TCRs as precision tools against B-cell and plasma cell malignancies

Meeuwsen, M.H.

Citation

Meeuwsen, M. H. (2024, October 1). *TCRs as precision tools against B-cell and plasma cell malignancies*. Retrieved from https://hdl.handle.net/1887/4093497

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4093497

Note: To cite this publication please use the final published version (if applicable).

TCRs as Precision Tools against B-cell and Plasma Cell Malignancies

Miranda Meeuwsen

HLA-A*01:01 HLA-C*15 HLA-C*07:02
HLA-C*08:02 HLA-A*03 HLA-B*08:01
HLA-C*07:01 HLA-A*02:01 HLA-B*15:01
HLA-A*32:01 HLA-A*03:01 HLA-C*04 HLA-A*02
HLA-C*03:04 HLA-B*07:02 HLA-A*23
HLA-B*44 HLA-B*40:02 HLA-C*02:02
HLA-B*27:05 HLA-B*40:01 HLA-B*15:17
HLA-A*03 HLA-B*39:01 HLA-A*02
HLA-A*24:02 HLA-C*03:07 HLA-C*03:03
HLA-A*11:01 HLA-A*03:01 HLA-B*07
HLA-B*14:02 HLA-C*07:01 HLA-C*02

TCRs as Precision Tools against B-cell and Plasma Cell Malignancies

Miranda Meeuwsen

ISBN: 978-94-6496-168-3
Cover design: Miranda Meeuwsen
Lay-out: Ilse Modder | www.ilsemodder.nl
Printed by: Gildeprint Enschede | www.gildeprint.nl

The research presented in this thesis was performed at the Department of Hematology of the Leiden University Medical Center and was financially supported by KWF kankerbestijding (grant number KWF UL-2014-6831) and Onderzoeksfonds Hematon (grant number FS 2018-2).

© 2024 M.H. Meeuwsen
All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means without prior written permission of the author.

TCRs as Precision Tools against B-cell and Plasma Cell Malignancies

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 1 oktober 2024
klokke 16.00 uur

door

Miranda Hester Meeuwsen
geboren te Zwijndrecht
in 1992

Promotores	Prof. dr. J.H.F. Falkenburg
	Prof. dr. M.H.M. Heemskerk
Leden promotiecommissie	Prof. dr. R.E.M. Toes
	Prof. dr. J.J.C. Neefjes
	Prof. dr. I.J.M. de Vries (Radboud Universiteit Nijmegen)
	Dr. I. Jedema (Nederlands Kanker Instituut)

TABLE OF CONTENTS

Chapter 1	General introduction and aim of this thesis	9
Chapter 2	A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy *Molecular therapy 2022 (published)*	31
Chapter 3	Broadly applicable TCR based therapy for multiple myeloma targeting the immunoglobulin J chain *Journal of Hematology & Oncology 2023 (published)*	73
Chapter 4	T cell receptor-based targeting of immune globulin constant domains for treatment of multiple myeloma *Manuscript submitted*	103
Chapter 5	Cutting Edge: Unconventional CD8$^+$ T-cell recognition of a naturally occurring HLA-A*02:01-restricted 20mer epitope *The Journal of Immunology 2022 (published)*	131
Chapter 6	Summary and general discussion	147
Appendices	Nederlandse samenvatting	169
	List of publications	175
	Curriculum vitae	179
	Dankwoord	181

CHAPTER 1

General introduction and aim of this thesis

GENERAL INTRODUCTION

The immune system and hematopoiesis

The immune system is our body's defense mechanism against pathogens. It comprises two primary arms: the innate and adaptive immune systems. The innate immune system serves as the first line of defense, providing rapid, non-specific protection against pathogens like bacteria and viruses. The adaptive immune system is highly specific and has a memory component. It recognizes and targets pathogens, allowing the body to remember and respond more effectively upon re-exposure. Both innate and adaptive immune cells originate from hematopoietic stem cells located in the bone marrow, a process known as hematopoiesis. This ensures the continuous renewal of immune cell populations, sustaining the body's safeguard against pathogens.

B cells

B-cell immunity

B cells play a crucial role in the adaptive immune response against pathogens by producing and releasing immunoglobulins. These immunoglobulins exist in two forms: as the B-cell receptor (BCR), acting as a membrane-bound antigen receptor for B cells, and as a secreted form, where they serve as the primary effector proteins released by mature B cells and plasma cells.

Immunoglobulins consist of two identical heavy and light chains, each containing constant and variable domains(1). The variable domain is responsible for binding to antigens, while the constant Fc domain facilitates effector functions. When antibodies bind to pathogens, they can coat the pathogen's surface, preventing them from binding and infecting host cells. The Fc tail-mediated effector mechanisms involve three main processes. Firstly, the Fc domains are recognized by innate immune cells through Fc receptors, resulting in the uptake and destruction of antibody-coated pathogens by phagocytic cells(2). Secondly, antibody binding via the Fc domain can trigger the activation of the classical complement cascade, leading to the recruitment of phagocytes or direct destruction of pathogens(3). Finally, Fc domains are bound by receptors that mediate active transport of immunoglobulins across mucosal membranes(4).

B-cell development

The initial phases of B-cell development occur independent of antigen and unfold within the bone marrow. This is where common lymphoid progenitor cells emerge from hematopoietic stem cells. Subsequently, these progenitors differentiate through stages, progressing from common lymphoid progenitors to pro-B cells, then pre-B cells, and eventually immature B cells. This differentiation process is linked to the stepwise formation of a functional B-cell receptor(5).

In the pro-B-cell phase, the initiation of BCR formation begins with VDJ recombination of the heavy chain variable domain. Following successful rearrangement, pro-B cells progress into pre-B cells. During the pre-B-cell stage, a surrogate light chain, comprising lambda5 and VPREB3, is expressed before the successful rearrangement of light chains(6). This surrogate light chain facilitates signaling through the pre-BCR, a necessity for proliferation and further differentiation. Subsequently, in the pre-B-cell stage, VJ recombination of the light chain transpires, leading to the expression of a functional IgM isotype BCR on the surface of immature B cells. These immature B cells then migrate to secondary lymphoid organs such as the lymph nodes and spleen, where they undergo further differentiation into mature B cells. Within secondary lymphoid organs, several developmental pathways are possible. In this context, the route that leads to formation of class-switched memory B cells or plasma cells from mature follicular B cells will be explained. This process is initiated upon native antigen encounter and relies on the assistance by helper T cells(7). Upon activation, follicular B cells progress into an activated state, leading to their differentiation into short-lived plasma blasts that produce IgM immunoglobulins or entry into germinal center reactions. Within these germinal center reactions, somatic hypermutation occurs, resulting in the production of antibodies with enhanced affinities for the specific antigen(8). This is followed by class switching to IgA, IgG, or IgE constant domains, broadening the effector functions of immunoglobulins. Within this context, B cells differentiate into memory B cells or plasma cells. Plasma cells secrete high levels of immunoglobulins, thereby providing direct protection against pathogens. While memory B cells establish immunological memory and can rapidly transform into plasma cells upon reinfection. Long-lived plasma cells return to and reside in the bone marrow.

Throughout each developmental stage, a distinct set of B-cell restricted genes is expressed. Some genes are only transiently expressed during specific stages, such as lambda5 and VPREB3 at the pre-B-cell stage(9, 10). Other genes, like CD19 and CD20, are more broadly expressed throughout the developmental process, but many of these genes are downregulated upon differentiation into plasma cells(11). In contrast, transcription factor POU2AF1 is expressed throughout the complete developmental process and also at the differentiated stage of plasma cell(12).

IgM, IgG and IgA functions and the role of the Jchain

Immunoglobulins come in a variety of isotypes, each with its unique function and specific distribution within the body. Among these isotypes, the most prevalent ones are IgM, IgG, and IgA.

IgM is predominantly secreted in a pentameric form by non-class-switched plasma cells. During an immune response, IgM antibodies are the initial responders, characterized by

their relatively lower affinity(7). Beyond their role in neutralization, IgM molecules serve as initiators of complement activation(13). The pentameric structure of IgM enables it to bind to multiple pathogens, inducing clumping, a process known as agglutination, which aids in their capture and destruction. Notably, IgM antibodies occur in the bloodstream as well as mucosal sites such as the gastrointestinal tract and lungs. In these locations, they can traverse epithelial barriers, providing protection against invading pathogens.

IgG antibodies are the predominant class found in both the blood and extracellular fluids. IgG plays a central role in pathogen neutralization and opsonization, rendering pathogens more susceptible to phagocytosis by immune cells, and initiation of the complement cascade.

IgA antibodies are encountered in both monomeric and dimeric forms, primarily functioning through pathogen neutralization. Consequently, IgA serves as a critical component of mucosal immunity in the gut and lungs.

Multimerization of IgM and IgA is facilitated by the joining chain (Jchain), a small polypeptide with binding sites for IgM and IgA monomers. Additionally, the Jchain acts as a ligand for the polymeric immunoglobulin receptor (pIgR) situated on the epithelium of mucosal tissues(14). Upon binding with the Jchain, the immunoglobulin complex is internalized and transported to the lumen, where IgA and IgM complexes exert their protective functions.

T cells

T-cell function

T cells play an essential role in the adaptive immune response by protecting against intracellular pathogens. They rely on a specific receptor called the T-cell receptor (TCR), which is essential to identify infected cells. Via the TCR, T cells can recognize foreign peptides that are presented in human leukocyte antigen (HLA) molecules on the surface of target cells(15). The TCR forms a complex together with various CD3 molecules. When the TCR binds to its peptide-HLA ligand, the CD3 complex functions as a signal transmitter triggering T-cell activation(16). Furthermore, CD3 plays an essential role in ensuring the TCR is expressed on the cell surface(17). In addition to the TCR and CD3, T cells express a CD4 or CD8 co-receptor. These co-receptors interact with HLA on the target cell and stabilize the interaction between the TCR on the T cell and the peptide-HLA complex on the target cell(7). This stabilization is often crucial for T-cell activation. The expression of either CD4 or CD8 divides T cells into two groups. CD4 positive T cells, known as helper T cells, assist in the activation and differentiation of other immune cell types like B cells and CD8 T cells by producing helper cytokines. CD4 T cells become activated when they recognize peptides presented by HLA class-II molecules. CD8

positive T cells have a cytotoxic function, causing lysis of target cells when their TCR binds to peptides displayed by HLA class-I molecules. When the TCR engages, CD8 T cells release perforin and granzyme from cytotoxic granules. Perforin creates pores in the target cell's membrane, allowing granzyme to enter and initiate a cascade that leads to cell death. In addition to foreign peptides presented by infected cells, T cells can recognize mutated peptides as non-self(18, 19). Mutated peptides can arise from genetic mutations that occur in tumor cells. Consequently, T cells can recognize and clear tumor cells that present such mutated peptides and contribute to tumor control(19).

T-Cell development and selection

Lymphoid progenitor cells undergo development and maturation within the thymus, resulting in the formation of mature naïve T cells. Within the thymus, developing T cells go through various developmental stages, each marked by the rearrangement of their TCR genes. Initially, the TCR beta chain undergoes rearrangement, followed by the alpha chain. During the TCR rearrangement process, an astounding degree of diversity in TCRs is achieved through the stochastic recombination of single V (D) and J gene segments drawn from a vast array of germline-encoded variants(20). Additionally, the rearrangement process allows for the introduction or removal of nucleotides at the V (D) J junctions, thereby generating what is termed "junctional diversity." This process significantly expands the repertoire of unique TCRs, theoretically allowing for a total number of approximately 1x10^18 distinct TCRs(7). In practical terms, the actual number of distinct TCRs is substantially more limited since the entire human T-cell repertoire encompasses approximately 10^12 T cells. In addition, some specific combinations occur more frequently at the population levels than would be expected if TCRs were rearranged randomly(21). These TCRs are referred to as public TCRs and demonstrate that total TCR repertoires are much more limited than theoretical calculations suggest. Studies have estimated that the pool of naïve T cells comprises approximately 1x10^8 unique TCRs(22).

Upon the completion of TCR rearrangement, T cells undergo a critical phase known as positive selection. This process assesses the ability of TCRs to interact with self-HLA molecules. HLA is a highly polymorphic gene for which thousands different genetic subtypes have been identified across the global population. Self-HLA refers to HLA-alleles expressed by an individual while HLA-alleles not expressed by an individual are foreign or non-self HLA-alleles. T cells that fail to bind to self-HLA molecules with sufficient affinity undergo programmed cell death (apoptosis), while those with TCRs proficient in binding to self-HLA molecules persist(23). Subsequently, following positive selection, T cells differentiate into either CD4 or CD8 single-positive subsets. The development continues with another crucial step: negative selection. During negative selection, T cells encounter thymic antigen-presenting cells (APCs) presenting self-peptides. T cells that

exhibit affinity for these self-peptides presented by APCs are removed from the T-cell repertoire(24). While T cells with a high affinity for self-peptides in self-HLA are deleted, no selection is made for T cells that recognize self-peptides in foreign/allogeneic HLA. As a result, many T cells in the T-cell repertoire will have the ability to recognize self-peptides in allogeneic (allo-) HLA molecules(25). T cells with allo-HLA reactive TCRs can mediate graft rejection after solid organ transplantation that occurs in absence of immunosuppressive therapies, as well as graft versus host or graft versus leukemia responses after HLA-mismatched allogeneic stem cell transplantation(26-28).

Positive and negative selection combined yield a finely tuned T-cell repertoire that possesses the capability to recognize foreign peptides presented within self-HLA molecules while maintaining tolerance toward self-peptides presented in self-HLA, thereby ensuring immune competence without autoimmunity.

Antigen presentation

Goal of antigen presentation

Within tissues, intracellular and extracellular contents are consistently processed and presented to T cells as a fundamental part of protection against pathogens. To this end, proteins are continuously degraded inside the cell, resulting in small protein fragments called peptides. These peptides are loaded in HLA molecules and transported to the cell membrane where peptide-HLA complexes can be recognized by T cells. This presentation serves the purpose of revealing intra- and extra-cellular contents to T cells, allowing them to initiate an immune response when foreign peptides are encountered. Two distinct categories of antigens exist, which are each primarily associated with a different type of HLA molecule. Peptides from intracellular proteins are presented in HLA class-I molecules, while extracellular proteins are taken up, processed, and presented in HLA class-II molecules. Beyond their mechanistic differences, HLA class-I and class-II molecules are expressed by different cell types in the body. HLA class-I molecules are found on the surfaces of most nucleated cells under normal conditions, HLA class-II molecules are primarily expressed by antigen-presenting cells (APCs) and may be upregulated in other cell types during inflammation(29).

Within the scope of this thesis, further focus will be on mechanisms underlying antigen presentation in HLA class-I molecules. Presentation of peptides by HLA class-I leads to the recognition of antigens by CD8 T cells and subsequent initiation of an immune reaction that contributes to the body’s defense.

Processing and presentation

Synthesis of HLA class-I molecules takes place in the endoplasmic reticulum (ER), where HLA class-I is subsequently loaded with peptides localized in the ER and transported

to the cell surface. The peptide repertoire in the ER is established via the classical and alternative pathways of peptide processing(30). In the classical pathway, proteins are degraded in the cytosol by proteasomes, after which they are transported into the ER by transported associated with antigen processing (TAP). TAP preferentially transports peptides between 8-16 amino acids in length, skewing the ER peptide repertoire to relatively short peptides(31). In the ER trimming of the N-terminus is mediated by ERAP1, generating peptides of length compatible with binding to HLA class-I. Studying cells with defects in components of the classical pathways has revealed various other mechanisms of peptide processing, together referred to as alternative pathways. One example of alternative peptide processing are type II proteins that are part of secretory pathways, these peptides are located in the cell membrane of the ER with their N-terminal side in the cytosol and their C-terminal side in the ER lumen(32). The C-terminal fraction of these proteins can be cleaved by signal peptide peptidases (SPP) releasing them into the ER independent of TAP and enabling their binding to HLA class-I and subsequent presentation to T cells on the cell membrane.

HLA variants and allele frequencies

HLA class-I molecules are composed of an HLA heavy chain and a light chain called beta 2-microglobulin (B2M). HLA-I heavy chains are encoded by three different genes, HLA-A, HLA-B and HLA-C. Each individual carries two variants of each gene resulting in a total of six HLA class-I alleles per person. At a population level, the diversity in peptide repertoires that arises from the variety in HLA alleles provides protection to mutated and newly arising pathogens(33). While the total number of HLA polymorphisms is huge, some alleles are frequently expressed. For example, HLA-A*02:01, HLA-A*24:01, HLA-B*35:01, and HLA-B*07:02 are expressed in 39%, 21%, 13%, and 8% of the world population, respectively(34). The frequency of each allele can be higher or lower within populations of a specific ethnic background.

HLA binding motifs

The peptide binding groove of HLA is flanked by two alpha helixes while the bottom of the groove is formed by beta pleated sheets. The N- and C-terminal sides of HLA class-I are closed restricting the length of peptides that can fit into the groove in a conventional manner to a size of 8-11 amino acids(35). In the peptide binding groove six pockets, termed A-F, can be identified. Pocket B and pocket F are the most dominant determinants of HLA specific peptide binding preferences(36). Pocket B binds peptides at position 2 (p2) and pocket F binds the C-terminal amino acid of peptides (pΩ). Amino acid residues at these positions of peptides are therefore most crucial for binding to specific HLA molecules. Different HLA molecules have amino acids variations in the peptide binding pockets, generating different motifs for preferred peptide binding. Over the past decades, data on peptide binding motifs of HLA alleles has become increasingly

available from mass spectrometry studies as well as peptide-HLA crystallography. These data underly peptide binding prediction algorithms and have increased the reliability of such predictions.

B-cell and plasma cell malignancies

Origin and treatment of B-cell and plasma cell malignancies

During B-cell development, malignancies can arise at any stage and the stage at which a malignancy develops determines the specific type of B-cell malignancy. B-cell malignancies form a heterogenous group of cancers that can be categorized in three main groups: leukemias, lymphomas and plasma cell malignancies. The development of B-cell malignancies is linked to B-cell intrinsic processes like V(D)J recombination, somatic hypermutation (SHM), and class-switch recombination (CSR)(37). These processes are critical to B-cell development and antibody diversification but carry risks due to the genetic alterations they induce. V(D)J recombination, the initial step in creating the diverse repertoire of B-cell receptors, involves the rearrangement of variable (V), diversity (D), and joining (J) gene segments. While essential for generating a wide array of antigen-specific receptors, this process can lead to chromosomal translocations or insertions/deletions if errors occur. Such genetic aberrations can disrupt the regulation of genes governing cell growth and survival, potentially leading to uncontrolled cellular proliferation and malignancy. Additionally, SHM and CSR can add to the development of B-cell malignancies, these processes occur in activated B cells within the germinal centers of lymphoid organs. SHM introduces point mutations in the variable region of the immunoglobulin genes to increase antibody affinity, while CSR alters the constant region of the heavy chain to change the antibody class. Both processes are mediated by the enzyme activation-induced cytidine deaminase (AID) and involve DNA modifications. While these modifications are crucial for critical for developing potent B-cell responses, they also harbor risks. The activity of AID, which is focused on immunoglobulin genes, can sometimes lead to off-target effects, causing mutations or DNA breaks in other genomic regions. This genomic instability, especially when coupled with a genetic predisposition of some individuals that may affect DNA repair mechanisms or the regulation of oncogenes and tumor suppressor genes, significantly elevates the risk of developing B-cell malignancies.

When pro- and pre-B cells undergo malignant transformation, this leads to development of acute lymphoblastic leukemia (ALL)(38). ALL is characterized by rapid proliferation of ALL cells in the bone marrow. The malignant growth leads to a reduction in healthy hematopoietic cell generation causing symptoms such as fatigue, increased susceptibility to infections, and bleeding. The treatment of ALL often involves intensive chemotherapy, which may be followed by stem cell transplantation, especially in high-risk cases or if the leukemia returns after initial treatment. Targeted therapies have also emerged

as effective options for certain subtypes of ALL. The prognosis for patients with ALL varies based on factors such as age, the presence of specific genetic aberrations, and the response to initial treatment(39). While advancements in treatment have improved outcomes, achieving a cure remains challenging and often requires aggressive treatment. Chronic Lymphocytic Leukemia (CLL) arises when mature B lymphocytes undergo malignant transformation(40). This type of leukemia is characterized by the slow accumulation of these malignant B cells in the bone marrow, blood, and lymphoid tissues. Unlike ALL, CLL typically has a more indolent course, but it can lead to a decrease in the production of healthy blood cells over time, resulting in symptoms like fatigue, increased susceptibility to infections, and anemia. The treatment of CLL in adults is less intensive than that for ALL and may not be immediately required in early stages. Treatment strategies include targeted therapies, such as monoclonal antibodies and kinase inhibitors, and may involve chemotherapy or immunotherapy, particularly in more advanced or aggressive cases. The prognosis for patients with CLL varies, influenced by factors such as the stage of the disease, the presence of specific genetic mutations, and the individual's overall health. Recent advancements in targeted treatments have significantly improved the management and outcomes for CLL patients(41). The disease is generally considered chronic and may require ongoing management rather than aiming for a cure.

Lymphomas are cancers that arise in the lymphatic system, containing various subtypes with different characteristics and treatment approaches(42). Lymphomas most frequently develop from B cells, but lymphomas of T-cell origin also exist. Indolent forms of B-cell Lymphoma, like Follicular Lymphoma, may not require immediate treatment and can be closely monitored, a strategy known as "watchful waiting." When treatment is necessary, options include chemotherapy, immunotherapy such as monoclonal antibodies, targeted therapy, and radiation therapy. Aggressive lymphomas like Diffuse Large B-Cell Lymphoma are usually treated with a combination of chemotherapy and immunotherapy. In some cases, especially for relapsed or refractory lymphomas, stem cell transplantation may be considered. The development of targeted therapies, which specifically target cancer cells based on their genetic characteristics, has significantly improved treatment outcomes and reduced side effects for many lymphoma patients(43). Personalized medicine, considering the specific characteristics of the lymphoma and the patient's overall health, is increasingly becoming the norm in lymphoma treatment strategies.

Multiple myeloma (MM) is the malignant counterpart of plasma cells. In this disease, a (precursor) plasma cell undergoes malignant transformation and clonally proliferates in the bone marrow(44). The uncontrolled growth interferes with production of healthy blood cells, leading to fatigue, anemia, and increased susceptibility to infections, but also results in the overproduction of a monoclonal antibody, known as paraprotein or

M-protein(44). The high levels of this abnormal protein can cause various complications, including kidney damage and impaired immunity. Additionally, the myeloma cells disrupt normal bone remodeling, leading to painful bone lesions and an increased risk of fractures. Treatment for MM typically involves chemotherapy, targeted therapy such as proteasome inhibitors and immunomodulatory agents, and in some cases high dose chemotherapy and autologous stem cell transplantation. The introduction of novel therapeutic agents has markedly improved patient outcomes, yet MM is still generally incurable, with treatment aimed at controlling disease progression and managing symptoms(45).

CAR T cell therapy for B-cell malignancies

In recent years innovative cellular therapies have emerged as treatment for patients with relapsed/refractory malignancies of B-cell origin. These new treatments use T cells genetically modified to express a receptor directing them to target the tumor. The first FDA approved therapies use chimeric antigen receptor (CAR) T cells. CARs are based on the structure of antibodies, they contain a single chain variable fragment (scFv) that recognizes a specific protein(46). The scFv is combined with co-stimulatory and signaling domains to induce T-cell activation after antigen binding. CAR T cells are designed to target specific surface proteins expressed on malignant cells. For malignancies of B-cell origin, broadly expressed tumor specific antigens are not available and therefore B-cell lineage antigens are targeted. B-cell lineage antigens are antigens that are exclusively expressed by cells of the B-cell lineage for which expression was maintained upon malignant transformation. An example is CD19, which has been used as a CAR target for various B-cell malignancies. CD19 CAR T-cell response rates are between 53% and 74% for patients with B-cell lymphoma(47). 39% to 55% of the patients reached complete responses, of which 49.5%- 80% were still complete responders after 24 months. For ALL initial complete response rates of CD19 CAR T cells are between 62% and 86%(48, 49). Many patients treated with CD19 CAR T cells received consolidative allogeneic stem cell transplantation, which hampers the interpretation of long-term complete responders. Overall, in ALL <50% of patients achieved long term event-free survival. Plasma cell malignancies cannot be treated with CD19 CAR because CD19 expression is lost upon B-cell differentiation to plasma cells. MM cells highly express B-cell maturation antigen (BCMA) and the expression of BCMA is restricted to B/plasma cells(50). BCMA-targeting CAR T cells induced complete response rates in 33-83% of patients, but despite high initial responses most patients eventually relapse(51). The studies on CAR T cells have highlighted the potential of T-cell based therapy for treatment of B-cell malignancies. Additionally, CAR T-cell therapy targeting B-cell lineage antigens resulted in B-cell aplasia as a side effect of therapy(48). While this scenario is not ideal due to subsequently compromised B-cell immunity, B-cell aplasia can be managed by treatment with soluble immunoglobulins. Overall, CAR T cells demonstrate that B-cell lineage antigens are good targets when treating B-cell malignancies using T-cell therapy.

Despite the successes, CAR T-cell therapy has revealed new challenges ahead as many patients relapse after therapy.

Challenges of CAR T-cell therapy

One prominent escape mechanism that has been observed after CAR T-cell therapy is antigen escape, where reduced or loss of antigen expression results in evasion from CAR T cells and subsequent tumor outgrowth(52). The most straight forward mechanism behind antigen escape is heterogenous antigen expression at the start of therapy, which leads to skewing and outgrowth of the antigen negative or low population(53, 54). Furthermore, complete loss of antigen can occur by genetic mutations(55). Besides complete antigen loss, various mechanisms can cause a reduction in antigen expression resulting in an expression level too low to be detected by CAR T cells. Reduced surface antigen expression can result from trogocytosis, a process in which the CAR T cells 'nibble' membrane fragments from the target cell. In the case of BCMA, active cleavage from the cell membrane mediated by gamma-secretase can also reduce BCMA surface expression(56). While patients can relapse due to antigen escape, a substantial fraction of patients display antigen-positive relapse from CAR T-cell therapy. Antigen-positive relapse is the result of incomplete tumor clearance potentially resulting from the inability of CAR T cells to reach all tumor cells or due to suboptimal CAR T-cell performance (52, 57). CAR T cells utilize artificial signaling that may render CAR T cells sensitive to dysfunction, particularly through tonic signaling or activation induced dysfunction(58). Tonic signaling is activation of CAR T cells that occurs in the absence of stimulatory antigen. This antigen-independent activation can result in progressive differentiation, upregulation of coinhibitory molecules, and antigen-independent proliferation, compromising CAR T-cell performance(59, 60). At the same time, CAR T cells have been described to be sensitive to antigen-dependent overactivation(60-62). Depending on the respective target antigen and CAR design, CAR T-cell exposure to antigen expressing target cells can lead to rapid exhaustion and antigen induced cell death, that may especially be at play in settings of high tumor burdens or solid tumors(60). Another challenge of CAR T cells is treatment related toxicity, particularly cytokine release syndrome. Upon activation, CAR T cells release significant amounts of proinflammatory cytokines that can trigger cytokine release syndrome in patients, a condition that can be life-threatening and frequently requires intensive care intervention(63). Furthermore, severe cases of neurotoxicity have been associated with CAR T-cell therapy, termed immune effector cell-associated neurotoxicity syndrome.

TCR T-cell therapy for B-cell malignancies

Rationale for TCR-therapy

While CAR T cells have shown clinical success for therapy of B-cell malignancies, they also revealed challenges that remain to be addressed. Two of these challenges are antigen

escape and CAR T-cell dysfunctionality. To combat antigen escape, combination therapies are likely needed, but the requirement for antigen surface expression limits possibilities of targetable CAR antigens(64). As a complementary modality to CAR T cells, transgenic TCR T cells could be of benefit. TCR T cells could be of unique value owing to their ability to recognize peptides from intracellular located proteins. This allows hypothetical targeting of any protein as long as the criteria for a favorable expression profile is met. This also means that proteins essential for survival of malignant cells, such as certain transcription factors that are located inside the cell can be targeted using TCR T cells(65-67). Furthermore, TCRs can be used to target peptides from proteins that are secreted by malignant cells, such as immunoglobulins. CAR T cells targeting these proteins are coated by target protein present in the circulation, which can render them insensitive(56, 68). Another potential benefit of TCR T cells over CAR T cells, is that TCRs are the natural receptors of T cells and TCR T cells might therefore be less sensitive to overstimulation, potentially leading to increased persistence and functionality compared to CAR T cells(58, 60).

While the recognition of peptides in the context of a specific HLA molecule provides advantages for TCR T-cell therapies, it should also be noted that it limits the applicability of a single receptor to patients expressing the targeted HLA allele.

Proposed antigens for TCR T-cell therapy

In the context of B-cell malignancies, CAR T-cell therapies have focused on targeting antigens specific to the B-cell lineage. As previously mentioned, targeting B-cell antigens induces B-cell aplasia, but this is generally considered an acceptable side effect. Consequently, B-cell lineage antigens present an interesting target for TCR T-cell therapies. A distinct advantage of TCR T cells lies in their capacity to target peptides derived from any protein, irrespective of its cellular localization. To increase the likelihood of successful TCR identification and to allow broad application of TCR therapy across a diverse patient group, identification of as many potential target proteins as possible is important. Gene expression datasets can be analyzed to identify proteins exclusively expressed in both malignant and healthy B-cell lineages(69). Ideal target proteins are characterized by high expression in tumors, coupled with homogeneous expression both within individual tumors and across patients. This homogeneity would ensure that a single receptor could potentially be used to treat a wide array of patients. After the identification of target proteins, it is critical to determine which peptides from these proteins are presented by specific HLA molecules on the surface of malignant B cells. Such peptides are potential candidates for recognition by B-cell antigen targeting TCRs.

Epitope identification

The identification of peptides derived from target proteins for which specific T cells can be identified is a crucial step in the development of TCR-based therapies for B-cell

malignancies. Online prediction algorithms can be used to identify which peptides derived from a protein are predicted to bind to an HLA-allele of interest(70). Identification of T cells which recognize peptides identified using prediction algorithms has been performed in the past. These studies observed that these T cells, despite having a high affinity for the target peptide, are often not able to recognize tumor cells expressing the respective target protein(71). This discrepancy demonstrates that predicted binding to HLA does not necessarily imply effective processing and presentation of the peptide in the respective HLA on the surface of malignant cells. To identified T-cell clones that could have the ability to recognize malignant cells expressing a specific target protein, it is therefore important to establish which peptides derived from a target protein are processed and presented in specific HLA molecules on the surface of malignant cells. To determine this, the HLA peptidome of B-cell malignancies can be analyzed(72). The HLA peptidome refers to the collection of peptides that are presented in HLA on the cell surface. To determine the HLA peptidome of B cell malignancies, peptide-HLA complexes can be isolated from malignant B cells, the peptides can then be eluted from HLA and peptide sequences determined by mass spectrometry. The peptidome can then be searched for peptides derived from a target protein of interest. Finally, the HLA origin of these peptides needs to be determined, this can be done by using HLA binding prediction tools together with the HLA typing of the cells used for peptide elution(73). In this manner peptides derived from target proteins of interest that are presented in a specific HLA molecule of choice can be identified. Using this method ensures that T cells with sufficiently affinity for the targeted peptide should be able to recognize malignant cells expressing the respective protein and HLA molecule. This approach has previously resulted in identification of T cells with TCRs valuable for TCR T-cell therapy of hematologic malignancies and will be applied in this study to identify promising TCRs(65, 74).

Identification of TCRs recognizing self-antigens

In the case of B-cell malignancies, many target antigens are non-mutated self-antigens. For these antigens immunological tolerance exists in the HLA-matched setting, and TCR identification from an HLA-matched individual could at best result in identification of intermediate affinity TCRs (27, 75). To circumvent this tolerance various methods can be applied. For example, the identification of TCRs recognizing human peptide-HLA complexes from mice(76). Alternatively, *ex vivo* affinity maturation can be performed to enhance the affinity of a TCR isolated from the autologous repertoire to ensure sufficient sensitivity to self-antigens(77). Furthermore, T cells can be isolated from HLA-mismatched healthy donors, which is based on the concept that the naïve T-cell repertoire hypothetically contains T cells specific for any foreign peptide-HLA complex(78, 79). While the frequencies of such T cells are extremely low, T-cell isolations over an HLA-barrier have resulted in identification of various tumor targeting TCRs in the past.

For example, identification of TCRs from HLA-mismatched healthy donors resulted in identification of potent TCRs with specificity for B-cell antigens in various HLA alleles (65, 80). To identify T cells which can recognize peptide-HLA complexes of choice, peptide-HLA (pHLA-) multimers can be used. pHLA-multimers contain multiple peptide-HLA complexes linked to beads. pHLA-multimers mimic natural peptide-HLA complexes as present on the surface of cells, and therefore pHLA-multimers can be used to bind T cells with TCRs able to bind the respective peptide-HLA complexes. pHLA-multimers coupled to a fluorochrome allow flow cytometry-based single-cell isolation of pHLA-multimer binding T cells. Sorted T cells can be clonally expanded, and T-cell clones can then be analyzed to determine recognition profiles, including on-target specificity and potency.

Safety of TCRs

As previously mentioned, the number of unique TCRs in the naïve repertoire has been estimated to be around 1x10^8. On the antigen side, calculations suggest that, considering the existence of 20 amino acids, the potential number of unique peptides that could be generated and presented within an individual's HLA molecules stands at approximately 12x10^11, focusing solely on 10-mer peptides (81). This vast surplus of potentially foreign peptides in comparison to the number of unique TCRs within the naïve T-cell repertoire reveals the necessity for TCRs to possess intrinsic cross-reactivity toward various peptides. This aspect should be considered when analyzing the 'specificity' of an individual TCR.

Since all TCR identification methods in the context of non-mutated peptides are based on avoidance of central tolerance or mutations to alter affinity, any of these approaches is inevitably linked with a risk of cross-reactivity with other peptides presented in the HLA allele of interest. In addition, cross-reactivity with any peptide in any other HLA allele could occur highlighting that extensive safety screening should always be performed. Even when safety screenings are performed thoroughly a risk for unidentified cross-reactivity will remain and should be considered. In the past unidentified cross-reactivities have had detrimental effects when such TCRs were used to treat patients, resulting in lethal toxicity(82, 83). Safety screenings to assess cross-reactivity with other peptides within the target HLA, can include extensive cell panels positive for target HLA but negative for antigen of interest and healthy cell subsets negative for the antigen of interest(84). An alternative strategy to assess safety is to first determine the peptide recognition motif of a specific TCR using alanine/serine substitutions or a peptide library scan(84, 85). This motif can then be used to perform a targeted search for peptides to which cross-reactivity might exist. Cross-reactivity with other HLA alleles can be assessed using an Epstein-Barr virus-transformed lymphoblastoid cell line (EBV-LCL) panel(86, 87). EBV-LCL panels contain EBV immortalized B cells generated from individuals with diverse HLA backgrounds. EBV-LCL panels can be designed in such a way that all common HLA-I alleles are included.

TCR T-cell therapy for B-cell malignancies

To generate multi-antigen-targeting T-cell therapy, TCR T cells can complement CAR T cells to prevent antigen negative immune escape(88). Additionally, the combination of CARs and TCRs can combine the advantages of both therapies and improve the overall efficacy. Multiple TCRs will be needed to allow therapy of patients with divers HLA typings and different types of B-cell or plasma cell malignancies. To identify as many TCRs as possible, all possible target proteins need to be identified and the HLA peptidome of B-cell malignancies can be screened for peptides derived from these proteins that are presented in HLA molecules of interest. T cells with specificity for B-cell lineage protein derived self-peptides can be identified from HLA-mismatched healthy donor PBMCs using pHLA-multimers(65, 80). The cross-reactive nature of TCRs means that thorough safety screenings need to be performed to prevent toxicity(83). Various efficacy screenings should be performed, ultimately including lysis of patient-derived malignant cells as well as *in vivo* anti-tumor efficacy experiments, to identify TCRs that would ultimately be able to induce potent anti-tumor responses in patients. Combining safety and efficacy profiles can reveal which TCRs have value to be further developed for treatment of B-cell and plasma cell malignancies.

AIM OF THIS THESIS

Over the past decades CAR T-cell therapies have been extensively studied and have even become part of standard healthcare in certain parts of the world. However, these advances have also highlighted the heterogeneity and plasticity of B-cell malignancies and demonstrated that multiple antigens should be targeted simultaneously to eliminate antigen escape as a potential way to evade T-cell therapy. TCR T cells could be a useful addition to CAR T cells based on their ability to recognize peptides originating from antigens located on the cell surface as well as inside the cell, allowing them to target antigens that cannot be targeted by CAR T cells. The HLA restricted recognition of TCRs implies that many TCRs need to be identified to have TCR availability for a large population. The aim of this thesis is to identify a large set of TCRs that can be used to treat patients suffering from B-cell malignancies.

