Introduction

T-cell-based immunotherapies for MM are on the advance, further improving the outcome of this still incurable disease. Beside strategies like allogeneic stem cell transplantation,[1] donor lymphocyte infusion,[2] immunomodulatory drugs,[3] and immune checkpoint inhibitors,[4] that induce a fairly general T-cell activation, several advanced approaches for targeting myeloma cells more specifically are in development. Such antigen-specific immunotherapy

approaches comprise mono- and bispecific antibodies,[5,6] CAR T cells,[7] TCR-engineered T cells,[8] and DC- or peptide-based vaccines.[9,10] A main prerequisite for such approaches is the selection of feasible targets for MM-directed T-cell responses. Ideally, such targets should be presented both, exclusively and frequently on myeloma cells. A particularly promising target for antigen-specific immunotherapy in MM and other B-cell malignancies is BCMA (TNFRSF17, CD269), a member of the tumor necrosis receptor superfamily. BCMA is a preferentially B-lineage-restricted differentiation transmembrane protein with exclusive presentation on myeloma cells, plasma blasts, differentiated plasma cells, and late memory B cells.[6]

Currently, several therapeutic approaches targeting BCMA are being evaluated in preclinical settings and clinical trials including CAR T cells,[7] bispecific antibodies and other T-cell engagers,[6,11] antibody-drug conjugates,[12] or immunotoxins.[13] Particularly BCMA-targeting CAR T cells showed promising results in heavily pretreated and refractory MM patients with an overall response rate of 81%.[7] However, despite these encouraging results, potential toxicities and immense costs should be taken into account, in particular prior to application of such immunotherapy approaches in elderly and comorbid MM patients or larger patient cohorts at an earlier disease setting.[14,15] Furthermore, all these substances are targeting the extracellular domain of BCMA with an associated risk of surface antigen loss.[16] Cytotoxic T cells bearing BCMA-specific TCRs might be an alternative approach to target MM cells. Such TCRs can also recognize intracellular proteins or domains, which are processed and presented *via* HLA molecules on the surface of tumor cells.[17] Antigen-specific T cells can either be induced *in vivo* by low side effect vaccination-based approaches or generated *ex vivo* as TCR-engineered cells. The main prerequisite for these approaches is the identification and characterization of naturally presented HLA-restricted peptides, which can serve as target structures for T cells.[18] In a previous study, we characterized the naturally presented immunopeptidome of MM using an MS-based approach and identified several novel MM-associated antigens.[19] Here, we evaluated this dataset for the presence of BCMA-derived peptides to provide a proof of concept for the feasibility to identify and target naturally presented T-cell epitopes from intracellular domains of highly promising tumor surface antigens.

Results

MS-based identification of BCMA-derived HLA-presented peptides in MM

Previously acquired MS datasets[19,20] of primary MM samples and MM cell lines (MCLs) were reprocessed using the search engine SequestHT and evaluated for the presence of naturally presented BCMA-derived peptides. Analysis of the immunopeptidome of seven primary MM samples and five MCLs revealed a total of 17 633 unique HLA class I ligands from 7 627 different source proteins as well as 9 482 unique HLA class II peptides from 2 371 source proteins. We identified two BCMA-derived HLA class I-restricted ligands, both derived from its intracellular domain (Fig. 1a).

The HLA-B*18-restricted peptide DEIILPRGL, referred to as P(BCMA)$_{B*18}$, was identified in 17% (2/12 samples, one primary MM patient sample and the MCL MM.1S) of the analyzed MM immunopeptidomes with a remarkably high allotype-adjusted frequency of 67% (2/3 HLA-B*18$^+$ samples). Notably, P(BCMA)$_{B*18}$ showed MM- and B-lineage-associated presentation and was solely detected on 1/5 benign B-cell (20%) and 2/17 benign lymph node samples (12%) according to our extensive benign immunopeptidome database (149 297 HLA class I ligands; 17 093 source proteins; 404 samples from various tissues). Additionally, P(BCMA)$_{B*18}$ could also be identified in the immunopeptidome of 2/3 (67%) primary HLA-B*18$^+$ CLL samples.[21] In contrast, the HLA-B*40-restricted P(BCMA)$_{B*40}$ ligand TEIEKSISA was detected solely in 1/12 (8%) MM-derived samples with an allotype-adjusted frequency of 33% (1/3 HLA-B*40$^+$ samples) but displayed no selective MM-association due to its representation in a variety of benign tissues. Furthermore, we identified two HLA class II restricted BCMA-derived antigens that showed MM-exclusive presentation according to our benign HLA class II immunopeptidome database (214 908 HLA class II peptides; 15 840 source proteins; 366 samples from various tissues). However, these HLA class II restricted BCMA-derived peptides were both detected only in MCLs but not in primary MM samples with a low representation frequency of 8% (1/12 samples) in our MM cohort.

a

Peptide ID	Peptide sequence	HLA restriction	Frequency in MM samples	Allotype-adjusted frequency in MM samples	Allotype-adjusted frequency in CLL samples	Identification in benign samples
$P(BCMA)_{B*18}$	DEIILPRGL	B*18	2/12 (17%)	2/3 (67%)	2/3 (67%)	B cell-containing tissues only
$P(BCMA)_{B*40}$	TEIEKSISA	B*40	1/12 (8%)	1/3 (33%)	0/3 (0%)	several benign samples/tissues
$P1(BCMA)_{class\ II}$	DEIILPRGLEY	class II	1/12 (8%)	n.a.	n.a.	no
$P2(BCMA)_{class\ II}$	LEKSRTGDEIILPRG	class II	1/12 (8%)	n.a.	n.a.	no

b

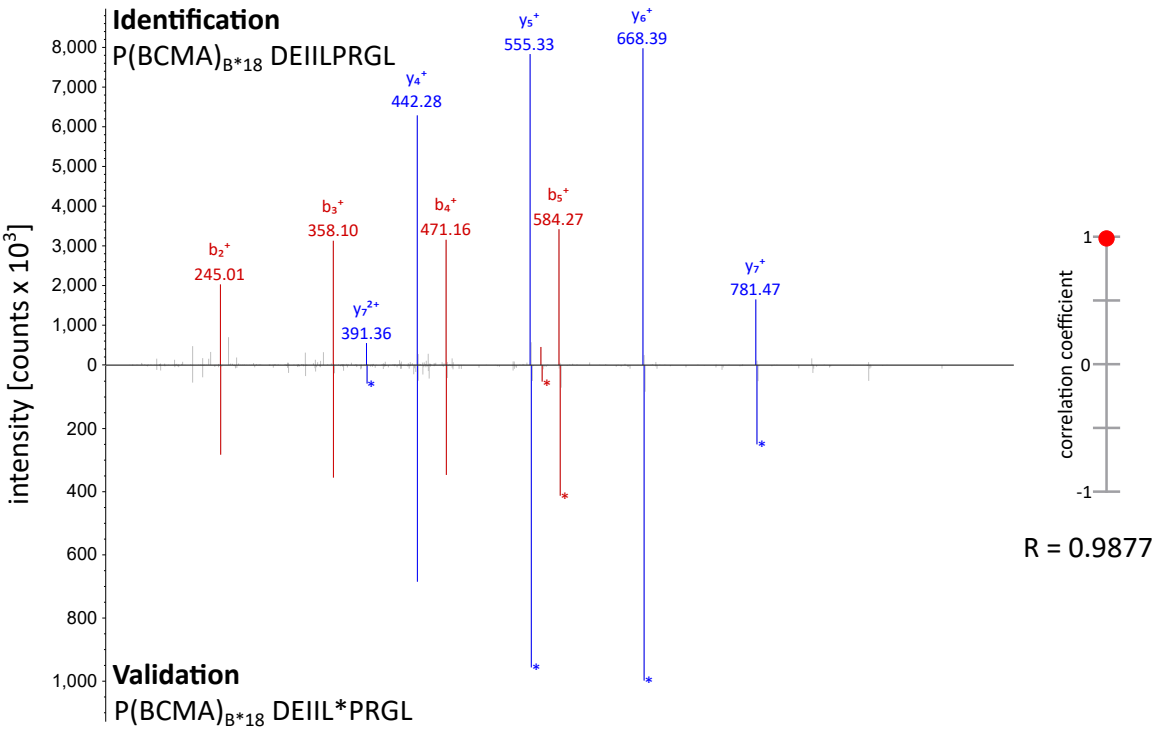


Figure 1: Identification of BCMA-derived peptides and validation of $P(BCMA)_{B*18}$ using a synthetic isotope-labeled peptide. (a) Identified BCMA-derived peptides with their respective sequence, HLA restriction, their total and allotype-adjusted frequency in the immunopeptidomes of the MM and CLL cohort, as well as their occurrence in the HLA peptidome of benign tissues. (b) Validation of the experimentally eluted $P(BCMA)_{B*18}$ peptide using the corresponding synthetic isotope-labeled peptide. Comparison of the fragment spectrum (m/z on the x-axis) of the $P(BCMA)_{B*18}$ peptide eluted from a primary MM patient sample (identification) with its corresponding synthetic peptide (validation). The spectrum of the synthetic peptide is mirrored on the x-axis. Identified b- and y-ions are marked in red and blue, respectively. Ions containing the isotope-labeled amino acid are marked with asterisks. The calculated spectral correlation coefficient is depicted on the right graph. Abbreviations: ID, identification; MM, multiple myeloma; CLL, chronic lymphocytic leukemia; n.a., not available.

Therefore, we selected the $P(BCMA)_{B*18}$ peptide due to its MM-association and the high representation frequency for further immunological characterization. Prior to immunogenicity testing, we validated the experimentally acquired spectrum of $P(BCMA)_{B*18}$ by comparison of MS/MS spectra as well as of the reversed-phase retention times of the precursor ions using an isotope-labeled synthetic peptide (Fig. 1b).

P(BCMA)$_{B*18}$ induced multifunctional peptide-specific T cells in healthy volunteers *in vitro*

To assess the immunogenicity of P(BCMA)$_{B*18}$, we performed *in vitro* aAPC-based priming experiments using CD8$^+$ T cells of HVs. Effective *de novo* priming and expansion of antigen-specific T cells was observed in 100% of analyzed HVs (n = 10) with frequencies of peptide-specific T cells ranging from 0.1 - 7.9% (mean 0.9%) within the viable CD8$^+$ T-cell population (Fig. 2a, Supplemental Table 1). The *de novo* induction of peptide-specific cells was further proven by the lack of preexisting memory T-cell responses after 12-day recall stimulation (Fig. 2a). Priming experiments with a control peptide frequently presented on HLA-B*18 in both, tumor and benign tissues (peptide presentation > 90% in HLA-matched sources), confirmed MM-specificity of the induced T-cell responses (Fig. 2b, Supplemental Table 2). Furthermore, multifunctionality of the induced P(BCMA)$_{B*18}$-specific T cells could be demonstrated in 6/6 HV samples. Using ICS, we observed a significant production of IFN-γ and TNF as well as upregulation of the degranulation marker CD107a upon P(BCMA)$_{B*18}$ stimulation (Fig. 2c). Moreover, cytotoxicity assays with polyclonal P(BCMA)$_{B*18}$-specific effector T cells revealed their capacity to induce antigen-specific lysis of autologous CD8$^-$ peptide-loaded target cells (Fig. 2d-f).

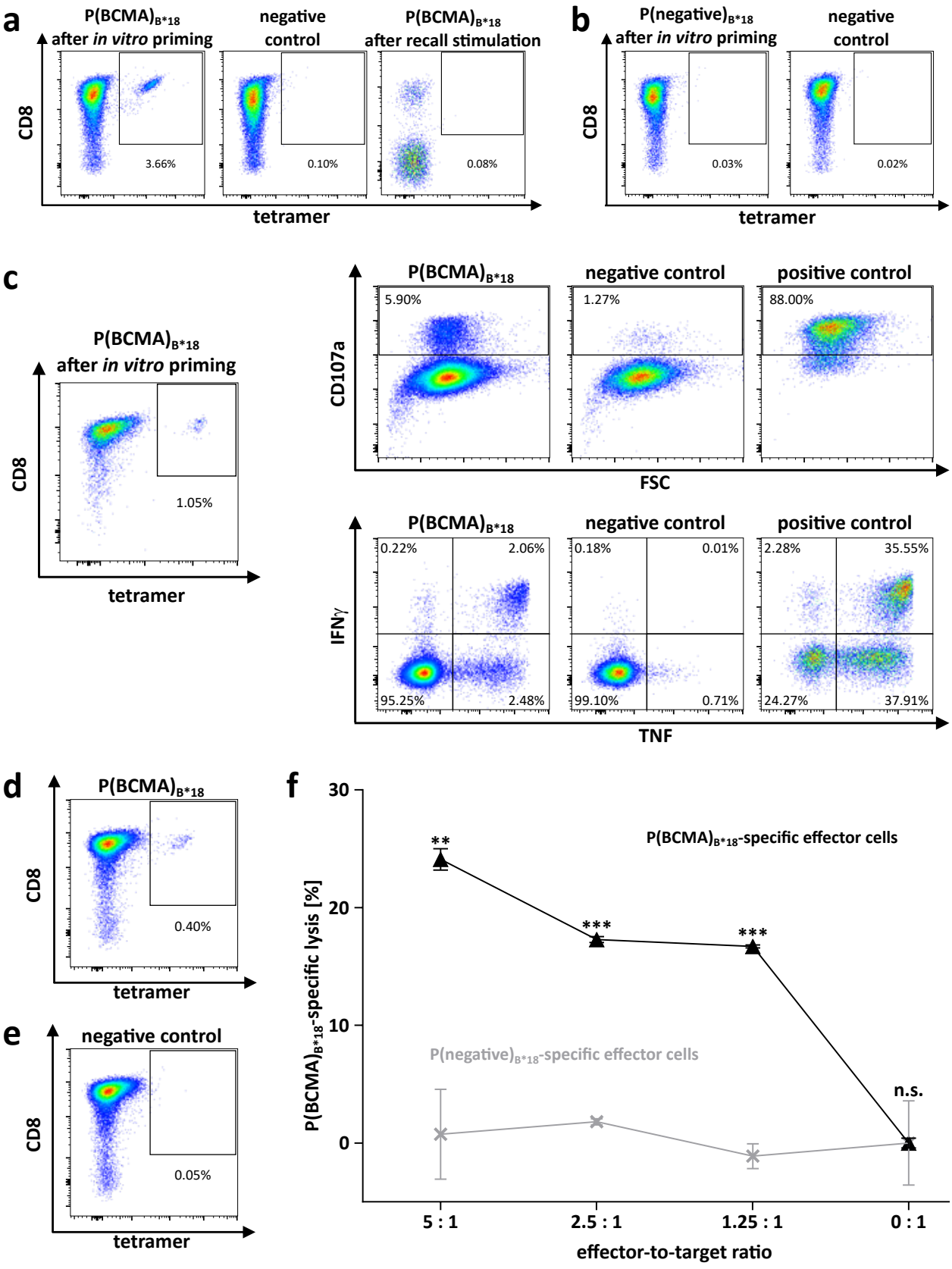

a
P(BCMA)B*18 after in vitro priming
negative control
P(BCMA)B*18 after recall stimulation
CD8
3.66%
0.10%
0.08%
tetramer
b
P(negative)B*18 after in vitro priming
negative control
CD8
0.03%
0.02%
tetramer
c
P(BCMA)B*18 after in vitro priming
CD8
1.05%
tetramer
P(BCMA)B*18
negative control
positive control
CD107a
5.90%
1.27%
88.00%
FSC
IFNγ
0.22%
2.06%
95.25%
2.48%
0.18%
0.01%
99.10%
0.71%
2.28%
35.55%
24.27%
37.91%
TNF
d
P(BCMA)B*18
CD8
0.40%
tetramer
e
negative control
CD8
0.05%
tetramer
f
P(BCMA)B*18-specific effector cells
P(negative)B*18-specific effector cells
P(BCMA)B*18-specific lysis [%]
30
20
10
0
**
***
***
n.s.
5 : 1
2.5 : 1
1.25 : 1
0 : 1
effector-to-target ratio

Figure 2: Induction and functional characterization of P(BCMA)$_{B*18}$-specific CD8$^+$ T cells from HVs. (a) Naïve CD8$^+$ T cells from HVs were primed *in vitro* using aAPCs. Graphs show single, viable cells stained for CD8 and PE-conjugated multimers of indicated specificity. Tetramer staining was performed after four stimulation cycles with peptide-loaded aAPCs. The left panel shows P(BCMA)$_{B*18}$-tetramer staining. The middle panel (negative control) depicts P(BCMA)$_{B*18}$-tetramer staining of T cells from the same donor primed with a control peptide. The right panel shows PBMCs from the same donor that were tested negative for preexisting memory T cells following 12-day recall stimulation. (b) Tetramer staining after four stimulation cycles with negative control peptide-loaded aAPCs. (c) Functional characterization of P(BCMA)$_{B*18}$-specific CD8$^+$ T cells by intracellular cytokine staining. Representative example of IFN-γ and TNF production as well as CD107a expression after stimulation of P(BCMA)$_{B*18}$-specific CD8$^+$ T cells with the P(BCMA)$_{B*18}$ peptide compared to a negative control peptide. PMA and ionomycin served as positive control. (d-f) Cytotoxicity of P(BCMA)$_{B*18}$-specific effector T cells analyzed in a VITAL assay with *in vitro* primed CD8$^+$ T cells. (d, e) Tetramer staining of polyclonal effector cells prior to the VITAL assay determined the amount of P(BCMA)$_{B*18}$-specific effector cells in the (d) population of successfully P(BCMA)$_{B*18}$-primed CD8$^+$ T cells and in the (e) population of control cells from the same donor primed with an HLA-matched control peptide. (f) Cell lysis by P(BCMA)$_{B*18}$-specific effector T cells (black) of P(BCMA)$_{B*18}$-loaded autologous CD8$^-$ target cells at various effector-to-target cell ratios in comparison to negative control peptide-loaded CD8$^-$ target cells. P(BCMA)$_{B*18}$-unspecific effector cells (grey) showed no peptide-specific lysis of the same targets. Results are shown as mean $\pm$SEM for three independent technical replicates. Significance was determined using two-tailed paired student's t-test. Abbreviations: FSC, forward scatter; n.s., not significant; ** $p<0.01$; *** $p<0.001$.

Detection of preexisting P(BCMA)$_{B*18}$-specific memory T-cell responses in MM patients

We further evaluated the existence of spontaneous preexisting memory T-cell responses directed against P(BCMA)$_{B*18}$ in IFN-γ ELISPOT assays using PBMCs of HLA-B*18$^+$ MM patients (Supplemental Table 3). We observed P(BCMA)$_{B*18}$-induced IFN-γ secretion in 1/4 (25%) samples of MM patients (Fig. 3a). Notably, we could not detect any preexisting immune responses against P(BCMA)$_{B*18}$ in samples from HVs (n = 12, Fig. 3b), further confirming the MM-specificity of P(BCMA)$_{B*18}$-directed immune responses.

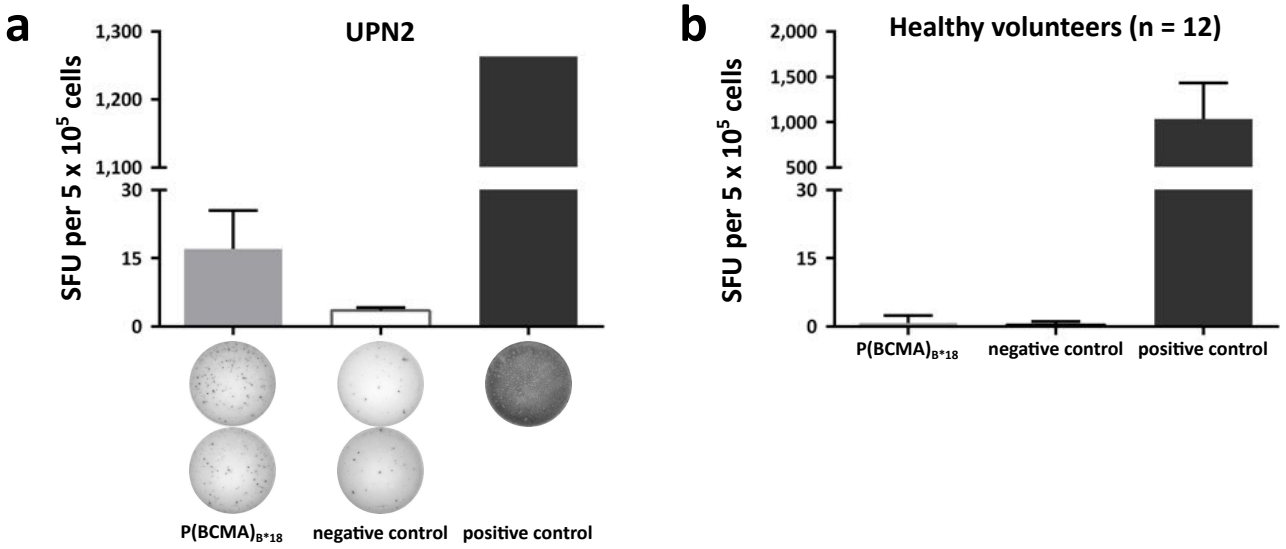


Figure 3: Preexisting memory T-cell responses directed against BCMA detected in IFN-γ ELISPOT assays. (a-b) Memory T-cell responses directed against P(BCMA)$_{B*18}$ were evaluated in IFN-γ ELISPOT assays after 12-day recall stimulation using PBMCs of (a) MM patients or (b) HVs. PHA was used as positive control. The peptide DEVRTLTY served as negative control. Data are expressed as mean $\pm$SD of two independent technical replicates for the MM patient and as mean $\pm$SD of all 12 HVs analyzed in two independent technical replicates each. Abbreviations: UPN, uniform patient number; HVs, healthy volunteers; SFU, spot forming unit.

Combination of immune checkpoint inhibitors and P(BCMA)$_{B*18}$ using cells of MM patients induced peptide-specific T cells with anti-myeloma activity

Next, we aimed to overcome the reported profound immune defects (including impaired function of immune effector cells) in MM,[22] and therefore evaluate the potential of P(BCMA)$_{B*18}$ to induce *de novo* functional T-cell responses using T cells of MM patients that displayed no preexisting memory immune responses against P(BCMA)$_{B*18}$ following 12-day recall stimulation. While no P(BCMA)$_{B*18}$-specific T cells could be induced in MM-derived samples using our standard priming protocol, we observed *de novo* induction of P(BCMA)$_{B*18}$-specific T cells with frequencies of 0.2 - 4.1% (mean 1.6%) within the CD8$^+$ T-cell population of an MM patient upon addition of CTLA-4 and PD-1 blocking antibodies (Fig. 4a). These P(BCMA)$_{B*18}$-specific T cells were multifunctional as demonstrated by IFN-γ and TNF production as well as upregulation of CD107a (Fig. 4b) and peptide-specific lysis of autologous P(BCMA)$_{B*18}$-loaded CD8$^-$ cells (Fig. 4c-e). Moreover, MM-derived P(BCMA)$_{B*18}$-specific CD8$^+$ T cells showed specific lysis of MM.1S cells (Fig. 4f-h), which naturally present P(BCMA)$_{B*18}$ as detected by MS-based immunopeptidomics.

Taken together, we identified a naturally presented myeloma-associated, BCMA-derived peptide, which constitutes a promising and highly immunogenic target for tailored T-cell-based immunotherapy and monitoring of immunotherapeutic approaches in MM and other B-cell malignancies.

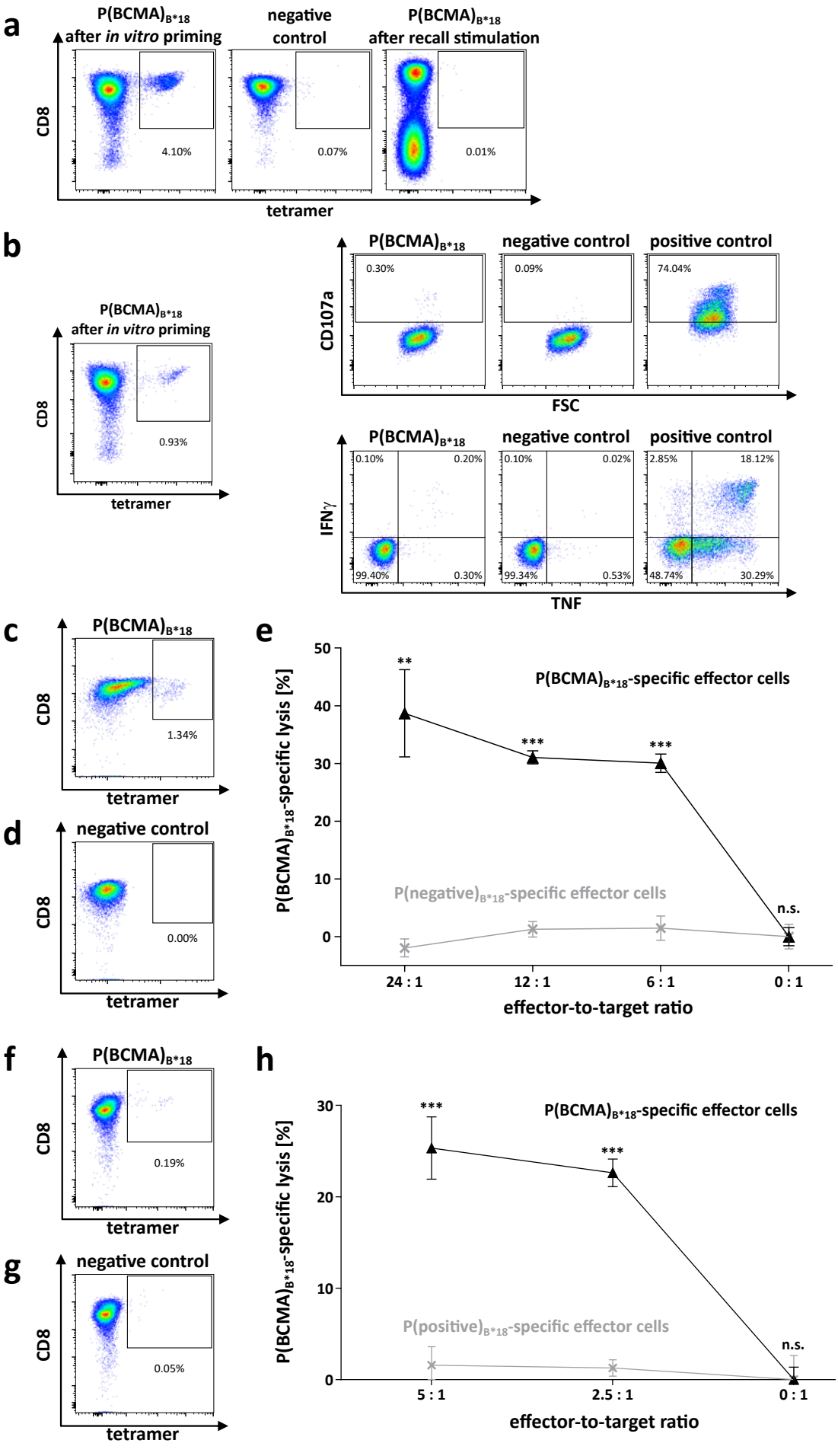

a
P(BCMA)B*18 after in vitro priming
negative control
P(BCMA)B*18 after recall stimulation
CD8
4.10%
0.07%
0.01%
tetramer
b
P(BCMA)B*18 after in vitro priming
CD8
0.93%
tetramer
P(BCMA)B*18
negative control
positive control
CD107a
0.30%
0.09%
74.04%
FSC
IFNγ
0.10%
0.20%
99.40%
0.30%
0.10%
0.02%
99.34%
0.53%
2.85%
18.12%
48.74%
30.29%
TNF
c
P(BCMA)B*18
CD8
1.34%
tetramer
d
negative control
CD8
0.00%
tetramer
e
P(BCMA)B*18-specific lysis [%]
P(BCMA)B*18-specific effector cells
P(negative)B*18-specific effector cells
**
***
***
n.s.
24 : 1
12 : 1
6 : 1
0 : 1
effector-to-target ratio
f
P(BCMA)B*18
CD8
0.19%
tetramer
g
negative control
CD8
0.05%
tetramer
h
P(BCMA)B*18-specific lysis [%]
P(BCMA)B*18-specific effector cells
P(positive)B*18-specific effector cells
***
***
n.s.
5 : 1
2.5 : 1
0 : 1
effector-to-target ratio

Figure 4: Induction and functional characterization of P(BCMA)$_{B*18}$-specific CD8$^+$ T cells from a myeloma patient. (a) Naïve CD8$^+$ T cells from UPN1 were stimulated four times with peptide-loaded aAPCs in addition to PD-1 and CTLA-4 blocking antibodies. Graphs show single, viable cells stained for CD8 and PE-conjugated multimers of indicated specificity. The left panel shows P(BCMA)$_{B*18}$-tetramer staining. The middle panel (negative control) depicts P(BCMA)$_{B*18}$-tetramer staining of respective T cells primed with a control peptide. The right panel shows T cells from the same donor that were tested for preexisting memory T cells after 12-d recall stimulation. (b) Example of IFN-γ and TNF production as well as CD107a expression following stimulation with the P(BCMA)$_{B*18}$ peptide compared to the negative control peptide using peptide-specific cells of UPN1. PMA and ionomycin served as positive control. (c, d, f, g) Tetramer staining of polyclonal effector cells prior to the VITAL assay determined the amount of P(BCMA)$_{B*18}$-specific cells in the (c, f) population of successfully P(BCMA)$_{B*18}$-primed CD8$^+$ T cells and in the (d, g) population of control cells from the same MM patient primed with an HLA-matched control peptide. (e) Cell lysis of P(BCMA)$_{B*18}$-specific effectors (black) of P(BCMA)$_{B*18}$-loaded autologous target cells at various effector-to-target cell ratios in comparison to P(negative)$_{B*18}$ control peptide-loaded target cells. P(BCMA)$_{B*18}$-unspecific effectors (grey) showed no peptide-specific lysis of the same targets. Results are shown as mean ±SEM for three independent replicates. (h) Cell lysis of P(BCMA)$_{B*18}$-specific effectors (black) of naturally P(BCMA)$_{B*18}$-presenting MM.1S target cells at various effector-to-target cell ratios in comparison to MV4-11 P(BCMA)$_{B*18}$ negative target cells. P(BCMA)$_{B*18}$-unspecific effectors (grey) showed no peptide-specific lysis of the same targets. Results are shown as mean ±SEM for three independent replicates. Significance was determined using student's t-test. Abbreviations: FSC, forward scatter; UPN, uniform patient number; n.s., not significant; **, $p < 0.01$; ***, $p < 0.001$.

Discussion

Valid antigen targets are the main prerequisite for the development of clinically effective antigen-specific cancer immunotherapy. These highly promising target structures are represented by HLA-independent surface antigens, such as BCMA in MM. However, the number of such antigens is limited due to the required exclusive presentation on the surface of tumor cells.[23] HLA-dependent antigens, in contrast, can originate from any intracellular protein or domain and are not restricted to cell surface proteins. Therefore, the amount of potential HLA-dependent targets for a given tumor entity is expected to be considerably higher. In recent years, numerous studies have defined HLA ligands derived from tumor-specific mutations as main targets of immune checkpoint inhibitor-induced T-cell responses in solid tumors.[24,25] Nonetheless, only a very small proportion of DNA-level mutations results in naturally presented, mutation-derived neoantigens detectable in the HLA ligandome.[26,27] Hence, the role of neoantigens for the development of broadly applicable immunotherapy,

especially in low mutational burden cancer entities such as MM, remains unclear. We and others have previously described several non-mutated HLA ligands as pathophysiologically relevant targets for T-cell-based immunotherapy approaches,[28] especially for tumors with a low mutational burden, such as hematological malignancies.[19,21,29–31]

In this study we aimed to identify HLA-dependent antigen targets derived from the intracellular domain of the established surface antigen BCMA using an MS-based approach. We identified P(BCMA)$_{B*18}$ as a highly immunogenic naturally presented epitope capable of inducing potent and multifunctional cytotoxic T-cell responses. This is in line with recent data reporting on the high immunogenicity of computationally predicted HLA*02-restricted T-cell epitopes derived from the extracellular surface domain of BCMA.[32,33] Our mass spectrometric approach further allowed us to validate P(BCMA)$_{B*18}$ additionally as a target for CLL, for which plasma BCMA-levels were described as a prognostic factor.[34] However, so far less data are available demonstrating the utility of BCMA-based immunotherapeutic approaches in CLL. As already shown on gene and protein expression level presentation of BCMA-derived peptides is not restricted to myeloma and CLL cells but is – albeit less frequently – detectable in benign B-cell containing tissues.[35] This is in line with other B-lineage-specific targets such as CD19, for which antigen-specific therapies, including CAR T cells and bispecific antibodies, are already approved for clinical use.[36,37] As the expression of CD19 is not limited to malignant B cells, targeting of CD19 can lead to on-target/off-tumor side effects including for example hypogammaglobulinemia, which however are manageable in the clinical setting.[38]

Our approach for the identification of BCMA-derived T-cell epitopes might be translated to other MM-associated antigens such as SLAMF7, CD38, CD74, or CD138,[39] providing naturally processed HLA-presented ligands derived from intracellular domains of established membrane-bound tumor-associated antigens. Such HLA ligands represent promising targets for (i) low side effect single agent vaccine approaches in elderly patients or early disease states,[21,28,40] (ii) TCR-engineered T cells,[41] (iii) antibody-based approaches,[42] or (iv) novel approaches combining HLA-dependent and -independent antigens and treatments.[43] In addition, such T-cell epitopes can be used for the assessment and monitoring of T-cell responses following various types of antigen-specific, or as in this example BCMA-specific, immunotherapy approaches. However, the HLA allotype restriction of HLA peptide targets,

in terms of HLA-B*18 covering only about 12% of the world population,[44] represents a limitation concerning the development of broadly applicable immunotherapy approaches. This calls for combinatorial approaches using peptides of various HLA restrictions or for patient (group)-individualized approaches. However, in this context it has to be taken into account, that different HLA ligands with distinct HLA allotype restrictions derived from the same source protein can show different tissue distribution as demonstrated exemplarily for the P(BCMA)$_{B*40}$ peptide in this study. The lack of correlation between protein expression and the immunopeptidome can be explained by the differential protein processing in malignant cells along with the complexity of HLA ligand formation, which is frequently altered in tumor cells.[45,46]

Besides the selection of optimal target antigens for the development of clinically effective T-cell-based immunotherapies in MM, one must consider the profound immune defects that prevail in these patients and might impair the efficacy of inducing antigen-specific T-cell responses *in vivo*.[22,47] A major immunosuppressive mechanism in MM is the upregulation of inhibitory immune checkpoint molecules on T cells.[22,39] By employing PD-1/CTLA-4 blockade, we were able to overcome this mechanism and to induce multifunctional cytotoxic P(BCMA)$_{B*18}$-specific T cells, even in MM-derived samples. These data are in line with reports from other solid tumors and hematological malignancies,[48–50] pointing towards a combination of antigen-specific immunotherapy with general approaches optimizing immune responses, as exemplified with immune checkpoint blockade.[51,52]

Taken together, this study provides first evidence that intracellular domain-derived HLA ligands from MM-associated membrane antigens can provide a novel category of highly immunogenic antigen targets for tailored combinatorial immunotherapies for patients suffering from MM or other B-cell malignancies.

