Aus der Arbeitsgruppe Molekulare Immuntherapie am

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DISSERTATION

Development of mutation-specific T-cell receptor gene therapy for treatment of B-cell malignancies

Entwicklung einer mutationsspezifischen T-Zell Rezeptor Gentherapie für die Behandlung von B-Zell Neoplasien

zur Erlangung des akademischen Grades

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Abbreviations

ABC-DLBCL: activated B cell-like diffuse large B-cell lymphoma
AGC: Automatic gain control
ANOVA: Analysis of variance
ATT: Adoptive T-cell therapy
BCMA: B-cell maturation antigen
CAR-T: T cells engineered with a CAR
CAR: Chimeric antigen receptors
CD3: Cluster of Differentiation 3
CD8: Cluster of Differentiation 8
CD19: Cluster of Differentiation 19
CD20: Cluster of Differentiation 20
CD22: Cluster of Differentiation 22
CID: Collision-induced dissociation
CNS: Central Nervous System
DC: Dendritic cells
DLBCL: Diffuse large B-cell lymphoma
EBV: Epstein-Barr virus
EC50: Half maximal effective concentration
ELISA: Enzyme-linked immunosorbent assay
FACS: Fluorescence-Activated Cell Sorting
FBS: Fetal bovine serum
Fmoc: N-(9- fluorenyl) methoxycarbonyl
GBC-DLBCL: germinal center B-cell like diffuse large B-cell lymphoma
GFP: Green florescent protein
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GvHD: Graft-versus-host disease
HCD: Higher-energy collisional dissociation
hIL2: Human Interleukin-2
HLA-B7: Human leukocyte antigen B7
HLA: Human leukocyte antigens
IFN-γ: Interferon gamma
IL-1: Interleukin 1
JAK: Janus kinase
L265P: Leucine at position 265 to proline
LC: Liquid Chromatography
LCL: Lymphoblastoid cell lines
MHC: Major histocompatibility complex
MyD88: Myeloid differentiation primary response 88
MS: Mass spectrometry
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NOG: NOD/Shi-scid/IL-2Rγnull mouse
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PD-L1: Programmed death-ligand 1
PMA: Phorbol myristate acetate
RPMI: Roswell Park Memorial Institute
TCR-T: T cells engineered with a TCR
TCR: T cell receptors
TRAC: Human T Cell Receptor alpha constant
TRAV: Human T cell receptor alpha variable
TRBC: Human T Cell Receptor beta constant
TRBV: Human T cell receptor beta variable

1. Abstract

1.1. English:

Lymphoid malignancies are a heterogeneous group of cancers that are responsible for one tenth of all cancer deaths in Europe. Immunotherapy using monoclonal antibodies has marked a major breakthrough in the therapy of lymphomas and leukemias since early 1990s, but cure is still difficult to achieve in a significant number of patients. As a next step, adoptive transfer of engineered T-cells has been shown to be a promising treatment. The current most common strategy of this form of cellular immunotherapy is targeting lineage-specific antigens, such as CD19, CD22 or BCMA for B-cell driven malignancies. These approaches still use antibody-fragments as recognition tools and are not truly tumor specific: Eliminating the entire cell lineage including normal, healthy B-cells can result in partial immunodeficiency. In addition, use of antibody-derivative chimeric receptors limits target repertoire to surface antigens, which frequently leads to relapse due to loss of target antigen expression on cell surface.

T-cell receptors recognize peptides derived from both intracellular and surface proteins when presented on the cell surface embedded in an HLA allele, and this recognition can be sensitive enough to recognize cancer specific alterations such as single nucleotide changes or even posttranslational modifications. In this study, we aimed to develop T-cell receptors (TCRs) targeting cancer specific recurrent neo-antigens in lymphoma and leukemia for precision cellular immunotherapy. With this purpose, we generated mutation-specific T-cell lines by autologous priming from multiple healthy donors, targeting one of the most common driver mutations found in B-cell lymphomas; MyD88 L265P. This distinct point mutation substituting a proline for a lysine amino acid in the adaptor protein MyD88 can be found in around one fifth of all lymphoid malignancies, and associates with aggressive/hard-to-treat cases such as primary central nervous system lymphomas (PCNSL) and “activated B-cell type” diffuse large B-cell lymphoma (ABC-DLBCL), as it leads to constitutive signaling through activation of the transcription factor NF-kB providing growth and survival benefit for tumor cells.

We generated several T-cell lines against the MyD88-L265P mutation, which were reactive selectively against the predicted mutant epitope in the context of HLA-B*07:02,

but not against the corresponding wild-type peptide expressed in normal B-cells. T-cell receptor sequences from these lines were cloned into the retroviral vector pMP71 and expressed on T-cells of healthy donors for further characterization. All cloned TCRs showed mutation-specific and HLA-B7 restricted reactivity with varying functional avidity. TCR-engineered T-cells (TCR-T cells) were able to selectively recognize and kill engineered target cells expressing MyD88 L265P, indicating that the mutant epitope is naturally processed and presented on HLA-B*07:02. In order to further evaluate the therapeutic potency, we tested TCR-T cells against non-Hodgkin lymphoma cell lines naturally harboring MyD88 L265P mutation, and again observed HLA-restricted and mutation-specific reactivity, as well as cytotoxic activity accompanied by antigen induced proliferation of TCR-T cells. Following these encouraging results in vitro, we developed two xenograft mouse models using human non-Hodgkin lymphoma cell lines growing in mice to assess therapeutic efficacy of adoptively transferred TCR-T cells in vivo. Treatment with a single dose of TCR-T cell injection led to durable regression of established tumors. Two models of immunodeficient mice were used. NOG mice (NOD/Shi-*scid*/IL-2Rγ^{null}) are derived by a further modification of Non-Obese-Diabetic (NOD) mice crossed with SCID (severe combined immunodeficiency) mice, by further introducing a defect in the gamma-chain of the Interleukin-2 receptor: they are routinely used for xenogenic tumor experiment, being unable to spontaneously reject grafted human cells. As a second model, NOG-mice genetically modified to express human Interleukin-2 were used: by endogenously producing human Interleukin-2 they provide better support for adoptively transferred T cells. Differences in treatment response between conventional NOG and human interleukin-2 expressing NOG mice used in our in vivo studies revealed important indications for T-cell persistence and exhaustion. Finally, initial safety screens to predict possible cross-reactivity or allo-reactivity did not show any sign of off-target reactivity by the mutation-specific TCR.

Taken together, our data suggest that mutation-specific TCRs can be used to target the MyD88 L265P mutation, and hold promise for precision therapy in a significant subgroup of B-cell malignancies.

1.2. Deutsch:

Lymphoide Malignome sind eine heterogene Gruppe von Krebserkrankungen, die für ein Zehntel aller Krebstodesfälle in Europa verantwortlich sind. Die Immuntherapie mit monoklonalen Antikörpern hat seit Anfang der 1990er Jahre einen Durchbruch in der Behandlung von Lymphomen und Leukämien gebracht, doch ist es nach wie vor schwierig, einen erheblichen Teil der Patienten dauerhaft zu heilen. Als nächster Schritt in der Entwicklung der Immuntherapie hat sich der adoptive Transfer von genetisch manipulierten T-Zellen als vielversprechende Behandlung angedeutet. Die derzeit gängigste Strategie dieser Form der zellulären Immuntherapie ist gerichtet gegen linienspezifische Antigene der B-zell Reihe wie CD19, CD22 oder BCMA. Diese Ansätze verwenden immer noch Antikörperfragmente als spezifische Erkennungsinstrumente und sind daher nicht wirklich tumorspezifisch: Die Eliminierung des gesamten Pools an Zellen, welche das Zielantigen tragen, einschließlich der normalen, gesunden B-Zellen, kann durchaus zu einer Immundefizienz mit Antikörpermangel führen. Darüber hinaus wird durch die Verwendung von chimären Antikörperrezeptoren das Zielrepertoire auf Oberflächenantigene beschränkt, was aufgrund des Verlusts der Expression von Zielantigenen auf der Zelloberfläche häufig zu Rückfällen führt.

Die meisten krebsinduzierenden Mutationen entstehen jedoch in intrazellulären Proteinen. Peptide, die von intrazellulären Proteinen stammen, werden erkannt, wenn sie auf der Zelloberfläche in einem HLA-Klasse-I-Molekül eingebettet den T-Zellen „präsentiert“ werden.

In dieser Arbeit haben wir T-Zell-Rezeptoren (TCRs) entwickelt, die auf krebsspezifische, wiederkehrende Neo-Antigene in Lymphomen und Leukämien abzielen und eine echte tumorspezifische, hochpräzise zelluläre Immuntherapie ermöglichen. Zu diesem Zweck haben wir durch autologes Priming von mehreren gesunden Spendern mutationsspezifische T-Zelllinien erzeugt, die auf eine der häufigsten Treibermutationen bei B-Zell-Lymphomen abzielen: MyD88 L265P. Diese Punktmutation, bei der eine Leucin- durch eine Prolin-Aminosäure im Adaptorprotein MyD88 ersetzt wird, kommt bei etwa einem Fünftel aller lymphatischen Neoplasien vor und ist mit einem aggressiven/schwer zu behandelnden Verlauf assoziiert, wie z. B. bei primären Lymphomen des zentralen Nervensystems (PCNSL) und bei diffus- großzelligen B-Zell-Lymphomen vom "aktivierten B-Zell-Typ" (ABC-DLBCL).

Wir haben mehrere T-Zelllinien gegen die MyD88-L265P-Mutation entwickelt. Diese T-Zell-Linien waren demnach selektiv gegen das vorhergesagte L265P Mutationsepitop im Kontext von HLA-B*07:02 reaktiv, nicht aber gegen das entsprechende Wildtyp-Peptid,

das in normalen B-Zellen exprimiert wird oder wenn das HLA-A-B*07:02 Molekül fehlt. T-Zell-Rezeptor-Sequenzen aus diesen Linien wurden in den retroviralen Vektor pMP71 kloniert und zur weiteren Charakterisierung auf T-Zellen gesunder Spender exprimiert. Alle klonierten TCRs zeigten eine mutationsspezifische und auf HLA-B7 beschränkte Reaktivität mit unterschiedlicher funktioneller Avidität. TCR-entwickelte T-Zellen (TCR-T-Zellen) waren in der Lage, MyD88 L265P exprimierende Zielzellen selektiv zu erkennen und zu killen als Beweis, dass das mutierte Epitop auf natürliche Weise prozessiert und auf HLA-B*07:02 präsentiert wird. Um die therapeutische Wirksamkeit solcher T-Zellen weiter zu untersuchen, haben wir TCR-T-Zellen gegen Non-Hodgkin-Lymphom-Zelllinien getestet, die auf natürliche Weise die MyD88 L265P-Mutation tragen, Erneut konnten wir eine HLA-gebundene und mutationsspezifische Reaktivität und zytotoxische Aktivität nachweisen, begleitet von einer antigeninduzierten Proliferation der genmodifizierten TCR-T-Zellen. Nach diesen ermutigenden Ergebnissen in vitro entwickelten wir zwei Xenograft-Mausmodelle mit in Mäusen wachsenden humanen Non-Hodgkin-Lymphom-Zelllinien, um die therapeutische Wirksamkeit von adoptiv transferierten TCR-T-Zellen in vivo zu beweisen. Die Behandlung mit einer einmaligen Injektion von TCR-T-Zellen führte zu einer dauerhaften Rückbildung der zuvor etablierten Tumore. Es wurden zwei Modelle von Immun defizienten Mäusen verwendet. NOG-Mäuse (NOD/Shi-scid/IL-2Rγnull) werden durch eine weitere Modifikation von „nicht-obesen diabetischen“ (NOD) Mäusen, die mit SCID-Mäusen (schwere kombinierte Immundefizienz) gekreuzt wurden, gewonnen, indem ein weiterer Defekt in der Gamma-Kette des Interleukin-2-Rezeptors eingeführt wird: Diese Zellen werden routinemäßig für xenotransplantierte Tumorexperimente verwendet, da sie nicht in der Lage sind, menschliche Zellen spontan abzustoßen. Als zweites Modell wurden NOG-Mäuse verwendet, die genetisch so verändert wurden, dass sie menschliches Interleukin-2 exprimieren: die endogene IL-2 Produktion bietet eine effektivere Unterstützung für adoptiv-transferierte T-Zellen. Unterschiede im Ansprechen auf die Behandlung zwischen herkömmlichen NOG-Mäusen und humanes Interleukin-2 exprimierenden NOG-Mäusen, die in unseren In-vivo-Experimenten verwendet wurden, ergaben wichtige Hinweise auf mögliche Mechanismen der Persistenz bzw. der Erschöpfung transferierter T-Zellen. Schließlich ergaben erste Sicherheitstests zur Vorhersage einer möglichen Kreuzreaktivität oder Allo-Reaktivität keine Anzeichen für eine „Off-Target“-Reaktivität des mutationsspezifischen TCR.

Insgesamt deuten unsere Daten darauf hin, dass mutationsspezifische TCRs zur Bekämpfung der MyD88-L265P-Mutation eingesetzt werden können und

vielversprechend für eine echte tumorspezifische, zielgerichtete Therapie bei einer großen Untergruppe von B-Zell-Malignomen sind.

2. Synopsis

2.1. Introduction, state of research, significance of the work for the discipline

The concept of immunity against illnesses was known since centuries and early forms of immunization have been developed and used by different civilizations long before we began to understand cellular and molecular components of immune system. Emil von Behring and Shibasaburo Kitasato, working in the Laboratory of Robert Koch at the University of Berlin in 1890, showed for the first time that there were protective factors in the serum of infected animals that provided resistance against infection when transferred to other animals (Behring and Kitasato, 1890). During the same years, William Bradley Coley in New York treated sarcoma patients with streptococcal organisms which lead to tumor shrinkage in some cases as a "bystander" effect of a strong immune response against the infectious agent (Coley, 1893). This study is considered as the first example of cancer immunotherapy. For many decades following it, however, efforts to develop effective immunization against cancer were mostly unsuccessful. Richmond T. Prehn and Joan M. Main of National Cancer institute stated in their publication in 1957: "The history of attempts to immunize against cancer is one of long frustration. As a result of apparent failure during the past half century, it is current consensus that immune mechanisms probably will be of little use in the control of this disease" (Prehn and Main, 1957). On the other hand, it was observed in early studies that mice can develop resistance to tumor transplantation following spontaneous recovery, and use of different mouse strains and sex affected efficiency of tumor transplantation, indicating a possible tumor-suppressor role for the immune system (Clowes and Baeslack, 1905; Gross, 1943, 1941). In 1959, Lewis Thomas suggested that immune system is capable of eliminating newly developing tumors through recognition of tumor-specific antigens (Thomas, 1959), and the theory of "cancer immunosurveillance" was debated extensively in following decades. After contradicting studies in mouse models and clinical findings, a renewed concept of "immunoediting" was proposed, which suggests that immune response can suppress tumorigenesis, but some cancerous cells could develop resistance to elimination by immune cells, and eventually adapt mechanisms of immune suppression and overcome

the control of immune system: elimination, equilibrium, and escape (Dunn et al., 2004; Swann and Smyth, 2007).

Development of monoclonal antibodies in 1975 (KÖHLER and MILSTEIN, 1975) resulted in acceleration of findings in many fields of biological and medical research. Characterization of the cells and the molecules of the immune system was made possible, generating a tremendous progress in knowledge and understanding of the mechanisms regulating immune responses.

First clinical trials using monoclonal antibodies were initiated in the early 1980s, pioneered by the group of Stuart F. Schlossman (Nadler et al., 1981; Ritz et al., 1980). Despite initial enthusiasm following the first therapeutic injection of a monoclonal antibody recognizing a B-cell leukemia-associated antigen by Lee Nadler in 1980 (Nadler et al., 1980), it took many more years to develop commercial production of clinical grade antibody, in 1992 Biogen filed an application for a new investigational drug (Rituximab), which was granted by the FDA in November 1997. Rituximab and several other Antibodies directed against the same structure (CD20, a B-cell lineage expressed in most B-cell leukemias and lymphomas) has become a standard part of the treatment in hematologic diseases as part of combined chemo-immunotherapy protocols). It can be assumed that over the past 30 years hundreds of thousands of lives have been saved thanks to the introduction of Rituximab as part of standard lymphoma-leukemia therapy considering the improvement in response rate, reduced death risk and remission duration induced by Rituximab (Dotan, Aggarwal and Smith, 2010).