In **chapter 2** we address this general aim using various sub-aims, the first aim is the identification of proteins that are exclusively expressed in the B-cell lineage and for which expression is maintained upon malignant information. To identify these genes, a previously established microarray database will be used. Next, we aim to identify which peptides derived from these genes are presented in HLA complexes of interest and can serve as targets for TCR T-cell therapy of B-cell and/or plasma cell malignancies. From a feasibility perspective, a selection of HLA alleles is made that includes HLA-A*01:01,

HLA-A*24:02, HLA-B*08:01 and HLA-B*35:01. Finally, we aim to identify T-cell clones from HLA-mismatched healthy donors that can recognize peptides derived from the selected genes that are presented in these HLA molecules using pHLA-multimer technology. Safety and efficacy screening will be performed to identify T-cell clones with promising recognition profiles. TCRs of these clones will be sequenced and transferred to CD8 T cells to investigate the potential of these TCRs in TCR T-cell therapy of B-cell malignancies.

In **chapter 3** we aim to identify TCRs that can recognize a protein that was identified in **chapter 2** as a promising target protein for treatment of MM, the joining chain (Jchain). The Jchain normally functions as a linker protein between monomer of IgA and IgM when secreted by plasma cells. The Jchain is highly expressed in most MM patient samples independent of the immunoglobulin isotype that is produced and therefore poses an attractive target for TCR T-cell therapy of MM. Next, we aim to identify peptides derived from the Jchain that are presented in HLA-A*01:01, HLA-A*03:01, HLA-A*11:01 and HLA-A*24:02 on the surface of malignant B-cell and/or plasma cells. Using peptide-HLA multimers, T cells will be isolated from PBMCs of HLA-mismatched healthy donors. Isolated T-cell clones will be analyzed for their ability to recognize the targeted peptide-HLA combinations. T-cell clones that demonstrate potent reactivity and high on-target specificity are candidates for TCR sequencing. After TCR sequencing and transfer to CD8 T cells, Jchain-TCR T cells will be screened for their lytic potential to patient derived MM samples as well as *in vivo* targeting potential of MM tumors to establish which Jchain-specific TCRs hold promise for TCR T-cell therapy of MM.

In **chapter 4** we aim to increase tumor specificity of MM-targeting TCRs. MM cells often produce high amounts of immunoglobulins either of IgG or IgA isotypes. In this chapter we therefore want to target IgG or IgA heavy chain constant domain derived peptides, since targeting these isotypes specifically will increase the tumor specificity of TCR T-cell therapy by preventing the depletion of healthy B cells expressing other immunoglobulin isotypes. Epitope and TCR identification will be performed using the same approach as used in **chapter 2** and **chapter 3**. After identification of immunoglobulin-specific T-cell clones, TCR sequencing and transfer, isotype dependent targeting of patient derived MM samples will be tested. Additionally, we will test if IgG- or IgA-targeting TCR T cells can specifically deplete B cells expressing the respective isotype while leaving B cells of other isotypes untouched.

In **chapter 5**, we aim to investigate the possibility that HLA class-I molecules present peptides that are of unconventional length. When selecting epitopes to target with TCR-based therapy from the peptidome of B-cell malignancies, we assume that peptides presented in HLA class-I are relatively short with typical lengths between 8-11 amino

acids. However, peptides with an unconventional length might also be potential targets. To investigate the presentation and subsequent T-cell recognition of unconventionally long peptides we will use a previously identified peptide that is derived from the IL27 receptor alfa. This is a 20-mer peptide that contains multiple amino acids that could be potential anker residues for binding to HLA-A*02:01. Peptide-HLA monomers will be generated and used to create crystal structures that will reveal the conformation of this specific peptide-HLA combination. T cells that recognize this peptide-HLA complex will be identified and used to explore how T cells can recognize such an unconventionally long peptide presented in HLA class-I.

REFERENCES

1. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S41-52.
2. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol. 2008;8(1):34-47.
3. Lu LL, Suscovich TJ, Fortune SM, Alter G. Beyond binding: antibody effector functions in infectious diseases. Nat Rev Immunol. 2018;18(1):46-61.
4. Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol. 2009;70(6):505-15.
5. Rajewsky K. Clonal selection and learning in the antibody system. Nature. 1996;381(6585):751-8.
6. Mårtensson I-L, Keenan RA, Licence S. The pre-B-cell receptor. Current Opinion in Immunology. 2007;19(2):137-42.
7. Murphy K. TP, Walport M. Janeway's Immunobiology. 7th edition ed: Garland Science; 2008 2008.
8. Kelsoe G. B cell diversification and differentiation in the periphery. J Exp Med. 1994;180(1):5-6.
9. Rodig SJ, Kutok JL, Paterson JC, Nitta H, Zhang W, Chapuy B, et al. The pre-B-cell receptor associated protein VpreB3 is a useful diagnostic marker for identifying c-MYC translocated lymphomas. Haematologica. 2010;95(12):2056-62.
10. Mårtensson IL, Melchers F. Pre-B cell-specific lambda 5 gene expression due to suppression in non pre-B cells. Int Immunol. 1994;6(6):863-72.
11. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood. 2008;112(5):1570-80.
12. Hess J, Nielsen PJ, Fischer KD, Bujard H, Wirth T. The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. Mol Cell Biol. 2001;21(5):1531-9.
13. Czajkowsky DM, Shao Z. The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. Proc Natl Acad Sci U S A. 2009;106(35):14960-5.
14. Brandtzaeg P, Prydz H. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. Nature. 1984;311(5981):71-3.
15. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytes choriomeningitis. Nature. 1974;251(5475):547-8.
16. Kane LP, Lin J, Weiss A. Signal transduction by the TCR for antigen. Curr Opin Immunol. 2000;12(3):242-9.
17. Kuhns MS, Davis MM, Garcia KC. Deconstructing the form and function of the TCR/CD3 complex. Immunity. 2006;24(2):133-9.
18. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348(6230):69-74.
19. Verdegaal EM, de Miranda NF, Visser M, Harryvan T, van Buuren MM, Andersen RS, et al. Neoantigen landscape dynamics during human melanoma-T cell interactions. Nature. 2016;536(7614):91-5.
20. Krangel MS. Mechanics of T cell receptor gene rearrangement. Curr Opin Immunol. 2009;21(2):133-9.
21. Turner SJ, Doherty PC, McCluskey J, Rossjohn J. Structural determinants of T-cell receptor bias in immunity. Nature Reviews Immunology. 2006;6(12):883-94.
22. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. Science. 1999;286(5441):958-61.
23. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. Annu Rev Immunol. 2003;21:139-76.
24. Wiegers GJ, Kaufmann M, Tischner D, Villunger A. Shaping the T-cell repertoire: a matter of life and death. Immunol Cell Biol. 2011;89(1):33-9.
25. DeWolf S, Grinshpun B, Savage T, Lau SP, Obradovic A, Shonts B, et al. Quantifying size and diversity of the human T cell alloresponse. JCI Insight. 2018;3(15).
26. Shaw BE, Potter MN, Mayor NP, Pay AL, Smith C, Goldman JM, et al. The degree of matching at HLA-DPB1 predicts for acute graft-versus-host disease and disease relapse following haematopoietic stem cell transplantation. Bone Marrow Transplant. 2003;31(11):1001-8.
27. Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD, et al. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin Cancer Res. 2011;17(17):5615-25.
28. Heeger PS. T-Cell Allorecognition and

Transplant Rejection: A Summary and Update. American Journal of Transplantation. 2003;3(5):525-33.
29. Klein J, Sato A. The HLA system. First of two parts. N Engl J Med. 2000;343(10):702-9.
30. Oliveira CC, van Hall T. Alternative Antigen Processing for MHC Class I: Multiple Roads Lead to Rome. Front Immunol. 2015;6:298.
31. van Endert PM, Tampé R, Meyer TH, Tisch R, Bach JF, McDevitt HO. A sequential model for peptide binding and transport by the transporters associated with antigen processing. Immunity. 1994;1(6):491-500.
32. Oliveira CC, Querido B, Sluijter M, de Groot AF, van der Zee R, Rabelink MJ, et al. New role of signal peptide peptidase to liberate C-terminal peptides for MHC class I presentation. J Immunol. 2013;191(8):4020-8.
33. Markov PV, Pybus OG. Evolution and Diversity of the Human Leukocyte Antigen(HLA). Evol Med Public Health. 2015;2015(1):1.
34. Gonzalez-Galarza FF, McCabe A, Santos E, Jones J, Takeshita L, Ortega-Rivera ND, et al. Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. Nucleic Acids Res. 2020;48(D1):D783-d8.
35. Remesh SG, Andreatta M, Ying G, Kaever T, Nielsen M, McMurtrey C, et al. Unconventional Peptide Presentation by Major Histocompatibility Complex (MHC) Class I Allele HLA-A*02:01: BREAKING CONFINEMENT. J Biol Chem. 2017;292(13):5262-70.
36. Nguyen AT, Szeto C, Gras S. The pockets guide to HLA class I molecules. Biochem Soc Trans. 2021;49(5):2319-31.
37. Küppers R, Klein U, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. N Engl J Med. 1999;341(20):1520-9.
38. Pui CH, Evans WE. Acute lymphoblastic leukemia. N Engl J Med. 1998;339(9):605-15.
39. Rowe JM. Prognostic factors in adult acute lymphoblastic leukaemia. Br J Haematol. 2010;150(4):389-405.
40. Zenz T, Mertens D, Küppers R, Döhner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. Nat Rev Cancer. 2010;10(1):37-50.
41. van der Straten L, Maas C, Levin MD, Visser O, Posthuma EFM, Doorduijn JK, et al. Long-term trends in the loss in expectation of life after a diagnosis of chronic lymphocytic leukemia: a population-based study in the Netherlands, 1989-2018. Blood Cancer J. 2022;12(4):72.
42. Thandra KC, Barsouk A, Saginala K, Padala SA, Barsouk A, Rawla P. Epidemiology of Non-Hodgkin's Lymphoma. Med Sci (Basel). 2021;9(1).
43. Wang L, Qin W, Huo Y-J, Li X, Shi Q, Rasko JEJ, et al. Advances in targeted therapy for malignant lymphoma. Signal Transduction and Targeted Therapy. 2020;5(1):15.
44. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med. 2011;364(11):1046-60.
45. Bobin A, Leleu X. Recent advances in the treatment of multiple myeloma: a brief review. Fac Rev. 2022;11:28.
46. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer Journal. 2021;11(4):69.
47. Sheikh S, Migliorini D, Lang N. CAR T-Based Therapies in Lymphoma: A Review of Current Practice and Perspectives. Biomedicines. 2022;10(8).
48. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. N Engl J Med. 2018;378(5):439-48.
49. Cappell KM, Kochenderfer JN. Long-term outcomes following CAR T cell therapy: what we know so far. Nature Reviews Clinical Oncology. 2023;20(6):359-71.
50. Dogan A, Siegel D, Tran N, Fu A, Fowler J, Belani R, et al. B-cell maturation antigen expression across hematologic cancers: a systematic literature review. Blood Cancer Journal. 2020;10(6):73.
51. Rodriguez-Otero P, Ailawadhi S, Arnulf B, Patel K, Cavo M, Nooka AK, et al. Ide-cel or Standard Regimens in Relapsed and Refractory Multiple Myeloma. The New England journal of medicine. 2023;388(11):1002-14.
52. Labanieh L, Mackall CL. CAR immune cells: design principles, resistance and the next generation. Nature. 2023;614(7949):635-48.
53. Brudno JN, Maric I, Hartman SD, Rose JJ, Wang M, Lam N, et al. T Cells Genetically Modified to Express an Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor Cause Remissions of Poor-Prognosis Relapsed Multiple Myeloma. J Clin Oncol. 2018;36(22):2267-80.
54. Cohen AD, Garfall AL, Stadtmauer EA, Melenhorst JJ, Lacey SF, Lancaster E, et al. B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma. J Clin Invest. 2019;129(6):2210-21.
55. Samur MK, Fulciniti M, Aktas Samur A, Bazarbachi AH, Tai YT, Prabhala R, et al. Biallelic loss of BCMA as a resistance mechanism to

CAR T cell therapy in a patient with multiple myeloma. Nat Commun. 2021;12(1):868.
56. Pont MJ, Hill T, Cole GO, Abbott JJ, Kelliher J, Salter AI, et al. γ-Secretase inhibition increases efficacy of BCMA-specific chimeric antigen receptor T cells in multiple myeloma. Blood. 2019;134(19):1585-97.
57. Good CR, Aznar MA, Kuramitsu S, Samareh P, Agarwal S, Donahue G, et al. An NK-like CAR T cell transition in CAR T cell dysfunction. Cell. 2021;184(25):6081-100.e26.
58. Wijewarnasuriya D, Bebernitz C, Lopez AV, Rafiq S, Brentjens RJ. Excessive Costimulation Leads to Dysfunction of Adoptively Transferred T Cells. Cancer Immunol Res. 2020;8(6):732-42.
59. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med. 2015;21(6):581-90.
60. Wachsmann TLA, Wouters AK, Remst DFG, Hagedoorn RS, Meeuwsen MH, van Diest E, et al. Comparing CAR and TCR engineered T cell performance as a function of tumor cell exposure. Oncoimmunology. 2022;11(1):2033528.
61. Feucht J, Sun J, Eyquem J, Ho YJ, Zhao Z, Leibold J, et al. Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. Nat Med. 2019;25(1):82-8.
62. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJC, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017;543(7643):113-7.
63. Bonifant CL, Jackson HJ, Brentjens RJ, Curran KJ. Toxicity and management in CAR T-cell therapy. Mol Ther Oncolytics. 2016;3:16011.
64. Huang R, Li X, He Y, Zhu W, Gao L, Liu Y, et al. Recent advances in CAR-T cell engineering. Journal of Hematology & Oncology. 2020;13(1):86.
65. Jahn L, Hombrink P, Hagedoorn RS, Kester MG, van der Steen DM, Rodriguez T, et al. TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1. Blood. 2017;129(10):1284-95.
66. Zhao C, Inoue J, Imoto I, Otsuki T, Iida S, Ueda R, et al. POU2AF1, an amplification target at 11q23, promotes growth of multiple myeloma cells by directly regulating expression of a B-cell maturation factor, TNFRSF17. Oncogene. 2008;27(1):63-75.
67. de Matos Simoes R, Shirasaki R, Downey-Kopyscinski SL, Matthews GM, Barwick BG, Gupta VA, et al. Genome-scale functional genomics identify genes preferentially essential for multiple myeloma cells compared to other neoplasias. Nature Cancer. 2023;4(5):754-73.
68. Ramos CA, Savoldo B, Torrano V, Ballard B, Zhang H, Dakhova O, et al. Clinical responses with T lymphocytes targeting malignancy-associated κ light chains. The Journal of clinical investigation. 2016;126(7):2588-96.
69. Pont MJ, Honders MW, Kremer AN, van Kooten C, Out C, Hiemstra PS, et al. Microarray Gene Expression Analysis to Evaluate Cell Type Specific Expression of Targets Relevant for Immunotherapy of Hematological Malignancies. PLoS One. 2016;11(5):e0155165.
70. Nielsen M, Lundegaard C, Worning P, Lauemøller SL, Lamberth K, Buus S, et al. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci. 2003;12(5):1007-17.
71. Hombrink P, Hadrup SR, Bakker A, Kester MG, Falkenburg JH, von dem Borne PA, et al. High-throughput identification of potential minor histocompatibility antigens by MHC tetramer-based screening: feasibility and limitations. PLoS One. 2011;6(8):e22523.
72. Hassan C, Kester MG, de Ru AH, Hombrink P, Drijfhout JW, Nijveen H, et al. The human leukocyte antigen-presented ligandome of B lymphocytes. Mol Cell Proteomics. 2013;12(7):1829-43.
73. Andreatta M, Nielsen M. Gapped sequence alignment using artificial neural networks: application to the MHC class I system. Bioinformatics. 2016;32(4):511-7.
74. van der Lee DI, Reijmers RM, Honders MW, Hagedoorn RS, de Jong RC, Kester MG, et al. Mutated nucleophosmin 1 as immunotherapy target in acute myeloid leukemia. J Clin Invest. 2019;129(2):774-85.
75. Roex MCJ, Hageman L, Veld SAJ, van Egmond E, Hoogstraten C, Stemberger C, et al. A minority of T cells recognizing tumor-associated antigens presented in self-HLA can provoke antitumor reactivity. Blood. 2020;136(4):455-67.
76. Li L-P, Lampert JC, Chen X, Leitao C, Popović J, Müller W, et al. Transgenic mice with a diverse human T cell antigen receptor repertoire. Nature Medicine. 2010;16(9):1029-34.
77. Border EC, Sanderson JP, Weissensteiner T,

Gerry AB, Pumphrey NJ. Affinity-enhanced T-cell receptors for adoptive T-cell therapy targeting MAGE-A10: strategy for selection of an optimal candidate. Oncoimmunology. 2019;8(2):e1532759.
78. Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S, et al. Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood. 2009;114(10):2131-9.
79. Stauss HJ. Immunotherapy with CTLs restricted by nonself MHC. Immunol Today. 1999;20(4):180-3.
80. Jahn L, Hombrink P, Hassan C, Kester MG, van der Steen DM, Hagedoorn RS, et al. Therapeutic targeting of the BCR-associated protein CD79b in a TCR-based approach is hampered by aberrant expression of CD79b. Blood. 2015;125(6):949-58.
81. Sewell AK. Why must T cells be cross-reactive? Nat Rev Immunol. 2012;12(9):669-77.
82. Cameron BJ, Gerry AB, Dukes J, Harper JV, Kannan V, Bianchi FC, et al. Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. Sci Transl Med. 2013;5(197):197ra03.
83. Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood. 2013;122(6):863-71.
84. Bijen HM, van der Steen DM, Hagedoorn RS, Wouters AK, Wooldridge L, Falkenburg JHF, et al. Preclinical Strategies to Identify Off-Target Toxicity of High-Affinity TCRs. Mol Ther. 2018;26(5):1206-14.
85. Foldvari Z, Knetter C, Yang W, Gjerdingen TJ, Bollineni RC, Tran TT, et al. A systematic safety pipeline for selection of T-cell receptors to enter clinical use. NPJ Vaccines. 2023;8(1):126.
86. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood. 2010;115(15):3146-57.
87. Huisman W, Leboux DAT, van der Maarel LE, Hageman L, Amsen D, Falkenburg JHF, et al. Magnitude of Off-Target Allo-HLA Reactivity by Third-Party Donor-Derived Virus-Specific T Cells Is Dictated by HLA-Restriction. Front Immunol. 2021;12:630440.
88. Teppert K, Wang X, Anders K, Evaristo C, Lock D, Künkele A. Joining Forces for Cancer Treatment: From "TCR versus CAR" to "TCR and CAR". Int J Mol Sci. 2022;23(23).

CHAPTER 2

A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy

Miranda H. Meeuwsen[1]
Anne K. Wouters[1]
Lorenz Jahn[1,3]
Renate S. Hagedoorn[1]
Michel G.D. Kester[1]
Dennis F.G. Remst[1]
Laura T. Morton[1]
Dirk M. van der Steen[1]
Christiaan Kweekel[1]
Arnoud H. de Ru[2]
Marieke Griffioen[1]
Peter A. van Veelen[2]
J.H. Frederik Falkenburg[1]
Mirjam H.M. Heemskerk[1]

[1]Department of Hematology, Leiden University Medical Center, The Netherlands; [2]Center for Proteomics and Metabolomics, Leiden University Medical Center, The Netherlands; [3]Department of Immunology, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, USA

Molecular therapy 2022
Vol. 30, Issue 2, Pages 564-578

ABSTRACT

CAR T-cell therapy has shown great promise for the treatment of B-cell malignancies. However, antigen-negative escape variants often cause disease relapse necessitating the development of multi-antigen-targeting approaches. We propose that a TCR-based strategy would increase the number of potential antigenic targets as peptides from both intracellular and extracellular proteins can be recognized. Here, we aimed to isolate a broad range of promising TCRs targeting multiple antigens for treatment of B-cell malignancies. As a first step, 28 target genes for B-cell malignancies were selected based on gene expression profiles. Twenty target peptides presented in either HLA-A*01:01, A*24:02, B*08:01 or B*35:01 were identified from the immunopeptidome of B-cell malignancies and used to form peptide-HLA-tetramers for T-cell isolation. Target-peptide specific CD8 T cells were isolated from HLA-mismatched healthy donors and subjected to a stringent stepwise selection procedure to ensure potency and eliminate cross-reactivity. In total, five T-cell clones specific for FCRL5 in HLA-A*01:01, VPREB3 in HLA-A*24:02 and BOB1 in HLA-B*35:01 recognized B-cell malignancies. For all three specificities, TCR gene transfer into CD8 T cells resulted in cytokine production and efficient killing of multiple B-cell malignancies. In conclusion, using this systematic approach we successfully identified three promising TCRs for T-cell therapy against B-cell malignancies.

INTRODUCTION

Adoptive T-cell therapy for the treatment of B-cell malignancies has shown great promise over the past decade. CD19-targeting chimeric antigen receptor (CAR) T-cell therapies have induced complete remission (CR) in 70-97% of patients with relapsed or refractory acute lymphoblastic leukemia (ALL).(1, 2) In aggressive relapsed or refractory diffuse large B-cell lymphoma (DLBCL) and transformed follicular lymphoma CR rates after CD19 CAR therapy are approximately 50%.(3, 4) In responding patients, antigen-loss escape variants are frequently observed, hampering long-term relapse-free survival.(5, 6) Multi-antigen-targeting T-cell therapy may reduce outgrowth of antigen-escape variants and enhance long-term remission rates of patients, as simultaneous loss of multiple antigens is more unlikely.(7, 8) However, in order to generate multi-antigen-targeting T-cell therapies, additional potent and safe T-cell therapies need to be developed.

Target antigens for immunotherapy of cancer should be expressed by the malignant cells while expression in essential healthy tissues must be absent.(9, 10) B-cells are considered a non-essential tissue, therefore antigens expressed by the healthy B-cell lineage could be safe targets for the treatment of B-cell malignancies. This was confirmed upon CD19 CAR T-cell therapy, which resulted in depletion of healthy B cells which was clinically managed by immunoglobulin administration.(11)

CAR T cells target epitopes of proteins located on the cell surface.(12) In contrast to CARs, T-cell receptors (TCRs) recognize peptides derived from proteins independent of cellular localization that are presented in HLA molecules on the cell surface.(13) Therefore, TCR based therapy theoretically allows targeting of all proteins, including those involved in essential intracellular pathways. So far, limited availability of safe and high-affinity TCRs has hampered clinical progress of TCR gene therapy. Furthermore, a large collection of high affinity TCRs targeting different peptide-HLA complexes would be required to allow TCR gene therapy for all patients.

T cells with high affinity TCRs for self-peptides presented in self-HLA alleles are deleted from the T-cell repertoire to prevent autoimmunity. Therefore, high avidity T cells targeting non-mutated peptides cannot be isolated from HLA-matched individuals.(14, 15) In contrast, high avidity T cells targeting non-mutated peptides have successfully been isolated from the T-cell repertoire of HLA-mismatched (allogeneic) healthy donors.(15-23) Previous efforts have mainly focused on identification of TCRs specific for peptides presented in HLA-A*02:01 and HLA-B*07:02.(15-19, 24) Here, to allow application of TCR gene therapy for individuals with other HLA genotypes, we aimed to identify TCR specific for peptides presented in HLA alleles A*01:01 (A1), A*24:02 (A24), B*08:01 (B8) and B*35:01 (B35). In this study, we performed a systematic approach for

simultaneous identification of clinically relevant TCRs targeting multiple epitopes. We started by identifying genes with expression restricted to B-cell malignancies and the healthy B-cell lineage. Target peptides derived from these genes presented in target HLA alleles HLA-A1, A24, B8 or B35 were selected from the immunopeptidome of B-cell malignancies. To allow identification of high avidity T cells targeting these epitopes, T cells were isolated from the allogeneic (allo) T-cell repertoire using HLA-mismatched healthy donor PBMCs. Multiple efficacy and safety screenings resulted in the selection of clinically relevant TCRs recognizing antigens in HLA-A1, HLA-A24 and HLA-B35 presented on B-cell malignancies. Finally, upon TCR gene transfer, selected TCRs induced specific lysis of different B-cell malignancies including a multiple myeloma (MM) cell line and patient-derived ALL, chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL) and hairy cell leukemia (HCL).

RESULTS

Target gene selection for treatment of B-cell malignancies

To ensure targeting of malignant B cells while limiting the risk of toxicity, data obtained from an in-house-generated microarray database were used to identify genes expressed in B-cell malignancies with expression restricted to the B-cell lineage. This database was previously generated and validated for selection of target antigens valuable for immunotherapy of hematological malignancies with lineage restricted expression. (25) Gene expression was measured by probe fluorescence represented as mean fluorescence intensity (MFI), with a lower detection limit of MFI=50. Potent T-cell recognition occurs when target antigens are highly expressed in B-cell malignancies, and therefore, a threshold of gene expression MFI≥250 in primary ALL, CLL or MM was set for each probe (**Figure S1**). To prevent recognition of healthy tissues (excluding B cells), a threshold was set at MFI=100 to define very low expression and all genes expressed MFI>100 in healthy tissues were excluded. In total, 28 genes highly expressed in primary B-cell malignancies with very low or no expression in healthy tissues were selected as target genes (**Figure 1**). Of these genes, 14 were highly expressed (MFI≥250) in either ALL, CLL or MM, while 13 genes were expressed in two types and the BOB1 encoding gene *POU2AF1* was expressed in all three types of primary B-cell malignancies.

Identification of candidate epitopes from the immunopeptidome of malignant B-cells

To identify target peptides for TCR based recognition of B-cell malignancy antigens, the HLA-presented peptide repertoire of B-cell malignancies was determined. Patient material was obtained from nine ALL patients, two CLL patients, one hairy cell leukemia (HCL) and one follicular lymphoma patient expressing HLA-A1, A24, B8 and/or B35 (**Table S1**).

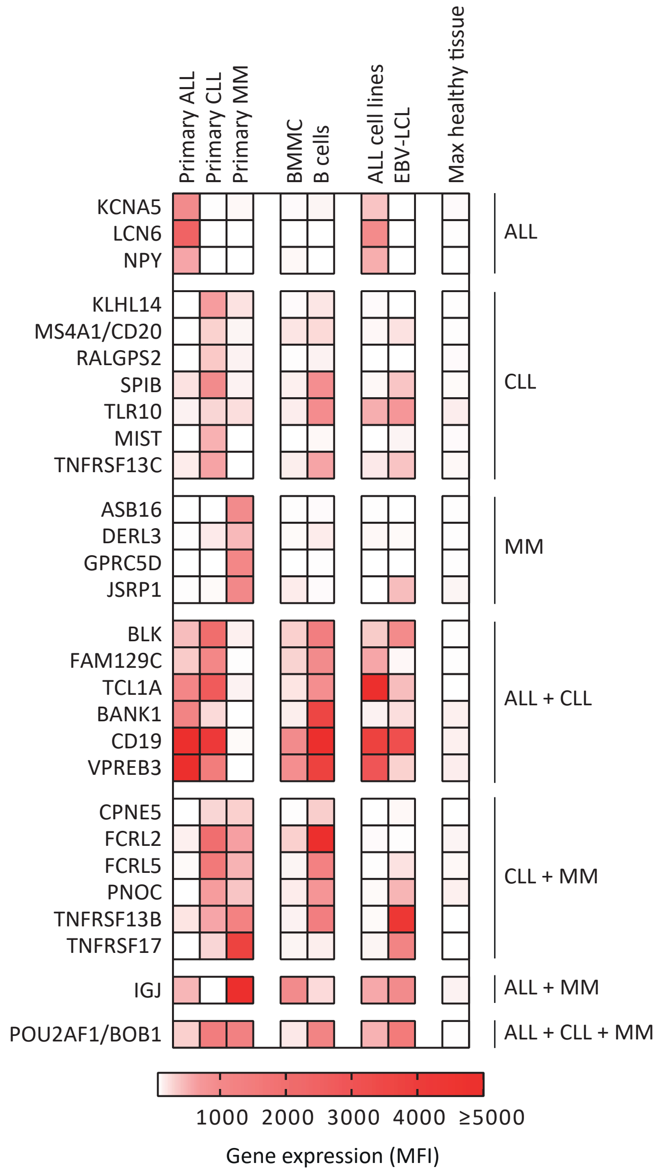


Figure 1. Gene expression of target genes selected for treatment of B-cell malignancies. Gene expression was retrieved from an Illumina HT12.0 microarray dataset.(25) Per gene the average mean fluorescence intensity (MFI) of samples is shown for different types of patient derived (primary) B-cell malignancies, BMMCs, B cells (CD19^{pos}), ALL cell lines and EBV-LCLs. The final column represents the highest gene expression as measured in any healthy tissue other than B cells, PBMCs and BMMCs as included in the microarray dataset and shown in supplementary figure 1. Genes are clustered according to the type of B-cell malignancy in which they are expressed (MFI≥250). Abbreviations: MFI, mean fluorescence intensity; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; BMMC, bone marrow mononuclear cells; EBV-LCL; Epstein-Barr virus-transformed lymphoblastoid cell lines.

Since materials from MM patients lacked sufficient cell numbers for peptide elution, the MM cell line UM9 was included instead. Cells from each malignant cell population were lysed, and peptides were eluted before subsequent separation by HPLC and mass spectrometry analysis. Per sample, between 539 and 82,504 unique peptides, with an Ion Score (IS) ≥20, were identified (**Table S1**). The Ion Score indicates the confidence of correctly matching the observed mass spectrum of a peptide to the reference database spectrum. Therefore, a cutoff of Ion score ≥20 was used to ensure a high chance of correct peptide identification. Eluted peptides were matched to HLA alleles by combining predicted HLA binding (netMHC3.4) with the HLA typing of the material from which the peptides originated. Twenty peptides were identified to be derived from one of the target genes and presented by target HLA alleles HLA-A1, A24, B8 or B35 (**Table S2, Table 1**). Peptide sequences of these peptides were validated by comparing mass spectra of eluted peptides to spectra of synthetically generated peptides (**Figure S2**). After peptide verification, target HLA binding was confirmed by stable peptide-HLA (pHLA)-monomer refolding (data not shown). From these monomers PE-labeled pHLA-tetramers were generated and used for T-cell isolation.

Table 1. Target gene derived peptides presented in HLA-A1, A24, B8 or B35 by B-cell malignancies

Protein	HLA	Assigned peptide nr	Sequence	Affinity (nM)[a]	SB/WB[a]
RALGPS2	A1	p242	LTDSEKGNSY	24	SB
FCRL5	A1	p243	LTEGHSGNYY	15	SB
FCRL5	A1	p263	TTENSGNYY	9	SB
MIST	A1	p248	ESEYADTHY	102	WB
TLR10	A1	p265	YLDHNSFDY	8	SB
IGJ	A1	p268	YTAVVPLVY	7	SB
BLK	A24	p246	AYIERMNSI	75	WB
VPREB3	A24	p269	YYCSVGYGF	37	SB
RALGPS2	A24	p258	QYIEELQKF	78	WB
TLR10	B8	p247	ELFKRTIQL	54	WB
TLR10	B8	p256	LPHLKTLIL	26	SB
IGLL1	B8	p251	HGLLRPTAA	588	<WB[b]
KLHL14	B8	p239	DMNTKRAIHTL	396	WB
FCRL2	B8	p252	IVKIKVQEL	252	WB
FAM129C	B8	p255	LPALRAQTL	32	SB
FAM129C	B8	p271	YLRLLDAL	726	<WB[b]
RALGPS2	B8	p261	TLKIRAEVL	43	SB
TNFRSF13B	B35	p259	SADQVALVY	11	SB
BOB1	B35	p233	APAPTAVVL	196	WB
BOB1	B35	p236	LPHQPLATY	6	SB

[a] According to NetMHC 3.4. [b] HLA-B8 most likely origin of peptides, highest predicted binding.

Isolation and selection of T-cell clones with on-target functional specificity

To isolate high avidity T cells recognizing target peptides, pHLA-tetramers were incubated with PBMCs from healthy donors negative for the target HLA alleles (**Table S3**). pHLA-tetramer bound cells were enriched and pHLA-tetramer^{pos} CD8^{pos} T cells were single-cell sorted and clonally expanded. In total 12,336 (Range; 192-2,640) T cells were sorted from 13 healthy donors (**Table 2**). On average 59% (Mean; range 14-83%) of the T-cell clones expanded. Expanded T-cell clones were initially screened for on-target functional specificity. The HLA-negative myeloid leukemia cell line K562 was transduced (Td) with target HLA alleles and mixed into two pools containing HLA-A1 and HLA-A24 or HLA-B8 and HLA-B35. All target peptides were mixed and loaded onto K562 before incubation with the T-cell clones (**Table 1**). As measured by cytokine production, unloaded K562 recognition was frequently observed revealing off-target recognition and these clones were discarded to prevent off-target toxicity (**Table 2**). Additionally, 34-98% of expanded T-cell clones were not reactive and were discarded due to lack of target peptide recognition. On-target recognition was defined when only peptide mix loaded K562 cells were recognized. From these 13 donors, 23 T-cell clones recognized only HLA-A1/A24^{pos} K562 cells loaded with the target peptide mix (**Table 2**). In addition, 23 T-cell clones only recognized target peptide loaded HLA-B8/B35^{pos} K562 cells (**Table 2**). In summary, 46 T-cell clones demonstrated specific, on-target recognition and were selected for further screening.

Defining T-cell specificity and confirming recognition of endogenously processed target antigen

To determine the peptide specificity of each selected T-cell clone, target peptides were assorted into combinatorial mixes revealing unique recognition patterns for all specificities. Mixes were loaded onto HLA-A1/A24^{pos} or HLA-B8/B35^{pos} K562 cells accordingly and incubated with each T-cell clone. For example, T-cell clones 1F3.4 and 6B10.12, recognized two peptide mixes loaded on HLA-A1/A24^{pos} K562 cells (**Figure 2A**). This recognition pattern revealed specificity for peptide 243 (p243) derived from FCRL5 presented in the context of HLA-A1 (**Table 1**). Following this approach, all 46 T-cell clones were tested revealing 11 different peptide specificities (**Table 2**). Since recognition of endogenously processed and presented antigen correlates with T-cell avidity and recognition of malignant cells (26), T-cell clones were also screened for recognition of K562 cells Td with target genes and target HLA alleles. T-cell clones demonstrating no or weak recognition of endogenously processed and presented antigen were discarded. In Figure 2, 23 T-cell clones, recognizing eight different B-cell specific peptides, that exhibited strong endogenous recognition are shown. These 23 T-cell clones were selected for further investigation of clinical relevance.

Table 2. Identification of target peptide specific T-cell clones from tetramer^{pos} CD8^{pos} sorted T cells using healthy donor PBMCs

			Reactivity of T-cell clones [d]				Peptide specificity of T-cell clones [e]										
Donor [a]	T cells sorted (n) [b]	Clones exp. (n) [c]	Not reactive (n)	Off-target (n)	HLA-A1/A24 (n)	HLA-B8/B35 (n)	p236 POU2AF1 B35	p243 FCRL5 A1	p247 TLR10 B8	p248 MIST A1	p252 FCRL2 B8	p256 TLR10 B8	p259 TNFRSF13B B35	p261 RALGPS2 B8	p263 FCRL5 A1	p265 TLR10 A1	p269 VPREB3 A24
3	288	239	233	6	0	0											
4	192	156	101	54	1	0		1x									
5	192	129	44	83	0	2							2x				
6	528	433	235	180	6	12	6x		1x	2x	3x		1x	1x		3x	1x
7	288	207	181	26	0	0											
8	384	311	289	22	0	0											
12	864	639	320	314	5	0		1x		2x					2x		
13	1728	1002	891	108	2	1						1x					2x
14	1296	384	330	53	0	1						1x					
15	864	449	399	49	0	1								1x			
16	1920	1018	907	105	3	3				1x			2x	1x			2x
17	2640	360	351	9	0	0											
18	1152	192	143	40	6	3				2x	2x	1x				2x	2x
Total	12336	5519	4424	1049	23	23	6x	2x	1x	7x	5x	3x	5x	3x	2x	5x	7x

[a] Arbitrary donor numbers assigned to healthy donors from which PBMCs were obtained, in accordance with Table S3. [b] Number of pHLA-tetramer^{pos} CD8^{pos} T cells single-cell sorted from each donor. [c] Number of single-cell sorted T cells that expanded and were tested for peptide specificity. [d] Expanded T-cell clones were stimulated with a 1:1 mixture of HLA-A1 and HLA-A24 transduced K562 cells or HLA-B8 and B35 transduced K562 cells, unloaded or loaded with a mixture of all target peptides (100nM). Reactivity was assessed after overnight co-culture based on IFN-γ ELISA, for T-cell clones not producing IFN-γ, GM-CSF was used instead. Recognition was categorized in; not reactive, off-target recognition, HLA-A1/A24 restricted peptide specific recognition (HLA-A1/A24) and HLA-B8/B35 restricted peptide specific recognition (HLA-B8/B35). [e] The number of peptide specific T-cell clones per donor is indicated, specificity was determined as demonstrated in Figure 2. No T-cell clones were identified for peptides not included in the table.