Methods

Patients, blood samples, and cell lines

PBMCs collected from MM patients at the Department of Hematology and Oncology (Tübingen, Germany), and from healthy volunteers, were isolated by density gradient centrifugation using Biocoll (Biochrom GmbH, Berlin, Germany). Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki protocol. The study was performed according to the guidelines of the local ethics committee. Patient characteristics are provided in Supplemental Table 3. The MM.1S (ATCC CRL-2974) and MV4-11 (DSMZ ACC102) cell lines were tested for mycoplasma contamination and cultured in the recommended cell media as described previously.[20]

MS data reevaluation

Reprocessing and reanalyzing of previously acquired and published MS data of primary MM patient samples and MCLs[19,20] was performed as described before.[53] The Proteome Discoverer (v1.3, Thermo Fisher Scientific, Waltham, MA, USA)[54] was used to integrate the search results of the SequestHT search engine (University of Washington, Seattle, WA, USA) against the human proteome (Swiss-Prot database[55]) without enzymatic restriction. Precursor mass tolerance was set to 5 ppm and fragment mass tolerance to 0.5 Da. Oxidized methionine was allowed as dynamic modification. The false discovery rate, estimated by the Percolator algorithm 2.04,[56] was limited to 5% for HLA class I and 1% for HLA class II. Peptide lengths were set to 8-12 or 8-25 amino acids for HLA class I and II, respectively. HLA class I annotation was performed using SYFPEITHI 1.0[57] and NetMHCpan4.0.[58]

Spectrum validation

Spectrum validation of the experimentally eluted peptide was performed by computing the similarity of the spectrum with the corresponding synthetic isotope-labeled peptide measured in a complex matrix. The spectral correlation coefficient was calculated between the spectra of the eluted and the synthetic peptide considering the mass shift of ions containing the isotope-labeled amino acid.[59] Peptides were synthesized as described previously.[30]

aAPC priming of naïve CD8$^+$ T cells

Priming of cytotoxic T lymphocytes was conducted using aAPCs following MACS for CD8$^+$ cells as described previously.[30] In detail, 800 000 streptavidin-coated microspheres were loaded with 200 ng biotinylated HLA:peptide monomer and 600 ng biotinylated anti-human CD28 mAb (clone 9.3, in-house production). These aAPCs were used for the stimulation of 1x10^6 CD8$^+$ T cells in four stimulation cycles in addition to 5 ng/mL of IL-12 (PromoKine, Heidelberg, Germany). For MM patient-derived CD8$^+$ cells the stimulation protocol comprised addition of 1 μg/mL purified anti-human PD-1 (CD279, #14-2799-80) and CTLA-4 (CD152, #16-1529-82, Invitrogen, Carlsbad, CA, USA) mAbs directly after PBMC isolation, after MACS, as well as for the first and third aAPC stimulation.

Cytokine and tetramer staining

Frequencies of peptide-specific CD8$^+$ T cells were determined by tetramer staining using the PE/Cy7 anti-human CD8 mAb (#737661, Beckman Coulter, Brea, CA, USA) and 5 μg/mL of PE-labeled HLA:peptide tetramer (in-house production). Tetramers of the same HLA allotype containing irrelevant control peptides were used as negative control (Supplemental Table 2). Functionality of peptide-specific T cells was analyzed by ICS using PE/Cy7 anti-human CD8, PacificBlue anti-human TNF (#502920), FITC anti-human CD107a (#328606, BioLegend, San Diego, CA, USA), and PE anti-human IFN-γ (#506507, BD, Franklin Lakes, NJ, USA) antibodies as described previously.[30] Results of tetramer staining were considered positive if the frequency of peptide-specific CD8$^+$ T cells was $\geq$0.1% of viable cells and at least three-fold higher than the frequency of peptide-specific cells in the negative control according to the harmonization guidelines for HLA-peptide multimer assays of the international Cancer Vaccine Consortium proficiency panel.[60] The same evaluation criteria were applied for ICS. All samples were analyzed using a FACS-Canto™ II cytometer and FlowJo software version 10.0.7 (BD).

Cytotoxicity assays

Peptide-specific CD8⁺ T cells were analyzed for their capacity to induce peptide-specific target cell lysis in the flow cytometry-based VITAL assay.[30] Autologous CD8⁻ target cells were either loaded with the P(BCMA)$_{B*18}$ peptide or an irrelevant control peptide (Supplemental Table 2) and labeled with CFSE or FarRed (life technologies, Carlsbad, CA, USA), respectively. The P(BCMA)$_{B*18}$-specific effector cells were added in the indicated effector-to-target ratios. Specific lysis of peptide-loaded CD8⁻ target cells was calculated relative to control targets. Additionally, we performed the VITAL assay using the P(BCMA)$_{B*18}$-presenting MM.1S cell line stained with CFSE as target cells. The HLA-B*18⁺ AML cell line MV4-11, negative for P(BCMA)$_{B*18}$ in MS-based HLA ligandome analysis, was stained with FarRed and served as negative target cell line.

Amplification of peptide-specific T cells and IFN-γ ELISPOT assay

PBMCs of MM patients and HVs were pulsed with 1 μg/mL per peptide and cultured for 12 days. In addition, cells were supplemented on day three, five, and seven with 20 U/mL IL-2 (Novartis, Basel, Switzerland). On day 12, PBMCs were analyzed in the IFN-γ ELISPOT assay as described previously[30] using an ImmunoSpot S5 analyzer (CTL Europe GmbH, Bonn, Germany). The negative control peptide P(negative)$_{B*18}$ (Supplemental Table 2) was used to exclude unspecific immune responses, for example from NK cells. Preexisting memory T-cell responses were considered positive if $\geq$10 spots/well were counted and the mean spot count was at least three-fold higher than the mean spot count of the negative control.

Declarations

Ethics approval and consent to participate

Informed consent was obtained in accordance with the Declaration of Helsinki protocol. The study was performed according to the guidelines of the local ethics committee (454/2016BO2).

Funding

This work was supported by the German Cancer Consortium (DKTK); the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Grant STI 704/1-1; the Deutsche Forschungsgemeinschaft under Germany's Excellence Strategy under Grant EXC2180-390900677; the Wilhelm Sander Stiftung under Grant 2016.177.1; the José Carreras Leukämie-Stiftung under Grant DJCLS 05 R/2017; the Bundesministerium für Bildung und Forschung (BMBF) under Grant 031A535A; the European Union (EU) under Grant ERC AdG339842 MUTAEDITING.

Authors' contributions

T.B., A.N., H.-G.R., S.S., J.S.W. designed the study; T.B., A.N., J.B., A.M. performed reanalysis of the previously acquired MS dataset; T.B., S.W., M.L., J.S. conducted *in vitro* T-cell experiments; S.W., M.R., H.R.S., K.W., B.B., M.C.N., J.S.W. provided new reagents, analytic tools, and samples; S.W., M.R., H.R.S., K.W., B.B., M.C.N., J.S.W. conducted patient data collection and medical evaluation; T.B., A.N., J.B., S.S., J.S.W. analyzed data; T.B., A.N., J.B., J.S.W. drafted the manuscript; H.-G.R., S.S., J.S.W. supervised the study.

Acknowledgments

We thank Ulrike Schmidt, Cécile Gouttefangeas, Claudia Falkenburger, Patricia Hrstić, Nicole Bauer, Beate Pömmerl, and Ulrich Wulle for excellent technical support.

Conflict of interest

Hans-Georg Rammensee is shareholder of Immatics Biotechnologies GmbH and Curevac AG. The other authors declare no competing financial interests.

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General discussion

The immune system and especially the immunological control are essential mechanisms to contain and restrain development and progress of neoplastic malignancies.[1,2] Several different strategies to harness the immune system against cancer have been examined and further sophisticated approaches are on advance. One particularly promising approach is antigen-specific immunotherapy with the major advantage of few adverse effects whilst specifically and effectively targeting malignant cells. Especially entity-specific and patient-individualized approaches are gaining more and more momentum. Such therapies include the tailored peptide-based vaccination: an attractive strategy with few adverse effects to reinstate immune responses and direct them against tumor-specific or -associated antigens. The indispensable prerequisite for a clinically effective peptide-based anti-cancer therapy is the identification and selection of immunogenic and valid tumor-associated targets. Such target structures are for example surface antigens with HLA-independent presentation, which however are limited due to their lack of tumor-exclusivity.[3] HLA-dependent antigens on the other hand can originate from any intracellular protein or domain, thereby increasing the proportion of potential targets. To this end, our group developed an immunopeptidomics-based identification pipeline to define potential tumor-associated T-cell epitopes that are frequently and naturally presented by HLA molecules on the surface of malignant cells.

Such targets for anti-cancer immunotherapy include mutation-derived neoantigens in addition to the classical cancer/testis and leukemia-associated antigens. Several studies revealed neoepitopes derived from tumor-specific mutations as the main anti-cancer T-cell specificities induced by immune checkpoint blockade in high mutational burden cancer entities.[4–6] However, only a small amount of such mutations on DNA level is detectable in the immunopeptidome in terms of naturally presented neoantigens.[7–9] MS-based immunopeptidome analysis of the low mutational burden CML, ET, PV, and PMF could not reveal any frequently presented neoepitopes (**CHAPTER 1 and 2**). This may be due to the differential protein processing and altered immunopeptidome genesis in malignant cells and the distorted correlation of gene expression and HLA-restricted antigen presentation.[10–16] The diversity of HLA allotypes might contribute to the restriction of HLA-dependent neoepitope presentation: not every mutation results in a peptide bearing the correct binding motif for

a distinct HLA allotype. The driver mutation V617F in JAK2 for example harbours only 22 peptides that might bind a certain HLA allotype (**CHAPTER 2**). Since absence of evidence does not mean that there is evidence of absence, we cannot preclude low abundant HLA-dependent neoepitope presentation in our patient cohorts.[17] In consequence, the relevance of neoepitopes remains ambiguous in the context of broadly applicable T-cell-based immunotherapy, especially for cancers with low mutational burden such as hematological malignancies. For such entities several non-mutated HLA ligands are indicated as pathophysiologically relevant targets for T-cell-based immunotherapy.[11,18–22]

This underlines all the more the importance of novel, non-mutated, tumor-specific, frequently and naturally presented antigens in the HLA ligandomes of our CML and PMF patient cohorts with ongoing analyses for the ET and PV patient cohorts. Within our MM patient cohort we identified the HLA-B*18 ligand P(BCMA)$_{B*18}$ derived from the intracellular domain of the established surface antigen BCMA, which we additionally validated as target for CLL.[23] Presentation of BCMA is not restricted to MM and CLL but is – albeit less frequently – detectable in benign B-cell containing tissues like other B-lineage-specific targets such as CD19.[24–26]

The here identified CML- and PMF-associated antigens and the MM- and CLL-associated P(BCMA)$_{B*18}$ can serve as targets for off-the-shelf single peptide-based immunotherapy approaches such as the adoptive transfer of TCR-engineered T cells or can be included in personalized multi-peptide vaccine cocktails. In addition, such T-cell epitopes can be used for the assessment and monitoring of T-cell responses following various types of antigen-specific immunotherapy approaches.[27,28] One major obstacle for broadly applicable off-the-shelf immunotherapy approaches is the HLA allotype restriction. Employment of multi-peptide cocktails combining different HLA allotype restrictions or combination of antigen-specific with antigen-unspecific approaches can circumvent these limitations. It is essential to activate both, cytotoxic CD8$^+$ and CD4$^+$ helper T cells for the induction of a clinically effective and long-lasting anti-cancer immune response.[29–33]

Besides the selection of optimal targets for immunotherapy in hematological malignancies, one must consider the profound immune defects that prevail in CML, MPN, and MM patients and might impair T-cell responses *in vivo*. In line with previous reports of a negative[34–37] or dysregulating[38] impact of TKI treatment on immune responses, the functionality of CD8$^+$ T cells in our CML$_{TKI}$ patient cohort was impaired, potentially explaining the reduced func-

tionality of T cells targeting CML-associated HLA class I ligands. The reduced T-cell functionality might also be due to a general immunosuppression in CML caused by HLA-G,[39] elevated MDSCs and Tregs,[40,41] or by increased PD-1 expression on immune cells.[40] In MPNs, immune dysregulation is reflected by the deregulated cytokine milieu and immune phenotype and thus the defective immune surveillance, which is believed to contribute to disease evolution and progress.[42–45] Reduced DCs, impaired monocyte differentiation, and a reduced Th1 compartment have been observed in advanced high-risk myelofibrosis patients.[46] The general hyperinflammatory state contributes to an enhanced PD-1/PD-L1 expression and thus to suppression of T-cell responses.[47–49] Increased numbers of immunosuppressive cells such as MDSCs - that are even more suppressive than the MDSCs in HVs - can further hamper immune responses.[50] Immune surveillance evasion can furthermore be reinforced by downregulation of HLA molecules.[51,52] In MM, particularly the upregulation of inhibitory immune checkpoint molecules on T cells represents a major immunosuppressive mechanism.[53–55]
For combinatorial approaches it is therefore crucial to select the optimal combination partners for an effective activation and reinstatement of anti-cancer T-cell responses. Reports from hematological and non-hematological malignancies are pointing towards a combination of antigen-specific immunotherapy with general approaches optimizing immune responses, such as IMiDs, IFNs, or checkpoint inhibitors.[56–62] Unspecific immunotherapy, such as alloSCT or IFN-α, enable long-lasting remissions in CML patients after TKI therapy discontinuation.[63–65] In MPN patients alloSCT, DLI, IFN-α, and JAK-inhibitors achieved promising results and revealed beneficial therapeutic effects, including the normalization of the cytokine milieu and depletion of malignant clones.[66–72] First studies combining antigen-specific immunotherapy with immune checkpoint blockade showed potent induction of anti-cancer T-cell responses.[60,62,73,74] In MM, checkpoint blockade can potently induce immune responses.[75,76] This is in line with our observations: we were able to induce multifunctional cytotoxic P(BCMA)$_{B*18}$-specific T cells, even in MM-derived samples, by employing PD-1/CTLA-4 blockade *in vitro*. In CML, immune responses against peptide-based vaccines were enhanced upon combination with IFN, TKIs, and IMiDs.[77,78] In MPNs, antigen-specific immunotherapy in combination with immune checkpoint blockade could equivalently induce and enhance T-cell responses.[79,80] Peptide-based approaches in MPN patients can also benefit from a direct combination with IFN-α,[81,82] which can normalize the cytokine

milieu, immune cell subsets, and immune phenotype, reduce tumor burden, and enhance HLA expression and expression of antigen processing- and presentation-related genes.[83–90] Another feasible combination partner are IMiDs, which can normalize cytokine production,[91] activate NKT cells,[92] regulate co-stimulation of T cells, hamper Tregs,[93] and reinstate the formation of the immunological synapse.[94] In contrast, a direct combination with JAK-inhibitors should be avoided due to observed myelosuppressive effects of Ruxolitinib such as impaired activation, differentiation, and proliferation of DCs and T cells.[95–97]

Upon effective reinstatement of immune responses against antigens that are included within and targeted by the vaccine cocktail, a further beneficial effect termed epitope spreading is believed to contribute to long-lasting remissions or even cure. Epitope spreading can occur in consequence of the immunotherapy-induced so-called immunogenic cell death: cancer cells lysed by induced cytotoxic T cells can release further tumor-associated antigens together with damage signals thereby providing immunostimulatory signals along with novel, individual, and tumor-specific target antigens.[98–102] This in turn enhances the repertoire of tumor-specific T cells targeting additional tumor-antigens that were not included in the vaccine, thereby further increasing clinical effectiveness of the immunotherapy.

In consequence, the major requirement for an effective and feasible target antigen is its immunogenicity - the capability to induce an immune response. Spontaneous preexisting memory T-cell responses against non-mutated antigens were described as pathophysiologically relevant in malignancies.[19,103,104] In **CHAPTER 1** we observed such preexisting T-cell responses against our newly defined CML-associated HLA class II peptides in samples of CML patients. Functional T cells directed against HLA class I antigens were detected with a low frequency in CML_{TKI} patient samples. To further prove the immunogenicity of all HLA class I antigens, we performed *in vitro* aAPC-based priming assays. Multifunctional cytotoxic CML-specific T cells were induced *de novo* using PBMCs from HVs as well as CML_{TKI} patients. In **CHAPTER 2** we assessed spontaneous preexisting T-cell responses against novel AML-associated antigens in PMF based on the biological proximity of PMF and AML and the observed overlap of tumor-exclusive antigens in these entities.[105] We observed low frequent T-cell responses directed by multifunctional $CD8^+$ T cells of one PMF patient against HLA class I targets as well as multifunctional $CD4^+$ memory T cells targeting HLA class II-restricted AML-associated antigens in ET and PMF patients. In **CHAPTER 3** we

identified P(BCMA)$_{B*18}$ as a highly immunogenic epitope capable of inducing multifunctional cytotoxic T-cell responses. This is in line with reports on the high immunogenicity of predicted T-cell epitopes derived from the extracellular surface domain of BCMA.[106,107] P(BCMA)$_{B*18}$ is thereby expanding the targeting of BCMA by including its intracellular domain.

Future investigations include clinical studies to further evaluate the CML-associated targets and to estimate and define the optimal therapy combinations. For Ph- MPNs, we aim to complete the data acquisition of our ET and PV patient cohorts and analyze the immunopeptidome and its alterations in correlation with disease progression, clinical parameters, as well as presence and functionality of MPN-associated peptide-specific T cells. Regarding MM, we aim to identify and characterize further T-cell epitopes, preferentially from the intracellular domain of BCMA and other MM-associated antigens. We further identified the TCR sequence of P(BCMA)$_{B*18}$-specific CD8$^+$ T-cell clones. The complementarity-determining region 3 (CDR3) including the VDJ recombination junctions is enclosing the major variations. CDR3 is also the TCR region recognizing the HLA:peptide complex.[108]

- CDR3α junction CLVGYGGSQGNLIF (TRAV4*01, TRAJ42*01)
- CDR3β junction CASSESEGVGTGGLFF (TRBV6-1*01, TRBD2*01, TRBJ2-4*01)

This represents a crucial step for the further development of our peptide target in terms of TCR-based immunotherapy approaches including the adoptive T-cell transfer, TCR-like antibodies, and even adaptor CAR T-cell systems.[24,109,110]

In conclusion, this thesis provides profound insights into the immunopeptidomes of myeloproliferative neoplasms. We delineated novel highly immunogenic CML-associated T-cell epitopes and took a first step towards the identification of ET-, PV-, and PMF-associated target antigens for immunotherapeutic approaches and subsequent immunomonitoring. In addition, we provided a proof-of-concept study for the natural HLA-dependent presentation of peptides derived from intracellular domains of established tumor-associated surface antigens as potential targets for immunotherapy by characterizing P(BCMA)$_{B*18}$ as a highly immunogenic T-cell epitope in B-cell malignancies. In general, we aim to provide a novel category of highly immunogenic antigen targets for tailored off-the-shelf T-cell-based and combinatorial immunotherapeutic approaches to target hematological malignancies and to circumvent leukemic transformation and progression of disease.

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Abbreviations

aa	amino acid
ADC	antibody-drug conjugate
ALL	acute lymphocytic leukemia
alloSCT	allogeneic stem cell transplantation
AML	acute myeloid leukemia
aAPC	artificial antigen-presenting cell
APC	antigen-presenting cell
BCMA	B-cell maturation antigen
CAR	chimeric antigen receptor
CALR	calreticulin
CD	cluster of differentiation
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CTA	cancer/testis antigen
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DLI	donor lymphocyte infusion
ELISPOT	enzyme-linked immunospot
ET	essential thrombocythemia
EPO	erythropoietin
FDA	Food and Drug Administration
FDR	false discovery rate
G-CSF	granulocyte colony-stimulating factor
HLA	human leukocyte antigen
HM	hematological malignancy
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
HU	hydroxyurea
HV	healthy volunteer
ICS	intracellular cytokine staining
IFN	interferon
IL	interleukin
IMiD	immunomodulatory drug
JAK2	Janus kinase 2
LAA	leukemia-associated antigen

mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MCL	myeloma cell line
MDS	myelodysplastic syndrome
MDSC	myeloid-derived suppressor cell
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
MM	multiple myeloma
MPL	myeloproliferative leukemia
MPN	myeloproliferative neoplasm
MR	molecular response
MRD	minimal residual disease
MS	mass spectrometry
NK	natural killer
PBMC	peripheral blood mononuclear cell
PD-L1	programmed cell death-ligand 1
PE	phycoerythrin
PET-MF	post-ET-MF
Ph-	Philadelphia chromosome-negative
Ph+	Philadelphia chromosome-positive
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
PMF	primary myelofibrosis
PPV-MF	post-PV-MF
PV	polycythemia vera
SCT	stem cell transplantation
SMM	smouldering multiple myeloma
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TCR	T-cell receptor
TFR	treatment-free remission
Th	helper T cell
TIL	tumor infiltrating lymphocyte
TKI	tyrosine kinase inhibitor
TLR	toll-like receptor
TNF	tumor necrosis factor
TPO	thrombopoietin
Treg	regulatory T cell
TSA	tumor-specific antigen

Appendix - CHAPTER 1

Supplemental methods

Supplemental Methods

Patients and blood samples

For HLA ligandome analysis, PBMCs from CML patients (Departments of Hematology and Oncology in Tübingen, Leipzig, and Aachen, Germany) at the time of diagnosis or in deep molecular remission (MR4.0, MR4.5, and MR5.0) as well as PBMCs, granulocytes, and bone marrow mononuclear cells from HVs were isolated by density gradient centrifugation (Biocoll, Biochrom) and erythrocyte lysis (EL buffer, Qiagen). B cells and HPCs of HVs were enriched by $CD19^+$ and $CD34^+$ MACS (Miltenyi), respectively. For T-cell-based assays PBMCs from HVs as well as CML patients at time of diagnosis or at different time points under therapy were isolated by density gradient centrifugation.

HLA surface molecule quantification

HLA surface expression on CML patient samples was determined using the QIFIKIT bead-based quantification flow cytometric assay (Dako) according to the manufacturer's instructions. Cells were stained with either pan-HLA class I-specific W6/32 mAb, HLA-DR-specific L243 mAb (produced in-house), or respective isotype control (BioLegend). Additional surface marker staining was carried out with directly labeled PE/Cy7 anti-human CD33, BV421 anti-human CD13, PE anti-human CD117 (BioLegend), and APC anti-human CD34 (BD) antibodies. Aqua fluorescent reactive dye (Invitrogen) was used as viability marker. Analysis was performed on a FACS Canto II cytometer (BD).

Isolation of HLA ligands

HLA class I and II molecules were isolated by standard immunoaffinity purification using the pan-HLA class I-specific W6/32, pan-HLA class II-specific Tü-39, and HLA-DR-specific L243 mAbs (produced in-house). For the immunoprecipitation of HLA class II-peptide complexes, we used a mixture of equal amounts of the L243 and Tü-39 mAbs. HLA-DR is known to be expressed at higher levels than the other class II allotypes. Therefore, the HLA-DR-specific L243 mAb was used at the given stoichiometry. Tü-39 mAb was utilized complementarily to pull-down the remaining class II complexes.

Spectrum validation

Spectrum validation of the experimentally eluted peptides was performed by computing the similarity of the spectra with corresponding synthetic peptides measured in a complex matrix. Because the synthetic peptides were isotope labeled, fragments containing an isotope label have a mass shift

compared to non-labeled fragments and therefore penalize spectral similarity score. To minimize such effects, the spectra of synthetic peptides were preprocessed by shifting isotope label containing single- or double-charged b- and y-ion peaks to the m/z position where their corresponding unlabeled fragment is supposed to be. The spectral correlation (Toprak *et al.* Mol Cell Proteomics 2014) was then calculated between eluted peptide spectra and preprocessed synthetic peptide spectra.

Amplification of peptide-specific T cells and IFN-γ ELISPOT assay

PBMCs from CML patients and HVs were pulsed with 1 μg/mL (class I) or 5 μg/mL (class II) per peptide and cultured for 12 days adding 20 U/mL IL-2 (Novartis) on days 3, 5, and 7. Peptide-stimulated PBMCs were analyzed by ELISPOT assay on day 12. Spots were counted using an ImmunoSpot S5 analyzer (CTL) and T-cell responses were considered positive when >10 spots/well were counted and the mean spot count was at least three-fold higher than the mean spot count of the negative control according to the cancer immunoguiding program guidelines.

Cytokine and tetramer staining

The frequency and functionality of peptide-specific CD8$^+$ T cells was analyzed by tetramer and ICS, respectively. For ICS, cells were pulsed with 10 μg/mL of individual peptide and incubated with 10 μg/mL Brefeldin A (Sigma-Aldrich) and 10 μg/mL GolgiStop (BD) for 12-16 h. Staining was performed using Cytofix/Cytoperm (BD), PerCP anti-human CD8, PacificBlue anti-human TNF, FITC anti-human CD107a (BioLegend), and PE anti-human IFN-γ antibodies (BD). PMA and ionomycin (Sigma-Aldrich) served as positive control. Negative control peptides are listed in Table S3. The frequency of peptide-specific CD8$^+$ T cells after aAPC-based priming was determined by PerCP anti-human CD8 mAb and HLA:peptide tetramer-PE. For negative control tetramers of the same HLA allotype containing irrelevant control peptides were used. The priming was considered successful if the frequency of peptide-specific CD8$^+$ T cells was >0.1% of CD8$^+$ T cells within the viable single cell population and at least three-fold higher than the frequency of peptide-specific CD8$^+$ T cells in the negative control. The same evaluation criteria were applied for the ICS results. All samples were analyzed on a FACS Canto II cytometer (BD).

Supplemental Table 1

UPN	Phase of disease	Experiment	Age	CML-specific therapy	WBC (10^3/µl)	HLA class I typing	HLA class II typing	HLA class I		HLA class II	
								HLA ligand IDs	Protein IDs	Peptide IDs	Protein IDs
1	chronic phase	L	71	no	n.a.	A*23, A*25, B*18, B*57, C*06, C*12	DRB1*07, DRB1*15, DQB1*03, DQB1*06	1,572	1,491	1,162	394
2	chronic phase	L	55	no	n.a.	A*03, B*35, B*52, C*04	DRB1*04, DRB1*13, DQB1*03, DQB1*06	1,385	1,360	n.a.	n.a.
3	chronic phase	L	72	no	n.a.	A*01, B*08, C*07	DRB1*03, DRB1*15, DQB1*02, DQB1*06	535	457	n.a.	n.a.
4	chronic phase	L	66	no	n.a.	A*03, A*68, B*07, B*44, C*07	DRB1*11, DRB1*15, DQB1*03, DQB1*06	1,032	994	390	208
5	chronic phase	L	61	no	n.a.	A*24, A*68, B*27, B*57	DRB1*11, DRB1*14, DQB1*03, DQB1*05	539	628	507	162
6	chronic phase	L	56	no	n.a.	A*01, A*03, B*07, B*08, C*07	DRB1*03, DRB1*15, DQB1*02, DQB1*06	657	516	1,044	379
7	chronic phase	L	70	no	n.a.	A*02, A*03, B*13, B*15, C*03, C*06	DRB1*07, DRB1*13, DQB1*02, DQB1*06	1,270	1,180	641	209
8	chronic phase	L	57	no	n.a.	A*01, A*02, B*08, B*50, C*06, C*07	DRB1*03, DRB1*07, DQB1*02	747	832	381	137
9	chronic phase	L	68	no	n.a.	A*03, B*07, B*35, C*04, C*07	DRB1*01, DRB1*15, DQB1*05, DQB1*06	701	541	172	121
10	chronic phase	L	60	no	169	A*02, A*11, B*35, B*44, C*04	n.a.	1,676	1,529	900	405
11	chronic phase	L	41	no	190	A*03, B*35, C*04	n.a.	1,228	1,187	1,142	279
		E, T, L_{MR}	47	nilotinib 600 mg	5			523	456	108	65
12	chronic phase	L	n.a.	no	124	A*02, A*11, B*07, C*07, C*17	n.a.	639	659	533	209
13	blast crisis	L	37	no	131	A*02, B*18, B*57, C*06, C*07	DRB1*07, DRB1*11, DQB1*03	1,075	1,109	699	398
14	chronic phase	L	n.a.	n.a.	106	A*02, A*11, B*35, B*44, C*04	n.a.	907	926	398	182
15	chronic phase	L	44	no	310	A*01, A*02, B*35, B*41	n.a.	835	696	555	327
16	chronic phase	L	78	no	128	A*66, B*40, B*44, C*03, C*05	n.a.	866	701	500	234
17	chronic phase	L	27	no	80	A*03:01, A*23:01, B*07:02, B*18:01, C*07:01, C*07:02	DRB1*14:01, DRB1*16:01, DQB1*05:02, DQB1*05:03	2,107	1,543	668	285
18	accelerated phase	L	21	no	483	A*02:20, A*11:01, B*27:05, B*44:02, C*01:02, C*05:02	DRB1*01:01, DRB1*08:01, DQB1*04:02, DQB1*05:01	1,823	1,263	1,157	273
19	chronic phase	L, Q	39	no	156	A*02, A*11, B*07, B*51, C*07	n.a.	1,476	1,362	484	199
20	blast crisis	L	83	no	235	A*01, A*03, B*07, B*08, C*07	DRB1*03, DRB1*07, DQB1*02	791	846	721	385
21	chronic phase	L, Q	73	no	130	A*02, B*57, C*06	n.a.	820	898	359	205
		P	74	imatinib 400 mg	-			n.a.	n.a.	n.a.	n.a.
22	chronic phase	L	63	no	140	A*02, A*33, B*14, B*40	n.a.	n.a.	n.a.	411	178
23	chronic phase	Q	19	no	-	A*02:01, A*23:01, B*18:01, B*35:01, C*04:01, C*07:01	DRB1*01:01, DRB1*11:04, DQB1*03:01, DQB1*05:01	n.a.	n.a.	n.a.	n.a.
		P, T	23	dasatinib 100 mg	-			n.a.	n.a.	n.a.	n.a.
24	chronic phase	Q	26	no	-	A*23:01, A*68:01, B*07:02, B*50:01, C*06:02, C*07:10	DRB1*03:01, DRB1*13:01, DQB1*02:01, DQB1*06:03	n.a.	n.a.	n.a.	n.a.

UPN	Phase of disease	Experiment	Age	CML-specific therapy	WBC (10^3/µl)	HLA class I typing	HLA class II typing	HLA class I		HLA class II	
								HLA ligand IDs	Protein IDs	Peptide IDs	Protein IDs
25	chronic phase	Q	49	no	-	A*24:02, A*26:01, B*51:01, B*55:01, C*03:03, C*15:02	DRB1*11:01, DRB1*16:01, DQB1*03:01, DQB1*05:02	n.a.	n.a.	n.a.	n.a.
		E	57	imatinib 400 mg	-			n.a.	n.a.	n.a.	n.a.
		L_{MR}	58	imatinib 400 mg	6			622	554	127	175
26	accelerated phase	Q	58	no	-	A*02:01, B*07:02, B*35:01, C*04:01, C*07:02	n.a.	n.a.	n.a.	n.a.	n.a.
27	chronic phase	Q	38	no	-	A*11, A*68, B*44, B*51, C*05, C*14	n.a.	n.a.	n.a.	n.a.	n.a.
		L_{MR}	41	no	7			904	975	232	153
28	chronic phase	P	54	no	-	A*02, A*03, B*18, B*44, C*12, C*16	n.a.	n.a.	n.a.	n.a.	n.a.
		L_{MR}	55	no	6			1,033	1,077	234	172
29	chronic phase	P, E	69	imatinib 400 mg	-	A*02, A*11, B*18, B*35, C*02, C*04	DRB1*01, DRB1*11, DQB1*03, DQB1*05	n.a.	n.a.	n.a.	n.a.
		L_{MR}	70	imatinib 400 mg	6			1,145	918	229	159
30	chronic phase	P, E	39	nilotinib 300 mg	-	A*02, A*03, B*18, B*73	n.a.	n.a.	n.a.	n.a.	n.a.
31	chronic phase	P, E	48	nilotinib 600 mg	-	A*03, A*24, B*07, B*49, C*07	n.a.	n.a.	n.a.	n.a.	n.a.
		L_{MR}	49	nilotinib 600 mg	6			417	498	193	257
32	chronic phase	E	55	imatinib 400 mg	-	A*11, A*32, B*27, B*47	n.a.	n.a.	n.a.	n.a.	n.a.
33	chronic phase	P, E, T	52	imatinib 400 mg	-	A*02:01, A*03:01, B*15:01, B*57:01, C*03:03, C*06:02	n.a.	n.a.	n.a.	n.a.	n.a.
34	chronic phase	E	80	nilotinib 600 mg	-	A*02, A*29, B*40, B*44, C*03, C*16	n.a.	n.a.	n.a.	n.a.	n.a.
		T, L_{MR}	81	nilotinib 800 mg	12			720	795	210	131
35	chronic phase	E	22	imatinib 400 mg	-	A*02, B*07, B*15	n.a.	n.a.	n.a.	n.a.	n.a.
36	chronic phase	P, E	58	nilotinib 800 mg	-	A*02:01, B*27:05, B*44:02, C*01:02, C*05:01	DRB1*04:08, DRB1*11:01, DQB1*03:01	n.a.	n.a.	n.a.	n.a.
37	chronic phase	E	57	dasatinib 100 mg	-	A*02:01, A*03:01, B*35:03, B*47:01, C*04:01, C*06:02	DRB1*04:05, DRB1*11:04, DQB1*03:01, DQB1*03:02	n.a.	n.a.	n.a.	n.a.
	accelerated phase	T	57	dasatinib 100 mg	-			n.a.	n.a.	n.a.	n.a.
38	chronic phase	E, T, L_{MR}	79	imatinib 400 mg	5	A*01, A*11, B*13, B*56, C*01, C*06	n.a.	311	289	122	129
39	chronic phase	E	46	imatinib 400 mg	-	A*01, A*32, B*15	n.a.	n.a.	n.a.	n.a.	n.a.
40	chronic phase	P, E	68	ponatinib 30 mg	-	A*03, A*24, B*18, B*58	n.a.	n.a.	n.a.	n.a.	n.a.
	accelerated phase	T	68	imatinib 400 mg, hydroxyurea 500 mg	-			n.a.	n.a.	n.a.	n.a.
41	chronic phase	E	72	nilotinib 600 mg	-	A*01, B*08, B*44	n.a.	n.a.	n.a.	n.a.	n.a.