In the meantime, several other Antibodies have been licensed and become a standard part of lymphoma and leukemia treatment: structure modified CD20 antibodies (e.g., Obinutuzumab), CD22 antibodies, CD19 antibodies, CD38 (Daratumumab), CD79 (Polatuzumab), BCMA antibodies, CD30 (Brentuximab). Furthermore, toxin-conjugated (Inotuzumab Ozogamicin, Polatuzumab-Vedotin, Brentuximab-Vedotin), radioimmunoconjugated (Iodine 131, Yttrium-90 and other explorative radioparticles), bispecific antibodies, (e.g., Blinatumomab) have been explored and partially established as part of relapsed- leukemia-lymphoma therapy strategies.

In 1982, James P. Allison first defined T-cell receptor (TCR) using monoclonal antibodies (Allison et al., 1982). Two years later, Tak W. Mak and Mark M. Davis described the cDNA

clones encoding the human and mouse TCRs (Hedrick et al., 1984; Yanagi et al., 1984). Studies in cancer immunology following their discovery have enabled a much better understanding of T-lymphocytes and their antigen-specific activities.

Immunotherapy using adoptive transfer of T-cells against tumor-associated antigen has been explored with particular efforts and partial success in melanoma and renal cell cancer for the past several decades, especially in the group of Steven Rosenberg at the National Institute of Health, Bethesda, MD (Topalian et al., 1987). Pivotal work investigating the role of tumor infiltrating lymphocytes (TILs) have contributed to the understanding of T-cell responses against tumors, including the role of MHC-expression, tolerance and escape mechanisms. Some success in clinical trials was achieved with the use of in vitro expanded tumor infiltrating lymphocytes (Dudley et al., 2002), but because of the toxicity associated with the treatment which included high-dose Interleukin-2 application to the patients and the difficulties in generating cell products of consistent quality, a wide-scale application of this strategy was never adopted.

A true breakthrough in cancer T-cell immunotherapy was achieved with the discovery of immune checkpoint signaling molecules (Ishida et al., 1992; Leach et al., 1996) and in particular with the development of antibodies able to target and inhibit these molecules with remarkable clinical success in some tumors, particularly in tumors with high mutational burden (Morad et al., 2021). A further step was the invention of chimeric antigen receptors (CAR) (Gross et al., 1989; Kuwana et al., 1987) combining variable regions from antibodies and constant/signaling regions of T-cell receptor became landmarks of a new era in immunotherapy, revolutionizing cancer treatment within last decade.

The introduction of T-cell engineering with chimeric antigen receptors against the B-cell antigen CD19 for the first time led to highly reproducible, clinically remarkable success in the 2010s, primarily in B-cell leukemia (Grupp et al., 2013; Kalos et al., 2011). In the following years, response in around 50% of patients with refractory and relapsed diffuse large B-cell lymphoma (DLBCL) was reported (Kochenderfer et al., 2015; Neelapu et al., 2017). Similar strategies have been developed targeting other B-cell lineage antigens such as CD20, CD22 and BCMA (Fry et al., 2018; Raje et al., 2019; Zhang et al., 2016). However, relapse of disease occurs in about 50% of treated patients, mostly due to loss

of surface expression of the target antigen (Braig et al., 2017; Sotillo et al., 2015). Furthermore, albeit being far more specific than chemotherapy, CAR-T cell therapy targets lineage specific antigens, therefore attacking the whole B-cell compartment including normal B-lymphocytes, frequently leading to severe B-cell aplasia that may require long term immunoglobulin substitution. So far, all the targets addressed by CAR-T cells involve lineage-specific or lineage-associated antigens that even if overexpressed in tumor cells, are expressed on a normal counterpart of antigen-bearing cells, which are potentially eliminated by the adoptively transferred T cells. Screening and evaluation of cellular antigens for T-cell therapy with higher specificity and efficacy is a rapidly progressing area of research, however, cancer-specific changes such as oncogenic mutations occur mostly in intracytoplasmic proteins regulating cell proliferation, survival

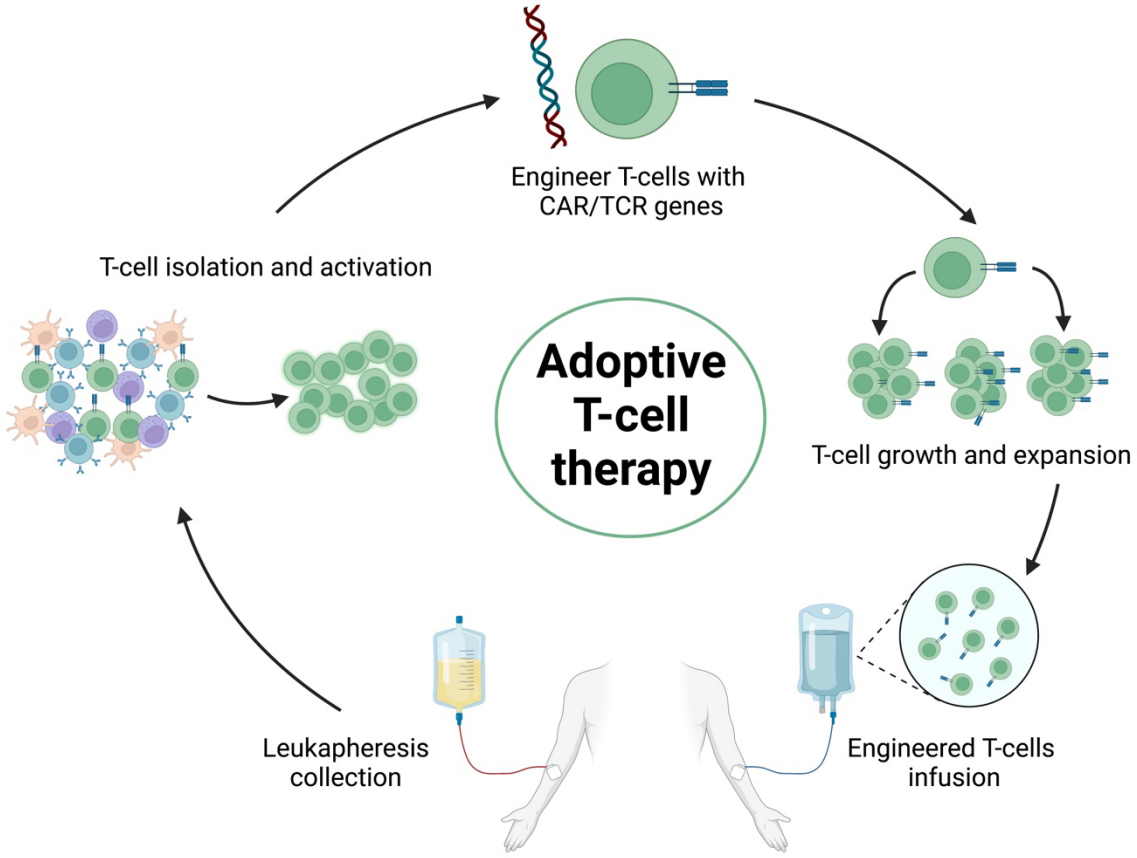

Figure 1: Schematic explanation of cellular immunotherapy using adoptive transfer of engineered T-cells. (Created with BioRender.com)

or sensitivity to drugs (Futreal et al., 2004). Targeting such intracellular antigens using antibodies, antibody fragments, or antibody derivatives like CARs and bispecific antibodies is very challenging (Slastnikova et al., 2018; Trenevska et al., 2017).

In contrast, TCR-based adoptive T-cell therapy relies on recognition of processed epitopes of any given antigen presented on MHC (Major Histocompatibility Complex; human leukocyte antigen–HLA for humans) molecules, independent of its cellular localization. Taking advantage of MHC presentation of epitopes greatly widens the spectrum of antigens that can be targeted, including truly cancer specific mutant antigens –so called “neoantigens”, derived from somatic mutations acquired during tumor development (Blankenstein et al., 2015). Oncogenic driver mutations among all cancer associated antigens are the most attractive targets for TCR gene therapy, if naturally processed epitopes harboring the mutation are presented on MHC molecules with high affinity (Malekzadeh et al., 2019; Tran et al., 2016). Many driver mutations however occur in highly variable positions of the target protein sequence, with many different hotspots and different amino acid substitutions, making immunotherapy highly individual and

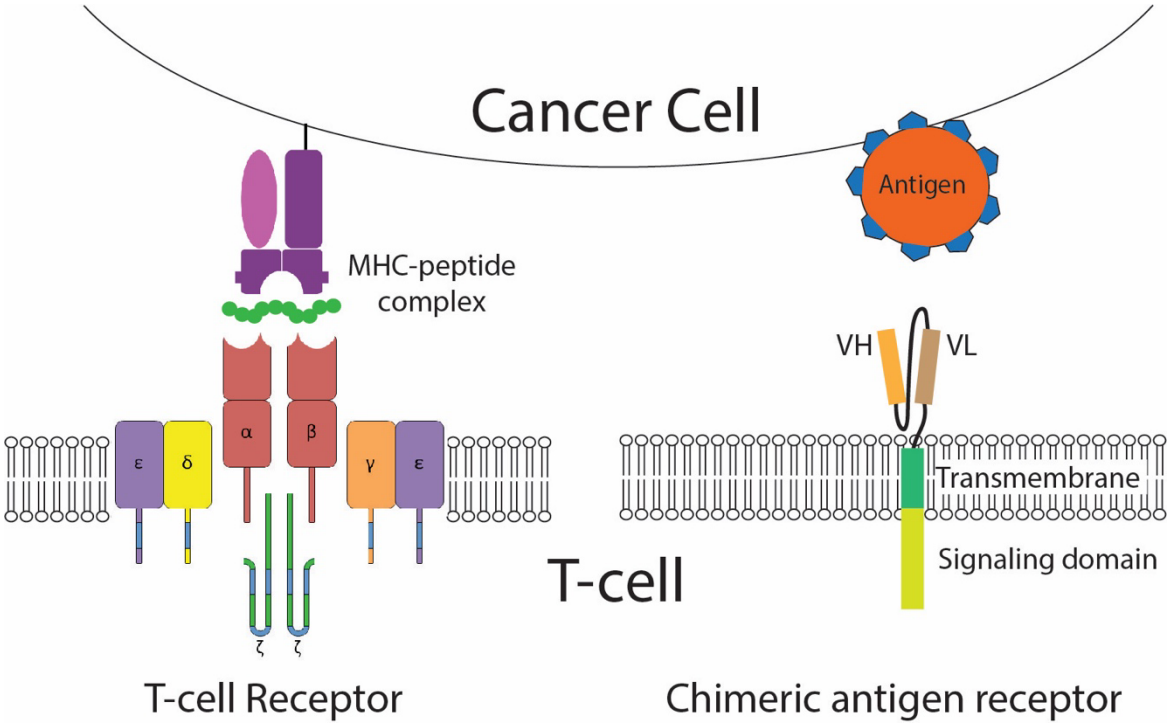

Figure 2: Differences in structure and target recognition between T cell receptor (TCR) and chimeric antigen receptor (CAR).

technically challenging. On the other hand, a handful of driver mutations can be shared by large groups of patients, which are also vital for survival of cancer cells.

Myeloid differentiation primary response 88 (MyD88) is an intracellular adaptor protein that mediates toll-like and interleukin (IL)-1 receptor signaling and has a central role in immunity. A missense mutation changing leucine in position 265 to proline (L265P) in MyD88 was first identified by Ngo et al. in diffuse large B-cell lymphomas (DLBCL) and found to be associated with more aggressive/less curable form called activated B cell-like (ABC)-DLBCL, providing genetic basis for constitutive nuclear factor (NF)-κB and JAK kinase signaling that promotes malignant cell survival in this disease (Ngo et al., 2011). Further studies revealed that MyD88 L265P is a gain-of-function mutation and one of the most common drivers of lymphomagenesis which can be found in over 90% of Waldenström Macroglobulinemia cases and around one-fifth of all lymphoid malignancies combined (Weber et al., 2018). Among high-grade lymphoma cases, MyD88 L265P is more frequently found in aggressive and therapy resistant forms (e.g., 30% of ABC-DLBCL and 45% of primary CNS lymphoma cases), which makes it an extremely valuable target for immunotherapy (Lee et al., 2017; Rovira et al., 2016).

Targeting such cancer specific genetic alterations might pave the way for an ideal targeted immunotherapy. We validated an epitope sequence harboring the MyD88 L265P mutation to be processed and presented in the context of MHC-I (HLA-B*07:02) by cells harboring the mutation, generated high-affinity TCRs recognizing this epitope with high specificity with the aim of eliminating lymphoma cells without harming healthy compartments, tested the specificity of the recognition and evaluated the safety of the TCR in preliminary tests.

2.2. Methodology

Mutations in MyD88 protein occur almost universally by substitution of a Leucine in position 265 with a Proline: MyD88 L265P. Peptides spanning this region have been analyzed for binding to common HLA alleles by in silico binding prediction using NetMHC 4.0 (Andreatta and Nielsen, 2016). A 10-mer sequence (R***P***IPIKYKAM) was predicted to

bind HLA-B*07:02 –one of the most commonly expressed HLA-B alleles globally– with high affinity, while corresponding wild-type sequence was not.

CD8+ naïve T-cells isolated from healthy HLA-B7-positive donors were primed with autologous dendritic cells pulsed with the mutant peptide. We have been successful in generating strong T-cell responses from multiple donors, detected using a custom MHC-peptide streptamer.

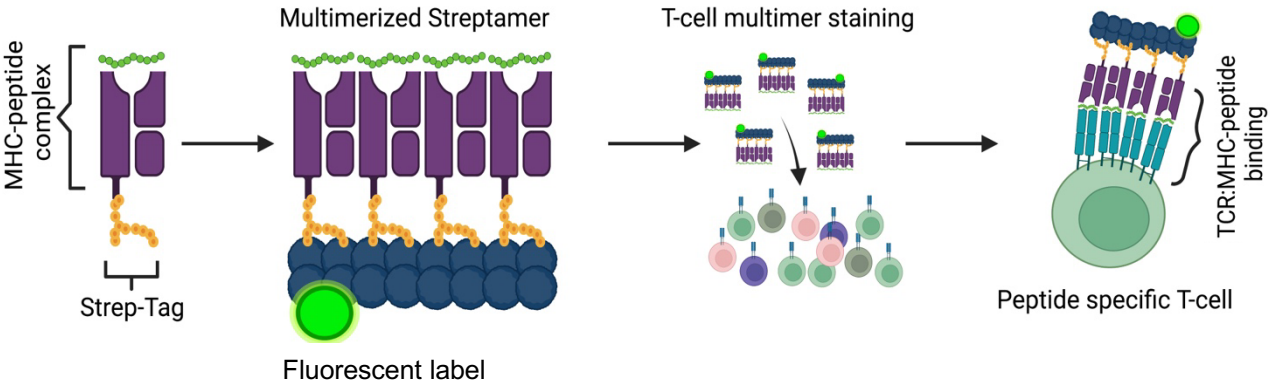

Figure 3: Schematic explanation of streptamer staining for selection of peptide-specific T-cells. (Created with BioRender.com)

After re-stimulation with peptide loaded autologous PBMCs, expanding streptamer-positive T-cells were tested for selective reactivity against mutant peptide and then sorted using FACS for analysis of TCR sequence.

Variable regions of TCR alpha and beta genes from 13 different T-cell lines have been combined with mouse TCR constant domains and cloned into a retroviral vector for further functional characterization. T-cells of HLA-B7-positive healthy donors were transduced to express these transgenic TCRs (TCR-T cells) and initially co-cultured with HLA*B07:02-positive artificial target cells (transduced K562 cell line) that are pulsed with different concentrations of mutant or wild-type peptide, for assessment of functional avidity. In order to test specificity of the T-cell response, TCR-T cells were then co-cultured first with engineered target cells that were stably transduced to express wild-type or mutant MyD88, with or without HLA-B*07:02 co-expression. Similarly, we repeated these in vitro tests using lymphoma cell lines that naturally harbor homozygous or heterozygous MyD88 L265P.

Following detailed in vitro characterization, we developed xenograft mouse models using non-Hodgkin lymphoma cell lines OCI-Ly3 and TMD8, with natural homozygous or heterozygous MyD88 L265P mutation respectively, to test therapeutic efficacy of our lead T-cell receptor; TCR2304. We used both conventional NOG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac) and hIL2-NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(CMV-IL2)4-2Jic/JicTac) mice -which due to their production of human IL-2 provide better support for the growth of adoptively transferred human T cells- to establish our in vivo models. Cells were subcutaneously injected to mice, and single dose of $1x10^7$ TCR-T or Mock T-cells was used as treatment. We used caliper and bioluminescence measurements to monitor tumor growth, and analyzed various tissues including tumor, blood, bone marrow, spleen and liver for tumor and T-cell infiltration via flow cytometry.
A detailed description of all methods can be found in the Materials and Methods section of Çınar et al., 2021 (Çınar et al., 2021).

