OncoImmunology

ISSN: (Print) 2162-402X (Online) Journal homepage: http://www.tandfonline.com/loi/koni20

HLA class I-restricted MYD88 L265P-derived peptides as specific targets for lymphoma immunotherapy

Annika Nelde, Juliane Sarah Walz, Daniel Johannes Kowalewski, Heiko Schuster, Olaf-Oliver Wolz, Janet Kerstin Peper, Yamel Cardona Gloria, Anton W. Langerak, Alice F. Muggen, Rainer Claus, Irina Bonzheim, Falko Fend, Helmut Rainer Salih, Lothar Kanz, Hans-Georg Rammensee, Stefan Stevanović & Alexander N. R. Weber

To cite this article: Annika Nelde, Juliane Sarah Walz, Daniel Johannes Kowalewski, Heiko Schuster, Olaf-Oliver Wolz, Janet Kerstin Peper, Yamel Cardona Gloria, Anton W. Langerak, Alice F. Muggen, Rainer Claus, Irina Bonzheim, Falko Fend, Helmut Rainer Salih, Lothar Kanz, Hans-Georg Rammensee, Stefan Stevanović & Alexander N. R. Weber (2017) HLA class I-restricted MYD88 L265P-derived peptides as specific targets for lymphoma immunotherapy, OncoImmunology, 6:3, e1219825, DOI: 10.1080/2162402X.2016.1219825

To link to this article: http://dx.doi.org/10.1080/2162402X.2016.1219825

© 2017 The Author(s). Published with license by Taylor & Francis Group, LLC© Annika Nelde, Juliane Sarah Walz, Daniel Johannes Kowalewski, Heiko Schuster, Olaf-Oliver Wolz, Janet Kerstin Peper, Yamel Cardona Gloria, Anton W. Langerak, Alice F. Muggen, Rainer Claus, Irina Bonzheim, Falko Fend, Helmut Rainer Salih, Lothar Kanz, Hans-Georg Rammensee, Stefan Stevanovic, and Alexander N. R. Weber

View supplementary material

Accepted author version posted online: 23 Dec 2016.
Published online: 23 Dec 2016.

Submit your article to this journal

Article views: 142

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=koni20

Download by: [Erasmus University]

Date: 07 April 2017, At: 06:17

ONCOIMMUNOLOGY
2017, VOL. 6, NO. 3, e1219825 (11 pages)
http://dx.doi.org/10.1080/2162402X.2016.1219825

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

OPEN ACCESS

HLA class I-restricted *MYD88* L265P-derived peptides as specific targets for lymphoma immunotherapy

Annika Nelde[a], Juliane Sarah Walz[b], Daniel Johannes Kowalewski[a], Heiko Schuster[a], Olaf-Oliver Wolz[a], Janet Kerstin Peper[a], Yamel Cardona Gloria[a], Anton W. Langerak[c], Alice F. Muggen[c], Rainer Claus[d], Irina Bonzheim[e], Falko Fend[e], Helmut Rainer Salih[b,f], Lothar Kanz[b], Hans-Georg Rammensee[a,g], Stefan Stevanović[a,g], and Alexander N. R. Weber[a]

[a]Department of Immunology, Institute for Cell Biology, University of Tübingen, Tübingen, Germany; [b]Department of Hematology and Oncology, University Hospital Tübingen, Tübingen, Germany; [c]Department of Immunology, Erasmus MC Rotterdam, Rotterdam, the Netherlands; [d]Department of Hematology, Oncology and Stem Cell Transplantation, University Medical Center Freiburg, Freiburg, Germany; [e]Department of Pathology, University Hospital Tübingen, Tübingen, Germany; [f]Clinical Cooperation Unit Translational Immunology, German Cancer Consortium (DKTK), DKFZ Partner Site Tübingen, Tübingen, Germany; [g]German Cancer Consortium (DKTK), DKFZ Partner Site Tübingen, Tübingen, Germany

ABSTRACT

Genome sequencing has uncovered an array of recurring somatic mutations in different non-Hodgkin lymphoma (NHL) subtypes. If affecting protein-coding regions, such mutations may yield mutation-derived peptides that may be presented by HLA class I proteins and recognized by cytotoxic T cells. A recurring somatic and oncogenic driver mutation of the Toll-like receptor adaptor protein *MYD88*, Leu265Pro (L265P) was identified in up to 90% of different NHL subtype patients. We therefore screened the potential of *MYD88*^{L265P}-derived peptides to elicit cytotoxic T cell responses as tumor-specific neoantigens. Based on *in silico* predictions, we identified potential *MYD88*^{L265P}-containing HLA ligands for several HLA class I restrictions. A set of HLA class I *MYD88*^{L265P}-derived ligands elicited specific cytotoxic T cell responses for HLA-B*07 and -B*15. These data highlight the potential of *MYD88*^{L265P} mutation-specific peptide-based immunotherapy as a novel personalized treatment approach for patients with *MYD88*$^{L265P+}$ NHLs that may complement pharmacological approaches targeting oncogenic MyD88 L265P signaling.

Abbreviations: aAPC, artificial antigen-presenting cell; APC, antigen-presenting cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; CIP, cancer immunoguiding program; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; E/T, effector to target ratio; FAM, fluorescein; Fmoc/tBu, 9-fluorenylmethyl-oxycarbonyl/tert-butyl; HBDs, healthy blood donors; HIV, human immunodeficiency virus; IOL, intraocular lymphoma; L265P, Leu265Pro; LPL, lymphoplasmacytic lymphoma; NHL, non-Hodgkin lymphoma; PBMCs, peripheral blood mononuclear cells; PCNHL, primary cerebral non-Hodgkin's lymphoma; PHA, phytohaemagglutinin; TLR, Toll-like receptor; WT, wild-type (i.e., non-mutated sequence); YAK, yakima yellow

ARTICLE HISTORY
Received 1 April 2016
Revised 25 July 2016
Accepted 28 July 2016

KEYWORDS
Cancer vaccine; HLA ligandome; immunotherapy; MyD88 L265P; neoantigen; non-Hodgkin lymphoma; peptide vaccination

Introduction

The immune system can recognize and to some extent eradicate tumor cells.[1,2] Nevertheless, this antitumor response is often inefficient.[3] Antigen-specific immunotherapy holds the potential to induce and boost clinically effective anticancer T cell responses[4] and might be used to guide and increase the specificity of cancer immunotherapy in future combination trials,[5] especially when combined with newly available immune checkpoint inhibitors.[6] For this purpose, the exact knowledge of tumor-associated or tumor-specific immunogenic T cell epitopes is crucial. Tumor-specific neoepitopes, derived from protein-altering mutational events like missense mutations, may be perceived as foreign by the immune system and elicit tumor-specific T cell immunity. [7,8]

Being tumor-specific, such neoantigens promise high specificity but are largely patient-specific, and therefore, hard to identify and mainly singular events in a patient cohort. Recurrent mutations could overcome this problem of patient-specificity and could be targeted in broadly applicable immunotherapeutic treatments of different types of cancer.

A recurring somatic and oncogenic driver mutation of the Toll-like receptor (TLR) adaptor protein *MYD88*, Leu265Pro (L265P) was identified in up to 90% of certain non-Hodgkin lymphoma (NHL) subtypes.[9-15] NHLs like diffuse large B cell lymphoma (DLBCL) or chronic lymphocytic leukemia (CLL) are the cause of death for more than 199,000 patients annually world-wide.[16] The success of established therapies varies strongly between these diseases. Whereas other DLBCL subtypes can be

CONTACT Alexander N. R. Weber alexander.weber@uni-tuebingen.de Institute for Cell Biology, Department. of Immunology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany.

Supplemental data for this article can be accessed on the publisher's website.

Published with license by Taylor & Francis Group, LLC © Annika Nelde, Juliane Sarah Walz, Daniel Johannes Kowalewski, Heiko Schuster, Olaf-Oliver Wolz, Janet Kerstin Peper, Yamel Cardona Gloria, Anton W. Langerak, Alice F. Muggen, Rainer Claus, Irina Bonzheim, Falko Fend, Helmut Rainer Salih, Lothar Kanz, Hans-Georg Rammensee, Stefan Stevanovic, and Alexander N. R. Weber. This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

effectively treated, the activated B cell-like lymphoma subtype, for example, is characterized by a less than 40% overall survival following standard therapy.[17] For CLL, there is currently no curative treatment and for some patients aggressive chemotherapies or allogenic stem cell transplantations are required.[18] Therefore, novel treatments are needed to further improve clinical outcome in these patients. Immunotherapy based on peptide-vaccines or adoptive immune cell transfers might represent ideal treatments, since they are well tolerated and have fewer side effects than chemotherapeutic agents. Growing evidence demonstrates that therapeutic peptide-vaccines can induce specific immune responses, impact clinical outcome[19] and even prolong overall survival in cancer patients.[4] Besides, peptide-vaccines experimental cancer immunotherapy using adoptive transfer of antigen-specific or genetically engineered T cells[20,21] as well as antigen-presenting dendritic cells[22] showed also high efficiency in different studies. Furthermore, vaccines may potentially yield a sustained antitumor effect by inducing immunological memory.[23] Thus, the identification of cancer-associated and cancer-specific antigens offers a great variety of treatment possibilities in cancer patients.