Recognition of B-cell malignancies identifies T-cell clones which are candidates for TCR-sequencing

To identify candidates for TCR sequencing, the 23 selected T-cell clones were screened for recognition of B-cell malignancies. T-cell clones were tested for cytokine production upon stimulation with patient derived CLL material, ALL cell lines or MM cell lines. Per specificity the appropriate cell types were selected based on gene expression profiles (**Figure 1**). Target gene expression in materials used was determined by qPCR (**Figure S3**). Despite recognition of endogenously processed antigen in target gene Td k562 cells, MIST HLA-A1, FCRL2 HLA-B8, RALGPS2 HLA-B8 and TNFRSF13B HLA-B35 restricted T-cell clones failed to produce cytokine upon co-culture with malignant B-cells (data not shown). FCRL5 HLA-A1 specific T-cell clones 1F3.4 and 7E8.12, recognizing p243 and p263 respectively, were co-cultured with *FCRL5* expressing patient derived CLL samples.

T-cell clone 6B10.12 did not expand and could therefore not be included. Recognition of HLA-A1^{pos} CLL samples was observed for clone 1F3.4 (**Figure 3A**), but not for clone 7E8.12 (data not shown). Allo-HLA T-cell clones were included as positive controls for stimulatory capacity and HLA expression of the target cells. Furthermore, 3 out of 6 VPREB3 HLA-A24 restricted T-cell clones (6A2.18, 6E2.18 and 10G3.16) efficiently recognized all *VPREB3* expressing HLA-A24 ^{pos} ALL cell lines, whereas HLA-A24 ^{neg} ALL cell lines were not recognized (**Figure 3B**). In addition, BOB1 HLA-B35 restricted T-cell clone 1C5.6, demonstrated efficient recognition of all *POU2AF1* (BOB1) expressing HLA-B35^{pos} ALL and MM cell lines tested (**Figure 3C**). BOB1 HLA-B35 restricted T-cell clone 4H5.6 did not recognize these B-cell malignancies (data not shown). Of note, malignant cell recognition by the VPREB3 HLA-A24 and BOB1 HLA-B35 T-cell clones correlated with a high avidity as measured by peptide titration (**Figure S4**). To summarize, 5 out of 22 tested T-cell clones recognized B-cell malignancies and were therefore promising candidates for TCR gene therapy. These T-cell clones were specific for FCRL5 in HLA-A1, VPREB3 in HLA-A24 and BOB1 in HLA-B35.

Safety screenings reveal high on-target specificity of selected T-cell clones

To study the specificity of the 5 selected T-cell clones, safety profiles were investigated. Cross-reactivity with other HLA-alleles was assessed using an Epstein-Barr virus transformed lymphoblastoid cell line (EBV-LCL) panel expressing all HLA-I alleles with an allele frequency >1% in the Caucasian population (**Figure 4, Table S4**).(27) Additionally, cross-reactivity with other peptides presented in target HLA alleles was determined by stimulating with HLA-A1 and B8 or HLA-A24 and B35 Td cell lines of non-B-cell origins (**Figure 4**). The respective allo HLA-A1, A24 and B35 T-cell clones recognized the HLA transduced cell lines (**Figure S5**), demonstrating that sufficient HLA was expressed to allow T-cell recognition. T-cell clones 1F3.4 (FCRL5 HLA-A1), 6A2.18 (VPREB3 HLA-A24), 10G3.16 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) did not demonstrate cross-reactivity with any of the target cells included in the screenings (**Figure 4A-C**). However, T-cell clone 6E2.18 (VPREB3 HLA-A24) produced IFN-γ when stimulated with EBV-LCLs from donor URN (EBV-LCL URN) and upon stimulation with HLA-A24/B35 Td CASKI cells (**Figure 4B**). This was a result of cross-reactivity with two possible peptide-HLA complexes. Recognition of EBV-LCL URN resulted from cross-reactivity with a peptide presented in HLA-B*08:01 or B*50:01, since these HLA alleles were expressed by EBV-LCL URN but not by other EBV-LCLs in the panel. Recognition of HLA-A24/B35 Td CASKI cells was caused by cross-reactivity with a peptide in HLA-A24 or B35, since untransduced CASKI cells were not recognized (data not shown). In summary, most T-cell clones targeting FCRL5 in HLA-A1, VPREB3 in HLA-A24 or BOB1 in HLA-B35 revealed promising safety profiles for clinical application in TCR gene therapy of B-cell malignancies.

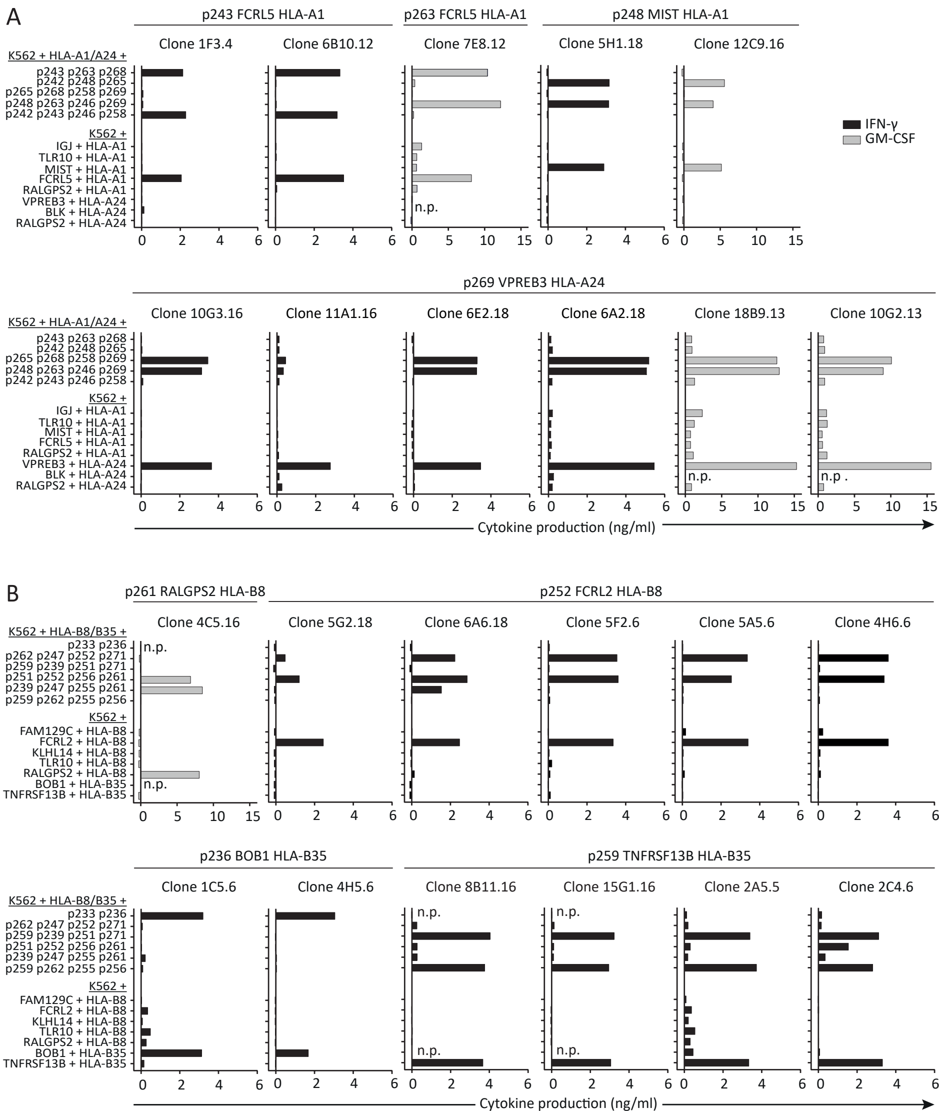


Figure 2. Peptide specificity and target gene recognition by selected T-cell clones. T-cell clones were stimulated with the appropriate HLA-expressing K562 cells in an effector:target ratio of 1:6 loaded with combinatorial combinations of target peptides to determine peptide specificity (upper part) and K562 cells Td with target gene and HLA (bottom part) to determine recognition of endogenously processed and presented peptide. IFN-γ (in black) or GM-CSF (in grey) production by T-cell clones categorized as high endogenous recognition. GM-CSF production is shown for clones that do not produce IFN-γ. T-cell clones isolated from different donors were tested in separate experiments. **A)** T-cell clones recognizing HLA-A1 or A24 presented target peptides. **B)** T-cell clones recognizing HLA-B8 or B35 presented target peptides, peptide 233 and 236 were not part of combinatorial mixes. For clone 1C5.6 and 4H5.6 specificity for p236 was determined by pHLA-tetramer stain (data not shown). Abbreviations: n.p., not performed

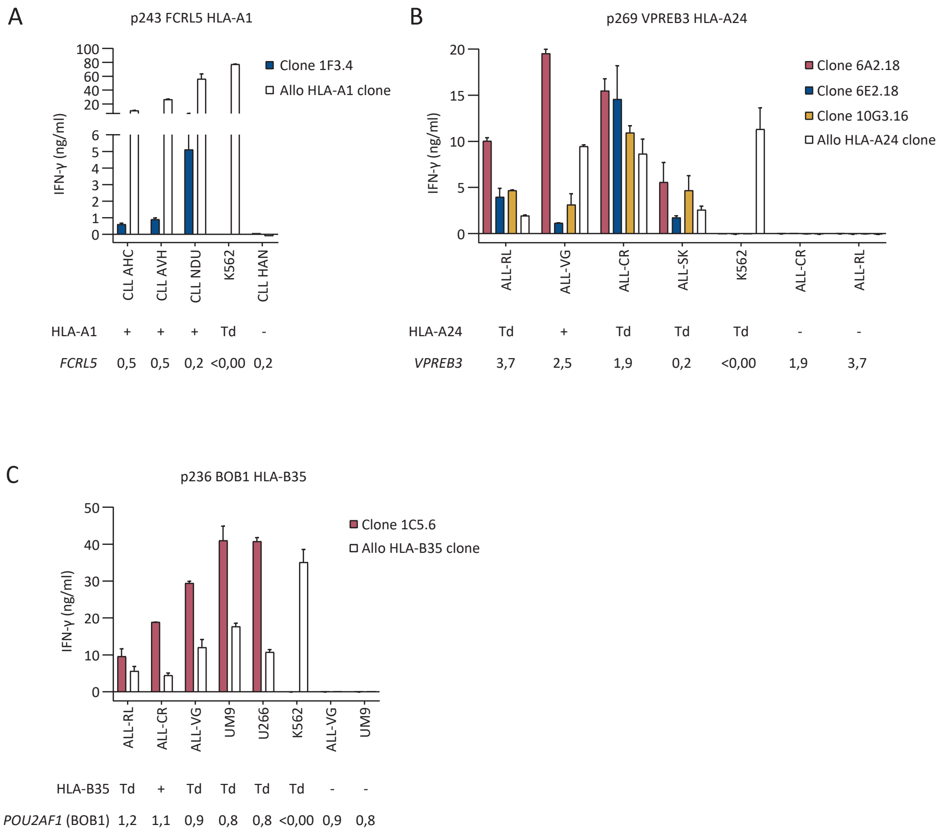


Figure 3. Recognition of malignant B cells by selected T-cell clones. IFN-γ production by T-cell clones after overnight stimulation with various target cells. Values and error bars represent mean and standard deviations of technical duplicates. Target cells were positive (+), negative (-) or transduced (Td) with target HLA. Target gene expression was measured by qPCR and is depicted below targets as expression relative to housekeeping genes (HKG set to 1). Allo HLA T-cell clones were included as positive controls for HLA expression. **A)** FCRL5 HLA-A1 specific T-cell clone stimulated with patient derived chronic lymphocytic leukemia (CLL) samples, in an effector:target (E:T) ratio of 1:20 to correct for cell size and negative control K562 cells in E:T ratio 1:6. T-cell clone 6B10.12 could not be tested due to lack of expansion. **B)** VPREB3 HLA-A24 clones stimulated with different acute lymphoblastic leukemia (ALL) cell lines and K562 cells in E:T ratio 1:6. **C)** BOB1 HLA-B35 clone stimulated with different ALL cell lines, multiple myeloma cell lines UM9 and U266 and K562 cells in E:T ratio 1:6.

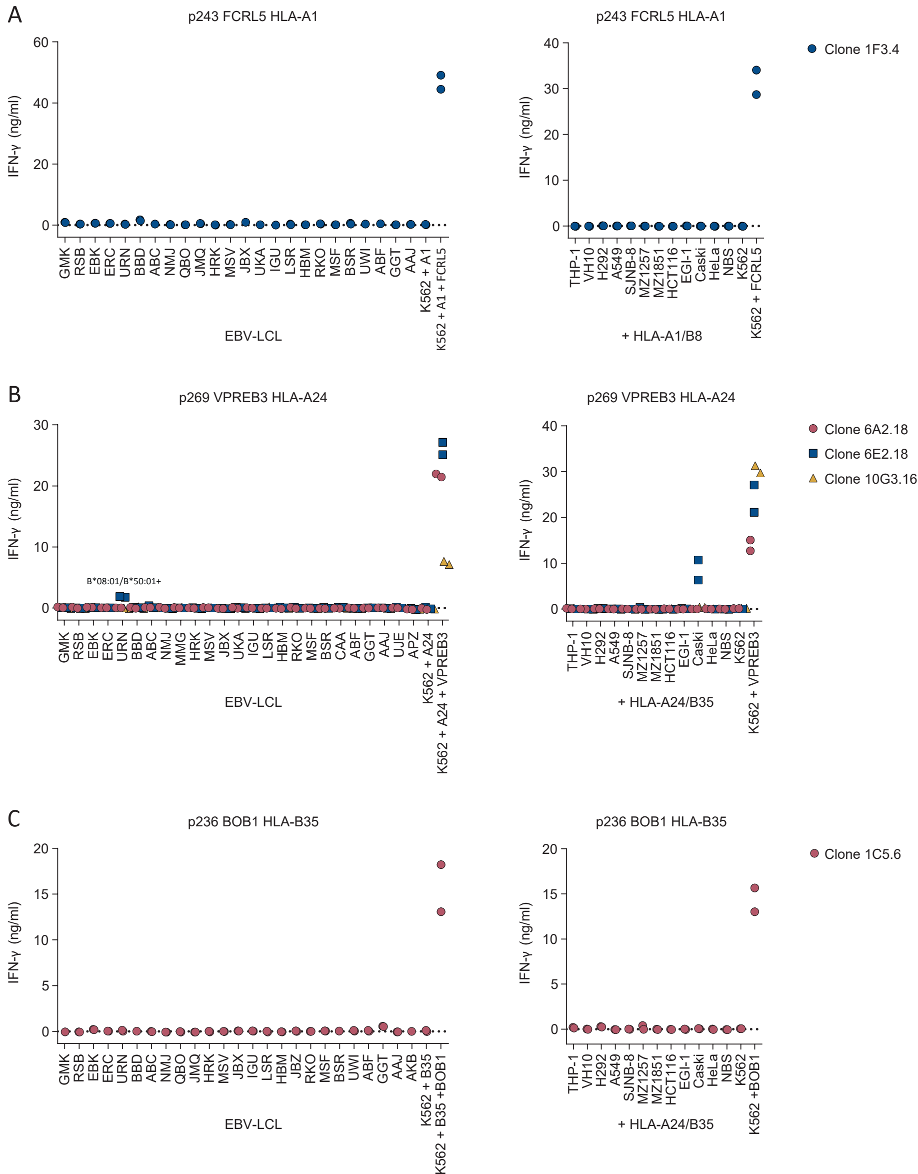


Figure 4. Safety screenings for selected T-cell clones. IFN-γ production by T cells measured by ELISA after overnight co-culture, technical duplicates are depicted. T-cell clones stimulated with an Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) panel not expressing target HLA alleles (left panels). Target gene and HLA transduced K562 cells were included as positive control for T-cell function. T cells were stimulated in effector:target (E:T) ratio 1:6. T-cell clones stimulated with NBS and VH10 fibroblast cell lines, Hela and Caski cervix carcinoma cell lines, EGI-1 bile duct carcinoma cell lines, HCT116 colon carcinoma cell line, MZ1851 and MZ1257 renal cell carcinoma cell lines, SJNB-8 neuroblastoma cell line, A549 and H292 lung carcinoma cell lines transduced with HLA-A1 and HLA-B8 (+HLA-A1/B8) or HLA-A24 and HLA-B35 (+HLA-A24/B35) (right panels). Lack of *FCRL5*, *VPREB3* and *POU2AF1* (BOB1) expression in these cell lines was confirmed by qPCR (data not shown). **A)** Safety screenings of FCRL5 HLA-A1 specific T-cell clones. **B)** Safety screenings of VPREB3 HLA-A24 T-cell clones. **C)** Safety screenings of the BOB1 HLA-B35 T-cell clone.

TCR gene transfer to CD8 T cells reveals promising candidates for TCR gene therapy of B-cell malignancies

To study the potential for clinical application in TCR gene therapy, TCRs of the five selected T-cell clones were sequenced and retrovirally transferred to healthy donor CD8 T cells. Additionally, the TCR of T-cell clone 6B10.12 (FCRL5 HLA-A1), which was not screened for recognition of B-cell malignancies due to lack of *in vitro* expansion, was included. All six identified TCRs, including the TCRs from T-cell clones 6A2.18 and 6E2.18 which were isolated from the same donor, had unique TCR sequences (data not shown). Moreover, all TCRs were functional upon transfer into CD8 T cells as demonstrated by pHLA-tetramer binding. The intensity of the staining, however, was weaker in TCR Td CD8 T cells than in the parental T-cell clones (**Figure 5A**). To determine clinical relevance of identified TCRs, cytokine production by TCR Td T cells after co-culture with primary B-cell malignancies of multiple origins was assessed. HCL, MCL, ALL and CLL patient derived samples were included depending on availability of material expressing target HLA in the LUMC biobank. TMD8 and U266 were used as representatives for DLBCL and MM respectively, as primary malignant cells were not available for these diseases.

The results depicted in Figure 5B demonstrate that FCRL5 HLA-A1 specific TCR 6B10.12 Td T cells efficiently recognized *FCRL5* expressing HLA-A1^{pos} patient derived HCL, MCL and CLL samples, as well as HLA-A1 Td DLBCL cell line TMD8. Reactivity by TCR 6B10.12 Td T cells was more efficient than by TCR 1F3.4 Td T cells (**Figure 5B**), which corresponded with the difference observed in tetramer binding (**Figure 5A**).

VPREB3 HLA-A24 specific TCR 6A2.18 Td T cells consistently outperformed TCRs 6E2.18 and 10G3.16 for recognition of HLA-A24^{pos} patient derived ALL and CLL samples, DLBCL cell line TMD8, and MM cell line U266 (**Figure 5B**), although, TCR 6E2.18 Td T cells showed higher tetramer binding than TCR 6A2.18 Td T cells (**Figure 5A**).

Finally, BOB1 HLA-B35 restricted TCR 1C5.6 Td T cells efficiently recognized patient derived HLA-B35^{pos} ALL, CLL and MCL samples as well as HLA-B35 Td MM and DLBCL cell lines (**Figure 5B**). Based on these results TCRs 6B10.12 (FCRL5 HLA-A1), 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) were selected to be the most potent TCRs identified. Since TCR 6B10.12 was the most potent FCRL5 HLA-A1 specific TCR, but safety of the parental T-cell clones had not been examined due to lack of *in vitro* expansion, safety screenings were performed using endogenous TCRαβ knock-out CD8 T cells Td with TCR 6B10.12 (**Figure S6**). Here, no cross-reactivity for TCR 6B10.12 was identified, therefore this TCR and TCRs 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) are promising candidates to further investigate relevance for TCR gene therapy of B-cell malignancies.

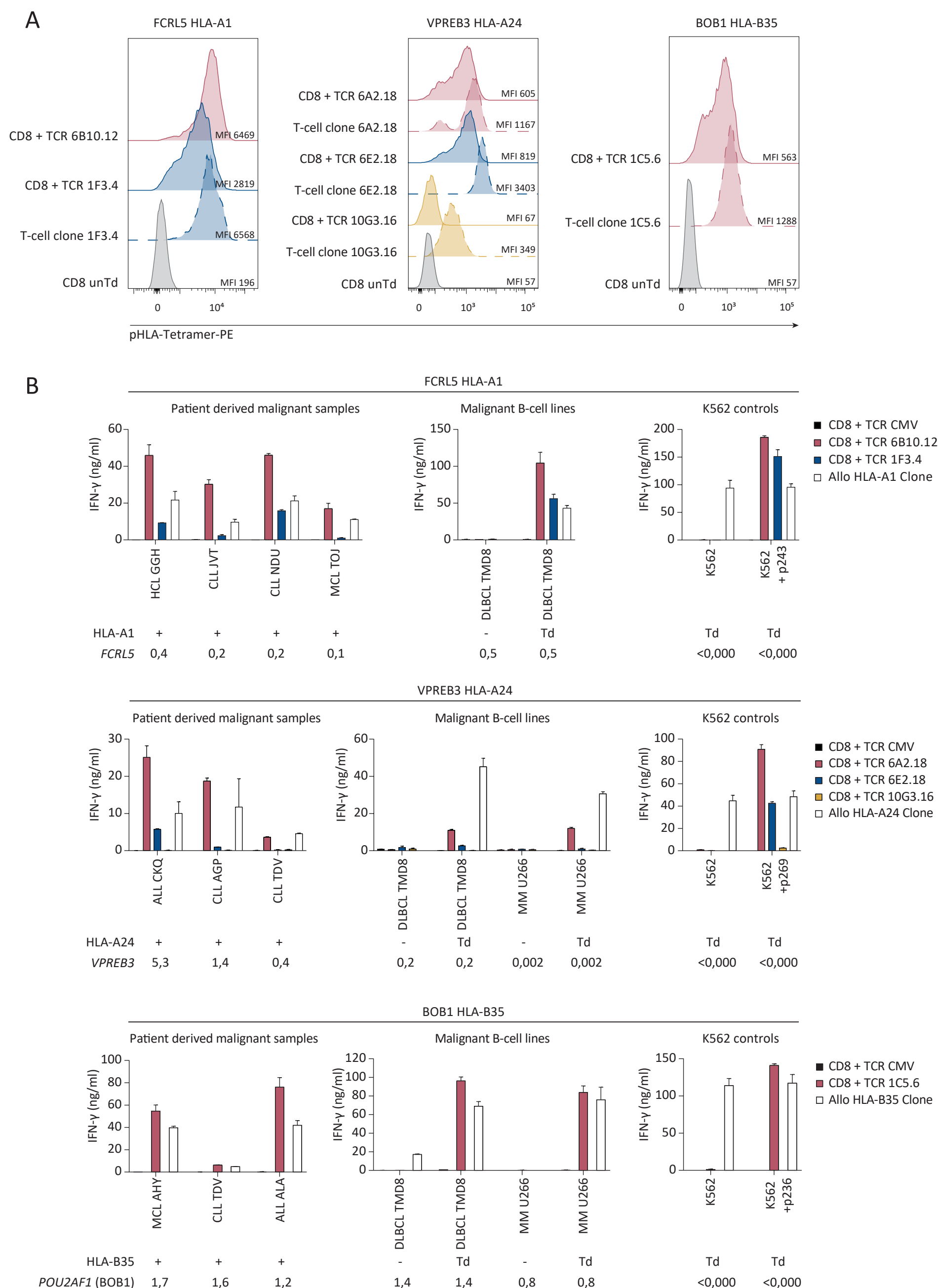


Figure 5. Target recognition by TCR transduced CD8 T cells. A) TCR transduced CD8 T cells and parental T-cell clones stained with pHLA-tetramer-PE, CD8 and mTCR. TCR transduced T cells were enriched for mTCR by MACS and gated on CD8 and mTCR expression. Parental T-cell clones were gated on CD8. Mean fluorescent intensity (MFI) of tetramer stains are depicted. Untransduced T cells were included as negative controls for tetramer staining. Left panel; FCRL5 HLA-A1 specific T cells, middle panel; VPREB3 HLA-A24 specific T cells, right panel; BOB1 HLA-B35 specific T cells. *[Legend continues on the next page]*

B) IFN-γ production after overnight co-culture of CD8 T cells Td with identified TCRs, CMV (pp65-NLV-HLA-A2) TCR as negative control and allo-HLA T-cell clones as positive controls. Target cells were patient derived B-cell malignancy samples, diffuse large B-cell lymphoma cell line TMD8 and multiple myeloma cell lines U266, HLA transduced K562 cells with (100nM) or without target peptide. Target cells were positive (+), negative (-) or transduced (Td) with target HLA. Target gene expression was measured by qPCR and is depicted below targets as expression relative to housekeeping genes (HKG set to 1). To correct for cell size different effector:target (E:T) ratios were used. E:T 1:12 for patient derived B-cell malignancies, E:T 1:6 for cell lines. Graphs are separated based on specificities, upper panels; FCRL5 HLA-A1 TCR T cells, middle panels; VPREB3 HLA-A24 TCR T cells, bottom panels; BOB1 HLA-B35 TCR T cells. Data is representative of two independent experiments, values and error bars represent mean and standard deviations of technical duplicates. Abbreviations: HCL, hairy cell leukemia; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia.

High avidity TCRs show strong promise for therapy of B-cell malignancies

To gain better insight in the value of the identified TCRs for TCR gene therapy of B-cell malignancies, TCR functionality was investigated further. Peptide titrations experiments demonstrated that the identified TCRs are of high avidity for the target peptide-HLA complexes, requiring between 364 and 1068 pg/ml peptide for a half-maximum response (**Figure S7**). Despite high-avidity interactions between the selected TCRs and target peptide-HLA complexes, TCR 6B10.12 (FCRL5 HLA-A1) and 1C5.6 (BOB1 HLA-B35) are dependent on expression of the CD8 co-receptor. Transfer into CD4 T cells induced pHLA-tetramer binding, but no IFN-γ was produced upon stimulation with antigen Td K562 cells (**Figure S8**). In contrast, TCR 6A2.18 (VPREB3 HLA-A24) Td CD4 T cells produced cytokine upon stimulation with antigen Td K562 cells in addition to pHLA-tetramer binding (**Figure S8**). However, stimulation with patient derived B-cell malignancy materials demonstrated reduced functionality of TCR 6A2.18 Td CD4 T cells compared to CD8 T cells (data not shown), therefore presence of the CD8 co-receptor was still beneficial for TCR 6A2.18 Td T cells.

To investigate the anti-tumor reactivity of the identified TCRs, *In vitro* cytotoxicity assays were performed with patient derived HCL, CLL, MCL and ALL samples, diffuse large B-cell lymphoma cell line TMD8 and multiple myeloma cell line U266. Demonstrating that TCR 6B10.12 (FCRL5 HLA-A1) Td T cells induced specific lysis of HCL, CLL, MCL and DLBCL (**Figure 6A**). Additionally, TCR 6A2.18 Td T cells (VPREB3 HLA-A24) mediated potent lysis of patient derived ALL, CLL, DLBCL and MM (**Figure 6B**) and TCR 1C5.6 Td T cells (BOB1 HLA-B35) efficiently killed ALL, CLL, MCL, DLBCL and MM (**Figure 6C**). No lysis of antigen negative target HLA Td K562 cells (**Figure 6**), or fibroblasts and keratinocytes endogenously expressing the HLA restriction alleles was observed (**Figure S9**). Demonstrating that no general *de novo* cross-reactivities were induced by transfer of the three selected TCRs into donor derived CD8 T cells.

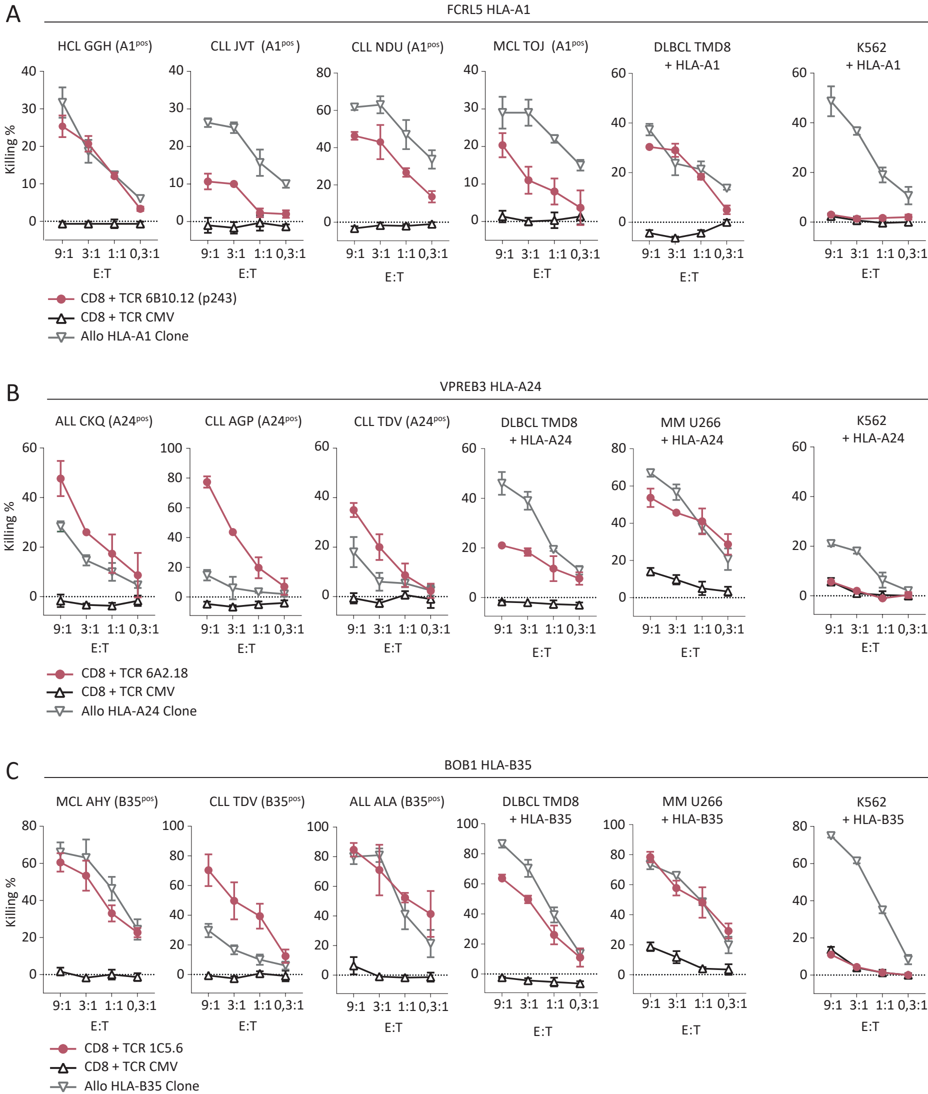


Figure 6. Antigen specific killing of B-cell malignancies by TCR transduced (Td) CD8 T cells. Killing by CD8 T cells Td with selected TCRs (in pink), CMV (pp65-NLV-HLA-A2) TCR Td CD8 T cells (in black) as negative control and allo-HLA-A1, A24 or B35 T-cell clones (in grey) as positive controls. Target cells were patient derived HCL, CLL, MCL and ALL, diffuse large B-cell lymphoma cell line TMD8, multiple myeloma cell line U266 and antigen negative target HLA Td K562 cells. Targets cells endogenously expressed target HLA (HLA^{pos}) or were transduced with target HLA alleles (+HLA). Killing was measured by 51CR release assay after 6-hour co-culture in different effector:target (E:T) ratios. Values and error bars represent mean and standard deviations of technical triplicates. Experiments are representative of two independent experiments. **A)** Killing by FCRL5 HLA-A1 specific TCR 6B10.12 Td T cells. **B)** Killing by VPREB3 HLA-A24 specific TCR 6A2.18 Td T cells. **C)** Killing by BOB1 HLA-B35 specific TCR 1C5.6 Td T cells. Abbreviations: HCL, hairy cell leukemia; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia.

Finally, we investigated the *in vivo* killing capacity of TCR 1C5.6 (BOB1 HLA-B35) Td CD8 T cells in a previously established xenograft model for treatment of established multiple myeloma.(16) NSG mice were inoculated with BOB1 expressing, HLA-B35 transduced multiple myeloma cell line U266. Upon treatment with BOB1 HLA-B35 restricted TCR 1C5.6 Td CD8 T cells a strong anti-tumor effect was observed (**Figure S10**). Tumors in TCR 1C5.6 treated mice reached their minimal size 6 days after T-cell infusion, when the mean tumor burden was 148-fold lower in 1C5.6 TCR treated mice compared to control TCR treated mice. Despite near complete tumor eradication, U266 regrows after day 6 post T cells likely due to absence of the required human cytokine environment.

In conclusion, TCR 6B10.12 (FCRL5 HLA-A1), 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (BOB1 HLA-B35) are promising TCRs for further clinical development for application in TCR gene therapy of B-cell malignancies.

DISCUSSION

In this study we aimed to extend the options for cellular immunotherapy of B-cell malignancies. We performed a broad and stepwise approach to identify multiple TCRs specific for different target genes and HLA alleles. As a first step, target genes with restricted expression in B-cell malignancies and the B-cell lineage were selected. Twenty HLA-A1, A24, B8 or B35 binding peptides derived from these genes were identified by mass spectrometry based immunopeptidomics. T-cell clones were isolated for 11 of these 20 peptides and multiple efficacy and safety screenings revealed the 6 most promising T-cell clones for which TCRs were sequenced. Despite confirmed presentation of all selected epitopes, only the most potent FCRL5, VPREB3 and BOB1 specific T-cell clones were of sufficient avidity to recognize antigen levels naturally expressed by B-cell malignancies. Upon transfer to CD8 T cells, three TCRs directed against peptides derived from FCRL5, VPREB3 and BOB1, demonstrated potent lytic activity against multiple B-cell malignancies.

The first selected TCR was specific for a peptide derived from FCRL5 presented in HLA-A1. FCRL5 is a cell surface receptor involved in regulation of BCR signaling through binding to IgG.(28) In this study, we aimed to identify TCRs targeting selected B-cell malignancy antigens regardless of their cellular localization. However, the cell surface localization of FCRL5 would also permit CAR mediated targeting which could additionally be exploited as a therapeutic strategy. In healthy B cells, *FCRL5* is expressed by naïve and memory peripheral blood B cells as well as on plasma cells, while expression in germinal center B cells is low.(29) *FCRL5* is expressed in almost all cases of CLL and has previously been proposed as immunotherapeutic target for CLL.(29) Similarly, in our study *FCRL5*

expression was observed in CLL and was additionally expressed in HCL, MCL and DLBCL. The second TCR targets a peptide derived from VPREB3 in HLA-A24. The exact function of VPREB3 is still unknown, but VPREB3 has been suggested to play a role in the intracellular assembly of the pre-B-cell receptor and thus in B-cell development. In healthy B cells, *VPREB3* is expressed in precursor B cells as well as in a subset of germinal center B cells.(30) *VPREB3* was also reported to be expressed in all cases of Burkitt lymphoma and in a subset DLBCL. In DLBCL, *VPREB3* expression co-occurred with c-MYC abnormalities and was therefore associated with an aggressive phenotype.(30) In this study, we additionally observed *VPREB3* expression and TCR targeting of ALL and CLL. Furthermore, despite low *VPREB3* expression, DLBCL cell line TMD8 and MM cell line U266 were lysed by TCR Td T cells, indicating broad applicability for VPREB3 restricted TCRs.

The third TCR targets a peptide in HLA-B35 derived from *POU2AF1* which encodes the BOB1 protein. BOB1 is an intracellular transcription factor regulating both B-cell development as well as function and is broadly expressed throughout the healthy B-cell lineage.(31, 32) Additionally, *POU2AF1* (BOB1) is expressed in all types of B-cell malignancies and has been suggested to play a role in survival of malignant cells.(33, 34) We previously identified a TCR targeting a BOB1 derived peptide in HLA-B*07:02 which demonstrated specific lysis of B-cell malignancies of multiple origins, including MM.(16) Here we similarly observed potent lysis of B-cell malignancies of multiple origins and strong *in vivo* anti-tumor efficacy by CD8 T cells Td with the HLA-B35 restricted TCR.