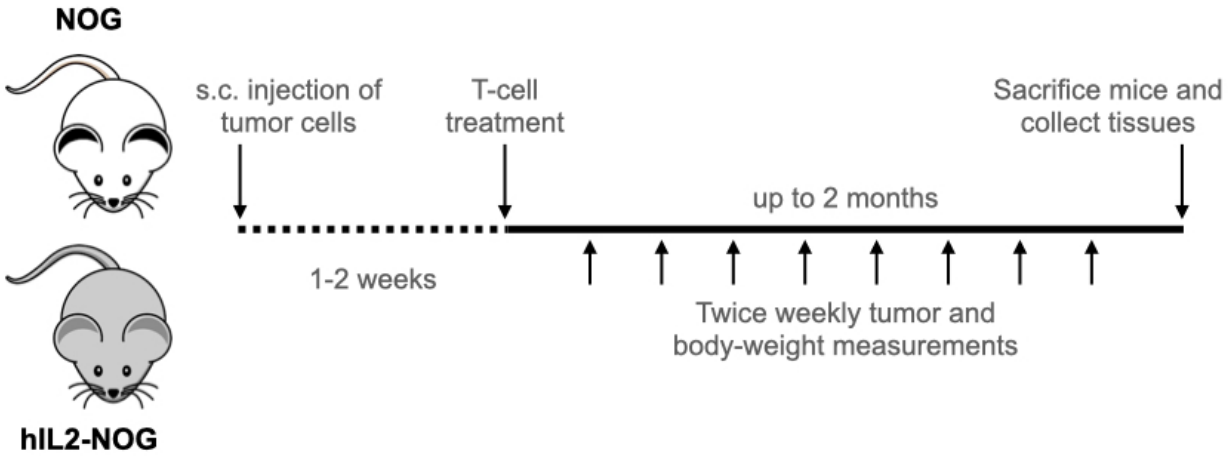

Figure 4: Study design for in vivo efficacy testing of TCR-T cells.

2.3. Results

In vitro autologous priming was performed from more than 20 healthy donors to generate mutation-specific T cell responses against MyD88 L265P, and 13 TCR α/β pairs were sequenced from 5 independent donors.

Table 1: CDR3 sequences and VDJ gene subtypes of MyD88 L265P mutation-specific TCRs.

TCR	Chain	CDR3	V-gene	J-gene	D-gene
1336	α	CAASGRYDYKLSF	TRAV13-1*02	TRAJ20*01	—
	β	CATASDLQGDRSTEAFF	TRBV15*02	TRBJ1-1*01	TRBD1*01
1605	α	CAEGTGSARQLTF	TRAV13-2*01	TRAJ22*01	—
	β	CASGPFRDSVLTLVANVLTF	TRBV28*01	TRBJ2-6*01	TRBD2*01
1610	α	CAPLGGGYNKLIF	TRAV21*01	TRAJ4*01	—
	β	CASRLPTTDEKLFF	TRBV6-6*02	TRBJ1-4*01	TRBD1*01
2202	α	CLSLSDSNYQLIW	TRAV4*01	TRAJ33*01	—
	β	CASSVGQGSYEQYF	TRBV9*01	TRBJ2-7*01	TRBD1*01
2205	α	CLVGRDGGSYIPTF	TRAV4*01	TRAJ6*01	—
	β	CASSAGQGAYEQYF	TRBV9*02	TRBJ2-7*01	TRBD1*01
2207	α	CAVDVGYSTLTF	TRAV1-2*01	TRAJ11*01	—
	β	CSARDRSGTLGGELFF	TRBV20-1*01	TRBJ2-2*01	TRBD2*02
2211	α	CIVRVMKTSYDKVIF	TRAV26-1*01	TRAJ50*01	—
	β	CASSEPRTSGISYNEQFF	TRBV10-1*02	TRBJ2-1*01	TRBD2*02
2219	α	CGTAHLRAGSYQLTF	TRAV30*01 / TRAV30*02	TRAJ28*01	—
	β	CASSSSSGGAFNEQFF	TRBV27*01	TRBJ2-1*01	TRBD2*01
2304	α	CAVRASGTYKYIF	TRAV1-2*01	TRAJ40*01	—
	β	CASQDSYEQYF	TRBV12-3*01	TRBJ2-7*01	*No result*
2705	α	CAMSGTGGFKTIF	TRAV12-3*01	TRAJ9*01	—
	β	CASSQDRPNYYGYTF	TRBV4-3*01	TRBJ1-2*01	TRBD1*01
2709	α	CILRDRYGGSQGNLIF	TRAV26-2*01	TRAJ42*01	—
	β	CASSYWPTTGESTDTQYF	TRBV6-2*01 / TRBV6-3*01	TRBJ2-3*01	TRBD1*01
2716	α	CAFMKPYSGGGADGLTF	TRAV38-1*01	TRAJ45*01	—
	β	CASSLAGTTVYNEQFF	TRBV13*01	TRBJ2-1*01	TRBD2*01
2719	α	CLVGADSNYQLIW	TRAV4*01	TRAJ33*01	—
	β	CASSPGGGAYEQYF	TRBV9*01	TRBJ2-7*01	TRBD2*01

Peripheral CD8+ T-cells from HLA-B7-positive healthy individuals were successfully transduced to express these mutation-specific TCRs, with no signs of fratricide, and co-cultured with K562 cells that were transduced with HLA*B07:02 and loaded with different concentrations of mutant or wild-type peptide. Response to mutant peptide was detectable below the concentration of 0.1 ng/ml with EC50 values within the nano molar (high-affinity) range. Mutation-specific TCRs showed more than 10,000-fold higher affinity to the mutant peptide than to the wild-type corresponding peptide (Çınar et al., 2021 Fig. 2D).

K562 cells with or without HLA-B*07:02 were then virally transduced to express complete length wild-type or mutant MyD88. When co-cultured with TCR-T cells for 16 hours, seven different TCRs led to recognition of target cells expressing the mutant MyD88 without prior peptide loading, suggesting that the epitope can successfully be processed and presented by human cells. Furthermore, target cells that express both the mutation and HLA*B07:02 were selectively killed by TCR-transduced T-cells (Çınar et al., 2021 Fig. 3).

To investigate the functional activity of the TCR against mutation-harboring cells in a more natural-like expression level, activation of T-cells transduced with the highest avidity TCR (TCR2304) was analyzed by flow cytometry after 16-h co-culture with OCI-Ly3 (ABC-like DLBCL cell line, homozygous for the MYD88 L265P mutation), TMD8 (ABC-like DLBCL cell line, heterozygous for MYD88-L265P) or as control SU-DHL-6 (GBC-DLBCL, wild-type MyD88) lymphoma cell lines. T-cells engineered with TCR2304 specifically recognized and lysed lymphoma cells expressing MyD88 L265P and HLA-B*07:02. In addition, TCR recognition of lymphoma cells lead to antigen induced proliferation of T-cells suggesting that in vivo expansion of adoptively transferred engineered T cells might occur upon contact with mutated lymphoma cells (Çınar et al., 2021 Fig. 4).

Furthermore, we established xenograft models in immunodeficient NOG and humanized immunodeficient hIL2-NOG mice using the OCI-Ly3 and TMD8 lines to evaluate the in vivo therapeutic potential of mutation-specific TCRs. The hIL-2 NOG mice produce human IL2 which is likely to help expansion of adoptively transferred human cells upon antigenic stimulation (Forsberg et al., 2019). Adoptive transfer of TCR2304 gene modified T-cells resulted in durable regression of lymphoma xenografts in both mouse models. In addition to the promising therapeutic efficacy, we were able to observe and analyze

differences in treatment response between common and humanized hIL2-NOG mice, and found indications for potential mechanisms of treatment escape. T-cells persisted longer in tissues of mice when there is cytokine support, moreover the treatment response was faster and more stable (Çınar et al., 2021 Fig. 5). However, it is worth mentioning that increased T-cell proliferation in hIL2-NOG mouse, especially with high dose treatment, lead to unspecific T-cell toxicity due to graft versus host disease, which may complicate experimental results.

Finally, we analyzed epitope binding motifs of mutation-specific TCRs using amino-acid replacement assays to assess potential cross-reactivity which might be of concern for immune damage of normal body constituents, and repeated testing revealed extreme specificity for the mutant epitope. We also checked for allo-reactivity against common HLA-alleles using a library of EBV-immortalized lymphoblastoid B-cell lines (LCLs): initial safety screening did not provide any sign of potential cross- or allo-reactivity risk (Çınar et al., 2021 Supp. Fig. 4-5).

Taken together, our data suggest that mutation-specific TCRs could be used to target MyD88 L265P mutation in vivo, holding promise for precision therapy for a significant subgroup of B-cell malignancies in HLA-B*:07:02-positive individuals.

2.4. Clinical applications, limitations of the work, further questions

Lymphoproliferative malignancies are a major group of cancers and responsible for one-tenth of all cancer deaths in Europe. Around 1500-2000 new cases of high-grade B-cell lymphoma are expected yearly in Germany and up to 40% of these patients relapse after initial standard treatment with chemoimmunotherapy, or will not respond to the first line treatment (primary refractory lymphomas) indicating the urgent need for alternative treatment options.

Chemotherapy is still the main treatment for most cancer types despite its limitations regarding toxicity and therapy resistance. Several chemotherapy regimens in combination with monoclonal antibodies targeting the B-cell antigen CD20 are widely used as first line treatment for Diffuse Large B-cell Lymphoma, with a cure rate of around 60% (Robert Koch Institute (ed.) and the Association of Population-based Cancer

Registries in Germany (ed.), 2020). Even high-dose chemotherapy with autologous stem cell rescue can salvage less than a third of patients with relapsed/refractory disease. Other diseases such as primary CNS lymphoma has an even poorer prognosis: only high-dose chemotherapy appears to be curative, but this is feasible only in a minority of patients because of age limitations and comorbidities, as this lymphoma sub-entity affects mainly elderly patients.

The idea of taking advantage of the host immune system for cancer treatment has been around for a long time, however immunotherapy has become an area of global interest in recent years only after remarkable success in clinical trials using checkpoint inhibitors or adoptive transfer of engineered T-cells.

All antibody-based immunotherapy strategies, and therefore even the CAR-T cell therapy which target cell surface antigens recognized by small antibody fragments are not truly tumor specific but rather tumor-associated, since all antigens targeted so far are normal differentiation antigens also expressed in a population of normal body cells.

Most oncogenic mutations originate within the cytoplasm or the nucleus of the cells and only in minority of cases cell surface proteins are involved. Rather, transcription factors, adapter proteins, and regulators of cell cycle and proliferation are affected by mutations that turn normal cells into malignant cells. Such mutations, which are truly tumor specific, can be potentially recognized by the immune system. This can happen only via presentation of protein fragments within the molecules of the Major Histocompatibility Complex (HLA). Theoretically, 6 different class-I alleles of these molecules (HLA-A, B, C) can be expressed in each body cell, with a genetic mendelian inheritance mechanism from both parents. The genes coding for the HLA molecules are highly heterogeneous, with some HLA alleles being overrepresented in ethnic populations (e.g., %35 of the Caucasian population in western Europe and USA expresse at least one copy of HLA-A2, with the subtype HLA-A*02:01 being the most frequent allele).

A major limitation for the success of mutation-specific immunotherapy is that in many cancers, mutations do not occur at the same “spot”, but at a highly variable number of positions along the sequence of a protein. For one of the proteins most frequently affected by cancerogenic mutation, like p53, a large number of different possible mutations has been described.

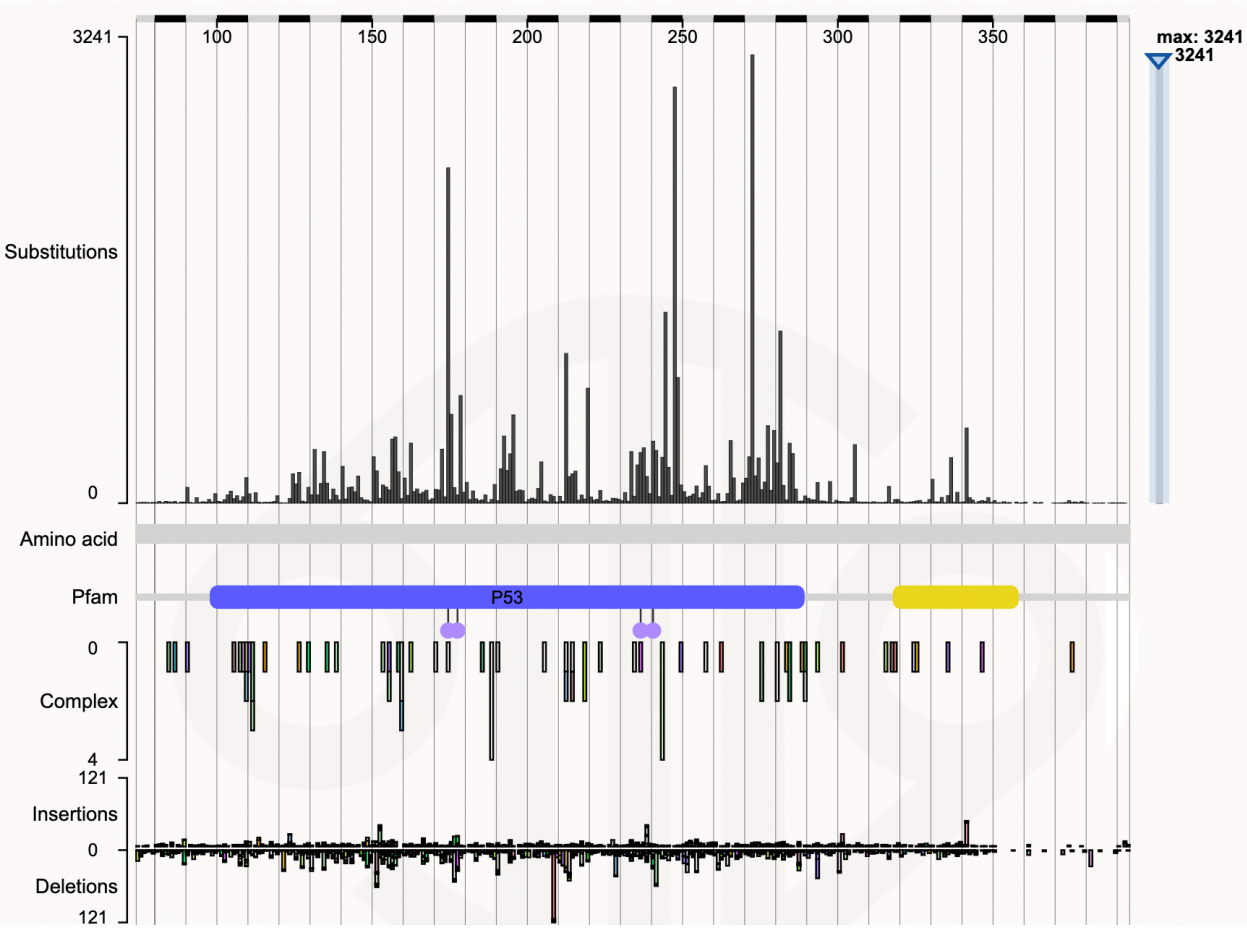

Figure 5: Graphical summary of identified cancer related mutations in P53 (https://cancer.sanger.ac.co.uk/cosmic - Tate et al., 2019).

It is possible and indeed frequent, that some of the mutations in p53 do not generate mutated peptides that able to fit the HLA-molecules of the host cell. For instance, none of the most recurrent p53 mutations bind to HLA-A2, making it impossible for individuals bearing this highly frequent allele to recognize mutated p53 peptides and generate mutation-specific T-cell responses, unless they also express some of the less common HLA alleles that bind to the mutant peptides.

Therefore, in a large number of cases induction of truly tumor-specific immune response remains impossible. This is particularly true, when the number of mutations leading to a cancerogenic phenotype is limited such in the case of p53 or k-RAS mutated tumors. On the contrary, the larger the number of mutations present in a given tumor, the higher is

the likelihood that a mutation is successfully presented in the available MHC molecules and a T-cell response can be generated.

In fact, a similar observation has been made with agents targeting the immune checkpoint: this therapy strategy is successful in tumors with high mutational burden, where a large number of mutated proteins are present and available for potential presentation by the MHC molecules of the patient and therefore for T-cell recognition.

In the first months of my doctoral work, I have evaluated several proteins for the presence of epitopes that bind to the most frequent HLA-alleles. I have tested some antigens specific for the B-cell lineage such as kappa and lambda immunoglobulins, as well as other commonly mutatated proteins such as STAT3. We finally decided to concentrate our efforts on MyD88, since it is the most common single point mutation overall in lymphoid malignancies and a 10-mer peptide harboring the mutation was predicted to bind with high affinity to HLA-B7, an HLA molecule broadly present in the European population.