As *MYD88*^{L265P} is a widely occurring and tumor-specific mutation, *MYD88*^{L265P}-based immunotherapy might constitute an ideal alternative or complementation to ongoing attempts to target this mutation pharmacologically[24] in NHLs. We, therefore, screened the potential of *MYD88*^{L265P}-containing peptides for CD8^{+} T cell-mediated immunotherapy and identified potential HLA ligands encompassing the *MYD88*^{L265P} mutation for several HLA class I restrictions based on *in silico* predictions. We focused on three HLA-B*07-restricted peptides and one HLA-B*15-restricted peptide to examine the immunogenicity of these tumor-specific neoantigens. The present study shows that *MYD88*^{L265P}-derived peptides can induce mutation-specific and functional immune responses *in vitro*, which may pave the way for developing new personalized immunotherapeutic strategies with broad applicability for different NHLs.

Results

In silico prediction yields several likely MYD88^{L265P}-derived HLA class I ligands

We identified 23 different *MYD88*^{L265P}-derived peptides predicted by SYFPEITHI and/or NetMHC 3.4 to bind one or more HLA class I allotypes with a score of $IC_{50} \leq$ 500 nM for NetMHC 3.4 or with $\geq$ 50% of the maximum allotype-specific score for SYFPEITHI. These algorithms predicted 8, 15, and 8 peptides restricted by 5, 17 and 5 different HLA-A, -B, and -C allotypes, respectively (Table S2). Notably, only four ligands were concordantly designated as ligands of the same HLA allotype by both algorithms (Fig. 1). This is mainly due to a different set of available HLA allotypes for the two algorithms (e.g., SYFPEITHI cannot predict 12mer peptides or HLA-B*30:01-restricted peptides).[25-29] For effective, on-target immunotherapy the identification of immunogenic tumor-specific antigens that do not elicit cross-reactivity with benign tissue is of paramount importance.[30] Therefore, we aimed at identifying mutated peptides with increased binding affinities compared with their non-mutated counterparts. This was realized by predicting the score for every potential mutation-derived ligand,

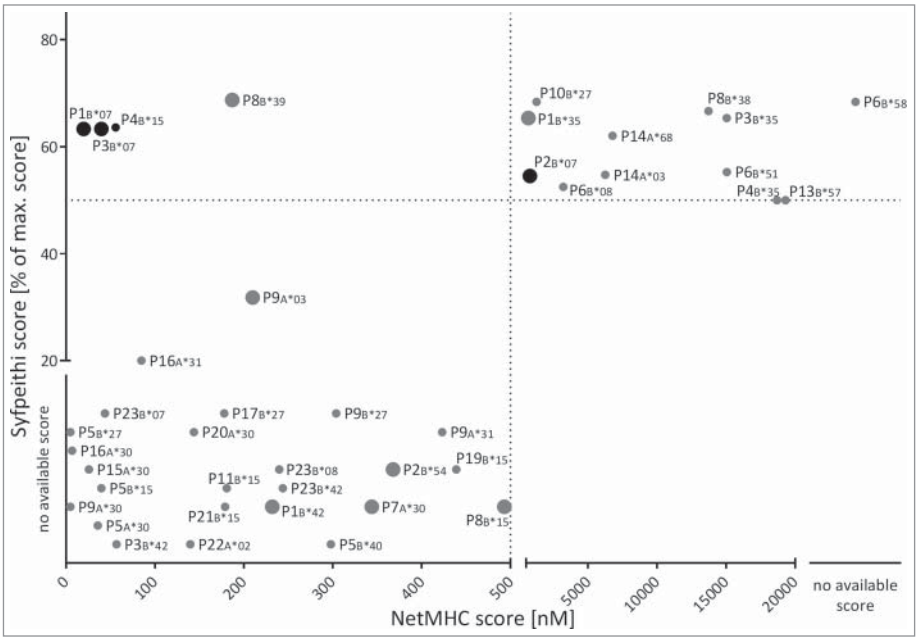

Figure 1. Prediction of MYD88^{L265P}-derived HLA class I ligands. 50 *MYD88*^{L265P}-derived 8- to 12-mer peptides were scored by the online prediction tools SYFPEITHI and NetMHC 3.4, as well as an extended in-house database. Peptides with NetMHC 3.4 $IC_{50} \leq$ 500 nM were defined as binders (region left of the dotted line). SYFPEITHI scores are displayed as percent of the maximum score for the respective HLA allotype. The threshold for binders is defined as $\geq$ 50% of the maximum score (above the dotted line). For some HLA:peptide combinations scoring was possible with only one of the prediction tools due to limited availability of predictors. The figure illustrates the predicted ligands for HLA-A and -B allotypes. Fold-change ratios in binding scores of mutated peptides compared with the corresponding WT peptides are indicated by the size of the respective dot: large dots indicate an at least 2-fold better score, mutated ligands illustrated by small dots exhibit no increased binding score in comparison to their corresponding WT ligand. Out of 50 unique peptide sequences, 23 were scored as potential HLA class I ligands. Four ligands were concordantly designated as ligands of the same HLA allotype by both algorithms with three of them having an at least 2-fold higher score as their corresponding WT peptide. The black dots indicate the peptides which were tested in aAPC-based *in vitro* primings in HBDs or CLL patients. Abbreviations: max., maximal.

as well as the score for the corresponding WT peptide on the same HLA class I allotype. We highlighted 16 of the 52 predicted mutated peptide:HLA combinations, which have a more than 2-fold higher predicted binding affinity compared with their non-mutated corresponding peptides. Among these, we identified two *MYD88*^{L265P}-derived gain-of-anchor antigens mutated in their P2-anchor position. These two peptides (P1 and P2) were therefore preferentially selected for further characterization and immunogenicity testing. In total, all 18 8–11mer peptides predicted as potential ligands for HLA class I were synthesized and subjected to functional characterization. For 13/18 predicted HLA class I ligands PBMCs of HLA-matched *MYD88*^{L265P}-mutated NHL patients were available and could therefore be tested in IFNγ ELISPOT assays for spontaneous memory T cell responses. To verify the ability of the predicted HLA ligands to indeed bind to the respective HLA class I allotypes, 10 of the predicted peptides were analyzed in *in vitro* monomer refolding assays. The peptides for the refolding experiments were selected according to their predicted binding score as well as available HLA molecules. Four peptide:HLA complexes ($P1_{B*07}$, $P2_{B*07}$, $P3_{B*07}$, and $P4_{B*15}$) were refolded successfully *in vitro* (Table S2). The successfully refolded HLA–peptide complexes were all used in experiments.

Spontaneous memory T cell responses targeting *MYD88*^{L265P}-derived peptides are very infrequent in NHL patients

Functional characterization of the predicted candidate HLA class I *MYD88*^{L265P}-derived NHL-specific ligands was performed by 12-d recall IFNγ ELISPOT assays using PBMCs obtained from *MYD88*^{L265P}-mutated and *MYD88*^{WT} patients (Table S1). In one (out of 22 tested) *MYD88*^{L265P}-mutated NHL patients, memory T cell responses targeting two different *MYD88*^{L265P}-derived HLA class I ligands were detected by IFNγ ELISPOT (Fig. 2). Importantly, one of the peptides (P5) is a predicted ligand for both, HLA-B*15 and -B*40, which are both expressed by the patient. Therefore, we could not resolve which peptide:HLA combination is responsible for the observed IFNγ secretion. Importantly, no IFNγ secretion was observed for any tested ligand in *MYD88*^{WT} patients. The frequency of memory T cell responses in 1/22 *MYD88*^{L265P}-mutated NHL patients appears low, but it is important to be aware of the HLA allotype-specific frequencies. The peptide P1, which is among others analyzed in further immunogenicity experiments, leads to a detectable memory T cell response in 1/3 (33%) tested HLA-matched *MYD88*^{L265P}-mutated NHL patients. For the other positive peptide P5 memory T cell responses could be detected in 1/4 (25%) HLA-B*15 patients and in 1/3 (33%) HLA-B*40 patients. Furthermore, only two-digit HLA typings were available for the NHL patients. Therefore, it is possible that a negative response is due to a different four-digit HLA restriction of the tested peptide and the HLA type of the patient. Table S3 summarizes all tested *MYD88*^{L265P}-derived HLA class I ligands.

