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ORIGINAL RESEARCH

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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]

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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

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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

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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,

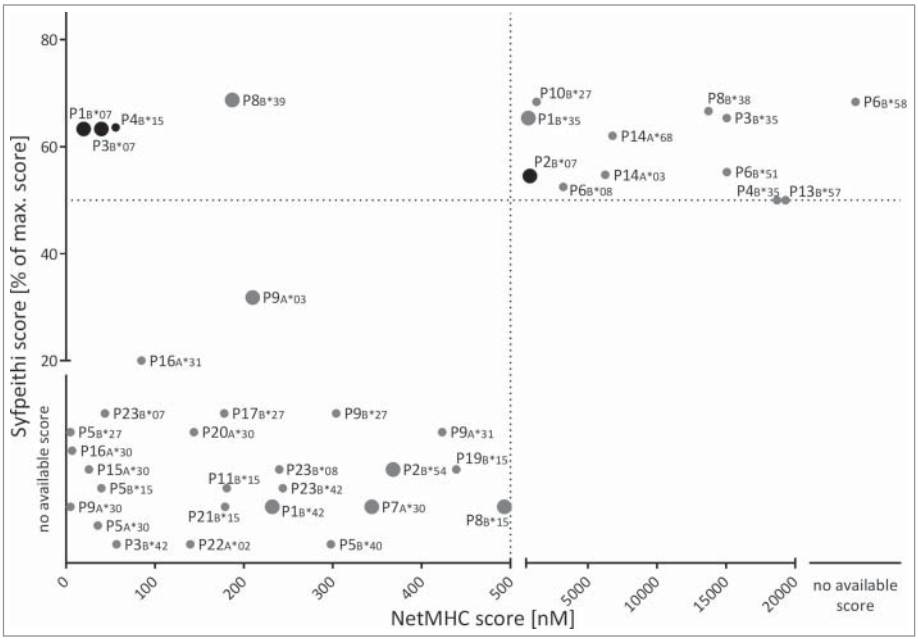

Figure 1. Prediction of MYD88^{L265P}-derived HLA class I ligands. 50 *MYD88*^{L265P}-derived 8- to 12-mer peptides were scored by the online prediction tools SYFPEITHI and NetMHC 3.4, as well as an extended in-house database. Peptides with NetMHC 3.4 $IC_{50} \leq$ 500 nM were defined as binders (region 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*

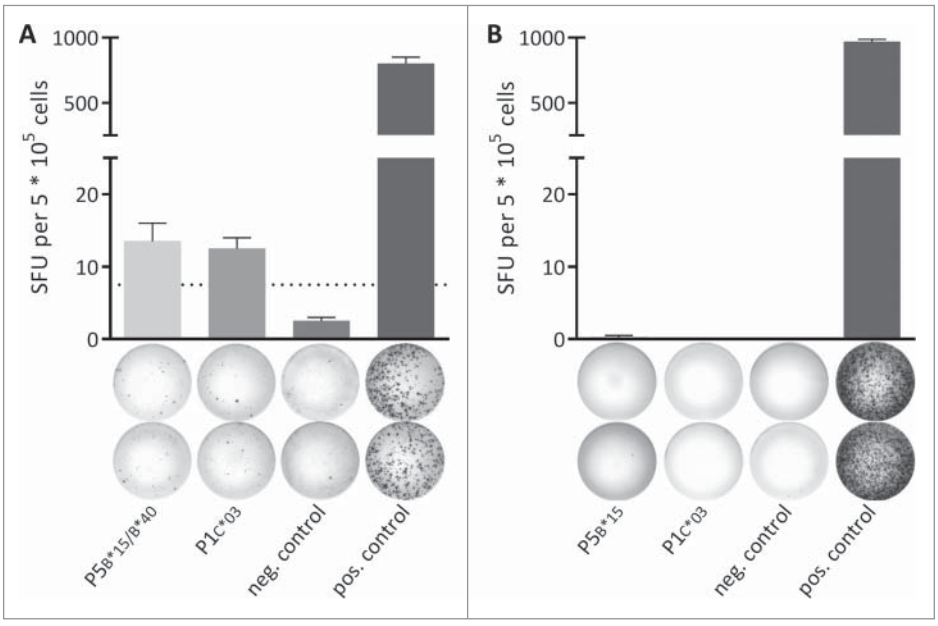

Figure 2. Spontaneous memory T cell responses are detectable in a leukemia patient. The presence of memory T cell responses in leukemia and lymphoma patients was analyzed using 12-d recall IFNγ ELISPOT assays. (A) In a single (out of 22 tested) *MYD88*$^{L265P+}$ NHL patients (CLL-05-R) IFNγ secretion 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.