UPN	Phase of disease	Experiment	Age	CML-specific therapy	WBC (10^3/µl)	HLA class I typing	HLA class II typing	HLA class I		HLA class II	
								HLA ligand IDs	Protein IDs	Peptide IDs	Protein IDs
42	chronic phase	E	40	imatinib 400 mg	-	A*02, B*27, B*56	n.a.	n.a.	n.a.	n.a.	n.a.
43	chronic phase	E	63	imatinib 400 mg	-	A*01, B*08	n.a.	n.a.	n.a.	n.a.	n.a.
44	chronic phase	E, T	39	nilotinib 600 mg	-	A*02, B*13, B*18	n.a.	n.a.	n.a.	n.a.	n.a.
45	chronic phase	E, T	58	imatinib 400 mg	-	A*02:01, A*23:01, B*44:02, B*44:03, C*04:01, C*05:01	DRB1*04:01, DQB1*02:02, DQB1*03:02	n.a.	n.a.	n.a.	n.a.
46	chronic phase	P, E	62	imatinib 400 mg	-	A*02, A*24, B*18, B*58	n.a.	n.a.	n.a.	n.a.	n.a.
47	chronic phase	E	77	imatinib 400 mg	-	A*01, A*26, B*08, B*55	n.a.	n.a.	n.a.	n.a.	n.a.
48	chronic phase	T	43	bosutinib 500 mg	-	A*02, A*68, B*44, B*55	n.a.	n.a.	n.a.	n.a.	n.a.
		E	44	dasatinib 70 mg	-			n.a.	n.a.	n.a.	n.a.
49	chronic phase	P, E, T	68	imatinib 300 mg	-	A*02, A*25, B*08, B*13, C*06, C*07	n.a.	n.a.	n.a.	n.a.	n.a.
50	chronic phase	E	77	nilotinib 600 mg	-	A*01, A*03, B*08, B*49	n.a.	n.a.	n.a.	n.a.	n.a.
51	chronic phase	E, T	53	nilotinib 600 mg	-	A*02:01, A*30:01, B*13:02, B*58:01, C*06:02, C*07:01	DRB1*07:01, DRB1*13:02, DQB1*02:02, DQB1*06:04	n.a.	n.a.	n.a.	n.a.
52	chronic phase	E	72	interferon	-	A*02, A*03, B*07, B*08	n.a.	n.a.	n.a.	n.a.	n.a.
53	chronic phase	E	55	nilotinib 600 mg	-	A*02:01, A*03:01, B*18:01, B*53:01, C*04:01, C*12:03	DRB1*04:01, DRB1*15:03, DQB1*03:02, DQB1*06:02	n.a.	n.a.	n.a.	n.a.
		L_{MR}	56	interferon	7			416	355	281	215
54	chronic phase	E	59	imatinib 200 mg	-	A*01:01, B*08:01, C*07:01	DRB1*01:01, DRB1*03:01, DQB1*02:01, DQB1*05:01	n.a.	n.a.	n.a.	n.a.
55	accelerated phase	P, E	62	dasatinib 140 mg	-	A*02:01, B*07:02, B*35:01, C*04:01, C*07:02	DRB1*11:01, DRB1*16:01, DQB1*03:01, DQB1*05:02	n.a.	n.a.	n.a.	n.a.
56	chronic phase	E	61	nilotinib 600 mg	-	A*26, A*33, B*14, B*56, C*01, C*15	n.a.	n.a.	n.a.	n.a.	n.a.
		L_{MR}	61	nilotinib 600 mg, interferon	5			544	648	74	116
57	chronic phase	E	50	dasatinib 100 mg	-	A*01:01, A*25:01, B*08:01, B*18:01, C*07:01, C*12:03	DRB1*03:01, DRB1*15:01, DQB1*02:01, DQB1*06:02	n.a.	n.a.	n.a.	n.a.
58	chronic phase	E, T	74	imatinib 400 mg	-	A*02, A*03, B*07, B*44, C*07, C*16	DRB1*07:01, DRB1*13:01, DQB1*02:02, DQB1*06:03	n.a.	n.a.	n.a.	n.a.
59	chronic phase	T	37	imatinib 400 mg	-	A*02:01, A*29:02, B*44:02, B*58:01, C*05:01, C*07:01	DRB1*08:04, DRB1*13:01, DQB1*04:02, DQB1*06:03	n.a.	n.a.	n.a.	n.a.
		P	38	imatinib 800 mg	-			n.a.	n.a.	n.a.	n.a.
60	chronic phase	P, L_{MR}	67	imatinib 400 mg	4	A*02, B*18, B*52, C*02	n.a.	576	512	88	124

UPN	Phase of disease	Experiment	Age	CML-specific therapy	WBC (10^3/µl)	HLA class I typing	HLA class II typing	HLA class I		HLA class II	
								HLA ligand IDs	Protein IDs	Peptide IDs	Protein IDs
61	chronic phase	L_{MR}	68	imatinib 400 mg, interferon	6	A*02, B*27, B*44, C*01, C*05	DRB1*01:03, DRB1*11:01, DQB1*03:01, DQB1*05:01	646	741	136	72
62	chronic phase	L_{MR}	57	interferon	7	A*24, A*31, B*18, B*40 , C*03, C*07	n.a.	768	863	180	168
63	chronic phase	T	55	dasatinib 100 mg	-	A*02, B*18, B*44	n.a.	n.a.	n.a.	n.a.	n.a.
64	chronic phase	T	75	imatinib 400 mg	-	A*02, A*24, B*40, B*44	n.a.	n.a.	n.a.	n.a.	n.a.
65	chronic phase	T	36	nilotinib 600 mg	-	A*02:01, A*30:01, B*13:02, B*40:01, C*03:04, C*06:02	DRB1*04:04, DRB1*04:05, DQB1*03:02	n.a.	n.a.	n.a.	n.a.
66	chronic phase	T	46	imatinib 400 mg	-	A*02, B*39, B*55	n.a.	n.a.	n.a.	n.a.	n.a.
67	chronic phase	L_{MR}	37	imatinib 400 mg	5	A*01:01, A*02:01, B*14:01, B*57:01, C*06:02, C*08:02	n.a.	467	404	147	152
68	chronic phase	L_{MR}	75	imatinib 400 mg	5	A*26, A*33, B*14, B*56, C*01, C*08	n.a.	735	823	101	129

Table S1: Patient characteristics. CML patients included in HLA ligandome analysis, quantification of HLA surface expression, *in vitro* T-cell priming experiments, and IFN-γ ELISPOT assays. CML-specific treatment at the time of sample collection is given as dose per day. Lymphocyte count at the time of sample collection is indicated for patients included in HLA ligandome analysis. Abbreviations: UPN, uniform patient number; WBC, white blood cell count; IDs, identifications; L, HLA ligandome analysis for the identification of CML-associated target antigens; L_{MR}, HLA ligandome analysis of CML patients in deep molecular remission (MR4.0 or better) as additional comparative benign dataset; Q, HLA quantification; P, *in vitro* priming of naïve $CD8^+$ T cells; E, IFN-γ ELISPOT assay; T, tetramer staining after 12-d stimulation; n.a., not available.

Supplemental Table 2

Protein	Protein accession	Sequence	HLA restriction
BCR-ABL fusion protein			
p210, X9	A1Z199, A9UF02	IPLTINKEEAL	B*07
e3a11	Q8NF93	LPQAGAQIRGL	B*07
		TPAIFSPRL	B*07, B*35
e8a2	A9YD18	LELLTSEAL	B*18, B*40
e8a2	B0ZRR0	EEITPRRPL	B*18
		LPEALQRPV	B*07
		RPLSLPEAL	B*07, B*35
		SLPEALQRPV	A*02
		TPRRPLSL	B*08
e8a2	E7E8T7	TVKKGELLNRK	A*03
		VPSIPYLEAL	B*07, B*35
e13a3	A2RQD3	NKEGEKLRVLGY	A*01
e14a2	B0ZRR1	KQSSVPTSSK	A*03
		VPTSSKENLL	B*35
e14a3	A2RQD4, Q16189	SSEKLRVLGY	A*01
e18-int1b-a2	B0ZRQ9	FLRKRPQEAL	B*08
		RPQEALQRPV	B*07
X3	A9UEZ6	KLASQLGVYRV	A*02
X5	A9UEZ8	ILASEEITL	A*02
X6	A9UEZ9	LELQGKPSRW	B*44
X7	A9UF00	LEMRKWDPV	B*40
X7, Y4	A9UF00, A9UF06	DPVKMTPTF	B*35
		DPVKMTPTFSL	B*07
		ILWPVEITL	A*02
X8	A9UF01	AEEVFQKLL	B*40, B*44
		HPGAAEEVF	B*35
X9	A9UF02	EETYLSHLQM	B*18, B*44
Y2	A9UF04	RPPHLTELPV	B*07
Y3	A9UF05	EALGEDPSF	B*35
		FPASDGPRH	B*35
		GEGAFHGDAVL	B*40
		GVRRARLSL	B*07
		HQGVRRARL	B*08
		RARLSLEAL	B*07
		VPHQGVRRARL	B*07
Y4	A9UF06	LDPVKMTPTF	B*35
		SELDPVKMTPTF	B*44
Y5	A9UF07	QIKSDIQREK	A*03
		SPSSSPHRQL	B*35
		SPSSSPHRQLL	B*07
		SSSPHRQLLK	A*03
		ATGFKQSSK	A*03
		KQSSKALQR	A*03
n.s.	A0A127KQ99	RISQNFLSKK	A*03
n.s.	A6MF66	GEGAFHGDAAL	B*40
n.s.	A6MF66, A6MF67	ALRLLREPL	B*08
n.s.	A6MF66, A6MF67, A6MF68	RLLREPLQH	A*03
		HPGRVGSSSF	B*07, B*35
n.s.	A6MF67	IPLTINKEAL	B*07, B*35
n.s.	A6MF68	TLRLLREPL	B*08
ABL-BCR fusion protein			
Iab3	n.s.	YLEDDESPGL	A*02
Iab3	n.s.	YLEDDESPGLY	A*01
Ibb3	n.s.	FVEHDDESPGLY	A*01

Table S2: BCR-ABL- and ABL-BCR-derived peptides targeted by parallel reaction monitoring. List of BCR-ABL- and ABL-BCR-derived peptide sequences with their corresponding HLA restriction targeted by parallel reaction monitoring. Abbreviation: n.s., not specified.

Supplemental Table 3

Source protein	Peptide sequence	HLA restriction
Positive control peptides		
BRLF1_EBVB9	YVLDHLIVV	A*02
EBNA3_EBVB9	RLRAEAQVK	A*03
EBNA3_EBVB9	RPPIFIRRL	B*07
BZLF1_EBVB9	RAKFKQLL	B*08
EBNA6_EBVB9	AEGGVGWRHW	B*44
PP65_HCMVA	YQEFFWDANDIYRIF	class II
EBNA1_EBVB9	KTSLYNLRRGTALA	class II
PP65_HCMVA	HPTFTSQYRIQGKLEYR	class II
PP65_HCMVA	KPGKISHIMLDVAFTSH	class II
GP350_EBVB9	STNITAVVRAQGLDV	class II
GP350_EBVB9	PRPVSRFLGNNSILY	class II
Negative control peptides		
POL_HV1BR	GSEELRSLY	A*01
DDX5_HUMAN	YLLPAIVHI	A*02
GAG_HV1BR	RLRPGGKKK	A*03
UBC_HUMAN	QIFVKTLTGK	A*03
NEF_HV1BR	TPGPGVRYPL	B*07
ANM1_HUMAN	DEVRTLTY	B*18
FLNA_HUMAN	ETVITVDTKAAGKGK	class II

Table S3: Positive and negative control peptides. Positive and negative control peptides with their respective HLA restrictions used for IFN-γ ELISPOT assays or aAPC-based *in vitro* priming experiments. Abbreviations: EBVB9, Epstein-Barr virus (strain B95-8); HCMVA, human cytomegalovirus (strain AD169); HV1BR, human immunodeficiency virus type 1 group M subtype B.

Supplemental Table 4

Protein	Sequence	Peptide length	HLA restriction	Representation frequency in CML cohort	Representation frequency in HLA-matched CML samples
All HLA ligands					
PLSL	HLLEQVAPK	9	A*03	29%	75%
ARP2, ACTB	RLDIAGRDI	9	C*04, C*05, C*17	29%	n.a.
NDC80	SINKPTSER	9	A*03	29%	63%
*	DMEKIWHHTFY	11	A*01, B*18, B*44	24%	n.a.
DHRS9	KIFWIPLSH	9	A*03	24%	63%
H12	AAKPKVVKPK	10	A*11	19%	80%
CSN6	ALHPLVILNI	10	A*02	19%	40%
BPI	AVNPGVVVR	9	A*11	19%	80%
RB27A	FRDAMGFLLL	10	C*04, C*05, C*07	19%	n.a.
PSB9	GTLGGMLTR	9	A*11, A*66	19%	n.a.
IQGA1	IFLLNKKFYGK	11	A*03	19%	50%
CEAM8	KLFIPNITTK	10	A*03	19%	50%
BEX1, BEX2	KLREKQLSH	9	A*03	19%	50%
GELS	KQGFEPPSFV	10	A*02	19%	40%
**	KQVHPDTGISSK	12	A*03	19%	50%
S10A8	NTDGAVNFQEF	11	A*01, C*05	19%	n.a.
CD24	RAMVARLGL	9	B*07	19%	57%
UTP20	RIAKLEAAY	9	A*03	19%	50%
ANLN	RLLLIATGK	9	A*03	19%	50%
PCNA	RLVQGSILKK	10	A*03	19%	50%
PTN7	RTAGHPLTR	9	A*03	19%	50%
GFI1B, GFI1	TLSTHLLIH	9	A*03	19%	50%
XPO5	YRPEFLPQVF	10	C*07, B*07	19%	n.a.
HLA-A*02					
C3AR	KLIPSIIVL	9	A*02	24%	50%
CSN6	ALHPLVILNI	10	A*02	19%	40%
GELS	KQGFEPPSFV	10	A*02	19%	40%
BPI	LLFGADVVYK	10	A*02	48%	40%
HLA-A*03					
PLSL	HLLEQVAPK	9	A*03	29%	75%
DHRS9	KIFWIPLSH	9	A*03	24%	63%
BPI	LLFGADVVYK	10	A*03	48%	63%
NDC80	SINKPTSER	9	A*03	29%	63%
IQGA1	IFLLNKKFYGK	11	A*03	19%	50%
CEAM8	KLFIPNITTK	10	A*03	19%	50%
BEX1, BEX2	KLREKQLSH	9	A*03	19%	50%
**	KQVHPDTGISSK	12	A*03	19%	50%
GELS	KTASDFITK	9	A*03	29%	50%
UTP20	RIAKLEAAY	9	A*03	19%	50%
ANLN	RLLLIATGK	9	A*03	19%	50%
PCNA	RLVQGSILKK	10	A*03	19%	50%
PTN7	RTAGHPLTR	9	A*03	19%	50%
CKAP5	STLPKSLLK	9	A*03	24%	50%
GFI1B, GFI1	TLSTHLLIH	9	A*03	19%	50%
GPAA1	ALVFPPLTQR	10	A*03	14%	38%
CEAM8	GTFQQYTQK	9	A*03	29%	38%
PERM	GVSEPLKRK	9	A*03	24%	38%
LARP1	HSNTQTLGK	9	A*03	14%	38%
SSPN	IQFSMKLLY	9	A*03	14%	38%

Protein	Sequence	Peptide length	HLA restriction	Representation frequency in CML cohort	Representation frequency in HLA-matched CML samples
PCNA	KIADMGHLKY	10	A*03	14%	38%
RIR1	KINGKVAER	9	A*03	14%	38%
BPI	KISGKWKAQK	10	A*03	14%	38%
GLE1	KLREAEQQRVK	11	A*03	14%	38%
DENR	KLTVENSPK	9	A*03	14%	38%
CQ085	KMISTPSPK	9	A*03	14%	38%
GTPB1	KMQSTKKGPLTK	12	A*03	14%	38%
PDIA1	KVHSFPTLK	9	A*03	14%	38%
KI67	RIQLPVVSK	9	A*03	33%	38%
HDGR2	RLKSRVLGPK	10	A*03	14%	38%
PDIP3	RLSDSPSMK	9	A*03	14%	38%
RHG25	SAFQGANSSK	10	A*03	14%	38%
TCO1	SLVEKILSEK	10	A*03	14%	38%
LPPR3	SVISDTTKLLK	11	A*03	33%	38%
S10A8	VIKMGVAAHKK	11	A*03	14%	38%
HLA-A*11					
H12	AAKPKVVKPK	10	A*11	19%	80%
BPI	AVNPGVVVR	9	A*11	19%	80%
PCNA	KIADMGHLK	9	A*11	33%	80%
HLA-B*07					
CD24	RAMVARLGL	9	B*07	19%	57%
XPO5	YRPEFLPQVF	10	B*07, C*07	19%	57%
STT3B	APESKHKSSL	10	B*07	14%	43%
ATPA	APGIIPRISV	10	B*07	14%	43%
CEBPE	APGQPLRVL	9	B*07	14%	43%
BPI	NPTSGKPTI	9	B*07	14%	43%
HTR5A, HTR5B	SPGPPTRKL	9	B*07	14%	43%
ANLN	SPVKSTTSI	9	B*07	14%	43%

Table S4: Identified CML-associated HLA class I antigens. Panel of naturally presented, CML-associated, HLA class I-restricted peptide targets identified by HLA class I ligandome profiling of primary CML samples (n = 21) and a hematological benign dataset (n = 108). Peptides mapping in multiple proteins are marked with asterisks: *ACTBM, ACTB, ACTC, ACTG, ACTS, POTEE, POTEF, POTEI, POTEJ and **H2B1A, H2B1B, H2B1C, H2B1D, H2B1H, H2B1J, H2B1K, H2B1L, H2B1M, H2B1N, H2B1O, H2B2E, H2B2F, H2B3B, H2BFS. Abbreviation: n.a., not applicable.

Supplemental Table 5

Protein	Sequence	Peptide length	Representation frequency	Peptide target	Protein target	Hotspot target
Peptide targets						
VAT1	**Synaptic vesicle membrane protein VAT-1 homolog**					
	VLFDFGNLQPGHSV	14	45%	x		x
TCPG	**T-complex protein 1 subunit gamma**					
	AMQVCRNVLLDPQLVPGG	18	25%	x		x
BPI	**Bactericidal permeability-increasing protein**					
	NEKLQKGFPLPTPARV	16	25%	x		x
KCY	**UMP-CMP kinase**					
	DEVVQIFDKEG	11	25%	x	x	x
MYO9B	**Unconventional myosin-IXb**					
	MEPAFIIQHF	10	25%	x		x
XRCC5	**X-ray repair cross-complementing protein 5**					
	EEASNQLINHIEQF	14	20%	x		x
Protein targets						
KCY	**UMP-CMP kinase**		25%			
	DEVVQIFDKEG	11	25%	x	x	x
TCPW	**T-complex protein 1 subunit zeta-2**		25%			
	ADALLIIPK	9	15%		x	x
	ADALLIIPKVL	11	10%		x	x
	ADALLIIPKVLA	12	5%		x	x
MCM2	**DNA replication licensing factor MCM2**		20%			
	SENVDLTEPIISRF	14	5%		x	
	GPRLEIHHRF	10	5%		x	
	IESIENLEDLKGHSV	15	5%		x	
	ANGFPVFATVIL	12	5%		x	
PTBP2	**Polypyrimidine tract-binding protein 2**		20%			
	GLPFGKVTNIL	11	10%		x	
	FGVYGDVQRV	10	10%		x	
Hotspot targets						
RB27A	**Ras-related protein Rab-27A**		20%			
	FDLTNEQSFLNV	12	15%			x
	FDLTNEQSFL	10	5%			x
	DLTNEQSFLNV	11	5%			x

Table S5: Identified CML-associated HLA class II antigens. Panel of naturally presented, CML-associated HLA class II antigens identified by HLA class II ligandome profiling of primary CML samples (n = 20) and a hematological benign dataset (n = 88). Peptides are assigned to the respective group of target antigens (peptide targets, protein targets, and hotspot targets).

Supplemental Table 6

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
CML-exclusive				
CRIS2	Cysteine-rich secretory protein 2	1	0	1
MAGA6	Melanoma-associated antigen 6	1	0	1
SPG17	Sperm-associated antigen 17	1	0	1
TULP2	Tubby-related protein 2	1	0	1
CML-overrepresented				
DNJB8	DnaJ homolog subfamily B member 8	1	1	1
FA46D	Putative nucleotidyltransferase FAM46D	1	1	1
KDM5B	Lysine-specific demethylase 5B	10	15	20
KIF2C	Kinesin-like protein KIF2C	4	5	7
MAGA1	Melanoma-associated antigen 1	1	1	2
NUF2	Kinetochore protein Nuf2	4	11	13
ODFP2	Outer dense fiber protein 2	2	5	5
SO6A1	Solute carrier organic anion transporter family member 6A1	1	1	1
Benign-overrepresented				
HEMGN	Hemogen	3	10	11
POTEA	POTE ankyrin domain family member A	1	2	2
POTEE	POTE ankyrin domain family member E	30	70	74
Benign-exclusive				
ACRBP	Acrosin-binding protein	0	4	4
BRDT	Bromodomain testis-specific protein	0	2	2
CCNA1	Cyclin-A1	0	1	1
CE290	Centrosomal protein of 290 kDa	0	1	1
CTCFL	Transcriptional repressor CTCFL	0	1	1
CTGE2	cTAGE family member 2	0	1	1
F133A	Protein FAM133A	0	1	1
GPAT2	Glycerol-3-phosphate acyltransferase 2, mitochondrial	0	1	1
LDHC	L-lactate dehydrogenase C chain	0	2	2
MAGAA	Melanoma-associated antigen 10	0	1	1
MAGC1	Melanoma-associated antigen C1	0	1	1
MORC1	MORC family CW-type zinc finger protein 1	0	3	3
MS18B	Protein Mis18-beta	0	1	1
PASD1	Circadian clock protein PASD1 (PAS domain containing 1)	0	1	1
PRS55	Serine protease 55	0	1	1

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
RGS22	Regulator of G-protein signaling 22	0	1	1
SSX1	Protein SSX1	0	1	1
SYCE1	Synaptonemal complex central element protein 1	0	1	1
TAF7L	Transcription initiation factor TFIID subunit 7-like	0	1	1
TSG10	Testis-specific gene 10 protein	0	1	1
XAGE2	X antigen family member 2	0	1	1
XAGE3	X antigen family member 3	0	1	1
XAGE5	X antigen family member 5	0	1	1
ZN165	Zinc finger protein 165	0	2	2

Table S6: Numbers of identified HLA class I peptides derived from cancer/testis antigens. Numbers of naturally presented unique HLA class I peptides derived from predescribed cancer/testis antigens (Almeida *et al.* Nucleic Acids Res. 2009, GTEx Consortium Cancer Immunol Immunother. 2011) identified in CML patient (n = 21) and hematological benign (n = 108) ligandomes. Antigens are assigned to their degree of CML-association determined by their representation frequencies in both cohorts (CML-exclusive, CML-overrepresented, benign-overrepresented, benign-exclusive).

Supplemental Table 7

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
CML-overrepresented				
SPG17	Sperm-associated antigen 17	1	1	2
SO6A1	Solute carrier organic anion transporter family member 6A1	1	1	1
POTEE	POTE ankyrin domain family member E	173	300	333
Benign-exclusive				
ACRBP	Acrosin-binding protein	0	1	1
AKAP3	A-kinase anchor protein 3	0	1	1
CTGE2	cTAGE family member 2	0	4	4
HEMGN	Hemogen	0	19	19
HSP1	Sperm protamine P1	0	2	2
KDM5B	Lysine-specific demethylase 5B	0	1	1
LUZP4	Leucine zipper protein 4	0	3	3
MAGA1	Melanoma-associated antigen 1	0	3	3
MAGA4	Melanoma-associated antigen 4	0	1	3
MAGC1	Melanoma-associated antigen C1	0	3	3
POTEA	POTE ankyrin domain family member A	0	1	1
SSX2	Protein SSX2	0	1	1
SYCP1	Synaptonemal complex protein 1	0	4	4
TAF7L	Transcription initiation factor TFIID subunit 7-like	0	1	1
TEX14	Inactive serine/threonine-protein kinase TEX14	0	2	2

Table S7: Numbers of identified HLA class II peptides derived from cancer/testis antigens. Numbers of naturally presented unique HLA class II peptides derived from predescribed cancer/testis antigens (Almeida *et al.* Nucleic Acids Res. 2009, GTEx Consortium Cancer Immunol Immunother. 2011) identified in CML patient (n = 20) and hematological benign (n = 88) ligandomes. Antigens are assigned to their degree of CML-association determined by their representation frequencies in both cohorts (CML-overrepresented, benign-exclusive).

Supplemental Table 8

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
CML-exclusive				
MAGA6	Melanoma-associated antigen 6	1	0	1
CML-overrepresented				
ABC3B	DNA dC->dU-editing enzyme APOBEC-3B	2	9	9
AURKA	Aurora kinase A	3	3	4
BMI1	Polycomb complex protein BMI-1	1	1	2
CATG	Cathepsin G	19	22	28
CLIC1	Chloride intracellular channel protein 1	7	14	14
CNTRL	Centriolin	3	12	13
COF1	Cofilin-1	21	29	33
CTDS1	Carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 1	1	5	5
DCTN3	Dynactin subunit 3	3	7	8
DDX6	Probable ATP-dependent RNA helicase DDX6	7	16	16
DEF1	Neutrophil defensin 1	14	18	26
DNLI3	DNA ligase 3	1	5	5
EF1A1	Elongation factor 1 alpha 1	28	37	48
EHD1	EH domain containing protein 1	6	25	26
ELNE	Neutrophil elastase	4	9	11
ENOA	Alpha-enolase	10	18	21
FES	Tyrosine-protein kinase Fes/Fps	2	11	12
GRK5	G protein-coupled receptor kinase 5	1	3	3
HBA	Hemoglobin subunit alpha	20	51	52
HBB	Hemoglobin subunit beta	20	39	41
HMMR	Hyaluronan mediated motility receptor	2	4	5
HNRPM	Heterogeneous nuclear ribonucleoprotein M	12	29	31
HSP7C	Heat shock cognate 71 kDa protein	15	46	46
IF1AY	Eukaryotic translation initiation factor 1A, Y-chromosomal	2	4	5
INAR1	Interferon alpha/beta receptor 1	1	1	2
KAT3	Kynurenine--oxoglutarate transaminase 3	1	5	5
MAF	Transcription factor Maf	1	2	2
MYBA	Myb-related protein A	1	1	1
PCNA	Proliferating cell nuclear antigen	18	26	29
PLIN2	Perilipin-2	3	17	17
PPIA	Peptidyl-prolyl cis-trans isomerase A	2	5	5

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
PRTN3	Myeloblastin	13	15	19
R51A1	RAD51 associated protein 1	3	2	3
RAB38	Ras-related protein Rab-38	1	2	2
RAF1	RAF proto-oncogene serine/threonine-protein kinase	2	10	11
RBBP4	Histone-binding protein RBBP4	9	24	24
RIMS2	Regulating synaptic membrane exocytosis protein 2	1	1	2
RL18A	60S ribosomal protein L18a	4	7	7
S10A9	Protein S100-A9	13	12	16
STMN1	Stathmin	3	8	8
SUH	Recombining binding protein suppressor of hairless	2	3	3
TBB5	Tubulin beta chain	43	76	89
TKT	Transketolase	15	30	31
TRYB1	Tryptase alpha/beta-1	2	3	4
TSP1	Thrombospondin 1	8	18	23
TYB4Y	Thymosin beta-4, Y-chromosomal	1	5	5
VPS4B	Vacuolar protein sorting-associated protein 4B	3	5	6
Benign-overrepresented				
ADIP	Afadin- and alpha-actinin-binding protein	1	7	7
ALDOA	Fructose-bisphosphate aldolase A	7	23	23
BCR	Breakpoint cluster region protein	1	5	5
CBL	E3 ubiquitin-protein ligase CBL	1	12	12
CDN2D	Cyclin-dependent kinase 4 inhibitor D	2	7	7
CHD2	Chromodomain-helicase-DNA-binding protein 2	2	10	11
CKS1	Cyclin-dependent kinases regulatory subunit 1	1	3	4
CSK	Tyrosine-protein kinase CSK	1	14	14
CUL4B	Cullin-4B	2	19	19
DDX5	Probable ATP-dependent RNA helicase DDX5	11	34	36
DEMA	Dematin	3	10	11
DNJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	3	12	12
DQB1	HLA class II histocompatibility antigen, DQ beta 1 chain	1	7	7
EM55	55 kDa erythrocyte membrane protein	5	22	22
FAK2	Protein tyrosine kinase 2 beta	10	22	24
G3P	Glyceraldehyde-3-phosphate dehydrogenase	5	28	29
GPKOW	G-patch domain and KOW motifs-containing protein	1	7	7
HS74L	Heat shock 70 kDa protein 4L	1	8	8

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
ICE2	Little elongation complex subunit 2	1	6	6
IRAK3	Interleukin-1 receptor-associated kinase 3	2	9	10
ITB5	Integrin beta-5	1	14	14
M3K1	Mitogen-activated protein kinase kinase kinase 1	4	21	22
MGRN1	E3 ubiquitin-protein ligase MGRN1	1	8	8
NAB2	NGFI-A binding protein 2	1	2	2
NOLC1	Nucleolar and coiled-body phosphoprotein 1	2	10	10
NU155	Nuclear pore complex protein Nup155	2	17	18
NUDC1	NudC domain containing 1	1	7	7
PARP1	Poly (ADP-ribose) polymerase 1	4	32	32
PHRF1	PHD and RING finger domain-containing protein 1	1	5	5
PIM1	Serine/threonine-protein kinase pim-1	5	13	16
RBM25	RNA binding protein 25	2	7	8
RHG04	Rho GTPase activating protein 4	1	15	15
RSSA	40S ribosomal protein SA	3	9	10
SEPT4	Septin-4	2	8	8
SP110	SP110 nuclear body protein	1	8	8
SPB9	Serpin B9	1	7	7
TPM1	Tropomyosin alpha-1 chain	1	5	5
UBP32	Ubiquitin carboxyl-terminal hydrolase 32	2	5	6
VPS51	Vacuolar protein sorting-associated protein 51 homolog	3	11	11
WASC5	WASH complex subunit 5	2	13	14
WT1	Wilms tumor protein	1	2	2
Benign-exclusive				
AFAD	Afadin	0	2	2
AGAP3	ArfGAP with GTPase, ANK repeat and PH domain-containing protein 3	0	2	2
ANR50	Ankyrin repeat domain-containing protein 50	0	1	1
ARI5A	AT-rich interactive domain-containing protein 5A	0	3	3
AVR2B	Activin receptor type-2B	0	1	1
BCL2	Apoptosis regulator Bcl-2	0	5	5
BIRC5	Baculoviral IAP repeat-containing protein 5	0	2	2
BRAP	BRCA1 associated protein	0	3	3
CD3Z	T-cell surface glycoprotein CD3 zeta chain	0	3	3
CD44	CD44 antigen	0	1	1
CD5	T-cell surface glycoprotein CD5	0	1	1
CNN3	Calponin 3	0	6	6
COR2A	Coronin-2A	0	2	2

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
CREL2	Cysteine-rich with EGF-like domain protein 2	0	1	1
DNJC2	DnaJ (Hsp40) homolog, subfamily C, member 2	0	1	1
ELMO3	Engulfment and cell motility protein 3	0	2	2
EXOS5	Exosome complex component RRP46	0	3	3
EZH2	Histone-lysine N-methyltransferase EZH2	0	5	5
FMO1	Dimethylaniline monooxygenase [N-oxide-forming] 1	0	1	1
GRAH	Granzyme H	0	2	2
IFT46	Intraflagellar transport protein 46 homolog	0	1	1
IFT88	Intraflagellar transport protein 88 homolog	0	5	5
JIP4	C-Jun-amino-terminal kinase-interacting protein 4	0	6	6
KS6C1	Ribosomal protein S6 kinase delta-1	0	4	4
MEMO1	Protein MEMO1	0	1	1
MVD1	Diphosphomevalonate decarboxylase	0	4	4
NDKB	Nucleoside diphosphate kinase B	0	4	4
OGFR	Opioid growth factor receptor	0	8	8
PASD1	Circadian clock protein PASD1	0	1	1
PEPD	Xaa-Pro dipeptidase	0	6	6
PTCD3	Pentatricopeptide repeat domain-containing protein 3, mitochondrial	0	1	1
RBBP5	Retinoblastoma binding protein 5	0	3	3
RET	Proto-oncogene tyrosine-protein kinase receptor Ret	0	4	4
RL12	60S ribosomal protein L12	0	6	6
RPC3	DNA-directed RNA polymerase III subunit RPC3	0	7	7
TBCE	Tubulin-specific chaperone E	0	2	2
TERT	Telomerase reverse transcriptase	0	2	2
TGFR2	TGF-beta receptor type-2	0	1	1
U119A	Protein unc-119 homolog A	0	5	5
UBR1	E3 ubiquitin-protein ligase UBR1	0	13	13
WDR4	tRNA (guanine-N(7)-)-methyltransferase non-catalytic subunit WDR4	0	4	4
ZN292	Zinc finger protein 292	0	14	14

Table S8: Numbers of identified HLA class I peptides derived from leukemia-associated antigens. Numbers of naturally presented unique HLA class I peptides derived from predescribed leukemia-associated antigens (Greiner *et al.* Eur J Haematol. 2008, Smahel Cancer Immunol Immunother. 2011) identified in CML patient (n = 21) and hematological benign (n = 108) ligandomes. Antigens are assigned to their degree of CML-association determined by their representation frequencies in both cohorts (CML-exclusive, CML-overrepresented, benign-overrepresented, benign-exclusive).