Generation of *MYD88*^{L265P}-specific T cells in vitro from naive T cells of CLL patients and HBDs

To assess whether *MYD88*^{L265P}-derived peptide-specific T cell responses can be induced from naive T cells *in vitro*, we isolated CD8$^+$ T cells from six HLA-B*07$^+$ and three HLA-B*15$^+$ HBDs as well as from two HLA-B*07$^+$ *MYD88*^{WT} CLL patients. We performed artificial antigen-presenting cell (aAPC)-based *in vitro*

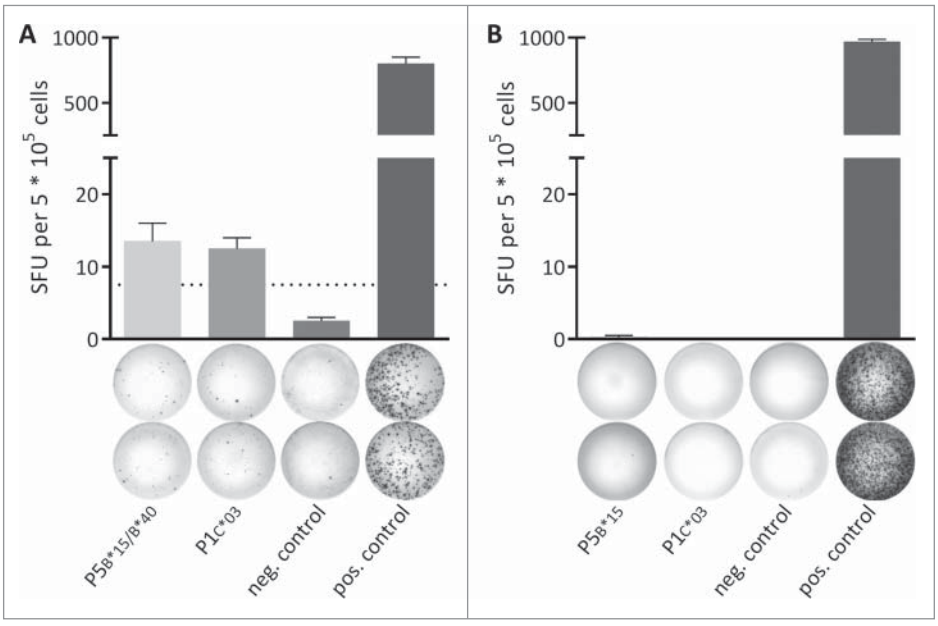

Figure 2. Spontaneous memory T cell responses are detectable in a leukemia patient. The presence of memory T cell responses in leukemia and lymphoma patients was analyzed using 12-d recall IFNγ ELISPOT assays. (A) In a single (out of 22 tested) *MYD88*$^{L265P+}$ NHL patients (CLL-05-R) IFNγ secretion was observed after stimulation with the *MYD88*^{L265P}-derived peptides $P5_{B*15/B*40}$ (HQKRPIPI) and $P1_{C*03}$ (RPIPIKYKAM). (B) Representative example of a *MYD88*$^{L265P+}$ patient (CLL-03-R) where no IFNγ secretion was observed after stimulation with the *MYD88*^{L265P}-derived peptides $P5_{B*15}$ (HQKRPIPI) and $P1_{C*03}$ (RPIPIKYKAM). An EBV epitope mix containing the frequently recognized peptides BRLF1 109–117 YVLDHLIVV (HLA-A*02) and EBNA3 247–255 RPPIFIRRL (HLA-B*07) served as positive control. Benign-tissue derived peptide DDX5 YLLPAIVHI (HLA-A*02) served as negative control. The dotted line indicates the 3-fold number of spot forming unit of the negative control. Error bars indicate ± SEM of two independent replicates. Abbreviations: SFU, spot forming unit; neg., negative; pos., positive; SEM, standard error of the mean.

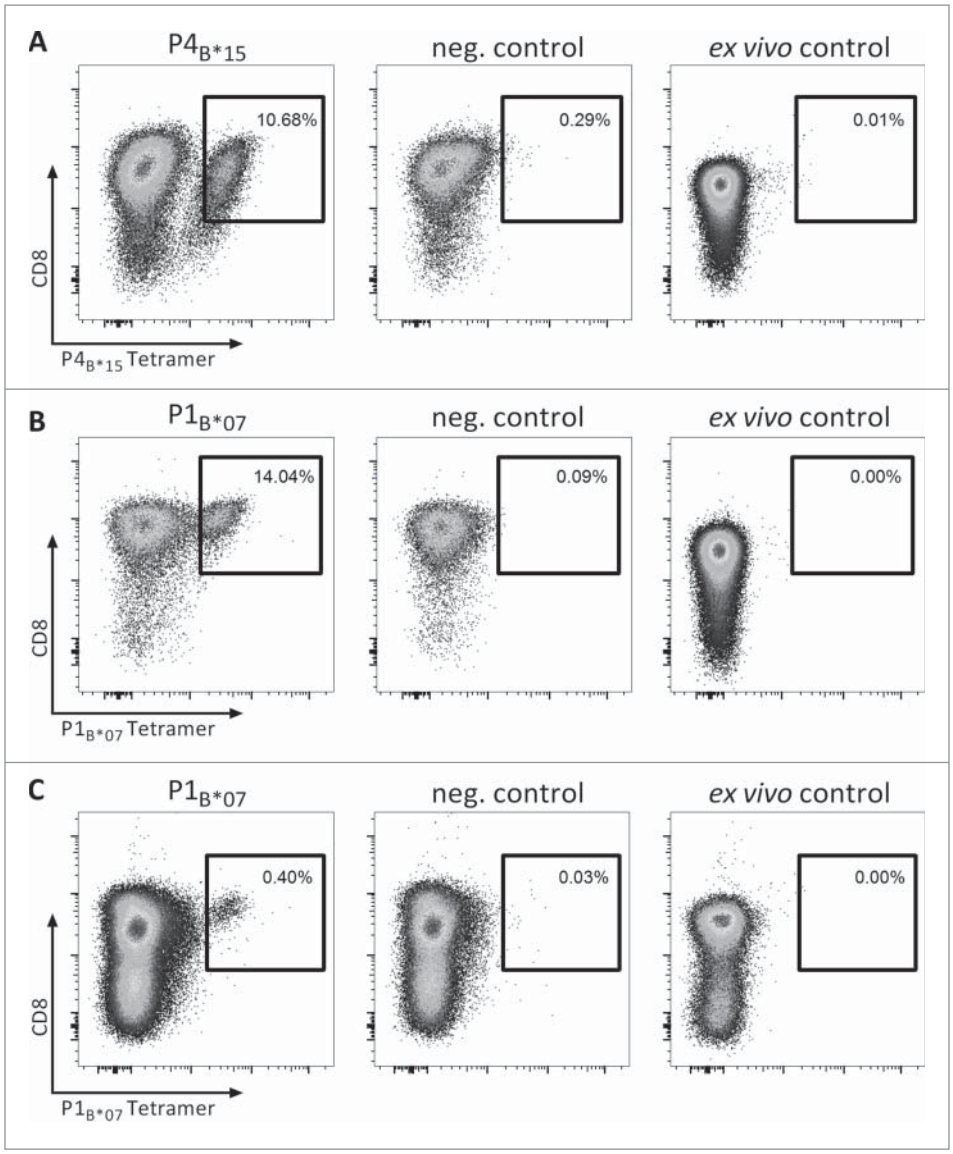

Figure 3. Efficient *in vitro* generation of $P4_{B*15}$- and $P1_{B*07}$-specific $CD8^+$ T cells from naive T cells of CLL patients and HBDs. Representative tetramer stainings of $CD8^+$ T cells after three cycles of aAPC-based *in vitro* priming using $CD8^+$ T cells derived from HLA-matched HBDs primed with (A) the HLA-B*15-restricted peptide HQKRPIPIKY ($P4_{B*15}$) and (B) the HLA-B*07-restricted peptide RPIPIKYKAM ($P1_{B*07}$) as well as from HLA-matched *MYD88*^{WT} CLL patient (CLL-05) primed with (C) the HLA-B*07-restricted peptide RPIPIKYKAM ($P1_{B*07}$): 1st column: tetramer staining of $CD8^+$ T cells primed with the *MYD88*^{L265P}-derived peptide; 2nd column: control staining with HLA-matched tetramer containing a non-relevant control peptide on $CD8^+$ T cells derived from the same population as T cells depicted in the 1st column; 3rd column: *ex vivo* tetramer staining of $CD8^+$ T cells. *In vitro* primings with HBD-derived PBMCs were performed in six ($P1_{B*07}$) and three ($P4_{B*15}$) independent replicates, respectively. For the *in vitro* priming with PBMCs of CLL patients two independent replicates were conducted. Abbreviations: neg., negative.

priming using the three HLA-B*07-restricted *MYD88*^{L265P}-derived peptides RPIPIKYKAM ($P1_{B*07}$), RPIPIKYKA ($P2_{B*07}$) and SPGAHQKRPI ($P3_{B*07}$) as well as the HLA-B*15-restricted peptide HQKRPIPIKY ($P4_{B*15}$). Using HBD-derived $CD8^+$ T cells, $P4_{B*15}$-tetramer-positive $CD8^+$ populations with frequencies of 1.50–10.68% of viable cells were detected in 2/3 HBDs after priming (Fig. 3A). For the HLA-B*07-restricted peptide $P1_{B*07}$, we observed tetramer-positive $CD8^+$ populations with frequencies of 0.47–14.04% of viable cells in 6/6 HBDs (Fig. 3B). For $P2_{B*07}$, 1/3 (33%) HBDs showed a tetramer-positive $CD8^+$ population with a frequency of 0.15% of viable cells (Fig. S1A). $P3_{B*07}$-tetramer positive $CD8^+$ populations with frequencies of 0.20–1.56% of viable cells were detected in 3/4 HBDs (Fig. S1B). Notably, after aAPC-based *in vitro* priming of $CD8^+$ T cells from *MYD88*^{WT} B*07$^+$ CLL patients without previous T cell reactivity for $P1_{B*07}$ (as detected by 12-day recall IFNγ ELISPOT assay and *ex vivo* tetramer staining), we observed in 1/2 patients a population of 0.40% $P1_{B*07}$-specific $CD8^+$ T cells within the viable cells (Fig. 3C). No tetramer-positive T cell populations >0.10% (>0.50%) were detectable in control stainings with an HLA-B*07 (HLA-B*15)-tetramer containing a control peptide. In *ex vivo* control stainings no tetramer-positive T cell populations >0.01% were detectable. Furthermore, Peper et al.[31] demonstrated that T cell responses observed after three rounds of aAPC-based stimulations were mediated by *in vitro* primed naive T cells rather than by pre-existing memory T cells, as short-time stimulation of the same PBMC did not result in the detection of specific T cell populations. Collectively, all 4/4 (100%) refolded *MYD88*^{L265P}-derived peptides thus are able to efficiently prime mutation-specific T cells in certain HLA contexts in HBDs as well as in CLL patients.