One possible question that may arise is: If the tumor-specific peptide harboring the mutation is naturally present, why does the tumor occur at all? Should the immune system not be able to immediately recognize the cells affected by the mutation and eliminate them? First of all, it should be evaluated in large databases whether individuals with HLA-B7*07:02 have a lower-than-expected frequency of MyD88 L265P positive tumors. Such studies are difficult to conduct but should be addressed. However, we assume that this is not the case. There are several explanations for the lack of an effective immune response even in the presence of mutated and presented peptides. It is commonly assumed that the immunosuppressive environment created by tumors induce the depletion or inhibition of immune cells recognizing putative tumor antigens with high affinity. Indeed, even in our experiments with xenotransplant tumors, we collected evidence that the quality of the T-cell response with the dominance of a particular type of T-cells, or the presence of inhibitory molecules in some of the T-cells may significantly affect their ability to eliminate the tumor. We are planning to address this aspect of the T cell therapy in a clinical trial. Theoretically, optimal affinity TCRs could be isolated from mice, which have not been in contact with the tolerogenic environment of a tumor (Obenaus et al., 2015). However, since murine TCRs are not negatively selected for human thymic antigens, the risk of

serious cross-reactivity is high, and indeed such toxic reaction have been observed in clinical trials, whereby severe autoreactivity against human colon mucosa had been observed (Parkhurst et al., 2011). For both safety and technical reasons therefore, significant amount of research is focused on generation of high-affinity TCRs in humans, e.g. by ex-vivo immunization of tumor-free hosts, where high-affinity T cells are supposed to be present (Ali et al., 2019).

A further reason for the failure of T-cell immune response against tumors is the possibility that a mutated epitope initially generated a potent T-cell response, potentially leading to killing of some the tumor cells harboring the mutation. However, if the mutation is a “secondary/passenger” mutation, originated in a second phase of tumor growth, it may not be fundamental for the tumor growth. Elimination of a subclone of tumor cells bearing the “secondary” mutation by host immune system will not eliminate the tumor.

MyD88 L265P is an important target not just because it is a recurrent mutation in many lymphoma cases, but also because it is the primary oncogenic driver event in many lymphomas. We assume that all tumor cells need the mutation and that the development of a mutation-negative subclone should be extremely unlikely. We have shown that mutation specific TCRs isolated form healthy donors can be used to specifically target cancer cells expressing this mutation, and hold promise for novel immunotherapy applications. We believe that besides CAR-T cell therapy, which has become an important tool for the treatment of relapsed and poor prognosis lymphomas and leukemias, there is a great potential in the use of TCR-T cell therapy both in hematological and solid tumors. Our study provides a strong base for such a highly tumor-specific, molecularly defined adoptive T-cell therapy. T cells expressing the TCR cloned in this work may represent a novel first in class reagent for cancer therapy. Accordingly, an international patent has been filed in collaboration with Charité Technology Transfer Office (international Application No. PCT/EP2020/051405, Publication No WO2020/152161 A1 (PEZZUTTO and CINAR, 2020) and a pilot phase I clinical trial for assessment of TCR2304 is being planned, having passed positive initial evaluation by the Paul-Ehrlich Institute in February 2022, and having received a positive first evaluation for funding for a clinical trial by the Research Ministry (Bundesministerium für Bildung und Forschung - BMBF) in July 2022 (PD Dr. Antonia Busse).

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3. Statutory Declaration

“I, Özcan Çınar, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic, “**Development of mutation-specific T-cell receptor gene therapy for treatment of B-cell malignancies**” **(Entwicklung einer mutationsspezifischen T-Zell Rezeptor Gentherapie für die Behandlung von B-Zell Neoplasien)**, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

4. Declaration of contribution to the top-journal publication for a PhD degree

Authors: Özcan Çınar, Bernadette Brzezicha, Corinna Grunert, Peter Michael Kloetzel, Christin Beier, Caroline Anna Peuker, Ulrich Keller, Antonio Pezzutto, Antonia Busse

Title: High-affinity T-cell receptor specific for MyD88 L265P mutation for adoptive T-cell therapy of B-cell malignancies

Journal: Journal for Immunotherapy of Cancer

Publication date: 30.07.2021

Author Contribution in details:

Özcan Çınar and Antonio Pezzutto developed the concept of the study, and they are inventors on a filed patent application for the T-cell receptors described in the publication (WO 2020/152161 A1).

Özcan Çınar (Figure 2, 3, 4, 5, and 6), Bernadette Brzezicha, Corinna Grunert, Christin Beier (Figure 1) and Caroline Anna Peuker performed the experiments for the study.

Analysis of the experiments were done by Özcan Çınar (Figure 2, 3, 4, 5 and 6) and Christin Beier (Figure 1). Özcan Çınar performed the statistical analysis and designed the figures.

Özcan Çınar, Peter Michael Kloetzel, Antonio Pezzutto and Antonia Busse interpreted the data. Antonio Pezzutto supervised the study.

Özcan Çınar and Antonio Pezzutto wrote the original manuscript, and all authors stated in author list read and revised the manuscript.

Berlin,

_____ Signature of the Student

_____ Signature, date and stamp of the Supervisor

5. Extract from the Journal Summary List "IMMUNOLOGY"

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE,SSCI
Selected Categories: **"Immunology"** Selected Category Scheme: WoS
Gesamtanzahl: 158 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS IMMUNOLOGY	42,168	40.358	0.068470
2	IMMUNITY	54,819	22.553	0.124040
3	NATURE IMMUNOLOGY	44,709	20.479	0.081870
4	Annual Review of Immunology	17,080	19.900	0.019310
5	Lancet HIV	3,301	14.813	0.018090
6	IMMUNOLOGICAL REVIEWS	15,973	13.939	0.026690
7	Science Immunology	2,516	13.440	0.015070
8	TRENDS IN IMMUNOLOGY	13,037	13.422	0.025020
9	JOURNAL OF EXPERIMENTAL MEDICINE	63,562	11.743	0.067350
10	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	52,417	10.228	0.077410
11	Journal for ImmunoTherapy of Cancer	4,557	9.913	0.016030
12	SEMINARS IN IMMUNOLOGY	5,345	9.186	0.010250
13	Cancer Immunology Research	6,969	8.728	0.026440
14	ALLERGY	18,217	8.706	0.024340
15	Cellular & Molecular Immunology	4,604	8.484	0.010020
16	CLINICAL INFECTIOUS DISEASES	66,656	8.313	0.123760
17	AUTOIMMUNITY REVIEWS	9,902	7.767	0.018300
18	Journal of Allergy and Clinical Immunology-In Practice	5,946	7.574	0.018100
19	Seminars in Immunopathology	3,734	7.480	0.006690
20	CURRENT OPINION IN IMMUNOLOGY	9,628	7.290	0.016900
21	JOURNAL OF CLINICAL IMMUNOLOGY	5,262	6.780	0.007610

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Journal for ImmunoTherapy of Cancer

High-affinity T-cell receptor specific for MyD88 L265P mutation for adoptive T-cell therapy of B-cell malignancies

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ABSTRACT

Background Adoptive transfer of engineered T cells has shown remarkable success in B-cell malignancies. However, the most common strategy of targeting lineage-specific antigens can lead to undesirable side effects. Also, a substantial fraction of patients have refractory disease. Novel treatment approaches with more precise targeting may be an appealing alternative. Oncogenic somatic mutations represent ideal targets because of tumor specificity. Mutation-derived neoantigens can be recognized by T-cell receptors (TCRs) in the context of MHC–peptide presentation.

Methods Here we have generated T-cell lines from healthy donors by autologous in vitro priming, targeting a missense mutation on the adaptor protein MyD88, changing leucine at position 265 to proline (MyD88 L265P), which is one of the most common driver mutations found in B-cell lymphomas.

Results Generated T-cell lines were selectively reactive against the mutant HLA-B*07:02-restricted epitope but not against the corresponding wild-type peptide. Cloned TCRs from these cell lines led to mutation-specific and HLA-restricted reactivity with varying functional avidity. T cells engineered with a mutation-specific TCR (TCR-T cells) recognized and killed B-cell lymphoma cell lines characterized by intrinsic MyD88 L265P mutation. Furthermore, TCR-T cells showed promising therapeutic efficacy in xenograft mouse models. In addition, initial safety screening did not indicate any sign of off-target reactivity.

Conclusion Taken together, our data suggest that mutation-specific TCRs can be used to target the MyD88 L265P mutation, and hold promise for precision therapy in a significant subgroup of B-cell malignancies, possibly achieving the goal of absolute tumor specificity, a long sought-after dream of immunotherapy.

INTRODUCTION

Recently, clinical studies of adoptive T-cell therapy (ATT) using chimeric antigen receptor T (CAR-T) cell therapy against the B-cell antigen CD19 have achieved remarkable success and have shown response in around 50% of refractory and relapsed patients with diffuse large B-cell lymphoma (DLBCL).[1 2] Similar strategies have been developed targeting other B-cell lineage antigens such as CD20, CD22 and BCMA (B-cell maturation antigen).[3–5] However, tumor escape by modulating surface expression of the target antigen is a limitation of this strategy, leading to relapse in up to 50% of patients treated with CD19 CAR-T cells.[6 7] Furthermore, although being much more specific than standard chemotherapy, CD19 CAR-based ATT is lineage-specific rather than truly cancer-specific, as major parts of the B-cell compartment including normal B lymphocytes are eliminated. This frequently leads to severe B-cell aplasia that may cause morbidity or requirement for long-term immunoglobulin substitution and/or antibiotic prophylaxis.[8]

Surface antigens druggable by antibodies or antibody derivatives are only rarely tumor-specific, as oncogenic mutations occur mostly in intracellular proteins regulating cell proliferation and survival.[9] In contrast to CAR-based strategies, T-cell receptor (TCR)-based ATT relies on classical TCR recognition of processed antigen-derived epitopes presented in the context of MHC (major histocompatibility complex) molecules. The ability to target any protein independent of cellular localization greatly widens the spectrum of target antigens including truly cancer-specific mutant antigens, so called 'neoantigens', derived from somatic mutations that are acquired in the course of tumor development.[10]

Among all cancer-associated somatic mutations, oncogenic driver mutations are obviously the most attractive targets for TCR gene therapy. A prerequisite for TCR-based therapies is that the mutation leads to generation of peptides presented on MHC molecules with high affinity.[11 12] Selection of antigen-loss variants within the tumor is unlikely, if a driver mutation is targeted that is crucial for

oncogenic transformation. However, many driver mutations such as common p53 mutations occur at many variable positions of a given protein, creating a large number of potential target epitopes, which would need to be addressed individually. In contrast, a missense mutation almost consistently changing leucine in position 265 to proline (L265P) in the MyD88 adaptor protein is one of the most common driver mutations found in about one-fifth of all lymphoid malignancies. MyD88 L265P is the hallmark mutation in Waldenström macroglobulinemia, but it is also frequently found in aggressive B-cell lymphomas, for example, 30% of activated B cell (ABC)-like DLBCL, 45%–60% of central nervous system lymphomas and testicular lymphomas,[13–16] diseases with a huge need for novel specific and well-tolerated therapies.

In this study, we developed high-affinity TCRs recognizing the mutant peptide sequence in MyD88 L265P with specific killing capacity towards the mutant malignant B-cell population. Targeting cancer-specific genetic alterations with highly specific immunotherapies represents a huge step towards precision cellular therapy in oncology.

MATERIALS AND METHODS

In vitro antigen processing and mass spectrometry

Before trying to generate TCRs specific for mutated MyD88, we checked whether a peptide spanning the mutation is processed by the proteasome and potentially presented by the MHC complex. The polypeptide substrate MyD88 $L265P_{256–281}$ was synthesized by the core facility of the Institute of Biochemistry using standard Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) methodology (0.1 mmol) on an Applied Biosystems 433A automated synthesizer. The peptide was purified by high-performance liquid chromatography and analyzed by mass spectrometry (ABI Voyager DE PRO). 20S proteasomes were purified from human red blood cells, in principle following the procedure as previously described by Textoris-Taube *et al*.[17] Proteasome digests of the synthetic MyD88 $L265P_{256–281}$ polypeptide were performed in 100 µL of TEAD buffer (20 mM Tris, 1 mM EDTA, 1 mM NaN_3, 1 mM Dithiothreitol, pH 7.2) over time at 37°C. For establishing a full-scale cleavage map, processing times were 48 hours. Proteasomal processing of the synthetic of polypeptides was performed at a substrate concentration of 40 µM in the presence of 4 µg 20S proteasome. Digested samples of 10 µL were loaded for 5 min onto a trap column (PepMap C18, 5 mm×300 µm×5 µm, 100 Å; Thermo Fisher Scientific, Massachusetts, USA) with 2:98 (v/v) acetonitrile:water containing 0.1% (v/v) Trifluoroacetic acid at a flow rate of 20 µL/min and analyzed by nanoscale LC-MS/MS using an Ultimate 3000 and LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The system comprises a 75 µm inner diameter ×250 mm nano LC column (Acclaim PepMap C18, 2 µm; 100 Å; Thermo Fisher Scientific) or a 200 mm PicoFrit analytical column (PepMap C18, 3 µm, 100 Å, 75 µm; New Objective). The mobile phase (A) is 0.1% (v/v) formic acid in water, and (B) is 80:20 (v/v) acetonitrile:water containing 0.1% (v/v) formic acid. For elution, a gradient 3%–45% B in 85 min with a flow rate of 300 nL/min was used. Full MS spectra (m/z 300–1800) were acquired on an Orbitrap instrument at a resolution of 60,000 (FWHM). At first, the most abundant precursor ion was selected for either data-dependent collision-induced dissociation (CID) fragmentation with parent list (1^+, 2^+ charge state included). Fragment ions were detected in an ion trap instrument. Dynamic exclusion was enabled with a repeat count of 2 and 60 s exclusion duration. Additionally, the theoretically calculated precursor ions of the expected spliced peptides were pre-elected for two Orbitrap CID (resolution 7500) and higher-energy collisional dissociation (HCD) (resolution 15,000) fragmentation scans. The maximum ion accumulation time for MS scans was set to 200 ms and that for tandem mass spectra (MS/MS) scans was set to 500 ms. Background ions at m/z 371.1000 and 445.1200 act as lock mass.

For LC-MS/MS runs using a Q Exactive Plus mass spectrometer coupled with an Ultimate 3000 RSLC-nano (Thermo Fisher Scientific), samples were trapped as described previously and then analyzed by the system that comprised a 250 mm nano LC column (Acclaim PepMap C18, 2 µm; 100 Å; 75 µm Thermo Fisher Scientific). A gradient of 3%–40% B (alternatively 3%–45% B) in 85 min was used for elution. Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B was 80% acetonitrile in water containing 0.1% (v/v) formic acid. The Q Exactive Plus instrument was operated in the data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Full MS spectra (m/z 200–2000) were acquired at a resolution of 70,000 (FWHM) followed by HCD MS/MS fragmentation of the top 10 precursor ions (resolution 17,500, 1^+, 2^+, 3^+, charge state included, isolation window of 1.6 m/z, normalized collision energy of 27%). The ion injection time for MS scans was set to a maximum of 50 ms, automatic gain control (AGCs) target value of 1×10^6 ions and that for MS/MS scans was set to 100 ms, AGCs 5×10^4; dynamic exclusion was set to 20 s. Background ions at m/z 391.2843 and 445.1200 act as lock mass.

Peptides were identified by PD2.1 software (Thermo Fisher Scientific) based on their merged MS/MS of CID and HCD. Based on the results, further work focused on the 10 mer peptide RPIPIKYKAM (figure 1).

Cell lines and generation of target cells

K562 leukemic cell line was cultured in RPMI with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. Non-Hodgkin's lymphoma cell lines SU-DHL-6, OCI-Ly3 and TMD8 were cultured in RPMI with 20% FBS and 100 U/mL penicillin/streptomycin. OCI-Ly3 and TMD8 cells were kindly supplied by groups of Professor Clemens Schmitt and Dr Martin Janz (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany).