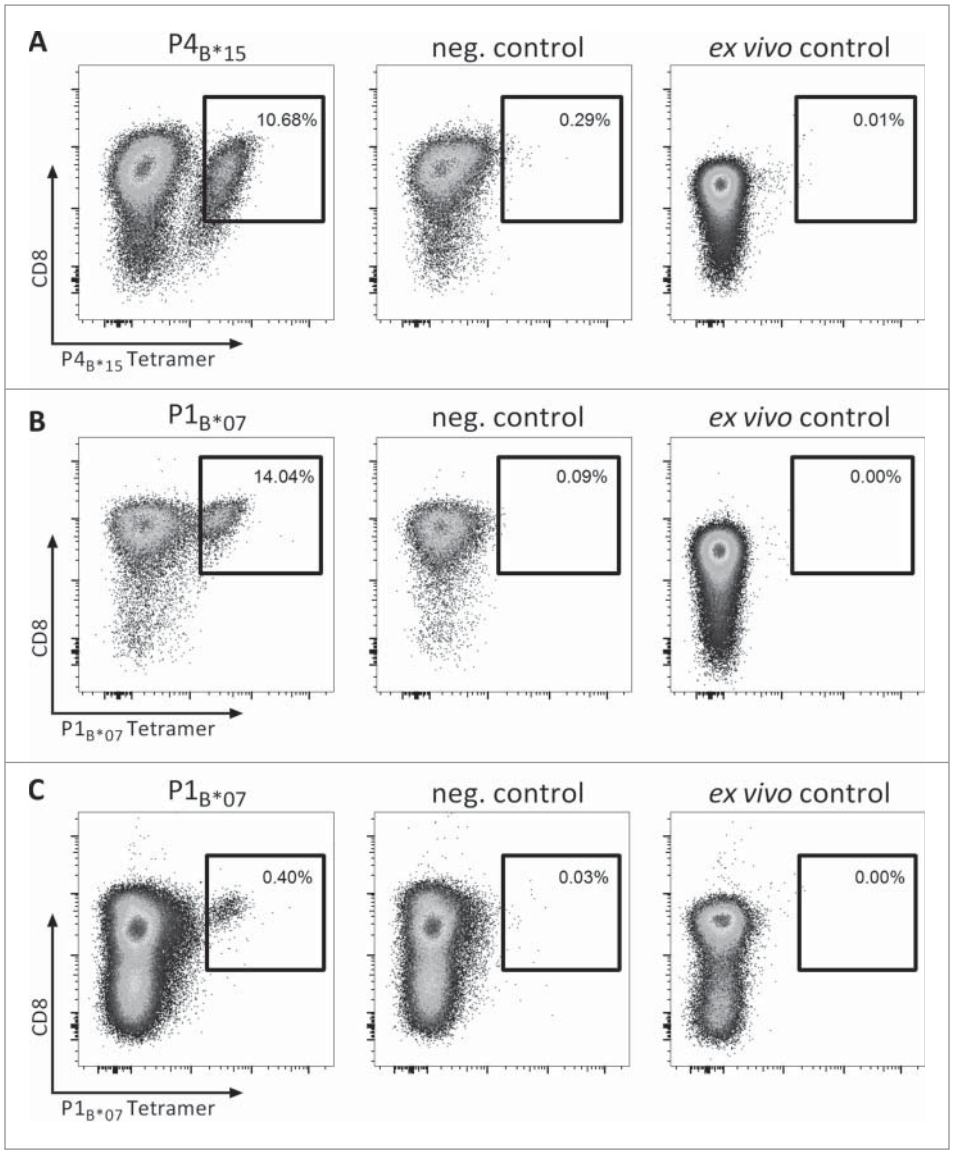

Figure 3. Efficient *in vitro* generation of $P4_{B*15}$- and $P1_{B*07}$-specific $CD8^+$ T cells from naive T cells of CLL patients and HBDs. Representative tetramer stainings of $CD8^+$ T cells after three cycles of aAPC-based *in vitro* priming using $CD8^+$ T cells derived from HLA-matched HBDs 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.