All three candidate TCRs demonstrated promising safety profiles, as no cross-reactivities were identified using parental T-cell clones 6A2.18 (VPREB3 HLA-A24) and 1C5.6 (POU2AF1 HLA-B35) or TCR 6B10.12 (FCRL5 HLA-A1) Td CD8 T cells. Cross-reactivities with peptides presented in target HLA alleles were investigated in a safety screening including tumor cell lines derived from a variety of cellular origins, thereby ensuring broad gene expression profiles. However, as not all genes will be expressed in the included cell lines, potential cross-reactivities could have remained unidentified. To further investigate the safety profile of the identified TCRs, peptide library scanning remains to be performed in additional preclinical studies. In addition to TCR intrinsic cross-reactivities, *de novo* cross-reactivities could potentially occur when transferring TCRs into CD8 T cells, resulting from mixed dimer formation between chains of the introduced TCR and endogenous TCR.(35, 36) Here, TCR transfer into CD8 T cells confirmed a lack of general *de novo* cross-reactivity when stimulated with antigen negative K562, fibroblast and keratinocytes. As the risk for *de novo* cross-reactivities remains a valid concern for the safety of TCR gene therapy, future TCR gene-therapy is expected to shift focus to generating T-cell products with a knock out of the endogenous TCR, or insertion of the introduced TCR into the TCR locus. Deletion of the endogenous TCR will additionally increase the expression of the introduced TCR by eliminating competition for CD3 and thereby lead to more potent

antitumor efficacy.(37)

The three TCRs identified in this study were specific for peptides presented in the context of HLA-A1, A24 or B35. These HLA-alleles have relative allele frequencies in the worldwide population of 17%, 21% and 8% respectively. Future clinical application of the TCRs is restricted to individuals expressing these HLA genotypes. However, since the target genes are expressed in a broad range of B-cell malignancies, a large group of patients is still eligible for therapy with these TCRs.

Since tumors are often of heterogenous nature, tumor cells negative for non-essential antigens can be present. Upon single-antigen-targeting immunotherapy regimens this can result in escape of antigen-negative variants.(5) This limitation could be overcome by multi-antigen-targeting therapy.(7, 8) CAR T cells can be used to treat all malignancies expressing the target antigen, independent of HLA genotype of patients. However, the number of suitable CAR antigens is limited and might not suffice to prevent escape variants. Additionally, for MM the number of cell-surface antigens expressed by malignant cells but not by healthy cell types of non-B-cell origin is low.

In contrast to CARs, TCRs are not limited by the cellular localization of antigens. In this study cellular localization was not included as a selection criteria for target antigens, which resulted in identification of TCRs recognizing peptides derived from cell surface as well as intracellular proteins. Moreover, targeting intracellular proteins that are essential for cell survival, like the transcription factor BOB1 for which we here identified an HLA-B35 restricted TCR, could prevent escape through antigen-loss. The benefits of CAR and TCR therapy can complement each other and should be combined in multi-antigen-targeting therapies. Generating a library of CARs and TCRs will allow selection of multiple relevant receptors based on antigen expression and HLA genotypes of patients. Our research has contributed to this goal by identification of three clinically relevant TCRs. The approach described in this study can similarly be applied to identify TCRs for treatment of solid tumors. For these cancers, tumor associated antigens and neo-antigens are promising targets and TCR-based therapy will likely be the most valuable option as cell-surface expression of tumor specific antigens is generally absent.

In summary, we applied a broad and systematic approach to identify clinically relevant TCRs for the treatment of B-cell malignancies. This resulted in identification of three TCRs that induced potent lysis of B-cell malignancies of different origins and are therefore promising TCRs for future application in multi-antigen-targeting T-cell therapy for B-cell malignancies.

MATERIALS AND METHODS

Target gene selection, HT12 microarray

A detailed description of protocols followed for sample collection, purification, RNA isolation and Illumina HT-12.0 microarray measurements can be found in Pont *et al*.(25) Gene expression analysis was performed using gene expression in patient derived ALL, CLL and MM, ALL cell lines, EBV-LCLs, healthy B-cells, BMMCs, total PBMCs, multiple healthy hematopoietic and non-hematopoietic cell types (Figure S1).

Peptide elution and candidate peptide identification

To identify peptides derived from candidate genes that are presented in HLA on the cell surface, the HLA peptidome of primary B-cell malignancy samples of different origins was established as previously described.(38) In short, after informed consent, apheresis material was obtained at time of diagnosis. HLA typing of the material was performed and cell pellets were stored at -80 until use. Cell pellets (0.1 x 10^9 to 610 x 10^9 cells) were lysed and peptide-HLA complexes were purified by immunoaffinity using the anti HLA-I W6/32 antibody. Peptides were separated from HLA molecules using acid and the peptide containing fraction was obtained by size filtration. Peptide containing fractions were separated by strong cation exchange chromatography and freeze dried. Peptide fractions were lyophilized, dissolved in 95/3/0.1 water/acetonitrile/formic acid v/v/v and subsequently analyzed with nanoHPLC-MS/MS. Peptide and protein identification from tandem mass spectra was performed by proteome discoverer version 2.1 (Thermo Fischer Scientific) using the mascot node and the UniProt Homo Sapiens database. Synthetic peptides were ordered for potential target peptides meeting the following selection criteria: 1) peptides derived from one of the candidate genes; 2) for which a cross query between patient HLA type and predicted HLA binding according to NetMHC version 3.4 indicates binding to HLA-A1, A24, B8 or B35; 3) with a minimal Mascot Ionsscore ≥ 20; 4) with an amino acid length between 8-11 amino acids; 5) that were rank 1 peptides; 6) cysteine containing peptides were excluded; 7) the peptide sequence had to be unique for the candidate gene. For peptides meeting all selection criteria, synthetic peptides were ordered, measured by mass spectrometry and spectra of the synthetic and eluted peptides were compared to confirm peptide identification. Peptides for which the spectra did not match were excluded from the selection.

Generation of pHLA-tetramers

Synthetic peptides were generated in house using standard Fmoc chemistry. Recombinant HLA-A1, A24, B8 or B35 heavy chains (HCs) and human beta-2 microglobulin (B2M) were produced in house in *Escherichia coli.* PE-labelled pHLA-tetramers were produced as previously described with minor modifications.(39)

T-cell isolation and culture

Buffy coats were obtained from healthy donors negative for HLA-A1, HLA-A24, HLA-B8 and HLA-B35 after informed consent (Sanquin). PBMCs were isolated using Ficoll gradient separation and incubated with pHLA-tetramers for 1 hour at 4°C or 15 minutes at 37°C. Cells were washed and pHLA-tetramer bound cells were enriched by magnetic associated cell sorting (MACS) using anti-PE beads (Miltenyi Biotec). The positive fraction was stained with CD8 Alexa fluor 700 (Invitrogen/Catlag) and FITC labelled CD4, CD14 and CD19 (BD pharmingen). pHLA-tetramer^{pos}, CD8^{pos} cells were single-cell sorted using an Aria III cell sorter (BD Biosciences) in a 96 well round bottom plate containing 5x10^4 irradiated PBMCs (35Gy) and 5x10^3 EBV-JY cells (50Gy) in 100ul T-cell medium (TCM) with 0.8 µg/ml phytohemagglutinin (PHA; Oxoid Microbiology Products, Thermo Fischer Scientific). TCM contains IMDM (Lonza), 1% Penicillin/Streptomycin (Pen/Strep; Lonza), 1.5% glutamine (Lonza), 100 IU/ml IL-2 (Proleukin; Novartis Pharma), 5% fetal bovine serum (FBS; Gibco, Life Technologies) and 5% human serum. T-cell clones were restimulated every 10-15 days with irradiated feeder cells and PHA or cryopreserved until further use.

Target cell culture and generation

Cell lines were cultured in IMDM (Lonza), 1% Pen/Strep (Lonza), 1.5% Glutamine (Lonza) and 10% FBS (Gibco, Life Technologies). ALL cell lines were cultured as described.(40) Primary malignant samples were defrosted and rested overnight at 37°C in medium containing 10% human serum before use in experiments. HLA and target gene transduced (Td) target cells were generated by retroviral transduction with HLA alone or with target gene and HLA combined. Retroviral transduction was performed as previously described.(19) Candidate genes and HLA alleles were expressed in MP71 retroviral backbone vectors with marker genes truncated nerve growth factor receptor (NGF-R), CD34 or mouse CD19 (mCD19). Transduced cells were MACS or FACS enriched for marker gene and/or HLA-I expression using HLA-ABC FITC (serotec), NGF-R PE (BD/Pharmingen), mCD19 PE (BD) or CD34 PE (BD/Pharmingen).

T-cell recognition assay

Target cell recognition was determined by incubating 5,000 T cells with target cells in Effector:Target (E:T) ratio 1:6, unless indicated otherwise, in a 384-well flat-bottom tissue culture plate. T cells were washed before use in experiments to remove expansion-related cytokines. After overnight (O/N) incubation recognition was determined by measuring IFN-γ and/or GM-CSF production in supernatants by ELISA (Sanquin and R&D systems). Supernatants were tested undiluted, 1:5 diluted (Figure 2, Figure S4) or in multiple dilutions to calculate concentrations using OD values in the linear part of the standard curve (all other experiment). Supernatants were transferred using a *Hamilton Microlab STAR* Liquid Handling System (Hamilton company). Peptide loaded target cells were

loaded with 100nM per peptide or decreasing peptide concentrations starting at 1µM for peptide titration experiments. Initial screenings to determine peptide recognition of pHLA-tetramer^{pos}, CD8^{pos} sorted and expanded T cells were performed in a high throughput manner. The 96-well T-cell cultures were split into 4 wells of 384-well plate using a *Hamilton Microlab STAR* Liquid Handling System (Hamilton company). Peptide mix loaded or unloaded target cells were added manually. T-cell clones were defined reactive when IFN-γ and/or GM-CSF production was > 100 pg/ml upon target cell stimulation. T-cell clones were considered peptide specific when no cytokine production (< 30 pg/ml) was measured for unloaded target cells. T cells meeting these selection criteria were picked manually and transferred to a 24 well plate for restimulation and further expansion. T-cell mediated cytotoxicity was measured using ^{51}Cr-release experiments. Target cells were incubated 1 hour at 37°C with 100 µCi $Na_2{}^{51}CrO_4$ (PerkinElmer). Target cells were washed and co-cultured with T cells at various E:T ratios for 6 hours in 96-well U-bottom culture plates. Supernatants were harvested and transferred to 96-well LumaPlates (PerkinElmer). Spontaneous and maximum ^{51}Cr-release was determined using TCM alone or TCM containing 1% Triton-X 100 (Sigma-Aldrich), respectively. ^{51}Cr-release was measured in counts per minute (CPM) using a 2450 Microbeta2 plate counter (PerkinElmer). Percentage target cell killing was calculated using

$$\%killing=((CPM_{test}-averageCPM_{spon})/(averageCPM_{max}-averageCPM_{spon}))*100.$$

Quantitative RT-PCR

Total RNA was isolated from 0.5-5x10^6 cells using the Small Scale Kit or ReliaPrep RNA cell mini prep system according to manufacturer's protocol (Ambion, Promega respectively). Total RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase and Oligo (dT) primer (Invitrogen by Thermo Fisher Scientific). Quantitative reverse transcription polymerase chain reaction (qPCR) was performed using Fast Start TaqDNA Polymerase (Roche) and EvaGreen (Biotum), gene expression was measured on the Lightcycler 480 (Roche). Forward and reverse primers used are depicted in Table S5. Target gene expression was calculated relative to the average expression of housekeeping genes: GUSB, PSMB4 and VPS29.

TCR identification

TCRα and TCRβ sequences of T-cell clones were identified as previously described with minor modifications.(41) mRNA was isolated from 1x10^6 cells using the Dynabeads mRNA DIRECT kit (Invitrogen by Thermo Fisher Scientific). TCR cDNA was generated using reverse primers in the TCR constant alfa and beta regions, SMARTScribe Reverse Transcriptase (Takara, Clontech) and a SA.rt template switching oligo forward primer.(42) Barcoded TCR PCR product was generated in two rounds of PCR. In the first PCR, alfa and beta TCR products were generated, in a second PCR the first PCR product was used to

include a barcode sequence that allowed discrimination between TCRs of different T-cell clones. PCR products of different T-cell clones were pooled after which TCR sequences were identified by HiSeq (GenomeScan). Hiseq data were analysed using MiXCR and ImMunoGeneTics (IMGT) database to determine the Vα /Vβ family. V(D)J segments of the TCRα and TCRβ were codon optimized and cloned into the modified MP71-TCR-flex retroviral vector. To increase expression and preferential pairing of the introduced TCRαβ chain, the MP71-TCR-flex vector contains codon-optimized and cysteine-modified murine TCRαβ constant domains and P2A sequence to link TCR chains.(43) Phoenix-AMPHO (ATCC) cells were transfected, after 48 and 72 hours virus supernatant was harvested and stored at -80°C.

TCR transfer to healthy donor T cells

CD8 and CD4 T cells were separately isolated from healthy donor PBMCs by MACS using anti-CD8 or anti-CD4 microbeads (Miltenyi Biotec). PBMCs were obtained after informed consent. T cells were activated with irradiated autologous PBMCs (35 Gy) and 0.8 µg/ml PHA. On day 2, retroviral supernatants were added to 24-well suspension culture plates (Greiner Bio-One) precoated with 30 mg/mL retronectin (Takara) and blocked with 2% human serum albumin (Sanquin). Plates were spun down for 20 min, 2000g at 4°C. Virus supernatant was removed and 0.3x10^6 activated T cells were transferred to each well. After O/N incubation T cells were transferred to a 24-well culture plate (Costar). On day 7 after T-cell activation TCR Td T cells were MACS enriched using anti-mouse TCR-Cβ (mTCR) APC antibody (BD Pharmingen) followed by anti-APC MicroBeads (Miltenyi Biotec) according to manufacturer's protocol. TCR Td T cells were functionally tested between day 10-12 after activation. For safety screening of TCR 6B10.12, endogenous TCRαβ knock out (KO) of healthy donor CD8 T cells was performed prior to TCR Td as described by Morton *et al*.(37) To asses TCR expression and tetramer binding cells were stained using mTCR APC antibody and PE labeled pHLA-tetramers. Cells were measured on the LSR II (BD Bioscience) and data were analysed with Flowjo software.

***In vivo* antitumor efficacy of BOB1 HLA-B35 TCR**

Female NSG mice (NOD.Cg-Prkdc(scid)Il2rg(tm1Wjl)/SzJ, The Jackson Laboratory) were injected i.v. with 2x10^6 U266 multiple myeloma cells, U266 were transduced with Luciferase- tdTomato Red and HLA-B35-NGFR and enriched to reach >98% purity. Tumor growth was measured 1-2 times per week after i.p. injection of 150 µl 7.5 mM D-luciferine (Cayman Chemical Co.) using a CCD camera (IVIS spectrum, PerkinElmer). On day 21 mice were i.v. injected with 5x10^6 T cells which were transduced with 1C5.6 (BOB1 HLA-B35) TCR (n=4) or irrelevant CMV TCR (n=3). The TCR transduced T cells were enriched for mTCR expression before infusion.

ACKNOWLEDGEMENTS

The authors thank the operators of the LUMC Flow cytometry Core Facility (Leiden University Medical Center, Leiden, The Netherlands) for providing expert technical assistance in flow cytometric cell sorting. The research in this study was funded by the Dutch Cancer Society (KWF kankerbestrijding, Amsterdam, The Netherlands) under grant number: KWF UL-2014-6831.

REFERENCES

1. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet (London, England). 2015;385(9967):517-28.
2. Turtle CJ, Hanafi LA, Berger C, Gooley TA, Cherian S, Hudecek M, et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. The Journal of clinical investigation. 2016;126(6):2123-38.
3. Locke FL, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. Lancet Oncol. 2019;20(1):31-42.
4. Abramson JS, Gordon LI, Palomba ML, Lunning MA, Arnason JE, Forero-Torres A, et al. Updated safety and long term clinical outcomes in TRANSCEND NHL 001, pivotal trial of lisocabtagene maraleucel (JCAR017) in R/R aggressive NHL. Journal of Clinical Oncology. 2018;36(15_suppl):7505-.
5. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. The New England journal of medicine. 2013;368(16):1509-18.
6. Park JH, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, et al. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. N Engl J Med. 2018;378(5):449-59.
7. Wang N, Hu X, Cao W, Li C, Xiao Y, Cao Y, et al. Efficacy and safety of CAR19/22 T-cell cocktail therapy in patients with refractory/relapsed B-cell malignancies. Blood. 2020;135(1):17-27.
8. Ruella M, Barrett DM, Kenderian SS, Shestova O, Hofmann TJ, Perazzelli J, et al. Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies. The Journal of clinical investigation. 2016;126(10):3814-26.
9. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Molecular therapy : the journal of the American Society of Gene Therapy. 2010;18(4):843-51.
10. Lamers CH, Klaver Y, Gratama JW, Sleijfer S, Debets R. Treatment of metastatic renal cell carcinoma (mRCC) with CAIX CAR-engineered T-cells-a completed study overview. Biochemical Society transactions. 2016;44(3):951-9.
11. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood. 2012;119(12):2709-20.
12. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(24):10024-8.
13. Vyas JM, Van der Veen AG, Ploegh HL. The known unknowns of antigen processing and presentation. Nat Rev Immunol. 2008;8(8):607-18.
14. Roex MCJ, Hageman L, Veld SAJ, van Egmond E, Hoogstraten C, Stemberger C, et al. A minority of T cells recognizing tumor-associated antigens presented in self-HLA can provoke antitumor reactivity. Blood. 2020;136(4):455-67.
15. Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD, et al. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin Cancer Res. 2011;17(17):5615-25.
16. Jahn L, Hombrink P, Hagedoorn RS, Kester MG, van der Steen DM, Rodriguez T, et al. TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1. Blood. 2017;129(10):1284-95.
17. Mensali N, Ying F, Sheng VO, Yang W, Walseng E, Kumari S, et al. Targeting B-cell neoplasia with T-cell receptors recognizing a CD20-derived peptide on patient-specific HLA. Oncoimmunology. 2016;5(5):e1138199.
18. Jahn L, Hagedoorn RS, van der Steen DM, Hombrink P, Kester MG, Schoonakker MP, et al. A CD22-reactive TCR from the T-cell allorepertoire for the treatment of acute lymphoblastic leukemia by TCR gene transfer. Oncotarget. 2016;7(44):71536-47.
19. Jahn L, Hombrink P, Hassan C, Kester MG, van der Steen DM, Hagedoorn RS, et al. Therapeutic targeting of the BCR-associated

protein CD79b in a TCR-based approach is hampered by aberrant expression of CD79b. Blood. 2015;125(6):949-58.
20. Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S, et al. Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood. 2009;114(10):2131-9.
21. Stronen E, Abrahamsen IW, Gaudernack G, Wälchli S, Munthe E, Buus S, et al. Dendritic cells engineered to express defined allo-HLA peptide complexes induce antigen-specific cytotoxic T cells efficiently killing tumour cells. Scand J Immunol. 2009;69(4):319-28.
22. Savage P, Gao L, Vento K, Cowburn P, Man S, Steven N, et al. Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen. Blood. 2004;103(12):4613-5.
23. Rammensee HG, Bevan MJ. Evidence from in vitro studies that tolerance to self antigens is MHC-restricted. Nature. 1984;308(5961):741-4.
24. Stanislawski T, Voss RH, Lotz C, Sadovnikova E, Willemsen RA, Kuball J, et al. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat Immunol. 2001;2(10):962-70.
25. Pont MJ, Honders MW, Kremer AN, van Kooten C, Out C, Hiemstra PS, et al. Microarray Gene Expression Analysis to Evaluate Cell Type Specific Expression of Targets Relevant for Immunotherapy of Hematological Malignancies. PLoS One. 2016;11(5):e0155165.
26. Jahn L, van der Steen DM, Hagedoorn RS, Hombrink P, Kester MG, Schoonakker MP, et al. Generation of CD20-specific TCRs for TCR gene therapy of CD20low B-cell malignancies insusceptible to CD20-targeting antibodies. Oncotarget. 2016;7(47):77021-37.
27. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood. 2010;115(15):3146-57.
28. Franco A, Kraus Z, Li H, Seibert N, Dement-Brown J, Tolnay M. CD21 and FCRL5 form a receptor complex with robust B-cell activating capacity. Int Immunol. 2018;30(12):569-78.
29. Polson AG, Zheng B, Elkins K, Chang W, Du C, Dowd P, et al. Expression pattern of the human FcRH/IRTA receptors in normal tissue and in B-chronic lymphocytic leukemia. Int Immunol. 2006;18(9):1363-73.
30. Rodig SJ, Kutok JL, Paterson JC, Nitta H, Zhang W, Chapuy B, et al. The pre-B-cell receptor associated protein VpreB3 is a useful diagnostic marker for identifying c-MYC translocated lymphomas. Haematologica. 2010;95(12):2056-62.
31. Hess J, Nielsen PJ, Fischer KD, Bujard H, Wirth T. The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. Mol Cell Biol. 2001;21(5):1531-9.
32. Kim U, Qin XF, Gong S, Stevens S, Luo Y, Nussenzweig M, et al. The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. Nature. 1996;383(6600):542-7.
33. Wang T, Birsoy K, Hughes NW, Krupczak KM, Post Y, Wei JJ, et al. Identification and characterization of essential genes in the human genome. Science (New York, NY). 2015;350(6264):1096-101.
34. Hodson DJ, Shaffer AL, Xiao W, Wright GW, Schmitz R, Phelan JD, et al. Regulation of normal B-cell differentiation and malignant B-cell survival by OCT2. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(14):E2039-46.
35. van Loenen MM, de Boer R, Amir AL, Hagedoorn RS, Volbeda GL, Willemze R, et al. Mixed T cell receptor dimers harbor potentially harmful neoreactivity. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(24):10972-7.
36. Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, Jorritsma A, et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nature medicine. 2010;16(5):565-70, 1p following 70.
37. Morton LT, Reijmers RM, Wouters AK, Kweekel C, Remst DFG, Pothast CR, et al. Simultaneous Deletion of Endogenous TCRalphabeta for TCR Gene Therapy Creates an Improved and Safe Cellular Therapeutic. Molecular therapy : the journal of the American Society of Gene Therapy. 2020;28(1):64-74.
38. van der Lee DI, Reijmers RM, Honders MW, Hagedoorn RS, de Jong RC, Kester MG, et al. Mutated nucleophosmin 1 as immunotherapy target in acute myeloid leukemia. J Clin Invest. 2019;129(2):774-85.
39. Burrows SR, Kienzle N, Winterhalter A, Bharadwaj M, Altman JD, Brooks A. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. J Immunol.

2000;165(11):6229-34.
40. Nijmeijer BA, Szuhai K, Goselink HM, van Schie ML, van der Burg M, de Jong D, et al. Long-term culture of primary human lymphoblastic leukemia cells in the absence of serum or hematopoietic growth factors. Exp Hematol. 2009;37(3):376-85.
41. van Bergen CA, van Luxemburg-Heijs SA, de Wreede LC, Eefting M, von dem Borne PA, van Balen P, et al. Selective graft-versus-leukemia depends on magnitude and diversity of the alloreactive T cell response. The Journal of clinical investigation. 2017;127(2):517-29.
42. Koning MT, Kielbasa SM, Boersma V, Buermans HPJ, van der Zeeuw SAJ, van Bergen CAM, et al. ARTISAN PCR: rapid identification of full-length immunoglobulin rearrangements without primer binding bias. British journal of haematology. 2017;178(6):983-6.
43. Linnemann C, Heemskerk B, Kvistborg P, Kluin RJ, Bolotin DA, Chen X, et al. High-throughput identification of antigen-specific TCRs by TCR gene capture. 2013;19(11):1534-41.

SUPPLEMENTARY MATERIAL

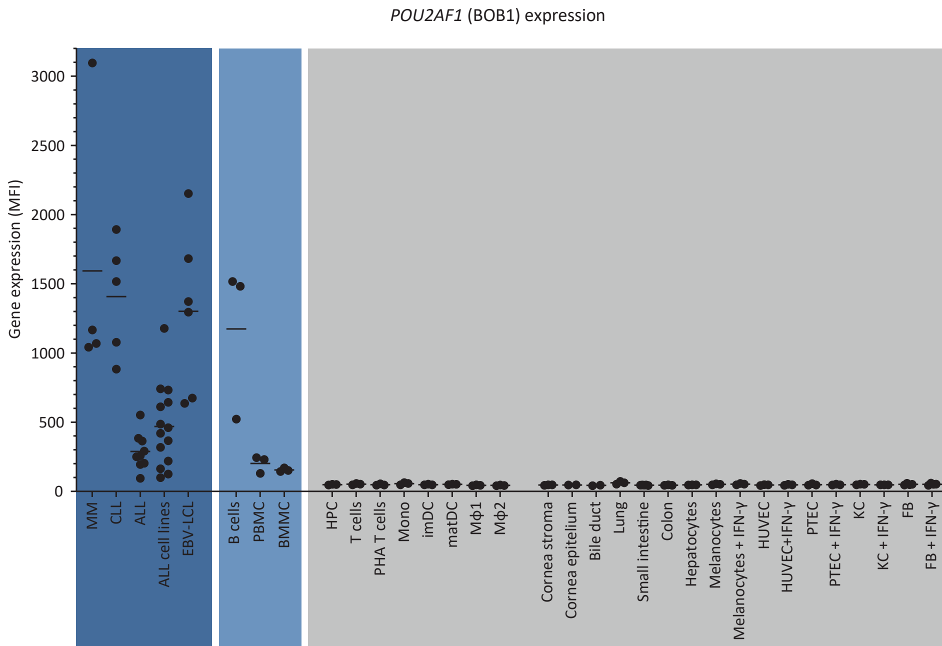


Figure S1. Example of a complete gene expression profile used to generate summary data in figure 1. Gene expression was retrieved from an Illumina HT12.0 microarray dataset.(25) *POU2AF1* (BOB1) gene expression (Mean Fluorescence Intensity; MFI) per cell type, individual samples and average (mean) gene expression is shown. Expression in patient derived B-cell malignancies or B-cell malignancy cell lines (dark blue), healthy B cells (CD19^{pos}) or B-cell containing PBMCs and BMMCs (light blue), healthy hematopoietic and non-hematopoietic cell types (grey). Abbreviations: MM, multiple myeloma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell lines; PBMC, peripheral blood mononuclear cells; BMMC, bone marrow mononuclear cells; HPC, hematopoietic precursor cells; Mono, monocytes; imDC, immature dendritic cells; matDC, mature dendritic cells; MΦ1, type 1 macrophages; MΦ2, type 2 macrophages; HUVEC, human umbilical vein endothelial cells; IFN-γ, interferon-γ; PTEC, proximal tubular epithelial cells; KC, keratinocytes; FB, fibroblasts.

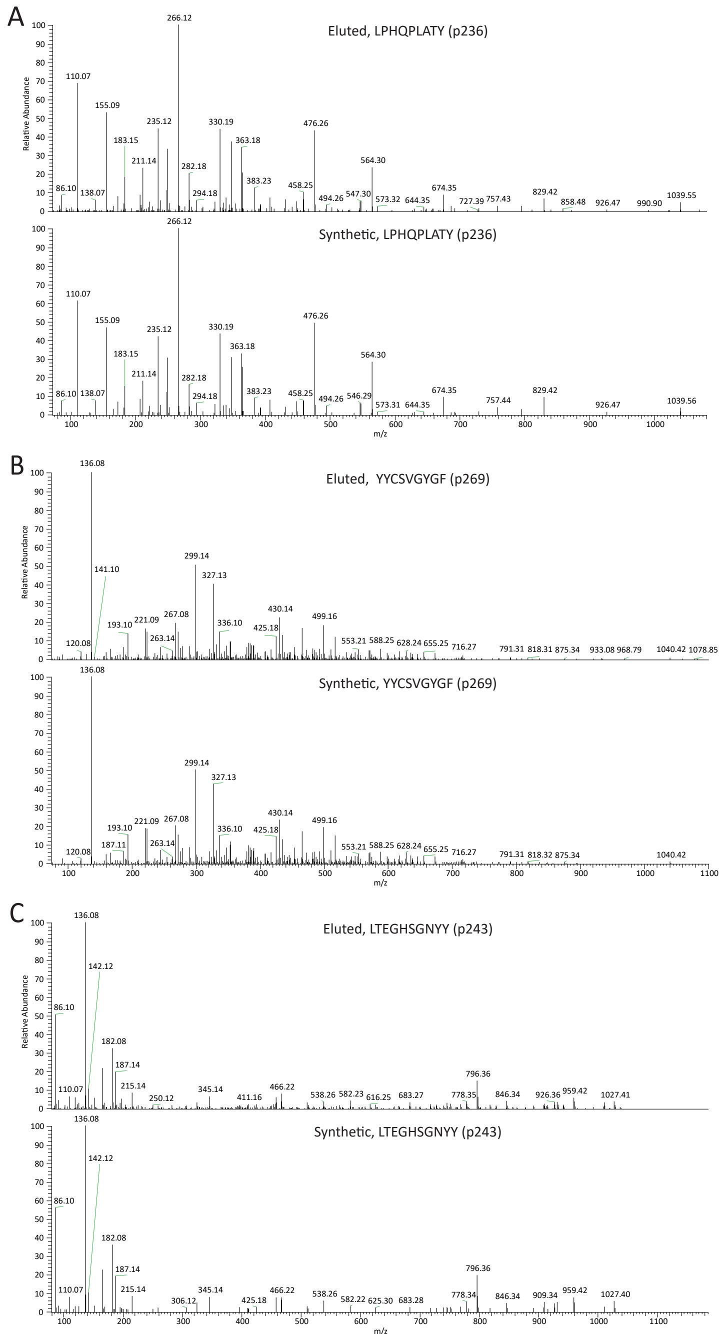


Figure S2. Examples of matching tandem mass spectra of eluted (top) and synthetic (bottom) peptides. A) Tandem mass spectra of peptide 236 (p236, LPHQPLATY) derived from BOB1 presented in HLA-B35. **B)** Tandem mass spectra of p269 (YYCSVGYGF) derived from VPREB3 presented in HLA-A24. **C)** Tandem mass spectra of p243 (LTEGHSGNYY) derived from FCRL5 presented in HLA-A1.

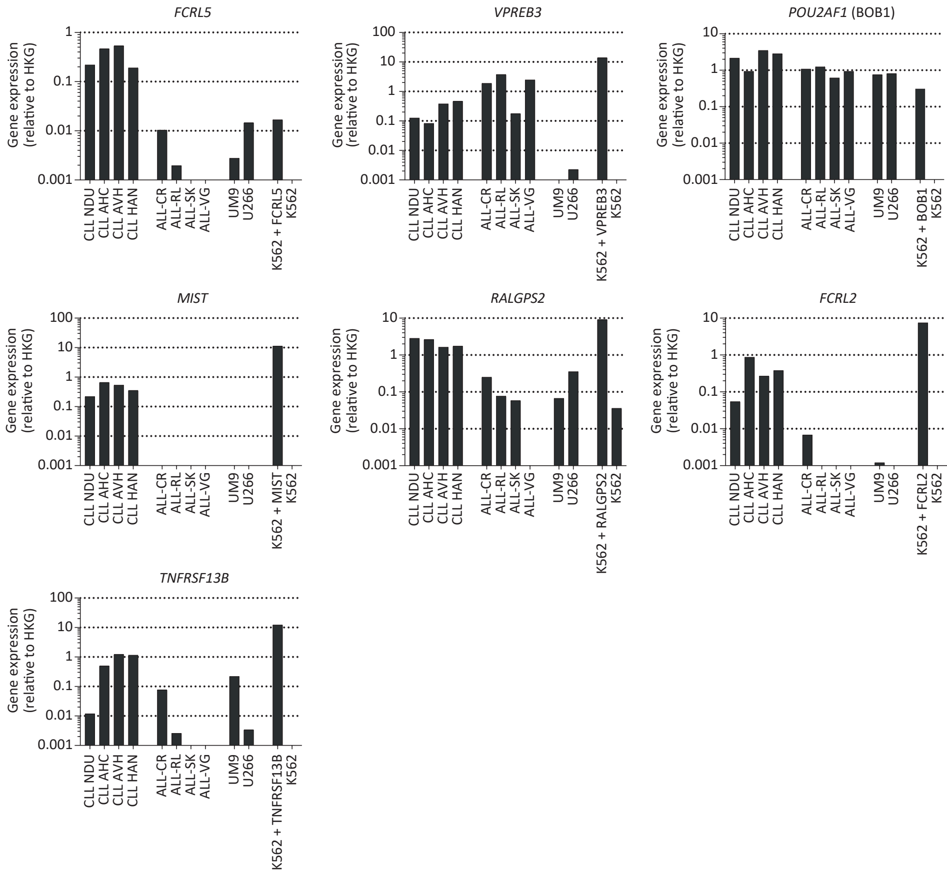


Figure S3. Target gene expression of targets used for T-cell selection. *FCRL5*, *VPREB3*, *POU2AF1*, *MIST*, *RALGPS2*, *FCRL2* and *TNFRSF13B* mRNA levels of patient derived chronic lymphocytic leukemia (CLL) samples, acute lymphoblastic leukemia (ALL) cell lines, multiple myeloma (MM) cell lines, target gene transduced K562 cells and K562 cells used in figure 4. Gene expression, measured by qPCR, depicted relative to housekeeping genes (HKG). Values not depicted are <0.001 relative gene expression.

Figure S4. Peptide titrations of T-cell clones selected to recognize gene transduced (Td) K562 cells. [*Figure on the next page*] IFN-γ (closed symbols) or GM-CSF (open symbols) production by T-cell clones from figure 3 after overnight stimulation with K562 cells Td to express target HLA, loaded with decreasing concentrations of target peptide in an effector:target ratio 1:6. Graphs are separated based on T-cell specificity and cytokine production. Values and error bars represent mean and standard deviations of technical duplicates.

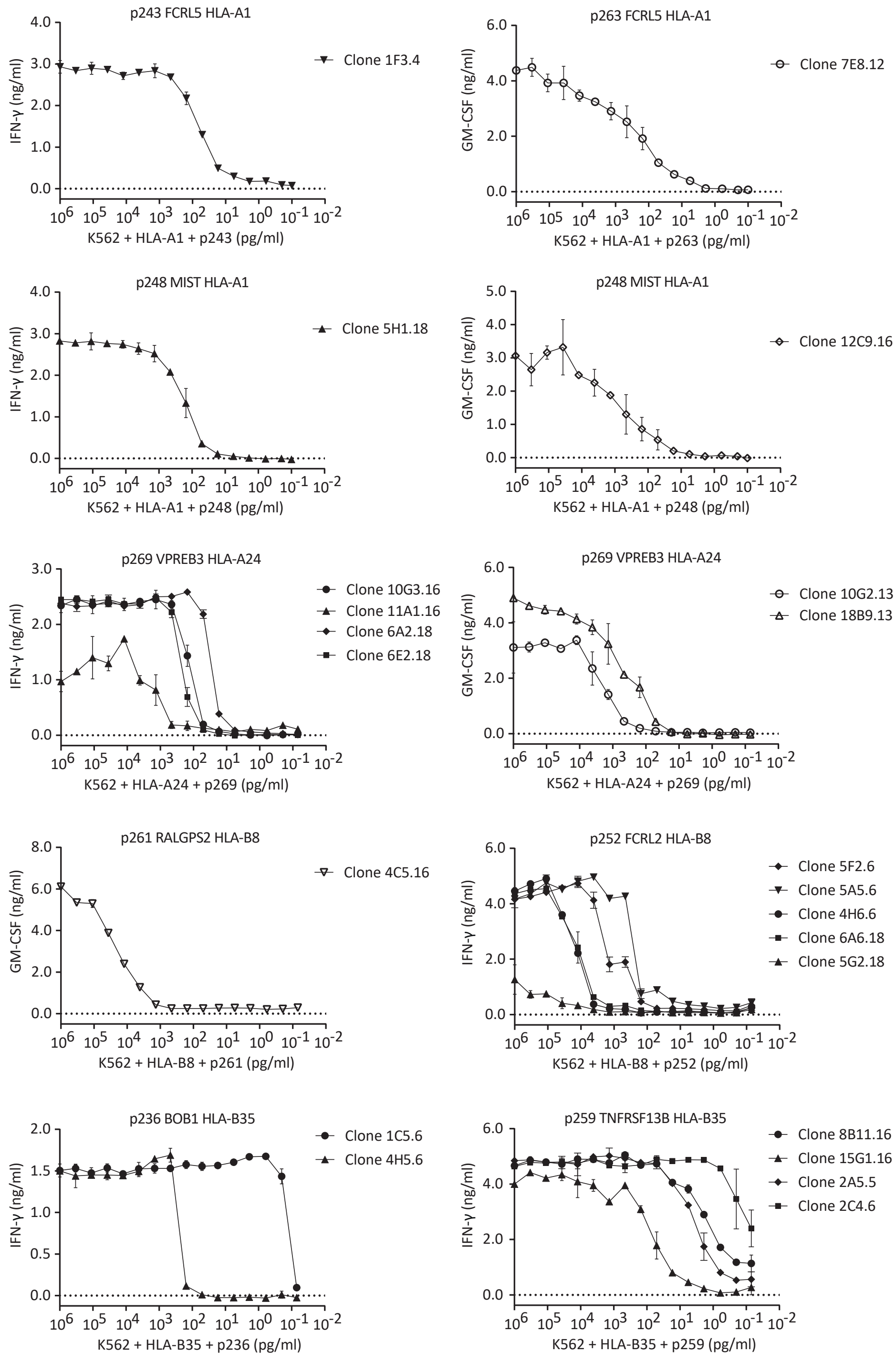


Figure S4 [*Legend on previous page*].