SU-DHL-6, OCI-Ly3, TMD8 and K652 cells do not naturally express HLA-B*07:02. To generate

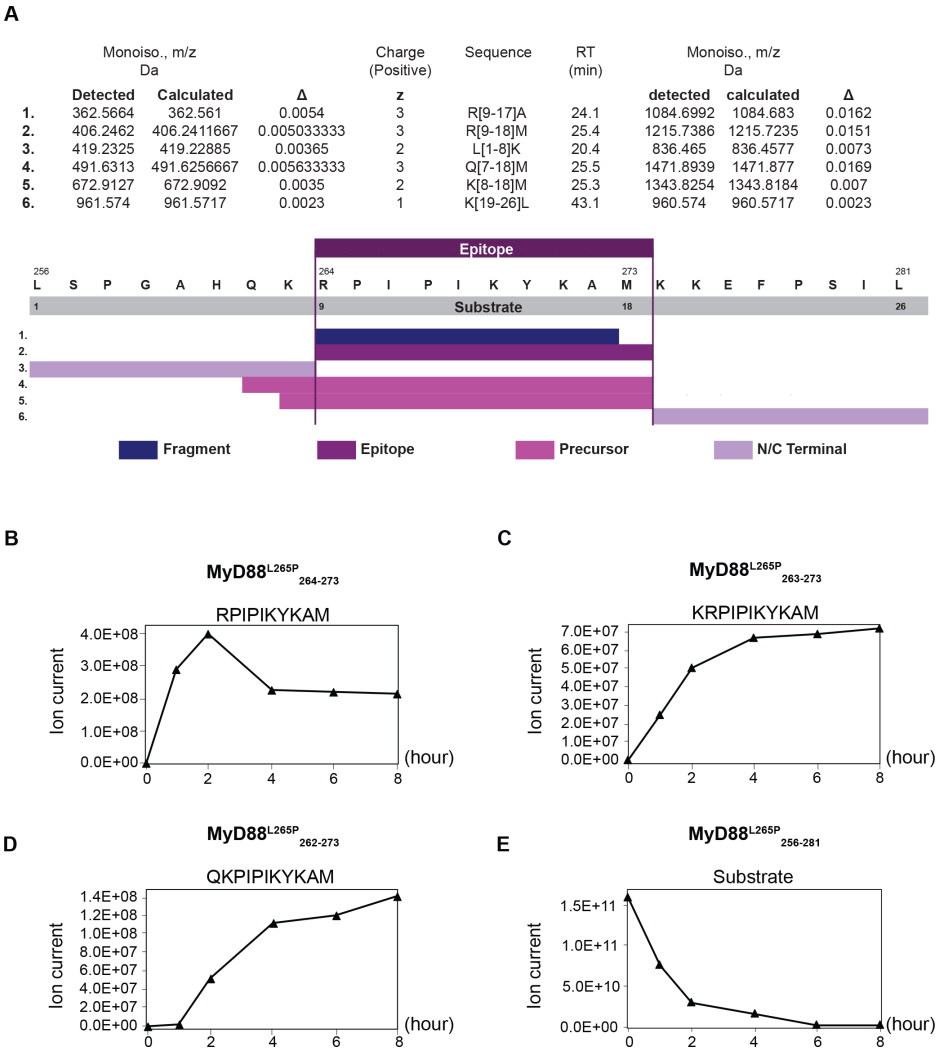

Figure 1 In vitro generation of the MyD88 L265P$_{264–273}$ neoepitope. Kinetic proteasomal antigen processing experiments were performed using the synthetic polypeptide substrate MyD88 L265P$_{256–281}$. (A) Summary of the peptides generated by the proteasome from the polypeptide substrate MyD88 L265P$_{256–281}$ in an in vitro digest. The complete list can be found in online supplemental table 4. (B–D) The generation kinetics of the MyD88 L265P-derived 10 mer neoepitope and its potential N-terminally extended precursor peptides are shown. (E) Kinetics of the degradation of the MyD88 L265P-derived polypeptide substrate MyD88 L265P$_{256–281}$.

HLA-B*07:02-positive target cells, we transduced the cell lines with HLA-B*07:02-coding gamma-retroviral vector MP71.[18] Moreover, K562 cells with or without HLA-B*07:02 were virally transduced to express the complete length wild-type or mutant (L265P) *MYD88*—coupled to the expression marker green florescent protein (GFP) via p2A—and used as artificial target cells.

Generation of mutation-specific T cells

All mutation-specific TCRs were cloned from peripheral blood mononuclear cells (PBMCs), isolated from HLA-B7-positive healthy donors. Monocytes were separated by plastic adherence for generation of dendritic cells (DCs). Following 3 days of culture with 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL interleukin (IL)-4 in RPMI with 1% human serum, immature DCs were cultured overnight with addition of 10 ng/mL LPS and 50 ng/mL interferon gamma (IFN-γ) for maturation. Mature DCs were then loaded with mutant peptide (RPIPIKYKAM) and used for priming autologous CD8+ naive T cells. After 10 days, cells from each well were stained with a streptamer (HLA*B07:02-RPIPIKYKAM; IBA GmbH, Germany) or stained for T-cell

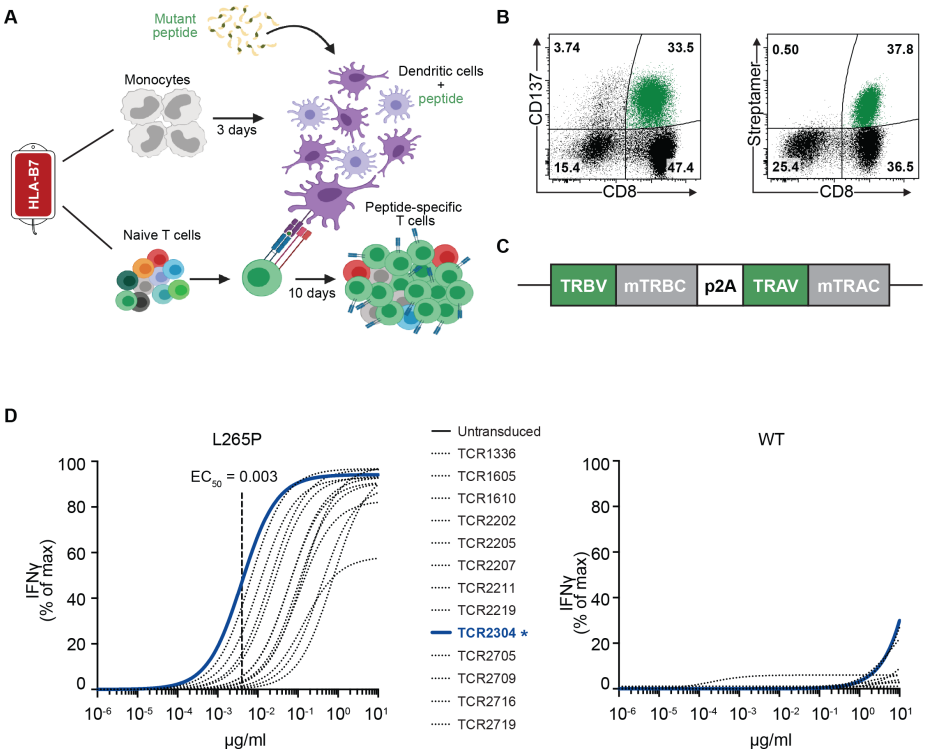

Figure 2 Generation of mutation-specific TCRs. (A) Schematic explanation of methodology for generation of mutation-specific T-cell lines. (B) Representative CD137 staining on 6 hours of restimulation with peptide, or streptamer staining for FACS isolation of peptide-specific T cells. (C) Construction of TCR gene cassettes. (D) Non-linear curve analysis of IFN-γ response by TCR-transduced CD8+ T cells from healthy donors, following coculture with K562 cells that were transduced with HLA*B07:02 and loaded with different concentrations of the mutant peptide (R**P**IPIKYKAM) or the corresponding WT peptide (RLIPIKYKAM). Response to the mutant peptide was detectable down to the concentration of 10^{-4} µg/mL with EC50 values within the nano molar (high affinity) range. Mutation-specific TCRs showed more than 10,000-fold higher affinity to the mutant peptide. IFN-γ response was measured by ELISA. FACS, fluorescence-activated cell sorting; IFN-γ, interferon gamma; TCR, T-cell receptor; WT, wild type.

activation markers such as CD137 (4-1BB) following a peptide restimulation. Positively stained wells were restimulated with peptide-loaded autologous PBMCs for expansion, in case it was necessary to obtain enough cells for fluorescence-activated cell sorting (FACS) isolation. The methodology for generating mutation-specific T cells is shown in figure 2A. This protocol was largely adapted from Wölfl and Greenberg.[19]

Identification of mutation-specific TCRs

Viable, CD8+ and streptamer-positive cells were sorted by FACS for total RNA isolation. A representative FACS plot is shown in figure 2B. TCR alpha and beta genes were amplified via 5′-RACE PCR (SMARTER RACE cDNA Amplification Kit; Clontech, Japan) and cloned (Zero Blunt TOPO Cloning Kit; Invitrogen/Thermo Fisher Scientific, Massachusetts, USA) in competent *Escherichia coli* cells. Multiple bacterial clones from each TCR chain were sequenced to analyze T-cell clonality, and dominant sequences were matched to create alpha-beta (αβ) TCRs for further characterization. Online supplemental table 1 shows CDR3 sequences and gene subtypes of MyD88 L265P mutation-specific TCRs. Identified variable domains (TRBV and TRAV) were combined with murine constant domain sequences (mTRBC and mTRAC) for experimental characterization of TCRs and synthesized on codon optimization (GeneArt, Thermo Fisher Scientific) for expression in human cells. TCR gene cassettes encoding the TRBV in combination with a murine TRBC and the TRAV in combination with a murine TRAC, separated by a p2A signal, were constructed as shown in figure 2C. TCR cassettes were cloned into the vector pMP71 by restriction site cloning. CD8+ T cells from healthy donors transduced with the TCRs were again stained with streptamer to test surface expression of the TCRs and functionality of αβTCR pairing (online supplemental figure 1).

T-cell activation and cytotoxicity analysis

Peripheral CD8+ T cells from HLA-B7-positive (not typed for HLA-B7 subtypes) healthy donors were transduced to express mutation-specific TCRs. Staining for murine TRBC (PE anti-mouse TCR β chain antibody; BioLegend, California, USA) was performed to check transduction efficiency before every experiment, which ranged between 25% and 65% of CD8+ T cells. Target cell lines were cocultured with TCR-transduced or control T cells for 16 hours. IFN-γ secretion was measured by ELISA (OptEIA Human IFN-γ ELISA Set; BD Biosciences, New Jersey, USA). Cytotoxicity was evaluated by flow cytometry analysis after staining of target cells for active caspase-3 (AF647 Rabbit Anti-Active Caspase-3, BD Biosciences) and fixable dead cell stain (LIVE/DEAD Fixable Violet Dead Cell Stain, Thermo Fisher Scientific). Target cells were gated on GFP-positive as reporter of wild-type or mutant MyD88 expression.

Xenograft models

All animal experiments were performed according to the institutional protocols and the national laws and regulations. Adult female NOG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac) and hIL2-NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(CMV-IL2)4-2Jic/JicTac) mice, which due to their production of human IL-2 provide better support for the growth of adoptively transferred human T cells, were acquired from Taconic Biosciences (New York, USA). In short, mice were inoculated subcutaneously with 5×10^6 OCI-Ly3 or luciferase-expressing TMD8 cells. Once the tumors reached the predetermined volume of 100 mm^3 (~2 weeks after injection), OCI-Ly3 tumor-bearing mice were treated with intravenous injection of 1×10^7 TCR-T cells, or mock T cells (untransduced) of the same donor, or phosphate buffered saline (PBS) as control. TMD8-bearing mice were treated similarly on day 7 after tumor cell injection. Caliper and bioluminescence measurements were used to monitor tumor growth. Tumor volume was calculated with the formula: length×width^2/2. Mice were sacrificed when tumor size exceeded 1500 mm^3 or signs of distress was observed as determined in the animal experimentation protocol.

Alanine scan for cross-reactivity

To define the TCR-binding motif, a list of peptides was created, in which every amino acid residue in the mutant epitope was exchanged one by one with alanine. All peptides were separately loaded on HLA-B*07:02-expressing K562 cells in the concentration of 10 µg/mL and cocultured with TCR-T cells for 16 hours. Response was measured via IFN-γ ELISA. Results were then analyzed using the online tool Expitope[20] to screen for motif similarities in the human proteome. Peptides identified to have a binding-motif similarity to the original epitope were loaded on HLA-B*07:02-expressing K562 cells in the concentration of 10 µg/mL for coculture with TCR-T cells from three different healthy donors. Reactivity was measured via IFN-γ ELISA.

Lymphoblastoid cell line (LCL) scan for alloreactivity

TCR-T cells from three different healthy donors were cocultured for 16 hours with immortalized B-LCLs expressing a variety of frequent HLA haplotypes (online supplemental table 2), with or without prior peptide loading. Response was measured via IFN-γ ELISA.

Data sharing

For further information, please contact oezcan.cinar@charite.de. Supplemental files can be found in the online version of this article. Complete length nucleic acid and amino acid sequences of TCRs can be found in the published patent application: WO 2020/152161 A1.

RESULTS

Mutation-encompassing peptide is processed by human proteasome and binds to HLA-B*07:02 with high affinity

We used NetMHC V.4.0[21] and NetMHCpan V.4.0[22] for screening of peptides spanning the mutation region on MyD88 for candidate epitopes, restricted to HLA haplotypes commonly found in the European population. A 10 mer peptide (RPIPIKYKAM) harboring a proline residue at position 2 which is highly preferred by HLA-B*07:02,[23] the most common HLA-B allele found in Europe and North America, was predicted to be a strong binder, while the wild-type sequence had very low predicted HLA binding affinity (online supplemental table 3). Since it is established that the in vitro generation of epitopes by 20S proteasomes reflects the in vivo situation with high fidelity,[24] we performed kinetic in vitro proteasome digestion experiments to study the generation of the predicted MyD88 L265P$_{264–273}$ neoepitope from the synthetic 26 mer polypeptide substrate MyD88 L265P$_{256–281}$. A summary of the different peptides generated by the proteasome in an in vitro digest is shown in figure 1A; the whole list can be found in online supplemental table 4. Giving confidence that the predicted 10 mer neoepitope is indeed generated, mass-spectrometric analysis of the proteasomal digests identified the generation of the predicted neoepitope MyD88 L265P $_{264–273}$ as well as the generation of two N-terminally extended neoepitope precursor peptides MyD88 L265P$_{263–273}$ and MyD88 L265P$_{262–273}$ requiring trimming by endoplasmic reticulum resident aminopeptidases. The kinetic generation of the three peptides is shown in figure 1B–D, while the degradation of the 26mer substrate is shown in figure 1E.

Isolated TCRs yielded mutation-specific and HLA-restricted T-cell activity

Next, we generated peptide-specific T-cell lines against this neoepitope by priming naive T cells with autologous peptide-loaded DCs from healthy HLA-B7-positive donors (figure 2A). These peptide-specific T-cell lines recognized the mutant peptide when restimulated as shown by CD137 expression and stained with custom MHC-peptide streptamer (figure 2B). We cloned 13 unique TCR alpha and beta genes from peptide-specific T cells

of five individual donors (first two digits in TCR names indicate donor number; eg, TCR1605 and TCR1610 are cloned from the same donor) into the gamma-retroviral vector MP71, for expression on primary CD8+ T cells (figure 2C). T cells transduced with these TCRs (TCR-T) recognized HLA-B*07:02-positive K562 cells loaded with mutant peptide with varying functional avidity, while the corresponding wild-type peptide was not recognized (figure 2D). In addition, the 11 and 12 mer precursor peptides (MyD88 L265P$_{263–273}$ and MyD88 L265P$_{262–273}$) detected in mass-spectrometric analysis were also recognized by TCR-T cells (online supplemental figure 2).

To further assess specificity and functionality, we cocultured TCR-T cells with engineered HLA-B*07:02-positive or HLA-B*07:02-negative K562 target cells expressing the complete wild-type or mutant *MYD88* gene. Flow cytometric analysis showed mutation-specific and HLA-restricted recognition of target cells without exogenous peptide loading, proving that the epitope can be generated from endogenously expressed MyD88 and presented by HLA-B*07:02 at the cell surface (figure 3A). Seven out of 13 peptide-specific TCRs showed strong reactivity against target cells (figure 3C). In addition, the amount of IFN-γ secreted by TCR-T cells on target recognition correlated with previously measured TCR avidity. The highly specific recognition of target cells observed here has also led to cytotoxic T-cell activity (figure 3B,D). The TCR with the highest overall activity in the comparative assessment was TCR2304. TCR2304 was thus chosen for further development. Hereafter, TCR-T cells refer to TCR2304-transduced T cells.