Supplemental Table 9

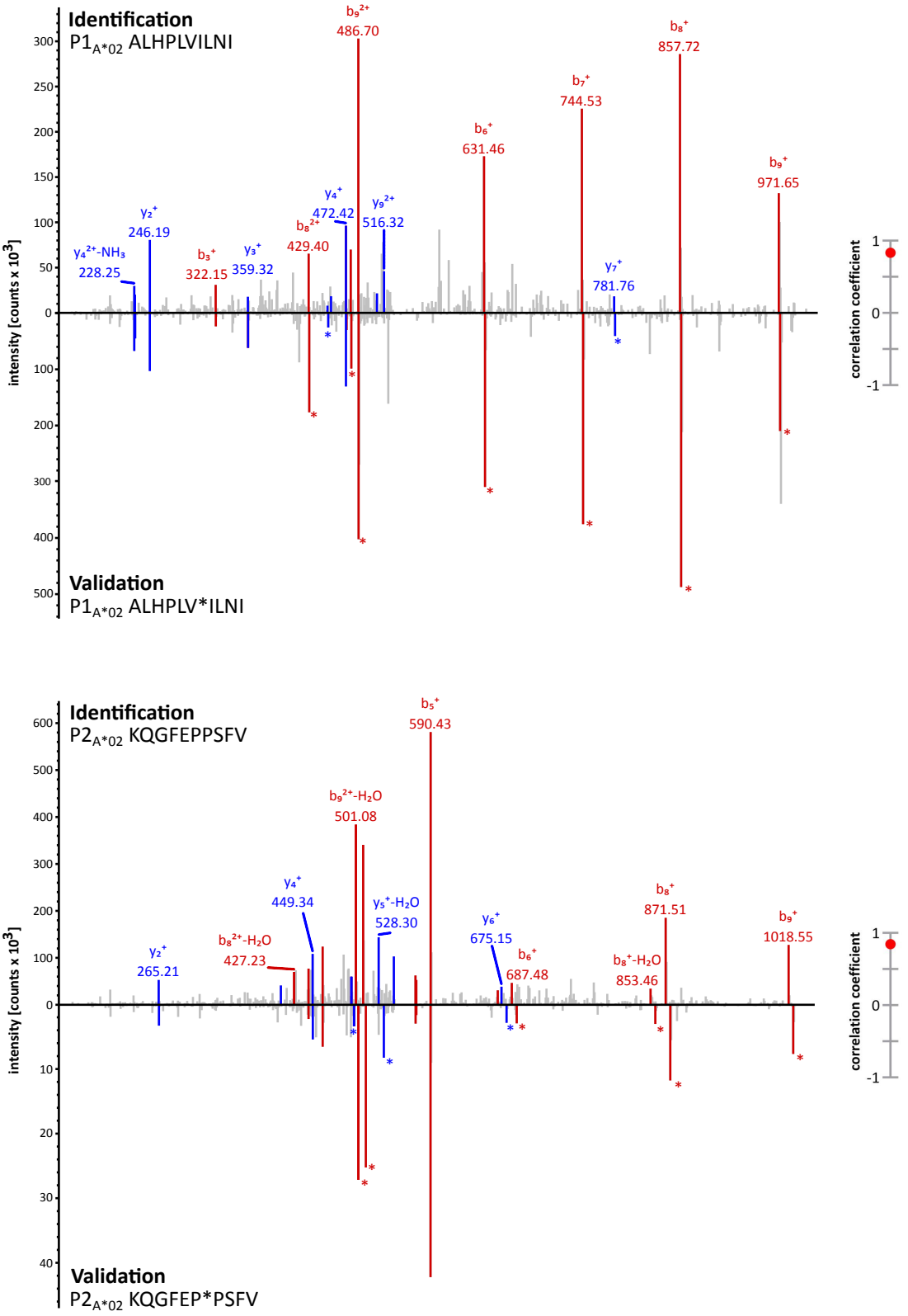
Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
CML-exclusive				
FAK2	Protein tyrosine kinase 2 beta	1	0	1
GPKOW	G-patch domain and KOW motifs-containing protein	1	0	1
OGFR	Opioid growth factor receptor	1	0	1
CML-overrepresented				
CATG	Cathepsin G	27	49	57
COF1	Cofilin 1	143	259	289
CSK	Tyrosine-protein kinase CSK	1	1	2
DDX5	Probable ATP-dependent RNA helicase DDX5	2	6	7
DEF1	Neutrophil defensin 1	25	57	62
EF1A1	Elongation factor 1-alpha 1	103	152	205
EHD1	EH domain-containing protein 1	6	14	17
PARP1	Poly (ADP-ribose) polymerase 1	1	4	4
PCNA	Proliferating cell nuclear antigen	6	22	27
RL12	60S ribosomal protein L12	1	10	11
S10A9	Protein S100-A9	62	114	130
STMN1	Stathmin	6	35	40
TBB5	Tubulin beta chain	110	303	333
TSP1	Thrombospondin-1	20	47	59
TYB4Y	Thymosin beta-4, Y-chromosomal	1	2	3
Benign-overrepresented				
DEMA	Dematin	2	10	12
DNLI3	DNA ligase 3	1	4	4
ELNE	Neutrophil elastase	5	34	35
ENOA	Alpha-enolase	53	298	307
G3P	Glyceraldehyde-3-phosphate dehydrogenase	22	335	338
HBA	Hemoglobin subunit alpha	86	412	417
HBB	Hemoglobin subunit beta	159	432	444
HNRPM	Heterogeneous nuclear ribonucleoprotein M	2	16	18
HSP7C	Heat shock cognate 71 kDa protein	6	162	163
PPIA	Peptidyl-prolyl cis-trans isomerase A	11	78	81
PRTN3	Myeloblastin	12	36	42
TGFR2	TGF-beta receptor type-2	2	12	14
TKT	Transketolase	1	20	21
TPM1	Tropomyosin alpha-1 chain	5	13	13
VPS4B	Vacuolar protein sorting-associated protein 4B	1	10	10
Benign-exclusive				
AFAD	Afadin	0	1	1
ALDOA	Fructose-bisphosphate aldolase A	0	55	55

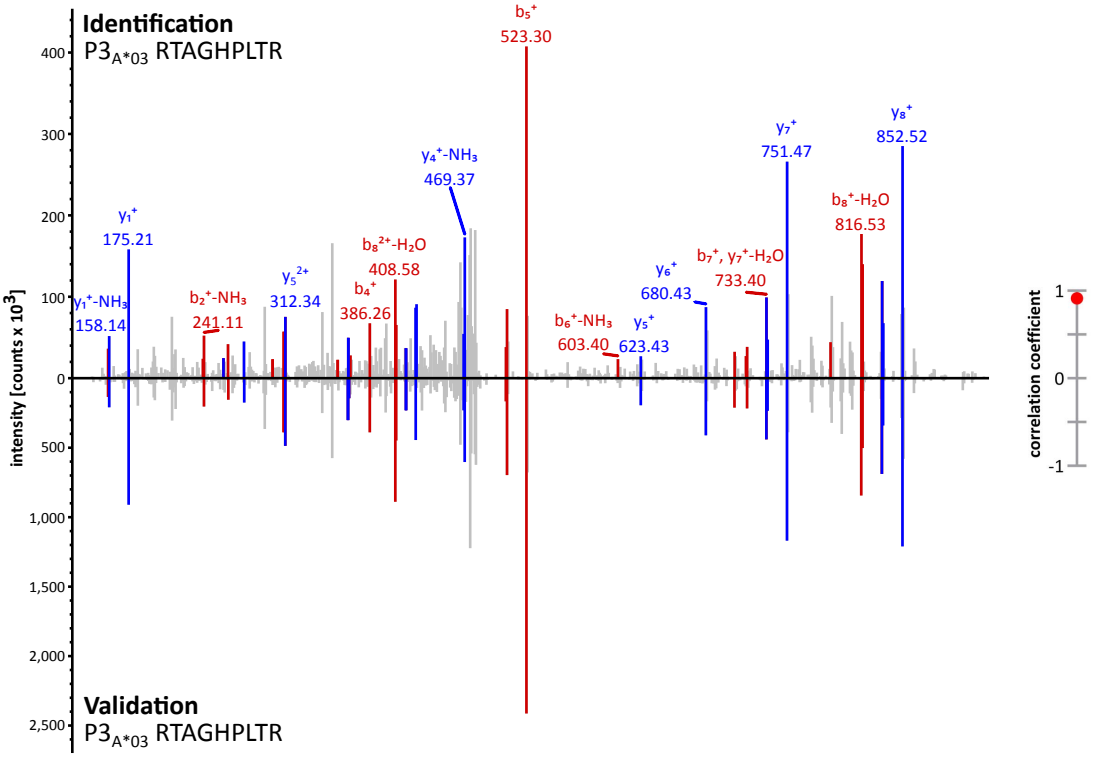
Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
BCL2	Apoptosis regulator Bcl-2	0	1	1
BIRC5	Baculoviral IAP repeat-containing protein 5	0	7	7
BRAP	BRCA1 associated protein	0	1	1
CBL	E3 ubiquitin-protein ligase CBL	0	2	2
CD3Z	T-cell surface glycoprotein CD3 zeta chain	0	2	2
CD44	CD44 antigen	0	7	7
CHD2	Chromodomain helicase DNA binding protein 2	0	1	1
CLIC1	Chloride intracellular channel protein 1	0	10	10
CNN3	Calponin-3	0	1	1
CNTRL	Centriolin	0	2	2
CSF2R	Granulocyte-macrophage colony-stimulating factor receptor subunit alpha	0	1	1
CTDS1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1	0	3	3
CUL4B	Cullin 4B	0	1	1
DDX6	Probable ATP-dependent RNA helicase DDX6	0	2	2
DNJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	0	7	7
DQB1	Major histocompatibility complex, class II, DQ beta 1	0	19	19
EM55	55 kDa erythrocyte membrane protein	0	3	3
GRAH	Granzyme H	0	6	6
GRL1A	DNA-directed RNA polymerase II subunit GRINL1A	0	1	1
HMMR	Hyaluronan mediated motility receptor	0	1	1
IF1AY	Eukaryotic translation initiation factor 1A, Y-chromosomal	0	4	4
IFT88	Intraflagellar transport protein 88 homolog	0	1	1
IL1AP	Interleukin 1 receptor accessory protein	0	5	5
INAR1	Interferon alpha/beta receptor 1	0	8	8
ITB5	Integrin beta-5	0	2	2
JIP4	C-Jun-amino-terminal kinase-interacting protein 4	0	8	8
KAT3	Kynurenine--oxoglutarate transaminase 3	0	5	5
KS6C1	Ribosomal protein S6 kinase delta-1	0	1	1
NDKB	Nucleoside diphosphate kinase B	0	7	7
NU155	Nuclear pore complex protein Nup155	0	2	2
NUDC1	NudC domain-containing protein 1	0	1	1

Protein		# of HLA peptides in CML patient ligandomes	# of HLA peptides in hematological benign ligandomes	Total # of unique HLA peptides
KS6C1	Ribosomal protein S6 kinase delta-1	0	1	1
NDKB	Nucleoside diphosphate kinase B	0	7	7
NU155	Nuclear pore complex protein Nup155	0	2	2
NUDC1	NudC domain-containing protein 1	0	1	1
PEPD	Xaa-Pro dipeptidase	0	3	3
PLIN2	Perilipin 2	0	4	4
PNPT1	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	0	1	1
RBBP4	Histone-binding protein RBBP4	0	1	1
RBM25	RNA binding protein 25	0	2	2
RET	Proto-oncogene tyrosine-protein kinase receptor Ret	0	1	1
RL18A	60S ribosomal protein L18a	0	40	40
RSSA	40S ribosomal protein SA	0	14	14
SPB9	Serpin B9	0	14	14
TRYB1	Tryptase alpha/beta-1	0	1	1
WT1	Wilms tumor protein	0	1	1

Table S9: Numbers of identified HLA class II peptides derived from leukemia-associated antigens. Numbers of naturally presented unique HLA class II peptides derived from predescribed leukemia-associated antigens (Greiner *et al.* Eur J Haematol. 2008, Smahel Cancer Immunol Immunother. 2011) identified in CML patient (n = 20) and hematological benign (n = 88) ligandomes. Antigens are assigned to their degree of CML-association determined by their representation frequencies in both cohorts (CML-exclusive, CML-overrepresented, benign-overrepresented, benign-exclusive).

Supplemental Figure 1

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b8 2+-H2O 408.58
y5 2+ 312.34
b4+ 386.26
y6+ 680.43
b7+, y7+-H2O 733.40
y1+-NH3 158.14
b2+-NH3 241.11
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y5+ 623.43
Validation
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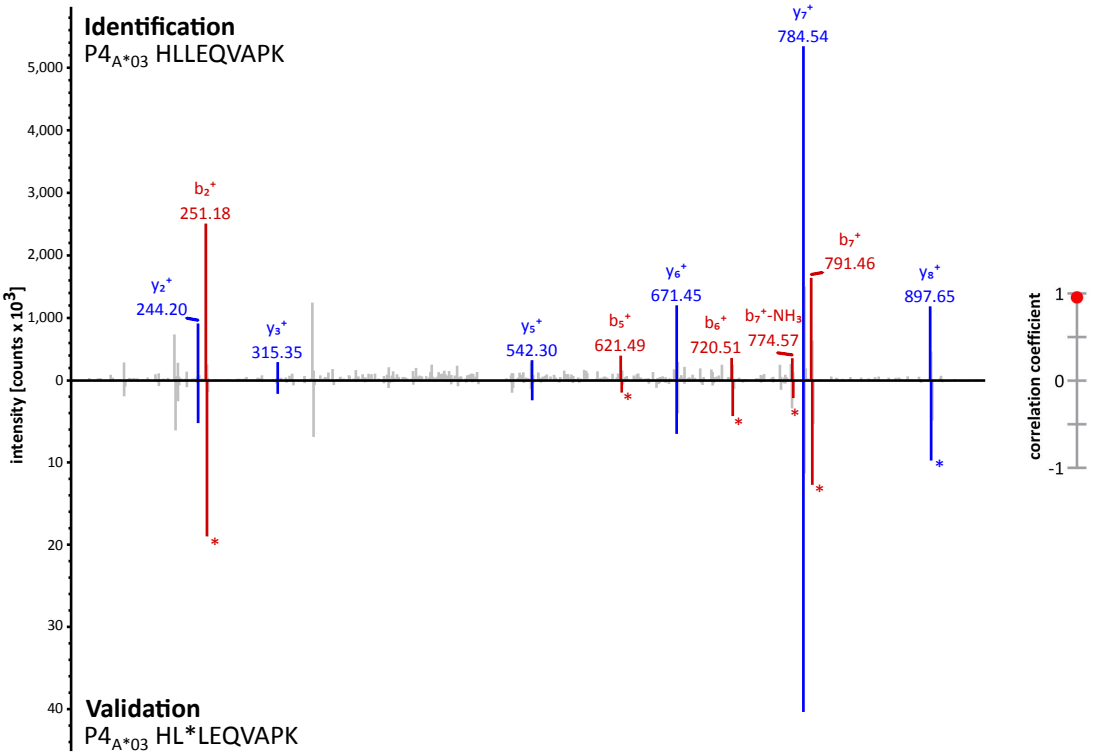
intensity [counts x 10^3]
correlation coefficient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b7+ 791.46
y2+ 244.20
y6+ 671.45
y8+ 897.65
y3+ 315.35
y5+ 542.30
b5+ 621.49
b6+ 720.51
b7+-NH3 774.57
Validation
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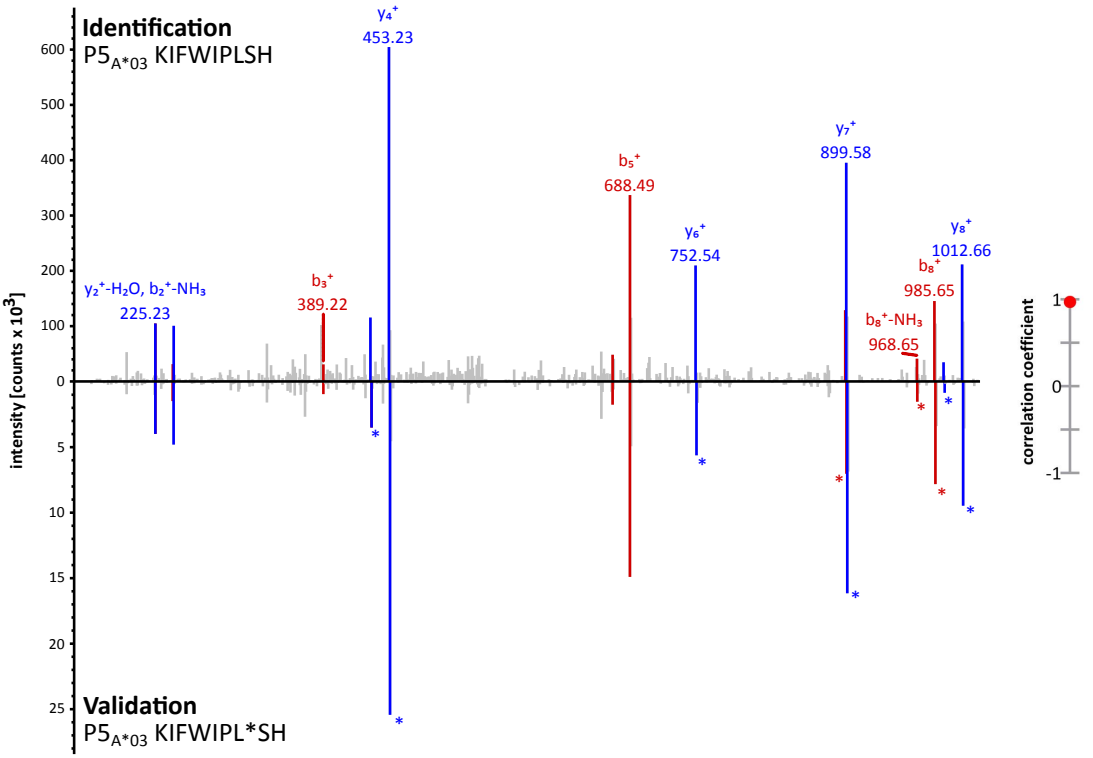
intensity [counts x 10^3]
correlation coefficient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b3+
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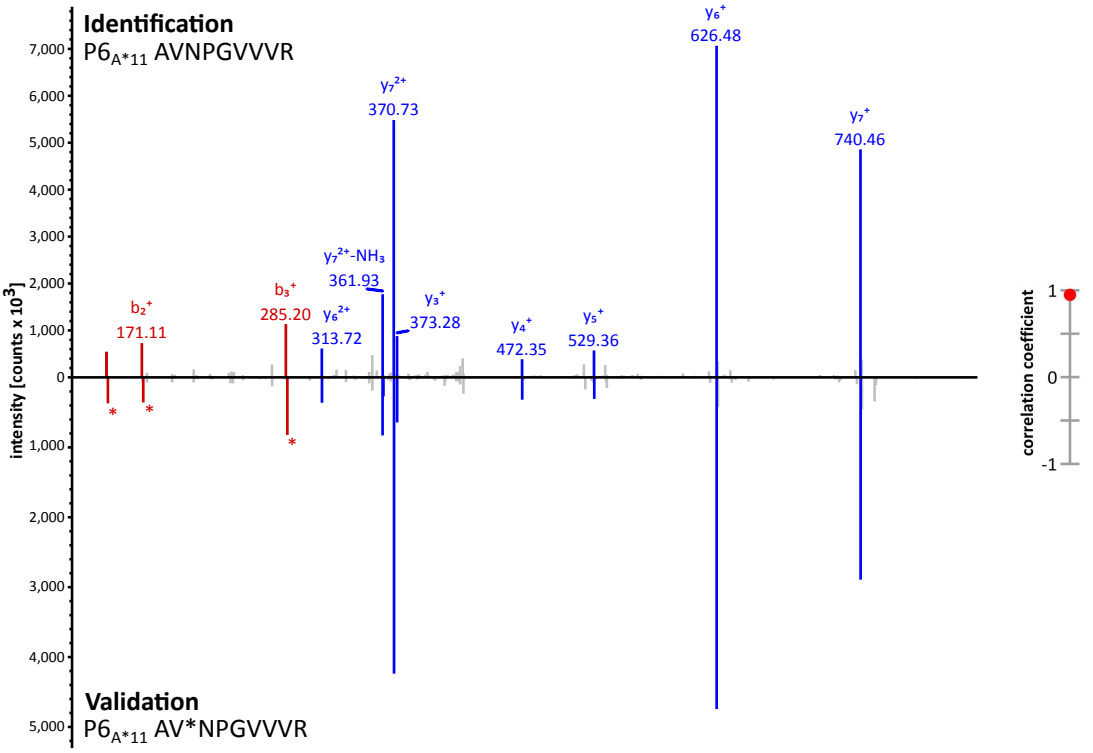
intensity [counts x 10^3]
correlation coefficient
Validation
P5A*03 KIFWIPL*SH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y5+
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intensity [counts x 10^3]
correlation coefficient
Validation
P6A*11 AV*NPGVVVR

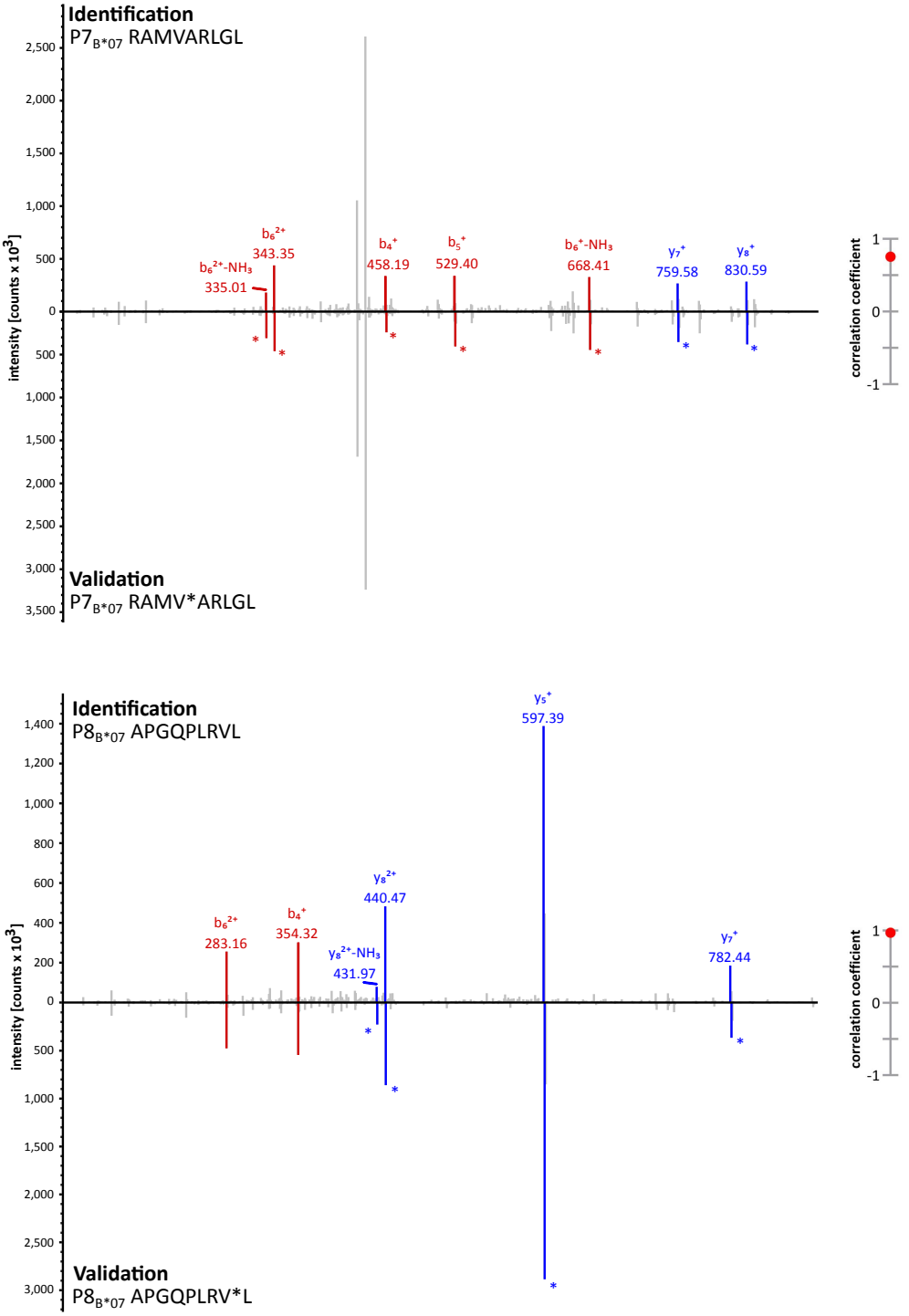
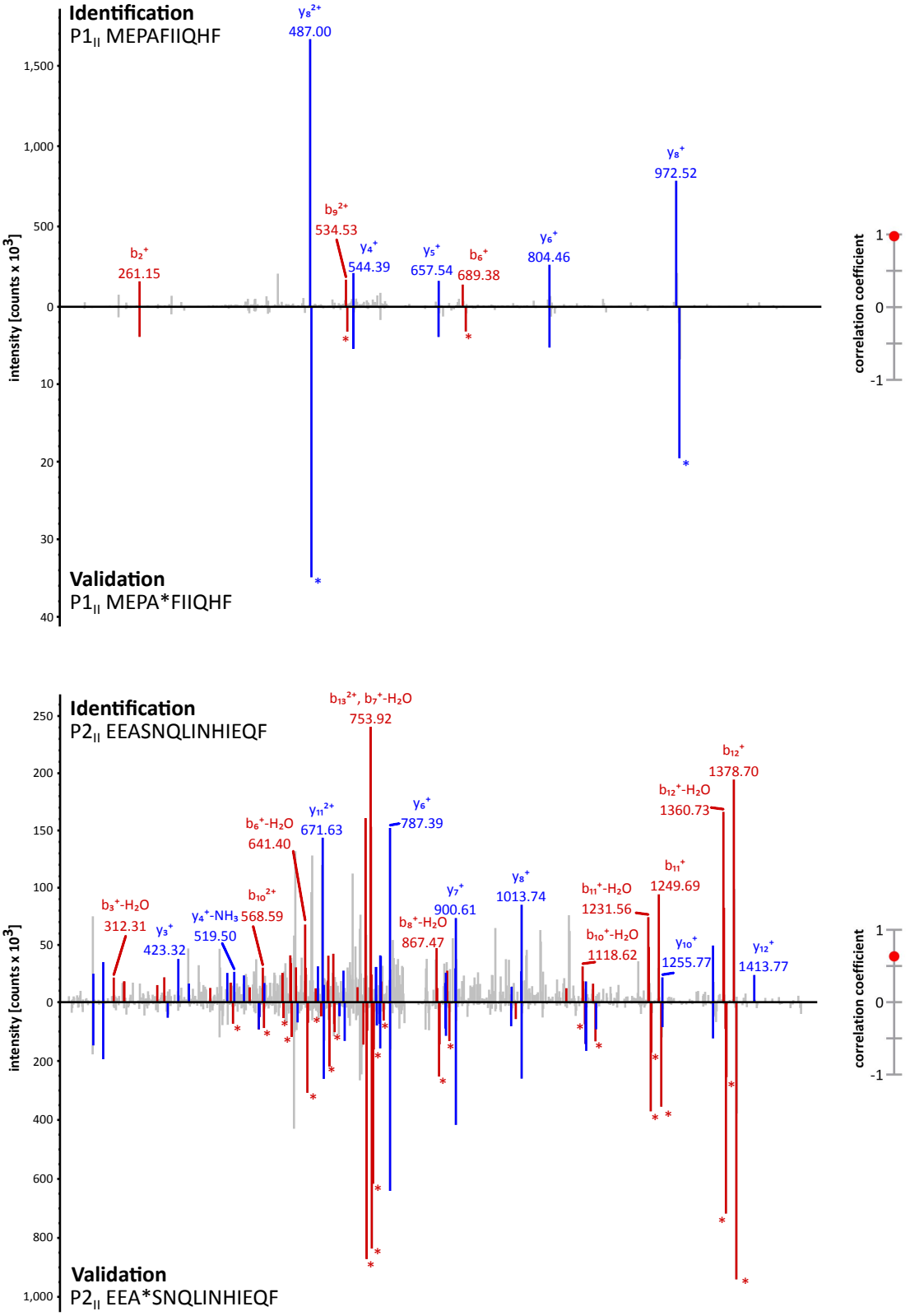
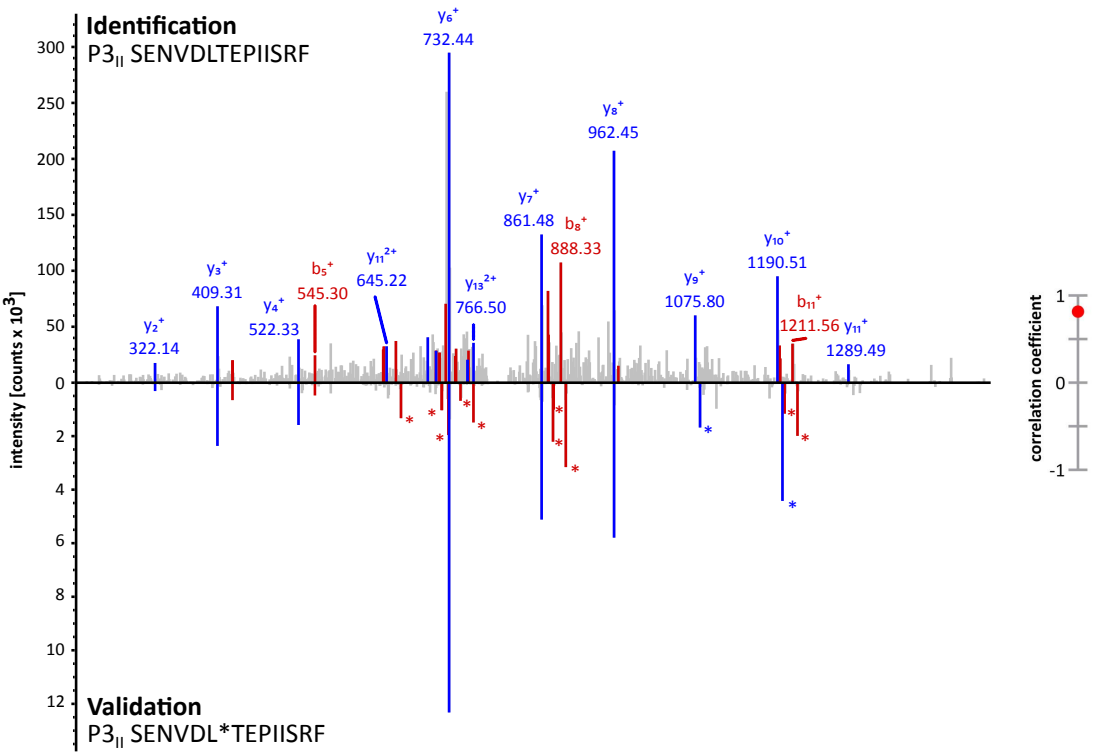


Figure S1: Validation of experimentally eluted HLA class I-restricted peptides by synthetic peptides. Comparison of fragment spectra (m/z on the x-axis) of HLA class I-restricted peptides eluted from primary CML patient samples (identification) to their corresponding synthetic peptides (validation). The spectra of the synthetic peptides are mirrored on the x-axis. Identified b- and y-ions are marked in red and blue, respectively. The calculated spectral correlation coefficients are depicted on the right graph, respectively. Ions containing isotopic labeled amino acids are marked with asterisks.

Supplemental Figure 2

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Validation
P3II SENVDL*TEPIISRF
intensity [counts x 10^3]
correlation coefficient
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y8+ 962.45
y7+ 861.48
b8+ 888.33
y10+ 1190.51
y9+ 1075.80
b11+ 1211.56
y11+ 1289.49
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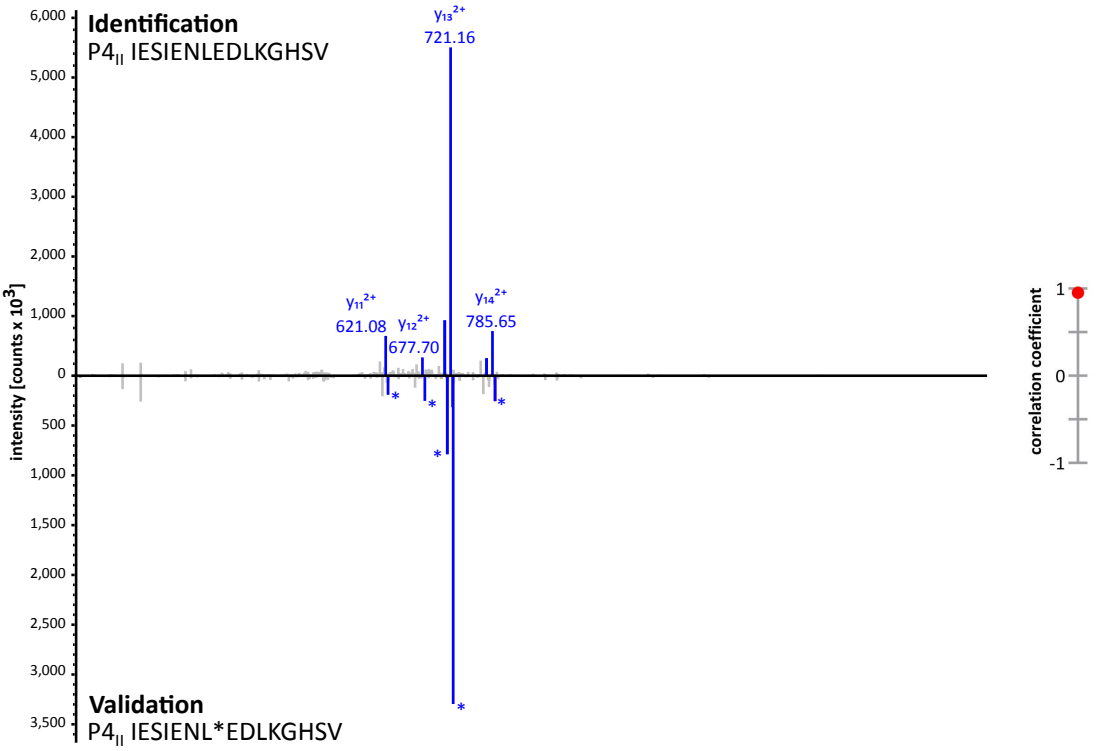
b5+ 545.30
y4+ 522.33
y11 2+ 645.22
y13 2+ 766.50
y2+ 322.14


Identification
P4II IESIENLEDLKGHSV
Validation
P4II IESIENL*EDLKGHSV
intensity [counts x 10^3]
correlation coefficient
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y11 2+ 621.08
y12 2+ 677.70
y14 2+ 785.65

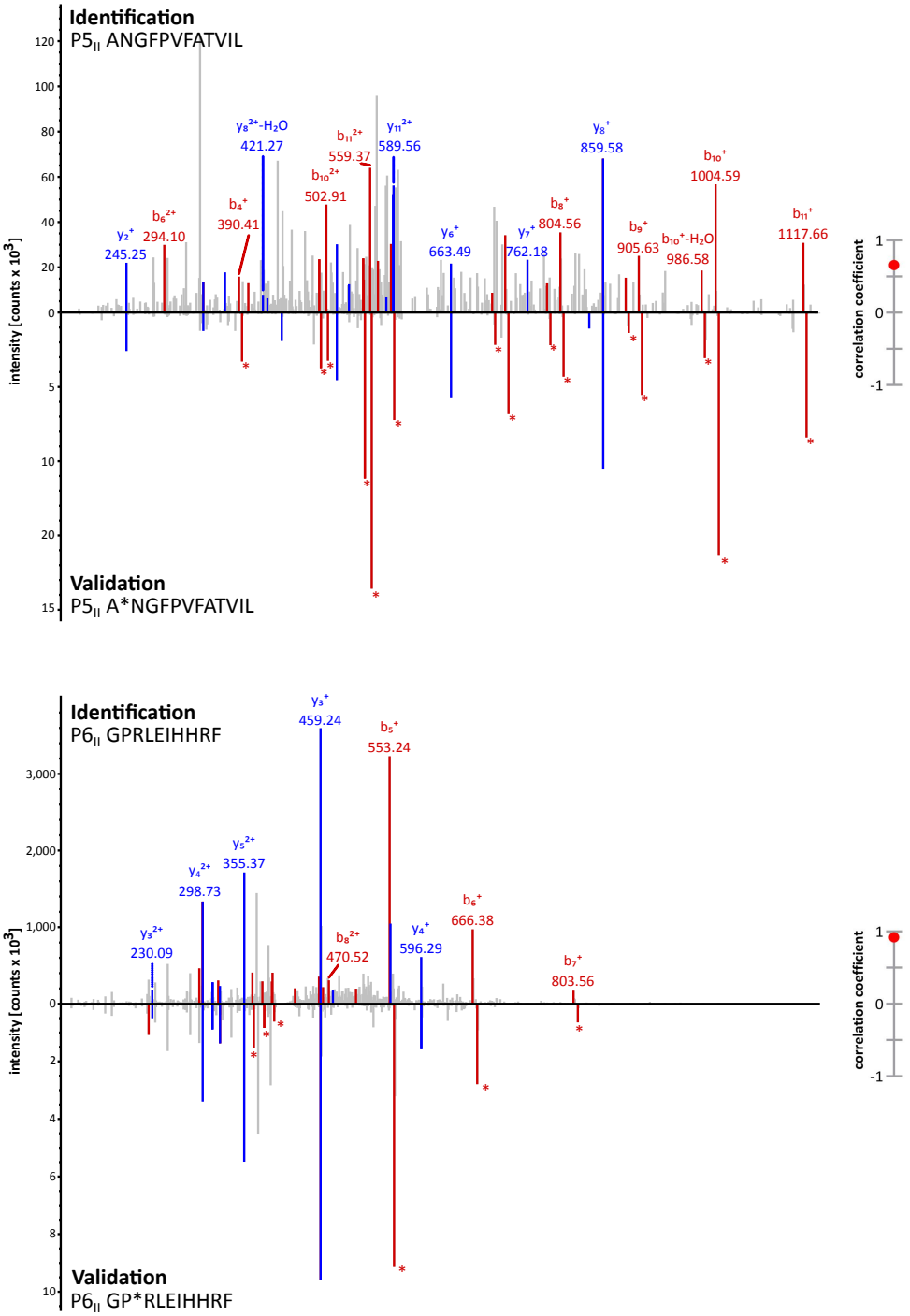

Figure S2: Validation of experimentally eluted HLA class II-restricted peptides by synthetic peptides. Comparison of fragment spectra (m/z on the x-axis) of HLA class II-restricted peptides eluted from primary CML patient samples (identification) to their corresponding synthetic peptides (validation). The spectra of the synthetic peptides are mirrored on the x-axis. Identified b- and y-ions are marked in red and blue, respectively. The calculated spectral correlation coefficients are depicted on the right graph, respectively. Ions containing isotopic labeled amino acids are marked with asterisks.