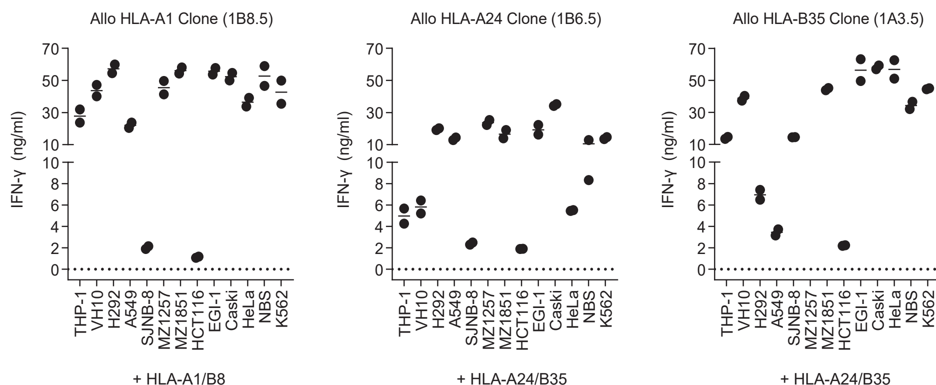


Figure S5. Recognition by allo HLA T-cell clones of cell lines used in safety screenings. IFN-γ production by allo HLA-A1 (left panel), A24 (middle panel) and B35 (right panel) T cell clones after overnight co-culture with HLA-A1 and HLA-B8 (+HLA-A1/B8) or HLA-A24 and HLA-B35 (+HLA-A24/B35) transduced cell lines. IFN-γ measured by ELISA, technical duplicates are depicted. Data obtained from the same experiment as shown in Figure 4.

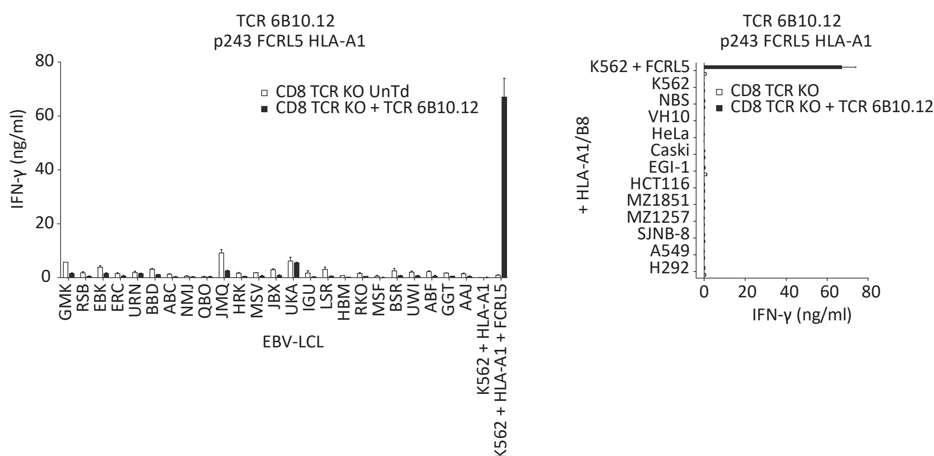


Figure S6. Safety screening of TCR 6B10.12 (FCRL5 HLA-A1). Endogenous TCR knock out CD8 T cells (5% residual huTCR^{pos} cells) untransduced or Td with TCR 6B10.12 (FCRL5 HLA-A1) were used because of lack of *in vitro* expansion of parental T-cell clone 6B10.12. Experiment performed as described in figure 5. Values represent means and standard deviations of technical duplicates.

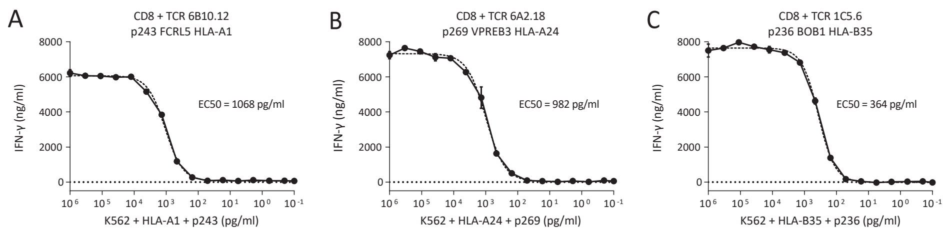


Figure S7. TCR avidity determined by peptide titrations. TCR transduced CD8 T cells were enriched for mTCR expression by MACS. IFN-γ production measured by ELISA after overnight co-culture with HLA transduced K562, loaded with decreasing concentrations of target peptide in an effector:target ratio 1:6. Sigmoidal curves (dotted lines) are plotted based on measured concentrations (solid lines). EC50 values were calculated based on sigmoidal curves and represent peptide concentrations required to induce 50% of the maximum cytokine production. Values represent means and standard deviations of technical duplicates. **A)** CD8 T cells transduced with FCRL5 HLA-A1 p243 restricted TCR 6B10.12. **B)** CD8 T cells transduced with VPREB3 HLA-A24 p269 restricted TCR 6A2.18. **C)** CD8 T cells transduced with BOB1 HLA-B35 restricted TCR 1C5.6.

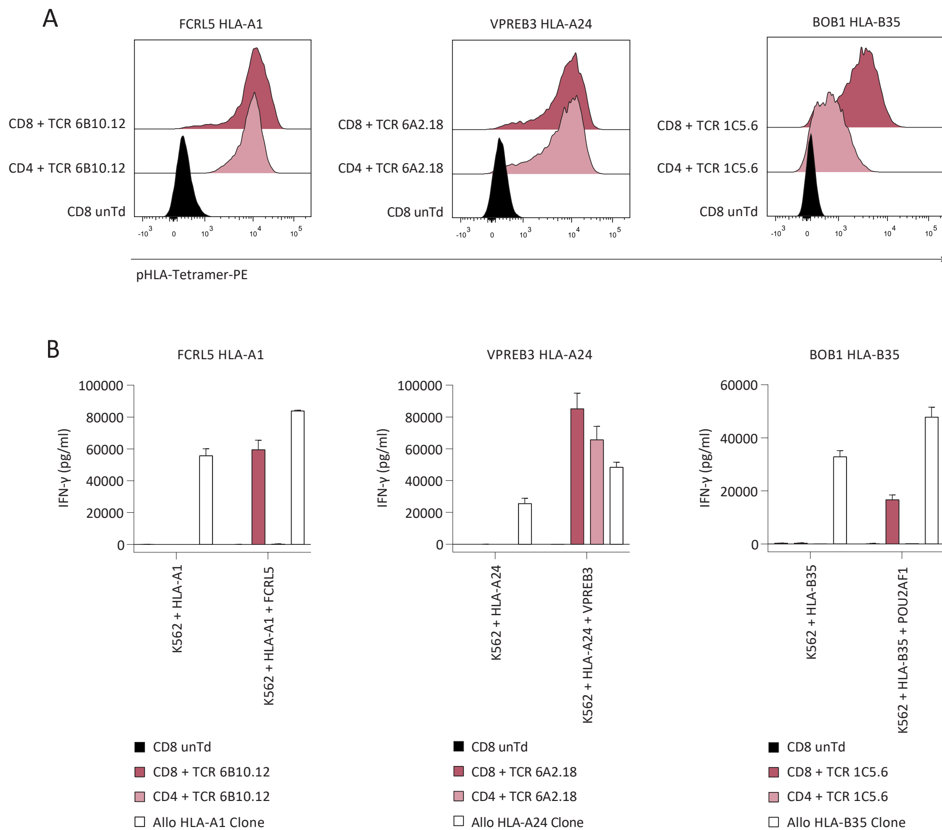


Figure S8. CD8 co-receptor dependence of identified TCRs. CD8 (dark pink) and CD4 (light pink) cells were isolated separately and transduced with identified TCRs or left untransduced (CD8 only, black). T cells were enriched for mTCR expression by MACS and used for analysis on day 10-12 after stimulation. T cells were transduced with FCRL5 HLA-A1 p243 restricted TCR 6B10.12. (left panels), VPREB3 HLA-A24 p269 restricted TCR 6A2.18. (middle panels) or BOB1 HLA-B35 restricted TCR 1C5.6 (right panels). **A)** FACS analysis of T cells stained with specific PE-labeled pHLA-tetramers, gated on CD8 or CD4 positive cells. **B)** IFN-γ production by T cells from experiment shown in A, after overnight co-culture with K562 cells transduced with target HLA alone or with target HLA and target gene combined in an effector:target ratio 1:6. Allo-HLA T-cell clones were included as positive control for HLA expression. Values represent means and standard deviations of technical duplicates.

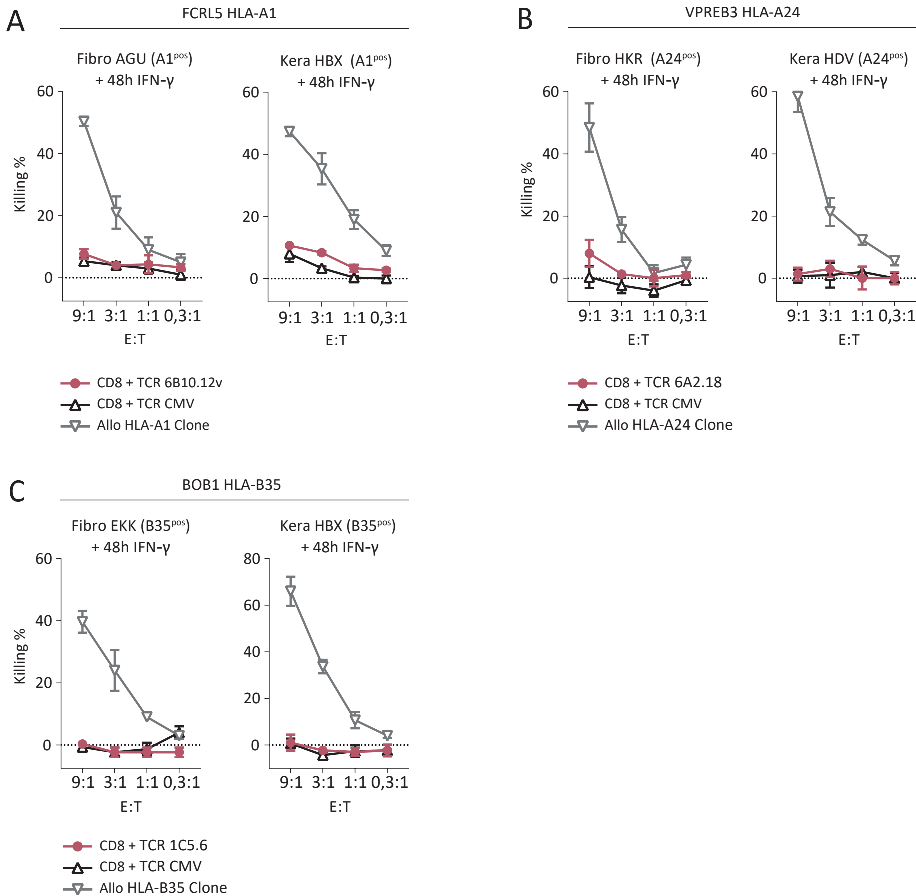
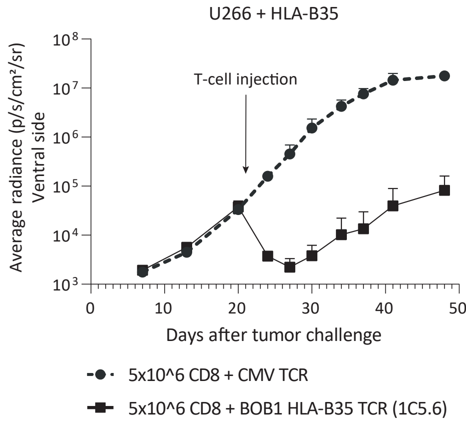


Figure S9. Cytotoxicity of antigen negative target cells by TCR transduced (Td) CD8 T cells. Killing by CD8 T cells Td with selected TCRs (pink), CMV (pp65-NLV-HLA-A2) TCR Td CD8 T cells (black) as negative control and allo-HLA-A1, A24 or B35 T-cell clones (grey) as positive controls. Target cells were fibroblasts and keratinocytes expressing target HLA alleles as indicated between brackets, pre-treated for 48 hours with 100 IU/ml IFN-γ to upregulate HLA expression. Killing was measured by 51CR release assay after 6-hour co-culture in different effector:target (E:T) ratios. Values and error bars represent mean and standard deviations of technical triplicates. Data was obtained from the same experiment as shown in figure 6. Fibroblasts and keratinocytes were negative (<0.0003 relative expression compared to house keeping genes) for *VPEB3*, *FCRL5* and *POU2AF1* expression measured by qPCR as described in figure S3. **A)** Killing by FCRL5 HLA-A1 specific TCR 6B10.12 Td CD8 T cells. **B)** Killing by VPREB3 HLA-A24 specific TCR 6A2.18 Td CD8 T cells. **C)** Killing by BOB1 HLA-B35 specific TCR 1C5.6 Td CD8 T cells. Abbreviations: Fibro, fibroblasts; Kera, Keratinocytes.

A



B

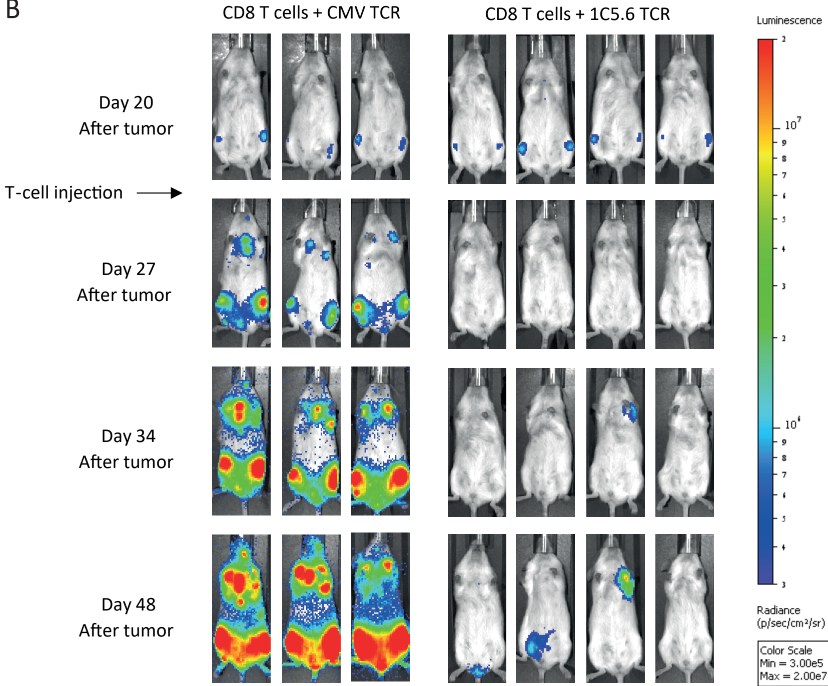


Figure S10. *In vivo* antitumor efficacy of BOB1 HLA-B35 restricted TCR transduced CD8 T cells. NSG mice engrafted with $2x10^6$ U266 multiple myeloma cells transduced with luciferase and HLA-B35, were i.v. injected with $5x10^6$ TCR transduced CD8 T cells after 21 days. T cells were transduced with BOB1 HLA-B35 restricted TCR 1C5.6 (n=4) or control CMV (pp65-NLV-HLA-A2) TCR (n=3) and enriched for mTCR expression by MACS. Tumor outgrowth was frequently tracked by bioluminescence imaging. **A)** Mean and standard deviations of tumor outgrowth over time on the ventral side of CMV TCR treated control mice (dashed line) and BOB1 HLA-B35 TCR (solid line) treated mice. **B)** Tumor outgrowth for individual CMV TCR (left) or BOB1 HLA-B35 TCR (right) treated mice measured on day 20, 27, 34 and 48 after tumor cell injection.

Table S1. Origin and HLA typing of HLA-A1, A24, B8 or B35[pos] B-cell malignancies used for peptide elution and the number of unique peptides that were identified by mass spectrometry with Ion scores ≥20 or ≥35

Patient Code	Diagnosis	Material	HLA-A	HLA-B	HLA-C	Experiment Tag	Cells used for peptide elution (x10^9)	Unique Peptides Ion Score≥20	Unique Peptides Ion Score≥35
AGP	ALL	Peripheral blood	A*01:01- A*02:01	B*08:01- B*38:01	C*07:01- C*12:03	Exp1	233	36773	17449
CDT	HCL	Spleen	A*02:01- A*29:02	B*35:01	C*04:01	Exp2	500	63562	26981
SWD	CLL	Spleen	A*01:01- A*02:01	B*08:01- B*40:01	C*03:04- C*07:01	Exp3	180	68892	30168
						Exp15	0,1	1351	415
						Exp22	0,2	4981	1645
MWY	ALL	Peripheral blood	A*11:01- A*NT	B*35:01- B*40:02	C*02:02- C*04:01	Exp4	610	24873	13655
						Exp14	0,1	539	322
WSG	ALL	Peripheral blood	A*01:01- A*03:01	B*18:01- B*35:08	C*04:01- C*12:03	Exp5	62	44098	23211
SLE	ALL	Peripheral blood	A*01:01- A*32:01	B*08:01- B*45:01	C*06:02- C*07:01	Exp6	512	32830	11242
NBA	ALL	Peripheral blood	A*24:02- A*26:01	B*38:01	C*12:03	Exp7	210	35330	17375
HJS	FL	Peripheral blood	A*24:02- A*32:01	B*44:02- B*44:05	C*02:02- C*05:01	Exp8	243	2696	1296
KYE	ALL	Peripheral blood	A*02:01- A*24:02	B*18:01- B*40:02	C*03:04- C*07:01	Exp10	284	45721	13332
AHC	CLL	Peripheral blood	A*01:01- A*03:01	B*07:02- B*08:01	C*07:01- C*07:02	Exp16	0,2	3476	1002
ALA	ALL	Peripheral blood	A*02- A*11	B*07- B*35	C*04- C*07	Exp17	0,2	3628	1233
EMY	ALL	Peripheral blood	A*11- A*32	B*35- B*44	C*03- C*04	Exp18	0,2	3800	1662
HAN	CLL	Bone marrow	A*02:01	B*08:01- B*15:01	C*03:03- C*07:01	Exp20	0,2	3257	895
HBP	ALL	Peripheral blood	A*02:01- A*24:02	B*39:01- B*57:01	C*06:02- C*12:03	Exp21	0,2	7335	2373
UM9 + A2	MM	Celline	A*01:01- A*11:01 (A*02:01 Td)	B*07:02- B*55:01	C*03:03- C*07:02	Exp107	30	82504	34115

Table S2. Peptides derived from selected genes identified by mass spectrometry from B-cell malignancy samples

				RALGPS2-LTDSEKGNSY-A1	FCRL5-LTEGHSGNYY-A1	FCRL5-TTENSGNYY-A1	MIST-ESEYADTHY-A1	TLR10-YLDHNSFDY-A1	IGJ-YTAVVPLVY-A1	BLK-AYIERMNSI-A24	VPREB3-YYCSVGYGF-A24	RALGPS2-QYIEELQKF-A24	TLR10-ELFKRTIQL-B8	TLR10-LPHLKTLIL-B8	IGLL1-HGLLRPTAA-B8	KLHL14-DMNTKRAIHTL-B8	FCRL2-IVKIKVQEL-B8	FAM129C-LPALRAQTL-B8	FAM129C-YLRLLDAL-B8	RALGPS2-TLKIRAEVL-B8	TNFRSF13B-SADQVALVY-B35	POU2AF1/BOB1-APAPTAVVL-B35	POU2AF1/BOB1-LPHQPLATY-B35
Patient Code	**Diag-nosis**	**Target HLA expressed**	**Experi-ment Tag**	**p 242**	**p 243**	**p 263**	**p 248**	**p 265**	**p 268**	**p 246**	**p 269**	**p 258**	**p 247**	**p 256**	**p 251**	**p 239**	**p 252**	**p 255**	**p 271**	**p 261**	**p 259**	**p 233**	**p 2 36**
AGP	ALL	A*01:01, B*08:01	Exp1					34,2	44,2						35,9			23,0		36,8			
CDT	HCL	B*35:01	Exp2																			39,9	40,0
SWD	CLL	A*01:01, B*08:01	Exp3	39,2	34,9	32,3	45,2	33,5	49,3				32,3	39,1		25,7			43,3	50,4			
SWD	CLL	A*01:01, B*08:01	Exp15						41,8											26,2			
SWD	CLL	A*01:01, B*08:01	Exp22	36,2					35,6											29,3			
MWY	ALL	B*35:01	Exp4																		21,8		35,7
MWY	ALL	B*35:01	Exp14																				27,5
WSG	ALL	A*01:01	Exp5	40,0				25,2															
SLE	ALL	A*01:01, B*08:01	Exp6					35,5	43,3				25,1				32,4			30,6			
NBA	ALL	A*24:02	Exp7							32,0		46,5											
HJS	FL	A*24:02	Exp8									44,6											
KYE	ALL	A*24:02	Exp10									22,1											

Table S2. Continued.

				RALGPS2-LTDSEKGNSY-A1	FCRL5-LTEGHSGNYY-A1	FCRL5-TTENSGNYY-A1	MIST-ESEYADTHY-A1	TLR10-YLDHNSFDY-A1	IGJ-YTAVVPLVY-A1	BLK-AYIERMNSI-A24	VPREB3-YYCSVGYGF-A24	RALGPS2-QYIEELQKF-A24	TLR10-ELFKRTIQL-B8	TLR10-LPHLKTLIL-B8	IGLL1-HGLLRPTAA-B8	KLHL14-DMNTKRAIHTL-B8	FCRL2-IVKIKVQEL-B8	FAM129C-LPALRAQTL-B8	FAM129C-YLRLLDAL-B8	RALGPS2-TLKIRAEVL-B8	TNFRSF13B-SADQVALVY-B35	POU2AF1/BOB1-APAPTAVVL-B35	POU2AF1/BOB1-LPHQPLATY-B35
Patient Code	**Diag-nosis**	**Target HLA expressed**	**Experi-ment Tag**	**p 242**	**p 243**	**p 263**	**p 248**	**p 265**	**p 268**	**p 246**	**p 269**	**p 258**	**p 247**	**p 256**	**p 251**	**p 239**	**p 252**	**p 255**	**p 271**	**p 261**	**p 259**	**p 233**	**p 2 36**
AHC	CLL	A*01:01, B*08:01	Exp16	34,7	23,9	27,1											32,4	21,8		30,5			
ALA	ALL	B*35	Exp17																				32,1
EMY	ALL	B*35	Exp18																				
HAN	CLL	B*08:01	Exp20																	31,6			
HBP	ALL	A*24:02	Exp21								22,3												
UM9	MM	A*01:01	Exp107						45,6														

* For each material, the patient code, diagnosis, expressed target HLA alleles and experiment tag as used in supplementary table 1 is shown. Columns indicate different identified target peptides. Values represent the highest detected Ion score per material, when a peptide was not detected in a material, no value is shown.

2

Table S3. HLA typing of healthy donor PBMCs used for T-cell isolation

Donor number	HLA-A	HLA-B	HLA-C
3	A*01- A*30	B*08- B*39	C*07- C*07
4	A*03- A*03	B*07- B*07	C*07- C*07
5	A*02- A*03	B*07- B*27	C*01- C*07
6	A*02- A*03	B*07- B*44	C*05- C*07
7	A*03- A*32	B*15- B*44	C*03- C*05
8	A*02- A*02	B*44- B*62	unknown
12	A*02- A*02	B*07- B*55	C*03- C*07
13	A*02- A*23	B*07- B*44	C*05- C*07
14	A*03- A*03	B*07- B*07	C*07- C*07
15[a]	A*24- A*29	B*07- B*55	C*03- C*03
16	A*23- A*30	B*44- B*49	C*04- C*07
17	A*02- A*03	B*07- B*44	C*01- C*07
18	A*03- A*11	B*07- B*56	C*01- C*07

[a] When target HLA is expressed, tetramers with this HLA restriction are excluded from experiment

Table S4. HLA typing of EBV-LCLs used in EBV-LCL panels

EBV-LCL	HLA-A	HLA-B	HLA-C
GMK	23:01:01- 02:01	41:01- 40:01	17:01:01:01- 03:04:01:01
RSB	02:01- 03:01/03:03/03:04	44:02- 57:01	06:02- 07:04/07:12/07:11
EBK	02:05- 02:05	58:01- 58:01	unknown
ERC	02:01- 02:01	13:02- 44:02	05:01- 06:02
BBD	02:01- 02:05	15:01- 45:01	01:02- 06:02
ABC	02:01:01- 11:01:01:01	44:05:01 - 51:01:01:01	02:02:02- 14:02:01
NMJ	02:01- 66:01/66:04	40:01/40:11/40:14 - 41:02	03:04/03:08/03:09- 17
HRK	03:01- 25:01	15:17 - 18:01/18:03/18:05	07:01/07:05/07:06- 12:03/12:06
MSV	03:01- 33:01	07:02- 14:02	07:02- 08:02
JBX	02:01- 30:02	15:01- 39:01	03:03- 12:03
IGU	03:01- 26:01	07:02:01- 14:01	07:02- 08:02
LSR	32:01- 68:01	35:03- 52:01	12:02- 12:03
HBM	02:01:01- 02:01:01	15:01:01:01 - 51:01:01	03:03:01- 15:02:01
RKO	02:05- 29:02	27:05- 44:03	01:02- 16:01:01
MSF	03:01/03:03/03:04- 30:01	07:02- 38:01	07:02/07:03/07:05- 12:03/12:06
BSR	02:01- 68:01	35:03- 37:01	04:01- 06:02
ABF	30:04- 68:02	38:01- 55:01	03:03- 12:03
GGT	26:01/26:08/26:02 - 31:01/31:02/31:06	14:01- 49:01	07:01/07:05/07:06- 08:02/08:07
AAJ	03:01/03:03/03:04 - 11:01/11:02/11:03	40:02/40:35/40:37 - 56:01	01:02/01:06/01:07 - 02:02/02:04/02:08
QBO	24:02:01:01- 31:01:02	07:02/07:61 - 35:08:01	04:01- 07:02

Table S4. Continued.

EBV-LCL	HLA-A	HLA-B	HLA-C
MMG	01:01:01- 32:01	35:08- 35:08	04:01- 04:01
UKA	03:01- 25:01	18:01- 35:01	04:01- 12:03
JBZ	01:01- 02:01	07:02- 18:01	07:01- 07:02
JMQ	02:01- 24:02:01:01	35:02- 44:02	04:01- 05:01
UJE	01:01:01:01- 33:03:01	44:03:02- 51:01:01	07:06/07:18- 14:02:01
UWI	02:01- 24:02	07:02:01- 40:02:01	02:02:02- 07:02:01
CAA	02:01- 02:01	40:02- 40:02	02:02- 02:02
URN	02:01- 03:01	08:01:01- 50:01:01	06:02:01- 07:01
AKB	01:01- 02:01	37:01- 39:01	06:02- 07:02
APZ	01:01- 68:01	44:02- 44:02	05:01- 07:04

Table S5. Primers used to measure target gene expression by qPCR

Gene	Forward primer	Reverse primer
GUSB	ACTGAACAGTCACCGACGAG	GGAACGCTGCACTTTTTGGT
PSMB4	GTTTCCGCAACATCTCTCGC	CATCAATCACCATCTGGCCG
VPS29	TGAGAGGAGACTTCGATGAGAATC	TCTGCAACAGGGCTAAGCTG
FCRL5	TGCAAATCCTAGAGGAGAAAATGTG	TAGGGGAACCCTTGTTCCTGA
VPREB3	GGGGACCTTCCTGTCAGTTTC	CGTAGTCCCTGATGGTGACG
POU2AF1	GACATGTATGTGCAGCCCGT	GAGCTTCTTGTCGTGACATTGG
MIST	GGACTCAGAGGAGATGAGAAGTT	GTTTCCTGTGGACCCCAGTT
RALGPS2	GCTGACTGACTCTGAGAAAGGAAA	CAGGCTGCACTCAAATGCTT
FCRL2	TCTCTGGGGACTGTTTGGTGT	GAAGCCCCTCTGGGTTCATTAGT
TNFRSF13B	GTGGCTATGAGATCCTGCCC	CAGCTGAGTGACCTGCAGAA

CHAPTER 3

Broadly applicable TCR-based therapy for multiple myeloma targeting the immunoglobulin J chain

Miranda H. Meeuwsen[1]
Anne K. Wouters[1]
Tassilo L.A. Wachsmann[1]
Renate S. Hagedoorn[1]
Michel G.D. Kester[1]
Dennis F.G. Remst[1]
Dirk M. van der Steen[1]
Arnoud H. de Ru[2]
Els P. van Hees[1]
Martijn Kremer[1]
Marieke Griffioen[1]
Peter A. van Veelen[2]
J.H. Frederik Falkenburg[1]
Mirjam H.M. Heemskerk[1]

[1]Department of Hematology, Leiden University Medical Center, Leiden 2333ZA, The Netherlands; [2]Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden 2333ZA, The Netherlands

Journal of Hematology & Oncology 2023
Vol. 16

ABSTRACT

Background

The immunoglobulin J chain (Jchain) is highly expressed in the majority of multiple myeloma (MM), and Jchain derived peptides presented in HLA molecules may be suitable antigens for T-cell therapy of MM.

Methods

Using immunopeptidomics, we identified Jchain derived epitopes presented by MM cells, and pHLA-tetramer technology was used to isolate Jchain specific T-cell clones.

Results

We identified T-cells specific for Jchain peptides presented in HLA-A1, -A24, -A3 and -A11 that recognized and lysed *JCHAIN* positive MM cells. TCRs of most promising T-cell clones were sequenced, cloned into retroviral vectors, and transferred to CD8 T-cells. Jchain TCR T cells recognized target cells when Jchain and the appropriate HLA restriction alleles were expressed, while *JCHAIN* or HLA negative cells, including healthy subsets, were not recognized. Patient derived *JCHAIN* positive MM samples were also lysed by Jchain TCR T-cells. In a pre-clinical *in vivo* model for established MM, Jchain-A1, -A24, -A3 and -A11 TCR T-cells strongly eradicated MM cells, which resulted in 100-fold lower tumor burden in Jchain TCR versus control treated mice.

Conclusions

We identified TCRs targeting Jchain derived peptides presented in four common HLA alleles. All four TCRs demonstrated potent preclinical anti-myeloma activity, encouraging further preclinical testing and ultimately clinical development.

INTRODUCTION

Multiple myeloma (MM) is a malignancy of the bone marrow (BM) characterized by uncontrolled expansion of plasma cells. Advances in treatment options have extended survival of MM patients, but curative therapies with an acceptable safety profile are lacking (1). To date, allogeneic stem cell transplantation (allo-SCT) has been the only curative therapy for MM, but allo-SCT is associated with high toxicity and treatment related mortality. Recently, B-cell maturation antigen (BCMA) targeting chimeric antigen receptor (CAR) T-cells have extended treatment options for MM patients. BCMA is expressed in a subset of memory B-cells and in plasma cells as well as in MM. BCMA CAR T-cells were effective and safe, but long-term complete responses were rare (2-4). Relapses often resulted from heterogenous BCMA expression within tumors, which led to outgrowth of antigen low or negative MM cells (5, 6), suggesting that targeting of a single antigen may be insufficient to induce durable complete responses in a majority of patients. A curative approach for treating multiple myeloma will likely require multi-antigen targeting. Alternative CAR targets like SLAMF7 and GPRC5D are currently being explored but safety and efficacy of targeting these antigens remains to be proven (7, 8). The requirement for CAR targets to be present on the cell surface largely restricts discovery of new CAR target antigens. Therefore, T-cell receptor (TCR) engineered T-cells could be of additional value since TCRs can recognize peptides presented in human leukocyte antigen (HLA) that can be derived from any protein. This includes antigens derived from intracellular proteins that would be inaccessible for conventional CAR T-cells, thereby accessing an additional pool of potential MM antigens.

Recently, we identified the immunoglobulin J chain (Jchain) as a new target antigen for MM (9). *JCHAIN* is highly expressed in the majority of patient MM samples whereas expression in healthy tissues of non-B-cell origin is absent (9). Physiologically, the Jchain protein links monomers in multimeric IgA and IgM when secreted by plasma cells. Furthermore, Jchain facilitates transport of dimeric IgA and pentameric IgM across mucosal barriers, where poly-Ig receptors bind to Jchain on the basolateral side of the membrane after which the complexes are secreted on the luminal side (10). Despite its functional role in multimerization of IgA and IgM, *JCHAIN* expression appears to be independent of the isotype as Jchain is also expressed in plasma cells secreting other isotypes (11, 12). *JCHAIN* expression was previously described in MM, and, importantly, *JCHAIN* expression was maintained with disease progression even when immunoglobulin production was lost (13). Similar to plasma cells, *JCHAIN* expression in MM was independent of the immunoglobulin isotype produced (14). These observations suggest Jchain as a promising antigen for T-cell-based targeting of MM. The intracellular expression of Jchain precludes CAR mediated recognition and would require TCR mediated targeting of Jchain derived epitopes presented in the context of HLA on

MM cells. Since Jchain derived peptide are non-mutated peptides, high affinity T-cells recognizing Jchain epitopes presented in self-HLA molecules are deleted during thymic development(15). To circumvent this immunogenic tolerance, the immunogenicity of self-peptides in foreign HLA molecules can be exploited using HLA mismatched donors(9, 16-21).

In this study, we identified Jchain derived epitopes presented by MM cells in HLA-A*01:01 (HLA-A1), HLA-A*02:01 (HLA-A2), HLA-A*03:01 (HLA-A3), HLA-A*11:01 (HLA-A11) and HLA-A*24:02 (HLA-A24). Respective peptide-HLA (pHLA) tetramers were generated and used to isolated Jchain specific T-cells from peripheral blood mononuclear cells (PBMCs) of HLA mismatched healthy donors (9, 22). T-cell clones recognizing peptides presented in HLA-A1, HLA-A3, HLA-A11 and HLA-A24 were identified. Upon sequencing and transfer of TCRs from selected T-cell clones, Jchain specific TCR transduced CD8 T-cells demonstrated potent killing of MM *in vitro* as well as *in vivo,* while no off-target effects were observed. Therefore, the TCRs identified in this study hold great promise for therapy of MM.

RESULTS

JCHAIN as target gene for MM and epitope discovery by mass spectrometry

Previously, we identified target genes for cellular therapy of B-cell malignancies using Illumina microarray data(9, 23). *JCHAIN* was identified as promising target since it was highly expressed in 4 out of 5 MM patient samples, and expression in healthy tissues of non-B-cell origin was absent (**Figure 1A**). To gain insight in the fraction of MM patients that could benefit from Jchain targeting therapy, gene expression data of 133 MM patient samples was extracted from a publicly available microarray dataset (GSE13591)(24). 81% of samples were clearly positive for *JCHAIN* expression (expression value>1000) (**Figure 1B**). To discover epitopes for T cell targeting of the Jchain, pHLA-I complexes were isolated from *JCHAIN* positive MM cell lines U266 and UM9 cells (**Figure S1A**) and peptides eluted from HLA-I were analyzed by mass spectrometry. Mass spectrometry data were analyzed for peptides originating from the Jchain protein according to the UniProt *Homo sapiens* database. To determine HLA-I origin of identified Jchain peptides, peptides were assessed for presence of anchor residues for binding to HLA-I alleles expressed by U266 and UM9 using netMHC4.0(25). Peptides with anchor residues for commonly expressed HLA-A alleles: HLA-A1, -A2, -A3, -A11, or -A24 were selected. This resulted in identification of 8 candidate peptides (**Table 1**). Peptide, ISDPTSPLRTR, was presented in HLA-A3 as well as HLA-A11. For the candidate peptides, correct sequence identification was confirmed by comparing mass spectra of eluted peptides to mass spectra of synthetic peptides (**Figure 1C**). For all 9 assigned peptide-HLA combinations

pHLA monomers could be refolded, indicating that the peptides bind their respective HLA-I alleles despite variable predicted binding affinities (**Table 1**).