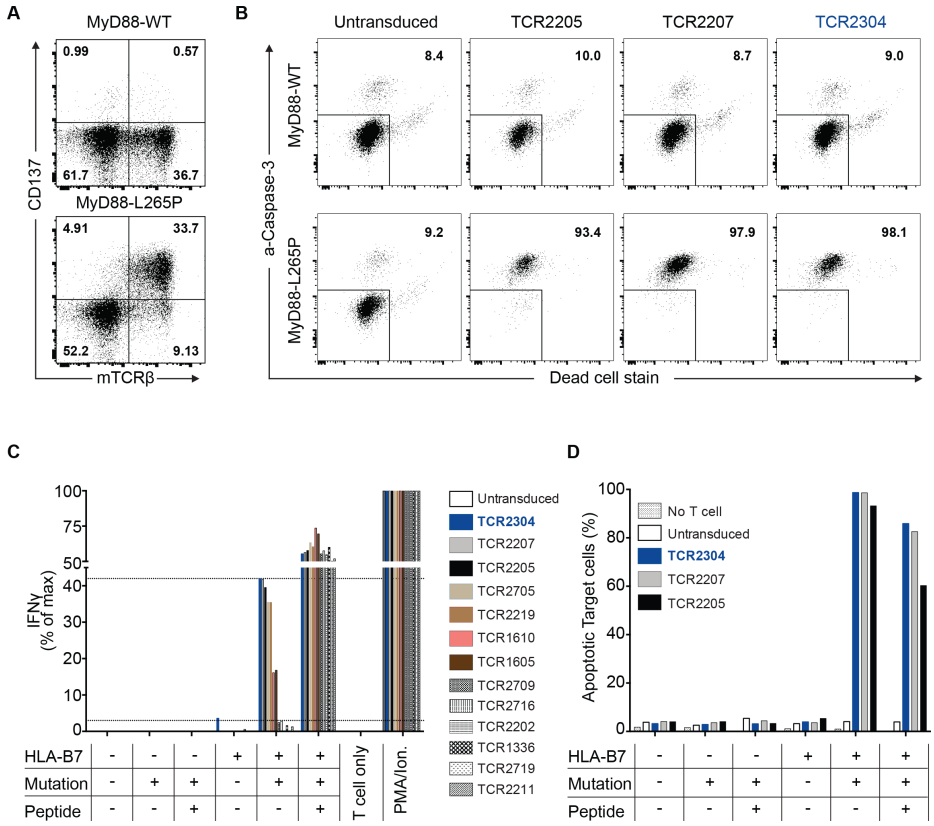

Figure 3 Mutation-specific activity of TCR-T cells. (A) Mutation-specific activation of TCR-T cells against K562 target cells—virally transduced to express complete length wild-type or mutant (L265P) MyD88 and HLA*B07:02, shown by flow cytometry analysis performed after 16 hours of coculture. (B) Viability of HLA*B07:02-positive target K562 cells that were cocultured for 16 hours with T cells expressing one of the three highest avidity TCRs, analyzed by flow cytometry. The proportions of apoptotic/dead cells are given in the upper right quadrant. (C) Comparative TCR-T cell response against target K562 cells. IFN-γ response measured by ELISA after coculture with K562 cells virally transduced to express complete length WT or mutant (L265P) MyD88 (mutation+) and/or HLA*B07:02 (HLA-B7+) as indicated. K562 cells loaded with the mutant peptide served as control (peptide+). Thirteen different TCRs were listed according to their respective EC50 values to the mutant peptide titration, from highest to the lowest. (D) Viability of target cells for cytotoxicity analysis of T cells transduced with three highest avidity TCRs. Strength of cytotoxicity as well as IFN-γ response against target cells strongly correlated with TCR avidity, previously measured by IFN-γ response to peptide titration. IFN-γ, interferon gamma; TCR, T-cell receptor; WT, wild type.

T cells engineered to express TCR2304 recognize and kill MyD88 L265P expressing lymphoma cells

To investigate the functional potential of TCR2304 when MyD88 L265P is expressed at natural expression levels, we analyzed the T-cell response against B-cell lymphoma cell lines. We used SU-DHL-6 (GCB-like DLBCL, wild-type MyD88), OCI-Ly3 (ABC-like DLBCL, homozygous L265P mutation) and TMD8 (ABC-like DLBCL, heterozygous L265P mutation) (figure 4A). Since all three cell lines originated from HLA-B7-negative patients, they were transduced to stably express the *HLA*B07:02* gene. Surface expression of HLA*B07:02 measured by flow cytometry was comparable to primary peripheral blood cells and to the HLA-B*07:02-positive B-LCL STA01 (figure 4B).

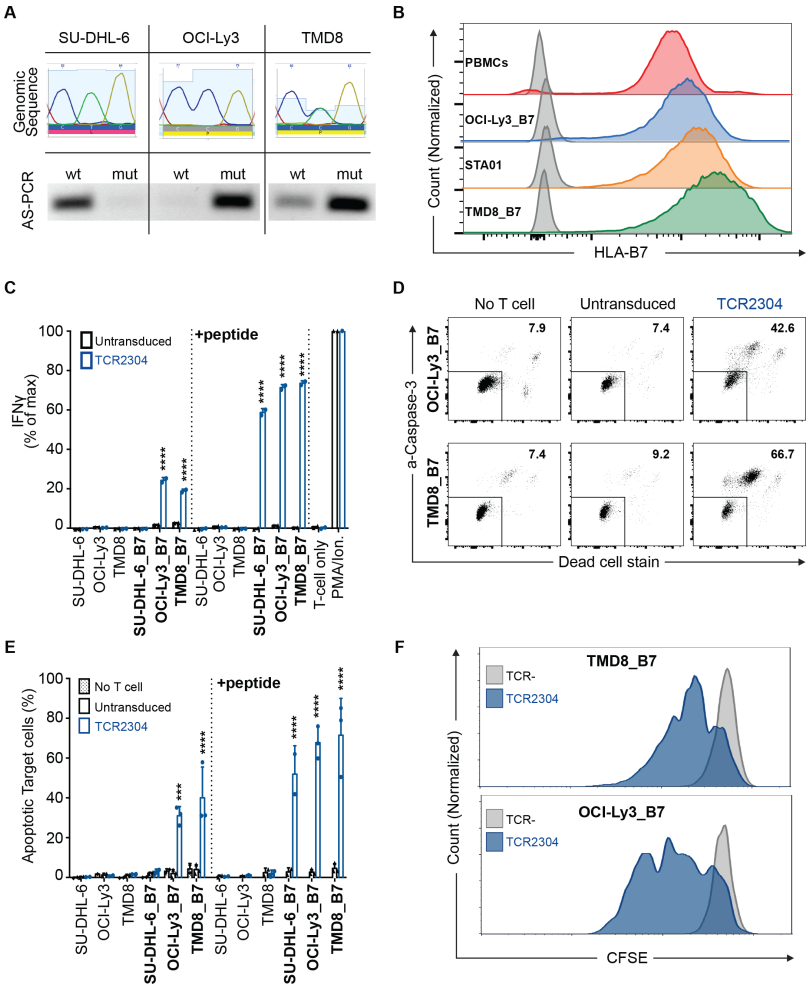

Figure 4 Mutation-specific activity of TCR-T cells against DLBCL cell lines with intrinsic MyD88 L265P expression. (A) Genomic sequence and allele-specific (AS) PCR analysis of lymphoma cell lines: SU-DHL-6 (GBC-like DLBCL, WT MyD88), OCI-Ly3 (ABC-like DLBCL, homozygous MyD88 L265P) and TMD8 (ABC-like DLBCL, heterozygous MyD88 L265P). (B) Side-by-side comparison of HLA*B07:02 expression of virally transduced lines versus natural expression in PBMCs isolated from an HLA*B07:02-positive donor and an HLA*B07:02-positive B-LCL line (STA01) measured by flow cytometric staining. (C) Activation analysis of TCR2304-transduced T cells via IFN-γ ELISA, after 16 hours of coculture with lymphoma cells. Cell lines virally transduced to express HLA*B07:02 are shown as 'cell line_B7'. Results from two different blood donors plotted with error bars showing SD significance analysis by two-way ANOVA: ****p<0.0001. (D) Representative flow cytometric viability analysis of lymphoma cells expressing HLA*B07:02 after 16 hours of coculture with T cells (as explained in figure 3). (E) Viability analysis of lymphoma cells with or without HLA*B07:02 expression. T cells of three different donors were used, error bars with SD significance analysis by two-way ANOVA: ***p<0.001, ****p<0.0001. (F) Antigen-induced proliferation of TCR2304-transduced T cells following 72 hours of coculture with HLA*B07:02-positive OCI-Ly3 and TMD8 cells. T cells were labeled with CFSE to trace proliferation prior to coculture. ANOVA, analysis of variance; DLBCL, diffuse large B-cell lymphoma; IFN-γ, interferon gamma; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; WT, wild type.

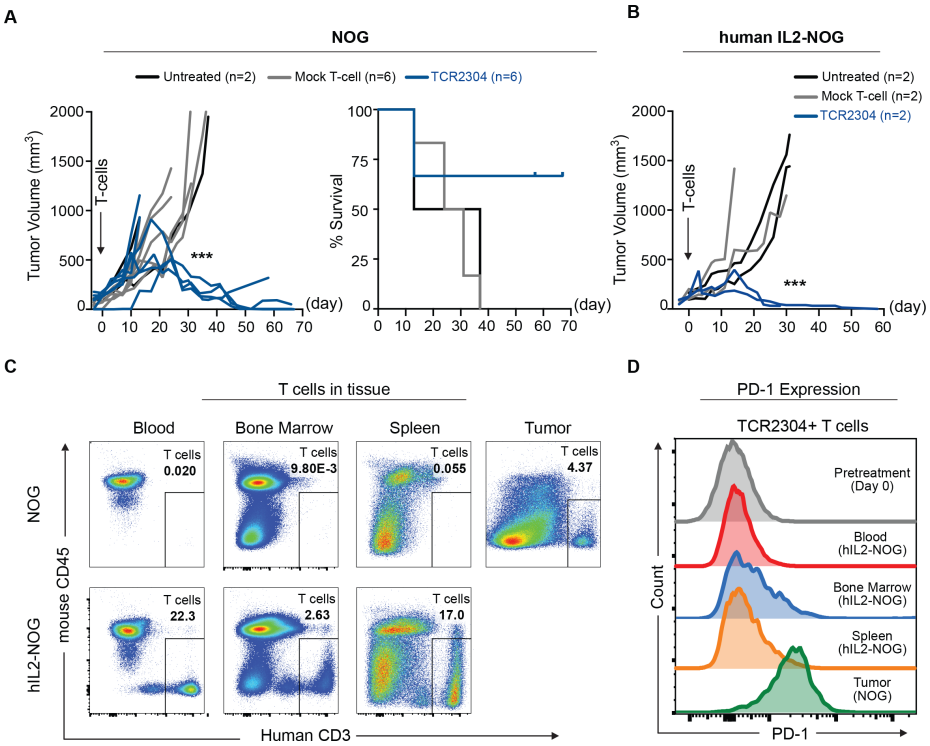

Figure 5 Therapeutic efficacy of TCR-T cells in OCI-Ly3 xenograft mouse models. NOG and hIL2-NOG mice were subcutaneously injected with 5×10^6 HLA*B07:02 expressing OCI-Ly3 cells. Treatment with 1×10^7 T cells was given by intravenous injection after tumors reached the predetermined size of 100 mm^3. (A) Change of tumor volume (significance analysis by two-way ANOVA, showing difference of tumor volume on day 31: ***p<0.001) and survival after treatment start in NOG mice. (B) Change of tumor volume after treatment start in hIL2-NOG mice. Significance analysis by two-way ANOVA, showing difference on day 30: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (C) Flow cytometric analysis of T cells in tissues of TCR-T cell-treated common NOG and hIL2-NOG mice, 58 days after treatment. (D) PD-1 expression in TCR-T cells in tissues of hIL2-NOG and common NOG mice on day 58 after treatment start, analyzed by flow cytometry. ANOVA, analysis of variance; IL, interleukin; TCR, T-cell receptor.

Overnight coculture with these lymphoma cells led to activation of TCR-T cells (figure 4C), which was accompanied by mutation-specific cytotoxicity (figure 4D,E) and T-cell proliferation (figure 4F). Specific T-cell response and cytotoxicity against naturally mutant lymphoma cells provided further evidence for the functional potential of TCR2304.

Myd88 L265P-redirected TCR-T cells induce durable regression of human lymphoma xenografts in mice

Having observed a strong specific T-cell response against MyD88 L265P mutant B-cell lymphoma lines in vitro, we tested TCR2304 in vivo for further assessment of its therapeutic potential. For this purpose, we generated a xenograft model by subcutaneously injecting HLA-B*07:02-expressing OCI-Ly3 cells into immunodeficient NOG mice. After tumors reached the predetermined size of 100 mm^3, mice were randomly treated with a single intravenous injection of 1×10^7 TCR-T or mock T cells, or PBS as untreated control. All untreated or mock-treated mice showed progressive tumor growth that required sacrificing the mice between days 13 and 37 (figure 5A). Four of the six mice that received TCR2304-T cells showed complete remission of the tumor, with tumor shrinking beginning between days 18 and 25 after T-cell application, although in one of these mice the tumor started to regrow around day 40. Two mice needed to be sacrificed early in the experiment due to rapid tumor progression and severe symptoms of disease before TCR-T cells could take effect. Since human T cells grow poorly without support from human cytokines, we also used the same tumor model in hIL2-NOG (immunodeficient NOG mouse expressing human IL-2 cytokine). This model is expected to have an enhanced T-cell engraftment and has been shown to improve the outcome from T-cell therapy.[25] Indeed, tumors in hIL2-NOG mice that received TCR-T cells disappeared completely after 3 weeks and did not relapse after up to 2 months following treatment (figure 5B).

To evaluate the mechanisms leading to tumor regrowth after initial response, the NOG mouse that had shown

tumor regrowth starting on day 40 was sacrificed on day 58 together with a mouse that had continuing response from the hIL2-NOG TCR-T treated group. We observed that the hIL2-NOG mouse still had a high amount of human T cells in blood, bone marrow and spleen, while the NOG mouse had only some T cells remaining in the tumor (figure 5C). TCR-T cells from tissues of the hIL2-NOG mouse did not show any significant change in the exhaustion marker PD-1 (programmed cell death protein 1), while TCR-T cells remaining in the growing tumor of the NOG mouse showed increased PD-1 staining in flow cytometric analysis (figure 5D).

However, in a second series of experiments using hIL2-NOG mice, some of the animals died independently of the size of the tumor. We had hints that these mice were developing severe graft-versus-host disease (GvHD) by proliferating human T cells, as reported by Ito *et al*.[26] The amount of GvHD-related toxicity indeed varied significantly using different human donors; therefore, we decided to stop further experiments in this model.

To validate our findings in a second model, luciferase-expressing HLA-B*07:02-positive TMD8 cells were subcutaneously injected into NOG mice, yet it caused some mice to die early due to strong systemic dissemination and aggressive tumor progression before treatment could be administered. Therefore, we decided to treat TMD8 bearing NOG mice 7 days after tumor inoculation. Indeed, on these experimental conditions, the T-cell treatment led to a significant reduction in tumor size starting at day 24 (figure 6A,B). Furthermore, TCR-T cell treatment prevented systemic dissemination of TMD8 cells (figure 6C,D and online supplemental figure 3).

Again, data in hIL2-NOG mice were complicated by T-cell toxicity caused by human versus mouse GvHD, which differed from experiment to experiment depending on different human donors, and although these experiments largely confirmed activity of transduced T cells in controlling tumor growth, they are not presented here.

TCR2304 is highly specific for the MyD88 L265P mutation without off-target reactivity

Even though all TCRs described in this study were isolated from healthy HLA-B7- positive donors and thus passed thymic selection, the risk of cross-reactivity against other human proteins is still potentially present. Peptides generated in the alanine scan were loaded separately on HLA-B*07:02-expressing K562 cells and cocultured with TCR-T cells to define the amino acids that are essential for the recognition (binding motif) by different TCRs (online supplemental figure 4). The proline in position 2, which reflects the amino acid substitution L265P in mutant MyD88, as well as the tyrosine in position 7, turned out to be essential for all TCRs, demonstrating the specificity of the TCRs for the mutation. Following detection of the binding motif (online supplemental figure 5A), we assessed the possibility of cross-reactivity to other human proteins, which might be caused by binding-sequence similarity, using the online tool Expitope.[20] In the case of TCR2304, the screen revealed 26 human peptides exhibiting motif similarity (xPxxIxYxxx) with up to five amino acid mismatches: 12 of these had some predicted binding affinity to HLA-B*07:02 on NetMHC V.4.0 and/or NetMHCpan V.4.0 (online supplemental figure 5). We tested TCR-T cells against all 12 peptides loaded on HLA-B*07:02-positive K562 cells in high concentration (10 µg/mL) in a coculture. However, no meaningful response was observed (online supplemental figure 5B).