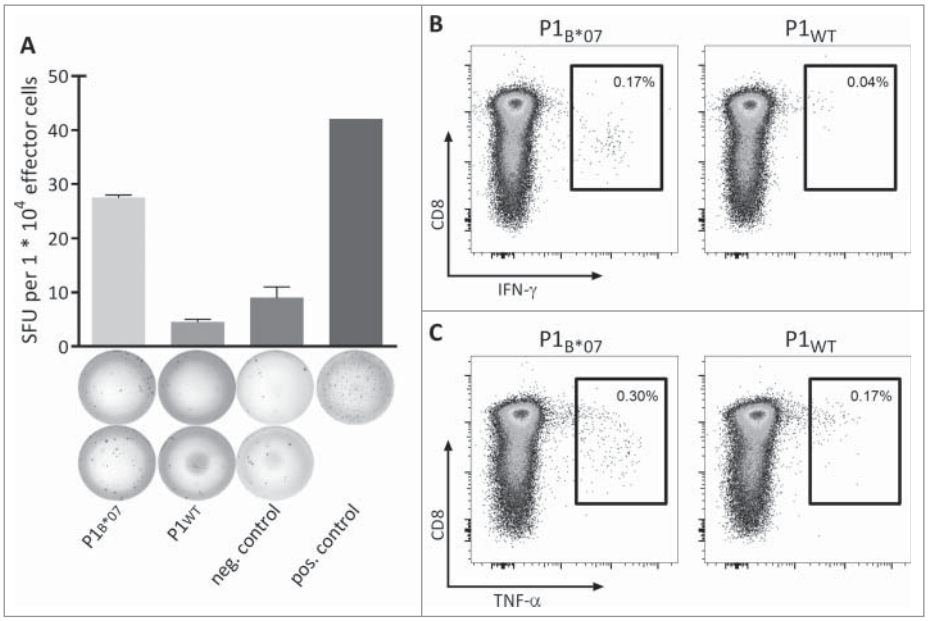

Figure 4. Functionality and specificity of *MYD88*^{L265P}-specific T cells. Functionality and specificity of *MYD88*^{L265P}-specific CD8$^+$ T cells were analyzed by (A) IFNγ ELISPOT assay or (B, C) intracellular cytokine staining. Both assays showed increased production of IFNγ or TNFα after stimulation with the mutation-derived peptide ($P1_{B^*07}$) in comparison with the corresponding WT peptide ($P1_{WT}$). Representative examples of two different donors are shown. The frequency of $P1_{B^*07}$-specific CD8$^+$ T cell populations was 2.69% (A) and 0.40% (B and C), respectively, as detected by tetramer staining (not shown). Error bars indicate ± SEM of two independent replicates. Abbreviations: SFU, spot forming unit; neg., negative; pos., positive; SEM, standard error of the mean.

MYD88^{L265P}*-specific T cells selectively recognize the mutated epitopes and are multi-functional*

To assess the functional potential of *MYD88*^{L265P}-specific CD8$^+$ T cells, the peptide-specific secretion of IFNγ and TNFα as well as the expression of the degranulation marker CD107a were analyzed after stimulation with mutation-derived peptides in comparison to the corresponding WT peptides. $P1_{B^*07}$-specific CD8$^+$ T cells of three different HBDs primed *in vitro* with aAPCs secreted IFNγ after stimulation with the peptide $P1_{B^*07}$ but not after stimulation with the corresponding WT peptide, as detected by IFNγ ELISPOT assay (Fig. 4A) or intracellular cytokine staining (Fig. 4B). $P1_{B^*07}$-specific CD8$^+$ T cells of two tested HBDs also showed an increased TNFα secretion in response to the mutation-derived peptide, but not in response to the corresponding WT peptide (Fig. 4C). Moreover, the $P1_{B^*07}$-specific CD8$^+$ T cells of 1/2 donors expressed the degranulation marker CD107a after stimulation with the peptide $P1_{B^*07}$ (data not shown). $P4_{B^*15}$-specific CD8$^+$ T cells showed IFNγ as well as TNFα secretion after stimulation with the peptide $P4_{B^*15}$ but not after stimulation with the respective WT peptide (data not shown).

To investigate the possibility that the observed T cell responses against the mutated *MYD88*^{L265P} peptides resulted from molecular mimicry, the *MYD88*^{L265P}-derived peptides were compared with proteins from microorganisms and viruses. Sequence homology screens against prokaryotic and virus protein sequences (using Smith–Waterman protein searcher), identified no homologous sequences for the peptides P1 (RPIPIKYKAM), P2 (RPIPIKYKA), P3 (SPGAHQKRPI) and P4 (HQKRPIPIKY). Thus indicating that the detected responses are not the result of cross-reacting microorganism- or virus-specific T cells.

MYD88^{L265P}*-specific CD8$^+$ T cells elicit mutation-restricted cytotoxicity*

To examine peptide recognition and antigen-specific cell lysis of $P1_{B^*07}$- and $P4_{B^*15}$-stimulated CD8$^+$ T cells, cytotoxicity assays (VITAL assays) with *in vitro* primed effector cells of HBDs were performed. The effector cells were polyclonal cell populations with 0.12% and 0.74% frequencies of $P1_{B^*07}$- and $P4_{B^*15}$-specific CD8$^+$ T cells, respectively (Fig. 5A, Fig. S2A). $P4_{B^*15}$-specific CD8$^+$ T cells showed 17.9% (±1.2%) *MYD88*^{L265P}-peptide-specific significant cell killing at an effector to target ratio (E/T) of 1:1 compared with 2.6% (±1.2%) of non-specific cell lysis of unspecific effector cells against the same targets in three independent replicates, respectively. The specific lysis showed E/T ratio dependent characteristics with specific lysis decreasing with reduced E/T ratios (Fig. 5C). $P1_{B^*07}$-specific CD8$^+$ T cells specifically killed 11.4% (±1.7%) of *MYD88*^{L265P}-loaded targets at an E/T ratio of 0.7:1 in comparison to 2.1% unspecific lysis of unspecific effector cells (Fig. S2C). These results demonstrated clearly *MYD88*^{L265P}-specificity and cytolytic potential of the *in vitro* primed CD8$^+$ T cells.

Discussion

T cell based immunotherapy combined with immune checkpoint modulation has enabled new treatment possibilities for a range of solid tumors.[32-35] Furthermore, the clinical investigation of T cell based immunotherapy for hematological malignancies has made significant progress over the past years.[36,37] Specific anticancer immune responses could be improved and guided further by antigen-specific immunotherapy. To this end, the identification and exact knowledge of immunogenic tumor-specific as well as tumor-associated T cell epitopes is essential.[8,38] In NHL, a multitude of

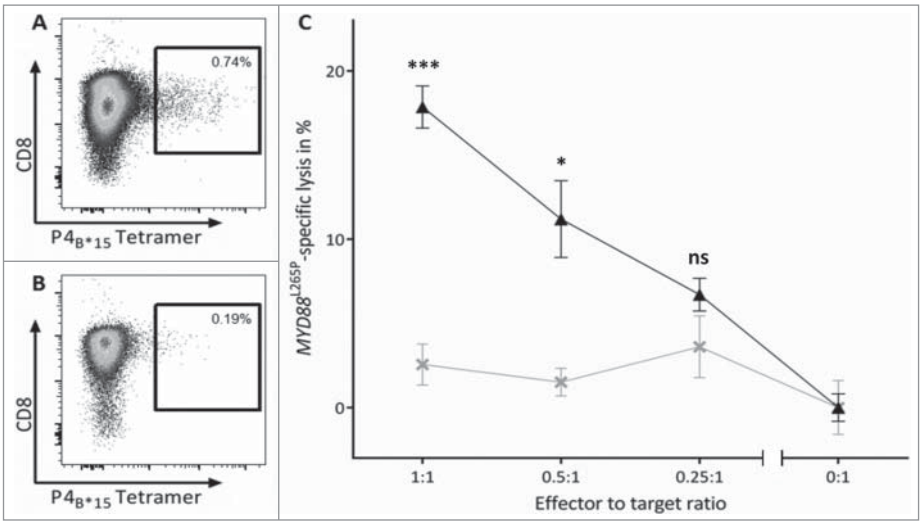

Figure 5. *MYD88*^{L265P}-selective cytotoxicity of $P4_{B*15}$-specific effector cells. The *MYD88*^{L265P}-specific cytotoxicity was analyzed in a VITAL cytotoxicity assay with CD8$^+$ effector cells of *in vitro* primed cells of HBDs. (A, B) Tetramer staining of polyclonal effector cells one day before the VITAL assay determined the number of $P4_{B*15}$-specific effector cells in the (A) population of successfully $P4_{B*15}$-primed CD8$^+$ T cells and in the (B) population of control cells primed with a HLA-matched non-relevant peptide. These control cells were used as unspecific effectors for the determination of the unspecific lysis of target cells. (C) At an effector to target ratio of 1:1 $P4_{B*15}$-specific effectors (▲) exerted 17.9% (±1.2%) *MYD88*^{L265P}-specific and significant higher lysis of $P4_{B*15}$-loaded autologous target cells in comparison to $P4_{WT}$-loaded cells. $P4_{B*15}$-unspecific effectors (×) only caused 2.6% (±1.2%) unspecific lysis of the same targets. Results are shown for three independent replicates. Error bars indicate ± SEM. Abbreviations: SEM, standard error of the mean; n.s., not significant; * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$.