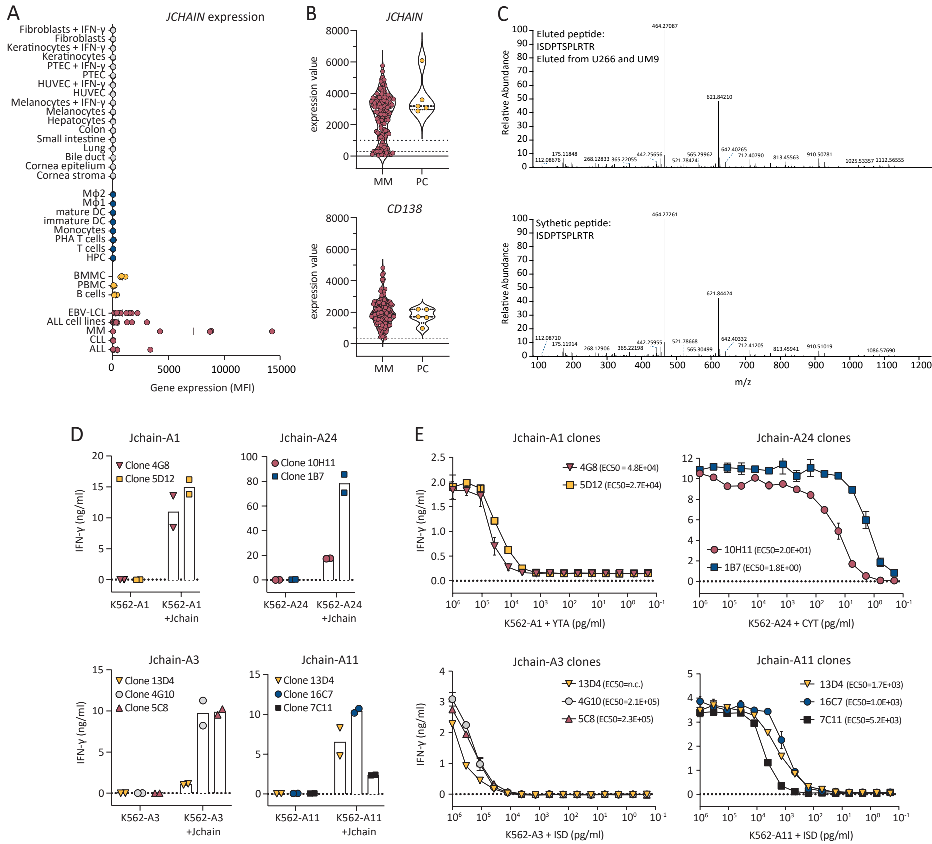


Figure 1. Jchain target identification and subsequent selection of promising T cell clones recognizing Jchain peptides in HLA-A1, A24, A3 and A11. A) *JCHAIN* microarray data (probe ILMN_2105441) from an Illumina HT12.0 microarray dataset (GSE76340) displaying gene expression levels in mean fluorescence intensities (MFI) in healthy tissue of non-hematopoietic origin (in grey), healthy tissue of hematopoietic origin (in blue), healthy subsets containing B cells (yellow) and B- and plasma cell malignancies (in red). Per healthy tissue gene expression was measured in 2-7 samples (mean; 3.4). Abbreviations: ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell lines; PBMC, peripheral blood mononuclear cells; BMMC, bone marrow mononuclear cells; HPC, hematopoietic precursor cells; DC, dendritic cells; MΦ1, type 1 macrophages; MΦ2, type 2 macrophages; IFN-γ, interferon-γ; HUVEC, human umbilical vein endothelial cells; PTEC, proximal tubular epithelial cells. **B)** Violin plot of *JCHAIN* expression (top graph) in 133 MM samples and 5 healthy plasma cell (PC) samples. Samples were enriched for CD138, therefore CD138 (*SCD1*) expression is displayed as positive control (bottom graph). Data was extracted from publicly available dataset GSE13591. Dashed lines indicate background level for negative expression values. Dotted line indicates an arbitrary cutoff for samples expressing *JCHAIN* (expression value >1000). *[Legend continues on the next page].*

3

C) Example of verification of peptide identification by mass spectrometry. Tandem mass spectra of ISDPTSPLRTR peptide eluted from HLA-A3 positive U266 MM cells as well as HLA-A11 positive UM9 MM cells (top graph). Corresponding tandem mass spectra of synthetic ISDPTSPLRTR peptide. **D-E)** IFN-γ production by Jchain specific T cells clones after overnight co-culture measured by ELISA. Graphs are separated based on peptide-HLA specificities. Averages of technical duplicates are depicted. **D)** Jchain T cell clones co-cultured with K562 target cells transduced with target HLA alleles (-A1, -A24, -A3 or –A11) without or with additional transduction of the *JCHAIN* gene (+Jchain). **E)** T cell clones overnight stimulated with antigen negative K562 cells transduced with target HLA molecules HLA-A1, -A24, -A3 or -A11 loaded with decreasing concentrations of Jchain peptides.

Table 1. Jchain derived peptides identified by peptide elution and mass spectrometry from MM cell lines UM9 or U266 presented in HLA-A1, A2, A3, A11 or A24

	Sequence	Jchain aa position[a]	Eluted from MM cell line[b]	HLA-I[c]	%Rank_EL[d]	WB/SB[e]	HLA binding confirmed[f]
YTA-A1	YTAVVPLVY	132-140	UM9	A1	0.02	SB	yes
TAV-A1	TAVVPLVY	133-140	UM9	A1	1.30	WB	yes
VLA-A2[1]	VLAVFIKAVHV	10-20	U266	A2	3.22	<WB	yes
VLA-A2[2]	VLAVFIKAV	10-18	UM9 and U266	A2	0.23	SB	yes
YTA-A2	YTAVVPLV	132-139	UM9	A2	4.17	<WB	yes
ISD-A3	ISDPTSPLRTR	72-82	U266	A3	3.16	<WB	yes
RII-A11	RIIVPLNNR	61-69	UM9	A11	0.24	SB	yes
ISD-A11	ISDPTSPLRTR	72-82	UM9	A11	1.90	WB	yes
CYT-A24	CYTAVVPLV	131-139	U266[g]	A24	0.96	WB	yes

[a]Amino acid (aa) position of identified peptides within the Jchain protein according to uniprot. [b]UM9 cells (HLA-A1, -A11, -B7, -B35, -C3 and -C7 positive) were HLA-A2 transduced and U266 cells (HLA-A2, -A3, -B7, -B40, -C3 and -C7 positive) were HLA-A24 transduced. [c]Most likely HLA-I origin of peptides based on HLA typing of MM cells from which peptides were eluted and peptide binding motifs of these HLA-alleles according to NetMHC4.0. [d]Rank of the predicted binding score for the eluted peptide to the respective HLA molecule compared to a set of random natural peptides. [e]Peptides were annotated as weak or strong binders using the netMHC4.1 default setting of 0.5% rank for strong binders (SB) and 2% rank for weak binders (WB). Peptides with %Rank >2.0 were annotated as <WB. [f]Peptide binding to the respective HLA allele was investigated by peptide-HLA monomer refolding. Yes: peptide-HLA monomers were succesfully refolded and remained stable. No: peptide-HLA monomers could not stably be refolded. [g]Epitope identified in HLA-peptide elution experiment using anti HLA-A1/A24 antibody but not in elution using pan HLA-I antibody W6-32.

Identification of Jchain specific T-cell clones from HLA mismatched healthy donors

To identify Jchain specific T-cell clones of high avidity we exploited T-cell immunogenicity in an HLA mismatched setting(9). PBMCs from donors that are negative for the HLA restriction alleles of the target peptides were incubated with PE-labelled pHLA-tetramers (**Table 1**) followed by single-cell sorting and clonal expansion of pHLA-tetramer^{pos} CD8^{pos} T-cells. In total, 26 buffy coats were used from which 17,000 T-cell clones could be expanded. Peptide specific T-cell clones were identified by high throughput screenings as previously described using the HLA-negative myeloid leukemia cell line K562 transduced with target HLA alleles loaded with Jchain peptides (9). In additional screenings, T-cell clones were tested for recognition of *JCHAIN* and HLA transduced K562 cells to identify clones that are able to recognize endogenously processed and presented peptide. Based

on recognition of endogenous antigen the 9 most potent T-cell clones were selected. T-cell clones 4G8 and 5D12 recognized Jchain peptide YTAVVPLVY in HLA-A1 (YTA-A1), T cell clones 10H11 and 1B7 recognized CYTAVVPLV in HLA-A24 (CYT-A24) and T cell clones 13D4, 4G10, 5C8 16C7 and 7C11 recognized ISDPTSPLRTR in HLA-A3 (ISD-A3) or in HLA-A11 (ISD-A11) (**Figure 1D**). Of note, T-cell clone 13D4 recognized ISD-A3 as well ISD-A11. For the other epitopes no peptide specific T-cell clones were identified, or recognition of *JCHAIN* transduced cells was low or absent. The avidities of the identified T-cell clones for target peptides YTA-A1, CYT-A24, ISD-A3 and ISD-A11 were tested in peptide titration experiments. T-cell clones that efficiently recognized *JCHAIN* transduced K562 cells exhibited different avidities between peptide specificities with average EC50 values ranging from 2.2x10^5 pg/ml for ISD-A3 to 1.1x10^1 pg/ml for YTA-A24 (**Figure 1E**). Per specificity, 2-3 promising T-cell clones were identified, from which the two most potent T-cell clones were selected for follow-up screenings of safety and potency. Given the weak recognition of ISD-A3 but strong recognition of ISD-A11, clone 13D4 was further analyzed for recognition of HLA-A11 positive targets.

Safety screenings revealed stringent on-target recognition of T-cell clones

As TCRs identified from the HLA mismatched repertoire are often promiscuous, specificity of selected T-cell clones needs to be assessed to identify potentially harmful cross-reactivities. First, recognition of look-a-like peptides presented in the target HLA alleles was investigated using a panel of *JCHAIN* negative (**Figure S1B**), target HLA allele positive cell lines from multiple tissue origins (**Figure 2A**). Allo-HLA T-cell clones, recognizing peptides derived from housekeeping proteins presented in respective HLA molecules, were used to verify that targets were generally susceptible to T-cell recognition (**Figure S2**). None of the Jchain specific T-cell clones revealed aberrant reactivity to *JCHAIN* negative cells expressing target HLA restriction alleles. As Jchain T-cell clones showed no off-target recognition when the target HLA is present, we continued to explore whether T-cell clones would cross-react with other HLA alleles. T-cell clones were stimulated with a panel of EBV-LCLs designed to express as many common HLA-I alleles as possible without expressing the target restriction allele (**Figure 2B, Table S1**). For Jchain-A1, both T-cell clones showed no recognition of any of the EBV-LCLs. For both Jchain-A24 and Jchain-A3, one clone per specificity showed recognition of multiple EBV-LCLs, leading to the exclusion of these T-cell clones. For Jchain-A11, both T-cell clones weakly recognized EBV-LCL VJY. While this could indicate cross-reactivity to an HLA-allele expressed by EBV-LCL VJY, recognition could alternatively be mediated by presentation of the Jchain derived ISD peptide presented in a lookalike HLA-allele. Indeed, EBV-LCL VJY expresses HLA-A*36:01 for which Jchain ISD is predicted to be a weak binder(25). Based on the recognition profiles, we continued with T cell clones 5D12 (Jchain HLA-A1), 10H11 (Jchain HLA-A24), 5C8 (Jchain HLA-A3) and 16C7 (Jchain HLA-A11) as these clones demonstrated acceptable safety profiles and highest potencies.

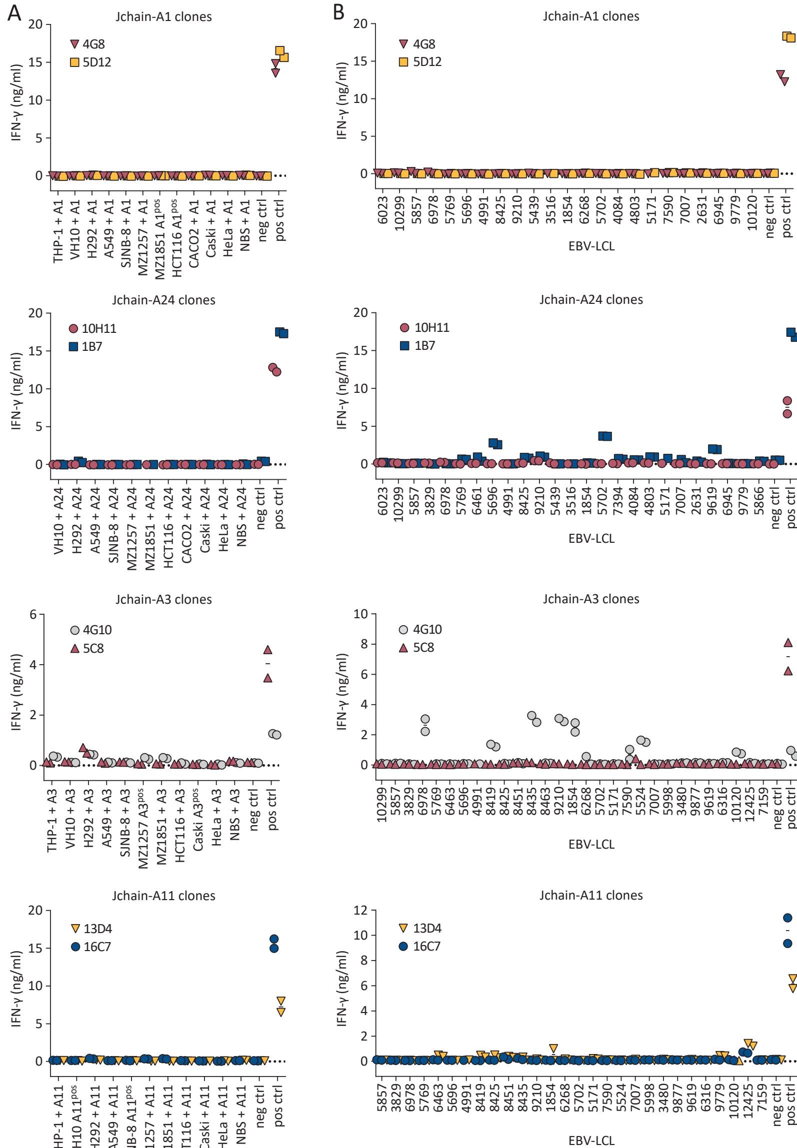


Figure 2. Investigation of cross-reactivity by Jchain specific T cells clones. IFN-γ production by T cell clones measured by ELISA after overnight co-culture, technical duplicates are depicted. Graphs are separated based on peptide-HLA specificities. **A)** T cell clones were stimulated with a panel of cell lines of non-B cell origins transduced with target HLA (+A1/A24/A3 or A11) or naturally expressing target HLA molecules ($A1^{pos}/A24^{pos}/A3^{pos}$ or $A11^{pos}$). HLA transduced K562 cells were included as negative control (neg ctrl), target gene and HLA transduced K562 cells were included as positive control for T-cell function (pos ctrl). THP-1 A24 was not recognized by allo HLA-A24 T cell clone, therefore this cell line was excluded from data as stimulatory capacity is lacking. **B)** T-cell clones were stimulated with an EBV-LCL panel containing EBV-LCLs expressing HLA-I alleles with an allele frequency over 1% that do not express target HLA alleles. Controls as in A).

Jchain specific T-cell clones potently recognize and lyse MM cell lines

To determine the ability of the selected Jchain clones to target MM cells, recognition and killing of MM cell line U266 was assessed. HLA-A1 and HLA-A24 Jchain specific T-cell clones 5D12 and 10H11 specifically lysed HLA-A1 or HLA-A24 transduced U266 cells respectively (**Figure 3A**). Jchain HLA-A3 T-cell clone 5C8 lysed WT HLA-A3^{pos} U266 cells, as well as U266 cells additionally transduced to express HLA-A11, while Jchain HLA-A11 T-cell clone 16C7 only recognized U266 transduced with HLA-A11 (**Figure 3A**). Target cell lysis by Jchain specific clones reached similar levels as allo-HLA T-cell clones that were used as positive controls. Additionally, target cell lysis by Jchain specific T-cell clones concurred with IFN-γ production upon co-culture (**Figure 3B**). Combined, these data demonstrate that Jchain specific T-cell clones recognized and lysed *JCHAIN* expressing MM cells in an HLA-dependent manner.

TCR gene transfer installs Jchain specific effector functions onto CD8 T-cells

To investigate the potential for TCR gene transfer, TCRs of Jchain specific T-cell clones were sequenced, cloned into retroviral expression vectors and introduced into CD4 and CD8 T-cells. For all four TCRs, introduction into healthy donor CD8 T-cells followed by TCR enrichment resulted in functional TCR expression as demonstrated by pHLA-tetramer binding (**Figure 4A**). Staining intensity was lower in CD8 T-cells compared to parental T-cell clones. TCR 16C7 (ISD-A11) transduced CD4 T-cells also bound pHLA-tetramer, but at a lower level than CD8 T-cells demonstrating dependence on the CD8 co-receptor for optimal pHLA-tetramer binding. To investigate the functionality of Jchain TCR transduced T-cells, Jchain TCR T-cells were co-cultured with *JCHAIN* transduced K562 cells. Jchain TCR CD8 T-cells produced cytokine when co-cultured with K562 cells transduced to express *JCHAIN* and the respective target HLA, but not when cultured with *JCHAIN* negative K562 cells (**Figure 4B**). Functionality of Jchain TCR transduced CD4 T-cells was absent or limited compared to CD8 T-cells, and Jchain TCR transduced CD4 T-cells were therefore not investigated further. Lytic potential of Jchain TCR transduced CD8 T-cells was studied using *JCHAIN* expressing MM cell lines U266 and UM9, and *JCHAIN* negative K562 cells as negative control. Jchain HLA-A1, -A24, -A3, and -A11 TCR transduced CD8 T-cells induced potent lysis of *JCHAIN* ^{pos} U266 as well as UM9 cells, while antigen negative K562 cells were not lysed (**Figure 4C**). Lysis of target cells was accompanied by antigen-specific cytokine production of TCR transduced CD8 T -cells (**Figure S3**). In conclusion, Jchain TCR transduced CD8 T-cells demonstrated Jchain specific effector functions.

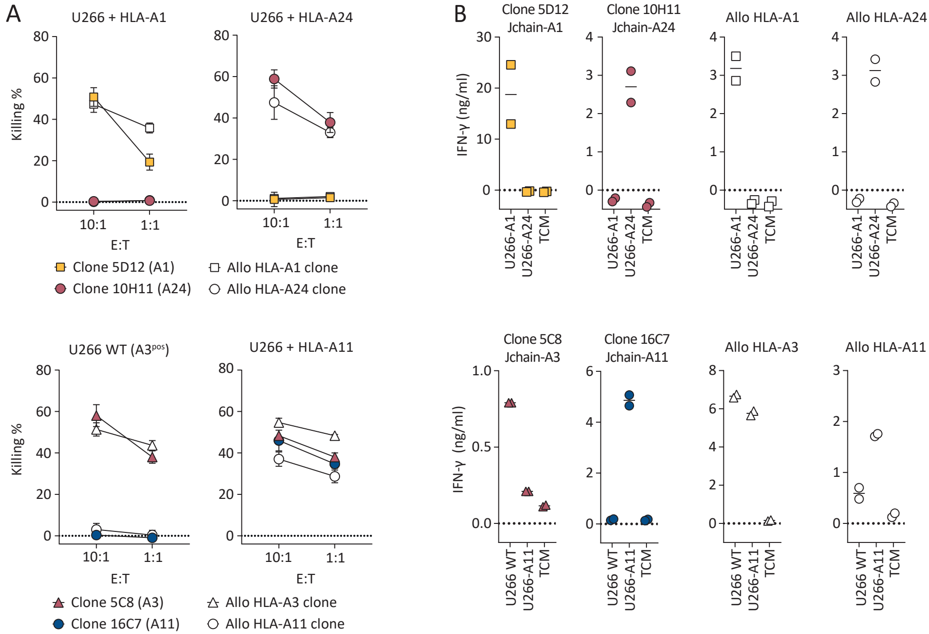


Figure 3. Killing of MM cell line U266 by Jchain specific T cell clones. A) Killing of *JCHAIN* expressing U266 cells transduced with HLA-A1 or A24 by Jchain A1 and A24 specific T cell clones in a 6-hour 51Cr release assay (top graphs). Killing of WT (HLA-A3 positive) or HLA-A11 transduced U266 cells by Jchain HLA-A3 or A11 specific T cell clones (bottom graphs). Killing assays were performed using E:T ratios 10:1 and 1:1. Allo-HLA T cell clones, recognizing peptides derived from housekeeping proteins in specific HLA alleles, were included as positive controls. Average values and standard deviations of technical triplicates are shown. **B)** IFN-γ production measured by ELISA after overnight co-culture using the same target cells as in A in an E:T of 1:6. Values and means of technical duplicates are shown.

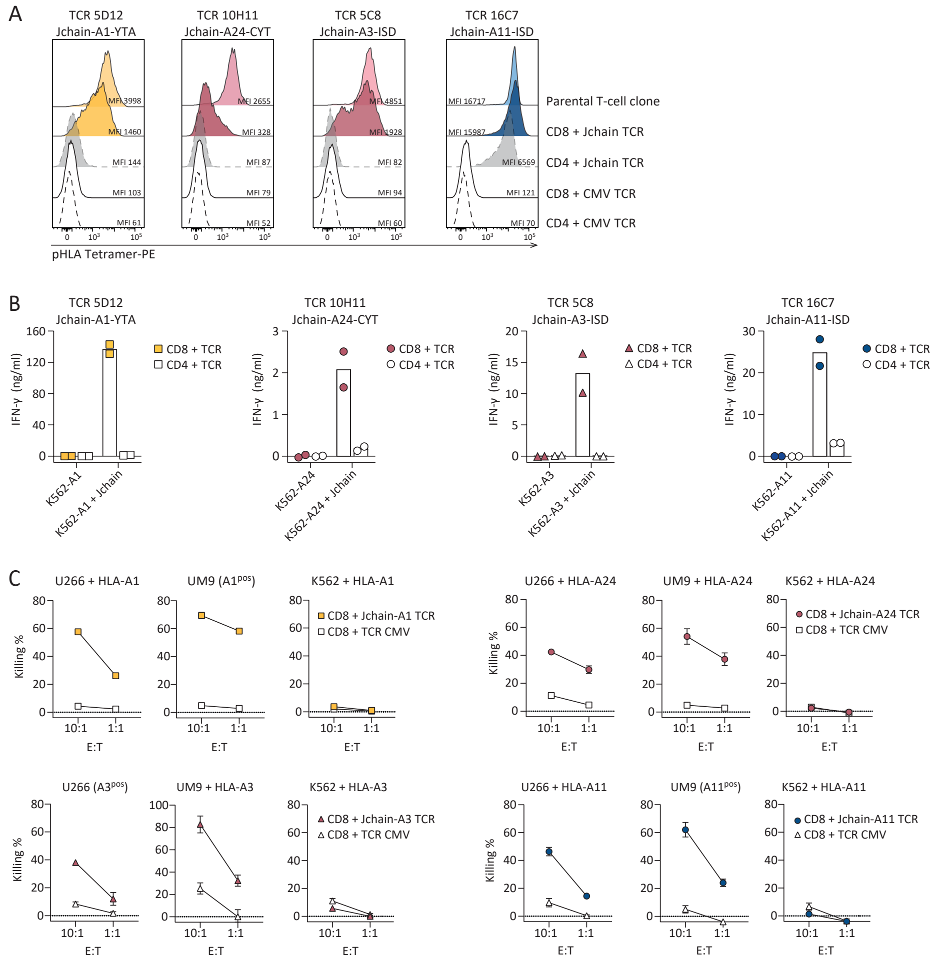


Figure 4. Functionality of Jchain TCRs in CD8 and CD4 T cells. A-C) Jchain A1, A24, A3 and A11 restricted TCRs were sequenced and introduced with murine constant beta domains (mTCR) into CD4 and CD8 T cells. After mTCR enrichment functionality was assessed. **A)** TCR T cells were stained with the respective Jchain pHLA-tetramers and analyzed by FACS. TCR T cells were gated on mTCR+. Parental T cell clones were included as positive controls and CMV TCR T cells were included as negative controls. **B)** Endogenous recognition of *JCHAIN* and HLA (A1, A24, A3 or A11) transduced K562 cells by Jchain TCR CD4 and CD8 T cells. HLA only transduced K562 cells were included as negative control. Values and means of technical duplicates are shown. C**)** TCR transduced CD8 T cells were used for 6-hour chromium release assays to study target cell lysis in E:T ratios 10:1 and 1:1. T cells were co-cultured with U266 MM cells, UM9 MM cells or antigen negative K562 cells transduced with (+HLA) or naturally expressing (HLA^{pos}) target HLA molecules. CMV TCR T cells were used as a negative control. Values and means of technical triplicates are shown.

Jchain TCR T-cells target healthy B-cells but not *JCHAIN* negative healthy tissues

To assess the safety profile of Jchain TCR T cells, Jchain TCR transduced T-cells were co-cultured with healthy subsets of hematopoietic and non-hematopoietic origin. Hematopoietic subsets including immature dendritic cells (DCs), mature DCs, PHA activated T-cells, and B-cells were tested. Fibroblasts and keratinocytes were used as non-hematopoietic cell types. Per healthy subset, cells from multiple donors positive or negative for HLA-A1, -A3, -A11 and -A24 were included. Stimulatory capacity of healthy subsets was confirmed upon recognition by allo-HLA T-cell clones (**Figure S4**) and *JCHAIN* expression was measured by qPCR (**Figure S1C**). Jchain TCR transduced CD8 T-cells did not recognize *JCHAIN* negative (<0.1 relative to HKG) primary cell subsets, but *JCHAIN* expressing peripheral blood B-cells were recognized when target HLA alleles were expressed (**Figure 5A**). To investigate if recognition of B-cells results in lysis, Jchain A1 and Jchain A24 TCR T-cells were co-cultured with B-cells derived from multiple donors. FACS analysis revealed specific lysis of B-cells positive for target HLA alleles (**Figure 5B, C**). These data demonstrate that Jchain TCR T-cells maintained Jchain specific reactivity as previously seen for parental T-cell clones. No off-target reactivity of *JCHAIN* negative heathy cell subsets was apparent, but on-target/off-tumor reactivity of *JCHAIN* expressing B-cells revealed that Jchain TCR gene therapy would likely result in depletion of the healthy B-cell compartment *in vivo*.

Jchain TCR T-cells lyse patient bone marrow derived MM cells

To analyze *ex vivo* lysis of primary MM cells, FACS based killing assays were performed using BM samples from MM patients. MM cell frequencies in BM samples varied between 13.3% and 85.4% (**Figure S5**). *JCHAIN* expression in positive MM samples was 9 to 416-fold higher than housekeeping genes (HKG), while 2 out of 8 included MM samples did not express *JCHAIN* (<0.1 relative to HKG) (**Figure S1D**). MM samples were incubated overnight with Jchain HLA-A1 and -24, or Jchain HLA-A3 and -A11 TCR T-cells or CMV TCR T-cells as negative controls. Jchain TCR T-cells efficiently lysed primary MM cells, as exemplified by co-culture of Jchain HLA-A1 and -A24 TCR T-cells with BM cells from HLA-A1^{pos}, A24^{pos} patient MM.J2 (**Figure 6A**). All tested Jchain TCR T-cells induced lysis of Jchain^{pos} target HLA allele expressing MM cells, while *JCHAIN*^{neg} samples or samples negative for HLA restriction alleles were not lysed (**Figure 6B**). However, *JCHAIN*^{pos} MM cells from HLA-A24^{pos} patient MM.J3 and patient MM.J4 were not lysed by Jchain A24 TCR T-cells (**Figure 6B**), while the allo-HLA-A24 T-cell clone induced potent lysis of these samples (data not shown), demonstrating that the CYT-A24 epitope is not always presented by MM cells at levels sufficient to mediate lysis by the TCR T-cells.

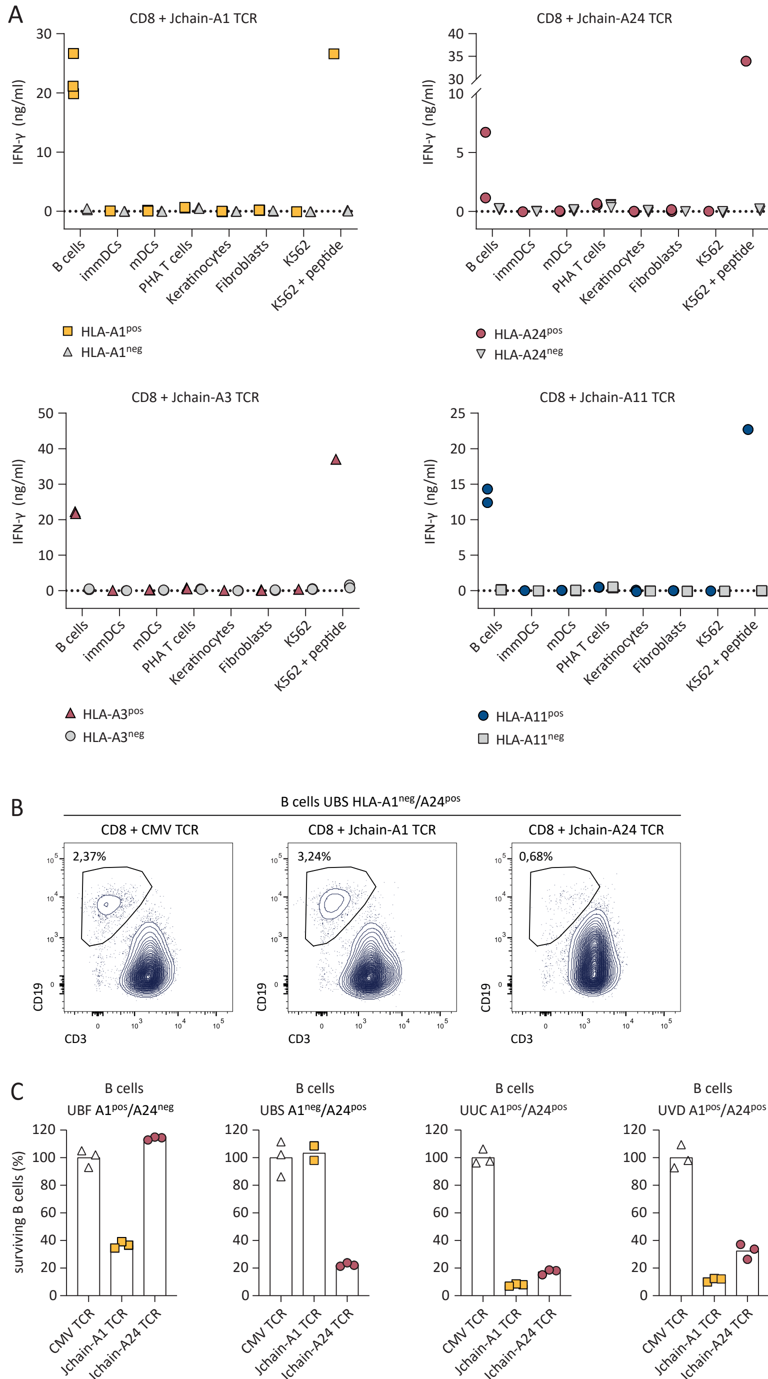


Figure 5. Recognition of healthy hematopoietic and non-hematopoietic subsets by Jchain TCR transduced CD8 T cells. A) IFN-γ production after overnight co-culture of Jchain TCR Td CD8 T cells with CD40L activated B cells, immature dendritic cells (immDCs), mature dendritic cells (mDCs), *[Legend continues on the next page]*

PHA activated T cells (PHA T-cells), and keratinocytes or fibroblasts pre-treated for 48h with 100 IU/ml IFN-γ. Symbols represent the average value (from technical duplicates) of target cells isolated from different donors. Target cells not expressing the relevant HLA restriction allele are depicted in grey, cells expressing the HLA restriction alleles are depicted in color. Per panel T cells with one of the Jchain TCRs are shown as indicated in the graph titles. K562 + HLA and peptide loaded K562 + HLA are included as negative and positive controls. **B-C)** FACS based killing experiment of CD40L activated peripheral blood B cells from healthy donors with Jchain A1 and Jchain A24 TCR T cells in an E:T ratio of 3:1, samples were measured using fixed acquisition times and fixed flow rates. **B)** Example of B cell survival and killing after overnight co-culture of HLA-A1neg/HLA-A24pos B cells with CMV TCR CD8 T cells (left), Jchain A1 TCR T cells (middle) and Jchain A24 TCR T cells (right). Gated on sytox blue-, single cells, CD3-, CD19+. **C)** Quantification of percentage surviving B cells of data in **B)** and additional donors, HLA-A1 and -A24 typing is indicated in graph titles. Percentage surviving cells was calculated relative to cells in the negative control CMV TCR T cell culture. Technical triplicates are shown.

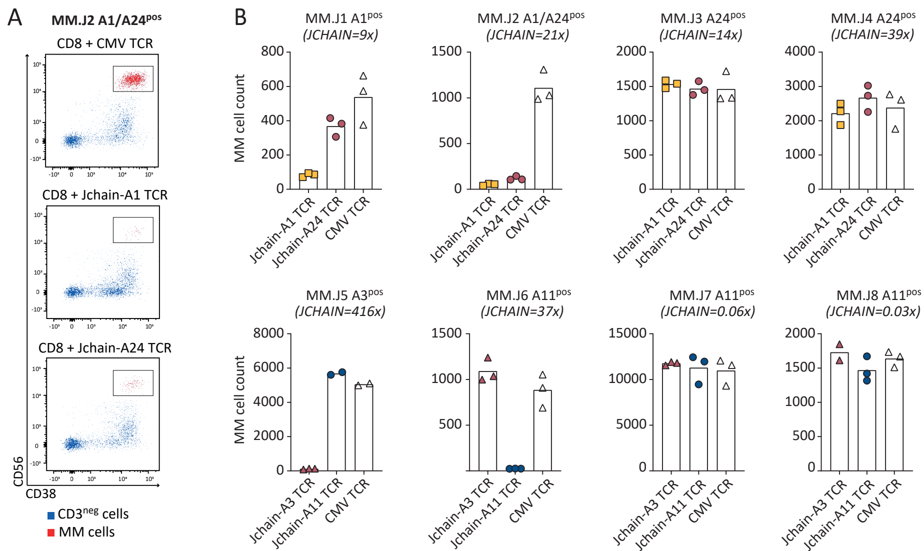


Figure 6. Killing of MM cells in patient derived bone marrow samples. Killing of patient derived bone marrow MM samples was assessed by FACS-based cytotoxicity experiments in which TCR Td T cells were co-cultured with patient BM samples in an E:T ratio 3:1. MM cell survival was analyzed after overnight co-culture. **A)** Example of survival of an HLA-A1pos/A24pos patient sample after co-culture with CD8 T cells transduced with a CMV (negative control), Jchain HLA-A1 or Jchain HLA-A24 restricted TCR. In red MM cells are displayed, highlighted by black boxes for clarity, MM cells were gated on: live cells → single cells → CD3 negative cells to exclude co-cultured T cells → CD45 negative-intermediate, CD19 negative → CD56 positive, CD38 positive. MM cells (in red) were backgated on total CD3 negative cells (in blue). **B)** MM.J1-MMJ.10 codes in graph titles represent different MM patients, additionally expression of target HLA molecules and *JCHAIN* expression as a fold increase relative to housekeeping genes is displayed. *JCHAIN* expression was measured by qPCR on sorted MM cells. Numbers of surviving MM cells acquired per 2500 Flow-Count Fluorospheres are displayed. Jchain HLA-A1 and HLA-A24 TCR Td T CD8 T cells co-cultured with MM patient samples from different individuals expressing HLA-A1, A24 or both (top row). Jchain HLA-A3 and HLA-A11 TCR Td T CD8 T cells co-cultured with MM patient sample from different individuals expressing HLA-A3 or A11 (bottom row). Technical triplicates are shown. Data representative of two independent experiments.

Jchain TCR T-cells potently eradicate established MM in a pre-clinical xenograft model

To test whether Jchain TCR T-cells would convey *in vivo* activity, we used a MM xenograft model. NSG mice were inoculated with WT HLA-A3^{pos} U266 cells or HLA-A1, HLA-A24, or HLA-A11 transduced U266 cells. Tumor cells were allowed to grow for 3 weeks to model established MM. On day 21, mice were infused with Jchain HLA-A1,-A24,-A3 or-A11 TCR or irrelevant CMV TCR transduced CD8 T-cells (**Figure 7**). All four Jchain TCRs substantially reduced MM tumor burden up to 6 days after infusion. Compared to control mice, tumor load in treated mice was approximately 100-fold lower, demonstrating strong *in vivo* anti-tumor responses (**Figure 7B**). To conclude, our data show that identified Jchain A1, A3 A11 and A24 TCRs demonstrate potent anti-MM responses when transferred to CD8 T-cells, both *in vitro* against patient derived primary MM samples as well as *in vivo* against *JCHAIN* ^{pos} MM cell line U266.