Supplemental Figure 3

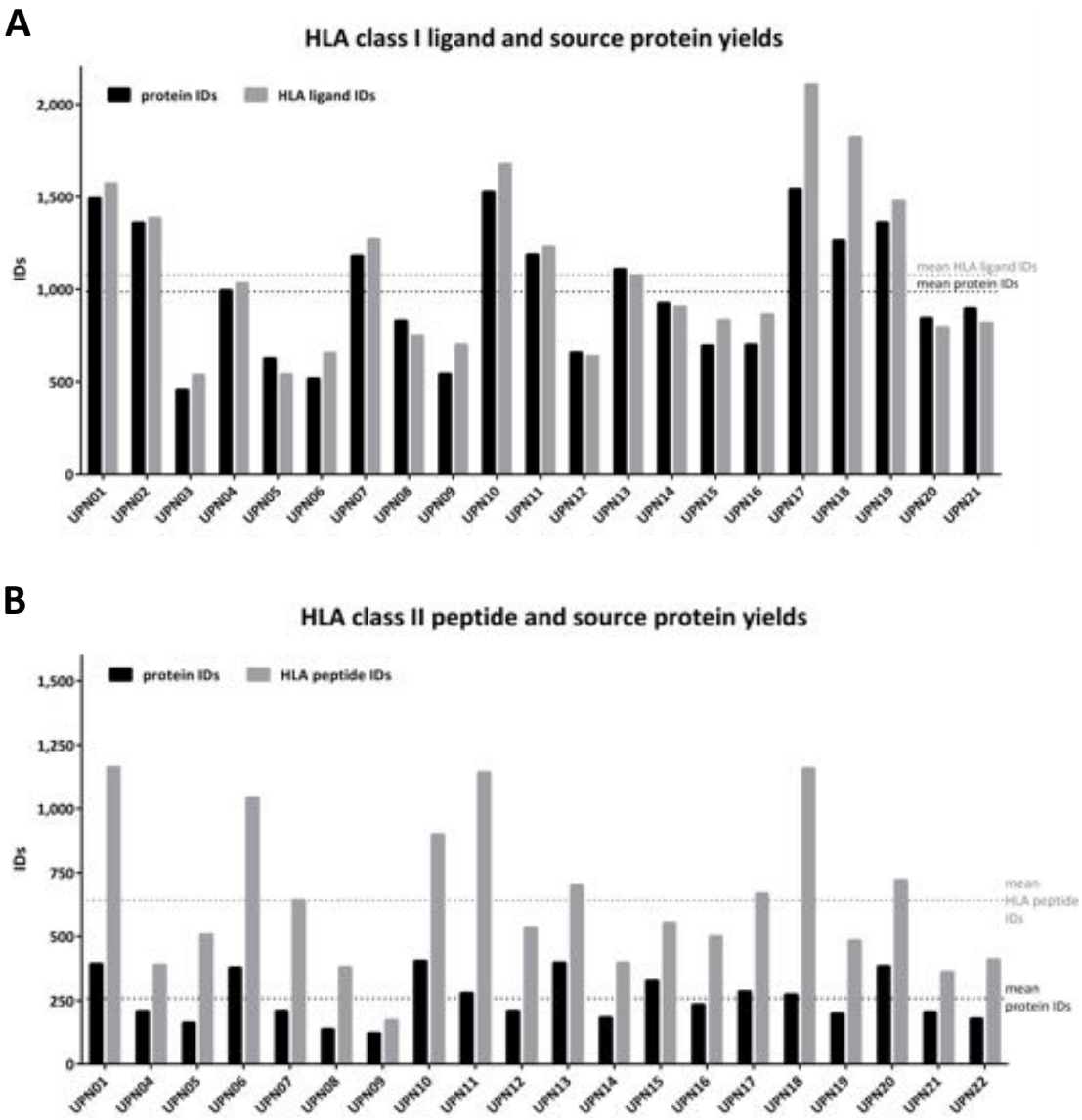

Figure S3: HLA class I and II peptide and source protein yields. (A) HLA class I ligand and respective source protein yields of primary CML samples (n = 21) as identified by mass spectrometry are indicated in grey and black bars, respectively. Mean HLA class I ligand and protein identifications are depicted by the grey and black dotted line, respectively. (B) Numbers of HLA class II peptides (grey bars) and source proteins (black bars) identified in primary CML samples (n = 20). Mean HLA class II peptide and protein identifications are illustrated by the grey and black dotted line, respectively. Abbreviations: IDs, identifications; UPN, uniform patient number.

Supplemental Figure 4

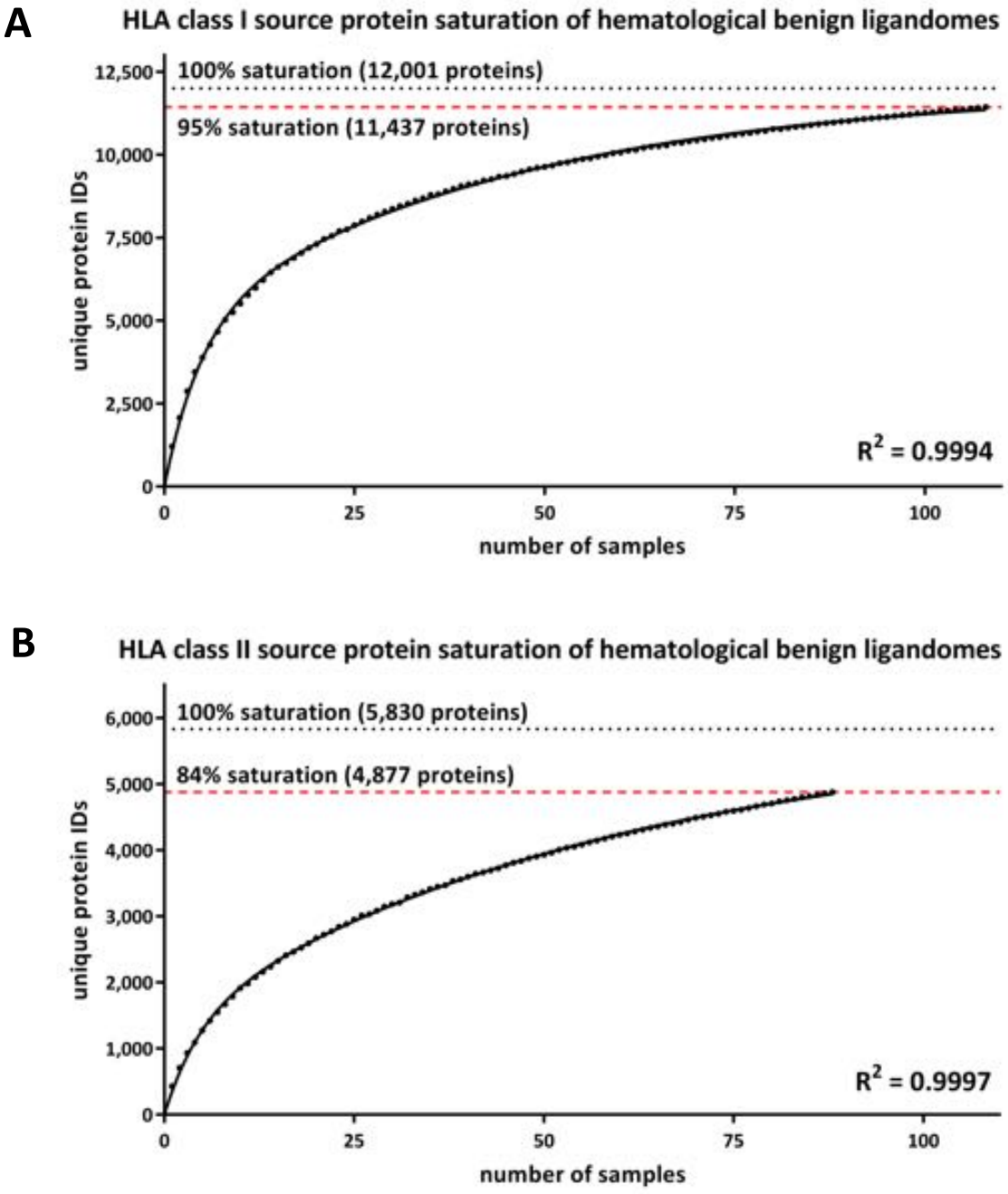

Figure S4: Saturation analysis of HLA peptide source proteins of hematological benign tissue cohorts. Saturation analysis of (A) HLA class I and (B) HLA class II peptide source proteins of the hematological benign tissue cohort. Number of unique HLA ligand source protein identifications shown as function of cumulative HLA ligandome analysis of hematological benign samples ($n = 108$ for HLA class I, $n = 88$ for HLA class II). Exponential regression allowed for the robust calculation ($R^2 = 0.9994$ for HLA class I, $R^2 = 0.9997$ for HLA class II) of the maximum attainable number of different source protein identifications (dotted line). The dashed red line depicts the source proteome coverage achieved in our hematological benign tissue cohort. Abbreviation: IDs, identifications.

Supplemental Figure 5

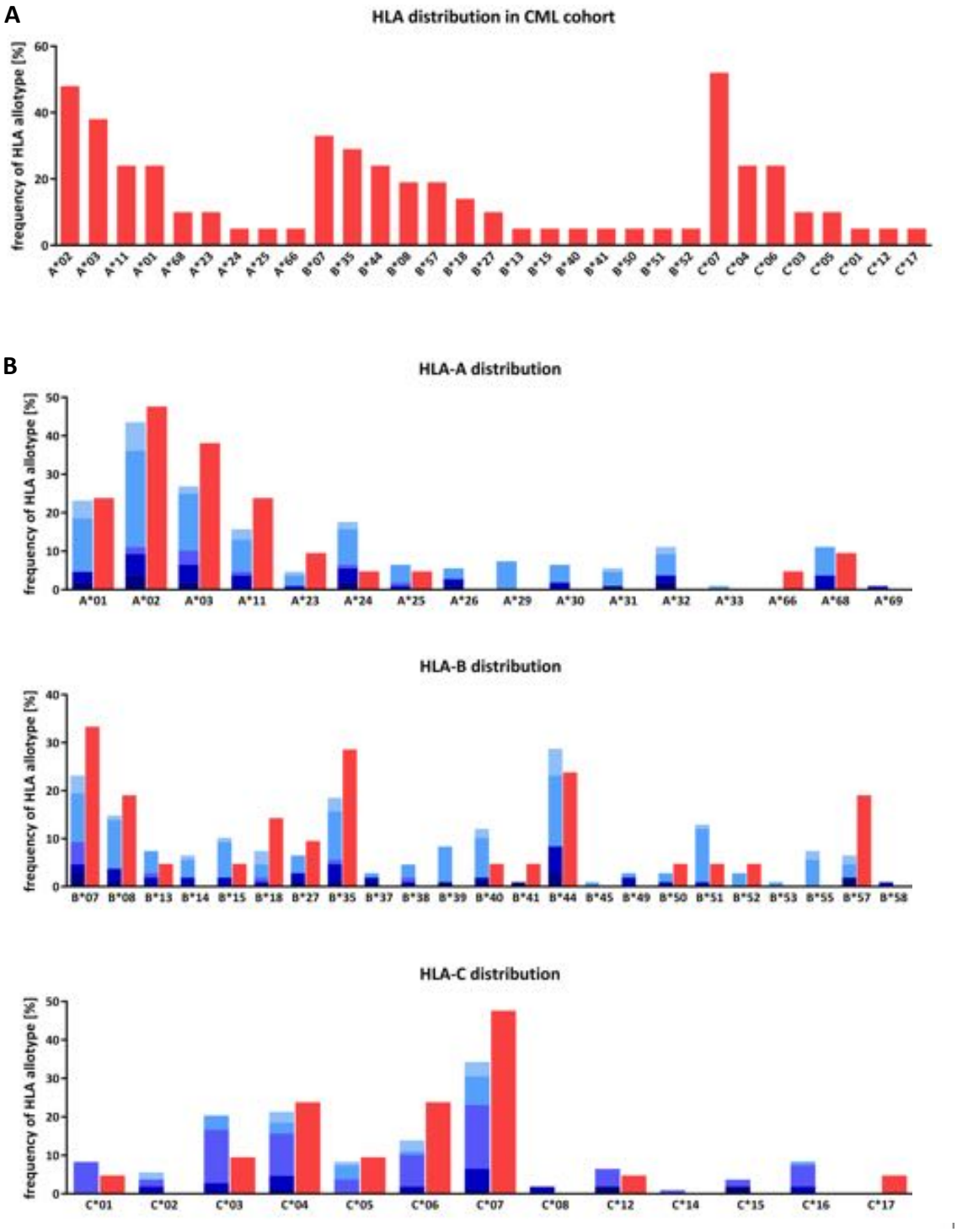

Figure S5: HLA allotype distribution in CML and hematological benign tissue cohorts. (A) HLA class I allotype frequencies in the CML patient cohort (n = 21) used for mass spectrometry-based analysis of naturally presented, CML-associated antigens. (B) HLA-A, -B, and -C allotype frequencies in CML patient (n = 21) and hematological benign tissue (n = 108) cohorts. Abbreviations: PBMCs, peripheral blood mononuclear cells; BM, bone marrow; HPCs, hematopoietic progenitor cells.

Supplemental Figure 6

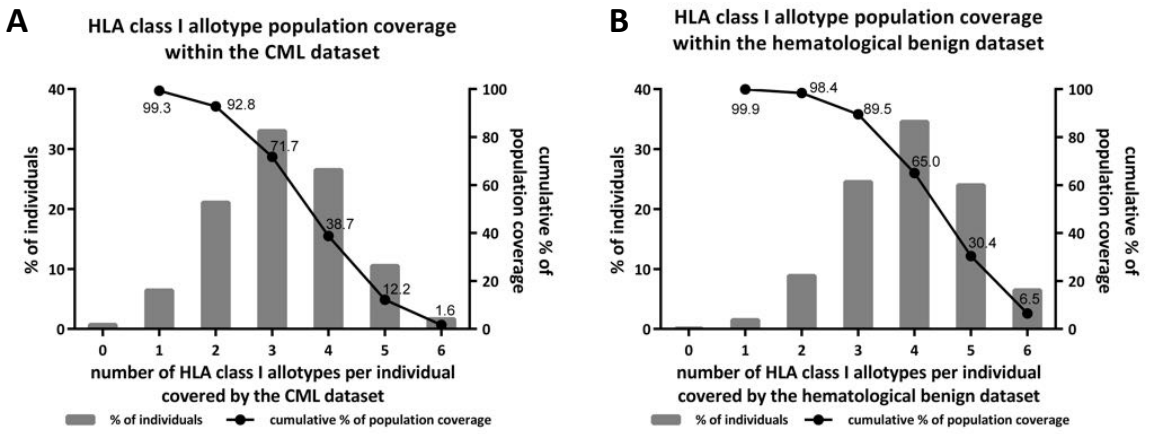

Figure S6: HLA class I allotype population coverage. HLA class I allotype population coverage within the (A) CML and (B) hematological benign tissue cohorts compared to the world population (calculated by the IEDB population coverage tool, www.iedb.org). The frequencies of individuals within the world population carrying up to six HLA allotypes (x-axis) of the respective CML or benign dataset are indicated as grey bars on the left y-axis. The cumulative percentage of population coverage is depicted as black dots on the right y-axis.

Supplemental Figure 7

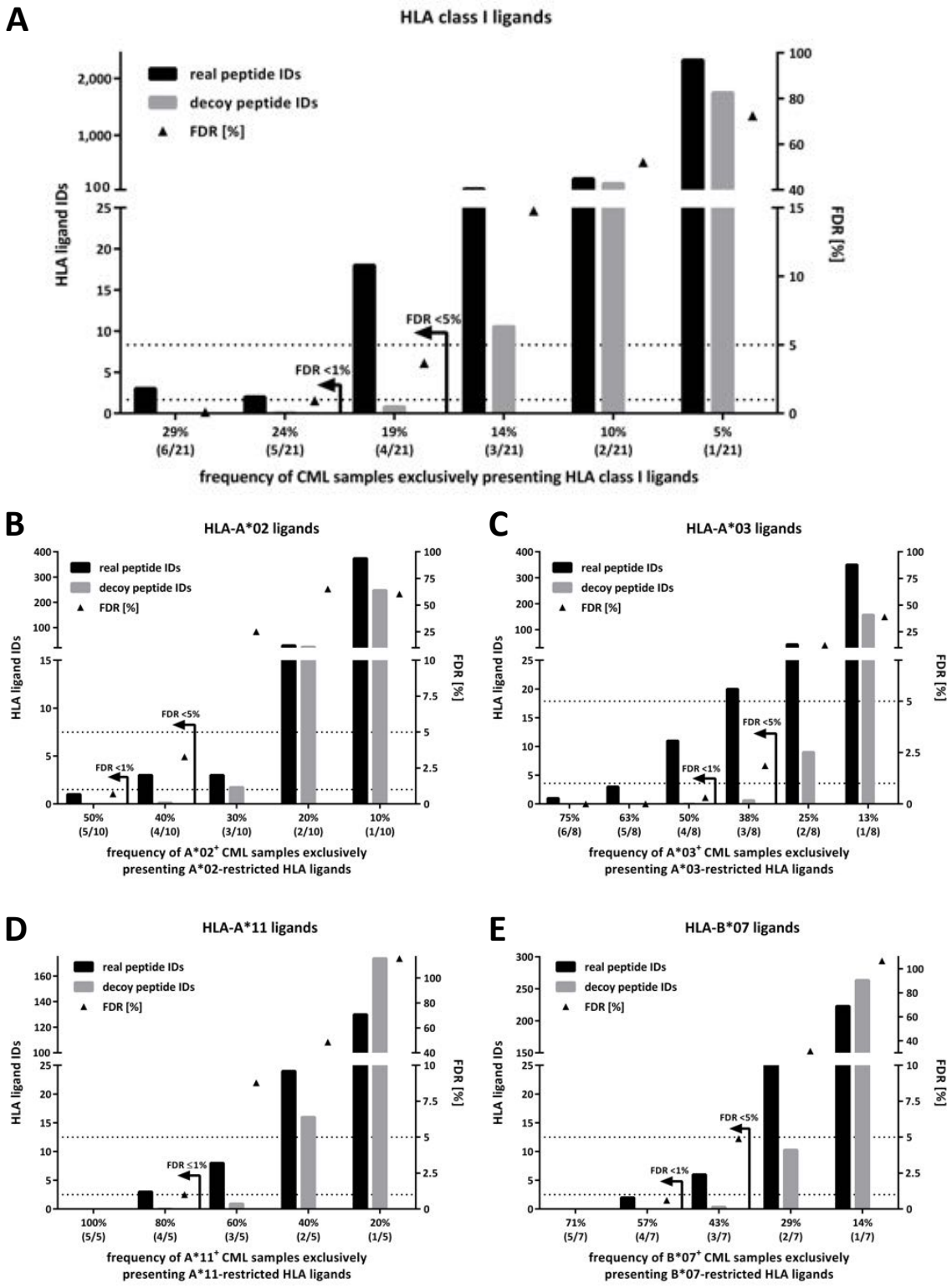

Figure S7: Statistical analysis of the proportion of false positive CML-associated HLA class I ligand identifications at different representation frequencies. The numbers of identified (A) HLA class I, (B) -A*02, (C) -A*03, (D) -A*11, and (E) -B*07 ligands based on the analysis of the CML and hematological benign tissue cohorts were compared with random virtual CML-associated HLA class I, -A*02, -A*03, -A*11, and -B*07 ligands (left y-axis), respectively. Virtual ligandomes of CML patient and hematological benign tissue samples were generated *in silico* based on random weighted sampling from the entirety of peptide identifications in both original cohorts. These randomized virtual ligandomes were used to define CML-associated antigens based on simulated cohorts of CML *versus* hematological benign tissue samples. The process of peptide randomization, cohort assembly, and CML-associated antigen identification was repeated 1,000 times and the mean value of resultant virtual CML-associated antigens was calculated and plotted for the different threshold values. The corresponding false discovery rates (right y-axis) for any chosen threshold (x-axis) were calculated and the 1% and 5% false discovery rates are indicated within the plot (dotted lines and arrows). Abbreviations: IDs, identifications; FDR, false discovery rate.

Supplemental Figure 8

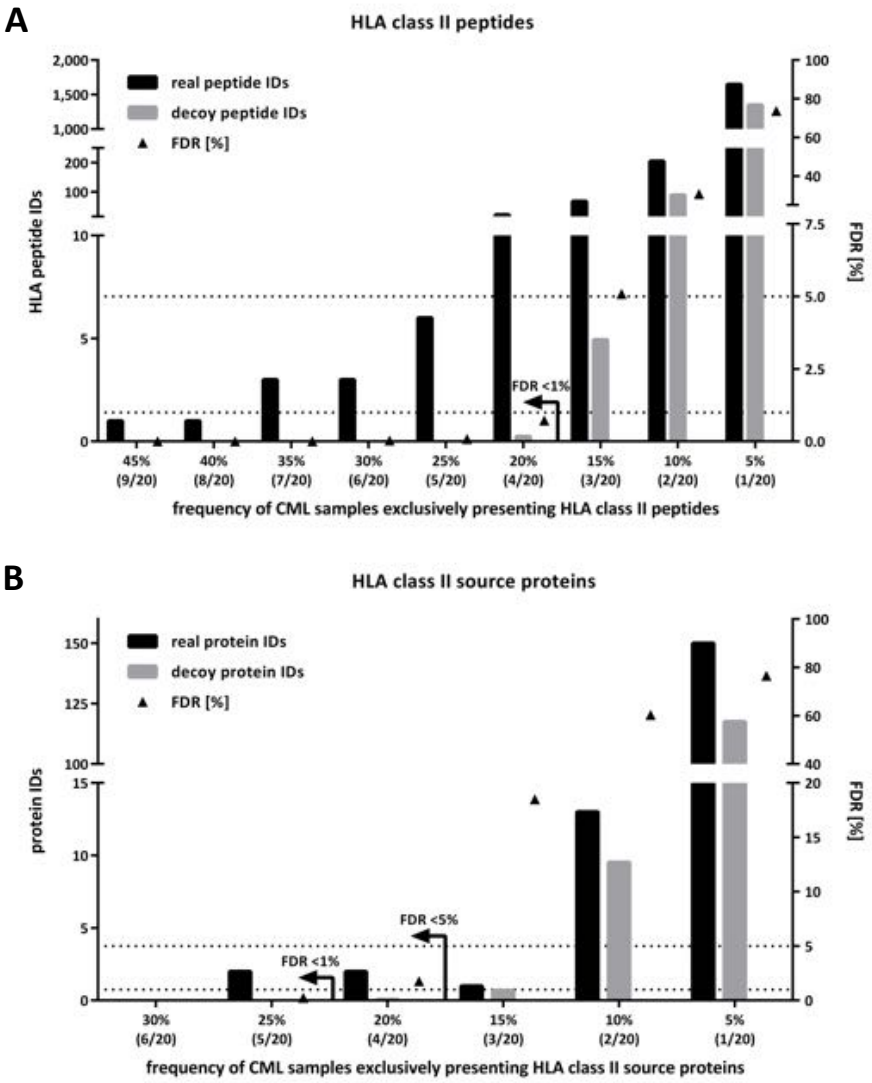

Figure S8: Statistical analysis of the proportion of false positive CML-associated HLA class II peptide and source protein identifications at different representation frequencies. The numbers of identified HLA class II (A) peptides and (B) source proteins based on the analysis of the CML and hematological benign tissue cohorts were compared with random virtual CML-associated HLA class II peptides and source proteins (left y-axis), respectively. Virtual ligandomes of CML patient and hematological benign tissue samples were generated *in silico* based on random weighted sampling from the entirety of peptide identifications in both original cohorts. These randomized virtual ligandomes were used to define CML-associated antigens based on simulated cohorts of CML *versus* hematological benign tissue samples. The process of peptide or source protein randomization, cohort assembly, and CML-associated antigen identification was repeated 1,000 times and the mean value of resultant virtual CML-associated antigens was calculated and plotted for the different threshold values. The corresponding false discovery rates (right y-axis) for any chosen threshold (x-axis) were calculated and the 1% and 5% false discovery rates are indicated within the plot (dotted lines and arrows). Abbreviations: IDs, identifications; FDR, false discovery rate.

Supplemental Figure 9

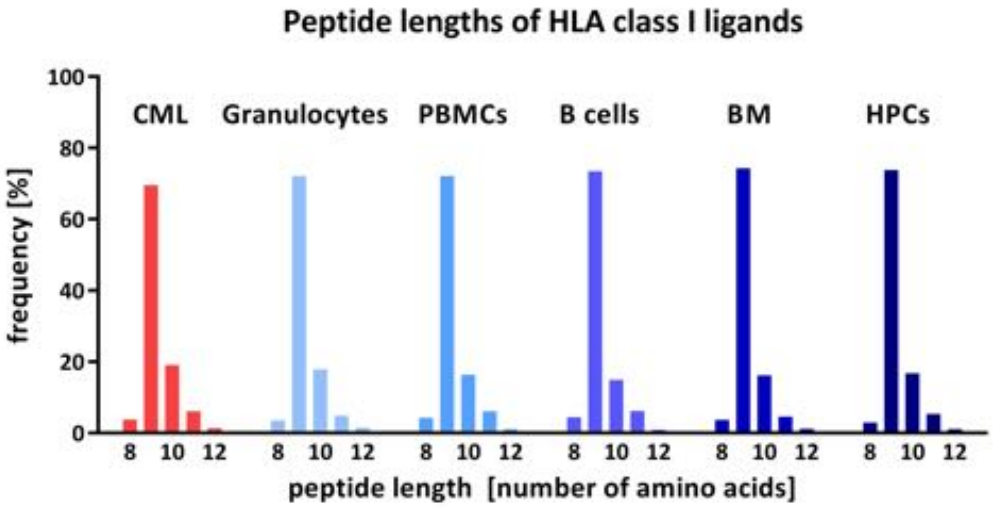

Figure S9: Peptide length distribution of HLA class I ligands. Tissue-specific HLA class I ligand length distribution (number of amino acids) of identified peptides on primary CML samples (n = 21), granulocytes (n = 14), PBMCs (n = 63), $CD19^+$ B cells (n = 5), bone marrow (n = 18), and $CD34^+$ HPCs (n = 8). Abbreviations: PBMCs, peripheral blood mononuclear cells; BM, bone marrow; HPCs, hematopoietic progenitor cells.

Supplemental Figure 10

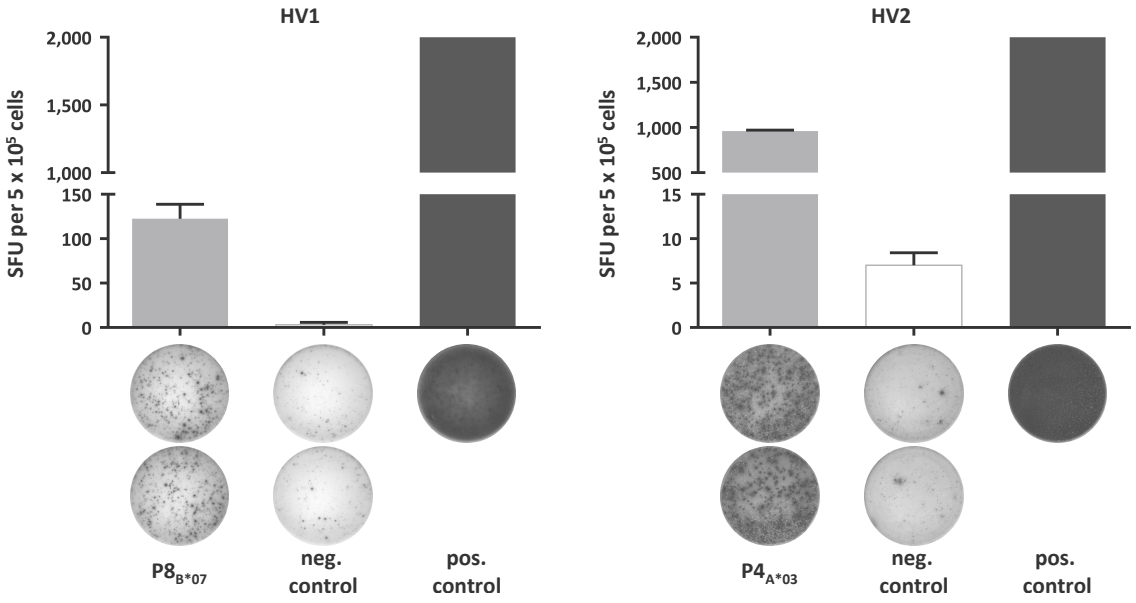

Figure S10: Preexisting T-cell responses of HLA class I-restricted, CML-associated antigens in healthy volunteers. Examples of CML-associated ligands evaluated in IFN-γ ELISPOT assays after 12-d stimulation using PBMCs of healthy volunteers. Results are shown for immunoreactive peptides only. PHA was used as positive control. The HLA-B*07-restricted NEF_HV1BR$_{128-137}$ peptide TPGPGVRYPL as well as the HLA-A*03-restricted GAG_HV1BR$_{20-28}$ peptide RLRPGGKKK served as negative controls, respectively. Data are expressed as mean $\pm$ SD of two independent replicates. Abbreviations: HV, healthy volunteer; SFU, spot forming unit; neg., negative; pos., positive.

Supplemental Figure 11

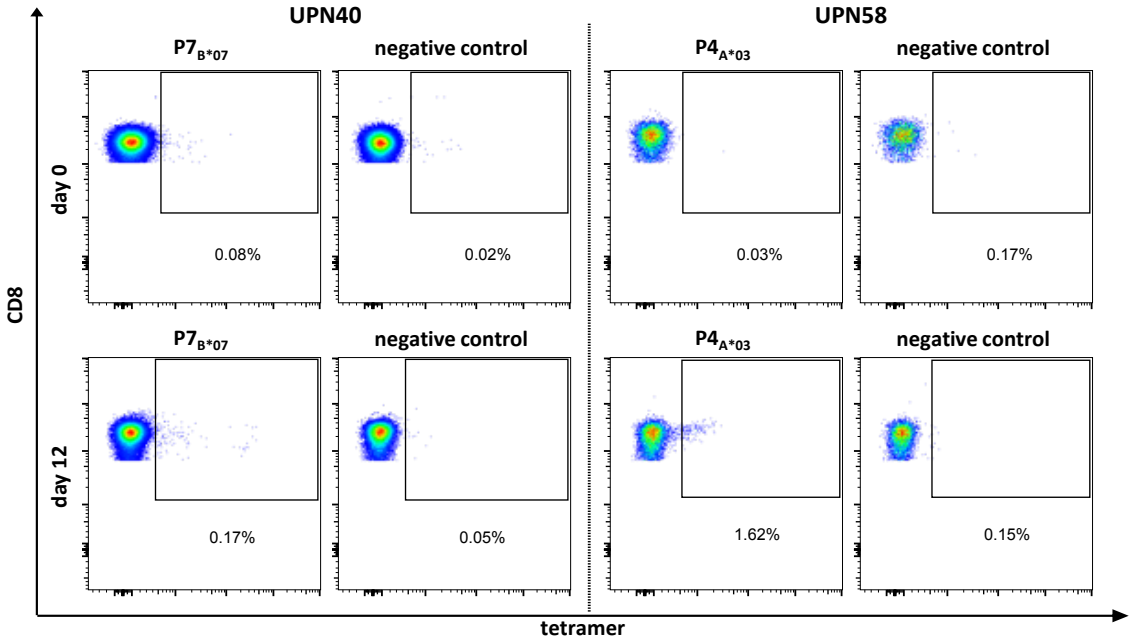

Figure S11: Examples of multimer stainings before and after 12-d stimulation. PBMCs of CML_{TKI} patients were stimulated *in vitro* for 12 days using CML-associated peptides. Graphs show single, viable cells stained for CD8 and PE-conjugated multimers of indicated specificity. HLA allotype-matched PE-conjugated multimers with irrelevant peptides served as negative controls, respectively. Stainings were performed *ex vivo* on day 0 with unstimulated cells as well as after 12-d stimulation. Abbreviation: UPN, uniform patient number.