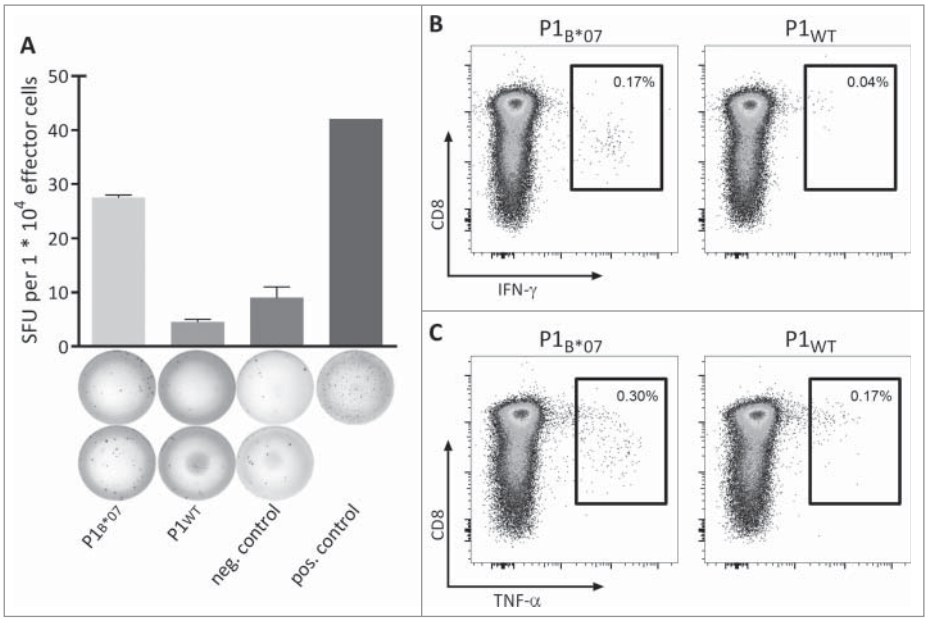

Figure 4. Functionality and specificity of *MYD88*^{L265P}-specific T cells. Functionality and specificity of *MYD88*^{L265P}-specific CD8$^+$ T cells were analyzed by (A) IFNγ ELISPOT assay or (B, C) intracellular cytokine staining. Both assays showed increased production of IFNγ or TNFα after stimulation 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

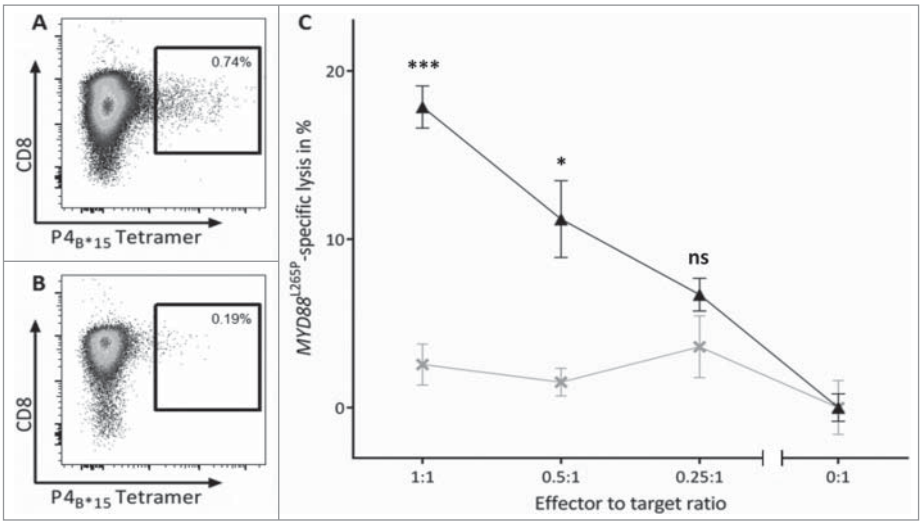

Figure 5. *MYD88*^{L265P}-selective cytotoxicity of $P4_{B*15}$-specific effector cells. The *MYD88*^{L265P}-specific cytotoxicity was analyzed in a VITAL cytotoxicity assay with CD8$^+$ effector cells of *in vitro* primed cells of HBDs. (A, B) Tetramer staining of polyclonal effector cells one day before 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.

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