For assessment of alloreactivity, we cocultured TCR-T cells with a panel of 16 Epstein-Barr virus-immortalized B-LCLs, which covered a broad spectrum of HLA haplotypes and most of the frequent HLA alleles found in Europe (Online supplemental table 2). When compared with untransduced control T cells, overnight coculture only demonstrated significant TCR-T cell response when HLA-B7-positive cell lines were externally loaded with mutant peptide (online supplemental figure 5C). Thus, we did not observe any sign of alloreactivity in this setting.

DISCUSSION

Contrary to CAR-T cell-based approaches, TCR-based cellular therapy has not yet led to breakthrough clinical results. However, because of the promise of cancer specificity, targeting tumor-specific neoepitopes by TCR gene therapy is continuing to attract attention. In solid tumors, TCRs directed against patient-individual mutations but also against a few recurrent mutations have been successfully isolated. First trials targeting mutant KRAS are currently recruiting.[27 28]

In this study, we present strong preclinical evidence that the lymphoma-associated L265P point mutation of MyD88 creates suitable neoepitopes for HLA-presentation, eliciting an efficient mutation-specific T-cell response. We have isolated a high-affinity TCR targeting a 10 mer neoepitope restricted to HLA-B*07:02, which mediates strong in vitro T-cell response against lymphoma cells with both homozygous and heterozygous L265P mutations. Adoptive transfer of gene-modified TCR-T cells shows promising therapeutic effect in preclinical experiments using human xenografts in immune-deficient mice. Our data indicate that the mutant epitope can indeed serve as a rejection antigen. In addition, preliminary safety screening does not indicate off-target activity of the TCR.

In a previous study by Nelde *et al* in 2017,[29] potential peptides derived from MyD88 L265P had been analyzed and shown to generate T-cell responses restricted to HLA-B*07:02 and HLA-B*15:01. Peptide-specific T cells reported in their study showed cytotoxic reactivity against peptide-loaded target cells, and it was suggested that these peptides may be used for a peptide-based immunotherapy approach, in case the peptides are naturally processed and presented. A separate study by Nielsen *et al*[30] in the same year confirmed that one of the mentioned peptides restricted to HLA-B*07:02 induced a mutation-specific T-cell response, and data suggested that the target epitope is endogenously processed. Peptide-specific T

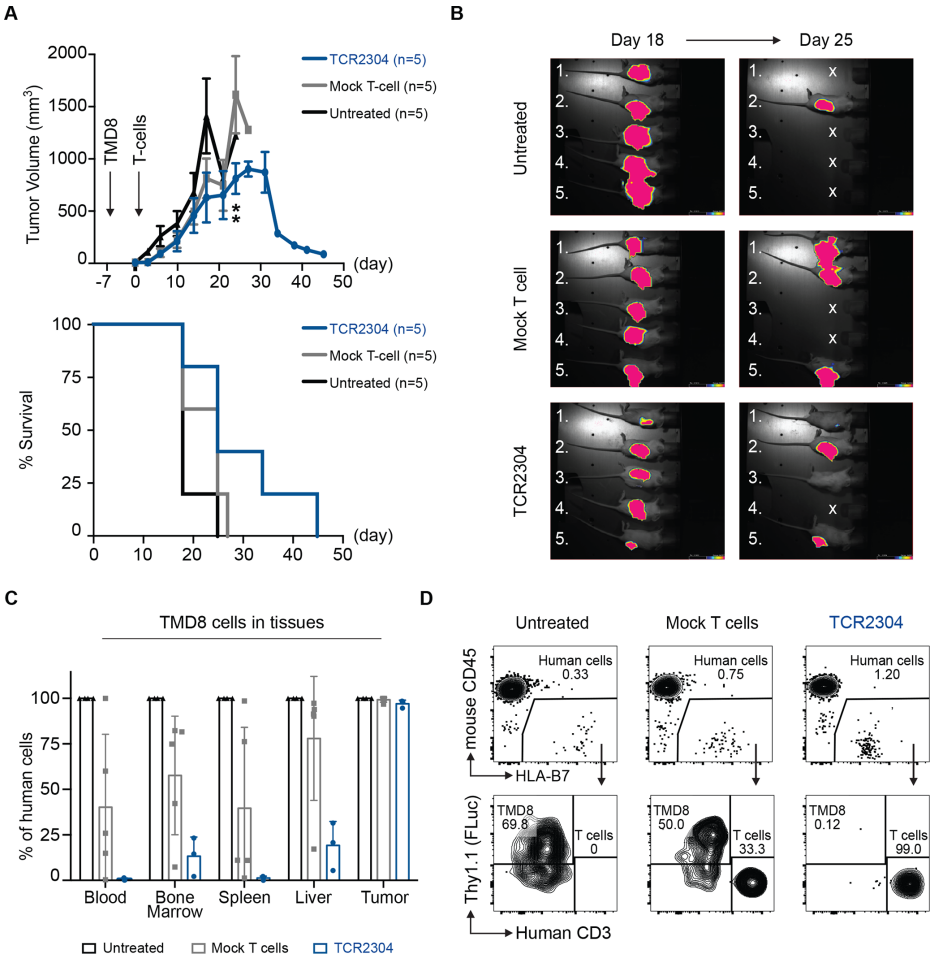

Figure 6 Therapeutic efficacy of TCR2304-T cells in TMD8 xenograft mouse model. NOG mice were suncutaneously injected with 5×10^6 TMD8 cells that are stably transduced with HLA*B07:02 and firefly luciferase. Tumor growth was detectable via bioluminescence imaging in all mice as early as day 2. (A) Change of tumor volume over time (error bars plotted with SEM significance analysis by two-way analysis of variance, showing difference on day 24: **p<0.01) and survival of NOG mice after treatment start. (B) Bioluminescence images of tumor-bearing mice at days 18 and 25 after treatment. Exposure: 60 s. (C) Flow cytometric analysis of systemic dissemination: proportion of TMD8 cells in tissues of tumor-bearing NOG mice. Human cells meaning sum of TMD8 lymphoma cells and human T cells. (D) Representative staining of blood samples from tumor-bearing NOG mice. Shown plots were pregated on single, viable cells.

cells against this HLA-B*07:02-restricted epitope were only detected in one out of six donors, hinting that a peptide-based immunotherapy approach may not elicit autologous immunity in the majority of patients.

In vitro antigen processing experiments, generating linear non-spliced peptides, are accepted to mirror the in vivo situation with high fidelity.[24] In our work, we show that the HLA-B*07:02 10 mer neoepitope is generated in vitro and that we also could generate strong T-cell responses in 5 out of 24 donors. However, only a few of 13 isolated and cloned TCRs from these five donors showed high affinity for the mutated peptide—demonstrating reactivity in the nanomolar range, which is thought to be crucial to achieve a therapeutic antitumor effect.[31] Our high-affinity TCR elicited strong therapeutic activity in a preclinical mouse model, suggesting that the sequence of a high-affinity TCR could be used 'off the shelf' to generate a highly specific, individualized yet broadly applicable ATT product usable in all HLA-B*07:02-positive patients whose tumors carry the L265P mutation. In our opinion, such a treatment could fulfill in an ideal way the criteria for precision cancer therapy.

A main limitation to TCR gene therapy is the impaired MHC–peptide presentation in cancer cells. Mutations in the processing and presentation machinery, or downregulation of MHC molecules on the cell surface, is described in solid cancers and also in lymphomas.[32 33] However, this rarely affects all malignant cells in a tumor and rarely

results in a total loss of HLA antigens. IFN-γ secretion by even a few specific T cells engaging tumor might be able to transform the tumor microenvironment,[34] inducing MHC upregulation and possibly enhancing the susceptibility of tumor cells to further TCR-T cells migrating into the tumor.[35]

In this respect, the difference we observed between hIL2-NOG and normal NOG mice is remarkable: NOG mice, in which T-cell proliferation is not supported by IL-2, showed some T-cell infiltration in tumors after adoptive transfer of MyD88 L265P-specific TCR-T cells, but tumors in some mice eventually regrew. Thus, it appears that besides lack of cytokine support for T-cell proliferation, upregulation of checkpoint inhibitory signals in T cells may have contributed to this result. Recent studies[36 37] have shown that MyD88-mutant lymphomas exhibit high level expression of the immune-checkpoint mediator PD-L1, thus possibly preventing their efficient clearance by adaptive host immunity. Conversely, these mutant-specific dependencies were therapeutically exploitable by anti-PD1 checkpoint blockade. On the other hand, mice where T-cell growth is supported by IL-2 stay in remission. Experiments in human IL-2 transgenic mice, however, are complicated by strong GVHD effect of human T cells with strong variations in the severity of GVHD from donor to donor; therefore, only a limited number of experiments were performed using this model. We think that in an appropriate clinical setting where autologous transduced T cells likely encounter cytokine support—particularly if administered after lymphodepletion—escape mechanisms could be avoided. A combination of adoptive transfer of mutation-specific TCR-T cells with immune-checkpoint blockade[38] might also be considered in a second phase of a pilot study.

We have tested our TCR in xenografted tumors but considered using a syngeneic lymphoma model with conditional MyD88 L265P mutation.[39] However, these mice would have to be crossed to humanized HLA-B*07:02 mice,[40] which is time consuming. More importantly, the information provided by such a model would be limited with respect to issues of safety and toxicity of the TCR in a clinical context, which is eventually crucial before moving to a clinical trial. This being our prior issue, we have extensively evaluated our TCR for potential toxicity. Based on our experience with generation of TCRs for ATT, we are confident that major reactivities can be excluded based on our LCL reactivity and alanine scan experiments. Moreover, while a number of TCRs are generated in transgenic mice for other targets with some of them already being approved by regulatory authorities for testing in clinical trials,[41 42] TCRs described in this study were generated from healthy humans—without further modifications to enhance affinity—and had undergone thymic selection, thus at least theoretically further reducing the risk of unwanted recognition of self-proteins. Further safety testing, such as reactivity studies using larger cell line and tissue libraries or the use of amino acids other than alanine in amino acid replacements,[43] has been proposed and will be discussed with safety authorities if required in preparation of a clinical trial.

We believe that besides CAR-T cell therapy, which has become a tremendous tool for the treatment of poor prognosis lymphomas and leukemias, there is a great potential in the use of TCR-T cell therapy. Our study provides a strong base for such a highly tumor-specific, molecularly defined TCR-based immunotherapy in selected hematological malignancies.

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Contributors ÖÇ designed and implemented studies, acquired, analyzed and interpreted data, and wrote the manuscript; BB designed and implemented animal studies and acquired and analyzed data; CG, CB and CAP acquired data; PMK interpreted the biochemistry data and reviewed and revised the manuscript; UK reviewed and revised the manuscript; AP designed and supervised the studies, interpreted the data, and reviewed and revised the manuscript; AB designed the studies, interpreted the data, and reviewed and revised the manuscript.

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Competing interests AP and ÖÇ are inventors on a filed patent application for the T-cell receptors described in the study (WO 2020/152161 A1).

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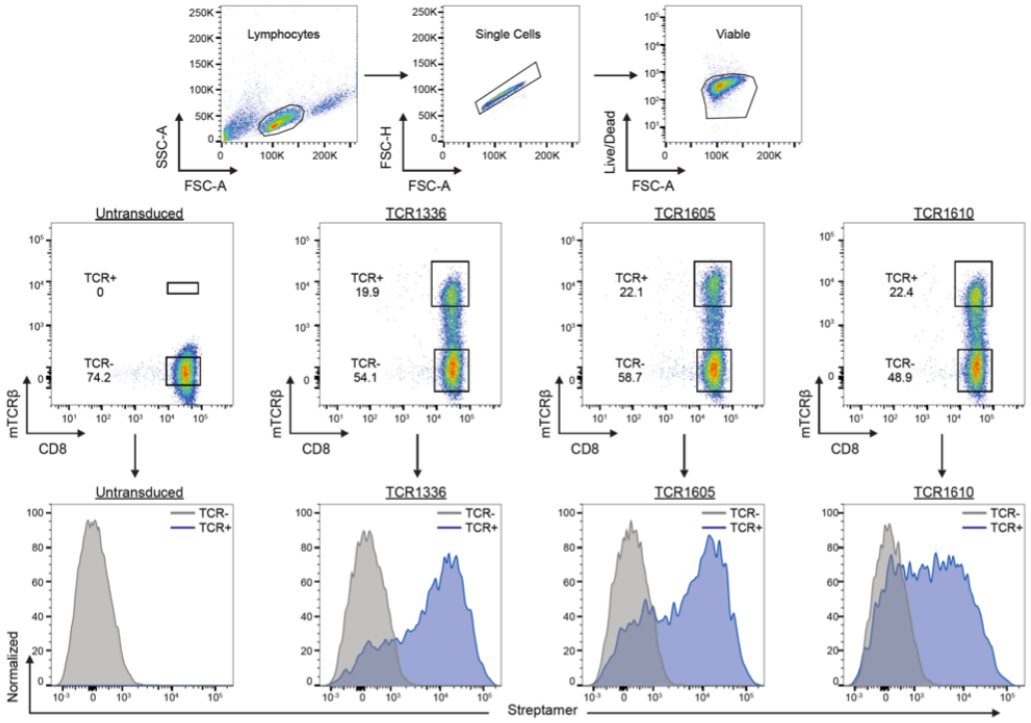


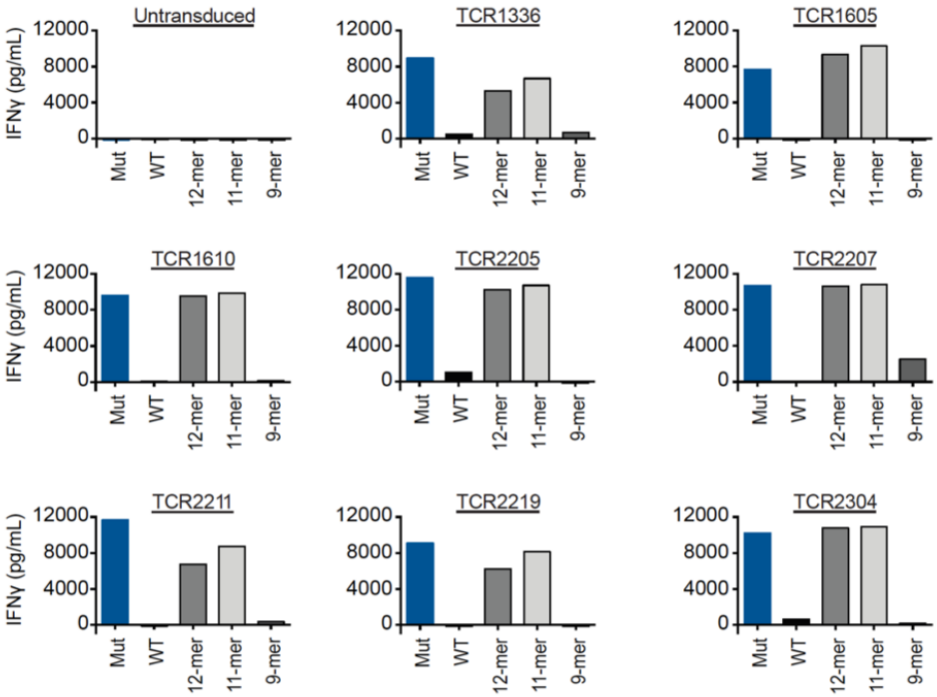
Supplementary Figure 1: Representative streptamer staining of TCR-transduced T cells

CD8+ T cells isolated from healthy donors transduced with the mutation-specific TCRs were stained with anti-mouse TCR β constant domain antibody and streptamer to test the surface expression the TCRs, and functionality of the alpha-beta TCR pairings from the sequencing results.