studies have examined tumor-associated antigens, and identified an array of promising targets.[39-41] Tumor-specific neoantigens, which are derived from protein-altering mutational events, are being viewed as the most attractive targets of T cell based immunotherapy because they may be recognized as foreign by the immune system and can indeed provoke tumor-specific T cell immunity. [7,8] Mutations in the nucleophosmin 1 gene, which are among the most common molecular alterations in acute myeloid leukemia, give rise to immunogenic peptides, which elicit spontaneous T cell responses in patients.[42] Furthermore, patients with immune responses to these peptides showed an improved overall survival.[43] For these reasons, the identification and characterization of further mutation-derived HLA ligands for T cell based immunotherapy is of great interest. The recurrent oncogenic driver mutation of the TLR adaptor protein *MYD88*, Leu265Pro (L265P) fulfills the criteria of being tumor-specific yet widely occurring with up to 90% frequency of different NHL subtype patients.[9-15] In the present study, we therefore screened peptides containing the L265P mutation (*MYD88*^{L265P}) for immunogenicity and thus as potential tools for tumor-specific immunotherapy.

In a reverse immunological approach we predicted potential *MYD88*^{L265P}-derived HLA class I ligands. Two of these peptides emerged as gain-of-anchor antigens carrying the mutation in their P2-anchor position. Based on other studies, such peptides have been proposed to be especially suited for immunotherapy since their WT counterpart would not be bound by the same HLA molecule.[30]

Out of 22 tested *MYD88*^{L265P}-mutated NHL patients, only one showed a weak preexisting immune response against *MYD88*^{L265P}-derived epitopes. We analyzed all predicted binders on every HLA-matched *MYD88*^{L265P}-mutated NHL patient matching only the two digit typing of patient and prediction to maximize the number of possible tests and to account for the inaccuracy of the predictions. Therefore, it is possible that a negative result in the IFNγ ELISPOT could be due to imprecise match with the four digit typing of the predictions and tested patients. However, the allotype-specific frequencies (25–33%) for detectable memory T cell responses in *MYD88*^{L265P}-mutated NHL patients are comparable to frequencies described in previous studies (CLL, Myeloma). Furthermore, in DLBCL, for example, mutations of genes controlling the immune recognition by T cells have been described.[13] However, the overall survival was better in patients carrying the *MYD88* mutation suggesting that *MYD88*-directed immune responses could be involved in tumor rejection.[44] Although this awaits formal proof, the observed recall response could be taken as an indication that L265P peptides can be naturally presented. Additionally, *in vitro* aAPC-based priming in HBDs and CLL patients showed clearly that a T cell response against *MYD88*^{L265P}-derived peptides could be effectively induced from naïve T cells. The strong immunogenicity of the mutation-derived peptides $P1_{B*07}$ and $P4_{B*15}$, as well as the mutation-specificity of the peptide-specific CD8$^+$ T cells was demonstrated by IFNγ and TNFα secretion, expression of the degranulation marker CD107a, and the specific cytotoxic activities of the effector cells. These results demonstrated clearly that the gain-of-function driver mutation[12] *MYD88*^{L265P} could be highly immunogenic. We present evidence that peptide-loaded target cells can be killed by *MYD88*^{L265P} peptide-primed T cells with an efficiency similar to other published studies.[42,45,46] Nevertheless future studies outside the scope of this screening approach should investigate whether killing of cells naturally expressing WT vs. L265P-mutated *MYD88* is as effective. Should this indeed be the case, *MYD88*^{L265P}-based peptide vaccination, alone or in combination with checkpoint inhibitors or additional immunogenic peptides, could maybe be used to induce or boost effective antitumor immune responses; as a complementation to current approaches to target *MYD88*^{L265P}-mutated NHL pharmacologically at the level of *MyD88* dimerization[47] or downstream Interleukin-1 receptor-associated kinase, Bruton's tyrosine kinase or TAK1.[48] This could either be done by vaccination of the patient using *MYD88*^{L265P}-derived peptides or *MyD88*^{L265P}-encoding RNA;[49] alternatively, for

example if priming in the patient is suboptimal, naïve T cells could be isolated, primed and expanded *ex vivo* and re-administered to the patient; third, the transduction of non-specific T cells with TCR α and β chains conferring specificity [50] for $MYD88^{L265P}$ peptide: HLA complexes could be envisaged. Further investigations will be necessary to fully explore and harness this potential.

In conclusion, our study shows that $MYD88^{L265P}$ can elicit specific $CD8^+$ T cell responses and therefore may emerge as a promising target for tumor-specific immunotherapy in a variety of different NHL subtypes.

Materials and methods

Patients and blood samples

CLL, DLBCL, lymphoplasmacytic lymphoma (LPL), primary cerebral NHL (PCNHL) or intraocular lymphoma (IOL) patients were recruited at the Erasmus Medical Center Rotterdam, the Department of Hematology, Oncology and Stem Cell Transplantation of the University Medical Center Freiburg, the Department of Hematology and Oncology of the University Hospital Tübingen as well as at the University Eye Hospital Tübingen in collaboration with the Department of Pathology, Tübingen. Informed written consent was obtained in accordance with the Declaration of Helsinki protocol. The study was performed according to the guidelines of and approval by the local ethics committees (373/2011BO2). Patient characteristics are provided in Table S1. Peripheral blood mononuclear cells (PBMCs) from patients as well as PBMCs from healthy blood donors (HBDs) were isolated by density gradient centrifugation (Biocoll, Biochrom GmbH, L 6113). PBMCs from patients were cryopreserved and stored at -80°C until analysis in ELISPOT assays. HBDs were recruited at the University Hospital Tübingen Blood transfusion unit and respective whole blood obtained from blood donations.

$MYD88^{L265P}$ genotyping of cancer cells and HLA typing of patients

For the identification of $MYD88^{L265P}$-mutated CLL patients, DNA of PBMCs was isolated with the QIAamp DNA Mini Kit (Qiagen, 51304). Each 5 μl reaction consisted of 2.5 μl of TaqMan Universal Master Mix II (life technologies, 4440047), oligonucleotides (0.5 μM of each primer GCAGACAGTGATGAACCTCAGGA and AAGGGCCTGATGCCAGC) from TIB MOLBIOL, 0.25 μM of each probe (YAK-AGCGACCGATCCCCATCA-Q and FAM-AGCGACTGATCCCCATCAAGT-Q) and 10 ng of sample DNA. The probes were labeled with the fluorescent dyes yakima yellow (YAK) and fluorescein (FAM). The PCR was performed in a QuantStudio 7 Flex instrument (life technologies, Carlsbad, California, USA) with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. In a cohort of n = 456 CLL patients, $MYD88^{L265P}$ mutation was detected in 10/456 specimens (2.2% $MYD88^{L265P+}$ CLL patients). In DLBCL, PCNHL, IOL and LPL the $MYD88^{L265P}$ mutation was determined as described previously.[51,52]

The HLA typings were performed by the Department of Hematology and Oncology, Tübingen and the German Red Cross blood donation center NSTOB, Institute Dessau (accredited by the European Federation for Immunogenetics). In total, the $MYD88^{L265P+}$ patient cohort comprised 24 patients covering 11 different HLA-A and 14 HLA-B alleles. HLA typing for HLA-C alleles was performed for 15 patients and revealed seven different HLA-C alleles (Table S1).

Prediction of peptide binding to HLA class I alleles

To discover peptide targets, HLA-binding affinity was predicted across all possible 8- to 12-mer peptides encoded by the $MYD88^{L265P}$ mutation by using the HLA-peptide binding prediction algorithms NetMHC 3.4,[26-28,53] SYFPEITHI[29] and an extended in-house database. This in-house database implements the NetMHC 3.4 prediction server as well as the latest SYFPEITHI matrices which are regularly updated by the newest results generated in our department. For the predictions by SYFPEITHI the threshold for binders was defined as 50% of the maximum score for each HLA allotype. Peptides predicted by NetMHC 3.4 with IC_{50}<50 nM were considered strong binders; peptides with IC_{50} values of 50–500 nM were considered weak binders. To verify the ability of the identified peptides to indeed bind to the predicted HLA class I molecules HLA:peptide monomers were produced.