Supplemental Figure 12

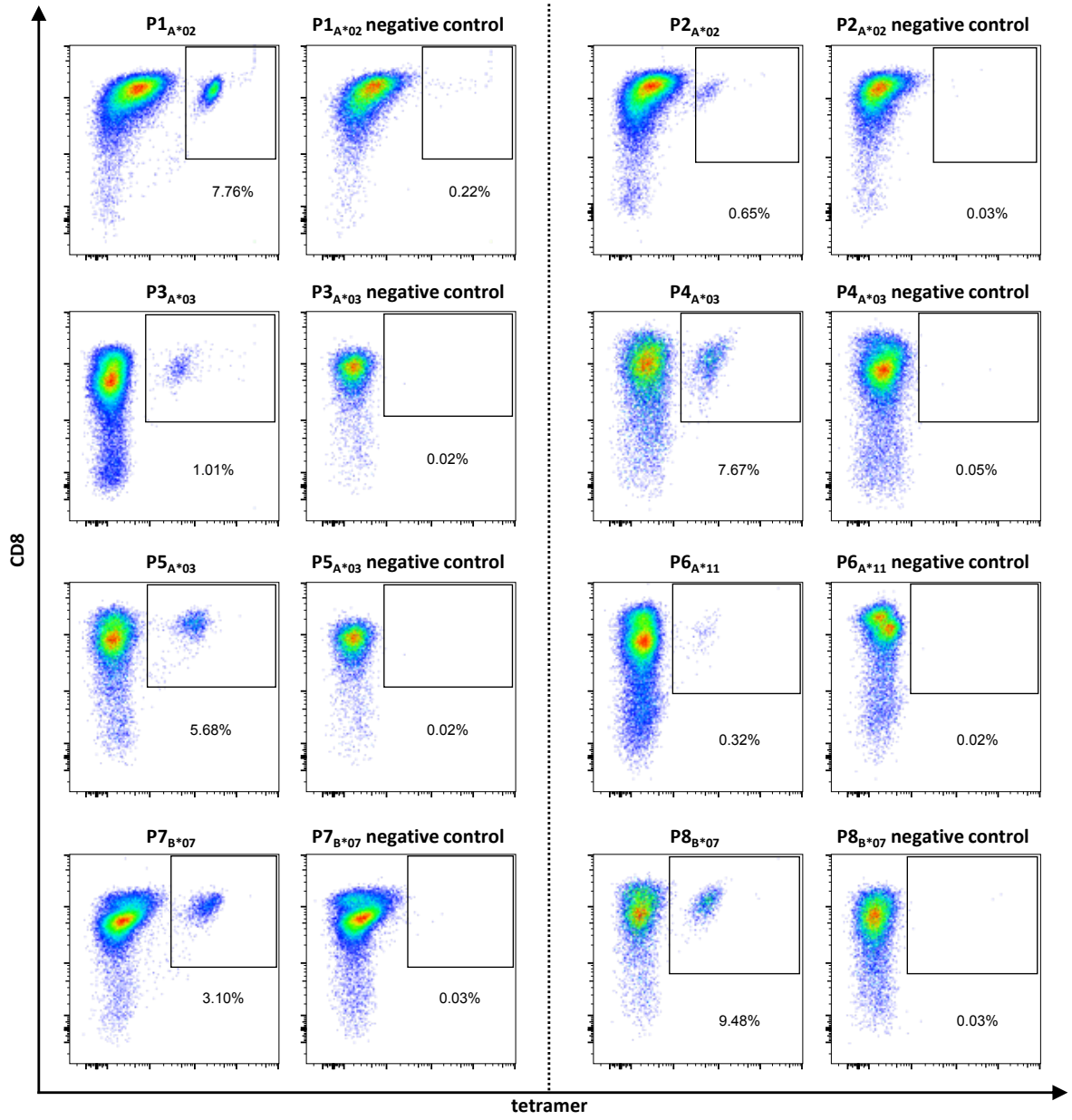

Figure S12: Representative examples of multimer stainings using HLA:peptide tetramers. Naïve CD8$^+$ T cells from healthy blood donors were primed *in vitro* using artificial APCs. Graphs show single, viable cells stained for CD8 and PE-conjugated multimers of indicated specificity. Tetramer staining was performed after four stimulation cycles with peptide-loaded aAPCs. Negative controls were performed using T cells from respective donors primed with irrelevant peptides and stained with the indicated tetramers.

Supplemental Figure 13

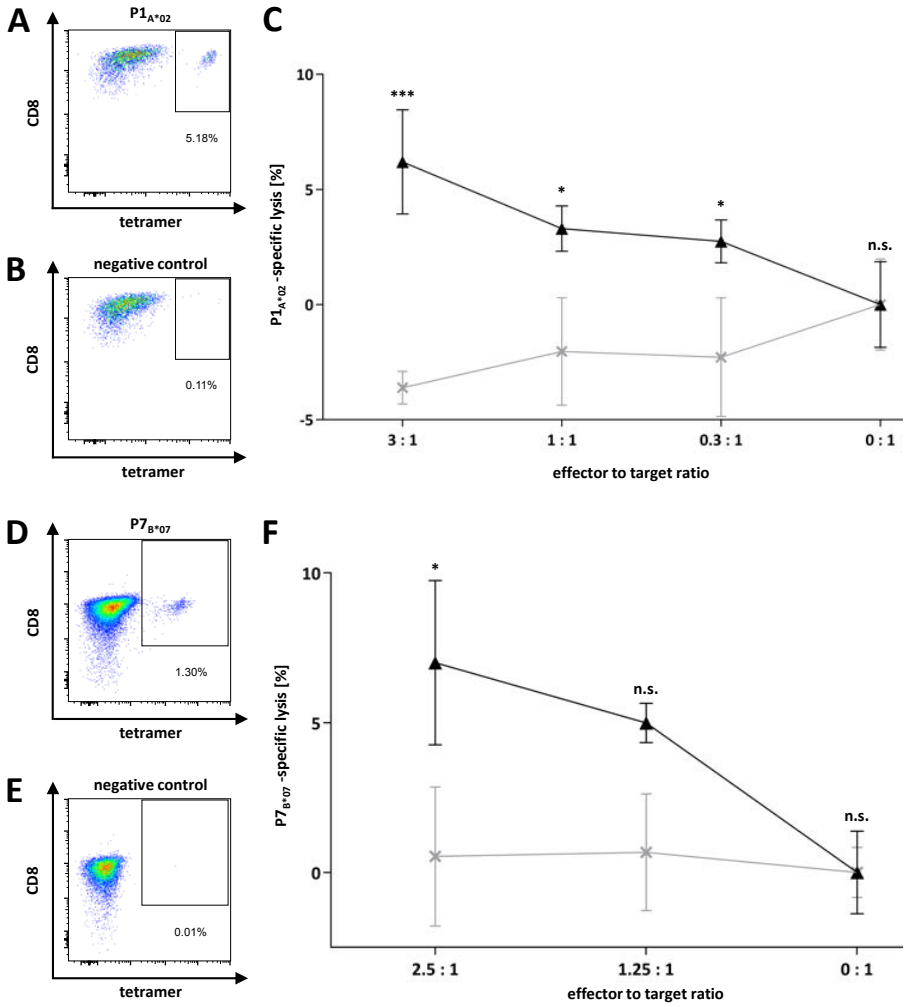

Figure S13: Cytotoxicity experiments using antigen-specific CD8$^+$ T cells. (A-C) Selective cytotoxicity of P1$_{A*02}$-specific effector T cells was analyzed in a VITAL cytotoxicity assay with *in vitro* primed CD8$^+$ effector cells of an HV. Tetramer staining of polyclonal effector cells before performance of the VITAL assay determined the amount of P1$_{A*02}$-specific effector cells in the (A) population of successfully P1$_{A*02}$-primed CD8$^+$ T cells and in the (B) population of control cells from the respective donor primed with an HLA-matched irrelevant peptide. (C) At an effector to target ratio of 3:1 P1$_{A*02}$-specific effectors (black) exerted 6.2% ($\pm$2.3%) P1$_{A*02}$-specific and significantly higher lysis of P1$_{A*02}$-loaded autologous target cells in comparison to control peptide-loaded target cells (HLA-A*02, YLLPAIVHI, DDX5_HUMAN$_{148-156}$). P1$_{A*02}$-unspecific effectors (grey) showed no unspecific lysis of the same targets. (D-F) Selective cytotoxicity of P7$_{B*07}$-specific effector T cells. Tetramer staining of polyclonal effector cells before performance of the VITAL assay determined the amount of P7$_{B*07}$-specific effector cells in the (D) population of successfully P7$_{B*07}$-primed CD8$^+$ T cells and in the (E) population of control cells from the respective donor primed with an HLA-matched irrelevant peptide. (F) At an effector to target ratio of 2.5:1 P7$_{B*07}$-specific effectors (black) exerted 7.0% ($\pm$2.7%) P7$_{B*07}$-specific and significantly higher lysis of P7$_{B*07}$-loaded autologous target cells in comparison to control peptide-loaded target cells (HLA-B*07, TPGPGVRYPL, NEF_HV1BR$_{128-137}$). P7$_{B*07}$-unspecific effectors (grey) only showed 0.5% ($\pm$2.3%) unspecific lysis of the same targets. Results are shown for three independent replicates. Error bars indicate $\pm$ SEM. Abbreviations: n.s., not significant; * $p < 0.05$; *** $p < 0.001$.

Appendix - CHAPTER 2

Supplemental Table 1

UPN	Entity	Experiment	Gender	Age	HLA class I	IPSET score - risk stratification	DIPSS+ score - risk stratification	Molecular genetics
01	ET	L, Q, E	female	66	A*02:01;A*25:01;B*18:01;B*57:01;C*06:02;C*07:01	1 - high	n.a.	JAK2 V617F
02	ET	E	female	62	A*02:02;A*33:01;B*14:02;B*41:02;C*08:02;C*17:01	1 - high	n.a.	JAK2 V617F
03	ET	L, E	female	69	A*03:01;B*13:02;B*44:03;C*04:01;C*06:02	2 - high	n.a.	JAK2 V617F
04	ET	E	male	67	A*02;A*03;B*27;B*44;C*01;C*07	2 - high	n.a.	triple-negative
05	ET	E	male	48	A*02:01;A*11:01;B*15:01;B*39:01;C*03:04;C*07:02	0 - low	n.a.	MPL
06	ET	Q	male	58	n.a.	low	n.a.	CALR
07	ET	L, Q, E	female	82	A*03:01;A*30:01;B*13:02;B*35:03;C*04:01;C*06:02	2 - intermediate	n.a.	CALR
08	ET	Q, E	male	20	n.a.	2 - intermediate	n.a.	triple-negative
09	ET	E	male	61	n.a.	2 - intermediate	n.a.	JAK2 V617F
10	ET	E	female	81	A*01:01;A*24:02;B*07:02;B*39:06;C*07:02	4 - high	n.a.	JAK2 V617F
11	ET	Q, E	female	65	n.a.	2 - intermediate	n.a.	MPL
12	ET	L, E	male	64	A*02:01;A*24:02;B*07:02;B*44:02;C*05:01;C*07:01	2 - intermediate	n.a.	CALR
13	ET	Q	female	60	n.a.	intermediate	n.a.	CALR
14	ET	L, Q, E	female	41	A*02:01;A*03:01;B*15:01;B*44:03;C*03:04;C*04:01	1 - intermediate	n.a.	JAK2 V617F
15	ET	Q, E	female	40	n.a.	1 - intermediate	n.a.	JAK2 V617F
16	ET	Q, E	male	63	n.a.	3 - high	n.a.	CALR
17	ET	L, Q	male	34	A*03:01;A*68:02;B*18:01;B*44:03;C*04:01;C*07:01	low	n.a.	CALR
18	ET	Q, E	female	27	n.a.	0 - low	n.a.	CALR
19	ET	Q	female	46	n.a.	1 - intermediate	n.a.	JAK2 V617F
20	ET	Q	female	43	n.a.	1 - intermediate	n.a.	JAK2 V617F
21	ET	Q	male	81	A*02:02;A*03:01;B*07:02;B*44:02;C*05:01;C*07:02	2 - intermediate	n.a.	JAK2 V617F-negative
22	ET	L, Q, E	male	48	A*02:01;A*24:02;B*18:01;B*35:01;C*04:01;C*07:01	0 - low	n.a.	CALR
23	PV	L	n.a.	n.a.	A*03:01;B*07:02;B*27:05;C*02:02;C*07:02	n.a.	n.a.	JAK2 V617F
24	PV	L	n.a.	n.a.	A*02:01;A*68:01;B*44:02;B*51:01;C*14:02	n.a.	n.a.	triple-negative
25	PV	L	n.a.	n.a.	A*03:01;A*24:02;B*07:02;B*35:01;C*04:01;C*07:02	n.a.	n.a.	JAK2 V617F
26	PV	L	n.a.	n.a.	A*11:01;B*15:01;B*35:01;C*03:04;C*04:01	n.a.	n.a.	JAK2 V617F
27	PV	L	n.a.	n.a.	A*01:01;A*03:01;B*13:02;B*57:01;C*04:01;C*06:02	n.a.	n.a.	triple-negative
28	PV	L	n.a.	n.a.	A*02:01;B*44:02;C*12:03;C*14:02	n.a.	n.a.	triple-negative
29	PV	L	n.a.	n.a.	A*02:01;B*38:01;B*51:01;C*12:03;C*14:02	n.a.	n.a.	JAK2 V617F

UPN	Entity	Experiment	Gender	Age	HLA class I	IPSET score - risk stratification	DIPSS+ score - risk stratification	Molecular genetics
30	PV	L	n.a.	n.a.	A*02:01;A*32:01;B*35:01;B*44:02;C*04:01;C*06:02	n.a.	n.a.	triple-negative
31	PV	L	female	58	A*03:01;A*31:01;B*07:02;C*07:02	n.a.	n.a.	JAK2 V617F
32	PV	L	female	67	A*03:01;A*26:01;B*40:02;B*44:02;C*02:06;C*03:03	n.a.	n.a.	JAK2 V617F
33	PV	Q	female	49	n.a.	n.a.	n.a.	JAK2 V617F
34	PV	L	male	52	A*31:01;A*33:01;B*14:02;B*57:01;C*04:01;C*08:02	n.a.	n.a.	JAK2 V617F
35	PV	L	male	47	A*02:01;A*03:01;B*07:02;B*27:02;C*02:02;C*07:02	n.a.	n.a.	JAK2 V617F
36	PV	Q	male	55	n.a.	n.a.	n.a.	JAK2 V617F
37	PV	L, Q	male	59	A*01:01;A*03:01;B*27:05;B*39:06;C*01:02;C*07:02	n.a.	n.a.	JAK2 V617F
38	PV	L	female	48	A*02:01;A*30:02;B*15:01;B*18:01;C*03:04;C*05:01	n.a.	n.a.	JAK2 V617F
39	PV	Q	female	55	A*24:02;A*25:01;B*35:03;B*52:01;C*04:01;C*12:02	n.a.	n.a.	JAK2 V617F
40	PV	Q	male	63	n.a.	n.a.	n.a.	JAK2 V617F
41	PV	Q	male	65	n.a.	n.a.	n.a.	JAK2 V617F
42	PV	Q	male	72	n.a.	n.a.	n.a.	JAK2 V617F
43	PMF	L, Q, E	female	63	A*03:01;A*24:02;B*15:01;B*35:01;C*03:03;C*04:01	n.a.	0	MPL
44	PMF	E	female	60	A*01;A*02;B*37;B*50	n.a.	(DIPSS 0)	JAK2 V617F
45	PMF	L, Q, E	female	77	A*01:01;A*11:01;B*40:01;B*51:01;C*03:03;C*07:01	n.a.	1	JAK2 V617F
46	PMF	E	male	57	A*02:01;A*24:02;B*07:02;B*14:02;C*07:02;C*08:02	n.a.	0	JAK2 V617F
47	PMF	L, Q	male	81	A*02:01;A*33:01;B*14:02;B*51:01;C*08:02;C*15:02	n.a.	(DIPSS int-2)	JAK2 V617F
48	PMF	L	male	75	A*02:01;B*07:02;B*44:02;C*07:02;C*05:01	n.a.	high	JAK2 V617F
49	PMF	L	male	74	A*02:01;A*11:01;B*13:02;B*56:01;C*06:02;C*01:02	n.a.	int-1	MPL
50	Post-ET-MF	L	female	73	A*01:01;A*23:01;B*44:03;B*57:01;C*04:01;C*06:02	n.a.	(DIPSS int-1)	JAK2 V617F
51	PMF	L, Q	male	55	A*11:01;A*68:01;B*51:01;B*52:01;C*03:03;C*12:02	n.a.	low	triple-negative
52	PMF	L, Q	female	78	A*24:02;A*68:01;B*15:01;B*51:01;C*03:03;C*14:02	n.a.	int-2	JAK2 V617F
53	PMF	L, Q	male	77	A*02:01;A*03:01;B*15:01;B*44:02;C*03:04;C*05:01	n.a.	(DIPSS high)	JAK2 V617F
54	PMF	E	male	26	n.a.	n.a.	0 - low	CALR
55	PMF	L, Q, E	male	77	A*02:01;A*66:01;B*51:01;C*01:02,C*14:02	n.a.	2 - int-2	JAK2 V617F
56	Post-ET-MF	E	female	65	n.a.	n.a.	2 - int-2	JAK2 V617F
57	Post-PV-MF	E	male	75	n.a.	n.a.	(DIPSS int-1)	JAK2 V617F
58	Post-PV-MF	L, E	female	79	A*02:01;A*68:01;B*35:03;B*51:01;C*02:02;C*04:01	n.a.	(DIPSS int-2)	JAK2 V617F

UPN	Entity	Experiment	Gender	Age	HLA class I	IPSET score - risk stratification	DIPSS+ score - risk stratification	Molecular genetics
59	PMF	L, Q, E	male	75	A*02:01;A*03:01;B*18:01;B*44:03;C*05:01;C*16:01	n.a.	2 - int-2	JAK2 V617F
60	PMF	L, Q	male	75	A*01:01;A*11:01;B*08:01;B*35:01;C*04:01;C*07:01	n.a.	high	JAK2 V617F
61	PMF	L, Q	male	87	A*02:01;A*24:02;B*07:02;B*13:02;C*06:02;C*07:02	n.a.	int-1	MPL
62	PMF	L, Q, E	male	42	A*02:05;A*11:01;B*07:02;B*15:03;C*07:02;C*12:03	n.a.	1 - int-1	JAK2 V617F
63	PMF	L, Q	male	68	A*03:01;A*32:01;B*14:02;B*35:01;C*08:02;C*04:01	n.a.	high	JAK2 V617F
64	PMF	L	male	76	A*02:01;A*25:01;B*18:01;B*40:01;C*12:03;C*03:04	n.a.	(DIPSS int-2)	JAK2 V617F
65	PMF	L, Q	female	82	A*24:02;A*29:02;B*15:01;B*44:03;C*03:03;C*16:02	n.a.	n.a.	JAK2 V617F
66	PMF	L, E	male	60	A*02:01;A*24:02;B*13:02;B*56:01;C*01:02;C*06:02	n.a.	(DIPSS 2 - int-1)	JAK2 V617F
67	PMF	L, Q, E	male	46	A*01:01;A*68:01;B*38:01;B*51:01;C*12:03;C*15:04	n.a.	0 - low	CALR
68	PMF	L, Q, E	female	65	A*02:01;A*24:02;B*07:02;B*40:01;C*03:04;C*07:02	n.a.	1	JAK2 V617F
69	PMF	Q	male	79	n.a.	n.a.	(DIPSS 1 - int-1)	JAK2 V617F-negative
70	PMF	L, Q	male	45	A*01:01;A*02:01;B*15:17;B*35:01;C*04:01;C*07:01	n.a.	1 - int-1	JAK2 V617F
71	PMF	Q	male	64	n.a.	n.a.	(DIPSS low)	JAK2 V617F

Table S1: Patient characteristics. ET, PV, and PMF patients included in HLA ligandome analysis, quantification of HLA surface expression, and *in vitro* IFN-γ ELISPOT assays. The International Prognostic Score for survival in ET (IPSET) or the Dynamic International Prognostic Scoring System plus scorers is indicated along with the risk stratification for ET or PMF patients, respectively. In case of a not available DIPSS+ score the respective DIPSS score is indicated instead. Molecular genetics indicate the present driver-mutation in JAK2, MPL, or CALR. Patients without mutations in any of these three proteins are indicated as triple-negative. Abbreviations: UPN, uniform patient number; L, HLA ligandome analysis; Q, HLA quantification, E, IFN-γ ELISPOT; n.a., not available.

Supplemental Table 2

UPN	Entity	Cell type	Cell number [x 10^6]	Cytometer	HLA class I ligands	HLA class I source proteins	HLA class I purity	HLA class II peptides	HLA class II source proteins
01	ET	PBMCs	95	Lumos	2567	2200	96%	1063	439
		granulocytes	160	Lumos	435	502	60%	2090	607
03	ET	PBMCs	600	Lumos	775	883	88%	1568	498
07	ET	PBMCs	130	Lumos	689	776	92%	1371	553
12	ET	PBMCs	780	Lumos	1743	1614	83%	1186	429
14	ET	PBMCs	150	Lumos	1384	1348	97%	568	329
17	ET	PBMCs	180	Lumos	1182	1203	94%	1106	489
22	ET	PBMCs	270	Lumos	1547	1458	91%	1325	523
23	PV	PBMCs	n.a.	Orbitrap XL	1672	1567	91%	excluded	excluded
24	PV	PBMCs	n.a.	Orbitrap XL	1318	1274	92%	excluded	excluded
25	PV	PBMCs	n.a.	Orbitrap XL	848	925	85%	excluded	excluded
26	PV	PBMCs	n.a.	Orbitrap XL	1328	1612	88%	excluded	excluded
27	PV	PBMCs	n.a.	Orbitrap XL	413	325	74%	excluded	excluded
28	PV	PBMCs	n.a.	Orbitrap XL	1599	1514	79%	excluded	excluded
29	PV	PBMCs	n.a.	Orbitrap XL	1869	1712	91%	excluded	excluded
30	PV	PBMCs	n.a.	Orbitrap XL	1566	1510	87%	excluded	excluded
31	PV	PBMCs	120	Lumos	1331	1355	83%	623	328
32	PV	PBMCs	500	Lumos	1798	1562	85%	2715	693
		granulocytes	250	Lumos	excluded	excluded	excluded	1623	502
34	PV	PBMCs	500	Lumos	2634	2181	81%	1935	601
		granulocytes	1.300	Lumos	977	990	79%	1287	455
35	PV	PBMCs	1.900	Lumos	excluded	excluded	excluded	749	185
37	PV	PBMCs	1.200	Lumos	excluded	excluded	excluded	1818	522
		granulocytes	1.300	Lumos	351	490	70%	1064	387
38	PV	PBMCs	100	Lumos	2719	2280	97%	1182	551
		granulocytes	100	Lumos	935	893	51%	1956	583
43	PMF	PBMCs	145	Lumos	4104	3034	91%	1145	511
		granulocytes	128	Lumos	630	663	63%	1729	575
45	PMF	PBMCs	600	Lumos	6678	4194	93%	2749	765
		granulocytes	1.500	Lumos	4935	2922	77%	3628	736
47	PMF	PBMCs	100	Lumos	815	938	93%	988	237
48	PMF	PBMCs	1.100	Lumos	2043	1712	75%	3543	760

UPN	Entity	Cell type	Cell number [x 10^6]	Cytometer	HLA class I ligands	HLA class I source proteins	HLA class I purity	HLA class II peptides	HLA class II source proteins
49	PMF	PBMCs	140	Lumos	572	674	82%	1192	474
50	Post-ET-MF	PBMCs	2.000	Lumos	4638	3276	89%	2378	686
		granulocytes	140	Lumos	1129	1048	78%	1378	378
51	PMF	PBMCs	200	Lumos	645	756	81%	excluded	excluded
52	PMF	PBMCs	2.120	Lumos	1576	1438	77%	1560	456
		granulocytes	940	Lumos	1084	1042	73%	1294	410
53	PMF	PBMCs	400	Lumos	610	723	89%	excluded	excluded
55	PMF	PBMCs	1.100	Lumos	2043	1821	86%	1283	492
58	Post-PV-MF	PBMCs	120	Lumos	1534	1419	91%	656	320
59	PMF	PBMCs	150	Lumos	excluded	excluded	excluded	1156	372
60	PMF	PBMCs	600	Lumos	1538	1489	90%	1002	422
61	PMF	PBMCs	190	Lumos	901	985	94%	799	389
62	PMF	PBMCs	410	Lumos	3166	2483	89%	2232	684
63	PMF	PBMCs	1.400	Lumos	2403	2063	77%	2250	622
		granulocytes	150	Lumos	684	772	64%	1187	352
64	PMF	PBMCs	1.500	Lumos	4778	3162	74%	5561	1302
65	PMF	PBMCs	140	Lumos	2510	2084	96%	975	449
		granulocytes	260	Lumos	3072	2044	78%	2237	556
66	PMF	PBMCs	530	Lumos	2433	2103	90%	1469	539
67	PMF	PBMCs	120	Lumos	632	707	83%	1424	308
68	PMF	PBMCs	210	Lumos	5123	3611	95%	2073	695
		granulocytes	150	Lumos	2554	1944	83%	2079	524
70	PMF	PBMCs	210	Lumos	2093	1872	94%	801	393
		granulocytes	190	Lumos	1289	1171	75%	1046	361

Table S2: Peptide and source protein yields. ET, PV, and PMF patients included in HLA ligandome analysis are indicated together with the respective cell type, lymphocyte counts at the time of sample collection, the cytometer used, as well as HLA class I and class II peptide and source protein yields. For HLA class I yields the purity is given indicating the percentage of ligands within the total HLA class I peptides in one sample. Samples were excluded from further analyses if the peptide yields were $< 30\%$ of the median from the respective cohort and/or if the purity was $< 50\%$ in granulocyte samples or $< 70\%$ in PBMC samples. Abbreviations: UPN, uniform patient number. n.a., not available

Supplemental Table 3

Source protein	Peptide sequence	Peptide length	HLA restriction	Representation frequency in PMF cohort	Representation frequency in HLA-matched PMF samples	Length variants on benign tissues
HLA-A*02						
LEG10	FQDGQEFEL	9	A*02	24%	33%	-
OR9K2	LLGNVGMMTI	10	A*02	19%	33%	-
*	ADIPEVVVSL	10	A*02	14%	25%	-
TKT	AISESNINL	9	A*02	19%	25%	both
PLF4	GLLLLPLVV	9	A*02	14%	25%	-
PDC6I	HAAELIKTV	9	B*51, C*03, C*12	24%	25%	-
XPO2	KLWTPLLQSL	10	A*02, A*32	19%	25%	-
**	YLAAVLEYL	9	A*02, B*15	19%	25%	-
HLA-B*51						
S23IP	DSFLGQTSI	9	B*51	24%	71%	-
C3AR	DAFLSTHL	8	B*51	19%	57%	-
MED24	DALLEQAMI	9	B*51	19%	57%	-
***	DFPVAMQI	8	B*51	19%	57%	hematological
TLK1	DGFAFQNLV	9	B*51	19%	57%	-
****	EALGRLLVV	9	B*51	19%	57%	non-hematological
CTR9	VPPEILNNV	9	B*51	19%	57%	-
ASNS	DAFRFENV	8	B*51	19%	43%	-
METL9	DAVFVLKPV	9	B*51	14%	43%	-
HXK3	DSLALRQV	8	B*51	14%	43%	-
UBP28	DSYGGLRNV	9	B*51	14%	43%	-
CATW	EAFKLFQI	8	B*51	14%	43%	-
ELNE	FAPVAQFV	8	B*51	14%	43%	-
IF44L	IPISNILMV	9	B*51	14%	43%	-
TAF7	LPPEYASTV	9	B*51	14%	43%	-
SC11C	LPYVGMVTI	9	B*51	14%	43%	-
SMHD1	TPIGALRI	8	B*51	14%	43%	-
HERC5	VGYRVTQI	8	B*51	14%	43%	-
LKHA4	VPYEKGFALL	10	B*51	14%	43%	hematological
HNRPU	YGYSLKGI	8	B*51	14%	43%	-

Table S3: Identified HLA allotype-adjusted PMF-associated ligands. Naturally presented, HLA allotype-adjusted HLA-A*02 and HLA-B*51 PMF-associated ligands identified by HLA class I ligandome profiling of primary PMF samples and a hematological and non-hematological benign tissues datasets. Peptides mapping in multiple proteins are marked with asterisks: *DEF1, DEF3; **H2A1A, H2A1B, H2A1C, H2A1D, H2A1H, H2A1J, H2A1, H2A1B, H2A3, H2AJ, H2AX, H2AB1, H2AB2; ***CLH1, CLH2; ****HBB, HBD, HBE.

Supplemental Table 4

Gene	Protein	Mutations							
ABL1	ABL1	M244V	G250E	Q252H	Y253H	E255K	E255V	V299L	T315I
		F317L	M351T	E355G	F359V	H396R	E459K		
BCORL1	BCORL	G209S							
CALR	CALR	type 1	(L367fs*46	52del)		type 2	(K385fs*47	5ins)	
CBL	CBL	Y371H	L380P	R420Q					
CSF3R	CSF3R	T618I							
DNMT3A	DNM3A	G543C	S714C	R882C	R882H	R882P	R882S		
FLT3	FLT3	E573insE	S574insS	Q575insQ	L576insL	Q577insQ	M578insM	V579insV	Q580insQ
		V581insV	T582insT	G583insG	S584insS	S585insS	D586insD	N587insN	E588insE
		Y589insY	F590insF	Y591insY	V592insV	D593insD	F594insF	R595insR	R595insRE
		E596insE	E596insEY	Y597insY	E598insE	Y599insY	D600insD	L601insL	K602insK
		W603insW	E604insE	F605insF	P606insP	R607insR	E608insE	N609insN	L610insL
		E611insE	F612insF	G613insG	K614insK	N676K	F691L	D835V	D835G
		D835Y	D835F	D835A	D835E	D835H	D385N	D835S	I836LD
		I836del							
		S584insSSDNEYFYVDFR		E588insEYFYVDFR		F594insFREY		R595insREYEYD	
		S585insSDNEYFYVDFR		Y591insYVDFREYE		F594insFREYE		R595insREYEYDL	
		D586insDNEYFYVDF		V592insVDFREY		F594insFREYEYDL		Y597insYEYDLKW	
		D586insDNEYFYVDFR		V592insVDFREYE		F594insFREYEYD		E598insEYDLKWEF	
		E588insEYFYVDF		D593insDFREY		R595insREY		Y599insYDLKWEFP	
		V581insVTGSSDNEYFYVDFREYEYDLKWEFPRENL				S584insSSDNEYFYVDFREYEYDLKWEFP			
		G583insGSSDNEYFYVDFREY				S584insSSDNEYFYVDFREYEYDLKWEFPRE			
		G583insGSSDNEYFYVDFREYE				S584insSSDNEYFYVDFREYEYDLKWEFPRENL			
		G583insGSSDNEYFYVDFREYEYDLKWEFPRE				S585insSDNEYFYVDFREYEYDLKWEFP			
		G583insGSSDNEYFYVDFREYEYDLKWEFPRENL				S585insSDNEYFYVDFREYEYDLKWEFPRE			
		G583insGSSDNEYFYVDFREYEYDLKWEFPRENLE				N586insDNEYFYVDFREYEYDLKWEFP			
		S584insSSDNEYFYVDFRE				N587insNEYFYVDFREYEYDLKWE			
		S584insSSDNEYFYVDFREY				N587insNEYFYVDFREYEYDLKWEFP			
		S584insSSDNEYFYVDFREYEYD				E588insEYFYVDFREYEYDLKWEFP			
GDF5	GDF5	S276A							
IDH1	IDHC	R132C	R132G	R132H	R132L	R132S			
IDH2	IDHP	R140Q	R140W	R172K					
JAK2	JAK2	FHK537-539L		HK538-539QL		K539L	K607N	V617I	V617F
KIT	KIT	D816H	D816V	D816Y	N822K				
KRAS	RASK	G12A	G12D	G12S	G12V	G13D	Q61H		
MPL	TPOR	P106L	S505N	W515A	W515K	W515L			
MYD88	MYD88	L252P							
NPM1	NMP1	type A	type B	type C	type D	type E			
NRAS	RASN	G12A	G12C	G12D	G12S	G12V	G13D	G13R	G13V
		Q61H	Q61K	Q61L	Q61R				
PTPN11	PTN11	G60V	D61V	D61Y	A72T	A72V	E76G	E76K	
SETBP1	SETBP	D868N	G870S	I871T					
SF3B1	SF3B1	E622D	H662Q	K666N	K666R	K666T	K700E		
SRSF2	SRSF2	P95H	P95L	P95R					
TET2	TET2	I1873T							
TP53	P53	R175H	V216M	Y220C	R248Q	R248W	R273C	R273H	C275Y
U2AF1	U2AF1	S34F	S34Y	R156H	Q157P	Q157R			

Table S4: Recurrent mutations in Ph- myeloproliferative neoplasms and AML. Recurrent mutations in the premalignant Ph- myeloproliferative neoplasms ET, PV, and PMF as well as in the established AML. The TOP100 missense mutations described in the COSMIC database, the most common mutations described for AML as well as the most common JAK2, MPL, and CALR mutations in MPNs are listed and were used for the identification of mutation-derived neoepitopes.