A

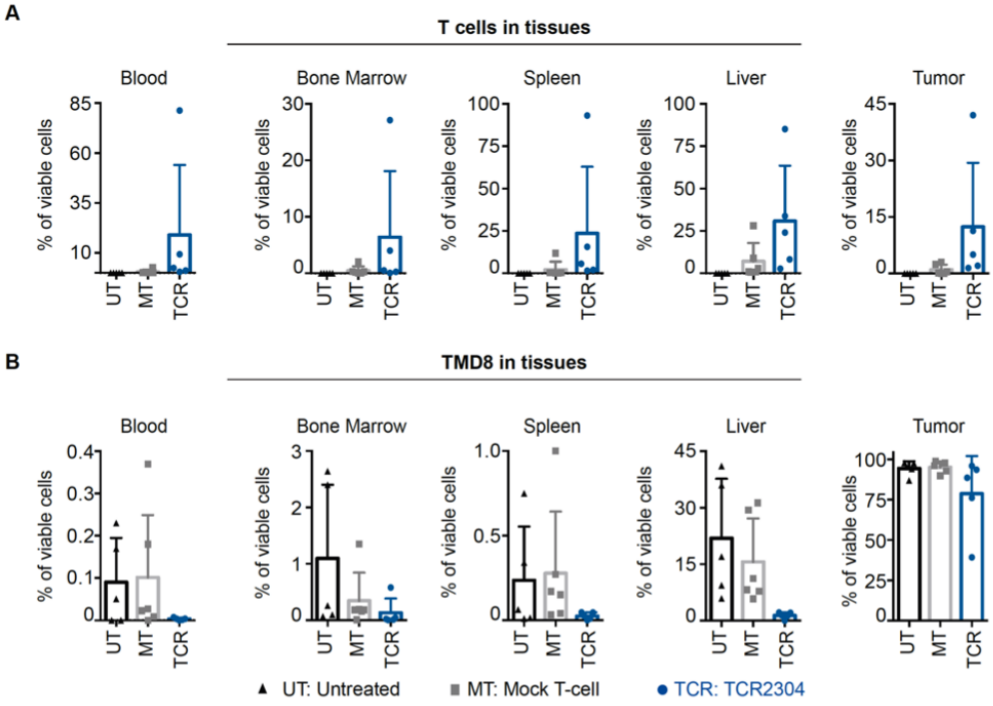
Peptide	HLA type	Sequence	Affinity (nM)	% Rank	Length
9mer	HLA-B07:02	**RPIPIKYKA**	613	1.30 (WB)	9mer
Mut	HLA-B07:02	**RPIPIKYKAM**	12	0.06 (SB)	10mer
Pre-1	HLA-B07:02	**KRPIPIKYKAM**	156	0.60 (SB)	11mer
Pre-2	HLA-B07:02	**QKRPIPIKYKAM**	219	0.70 (SB)	12mer

B



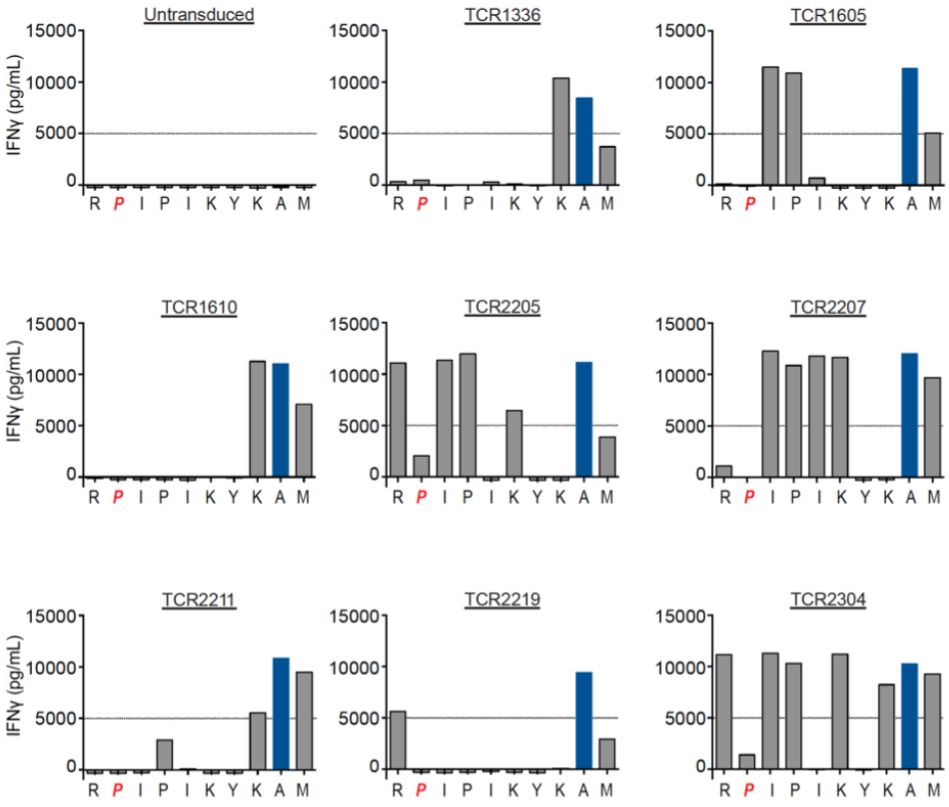
Supplementary Figure 2: Precursor peptides and epitope recognition

A. HLA binding prediction for 9-12mer peptides detected in the mass-spectrometric analysis of the mutant epitope (NetMHC 4.0). *B.* HLA-B*07:02 expressing K562 cells were loaded with peptides and co-cultured with TCR-T cells for 16 hours. IFNγ response was measured by ELISA. Mutation-specific TCRs recognized precursor peptides similarly to the 10mer epitope.



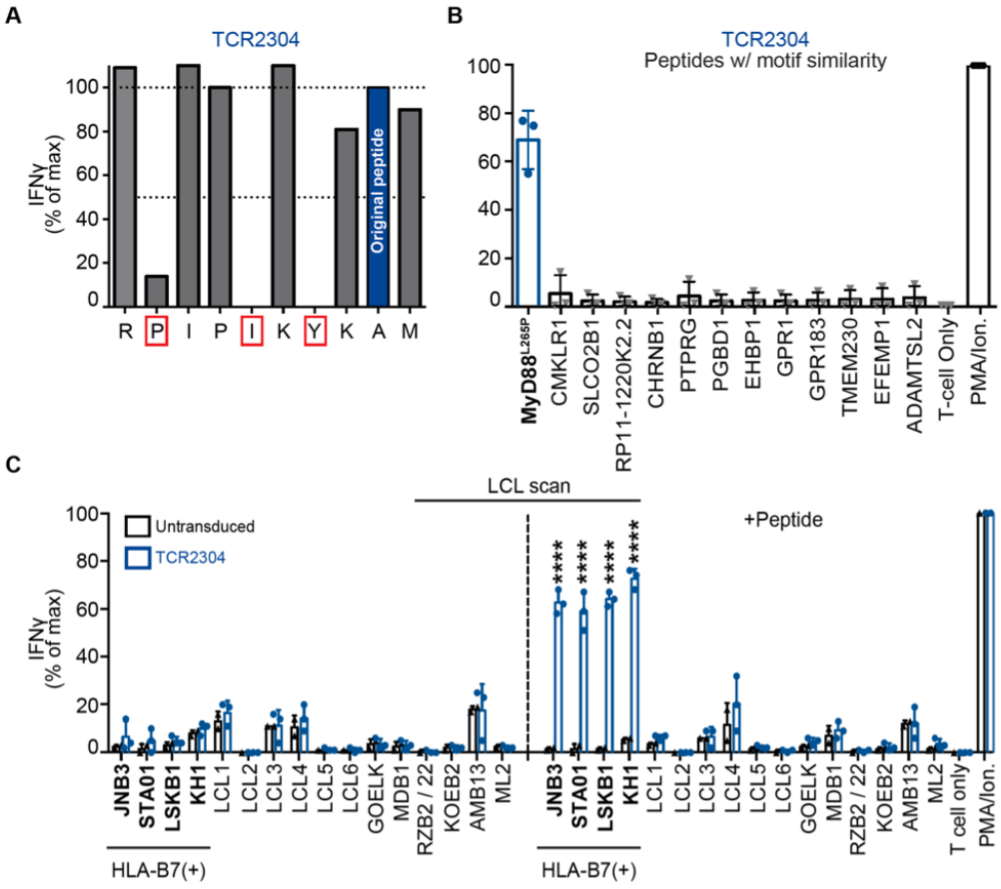
Supplementary Figure 3: Flow cytometric analysis of systemic dissemination of TMD8 cells

NOG mice were s.c. injected with 5x10^6 HLA-B*07:02 positive luciferase expressing TMD8 cells. *A.* Proportion of T cells in tissues of tumor bearing NOG mice. *B.* Proportion of TMD8 cells in tissues of tumor bearing NOG mice. Analysis from single, viable cells. Each symbol represents an individual mouse.



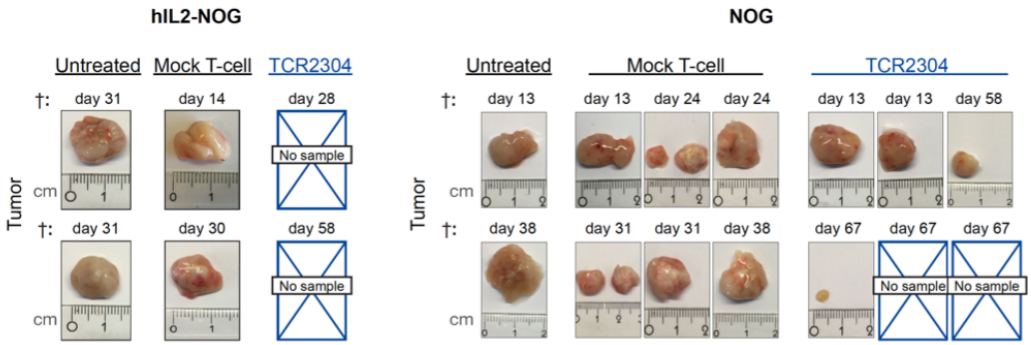
Supplementary Figure 4: Unique alanine scan results of mutation-specific TCRs

HLA-B*07:02 positive K562 cells were loaded either with 10mer mutant epitope or one of the 9 alanine scan peptides in high concentration (10 µg/ml) –to determine the most crucial amino acid positions for recognition, and co-cultured with TCR-T cells for 16 hours. IFNγ response was measured by ELISA.



Supplementary Figure 5: Safety analysis of TCR2304

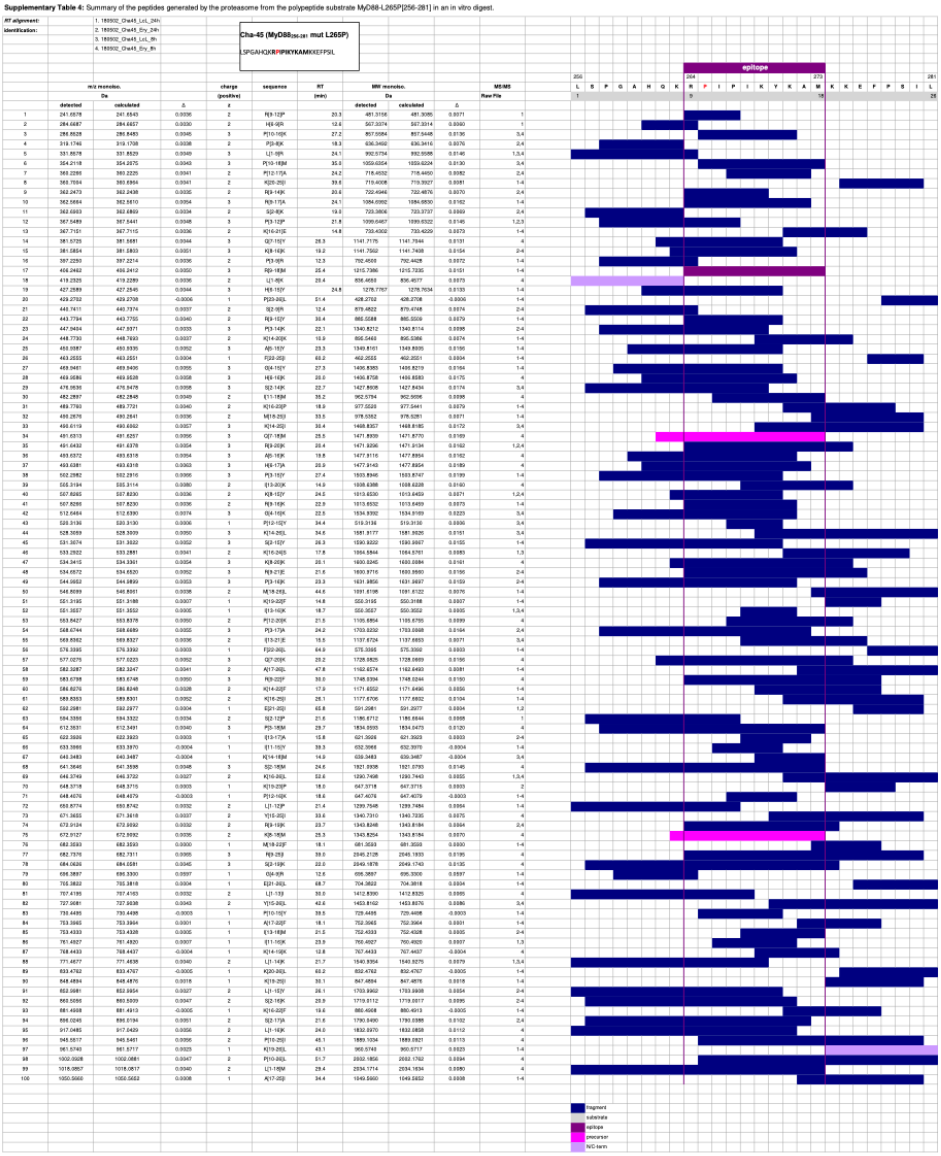
A. HLA-B*07:02 expressing K562 cells were loaded either with 10mer mutant epitope or one of the 9 alanine scan peptides at the concentration of 10 µg/ml, and co-cultured with TCR2304-T cells for 16 hours. IFNγ response was measured by ELISA. Amino acid positions that negatively affected IFNγ response more than 50% were considered vital for recognition of the mutant epitope by the TCR. *B.* Expitope online tool [19] was used to scan human proteome for peptides with sequence similarity to binding motif of TCR2304 (xPxxIxYxxx) with up to 5 mismatch positions. Resulting peptides with any binding prediction to HLA*B07:02 were loaded on target cells similarly to alanine scan (10 µg/ml), and co-cultured with TCR2304-T cells from 3 different donors for 16 hours. IFNγ response was measured by ELISA. Error bars showing SD. *C.* A library of EBV-immortalized B-LCLs were co-cultured with TCR2304-T cells from 3 different donors for 16 hours, with or without prior peptide loading. IFNγ response was measured by ELISA. Error bars showing SD. Difference between untransduced and TCR-transduced T cells in with or without peptide conditions analyzed by 2-way ANOVA: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.



Supplementary Figure 6: OCI-Ly3 tumors from sacrificed hIL2-NOG and common NOG mice

Mice were s.c. inoculated with $5x10^6$ HLA-B*07:02 positive OCI-Ly3 cells. Once the tumors reached predetermined size of $100mm^3$ (~2weeks after injection), tumor bearing mice were treated with i.v. injection of $1x10^7$ TCR2304-T cells, or mock (untransduced) T cells of the same donor, or PBS as untreated control. Mice were sacrificed with cervical dislocation when tumor size exceeded $1500mm^3$, or or signs of distress was observed.

Supplementary Table 4: Summary of the peptides generated by the proteasome from the polypeptide substrate MyD88-L265P[256-281] in an in vitro digest.



7. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

8. List of publications

Özcan Çınar, Bernadette Brzezicha, Corinna Grunert, Peter Michael Kloetzel, Christin Beier, Caroline Anna Peuker, Ulrich Keller, Antonio Pezzutto, Antonia Busse: “High-affinity T- cell receptor specific for MyD88 L265P mutation for adoptive T-cell therapy of B-cell malignancies” *Journal for ImmunoTherapy of Cancer* 07/2021 (Impact Factor: 13.751) 2021;9:e002410. doi:10.1136/ jitc-2021-002410

Antonio Pezzutto and **Özcan Çınar:** “Specific T cell receptors against epitopes of mutant MyD88^{L265P} protein for adoptive T cell therapy” *World Intellectual Property Organization* 07/2020 International application No: PCT/EP2020/051405, Publication No: WO 2020/152161 A1

Alexander N. R. Weber, Yamel Cardona Gloria, **Özcan Çınar**, H. Christian Reinhardt, Antonio Pezzutto, Olaf-Oliver Wolz: “Oncogenic MYD88 mutations in lymphoma: novel insights and therapeutic possibilities” *Cancer Immunology, Immunotherapy* 09/2018 (Impact Factor: 6.968) 67(11):1797–1807

Maarten A. Ligtenberg, **Özcan Çınar**, Rikard Holmdahl, Dimitrios Mougiakakos, Rolf Kiessling: “Methylcholanthrene-Induced Sarcomas Develop Independently from NOX2-Derived ROS” *PLoS ONE* 06/2015 (Impact Factor: 3.240) 10(6): e0129786.

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This thesis is dedicated to my wife Dr. Dilansu Güneykaya, and my brother Özgür Çınar – my first best friend and hero, whom we lost to leukemia when he was only sixteen. It is my greatest hope that the findings in this thesis one day will be the tiniest part of the victory against cancer, and no one will have to say goodbye to their loved ones before they could play all the games in this world together.

Özcan Çınar