3

DISCUSSION

BCMA targeting CAR T cell therapy can induce deep remissions in relapsed/refractory MM but durable responses remain rare (26). Multi-antigen targeting of MM is a suitable approach to tackle antigen heterogeneity and immune escape in the context of cellular therapy. Alternative MM antigens that are being explored as targets for CAR therapy are CD38, SLAMF7 and CD138, however, on-target off-tumor expression of these antigens on other hematopoietic or non-hematopoietic cells compromises their safety profile. CD38 is expressed on activated lymphocytes and on a subset of CD34+ hematopoietic stem cells (27). Similarly, SLAMF7 is expressed on a subset of T cells, and conclusively T cells engineered to target SLAMF7 undergo limited levels of fratricide (28). CD138, a general marker for MM, is also expressed on other lymphocytes as well as some epithelial cells (29). More recently, GPRC5D was suggested as an additional surface antigen with an expression profile more comparable to that of BCMA (8). A first clinical trial with GPRC5D targeting CAR T cells demonstrated clinical activity and, similarly to the experience with BCMA targeting CAR T cells, relapse was associated with antigen loss (30). Here, we present Jchain as promising additional target with an attractive expression profile, i.e. expression is strictly limited to B- and plasma cells as well as MM. Intracellular expression of Jchain precludes CAR mediated targeting and therefore requires TCR mediated targeting of Jchain derived peptides presented in HLA.

Using immunopeptidomics, pHLA tetramer technology and by exploiting the immunogenicity of non-mutated epitopes in foreign HLA alleles, we successfully identified specific T-cell clones recognizing Jchain peptides in the context of HLA-A1,-A24,-A3 and -A11.Jchain directed recognition was maintained upon TCR sequencing and transfer to

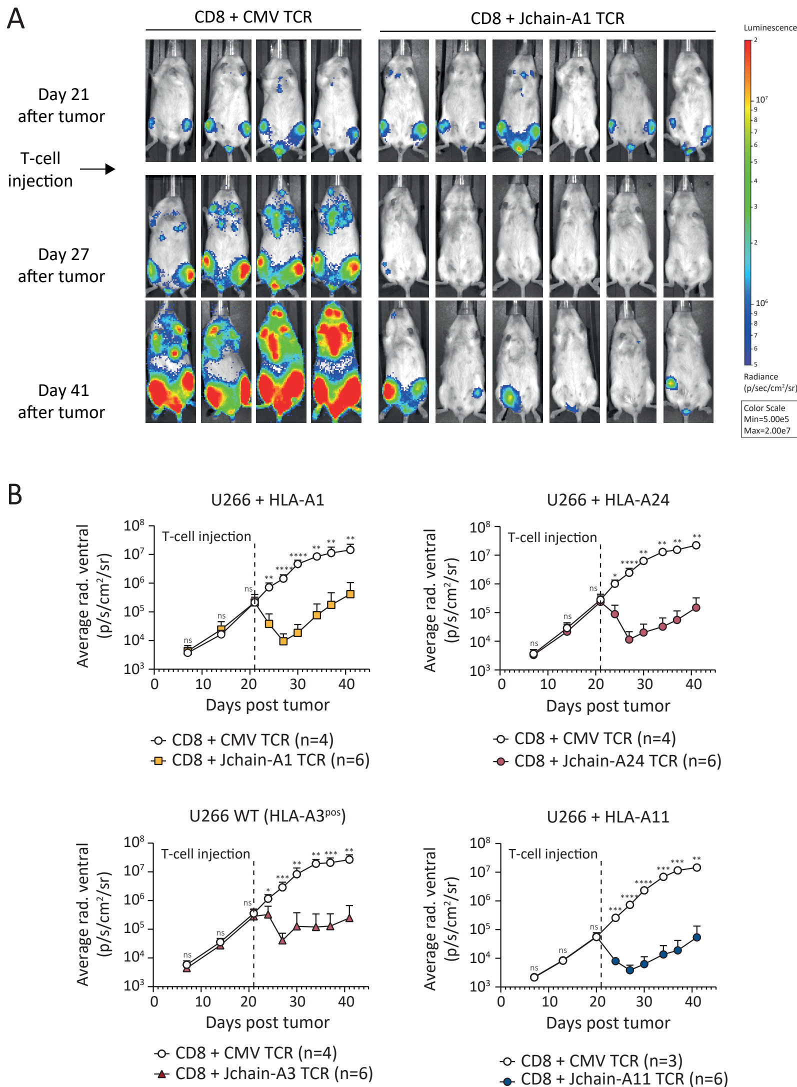


Figure 7. ***In vivo*** **antitumor efficacy of Jchain HLA-A1, A24, A3 and A11 restricted TCR transduced CD8 T cells.** NSG mice engrafted with 2x10^6 U266 multiple myeloma cells transduced with *Luc2* luciferase and HLA-A1,-A11,-A24 or wildtype (WT) were i.v. injected with 3-6x10^6 TCR transduced CD8 T cells after 21 days. CD8 T cells were separately transduced with Jchain HLA-A1 (5D12), -A3 (5C8), -A11 (16C7), -A24 (A24) or control CMV (pp65-NLV-HLA-A2) TCR and enriched for mTCR expression by MACS. T cells were infused 10 days after re-stimulation. Tumor outgrowth was frequently tracked by bioluminescence imaging. Mean and standard deviations of tumor outgrowth (average radiance) over time on the ventral side of CMV (n=4) versus Jchain TCR (n=6) treated mice is shown. Data representative of two independent experiments. A) Jchain HLA-A1 TCR 5D12.9 treated mice. B) Jchain HLA-A3 TCR 5C8.8 treated mice. C) Jchain HLA-A11 TCR 16C7.9 treated mice. D) Jchain HLA-A24 TCR 10H11.11 treated mice. Statistics depict 2-way ANOVA comparing groups per timepoint with Sidak's multiple comparisons post-hoc test.

CD8 T-cells. Jchain TCR T-cells did not recognize healthy tissue subsets other than B-cells. Potent eradication of MM cells from patient derived BM was observed for 6 out of 8 *JCHAIN* expressing MM samples. Finally, we demonstrated that Jchain TCR T-cells were able to largely reduce MM tumor burden in an *in vivo* xenograft model. Combined, the HLA-A1,-A24,-A3 and-A11 Jchain specific TCRs result in an HLA allele coverage of 60.7% of the average worldwide population, allowing for a broadly applicable TCR therapy for the treatment of MM.

Epitope selection in this study was performed based on HLA-peptide elution studies using MM cell lines to ensure targeting of epitopes that are naturally processed and presented on MM rather than relying solely on prediction algorithms. Despite this, a discrepancy between *JCHAIN* expression and killing of HLA-A24 positive MM cells by Jchain TCR T-cells was observed, indicating that the CYT-A24 epitope is not sufficiently presented on all primary materials for TCR T-cell recognition. Similar mechanisms might play a role in presentation of the Jchain YTA-A1, ISD-A3 and ISD-A11 epitopes, but could have remained undetected since limited primary materials were tested in this study. Investigation of Jchain epitope presentation in a larger MM cohort will be needed to explore mechanisms of aberrant Jchain CYT-A24 epitope presentation and to clarify the proportion of patients that would benefit from clinical development of Jchain TCRs. For clinical application, it will be essential to include diagnostics tools to ensure Jchain epitope presentation in addition to *JCHAIN* and respective HLA expression prior to therapy of MM patients.

Safety aspects of transgenic T-cell therapy remain a major concern since both off-target and on-target/off-tumor reactivities can result in lethal toxicity(31, 32). While off-target reactivity screenings were performed in this study, cross-reactivities might have remained undetected, and a more detailed mapping of potential cross-reactivities using peptide library scanning should be performed in additional pre-clinical studies. Besides off-target toxicity, on-target/off-tumor toxicity is a crucial safety aspect of engineered T-cell therapy. *JCHAIN* expression profile strongly overlaps with the expression profile of *BCMA* (proteinatlas.org version 21.0)(33, 34). While Jchain has not been explored as a therapeutic target for MM therapy, BCMA CAR T-cell therapy have demonstrated safe application. BCMA CAR T-cell therapy was well tolerated, but *BCMA* expression in healthy plasma cells resulted in plasma cell aplasia thereby compromising humoral immunity(35). While off-target toxicity can be subjective to affinities of the individual CAR or TCR used(36), side effects due to on-target/off-tumor toxicity observed after BCMA CAR T might be of predictive value on the potential off-tumor effects exerted by Jchain TCR T-cells.

To conclude, we identified promising Jchain TCRs targeting epitopes presented in HLA-A1,-A24,-A3 and-A11 resulting in a total HLA-allele coverage of more than 60%. TCR

transduced CD8 T-cells demonstrated stringent specificity for *JCHAIN* expressing target cells with the relevant HLA restriction alleles. Potent eradication of MM cells *in vitro* as well as *in vivo* demonstrates high promise of Jchain TCR T-cells for clinical development as a novel therapy of MM.

MATERIALS AND METHODS

JCHAIN microarray data

Log2 transformed expression values of *JCHAIN* (probe: 212592_at) and *SDC1* (probe: 201286_at) were retrieved from publicly available microarray dataset (GSE: 13591; https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE13591)(24). The dataset consists of expression profiles of 133 multiple myeloma patient samples and 5 healthy donor plasma cell samples (sorted on CD138+). Expression values were back transformed using the function: y=2^x. The cutoff for expression was defined using a selection of genes not expressed in MM (*GFAP*_203540_at, *INS*_ 206598_at, *MUC16*_ 220196_at, *MYH11*_ 201496_x_at, *TRAC*_ 209670_at) calculated as mean values of the selected negative genes + (3xSTDEV).

JCHAIN expression

JCHAIN expression was measured by quantitative real-time polymerase chain reaction (qPCR) as previously described(9). *JCHAIN* forward primer: 5' GTACCATTTGTCTGACCTCTGT 3' and reverse primer: 5' AGCAGGTCTCTGTAGCACTG 3'. *GUSB*, *VPS29* and *PSMB4* were included as housekeeping genes (HKGs). *JCHAIN* expression was calculated relative to HKGs.

Epitope discovery

MM cell lines U266 (HLA-A*02:01,-A*03:01,-B*07:02,-B*40:01,-C*03:04, and-C*07:02) and UM9 (HLA-A*01:01, -A*11:01, -B*07:02, -B*55:01, -C*03:04, and -C7*07:02) were used for Jchain epitope discovery. U266 cells were retrovirally transduced with HLA-A24 and UM9 cells were transduced with HLA-A2. Retroviral transduction was performed as previously described (37). pHLA-class I complexes were isolated from MM cell lysates from 30x10^9 cells using anti HLA class-I (Clone W6/32) or an anti-HLA-A1/A24 (Clone GV5D1, provided by Dr. Arend Mulder, Leiden University Medical Center, the Netherlands) antibodies after which peptide elution, mass spectrometry analysis and epitope selection were performed as previously described (9). Peptide elution data was investigated for peptides originating from the Jchain protein according to the Uniprot *Homo sapiens* database. Peptides were assigned to HLA class I (HLA-I) alleles expressed by cells of origin for which the peptides contain anchor residues for binding the respective HLA allele according to netMHC4.0(25). Mass spectra of identified peptides

were compared to mass spectra of synthetically generated peptides to confirm correct identification. Peptide-HLA monomers were refolded, and successful refolding was used as criterium for stable binding of identified peptides to designated HLA-I molecules.

Cell culture and target cell generation

T cells were cultured in TCM consisting of IMDM (Lonza) supplemented with 5% fetal bovine serum (FBS; Gibco, Life Technologies), 5% human serum 1.5% glutamine (Lonza) and 1% penicillin/streptomycin (Lonza) and 100IU/ml IL2 (Proleukin; Novartis Pharma). Expanded T-cell clones were cryopreserved or restimulated every 10-15 days with feeder mix containing 1x10^6 PBMC's, 0.1x10^6 Epstein-Barr virus transformed lymphoblastoid cell line (EBV-LCL) JY cells and 0.8 mg/ml phytohemagglutinin (PHA; Oxoid Microbiology Products, Thermo Fisher Scientific) . Cell lines were cultured in IMDM with 10% FBS, 1.5% glutamine and 1% penicillin/streptomycin. Cell lines negative for target HLA alleles were retrovirally transduced to express HLA-A1, A24, A3 or A11. Viral vectors encoded murine CD19 or tNGFR as transduction markers. K562 cells were additionally transduced to express *JCHAIN*. Transduced cells were purified for transgene expression by MACS enrichment of marker gene positive cells. PBMCs from healthy donors expressing one or two target HLA alleles were used to derive hematopoietic subsets as previously described (22). Fibroblasts and keratinocytes were pre-treated for 48h with 100IU/ml IFN-γ (Immukine) prior to experiments. MM Patient derived BM samples were thawed and rested overnight in medium containing 10% human serum before use in cytotoxicity experiments.

T-cell assays

pHLA-tetramer sorted T-cells were initially screened for peptide reactivity in a high-throughput manner as previously described(9). Peptide reactive T-cell clones were selected and screened for recognition of endogenously processed and presented peptide using *JCHAIN* transduced K562 cells. T-cell clones producing >2ng/ml IFN-y were selected for further investigation. For cytokine production experiments, 5,000 T-cells were co-cultured with 30,000 target cells. IFN-y production after overnight co-culture was measured by ELISA (R&D systems). In peptide titration experiments, target cells were loaded with decreasing peptide concentrations starting at 1uM. Supernatants were tested in 5x and 125x dilution. For tetramer binding experiments, T-cells were stained with 2ug/ml PE-labelled Jchain pHLA-tetramers and analyzed by fluorescence-activated cell sorting (FACS).

Cytotoxicity experiments

Killing of cell lines was tested in standard ^{51}chromium release assays as described previously(9). Killing of primary material was analyzed in FACS based cytotoxicity assays. 50,000 target cells were co-cultured with T-cells in effector:target (E:T) ratio 3:1

overnight. After overnight culture cells were stained with specific antibody panels and SYTOX Blue Dead Cell Stain (Invitrogen by Thermo Fisher Scientific) was added prior to acquisition to identify living cells. Samples were acquired by FACS using fixed flowrates and stable acquisition was validated using Flow-Count Fluorospheres (Beckman Coulter). Co-cultures with activated B-cells were stained using anti-CD3 (Alexa Fluor 700, BD pharmingen), anti-CD19 (PE-Cy7, BD pharmingen), anti-IgA (PE, Miltenyi), anti-IgG (FITC, DAKO) and anti-IgM (APC, BD). For primary MM, 50,000 patient derived BM mononuclear cells were used as target cells. Co-cultures were stained with anti-CD3, anti-CD19 (APC, BD pharmingen), anti-CD45 (FITC, BD), anti-CD38 (PE-Texas Red, Invitrogen) and anti-CD56 (PE-Cy7, BD pharmingen). MM cells were defined as $CD3^{neg}$, $CD19^{neg}$, $CD45^{neg\text{-}int}$, $CD38^{pos}$, $CD56^{pos}$.

3

TCR sequencing and transfer

TCRα and TCRβ sequences of selected T-cell clones were identified as previously described(9). TCRα and β variable chains were codon optimized and introduced into MP71-TCR-flex retroviral vectors encoding cysteine modified murine TCRαβ constant domains. CD4 and CD8 T-cells were separately isolated, activated and transduced with TCR as previously described(9). On day 7 post activation, TCR transduced T-cells were enriched for expression of introduced TCRs using indirect MACS and APC labelled mouse TCR-Cβ (mTCR; BD Pharmingen). Purified T-cells were used in experiments between day 10-14 after activation.

pHLA Tetramer based T-cell isolations

PE-labelled pHLA-tetramers were produced as previously described with minor modifications(9, 38). PBMCs isolated from complete buffy coats (Sanquin) obtained from healthy donors negative for HLA-I alleles of interest were used to isolate Jchain specific T cell clones as previously described(9). In short, PBMCs were incubated with pooled pHLA-tetramers and pHLA-tetramer bound cells were enriched by MACS using anti-PE MicroBeads (Miltenyi Biotec). Enriched fractions were single-cell sorted for pHLA-$tetramer^{pos}$ $CD8^{pos}$ T-cells in 96-well round bottom plates containing feeder mix consisting of 5x10^5 irradiated PBMCs, 5x10^4 irradiated EBV-LCL JY cells and 0.8 mg/ml PHA in T-cell medium (TCM).

***In vivo* MM model**

U266 cells were transduced with and enriched for Luciferase-tdTomato Red and target HLA-A alleles when indicated. NOD-scid-$IL2Rgamma^{null}$ (NSG) mice (The Jackson Laboratory) were intravenously (i.v.) injected with 2x10^6 U266 cells. After 21 days, mice were treated i.v. with 3-6x10^6 purified Jchain TCR transduced CD8 T-cells. CMV TCR T-cells were used as negative control. Tumor outgrowth was measured at regular intervals after subcutaneous (s.c.) injection of 150 µL 7.5 mM D-luciferine

(Cayman Chemical) using a CCD camera (IVIS Spectrum, PerkinElmer). All mice were sacrificed when control mice reached an average luminescence of 1x10^7 p/s/cm^2/sr. This study was approved by the national Ethical Committee for Animal Research (AVD116002017891) and performed in accordance with Dutch laws for animal experiments. Statistical analysis was performed using GraphPad Prism version 9.3.1. *In vivo* experiments were analyzed using 2-way ANOVA comparing groups per timepoint with Sidak's multiple comparisons post-hoc test.

AKNOWLEDGEMENTS

The authors thank the Flow cytometry Core Facility (FCF) of Leiden University Medical Center (LUMC) in Leiden, the Netherlands (https://www.lumc.nl/research/facilities/fcf), coordinated by dr. K. Schepers and M. Hameetman, run by the FCF Operators E.F.E de Haas, J.P. Jansen, D.M. Lowie, S. van de Pas, and G.IJ. Reyneveld (Directors: Prof. F.J.T. Staal and Prof. J.J.M. van Dongen) for cell sorting assistance.

REFERENCES

1. Nandakumar B, Binder M, Dispenzieri A, Kapoor P, Buadi F, Gertz MA, et al. Continued improvement in survival in multiple myeloma (MM) including high-risk patients. Journal of Clinical Oncology. 2019;37(15):8039-.
2. Raje N, Berdeja J, Lin Y, Siegel D, Jagannath S, Madduri D, et al. Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or Refractory Multiple Myeloma. The New England journal of medicine. 2019;380(18):1726-37.
3. Zhao WH, Liu J, Wang BY, Chen YX, Cao XM, Yang Y, et al. A phase 1, open-label study of LCAR-B38M, a chimeric antigen receptor T cell therapy directed against B cell maturation antigen, in patients with relapsed or refractory multiple myeloma. Journal of hematology & oncology. 2018;11(1):141.
4. Berdeja JG, Madduri D, Usmani SZ, Jakubowiak A, Agha M, Cohen AD, et al. Ciltacabtagene autoleucel, a B-cell maturation antigen-directed chimeric antigen receptor T-cell therapy in patients with relapsed or refractory multiple myeloma (CARTITUDE-1): a phase 1b/2 open-label study. Lancet (London, England). 2021;398(10297):314-24.
5. Cohen AD, Garfall AL, Stadtmauer EA, Melenhorst JJ, Lacey SF, Lancaster E, et al. B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma. J Clin Invest. 2019;129(6):2210-21.
6. Samur MK, Fulciniti M, Aktas Samur A, Bazarbachi AH, Tai YT, Prabhala R, et al. Biallelic loss of BCMA as a resistance mechanism to CAR T cell therapy in a patient with multiple myeloma. Nat Commun. 2021;12(1):868.
7. Bouchon A, Cella M, Grierson HL, Cohen JI, Colonna M. Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. Journal of immunology (Baltimore, Md : 1950). 2001;167(10):5517-21.
8. Smith EL, Harrington K, Staehr M, Masakayan R, Jones J, Long TJ, et al. GPRC5D is a target for the immunotherapy of multiple myeloma with rationally designed CAR T cells. Science translational medicine. 2019;11(485):eaau7746.
9. Meeuwsen MH, Wouters AK, Jahn L, Hagedoorn RS, Kester MGD, Remst DFG, et al. A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy. Mol Ther. 2022;30(2):564-78.
10. Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol. 2009;70(6):505-15.
11. Xu AQ, Barbosa RR, Calado DP. Jchain-driven cre enables specific genetic manipulation and timestamping of plasma cell in their niche. bioRxiv. 2020:2020.04.12.038380.
12. Nilssen DE, Brandtzaeg P, Frøland SS, Fausa O. Subclass composition and J-chain expression of the 'compensatory' gastrointestinal IgG cell population in selective IgA deficiency. Clin Exp Immunol. 1992;87(2):237-45.
13. Lokhorst HM, Dekker AW, Baarlen JV, Bast EJ. J chain disease: an aggressive evolution of multiple myeloma. Am J Med. 1990;88(4):417-20.
14. Kelényi G. Intracellular J chains in lymphoproliferative diseases. Virchows Archiv A. 1985;405(3):365-78.
15. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol. 2014;14(6):377-91.
16. Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD, et al. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin Cancer Res. 2011;17(17):5615-25.
17. Jahn L, Hagedoorn RS, van der Steen DM, Hombrink P, Kester MG, Schoonakker MP, et al. A CD22-reactive TCR from the T-cell allorepertoire for the treatment of acute lymphoblastic leukemia by TCR gene transfer. Oncotarget. 2016;7(44):71536-47.
18. Wilde S, Sommermeyer D, Frankenberger B, Schiemann M, Milosevic S, Spranger S, et al. Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood. 2009;114(10):2131-9.
19. Stronen E, Abrahamsen IW, Gaudernack G, Wälchli S, Munthe E, Buus S, et al. Dendritic cells engineered to express defined allo-HLA peptide complexes induce antigen-specific cytotoxic T cells efficiently killing tumour cells. Scand J Immunol. 2009;69(4):319-28.
20. Savage P, Gao L, Vento K, Cowburn P, Man S, Steven N, et al. Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen.

Blood. 2004;103(12):4613-5.
21. Rammensee HG, Bevan MJ. Evidence from in vitro studies that tolerance to self antigens is MHC-restricted. Nature. 1984;308(5961):741-4.
22. Jahn L, Hombrink P, Hagedoorn RS, Kester MG, van der Steen DM, Rodriguez T, et al. TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1. Blood. 2017;129(10):1284-95.
23. Pont MJ, Honders MW, Kremer AN, van Kooten C, Out C, Hiemstra PS, et al. Microarray Gene Expression Analysis to Evaluate Cell Type Specific Expression of Targets Relevant for Immunotherapy of Hematological Malignancies. PLoS One. 2016;11(5):e0155165.
24. Agnelli L, Mosca L, Fabris S, Lionetti M, Andronache A, Kwee I, et al. A SNP microarray and FISH-based procedure to detect allelic imbalances in multiple myeloma: an integrated genomics approach reveals a wide gene dosage effect. Genes Chromosomes Cancer. 2009;48(7):603-14.
25. Jurtz V, Paul S, Andreatta M, Marcatili P, Peters B, Nielsen M. NetMHCpan-4.0: Improved Peptide-MHC Class I Interaction Predictions Integrating Eluted Ligand and Peptide Binding Affinity Data. Journal of immunology (Baltimore, Md : 1950). 2017;199(9):3360-8.
26. Teoh PJ, Chng WJ. CAR T-cell therapy in multiple myeloma: more room for improvement. Blood Cancer J. 2021;11(4):84.
27. Drent E, Groen RWJ, Noort WA, Themeli M, Lammerts van Bueren JJ, Parren PWHI, et al. Pre-clinical evaluation of CD38 chimeric antigen receptor engineered T cells for the treatment of multiple myeloma. Haematologica. 2016;101(5):616-25.
28. Gogishvili T, Danhof S, Prommersberger S, Rydzek J, Schreder M, Brede C, et al. SLAMF7-CAR T cells eliminate myeloma and confer selective fratricide of SLAMF7(+) normal lymphocytes. Blood. 2017;130(26):2838-47.
29. Ormhoj M, Bedoya F, Frigault MJ, Maus MV. CARs in the Lead Against Multiple Myeloma. Curr Hematol Malig Rep. 2017;12(2):119-25.
30. Mailankody S, Devlin SM, Landa J, Nath K, Diamonte C, Carstens EJ, et al. GPRC5D-Targeted CAR T Cells for Myeloma. The New England journal of medicine. 2022;387(13):1196-206.
31. Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood. 2013;122(6):863-71.
32. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Molecular therapy : the journal of the American Society of Gene Therapy. 2010;18(4):843-51.
33. Uhlen M, Karlsson MJ, Zhong W, Tebani A, Pou C, Mikes J, et al. A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. Science (New York, NY). 2019;366(6472).
34. Karlsson M, Zhang C, Méar L, Zhong W, Digre A, Katona B, et al. A single-cell type transcriptomics map of human tissues. Sci Adv. 2021;7(31).
35. Wang Y, Li C, Xia J, Li P, Cao J, Pan B, et al. Humoral immune reconstitution after anti-BCMA CAR T-cell therapy in relapsed/refractory multiple myeloma. Blood Adv. 2021;5(23):5290-9.
36. Drent E, Themeli M, Poels R, de Jong-Korlaar R, Yuan H, de Bruijn J, et al. A Rational Strategy for Reducing On-Target Off-Tumor Effects of CD38-Chimeric Antigen Receptors by Affinity Optimization. Molecular therapy : the journal of the American Society of Gene Therapy. 2017;25(8):1946-58.
37. Jahn L, Hombrink P, Hassan C, Kester MG, van der Steen DM, Hagedoorn RS, et al. Therapeutic targeting of the BCR-associated protein CD79b in a TCR-based approach is hampered by aberrant expression of CD79b. Blood. 2015;125(6):949-58.
38. Burrows SR, Kienzle N, Winterhalter A, Bharadwaj M, Altman JD, Brooks A. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. J Immunol. 2000;165(11):6229-34.

SUPPLEMENTARY MATERIAL

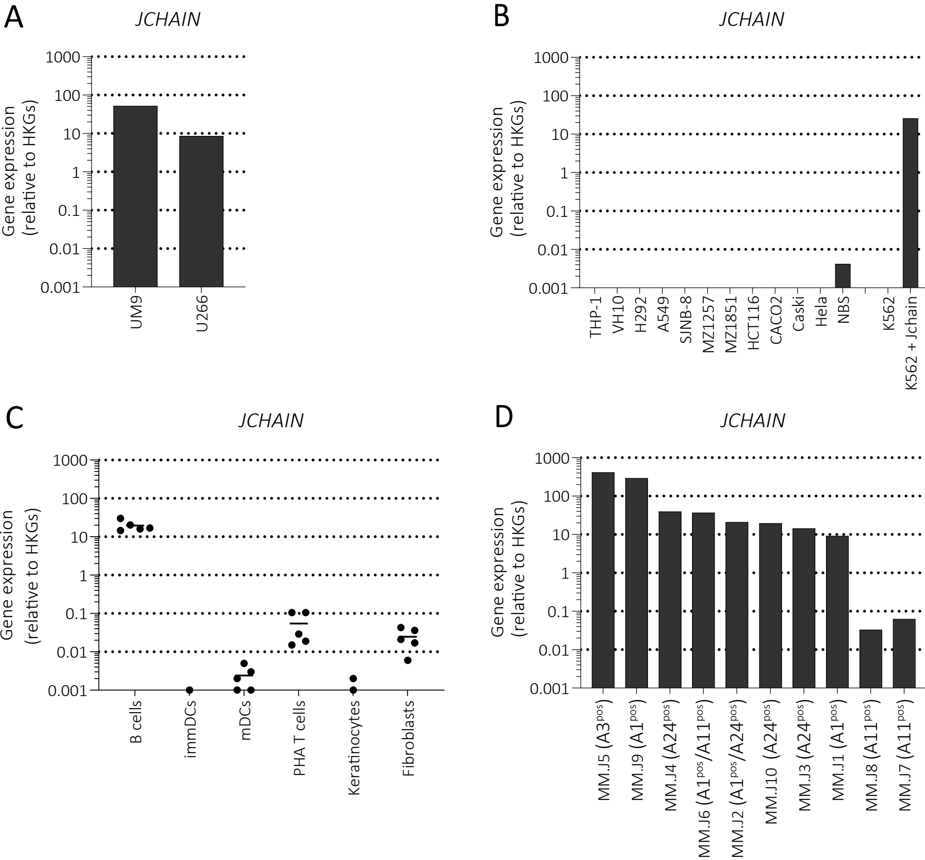


Figure S1. qRT-PCR of *JCHAIN* expression relative to housekeeping genes (HKGs) in cells used in this study. A) MM cell lines U266 and UM9. **B)** Cell lines of non-B cell origin including K562 cells and *JCHAIN* transduced K562 cells. **C)** Healthy tissues of various origins used in figure 5 each dots represents cells from another donor. **D)** Expression in MM cells sorted from various MM patient bone marrow samples. A selection of these materials was used in figure 6.

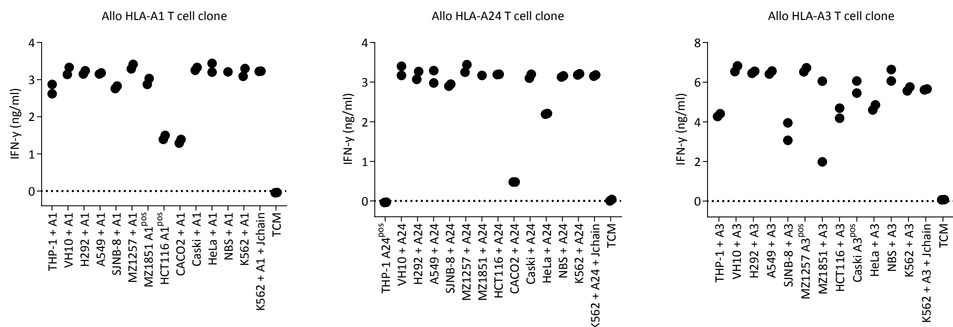


Figure S2. T cell stimulatory capacity of non-B cell lines used in safety panel. Tumor cell lines used in figure 2A co-cultured with HLA-A1 (left graph), HLA-A24 (middle graph) or HLA-A3 (right graph) allo HLA reactive T cell clones. IFN-γ concentration in supernatant was tested by ELISA using a 5-fold dilution only.

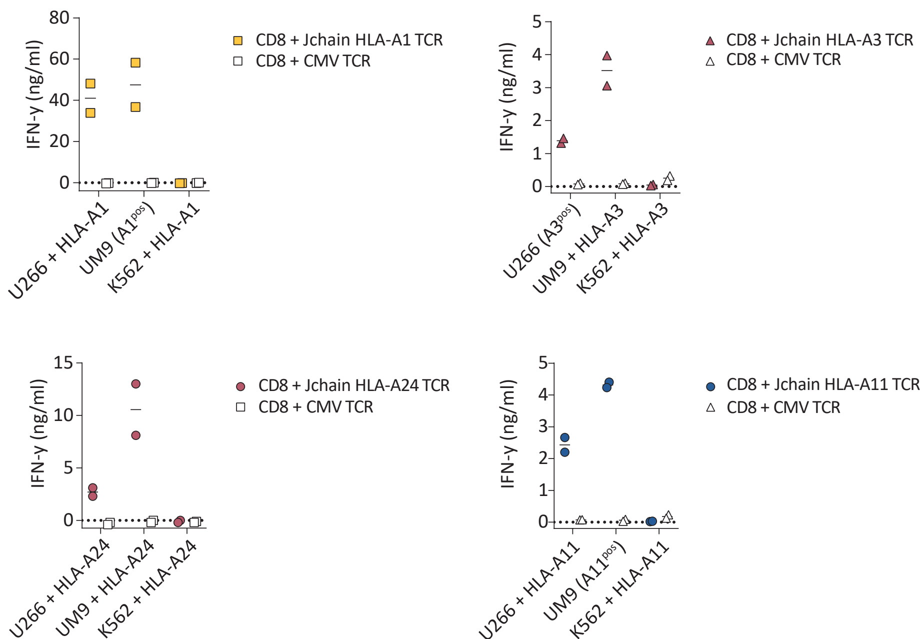
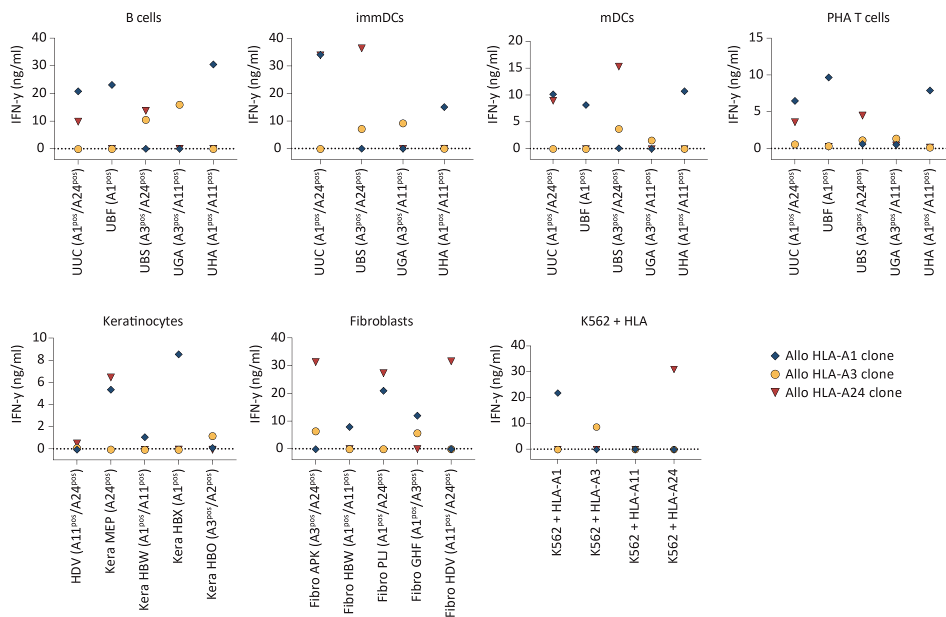


Figure S3. Antigen specific killing of MM cells by Jchain TCR CD8 T cells is associated with cytokine production. IFN-γ production measured after overnight co-culture of T cells and target cells used in figure 4C, data obtained in the same experiment. CMV TCR transduced CD8 T cells were used as a control for background cytokine production.



3

Figure S4. IFN-γ production after co-culture of AlloHLA-A1 (in blue), -A3 (in yellow) and -A24 (in red) T cell clones with all target cells used in figure 5. Three letter codes represent target cell donors and expression of HLA-A1,-A3,-A11 or-A24 is indicated. Data points represent means of technical duplicates. Graphs are separated based on cell type.

Figure S5. Phenotype of patient derived MM samples analyzed by FACS. [*Figure on the next page*]. Data obtained from the target cell only condition of the experiment shown in figure 6. Three letter codes in titles represent different patients and expression of HLA-A1,-A3,-A11 or A24 is indicated. MM patient BM samples were stained with SYTOX blue Dead Cell Stain, anti-CD3, anti-CD19, anti-CD45, anti-CD38 and anti-CD56. MM cells were gated on: SYTOX blue negative, live cells →single cells →CD3 negative, CD19 negative →CD38 positive, CD45 negative-intermediate→CD56 positive. Percentages depicted indicated the percentage of cells of the parent gate. To calculate the frequency of MM cells in BM samples, percentage in subsequent gates were multiplied.