Peptide and HLA peptide monomer synthesis

Peptides were synthesized using the automated peptide synthesizer EPS221 (Abimed, Langenfeld, Germany) by applying standard 9-fluorenylmethyl-oxycarbonyl/tert-butyl (Fmoc/tBu) strategy.[54,55] Purity was assessed by reversed phase HPLC (e2695; Waters, Eschborn, Germany) and identity affirmed by mass spectrometry. Lyophilized peptides were dissolved at 10 mg/mL in DMSO (WAK Chemie, WAK-DMSO) and further diluted in bidestilled H_2O. Biotinylated recombinant HLA molecules, HLA:peptide monomers and fluorescent HLA:peptide tetramers were produced as described previously.[56,57]

Amplification of peptide-specific T cells for IFNγ ELISPOT assays

PBMCs from patients were cultured as described previously.[41,58] In brief, for $CD8^+$ T cell stimulation, PBMCs were pulsed with 1 μg/mL per peptide 24 h after thawing and cultured for 12 days, adding 5 ng/mL human IL-4 (PeproTech, 200–04) and 5 ng/mL human IL-7 (PromoKine, C-61712) on day 0 and 1 as well as 2 ng/mL human IL-2 (R&D Systems, 202-IL) on day 3, 5, 7 and 9, respectively. Peptide-stimulated PBMCs were analyzed by IFNγ ELISPOT assays on day 12.

IFNγ ELISPOT assay

IFNγ ELISPOT assays were performed as described previously.[59] In brief, 96-well nitrocellulose plates (Merck Millipore, MSHAN4B50) were coated with 1 mg/mL IFNγ mAb (Mabtech, 3420–3–250) and incubated overnight at 4°C. Plates were blocked with 10% human serum for 2 h at 37°C. 2.5 – 5 × 10^5

cells/well of pre-stimulated PBMCs were pulsed with 1 μg/mL peptide and incubated for 24 h. For IFNγ ELISPOT assays implementing $CD8^+$ effector cells obtained by aAPC-based *in vitro* priming, autologous PBMCs depleted of monocytes and $CD8^+$ T cells were used as antigen-presenting cells (APCs). For this purpose, APCs were pulsed before the ELISPOT assay for 2 h with 2 μg/mL of the respective peptide. Secretion of IFNγ was detected using an ELISPOT kit (Mabtech, 3420–2A) according to manufacturer's instructions. Phytohaemagglutinin (PHA; Sigma Life Science, L1668) as well as an EBV epitope mix containing the frequently recognized peptides YVLDHLIVV (BRLF1, HLA-A*02), RLRAEAQVK (EBNA3, HLA-A*03), RPPIFIRRL (EBNA3, HLA-B*07), RAKFKQLL (BZLF1, HLA-B*08), and AEGGVGWRHW (EBNA6, HLA-B*44) served as positive controls. HLA-A*01 (GSEELRSLY, POL_HV1H2), -A*02 (YLLPAIVHI, DDX5_HUMAN), -A*03 (RLRPGGKKK, GAG_HV1BR), -A*24 (AYVHMVTHF, BI1_HUMAN), -B*07 (TPGPGVRYPL, NEF_HV1H2), and -B*08 (DIAARNVL, FAK1_HUMAN) -restricted control peptides derived from the human immunodeficiency virus (HIV) or benign tissues served as negative controls. Spots were counted using an ImmunoSpot S5.0.9.21 analyzer (CTL, Shaker Heights, Ohio, USA). T cell responses were evaluated according to the cancer immunoguiding program (CIP) guidelines[60] and considered to be positive when >10 spots/well were counted and the mean spot count per well was at least 3-fold higher than the mean number of spots in the negative control wells.

Generation of peptide-specific $CD8^+$ T cells by aAPC-based in vitro priming

For generation of aAPC, 5.6-μm-diameter streptavidin-coated polystyrene beads (Bangs Laboratories, CP01N) were re-suspended at 2×10^6 particles per mL, incubated with 200 pM of biotinylated HLA:peptide monomer and 20 nM biotinylated anti-CD28 antibody (produced in-house) for 30 min at room temperature.[61] $CD8^+$ T cells from patients and HBDs were enriched from PBMCs by positive selection using magnetic cell sorting (Miltenyi Biotec, 130–045–201). 1×10^6 $CD8^+$ T cells per well were cultured in round bottom 96-well plates (Costar/Corning) and stimulated with 2×10^5 aAPCs and 5 ng/mL human IL-12 (PromoKine, C-62213) three times with a 7 d stimulation interval. 65 U/μL IL-2 (R&D Systems, 202-IL) were added 2 d after stimulation.[31] After priming the cells were expanded over 2–6 weeks, fed every 3–4 d with medium containing 150 U/mL IL-2 as well as every 2 weeks with feeder cells (freshly isolated PBMCs of HBDs plus LG2-EBV cells) together with 1 μg/mL PHA-L (Sigma-Aldrich, 11249738001) and 150 U/mL IL-2.

Tetramer staining

The frequency of peptide-specific $CD8^+$ T cells after aAPC priming was determined on a FACS Canto II cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA) by staining with anti-CD8-PerCP (BioLegend, 301030) and HLA:peptide-tetramer-PE as described previously.[61] Stainings with tetramers of the same HLA allotype containing irrelevant control peptides served as negative controls. The priming was considered successful if the frequency of peptide-specific $CD8^+$ T cells was ≥ 0.1% of sorted $CD8^+$ T cells and at least 3-fold higher than the frequency of peptide-specific $CD8^+$ T cells in the negative control.

Intracellular cytokine staining

The functionality of peptide-specific $CD8^+$ T cells was analyzed by intracellular IFNγ and TNFα staining as well as staining of the degranulation marker CD107a as described previously.[59,62] PBMCs were pulsed with 1 μg/mL of individual peptide and incubated in the presence of 10 μg/mL brefeldin A (Sigma-Aldrich, B6542) and 10 μg/mL GolgiStop (BD Bioscience, 51–2092KZ) for 12–16 h. Cells were labeled using anti-CD107a-FITC (BD Bioscience, 555800), anti-CD8-PerCP (BioLegend, 301030), Cytofix/Cytoperm (BD Bioscience, 554722), anti-TNFα-Pacific Blue (BioLegend, 502920), and anti-IFNγ-PE (BD Bioscience, 559327). Samples were analyzed on a FACS Canto II cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA). Cells stimulated with the corresponding non-mutated (WT) peptide served as negative control.

Cytotoxicity assay (VITAL assay)

The cytolytic capacity of peptide-specific $CD8^+$ T cells was tested using the flow cytometry-based VITAL assay, essentially as described previously.[63] Autologous target cells (2×10^6 PBMCs depleted of monocytes and $CD8^+$ T cells) were loaded with peptides by overnight incubation in T cell medium supplemented with 10 μg/mL of the respective peptide. Cells loaded with mutation-derived peptides and the corresponding WT controls were labeled with 0.5 μM of the fluorescent dyes carboxyfluorescein diacetate succinimidyl ester (CFSE) and Far Red (both Invitrogen, C34554, L34973), respectively. The differentially labeled targets were combined in a 1:1 ratio and 6,000 cells of each fluorescent population were plated in a 96-well round-bottomed plate. Effector cells were added in the indicated effector to target ratios (E/T) in triplicates. Following incubation for 24 h at 37°C, all cells were assessed by FACS analysis using a FACS Canto II cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA).

The specific lysis of mutated peptide-loaded target cells was calculated relative to WT peptide-loaded control targets. Effector-independent lysis was assessed in parallel in wells containing no effector cells and the specific lysis was normalized according to the following equation:

$$\%\ \text{specific lysis} = \left[1 - \left(\frac{\text{targets loaded with } MYD88^{L265P} \text{ peptide/ targets loaded with } MYD88^{WT} \text{ peptide}}{\text{mean \% survival in absence of effectors}}\right)\right] \times 100$$

Search for homologous peptide sequences

Screening sequence homology of *MYD88*^{L265P} peptides with prokaryotic and virus proteins was performed for P1 (RPIPIKYKAM), P2 (RPIPIKYKA), P3 (SPGAHQKRPI), and P4 (HQKRPIPIKY), using the Smith–Waterman protein searcher application (SSEARCH) on the European Molecular Biology Laboratory server (http://www.ebi.ac.uk/Tools/sss/fasta/), using default settings.

Software and statistical analysis

Flow cytometric data analysis was performed using FlowJo 10.0.7 (Treestar, Ahland, Oregon, USA). Taqman assay data analysis was performed using the QuantStudio™ Real-Time PCR software (ThermoFisher, Waltham, Massachusetts, USA). GraphPad Prism 6.0 (GraphPad Software, La Jolla, California, USA) was used for the generation of plots and for statistical analysis. Statistical analysis of *MYD88*^{L265P}-specific lysis was based on unpaired *t* tests.

Disclosure of potential conflicts of interest

The authors declare no competing financial interests. A.N., J.S.S., O.O.W., D.J.K., H.-G.R., and A.N.R.W. are listed as inventors in a filed patent application.

Acknowledgments

We thank Sabine Dickenhöfer, Patricia Hrstic, Nicole Zuschke and Katharina Graf for excellent technical support. Dr med. Christoph Deuter and Dr med. Daniela Süsskind (Eye Hospital, University of Tübingen) kindly provided us further PBMC samples of lymphoma patients.

Funding

This work was supported by the German Research Foundation (DFG) funded Collaborative Research Center (SFB) 685 "Immunotherapy," the Else-Übelmesser-Stiftung, the European Union (EU; ERC AdG339842 MUTAEDITING), the German Cancer Consortium (DKTK), the University Hospital and the University of Tübingen.