Supplemental Figure 1

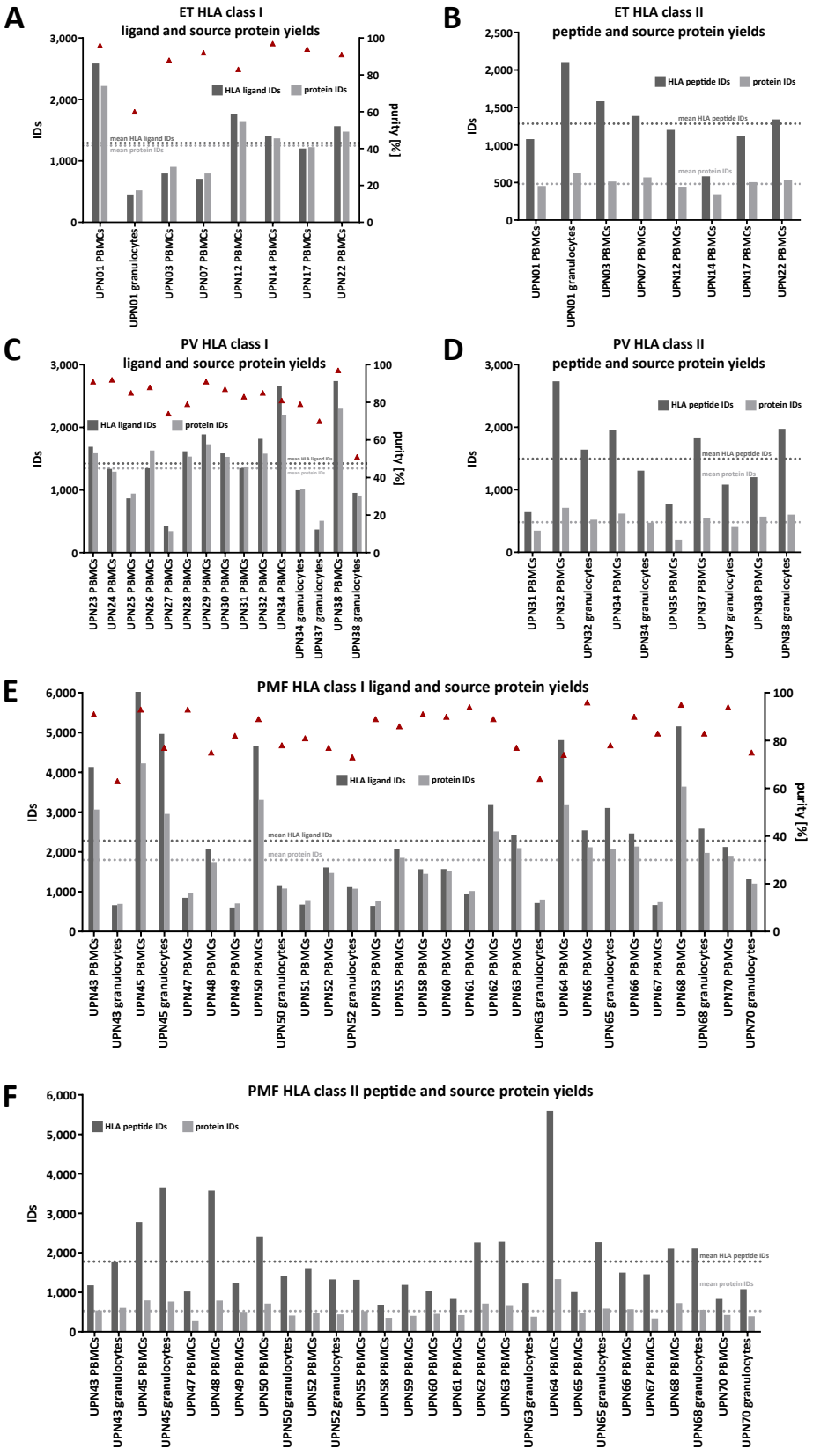

Figure S1: HLA class I and II peptide and source protein yields. (A) HLA class I ligand and respective source protein yields of primary ET samples (n = 7) as identified by mass spectrometry are indicated in dark grey and light grey bars, respectively. Mean HLA class I ligand and protein identifications are depicted by the dark grey and light grey dotted line, respectively.
(B) Numbers of HLA class II peptides (dark grey bars) and source proteins (light grey bars) identified in primary ET samples (n = 7). Mean HLA class II peptide and protein identifications are illustrated by the dark grey and light grey dotted line, respectively.
(C) HLA class I ligand and respective source protein yields of primary PV samples (n = 13) as identified by mass spectrometry are indicated in dark grey and light grey bars, respectively. Mean HLA class I ligand and protein identifications are depicted by the dark grey and light grey dotted line, respectively.
(D) Numbers of HLA class II peptides (dark grey bars) and source proteins (light grey bars) identified in primary PV samples (n = 6). Mean HLA class II peptide and protein identifications are illustrated by the dark grey and light grey dotted line, respectively.
(E) HLA class I ligand and respective source protein yields of primary PMF samples (n = 21) as identified by mass spectrometry are indicated in dark grey and light grey bars, respectively. Mean HLA class I ligand and protein identifications are depicted by the dark grey and light grey dotted line, respectively.
(F) Numbers of HLA class II peptides (dark grey bars) and source proteins (light grey bars) identified in primary PMF samples (n = 20). Mean HLA class II peptide and protein identifications are illustrated by the dark grey and light grey dotted line, respectively.
Abbreviations: IDs, identifications; UPN, uniform patient number.

Supplemental Figure 2

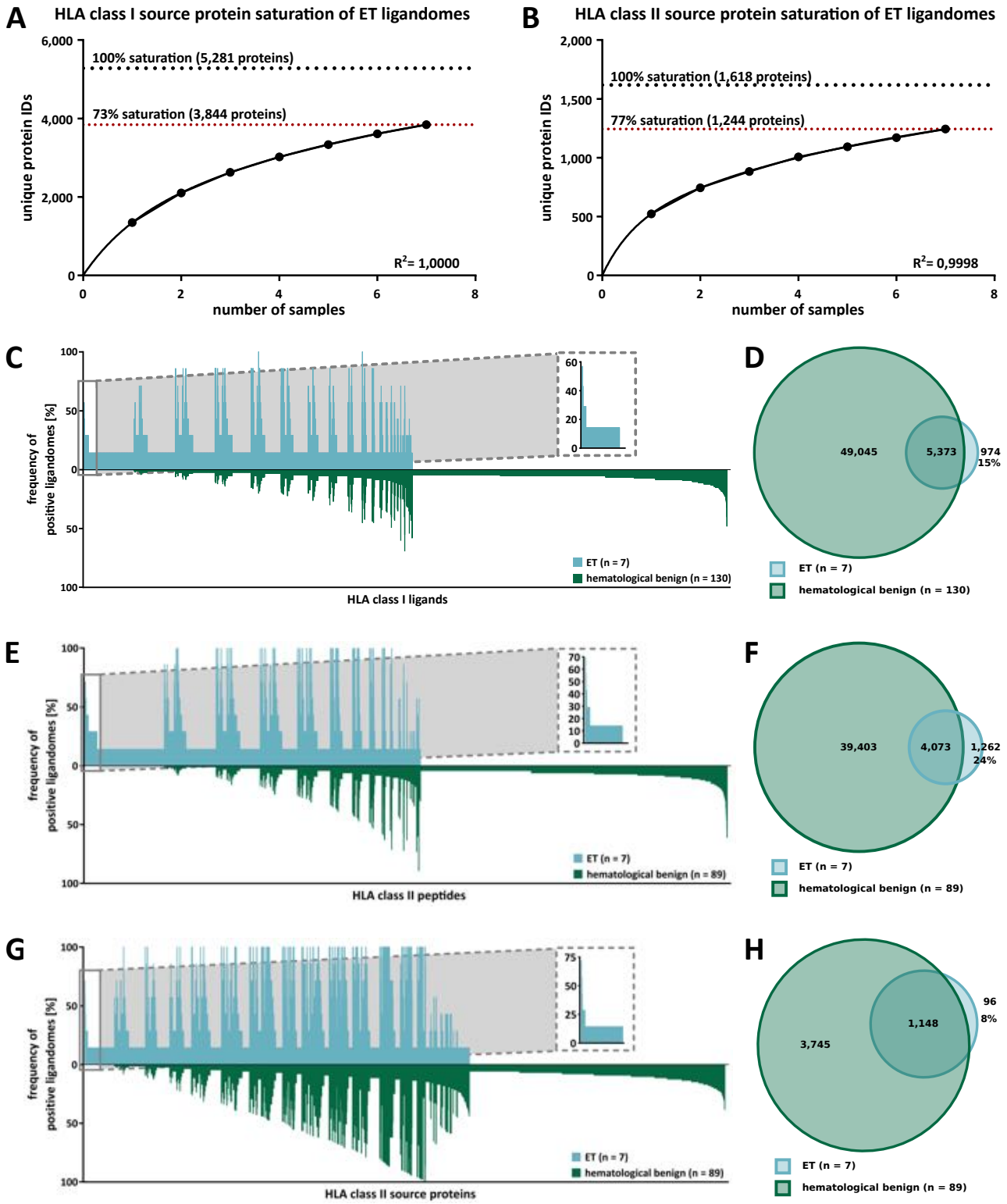

Figure S2: Comparative ligandome profiling and identification of ET-associated antigens. (A) Saturation analysis of HLA class I ligand source proteins and (B) HLA class II peptide source proteins of the ET patient cohort. Number of unique source protein identifications are shown as a function of cumulative HLA ligandome analysis of ET samples (n = 7 for HLA class I and II). Exponential regression allowed for the robust calculation ($R^2 = 1.0000$ for HLA class I and $R^2 = 0.9998$ for HLA class II) of the maximum attainable number of different source protein identifications (dotted black line). The dotted red line depicts the source proteome coverage achieved in our ET patient cohort.
Comparative profiling based on the presentation frequency of (C) HLA class I-restricted ligands, (E) HLA class II-restricted peptides, and (G) HLA class II source proteins in the ligandomes of primary ET samples (n = 7) and hematological benign samples (n = 130 for HLA class I), including PBMCs (n = 63), granulocytes (n = 32), $CD19^+$ B cells (n = 6), T cells (n = 1), bone marrow (n = 20), and $CD34^+$ HPCs (n = 8). Hematological benign samples for HLA class II analysis (n = 89) comprised PBMCs (n = 36), granulocytes (n = 20), $CD19^+$ B cells (n = 10), bone marrow (n = 15), and $CD34^+$ HPCs (n = 8). Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. The boxes on the left and the magnifications within highlight the subset of ET-associated antigens showing ET-exclusive presentation. To allow for better readability, HLA class I ligands identified on $\leq$3.5% of the samples, HLA class II peptides identified on $\leq$4.5%, and HLA class II source proteins identified on $\leq$5% of the samples within the hematological benign cohort were not depicted in the waterfall plots.
Overlap analyses of (D) HLA class I ligands, (F) HLA class II peptides, and (H) HLA class II source proteins identified in primary ET samples (n = 7) and hematological benign samples (n = 130 for HLA class I and n = 89 for HLA class II). (D,F,H) Created using BioVenn (Hulsen *et al.* 2008). Abbreviation: IDs, identifications.

Supplemental Figure 3

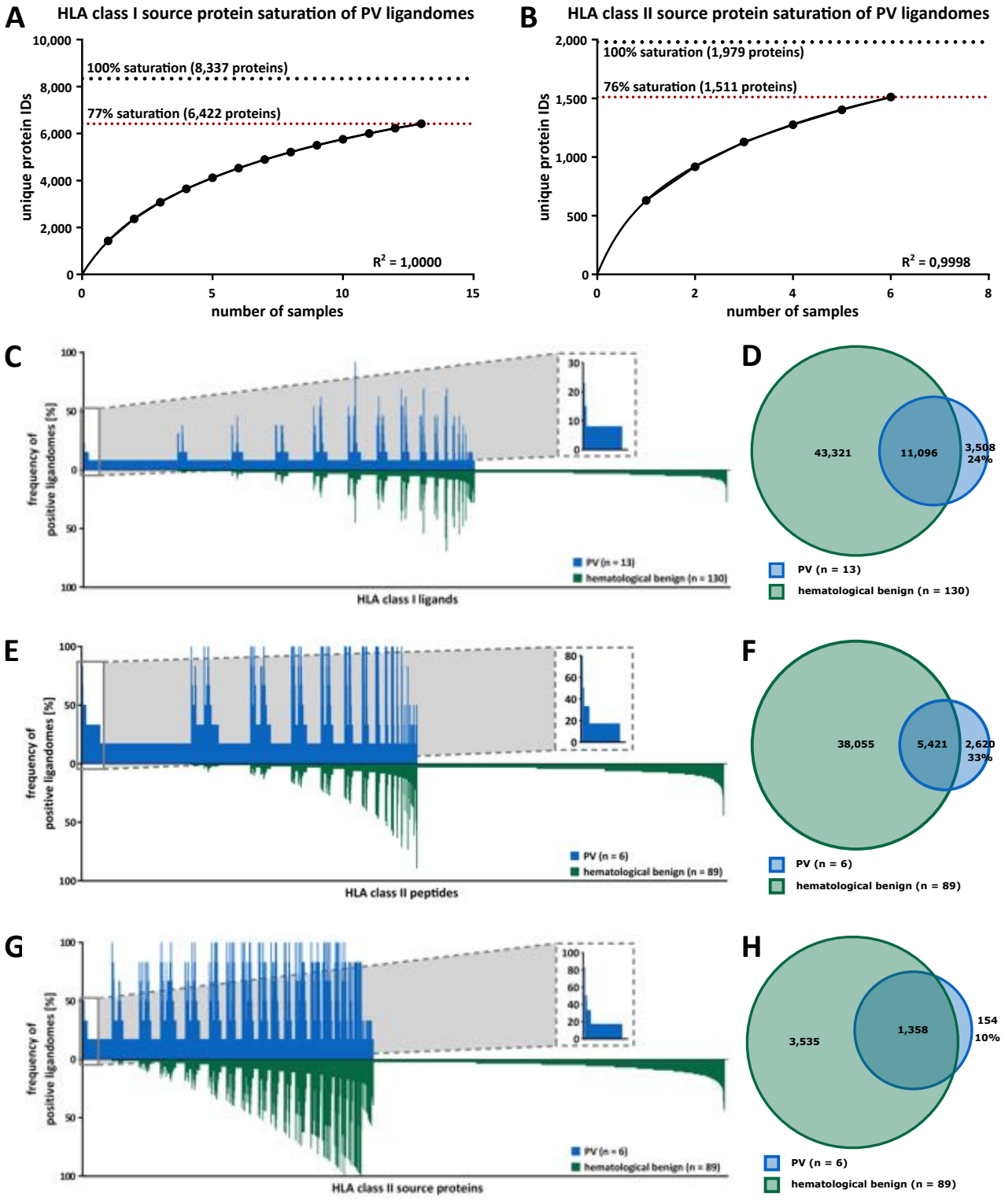

Figure S3: Comparative ligandome profiling and identification of PV-associated antigens. (A) Saturation analysis of HLA class I ligand source proteins and (B) HLA class II peptide source proteins of the PV patient cohort. Number of unique source protein identifications are shown as a function of cumulative HLA ligandome analysis of PV samples (n = 13 for HLA class I and n = 6 for HLA class II). Exponential regression allowed for the robust calculation ($R^2 = 1.0000$ for HLA class I and $R^2 = 0.9998$ for HLA class II) of the maximum attainable number of different source protein identifications (dotted black line). The dotted red line depicts the source proteome coverage achieved in our PV patient cohort.
Comparative profiling based on the presentation frequency of (C) HLA class I-restricted ligands, (E) HLA class II-restricted peptides, and (G) HLA class II source proteins in the ligandomes of primary PV samples (n = 13 for HLA class I and n = 6 for HLA class II) and hematological benign samples (n = 130 for HLA class I), including PBMCs (n = 63), granulocytes (n = 32), $CD19^+$ B cells (n = 6), T cells (n = 1), bone marrow (n = 20), and $CD34^+$ HPCs (n = 8). Hematological benign samples for HLA class II analysis (n = 89) comprised PBMCs (n = 36), granulocytes (n = 20), $CD19^+$ B cells (n = 10), bone marrow (n = 15), and $CD34^+$ HPCs (n = 8). Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. The boxes on the left and the magnifications within highlight the subset of PV-associated antigens showing PV-exclusive presentation. To allow for better readability, HLA class I ligands identified on $\leq$2% of the samples, HLA class II peptides identified on $\leq$3%, and HLA class II source proteins identified on $\leq$2% of the samples within the hematological benign cohort were not depicted in the waterfall plots.
Overlap analyses of (D) HLA class I ligands, (F) HLA class II peptides, and (H) HLA class II source proteins identified in primary PV samples (n = 13 for HLA class I and n = 6 for HLA class II) and hematological benign samples (n = 130 for HLA class I and n = 89 for HLA class II). (D,F,H) Created using BioVenn (Hulsen *et al.* 2008). Abbreviation: IDs, identifications.

Supplemental Figure 4

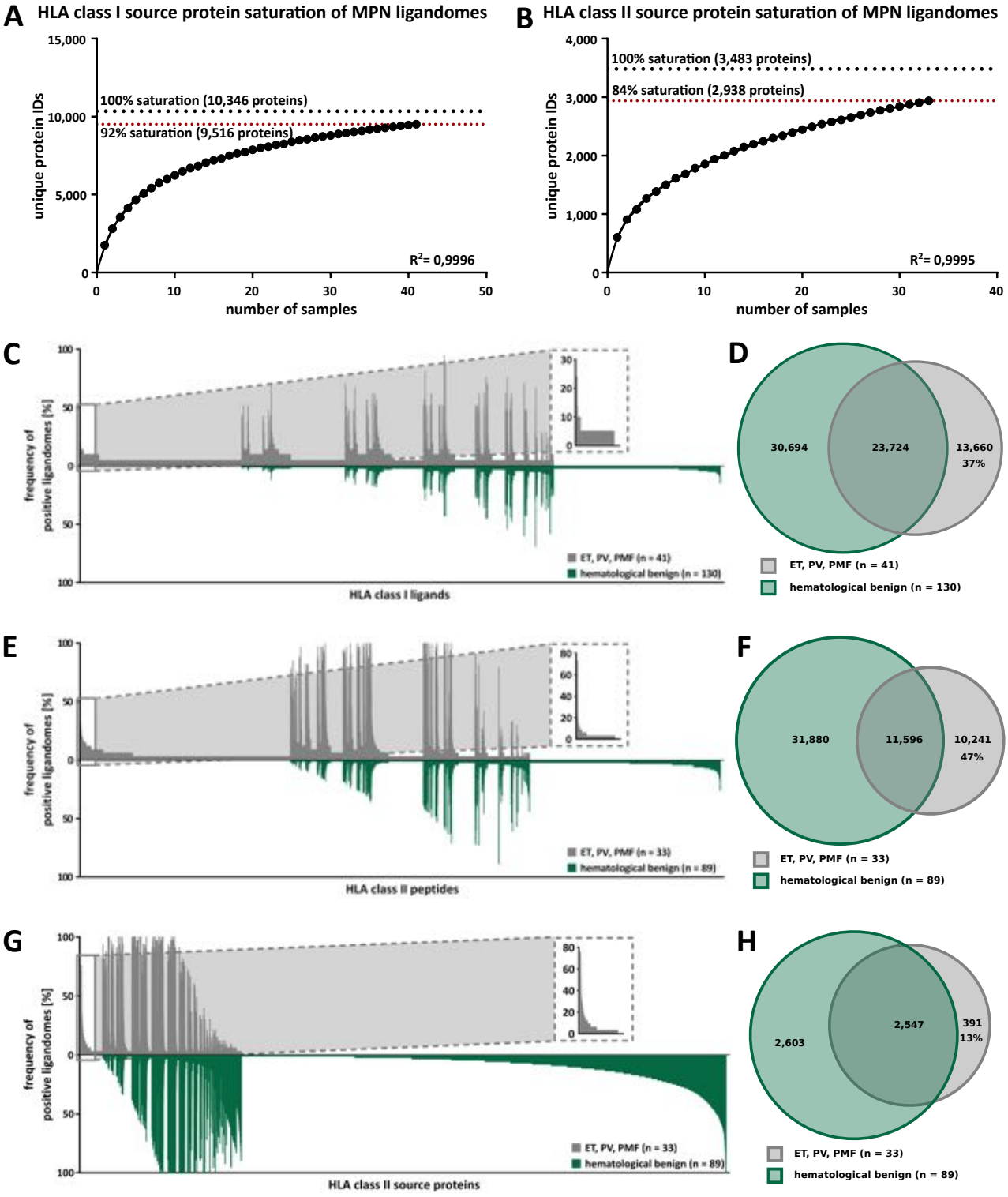

Figure S4: Comparative ligandome profiling and identification of MPN-associated antigens. (A) Saturation analysis of HLA class I ligand source proteins and (B) HLA class II peptide source proteins of the MPN patient cohort (ET, PV, and PMF samples). Number of unique source protein identifications are shown as a function of cumulative HLA ligandome analysis of MPN samples (n = 41 for HLA class I and n = 33 for HLA class II). Exponential regression allowed for the robust calculation ($R^2 = 0.9996$ for HLA class I and $R^2 = 0.9995$ for HLA class II) of the maximum attainable number of different source protein identifications (dotted black line). The dotted red line depicts the source proteome coverage achieved in our MPN patient cohort.
Comparative profiling based on the presentation frequency of (C) HLA class I-restricted ligands, (E) HLA class II-restricted peptides, and (G) HLA class II source proteins in the ligandomes of primary MPN samples (n = 41 for HLA class I and n = 33 for HLA class II) and hematological benign samples (n = 130 for HLA class I), including PBMCs (n = 63), granulocytes (n = 32), $CD19^+$ B cells (n = 6), T cells (n = 1), bone marrow (n = 20), and $CD34^+$ HPCs (n = 8). Hematological benign samples for HLA class II analysis (n = 89) comprised PBMCs (n = 36), granulocytes (n = 20), $CD19^+$ B cells (n = 10), bone marrow (n = 15), and $CD34^+$ HPCs (n = 8). Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. The boxes on the left and the magnifications within highlight the subset of MPN-associated antigens showing MPN-exclusive presentation. To allow for better readability, HLA class I ligands identified on $\leq$2% of the samples and HLA class II peptides identified on $\leq$2% of the samples within the hematological benign cohort were not depicted in the waterfall plots.
Overlap analyses of (D) HLA class I ligands, (F) HLA class II peptides, and (H) HLA class II source proteins identified in primary MPN samples (HLA class I n = 41 including 7 ET, 13 PV, and 21 PMF samples; HLA class II n = 33 including 7 ET, 6 PV, and 20 PMF samples) and hematological benign samples (n = 130 for HLA class I and n = 89 for HLA class II). (D,F,H) Created using BioVenn (Hulsen *et al.* 2008). Abbreviation: IDs, identifications.

Supplemental Figure 5

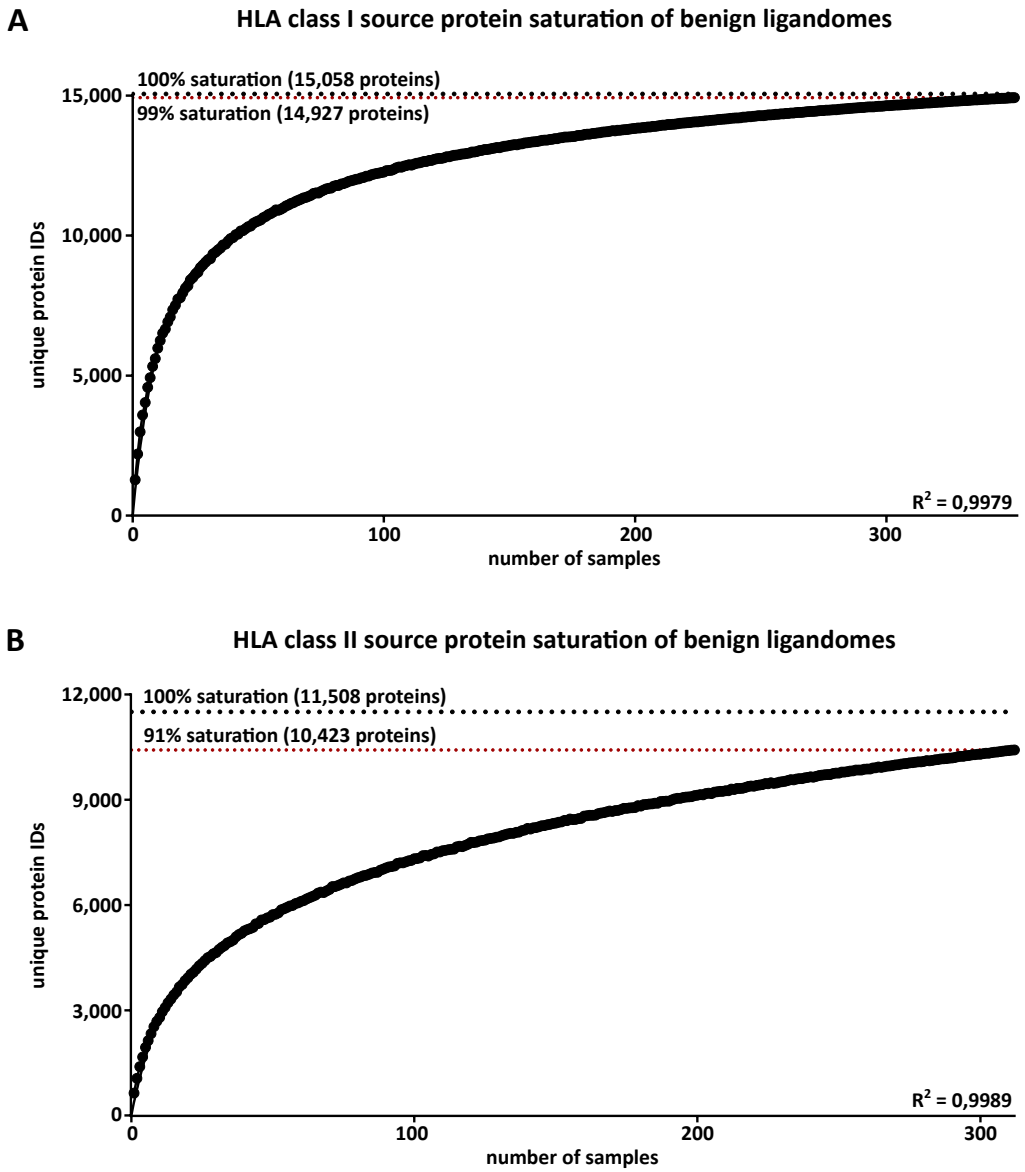

Figure S5: Saturation analysis of HLA peptide source proteins of hematological and non-hematological benign tissue cohorts. Saturation analysis of (A) HLA class I and (B) HLA class II peptide source proteins of the benign tissue cohort. Number of unique HLA ligand source protein identifications shown as function of cumulative HLA ligandome analysis of benign tissue samples ($n = 351$ for HLA class I, $n = 312$ for HLA class II). Exponential regression allowed for the robust calculation ($R^2 = 0.9979$ for HLA class I, $R^2 = 0.9989$ for HLA class II) of the maximum attainable number of different source protein identifications (dotted black line). The dotted red line depicts the source proteome coverage achieved in our benign tissue cohort. Abbreviation: IDs, identifications.

Supplemental Figure 6

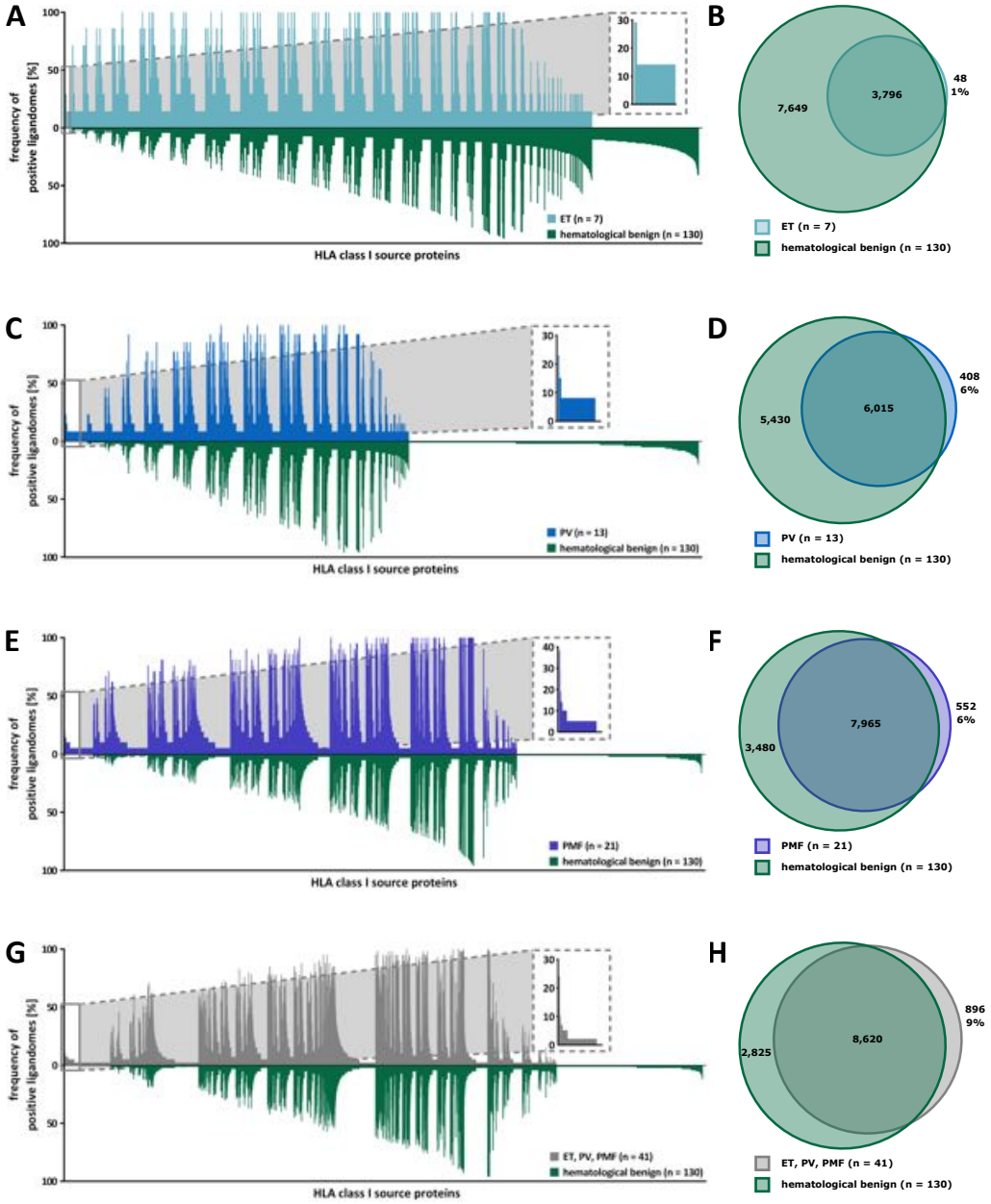

Figure S6: Comparative HLA class I ligandome profiling on source protein level. Comparative profiling of HLA class I source proteins based on the frequency of HLA-restricted presentation in the ligandomes of (A) ET (n = 7), (C) PV (n = 13), (E) PMF (n = 21), (G) MPN (ET, PV, PMF, n = 41), and hematological benign (n = 130) cohorts. Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. The boxes on the left and their magnifications highlight the subset of (A) ET-, (C) PV-, (E) PMF-, and (G) MPN-associated antigens showing tumor-exclusive presentation. (A) To allow for better readability, HLA ligand source proteins identified on $\leq$10% of the samples within the hematological benign cohort were not depicted in the ET waterfall plot. (B, D, F, H) Overlap analysis of HLA class I source proteins identified in primary (B) ET, (D) PV, (F) PMF, (H) MPN samples, and hematological benign samples (n = 130), including PBMCs (n = 63), granulocytes (n = 32), $CD19^+$ B cells (n = 6), T cells (n = 1), bone marrow (n = 20), and $CD34^+$ HPCs (n = 8). (B,D,F,H) Created using BioVenn (Hulsen *et al.* 2008).

Supplemental Figure 7

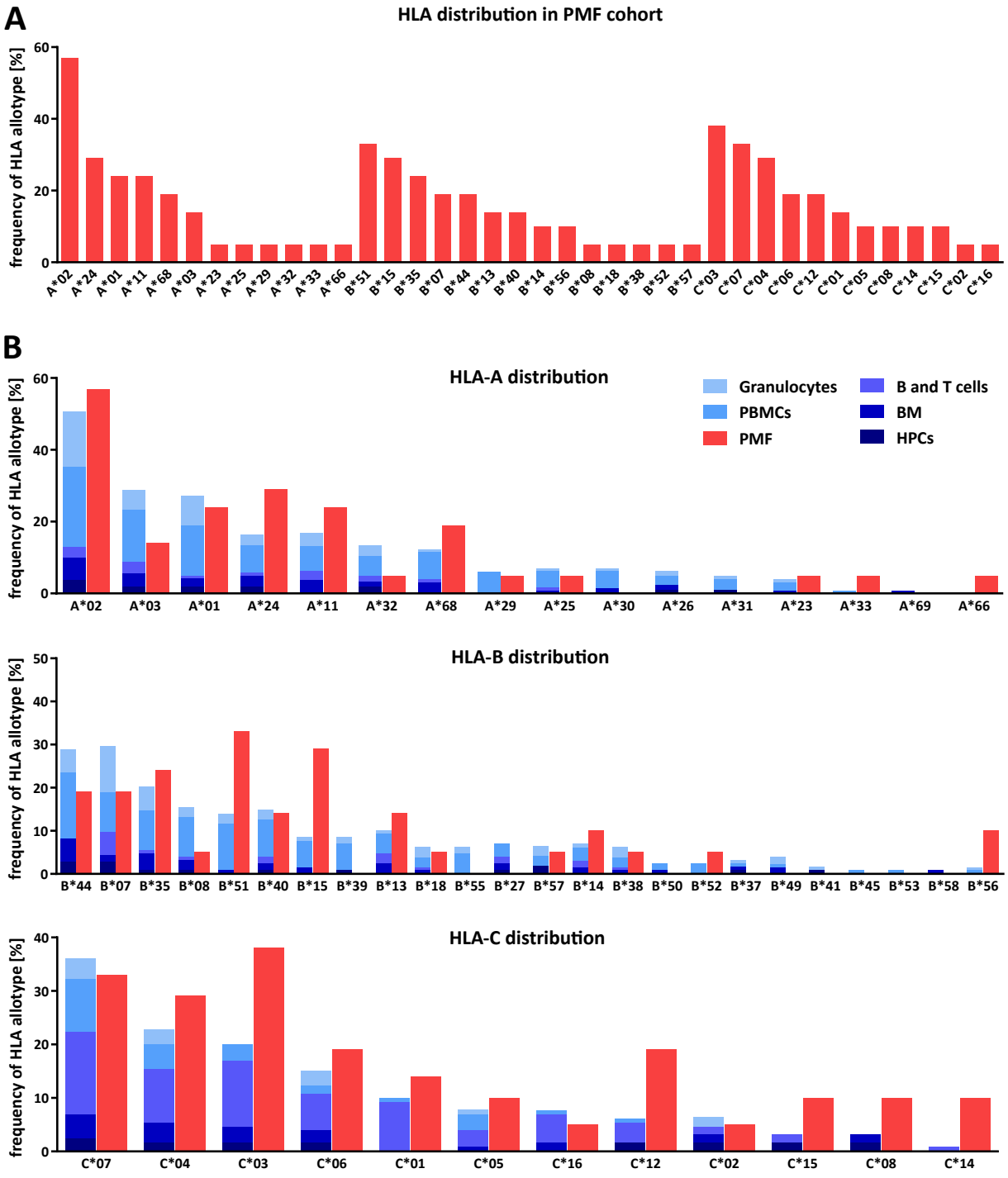

Figure S7: HLA allotype distribution in PMF and hematological benign tissue cohorts. (A) HLA class I allotype frequencies in the PMF patient cohort (n = 21) used for mass spectrometry-based analysis of naturally presented, PMF-associated antigens. (B) HLA-A, HLA-B, and HLA-C allotype frequencies in PMF patient (n = 21) and hematological benign tissue (n = 130) cohorts. Abbreviations: PBMCs, peripheral blood mononuclear cells; BM, bone marrow; HPCs, hematopoietic progenitor cells.