References

1. Boon T, Cerottini J-C, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 1994; 12:337-65; PMID:8011285; http://dx.doi.org/10.1146/annurev.iy.12.040194.002005
2. Ostrand-Rosenberg S. Tumor immunotherapy: the tumor cell as an antigen-presenting cell. Curr Opin Immunol 1994; 6:722-7; PMID:7826527; http://dx.doi.org/10.1016/0952-7915(94)90075-2
3. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8$^+$ T cells by B7-transfected melanoma cells. Science 1993; 259:368-70; PMID:7678351; http://dx.doi.org/10.1126/science.7678351
4. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, Staehler M, Brugger W, Dietrich PY, Mendrzyk R et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 2012; 18:1254-61; PMID:22842478; http://dx.doi.org/10.1038/nm.2883
5. Perez-Gracia JL, Labiano S, Rodriguez-Ruiz ME, Sanmamed MF, Melero I. Orchestrating immune check-point blockade for cancer immunotherapy in combinations. Curr Opin Immunol 2014; 27:89-97; PMID:24485523; http://dx.doi.org/10.1016/j.coi.2014.01.002
6. Ansell SM. Targeting immune checkpoints in lymphoma. Curr Opin Hematol 2015; 22:337-42; PMID:26049755; http://dx.doi.org/10.1097/MOH.0000000000000158
7. Coulie PG, Lehmann F, Lethe B, Herman J, Lurquin C, Andrawiss M, Boon T. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. Proc Natl Acad Sci U S A 1995; 92:7976-80; PMID:7644523; http://dx.doi.org/10.1073/pnas.92.17.7976
8. van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJ, Behjati S, Hilkmann H, El Atmioui D et al. Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 2013; 31:e439-42; PMID:24043743; http://dx.doi.org/10.1200/JCO.2012.47.7521
9. Treon SP, Xu L, Yang G, Zhou Y, Liu X, Cao Y, Sheehy P, Manning RJ, Patterson CJ, Tripsas C et al. MYD88 L265P somatic mutation in Waldenstrom's macroglobulinemia. N Engl J Med 2012; 367:826-33; PMID:22931316; http://dx.doi.org/10.1056/NEJMoa1200710
10. Varettoni M, Arcaini L, Zibellini S, Boveri E, Rattotti S, Riboni R, Corso A, Orlandi E, Bonfichi M, Gotti M et al. Prevalence and clinical significance of the MYD88 (L265P) somatic mutation in Waldenstrom's macroglobulinemia and related lymphoid neoplasms. Blood 2013; 121:2522-8; PMID:23355535; http://dx.doi.org/10.1182/blood-2012-09-457101
11. Xu L, Hunter ZR, Yang G, Zhou Y, Cao Y, Liu X, Morra E, Trojani A, Greco A, Arcaini L et al. MYD88 L265P in Waldenstrom macroglobulinemia, immunoglobulin M monoclonal gammopathy, and other B-cell lymphoproliferative disorders using conventional and quantitative allele-specific polymerase chain reaction. Blood 2013; 121:2051-8; PMID:23321251; http://dx.doi.org/10.1182/blood-2012-09-454355
12. Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH, Kohlhammer H, Xu W, Yang Y, Zhao H et al. Oncogenically active MYD88 mutations in human lymphoma. Nature 2011; 470:115-9; PMID:21179087; http://dx.doi.org/10.1038/nature09671
13. Pasqualucci L, Trifonov V, Fabbri G, Ma J, Rossi D, Chiarenza A, Wells VA, Grunn A, Messina M, Elliot O et al. Analysis of the coding genome of diffuse large B-cell lymphoma. Nat Genet 2011; 43:830-7; PMID:21804550; http://dx.doi.org/10.1038/ng.892
14. Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N, Escaramis G, Jares P, Bea S, Gonzalez-Diaz M et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature 2011; 475:101-5; PMID:21642962; http://dx.doi.org/10.1038/nature10113
15. Landau DA, Carter SL, Stojanov P, McKenna A, Stevenson K, Lawrence MS, Sougnez C, Stewart C, Sivachenko A, Wang L et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell 2013; 152:714-26; PMID:23415222; http://dx.doi.org/10.1016/j.cell.2013.01.019
16. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0. Cancer Incidence and Mortality Worldwide: IARC CancerBase No 11, 2013:International Agency for Research on Cancer.
17. Lenz G, Wright G, Dave SS, Xiao W, Powell J, Zhao H, Xu W, Tan B, Goldschmidt N, Iqbal J et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med 2008; 359:2313-23; PMID:19038878; http://dx.doi.org/10.1056/NEJMoa0802885
18. Tsimberidou A-M, Keating MJ. Treatment of patients with fludarabine-refractory chronic lymphocytic leukemia: need for new treatment options. Leuk Lymphoma 2010; 51:1188-99; PMID:20545582; http://dx.doi.org/10.3109/10428194.2010.486089
19. Schmitt M, Schmitt A, Rojewski MT, Chen J, Giannopoulos K, Fei F, Yu Y, Gotz M, Heyduk M, Ritter G et al. RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. Blood 2008; 111:1357-65; PMID:17978170; http://dx.doi.org/10.1182/blood-2007-07-099366
20. Porter DL, Levine BL, Bunin N, Stadtmauer EA, Luger SM, Goldstein S, Loren A, Phillips J, Nasta S, Perl A et al. A phase 1 trial of donor