Supplemental Figure 8

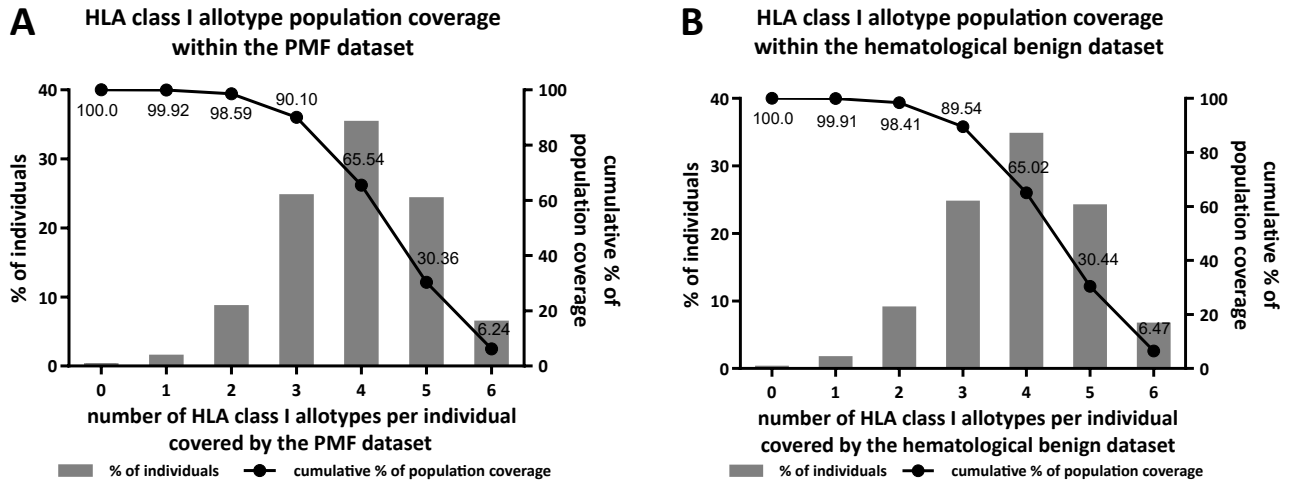

Figure S8: HLA class I allotype population coverage. HLA class I allotype population coverage within the (A) PMF and (B) hematological benign tissue cohorts compared to the world population (calculated by the IEDB population coverage tool, www.iedb.org). The frequencies of individuals within the world population carrying up to six HLA allotypes (x-axis) of the respective PMF or benign dataset are indicated as grey bars on the left y-axis. The cumulative percentage of population coverage is depicted as black dots on the right y-axis.

Supplemental Figure 9

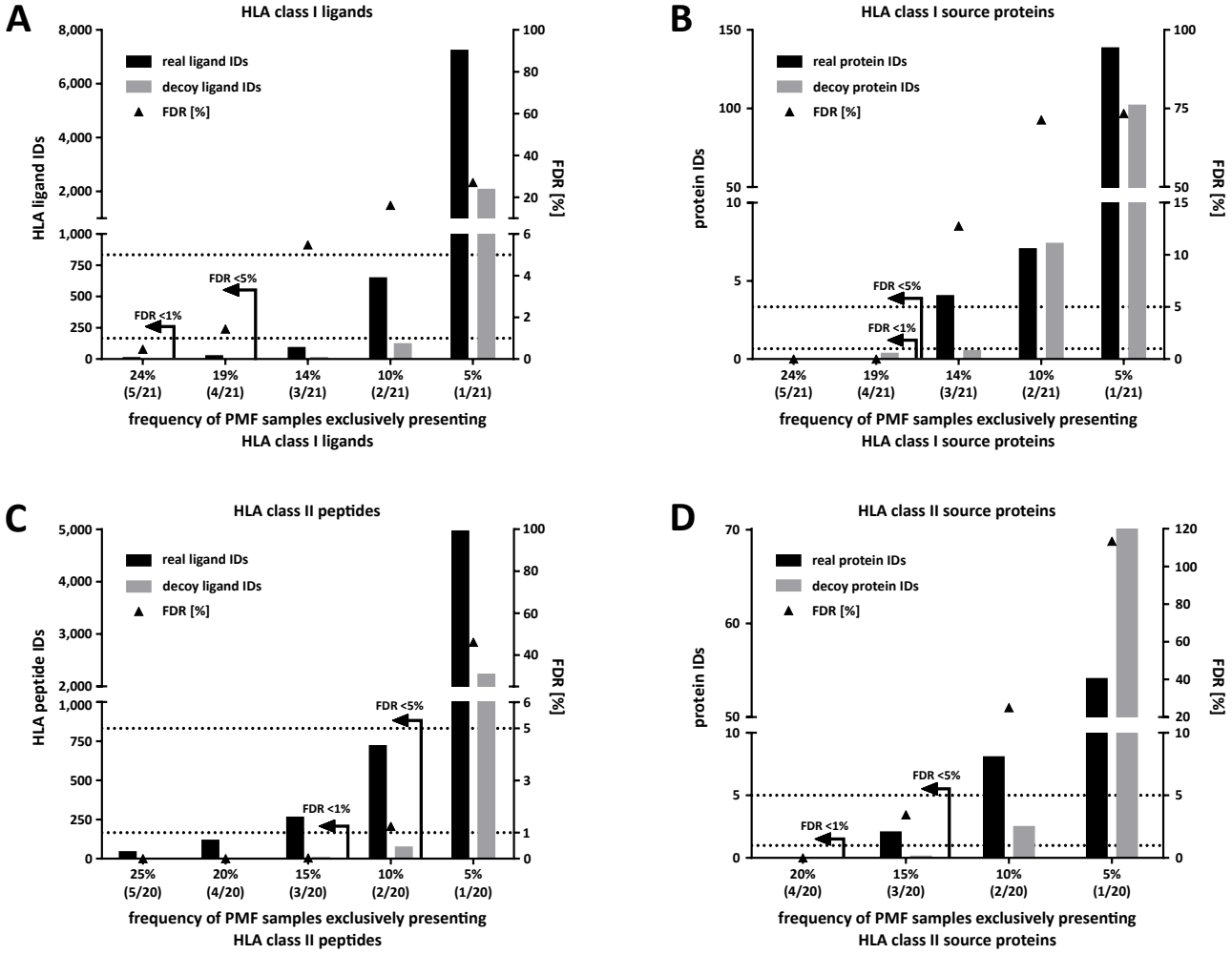

Figure S9: Statistical analysis of false positive PMF-associated antigen identifications. The numbers of identified (A) HLA class I ligands and (B) source proteins, (C) HLA class II peptides and (D) source proteins based on the analysis of the PMF and total benign tissue cohorts were compared with random virtual PMF-associated HLA class I and II peptides or source proteins (left y-axis), respectively. Virtual ligandomes of PMF and benign tissue samples were generated *in silico* based on random weighted sampling from the entirety of peptide identifications in both original cohorts. These randomized virtual ligandomes were used to define PMF-associated antigens based on simulated cohorts of PMF *versus* benign tissue samples. The process of peptide randomization, cohort assembly, and PMF-associated antigen identification was repeated 1,000 times and the mean value of resultant virtual PMF-associated antigens was calculated and plotted for the different threshold values. The corresponding FDRs (right y-axis) for any chosen threshold (x-axis) were calculated and the 1% and 5% FDRs are indicated within the plot (dotted lines and arrows). Abbreviations: IDs, identifications; FDR, false discovery rate.

Supplemental Figure 10

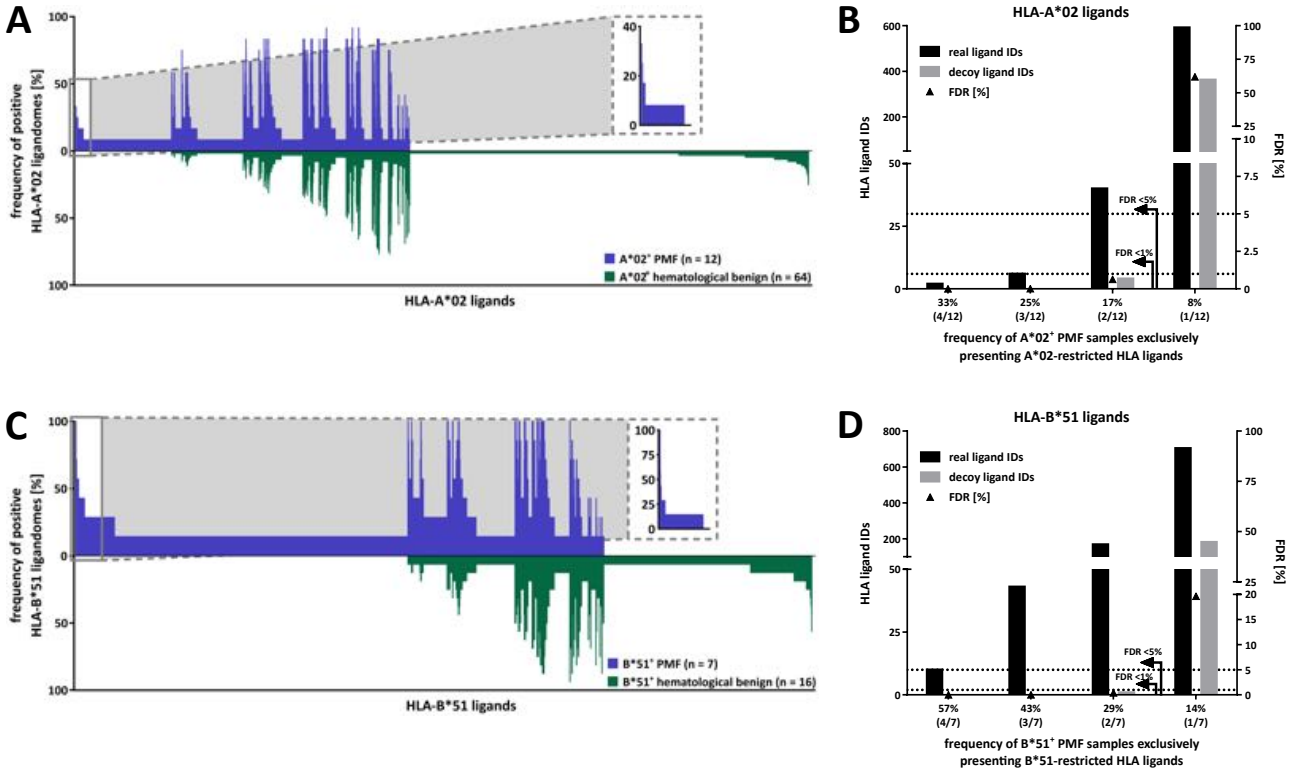

Figure S10: Comparative ligandome profiling of PMF-associated HLA-A*02 or -B*51 ligands. Comparative profiling of naturally presented (A) HLA-A*02 and (C) -B*51 ligands based on the frequency of HLA-restricted presentation in the immunopeptidomes of primary PMF samples and hematological benign tissues. Frequencies of positive immunopeptidomes for the respective HLA ligands (x-axis) are indicated on the y-axis. The boxes on the left and their magnifications highlight the subset of PMF-associated antigens showing tumor-exclusive presentation. Statistical analysis of the proportion of false positive PMF-associated antigen identifications within the (B) HLA-A*02$^+$ and (D) HLA-B*51$^+$ PMF samples. The numbers of identified HLA ligands based on the analysis of the PMF and benign tissue cohorts were compared with random virtual PMF-associated ligands (left y-axis), respectively. Virtual ligandomes of PMF and benign tissue samples were generated *in silico* based on random weighted sampling from the entirety of peptide identifications in both original cohorts. These randomized virtual ligandomes were used to define PMF-associated antigens based on simulated cohorts of PMF *versus* benign tissue samples. The process of peptide randomization, cohort assembly, and PMF-associated antigen identification was repeated 1,000 times and the mean value of resultant virtual PMF-associated antigens was calculated and plotted for the different threshold values. The corresponding FDRs (right y-axis) for any chosen threshold (x-axis) were calculated and the 1% and 5% FDRs are indicated within the plot (dotted lines and arrows). Abbreviations: IDs, identifications; FDR, false discovery rate.

Supplemental Figure 11

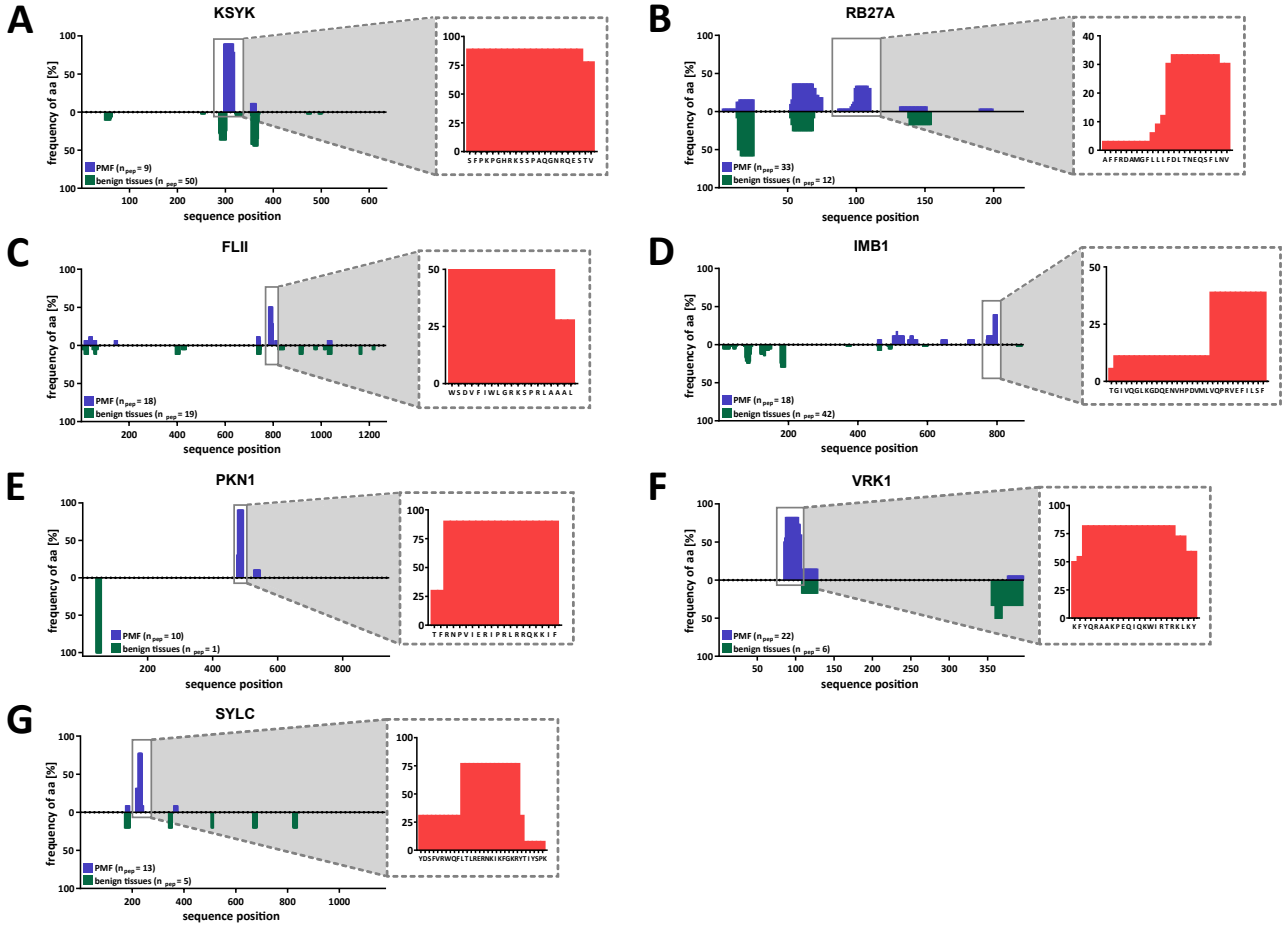

Figure S11: Hotspot analysis of the hotspot targets. Hotspot analysis of the protein (A) KSYK, (B) RB27A, (C) FLII, (D) IMB1, (E) PKN1, (F) VRK1, and (G) SYLC generated by peptide clustering. Identified peptides were mapped to their amino acid positions within the source protein. Representation frequencies of amino acid counts within each cohort for the respective amino acid position (x-axis) were calculated and are indicated on the y-axis. The boxes on the left and their magnifications highlight the identified hotspot with the respective amino acids on the x-axis. Abbreviations: aa, amino acids; n_{pep}, number of peptides.

Supplemental Figure 12

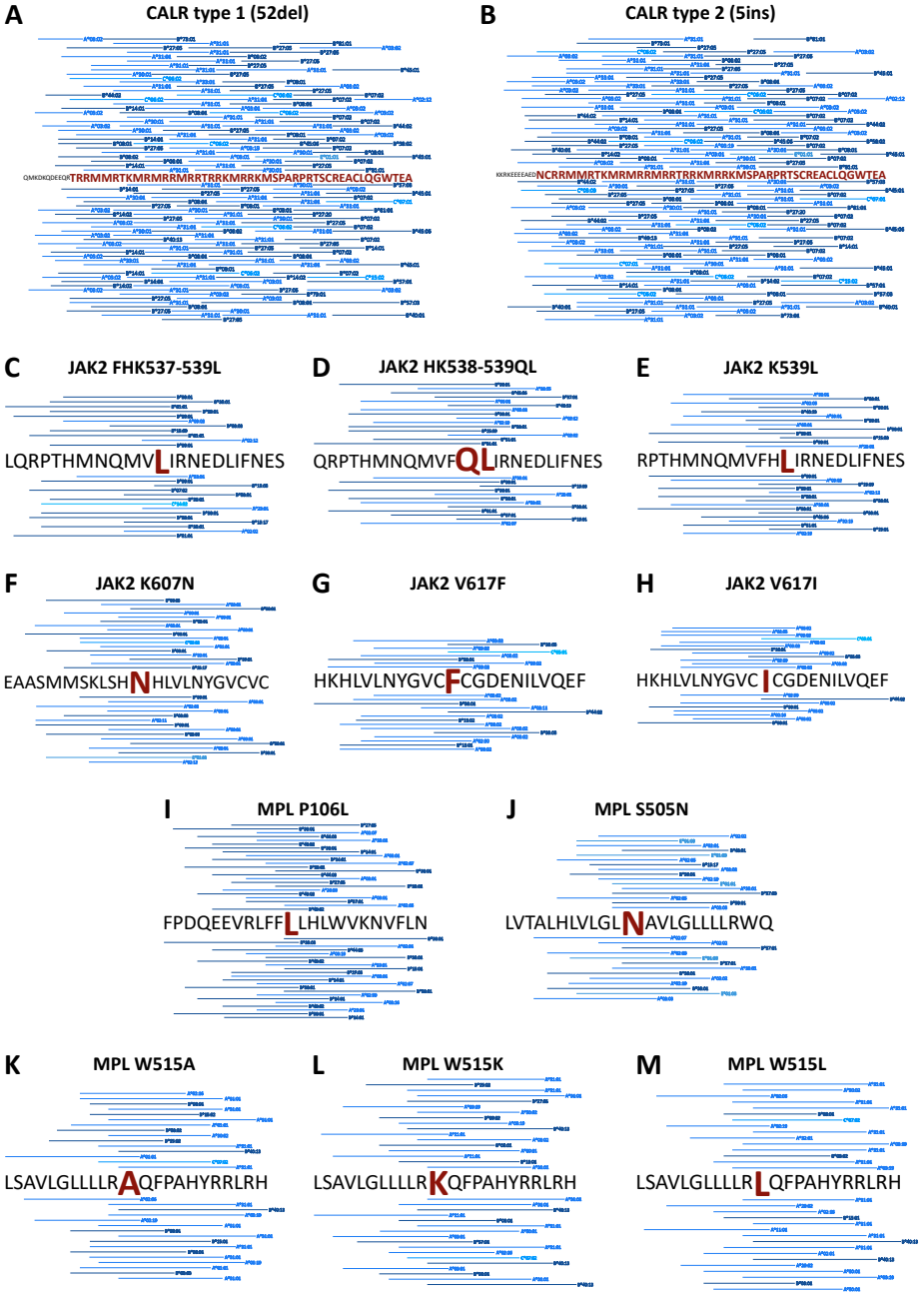

Figure S12: Neoepitope prediction of the most common CALR, JAK2, and MPL mutations. Annotation of potential HLA class I-restricted peptides (8-12 amino acids) was performed using SYFPEITHI 1.0 (Schuler *et al.*, Methods Mol Biol., 2007) and NetMHC 4.0 (Nielsen *et al.*, Protein Sci., 2003). Possible neoepitopes were considered as HLA class I-restricted ligands if the SYFPEITHI score yielded $\geq$60% and/or NetMHC rank value was $\leq$2.0. Each line represents one peptide spanning the protein region comprising the mutated amino acids, which are indicated in red letters. The predicted best fitting HLA allotype is indicated at the right side of each line, respectively. The total number of potential HLA ligands is (A) 178, (B) 176, (C) 25, (D) 27, (E) 28, (F) 32, (G) 22, (H) 20, (I) 45, (J) 29, (K)31, (L) 34, and (M) 31.

Supplemental Figure 13

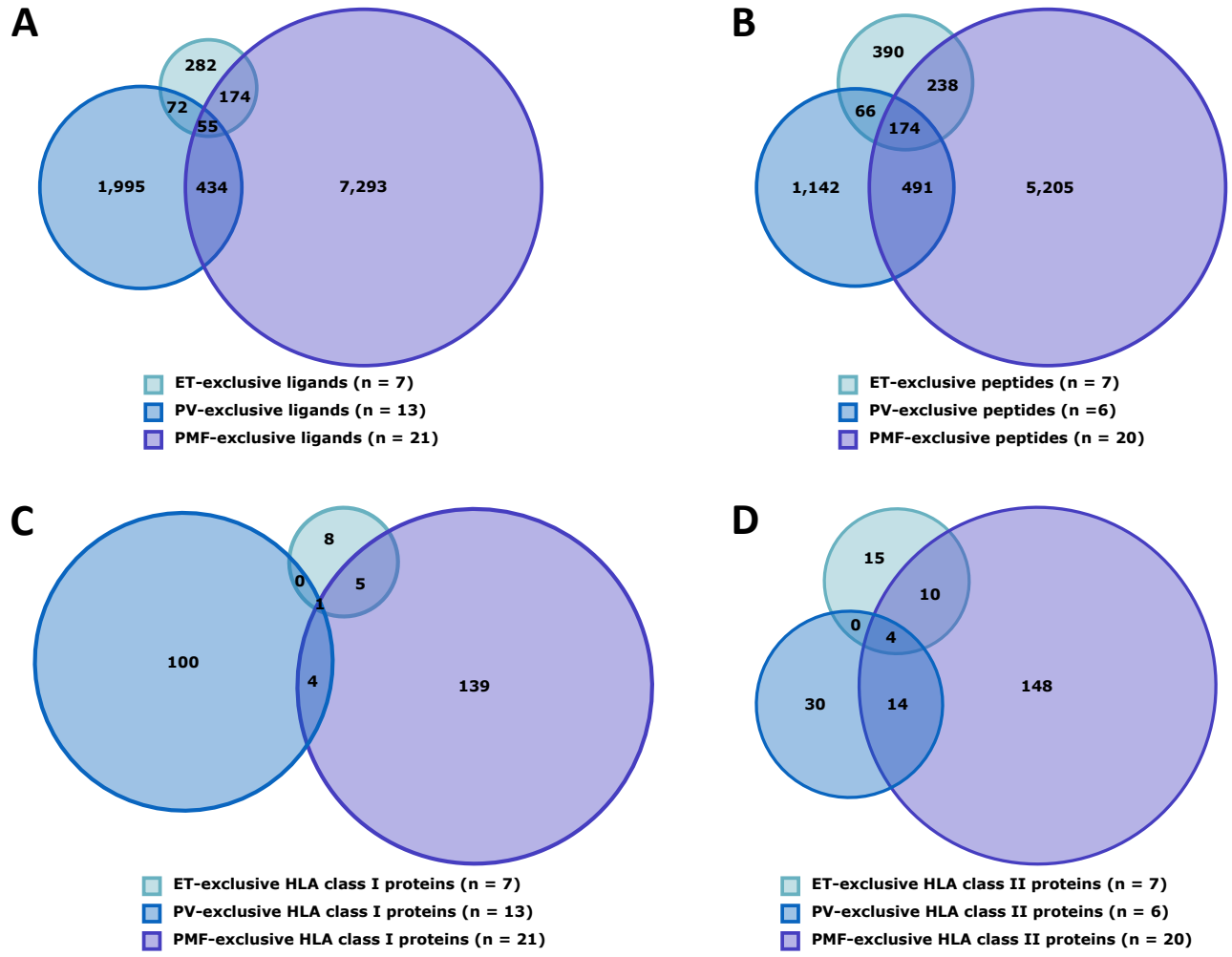

Figure S13: Overlap analysis of ET-, PV-, and PMF-exclusive peptides and source proteins. (A) Overlap analysis of ET-, PV-, and PMF-exclusive HLA class I ligands identified in samples from 7 ET, 13 PV, and 21 PMF patients. (B) Overlap analysis of ET-, PV-, and PMF-exclusive HLA class II peptides identified in samples from 7 ET, 6 PV, and 20 PMF patients. (C) Overlap analysis of ET-, PV-, and PMF-exclusive HLA class I ligand source proteins identified in samples from 7 ET, 13 PV, and 21 PMF patients. (D) Overlap analysis of ET-, PV-, and PMF-exclusive HLA class II peptide source proteins identified in samples from 7 ET, 6 PV, and 20 PMF patients. Created using BioVenn (Hulsen *et al.* 2008).

Supplemental Figure 14

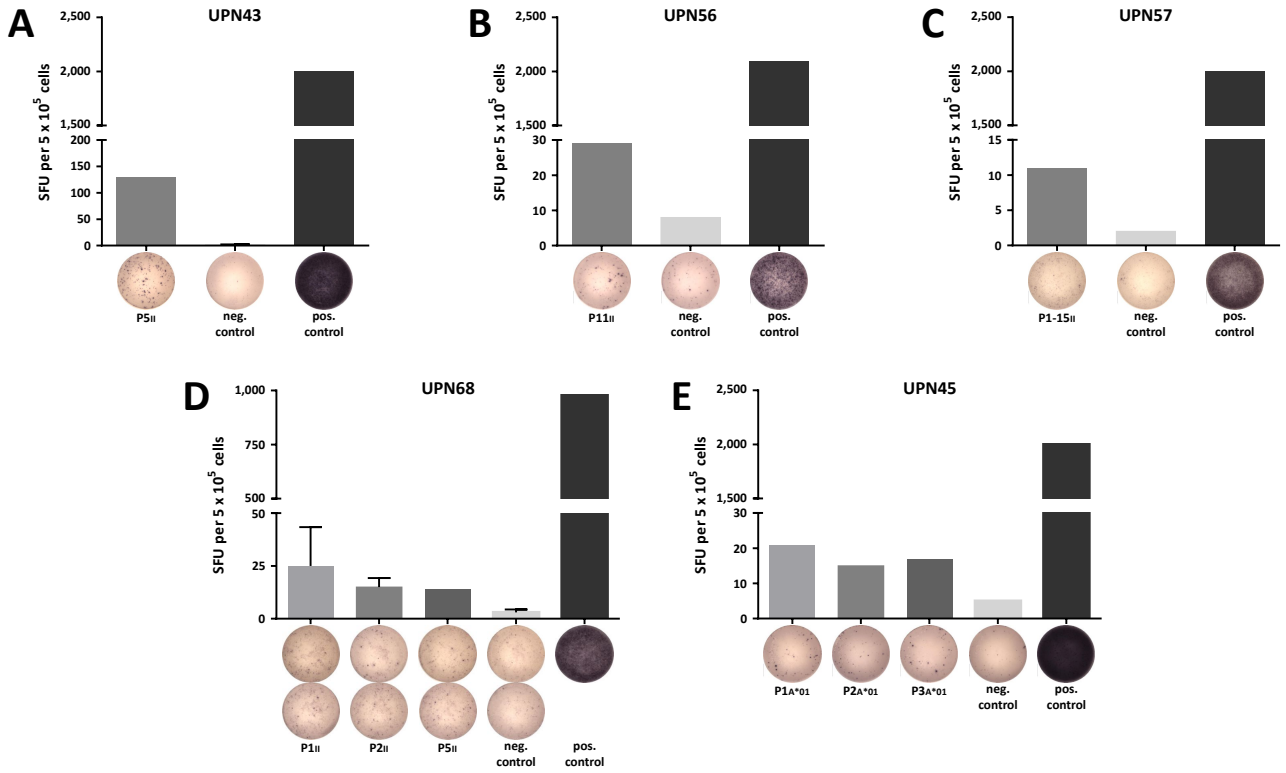

Figure S14: IFN-γ ELISPOT results of ET and PMF using AML-associated antigens. Exemplary IFN-γ ELISPOT results for AML-associated peptides after 12-day stimulation using PBMCs from ET or PMF patients. Results are shown for immunoreactive peptides only. Phytohemagglutinin was used as positive control. (A-D) The HLA class II–restricted FLNA_HUMAN$_{1669-1683}$ peptide ETVITVDTKAAGKGK served as negative control. (D) Data are expressed as mean $\pm$ standard deviation of 2 independent replicates. (E) The HLA-A*01–restricted GAG_HIV$_{71-79}$ peptide GSEELRSLY served as negative control. Abbreviations: UPN, uniform patient number; neg., negative; pos., positive; SFU, spot-forming unit.

Appendix - CHAPTER 3

Supplemental Table S1

Healthy volunteer ID	Mean frequency of P(BCMA)$_{B*18}$-specific CD8$^+$ T cells	Multifunctionality of peptide-specific T cells
HV01	0.76%	IFNγ^+ TNF$^+$ CD107a$^+$
HV02	0.71%	IFNγ^+ TNF$^+$ CD107a$^+$
HV03	0.40%	IFNγ^+ TNF$^+$ CD107a$^+$
HV04	0.22%	IFNγ^+ TNF$^+$ CD107a$^+$
HV05	2.01%	IFNγ^+ TNF$^+$ CD107a$^+$
HV06	0.99%	not tested
HV07	1.42%	not tested
HV08	0.74%	not tested
HV09	0.40%	not tested
HV10	0.42%	not tested

Supplemental Table 1: Results overview of T-cell experiments using cells of healthy volunteers. Results of T-cell-based assays using cells of HVs. Mean frequency of *de novo* induced P(BCMA)$_{B*18}$-specific CD8$^+$ T cells using *in vitro* aAPC-based priming experiments and assessment of cytokine production as well as CD107a expression using intracellular cytokine staining of peptide-specific cells. Abbreviations: ID, identification; HV, healthy volunteer.

Supplemental Table S2

Peptide ID	Gene name of source protein	Peptide sequence	Position	HLA restriction
P(BCMA)$_{B*18}$	BCMA	DEIILPRGL	111 - 119	B*18
P(negative)$_{B*18}$	ANM1	DEVRTLTY	69 - 76	B*18
P(positive)$_{B*18}$	MUC16	TETEAIHVF	9 507 - 9 515	B*18

Supplemental Table 2: Peptides used for T-cell experiments. HLA-B*18-restricted BCMA-derived peptides as well as positive and negative control peptides used for IFN-γ ELISPOT assays and aAPC-based *in vitro* priming experiments. Abbreviations: ID, identification; aAPC, artificial antigen-presenting cell.

Supplemental Table S3

UPN	Experiment	Sex	Age	ISS stage	Durie & Salmon	Cytogenetic risk	HLA class I typing
1	E, P	male	72	1	3A	unknown	A*03:01, A*33:01, B*14:02, B*18:01
2	E	male	74	1	2A	low-risk	A*24:02, A*66:02, B*15:09, B*18:01
3	E	female	73	3	3B	high-risk	A*02:01, A*11:01, B*15:01, B*18:01
4	E, P	male	53	3	3A	high-risk	A*03:01, A*11:01, B*18:01, B*35:01

Supplemental Table 3: Patient characteristics. Patients included in T-cell-based assays. Age is given in years at time of sample collection. Cytogenetic risk was assessed according to the International Myeloma Working Group (IMWG) risk stratification (Chng *et al.*, 2014, Leukemia). Abbreviations: UPN, uniform patient number; E, IFN-γ ELISPOT; P, aAPC-based priming; ISS, International Staging System.

Acknowledgements

A dissertation is not an endeavour of an individual alone: the author needs to draw on inspiration and support from his surroundings. I've experienced support of various sorts from many people around me, and here I would like to thank them for the past years and simply for everything!

First of all I have to thank my husband - my person - for the relentless support, for always believing in me, and for never giving up on me. For not once complaining about the countless night shifts and the countless weekends and holidays working in the lab. For listening to my sorrows and for always understanding. You have made it so easy and all of this would have been impossible without you being there for me!

I also thank my supervisors Hans-Georg Rammensee and Stefan Stevanović for giving me the opportunity to study and work in this amazing environment they provided. I felt at home the first time I've set foot in the lab. I thank Juliane Walz for taking me in, letting me become a part of her expanding group, and always taking precious time to help out with ideas and support.

I thank my colleagues that became my dearest friends: thank you Jules, Maren, and Ana for being my friends, for listening and helping, for all the sessions and girls nights and so much more!!! You have made the time in the lab the greatest.
I thank my colleagues of team Walz: Annika, Yacine, Jens, Jonas, and Malte for standing together and helping each other, for listening and supporting each other. Special thanks to Yacine for becoming a T-cell mom and supporting me so much.
The group of Stefan for always being there and supporting: Thank you Moni, Nici, Patricia, Ulli, Camille, Miri, and especially Marion for the chocolate brownies in times of despair.
From the group Gouttefangeas I thank Cécile, Sonja, Elisa, and Jenny for their expertise and for always being there whenever I was in need of help.
I also want to thank our technical support team, especially Beate, Claudia, and Franziska. You are the kind souls of the lab. Thank you for always helping out, for always having time to support and to listen, and for making the place better for all of us!
Not to forget I thank the alumni for taking me in as a student: Daniel ("D.K."), Heiko, and Janet. And of course I thank all the elks.

I thank my friends, especially my besty Anastasiya, her life partner Laurens and her dancing partner Jesper. Thank you guys for your constant support, belief, interest and your understanding! Also Kai, Miri, Magda, and the rest of my big dancing family. With you everything is simply better!

I thank my mom for making everything possible and my parents in law. Thank you for everything!

Nature is the source of all true knowledge. She has her own logic, her own laws, she has no effect without cause nor invention without necessity. **Leonardo da Vinci**

Science moves with the spirit of an adventure characterized both by youthful arrogance and by the belief that the truth, once found, would be simple as well as pretty. **James D. Watson**

The most beautiful thing we can experience is the mysterious. It is the source of all true art and science. **Albert Einstein**

Science is magic that works. **Kurt Vonnegut**

Everything is theoretically impossible, until it is done. **Robert A. Heinlein**