lymphocyte infusions expanded and activated ex vivo via CD3/CD28 costimulation. Blood 2006; 107:1325-31; PMID:16269610; http://dx.doi.org/10.1182/blood-2005-08-3373
21. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 2011; 17:4550-7; PMID:21498393; http://dx.doi.org/10.1158/1078-0432.CCR-11-0116
22. Timmerman JM, Czerwinski DK, Davis TA, Hsu FJ, Benike C, Hao ZM, Taidi B, Rajapaksa R, Caspar CB, Okada CY. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. Blood 2002; 99:1517-26; PMID:11861263; http://dx.doi.org/10.1182/blood.V99.5.1517
23. Park HJ, Neelapu SS. Developing idiotype vaccines for lymphoma: from preclinical studies to phase III clinical trials. Brit J Haematol 2008; 142:179-91; PMID:18422783; http://dx.doi.org/10.1111/j.1365-2141.2008.07143.x
24. Wilson WH, Young RM, Schmitz R, Yang Y, Pittaluga S, Wright G, Lih C-J, Williams PM, Shaffer AL, Gerecitano J. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. Nat Med 2015; 21:922-6; PMID:26193343; http://dx.doi.org/10.1038/nm.3884
25. Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. Nucleic Acids Research 2008; 36:W509-W12; PMID:18463140; http://dx.doi.org/10.1093/nar/gkn202
26. Lundegaard C, Lund O, Nielsen M. Accurate approximation method for prediction of class I MHC affinities for peptides of length 8, 10 and 11 using prediction tools trained on 9mers. Bioinformatics 2008; 24:1397-8; PMID:18413329; http://dx.doi.org/10.1093/bioinformatics/btn128
27. Nielsen M, Lundegaard C, Worning P, Hvid CS, Lamberth K, Buus S, Brunak S, Lund O. Improved prediction of MHC class I and class II epitopes using a novel Gibbs sampling approach. Bioinformatics 2004; 20:1388-97; PMID:14962912; http://dx.doi.org/10.1093/bioinformatics/bth100
28. Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci 2003; 12:1007-17; PMID:12717023; http://dx.doi.org/10.1110/ps.0239403
29. Schuler MM, Nastke MD, Stevanovikc S. SYFPEITHI: database for searching and T-cell epitope prediction. Methods Mol Biol 2007; 409:75-93; PMID:18449993; http://dx.doi.org/10.1007/978-1-60327-118-9_5
30. Duan F, Duitama J, Al Seesi S, Ayres CM, Corcelli SA, Pawashe AP, Blanchard T, McMahon D, Sidney J, Sette A et al. Genomic and bioinformatic profiling of mutational neoepitopes reveals new rules to predict anticancer immunogenicity. J Exp Med 2014; 211:2231-48; PMID:25245761; http://dx.doi.org/10.1084/jem.20141308
31. Peper JK, Bösmüller H-C, Schuster H, Gückel B, Hörzer H, Roehle K, Schäfer R, Wagner P, Rammensee H-G, Stevanović S et al. HLA ligandomics identifies Histone Deacetylase 1 as target for Ovarian Cancer immunotherapy. OncoImmunology 2016; 5(5): e1065369; PMID:27467910; http://dx.doi.org/10.1080/2162402X.2015.1065369
32. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013; 369:122-33; PMID:23724867; http://dx.doi.org/10.1056/NEJMoa1302369
33. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012; 366:2443-54; PMID:22658127; http://dx.doi.org/10.1056/NEJMoa1200690
34. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, Weber JS, Joshua AM, Hwu WJ, Gangadhar TC et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet 2014; 384:1109-17; PMID:25034862; http://dx.doi.org/10.1016/S0140-6736(14)60958-2
35. Lynch TJ, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, Sebastian M, Neal J, Lu H, Cuillerot JM et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. J Clin Oncol 2012; 30:2046-54; PMID:22547592; http://dx.doi.org/10.1200/JCO.2011.38.4032
36. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, Schuster SJ, Millenson MM, Cattry D, Freeman GJ et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N Engl J Med 2015; 372:311-9; PMID:25482239; http://dx.doi.org/10.1056/NEJMoa1411087
37. Martin P, Furman RR, Rutherford S, Ruan J, Ely S, Greenberg J, Coleman M, Goldsmith SJ, Leonard JP. Phase I study of the anti-CD74 monoclonal antibody milatuzumab (hLL1) in patients with previously treated B-cell lymphomas. Leuk Lymphoma 2015; 56(11):3065-70; PMID:25754579; http://dx.doi.org/10.3109/10428194.2015.1028052
38. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 2014; 371:2189-99; PMID:25409260; http://dx.doi.org/10.1056/NEJMoa1406498
39. Brossart P, Schneider A, Dill P, Schammann T, Grunebach F, Wirths S, Kanz L, Buhring HJ, Brugger W. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. Cancer Res 2001; 61:6846-50; PMID:11559560.
40. Walz S, Stickel J, Kowalewski DJ, Schuster H, Weisel K, Backert L, Kahn S, Nelde A, Stroh T, Handel M et al. The antigenic landscape of multiple myeloma: mass spectrometry (re-)defines targets for T-cell based immunotherapy. Blood 2015; 126(10):1203-13; PMID:26138685; http://dx.doi.org/10.1182/blood-2015-04-640532
41. Berlin C, Kowalewski DJ, Schuster H, Mirza N, Walz S, Handel M, Schmid-Horch B, Salih HR, Kanz L, Rammensee HG et al. Mapping the HLA ligandome landscape of acute myeloid leukemia: a targeted approach toward peptide-based immunotherapy. Leukemia 2015; 29, 647-59; PMID:25092142; http://dx.doi.org/10.1038/leu.2014.233
42. Greiner J, Ono Y, Hofmann S, Schmitt A, Mehring E, Gotz M, Guillaume P, Dohner K, Mytilineos J, Dohner H et al. Mutated regions of nucleophosmin 1 elicit both CD4(+) and CD8(+) T-cell responses in patients with acute myeloid leukemia. Blood 2012; 120:1282-9; PMID:22592607; http://dx.doi.org/10.1182/blood-2011-11-394395
43. Greiner J, Schneider V, Schmitt M, Götz M, Döhner K, Wiesneth M, Döhner H, Hofmann S. Immune responses against the mutated region of cytoplasmatic NPM1 might contribute to the favorable clinical outcome of AML patients with NPM1 mutations (NPM1mut). Blood 2013; 122:1087-8; PMID:23929838; http://dx.doi.org/10.1182/blood-2013-04-496844
44. Martinez-Trillos A, Pinyol M, Navarro A, Aymerich M, Jares P, Juan M, Rozman M, Colomer D, Delgado J, Gine E et al. Mutations in TLR/MYD88 pathway identify a subset of young chronic lymphocytic leukemia patients with favorable outcome. Blood 2014; 123:3790-6; PMID:24782504; http://dx.doi.org/10.1182/blood-2013-12-543306
45. Stanke J, Hoffmann C, Erben U, von Keyserling H, Stevanovic S, Cichon G, Schneider A, Kaufmann AM. A flow cytometry-based assay to assess minute frequencies of $CD8^+$ T cells by their cytolytic function. J Immunological Methods 2010; 360:56-65; PMID:20558172; http://dx.doi.org/10.1016/j.jim.2010.06.005
46. Andersen MH, Reker S, Kvistborg P, Becker JC, Straten Pt. Spontaneous Immunity against Bcl-xL in Cancer Patients. J Immunol 2005; 175:2709-14; PMID:16081848; http://dx.doi.org/10.4049/jimmunol.175.4.2709
47. Avbelj M, Wolz O-O, Fekonja O, Benčina M, Repič M, Mavri J, Krüger J, Schärfe C, Garcia MD, Panter G. Activation of lymphoma-associated MyD88 mutations via allostery-induced TIR-domain oligomerization. Blood 2014; 124:3896-904; PMID:25359991; http://dx.doi.org/10.1182/blood-2014-05-573188
48. Ansell S, Hodge L, Secreto F, Manske M, Braggio E, Price-Troska T, Ziesmer S, Li Y, Johnson S, Hart S. Activation of TAK1 by MYD88

L265P drives malignant B-cell Growth in non-Hodgkin lymphoma. Blood Cancer J 2014; 4:e183; PMID:24531446; http://dx.doi.org/10.1038/bcj.2014.4

49. Rittig SM, Haentschel M, Weimer KJ, Heine A, Muller MR, Brugger W, Horger MS, Maksimovic O, Stenzl A, Hoerr I. Intradermal Vaccinations With RNA Coding for TAA Generate $CD8^+$ and $CD4^+$ Immune Responses and Induce Clinical Benefit in Vaccinated Patients. Molecular Therapy 2011; 19:990-9; PMID:21189474; http://dx.doi.org/10.1038/mt.2010.289
50. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, Badros AZ, Garfall A, Weiss B, Finklestein J et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat Med 2015; 21:914-21; PMID:26193344; http://dx.doi.org/10.1038/nm.3910
51. Bonzheim I, Giese S, Deuter C, Süsskind D, Zierhut M, Waizel M, Szurman P, Federmann B, Schmidt J, Quintanilla-Martinez L et al. High frequency of MYD88 mutations in vitreoretinal B-cell lymphoma: a valuable tool to improve diagnostic yield of vitreous aspirates. Blood 2015; 126:76-9; PMID:25900979; http://dx.doi.org/10.1182/blood-2015-01-620518
52. Schmidt J, Federmann B, Schindler N, Steinhilber J, Bonzheim I, Fend F, Quintanilla-Martinez L. MYD88 L265P and CXCR4 mutations in lymphoplasmacytic lymphoma identify cases with high disease activity. Brit J Haematol 2015; 169:795-803; PMID:25819228; http://dx.doi.org/10.1111/bjh.13361
53. Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. Nucleic Acids Res 2008; 1;36:W509-12; PMID:18463140; http://dx.doi.org/10.1093/nar/gkn202
54. Merrifield B. Solid phase synthesis. Science 1986; 232:341-7; PMID:3961484; http://dx.doi.org/10.1126/science.3961484
55. Atherton E, Fox H, Harkiss D, Logan C, Sheppard R, Williams B. A mild procedure for solid phase peptide synthesis: use of fluorenylmethoxycarbonylamino-acids. J Chem Soc Chem Commun 1978; 13:537-9; http://dx.doi.org/10.1039/c39780000537
56. Garboczi DN, Hung DT, Wiley DC. HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in Escherichia coli and complexed with single antigenic peptides. Proc Natl Acad Sci U S A 1992; 89:3429-33; PMID:1565634; http://dx.doi.org/10.1073/pnas.89.8.3429
57. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996; 274:94-6; PMID:8810254; http://dx.doi.org/10.1126/science.274.5284.94
58. Kowalewski DJ, Schuster H, Backert L, Berlin C, Kahn S, Kanz L, Salih HR, Rammensee HG, Stevanovic S, Stickel JS. HLA ligandome analysis identifies the underlying specificities of spontaneous antileukemia immune responses in chronic lymphocytic leukemia (CLL). Proc Natl Acad Sci U S A 2015; 112.2:E166-E75; PMID:25548167; http://dx.doi.org/10.1073/pnas.1416389112
59. Widenmeyer M, Griesemann H, Stevanovic S, Feyerabend S, Klein R, Attig S, Hennenlotter J, Wernet D, Kuprash DV, Sazykin AY et al. Promiscuous survivin peptide induces robust $CD4^+$ T-cell responses in the majority of vaccinated cancer patients. Int J Cancer 2012; 131:140-9; PMID:21858810; http://dx.doi.org/10.1002/ijc.26365
60. Britten CM, Gouttefangeas C, Welters MJ, Pawelec G, Koch S, Ottensmeier C, Mander A, Walter S, Paschen A, Muller-Berghaus J et al. The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific $CD8^+$ T lymphocytes by structural and functional assays. Cancer Immunol Immunother 2008; 57:289-302; PMID:17721783; http://dx.doi.org/10.1007/s00262-007-0378-0
61. Rudolf D, Silberzahn T, Walter S, Maurer D, Engelhard J, Wernet D, Buhring HJ, Jung G, Kwon BS, Rammensee HG et al. Potent costimulation of human CD8 T cells by anti-4-1BB and anti-CD28 on synthetic artificial antigen presenting cells. Cancer Immunol Immunother 2008; 57:175-83; PMID:17657490; http://dx.doi.org/10.1007/s00262-007-0360-x
62. Neumann A, Horzer H, Hillen N, Klingel K, Schmid-Horch B, Buhring HJ, Rammensee HG, Aebert H, Stevanovic S. Identification of HLA ligands and T-cell epitopes for immunotherapy of lung cancer. Cancer Immunol Immunother 2013; 62:1485-97; PMID:23817722; http://dx.doi.org/10.1007/s00262-013-1454-2
63. Hermans IF, Silk JD, Yang J, Palmowski MJ, Gileadi U, McCarthy C, Salio M, Ronchese F, Cerundolo V. The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. J Immunol Methods 2004; 285:25-40; PMID:14871532; http://dx.doi.org/10.1016/j.jim.2003.10.017