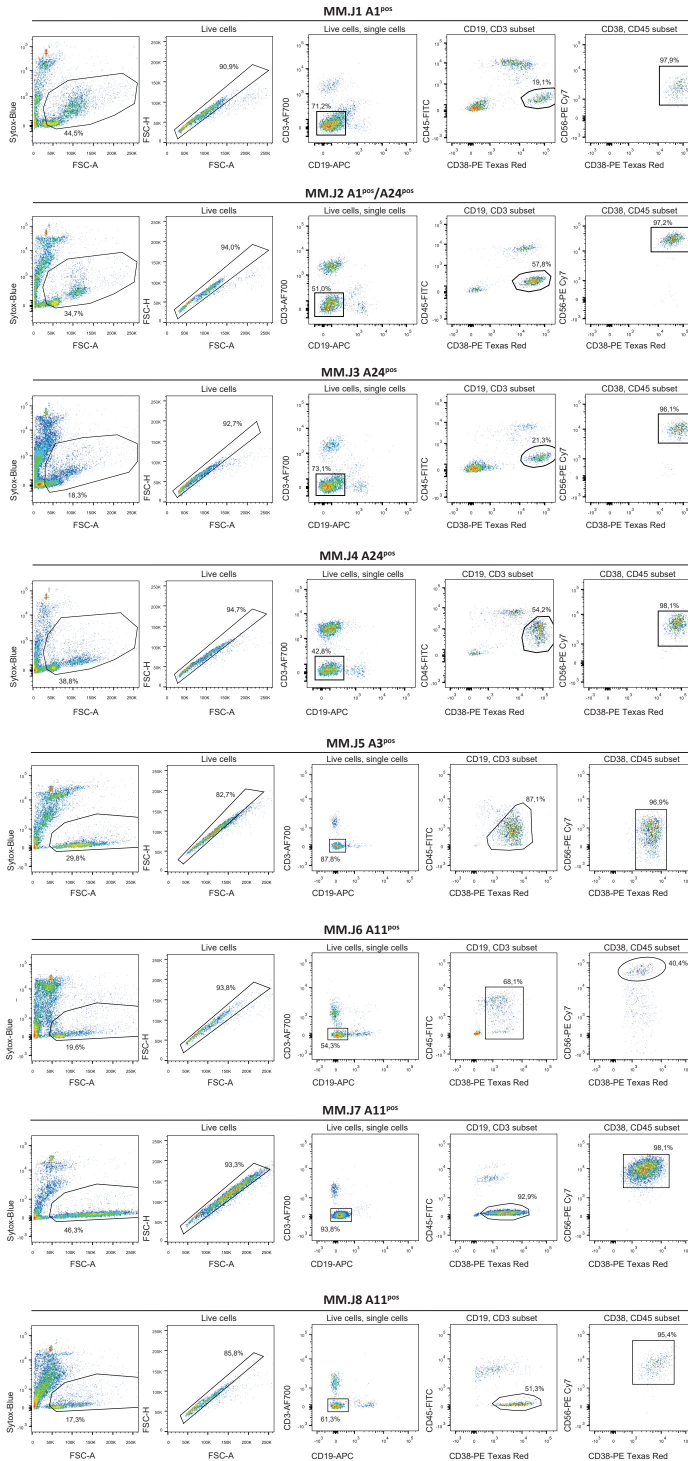


Figure S5 [*Legend on previous page*].

3

Table S1. HLA typing of EBV-LCLs used in EBV-LCL panels

	Included in Jchain EBV-LCL panel				HLA typing		
EBV-LCL	**HLA-A1**	**HLA-A24**	**HLA-A3**	**HLA-A11**	**HLA-A**	**HLA-B**	**HLA-C**
6023	x	x			A*03:01/03:03/03:04 - A*11:01/11:02/11:03	B*40:02/40:35/40:37 - B*56:01	C*01:02/01:06/01:07- C*02:02/02:04/02:08
10299	x	x	x		A*02:01- A*11:01	B*44:05 - B*51:01	C*02:02- C*14:02
5857	x	x	x	x	A*30:04 - A*68:02	B*38:01 - B*55:01	C*03:03 - C*12:03
3829		x	x	x	A*01:01 - A*68:01	B*44:02 - B*44:02	C*05:01 - C*07:04
6978	x	x	x	x	A*02:01 - A*02:05	B*15:01 - B*45:01	C*01:02 - C*06:02
5769	x	x	x	x	A*02:01 - A*68:01	B*35:03 - B*37:01	C*04:01 - C*06:02
6461		x			A*02:01- A*02:01	B*40:02- B*40:02	C*02:02- C*02:02
6463			x	x	A*02:01 - A*02:01	B*57:01 - B*57:01	C*06:02 - C*06:02
5696	x	x	x	x	A*02:05 - A*02:05	B*58:01 - B*58:01	undetermined
4991	x	x	x	x	A*26:01/26:08/26:02- A*31:01/31:02/31:06	B*14:01 - B*49:01	C*07:01/07:05/07:06 - C*08:02/08:07
8419			x	x	A*02:01- A*01:01	B*50:01- B*07:02	C*07:02- C*06:02
8425	x	x	x	x	A*23:01- A*02:01	B*41:01 - B*40:01	C*17:01- C*03:04
8451			x	x	A*02:01- A*01:01	B*35:01- B*08:01	C*07:01- C*04:01
8435			x	x	A*25:01- A*02:01	B*44:02- B*07:02	C*07:02- C*05:01
8463			x		A*11:01 - A*01:01	B*51:01 - B*50:01	C*15:02 - C*06:02
9210	x	x	x	x	A*02:01- A*02:01	B*15:01- B*51:01	C*03:03- C*15:02
5439	x	x			A*03:01 - A*25:01	B*15:17 - B*18:01/18:03/18:05	C*07:01/07:05/07:06 - C*12:03/12:06
3516	x	x			A*03:01 - A*26:01	B*07:02- B*14:01	C*07:02 - C*08:02
1854	x	x	x	x	A*02:01 - A*30:02	B*15:01 - B*39:01	C*03:03 - C*12:03
6268	x		x	x	A*02:01 - A*24:02	B*35:02 - B*44:02	C*04:01 - C*05:01
5702	x	x	x	x	A*32:01 - A*68:01	B*35:03 - B*52:01	C*12:02 - C*12:03
7394		x			A*01:01- A*32:01	B*35:08 - B*35:08	C*04:01 - C*04:01
4084	x	x			A*03:01/03:03/03:04- A*30:01	B*07:02 - B*38:01	C*07:02/07:03/07:05- C*12:03/12:06

Table S1. Continued.

	Included in Jchain EBV-LCL panel				HLA typing		
EBV-LCL	HLA-A1	HLA-A24	HLA-A3	HLA-A11	HLA-A	HLA-B	HLA-C
4803	x	x			A*03:01 - A*33:01	B*07:02 - B*14:02	C*07:02 - C*08:02
5171	x	x	x	x	A*02:01 - A*66:01/66:04	B*40:01/40:11/40:14- B*41:02	C*03:04/03:08/03:09 - C*17
7590	x		x	x	A*24:02- A*31:01	B*07:02/07:61 - B*35:08	C*04:01 - C*07:02
5524			x	x	A*02:01/02:07/02:09- A*31:01/31:02/31:06	B*15:01/15:33/15:34- B*15:17	C*03:04/03:08/03:09 - C*07:01/07:05/07:06
7007	x	x	x	x	A*02:05 - A*29:02	B*27:05 - B*44:03	C*01:02 - C*16:01
2631	x	x			A*02:01 - A*03:01/03:03N/03:04	B*44:02 - B*57:01	C*06:02 - C*07:04/07:12/07:11
5998			x	x	A*24:02- A*68:02	B*14:02- B*38:01	C*08:02- C*12:03
3480			x	x	A*26:01 - A*01:01	B*38:01 - B*18:01	C*12:03 - C*07:01/07:06
9877			x	x	A*01:01- A*23:01/23:17	B*08:01- B*41:02	C*07:01- C*17:03
9619		x	x	x	A*01:01- A*33:03	B*44:03- B*51:01	C*07:06/07:18 - C*14:02
6945	x	x			A*03:01 - A*25:01	B*18:01 - B*35:01	C*04:01 - C*12:03
6316			x	x	A*29:02- A*30:01	B*13:02- B*44:03	C*06:02- C*16:01
9779	x	x		x	A*02:01 - A*03:01	B*08:01- B*50:01	C*06:02- C*07:01
10120	x		x	x	A*02:01 - A*24:02	B*07:02- B*40:02	C*02:02- C*07:02
12425			x	x	A*23:01- A*36:01	B*15:03- B*53:01	C*02:10- C*04:01
7159			x	x	A*02:01- A*02:01	B*13:02- B*44:02	C*05:01- C*06:02
5866		x			A*02:01 - A*11:01	B*35:01 - B*51:01	C*04:01 - C*14:02

CHAPTER 4

T-cell receptor-based targeting of immunoglobulin constant domains for treatment of multiple myeloma

Miranda H. Meeuwsen[1]
Anne K. Wouters[1]
Tassilo L.A. Wachsmann[1]
Renate S. Hagedoorn[1]
Michel G.D. Kester[1]
Dennis F.G. Remst[1]
Dirk M. van der Steen[1]
Arnoud H. de Ru[2]
Els P. van Hees[1]
Peter A. van Veelen[2]
J.H. Frederik Falkenburg[1]
Mirjam H.M. Heemskerk[1]

[1]Department of Hematology, Leiden University Medical Center, Leiden 2333ZA, the Netherlands; [2]Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden 2333ZA, the Netherlands

Manuscript submitted

ABSTRACT

In multiple myeloma (MM), transformed plasma cells produce high levels of monoclonal immunoglobulins, most of which are of IgG or IgA subtype. While surface expression of immunoglobulins on MM is usually absent, we hypothesized that peptides derived from immunoglobulins presented in HLA would pose suitable targets for T-cell receptor (TCR-) based therapy of MM. Based on a previously established HLA-peptidome database of B-cell malignancies, we identified three IgG- and four IgA-constant-domain-derived peptides presented in the commonly expressed HLA-A2 and HLA-B7. Using peptide-HLA tetramers, we isolated high-avidity CD8 T-cell clones from the allo-HLA repertoire. In total, we characterized one T-cell clone recognizing an endogenously processed and presented IgG peptide in HLA-A2, and seven T-cell clones recognizing endogenously processed and presented IgA peptides in HLA-B7. Based on recognition of isotype positive MM cell lines, TCRs of the most potent T-cell clones were sequenced, cloned and retrovirally expressed in healthy donor T cells. Upon TCR gene transfer, immunoglobulin-TCR T cells demonstrated potent and isotype-specific recognition and lysis of MM cell lines as well as MM cells in patient bone marrow samples. TCR-transduced CD8 T cells specifically depleted healthy IgA or IgG B cells in an HLA-and isotype-dependent manner. Importantly, healthy cells from non-B-cell lineages were not recognized. Furthermore, IgA-TCR T cells induced anti-tumor responses in an *in vivo* xenograft model of established IgA^{positive} MM, demonstrating potential for clinical development of immunoglobulin-targeting TCR therapy.

INTRODUCTION

Multiple myeloma (MM) is a malignancy caused by uncontrolled expansion of plasma cells and is largely incurable(1, 2). Chemotherapy, immunomodulatory drugs like proteasome inhibitor, anti-CD38 monoclonal antibodies and autologous stem cell transplantation extended life expectancy of patients, but patients relapsing after treatment have extremely poor overall survival(3). The only truly curative therapy for MM to date has been allogeneic stem cell transplantation (allo-SCT), but allo-SCT is generally not generally performed anymore due to high treatment related mortality(4). In recent years, the development of chimeric antigen receptor (CAR) T cells that target antigens expressed on the surface of malignant cells generated promising treatment options for relapsed/refractory MM (RRMM). Notably, B-cell maturation antigen (BCMA-)targeting CAR T cells induce responses in a majority of RRMM patients(5, 6). Despite high initial response rates and median progression free survival in RRMM exceeding one year, most patients eventually relapse(7, 8). Relapses after CAR T-cell therapy can often be attributed to immune escape of antigen low or negative tumor cells, as a consequence of single-antigen-targeting(8-11). In contrast, the effectiveness of allo-SCT has been attributed to polyclonal T-cell responses, illustrating that T-cell responses targeting multiple antigens are likely needed to induce durable complete remissions(12). Consequently, identification of additional antigens suitable for targeted T-cell therapy will be crucial for a tolerable and curative approach(11).

As MM originates from a malignant plasma cell clone, most MM cases are characterized by production of monoclonal immunoglobins(13). We hypothesized that the constant domains of immunoglobulin heavy chains could serve as targets for immunotherapy. IgG immunoglobulins are produced in 54% of MM cases, while IgA is produced in 22% of MM and is associated with a worse prognosis(14). Other subtypes such as IgM isotypes are rare, and in 20% of cases MM cells secrete immunoglobulin light chains only(15). During B-cell development, formation of plasma cells is marked by loss of BCR surface expression. Consequently, malignant plasma cells do not express a BCR on the surface, hindering recognition by CAR T cells. However, peptides derived from IgG and IgA heavy chains presented in HLA could be targeted using T-cell receptors (TCRs). The constant domains of immunoglobulin heavy chains are regions conserved within immunoglobulin isotypes and could thus serve as a universal target for malignancies expressing the respective immunoglobulin. IgG heavy chain constant domains are encoded by the *IGHG* genes and IgA heavy chain constant domains are encoded by the *IGHA* genes.

In this study, we aimed to create new options for T-cell therapy of MM by identification of TCRs recognizing peptides from the constant domains of IgG and IgA presented in the common HLA alleles HLA-A*02:01 (HLA-A2) and HLA-B*07:02 (HLA-B7). We

demonstrate that IgG- and IgA-specific T-cell clones with high functional avidity can be identified from PBMCs of HLA-mismatched healthy donors, and that gene transfer of immunoglobulin-targeting TCRs (immunoglobulin-TCRs) to healthy donor T cells installs potent anti-myeloma activity *in vitro* and *in vivo* without off-target reactivity towards immunoglobulin negative cells.

RESULTS

IgG- and IgA-constant-domain-derived peptide identification for HLA-A2 and HLA-B7

To identify epitopes suitable for TCR-mediated-targeting of *IGHG* or *IGHA* expressing malignancies, we made use of a previously established HLA class-I peptidome database. This database consists of the peptidomes of various patient-derived B-cell malignancy samples, EBV-LCL cell lines and the MM cell line U266(16-18). We analyzed the peptidomes of HLA-A2 and/or HLA-B7 expressing materials for peptides derived from IgG or IgA constant domain proteins according to the UniProt *homo sapiens* database, and selected those peptides for which binding to HLA-A2 or HLA-B7 was predicted by netMHC4.0. Furthermore, to allow targeting of IgG or IgA producing cells independent of additional subtyping (IGHG1-IGHG4 for IgG and IGHA1 and IGHA2 for IgA), peptides were only selected when present in most variants. In total, this resulted in identification of seven peptides: two IgG-derived peptides presented in HLA-A2 and, one IgG-derived peptide presented in HLA-B7, one IgA-derived peptide presented in HLA-A2, and three IgA peptides presented in HLA-B7 (**Table 1**). IgG- and IgA-derived peptides as identified by mass spectrometry were synthetically generated and refolded with their cognate HLA molecules to generate peptide-HLA (pHLA-) monomers. Biotinylated pHLA-monomers were combined with streptavidin to form phycoerythrin (PE)-labelled pHLA-tetramers that were used for T-cell identification.

Identification and selection of IgG- and IgA-specific T-cell clones recognizing MM cells

To identify high avidity T-cell clones recognizing IgG- or IgA-constant-domain-derived peptides in HLA-A2 or HLA-B7, we exploited the immunogenicity of self-peptides in the HLA-mismatched repertoire (19). To this end, peripheral blood mononuclear cells (PBMC’s) from 26 HLA-A2^{negative} and HLA-B7^{negative} healthy donors were isolated from buffy coats. pHLA-tetramer^{positive}, CD8^{positive} T cells were single-cell sorted, and T-cell clones were expanded. Peptide reactivity and specificity of T-cell clones were screened in a high-throughput manner as previously described (17) using antigen negative HLA-A2 or HLA-B7 transduced K562 target cells loaded with IgG and IgA peptides. Peptide specific T-cell clones were expanded further, and reactivity to endogenously processed and presented peptide was tested using HLA-A2 or HLA-B7 and *IGHG* or *IGHA* transduced

target cells. From 26 buffy coats, we identified 8 T-cell clones specific for target epitopes $IgG^{LMI/A2}$, $IgA^{HPR/B7}$ or $IgA^{SPK/B7}$ that recognized HLA-A2 or HLA-B7 positive and IgG or IgA positive K562 cells to varying degrees (**Figure 1A**). Peptide titration experiments revealed a high functional avidity of these clones with EC_{50} values ranging between 34.2nM and 2.2nM (**Figure 1B**). The identified T-cell clones were then tested for their capability to recognize *IGHG* or *IGHA* expressing MM cell lines. The $IgG^{LMI/A2}$ specific T-cell clone 11B7 potently recognized $IGHG^{positive}$ MM cell line UM3 and $IGHG^{negative}$ cell line ALL GD was not recognized (**Figure 1C**). All IgA HLA-B7 specific T-cell clones recognized $IGHA^{positive}$ UM6 cells while $IGHA^{negative}$ UM3 MM cells were not recognized (**Figure 1C**). $IgA^{SPK/B7}$ specific T-cell clones 6C4 and 8E9 recognized HLA-$B7^{negative}$ wild-type UM6 cells in addition to HLA-B7 transduced UM6 cells (**Figure S1**), demonstrating off-target reactivity outside the HLA-B7 molecule for these T-cell clones. $IgA^{HPR/B7}$ specific T-cell clone 1A8 and $IgA^{SPK/B7}$ specific T-cell clone 3C12 demonstrated the most potent recognition of UM6 cells corresponding with the peptide affinities of these clones. Based on these data, clones 11B7 ($IgG^{LMI/A2}$), 1A8 ($IgA^{HPR/B7}$) and (3C12 $IgA^{SPK/B7}$) were selected for further analysis.

Table 1. IgA (*IGHA*) and IgG (*IGHG*) derived peptides identified in HLA-I peptidome data of MM cell line U266, EBV-LCLs or patient B-cell malignancy samples presented in HLA-A2 or HLA-B7

Immunoglobulin gene	Sequence	HLA-I[a]	Peptide code	HLA binding confirmed[b]	Eluted from cell type[c]
IGHG	GLYSLSSVVTV	A2	$IgG^{GLY/A2}$	yes	EBV-LCL
IGHG	LMISRTPEV	A2	$IgG^{LMI/A2}$	yes	EBV-LCL, CLL
IGHG	QPREPQVYTL	B7	$IgG^{QPR/B7}$	yes	EBV-LCL, CLL
IGHA	FAVTSILRV	A2	$IgA^{FAV/A2}$	yes	ALL, MM
IGHA	HPRLSLHRPAL	B7	$IgA^{HPR/B7}$	yes	MM
IGHA	SPKVFPLSL	B7	$IgA^{SPK/B7}$	yes	EBV-LCL
IGHA	KPTHVNVSV	B7	$IgA^{KPT/B7}$	yes	EBV-LCL

[a] HLA allele of origin of identified peptides. HLA alleles of origin were determined based on HLA-A*02:01 (HLA-A2) or HLA-B*07:02 (HLA-B7) expression of materials for which peptides were identified in the peptidome combined with predicted binding to HLA-A2 or HLA-B7 according to netMHC 4.0. [b] Peptide binding to the respective HLA allele was investigated by peptide-HLA monomer refolding. Yes: peptide-HLA monomers were successfully refolded and remained stable. No: peptide-HLA monomers could not stably be refolded. [c] cell type of origin from which peptides were eluted. Abbreviations: ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell lines.

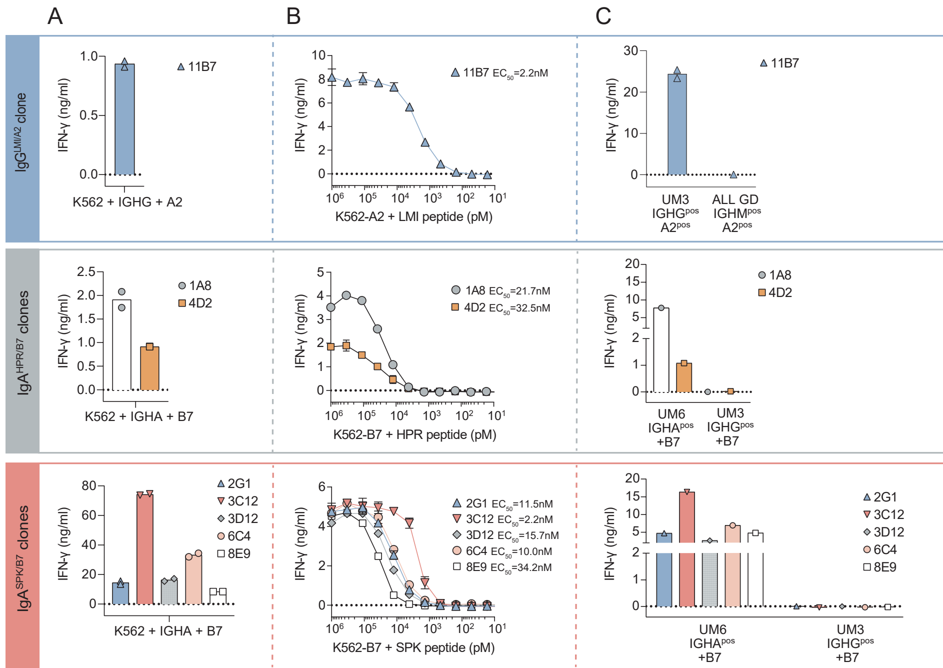


Figure 1. Selection of immunoglobulin-specific T-cell clones that recognize multiple myeloma cell lines. T-cell clones were co-cultured overnight with target cells and IFN-γ production was measured by ELISA. Data is representative of two independent experiments. **A)** T-cell clones that recognize LMI peptide from IgG in HLA-A2 (upper panel), HPR peptide from IgA in HLA-B7 (middle panel) or SPK peptide from IgA in HLA B7 (bottom panel) were stimulated with K562 cells transduced with *IGHG* or *IGHA* genes encoding for IgG and IgA, respectively. K562 cells were additionally transduced with HLA-A2 (+A2) or HLA-B7 (+B7). An effector:target ratio of 1:6 was used. Technical duplicates are shown. **B)** T-cell clones were co-cultured with target HLA transduced K562 cells loaded with decreasing concentrations of immunoglobulin peptides, starting at 1uM. Data from A) and B) were obtained in the same experiment. Values represent means of technical duplicates. **C)** The IgG$^{LMI/A2}$ specific clone was stimulated with IgG expressing, HLA-A2 positive UM3 cells and IgM expressing HLA-A2 positive acute lymphoblastic leukemia cell line (ALL GD) as a negative control (right panel). IgA$^{HPR/B7}$ and IgA$^{SPK/B7}$ specific T cell clones were co-cultured with IgA positive UM6 multiple myeloma cells or IgG positive UM3 multiple myeloma cells as a negative control. Stimulator cells were transduced with HLA-B7 (+B7).

Safety profiling of candidate immunoglobulin-targeting T-cell clones

To gain insight into the target specificity of the selected T-cell clones, we tested cross-reactivity with other peptides within the same restriction element, as well as reactivity towards peptides in other HLA alleles. Cross-reactivity with peptides presented by the HLA restriction alleles was assessed by stimulating with HLA-restriction-allele positive cells from various tissue origins that were negative for immunoglobulin expression. None of the tested clones reacted towards the cell line panel, while gene transduced K562 cells (pos ctrl) induced recognition (**Figure 2A**). General T-cell stimulatory capacity

of the target cells used was verified using allo-HLA-A2 and allo-HLA-B7 T-cell clones (**Figure S2**). To test cross-reactivity with other HLA restriction alleles, immunoglobulin-targeting T-cell clones were co-cultured with a panel of Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) expressing common HLA class-I alleles but not the target HLA restriction allele (**Table S1**). T-cell clones 11B7 ($IgG^{LMI/A2}$) and 1A8 ($IgA^{HPR/B7}$) did not recognize any EBV-LCL (**Figure 2B**), indicating no cross-reactivity towards any of the expressed HLA class-I alleles. T-cell clone 3C12 ($IgA^{SPK/B7}$) recognized one EBV-LCL in the panel, namely EBV-LCL MMG expressing HLA-A*01:01, -A*32:01, -B*35:08 and -C*04:01 (**Table S1**). All alleles except HLA-B*35:08 were also expressed by at least one other EBV-LCLs in the panel. Since the other EBV-LCLs were not recognized, reactivity of clone 3C12 is most likely directed to HLA-B*35:08. While recognition could be directed to an unrelated peptide in HLA-B*35:08, EBV-LCLs can express *IGHA,* and recognition might be directed to the SPK peptide presented in HLA-B*35:08, which is highly similar to the target allele HLA-B*07:02. Future research needs to be performed to identify the peptide-HLA complex inducing recognition. However, the HLA-allele frequency in the world population of HLA-B*35:08 is 0.7%, therefore we do not consider this potential cross-reactivity detrimental to further investigation of this clone for TCR gene therapy.

Immunoglobulin-TCR transfer redirects CD8 T cells to antigen positive targets

Having established general target specificity and safety profiles of immunoglobulin-targeting T-cell clones, we continued to study their potential for TCR gene transfer. The TCRs of the identified T-cell clones were sequenced and cloned intro retroviral expression vectors. Third-party CD8 and CD4 T cells were retrovirally transduced and enriched for transgenic-TCR expression. CD8 T cells transduced with immunoglobulin-TCRs 11B7, 1A8 or 3C12 showed specific binding to respective pHLA-tetramers, demonstrating surface expression and retainment of specificity of virally expressed immunoglobulin-TCRs (**Figure 3A**). Although mean fluorescence intensities (MFI) of bound pHLA-tetramer to TCR-CD8 T cells was lower than for parental T-cell clones, the peptide sensitivity was comparable to that of parental T-cell clones (**Figure 3B**). In TCR-CD4 T cells, high pHLA-tetramer binding was observed for TCRs 1A8 and 3C12, but in peptide titrations experiments antigen specific activation of these CD4 T cells was not observed (**Figure 3B**), suggesting that CD8 is required for functional sensitivity. Successful introduction of immunoglobulin-TCRs into CD8 T cells was reproducible for multiple donors (**Figure 3C**), and immunoglobulin-TCR T cells were screened for recognition of *IGHG* or *IGHA* expressing MM cell lines and an *IGHG/IGHA* double negative acute lymphoblastic leukemia (ALL) control cell line. IgG-TCR T cells specifically recognized $IGHG^{positive}$ UM3 cells, and IgA-TCR T cells specifically recognized $IGHA^{positive}$ UM6 cells (**Figure 3D**). To further analyze the functionality of immunoglobulin-TCR T cells, lysis of MM cell lines was analyzed after short term co-culture experiments. Immunoglobulin-TCR CD8 T cells specifically lysed MM cells in an antigen dependent manner (**Figure 3E**). Together,

these data demonstrate that immunoglobulin-TCRs T cells were highly functional while specificity was maintained.

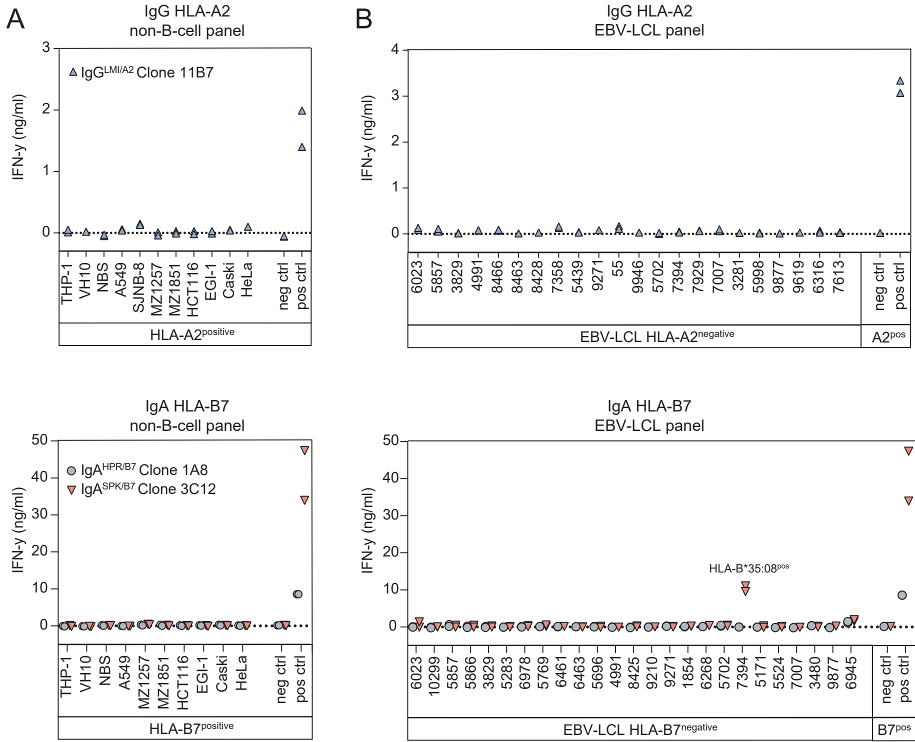


Figure 2. Safety screenings of IgG$^{LMI/A2}$-, IgA$^{HPR/B7}$-, and IgA$^{SPK/B7}$-targeting T-cell clones. T-cell clones were co-cultured overnight with target cells in an E:T ratio 1:6 and IFN-y production was measured by ELISA. Technical duplicates are shown. **A)** T-cell clones were stimulated with cell lines of various non-B-cell origins, namely AML (THP-1), fibroblast (VH10 and NBS), lung carcinoma (A549), neuroblastoma (SJNB-8), renal cell carcinoma (MZ1257 and MZ1851), colon carcinoma (HCT116), bile duct carcinoma (EGI-1) and cervix carcinoma (Caski and HeLa). IgG$^{LMI/A2}$ reactive clone was co-cultured with HLA-A2 expressing stimulator cells (upper panel), IgA$^{HPR/B7}$ and IgA$^{SPK/B7}$ reactive clones were co-cultured with HLA-B7 expressing stimulator cells (lower panel). Cells either naturally expressed HLA-A2 or-B7 or were transduced with HLA restriction alleles. Target HLA transduced K562 cells were included as a negative control (neg ctrl) and HLA transduced K562 cells additionally transduced with *IGHA* or *IGHG* were included as positive control (pos ctrl). **B)** T-cell clones from A) were co-culture with an EBV-LCL panel. EBV-LCL panels were designed to not express the target HLA restriction alleles. Controls as in A).

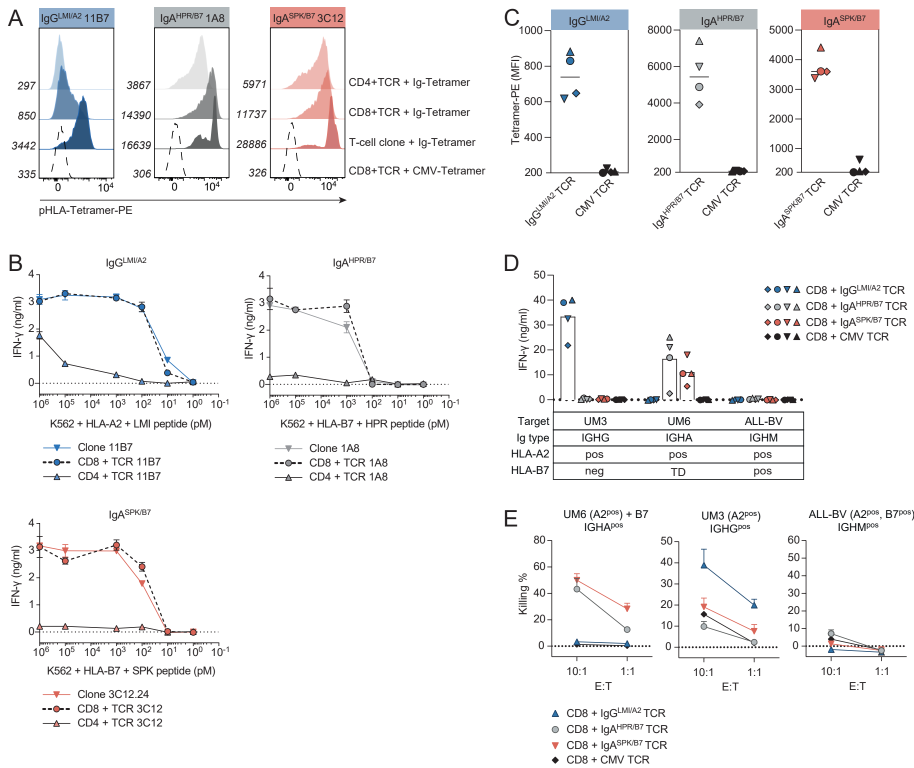


Figure 3. Functionality of IgG- and IgA-specific TCR transduced T cells. A) MACS isolated CD4 and CD8 T-cell populations were separately transduced with TCR 11B7 specific for IgG$^{LMI/A2}$, TCR 1A8 specific for IgA$^{HPR/B7}$ or TCR 3C12 specific for IgA$^{SPK/B7}$. TCR T cells were stained with the respective immunoglobulin pHLA-tetramers. Parental T-cell clones were included as positive controls. Immunoglobulin-TCR CD8 T cells were stained with CMV pp65-HLA-A2 tetramer as a negative control. Cells were analyzed by flowcytometry and TCR T cells were gated on mTCR+. **B)** Parental T-cell clones and TCR-transduced CD8 and CD4 T cells from one donor were co-cultured overnight with target-HLA transduced K562 cells loaded with decreasing concentrations of IgG or IgA peptides, starting at 1uM. IFN-y production was measured by ELISA. Values represent means of technical duplicates. **C)** CD8 T cells from 4 donors transduced with and enriched for immunoglobulin TCRs or CMV pp65-HLA-A2 TCR as a negative control. Cells were stained with immunoglobulin pHLA-tetramers as indicated in graph titles and analyzed by flowcytometry. Mean fluorescent intensities are plotted. Each symbol represents CD8 T cells isolated from a different donor. **D)** TCR transduced CD8 T cells from C) were co-cultured overnight with IgG positive HLA-A2 positive UM3 multiple myeloma cells, IgA positive, HLA-A2 positive UM6 multiple myeloma cells additionally transduced with HLA-B7 (TD) or and IgM expressing HLA-A2 and HLA-B7 positive acute lymphoblastic leukemia cell line (ALL BV) as a negative control. IFN-y production was measured by ELISA. **E)** Target cell killing by immunoglobulin-TCR CD8 T cells was analyzed in a 6-hour chromium release assay using the same targets as in D). CMV TCR CD8 T cells were included as a negative control. Percentage target cell lysis is displayed, values represent means and standard deviations of technical triplicates.

Immunoglobulin-TCR T cells recognize isotype specific B cells and immunoglobulin cross-presenting APCs

As immunoglobulin-TCR T cells were highly reactive towards MM cells, we set out to gain further insight into potential on-target off-tumor reactivity. TCR T cells were co-cultured with healthy tissue subsets of both non-hematopoietic and hematopoietic origin, including peripheral blood B cells. Immunoglobulin-TCR T cells did not recognize T cells, immature dendritic cells, mature dendritic cells, fibroblasts or keratinocytes, regardless of target HLA restriction alleles being expressed (**Figure 4A**). Positive control T cells recognized all included subsets when target HLA was expressed (**Figure S3**). Peripheral blood-derived B cells were recognized by immunoglobulin-TCR T cells, but only when target HLA alleles were expressed. Recognition of mixed B cells was relatively limited compared to positive control cells, especially by IgA-TCR T cells. Limited recognition could be caused by lower frequency of $IgA^{positive}$ B cells in peripheral blood. To investigate if immunoglobulin-TCR T-cell reactivity was specifically directed to IgG or IgA positive B cells, we co-cultured total B cells with immunoglobulin-TCR T cells and analyzed the subtype-specific survival of IgM, IgA or IgG positive B cells by flowcytometry. IgG-TCR T cells specifically depleted IgG B cells, while IgA B cells survived (**Figure 4B**). Vice versa, when cultured with IgA-TCR 1A8 and 3C12 T cells, IgA B cells were completely eradicated whereas IgG B cells survived (**Figure 4B**). We quantified the survival of B cells isolated from 3 additional donors by flowcytometry, confirming preferential lysis of isotype positive B cells, although we observed some degree of bystander killing (**Figure 4C**). IgA or IgG B cells that did not express target HLA restriction alleles were not lysed. Together, these data demonstrate that immunoglobulin-TCR T cells can be expected to lyse healthy B cells expressing the respective immunoglobulins, while antigen negative subsets are unlikely to be targeted.

Since MM is associated with high serum immunoglobulin concentrations, we considered the possibility that this could lead to immunoglobulin uptake and cross-presentation of immunoglobulin peptides by antigen presenting cells (APCs). APCs from $HLA\text{-}A2^{positive}$ donors were cultured with Nanogam (normal serum immunoglobulins) at concentrations between 3.3 mg/ml and 33 mg/ml. A concentration of 33mg/ml corresponds with serum M-protein levels observed in MM patients(20). Nanogam loading of DCs induced recognition by IgG-targeting T cells in a concentration dependent manner (**Figure 4D**). While recognition of cross-presenting APCs might be beneficial for *in vivo* T-cell activation, cross-presentation by other antigen negative cells could potentially be harmful. To explore if non-APC cells would cross-present immunoglobulin peptides under inflammatory conditions, cell lines of various origins were pre-treated with IFN-γ and loaded with 33 mg/ml Nanogam. Six out of seven target cells did not induce any recognition, whereas one stimulator cell was weakly recognized by IgG-targeting T cells (**Figure 4E**).

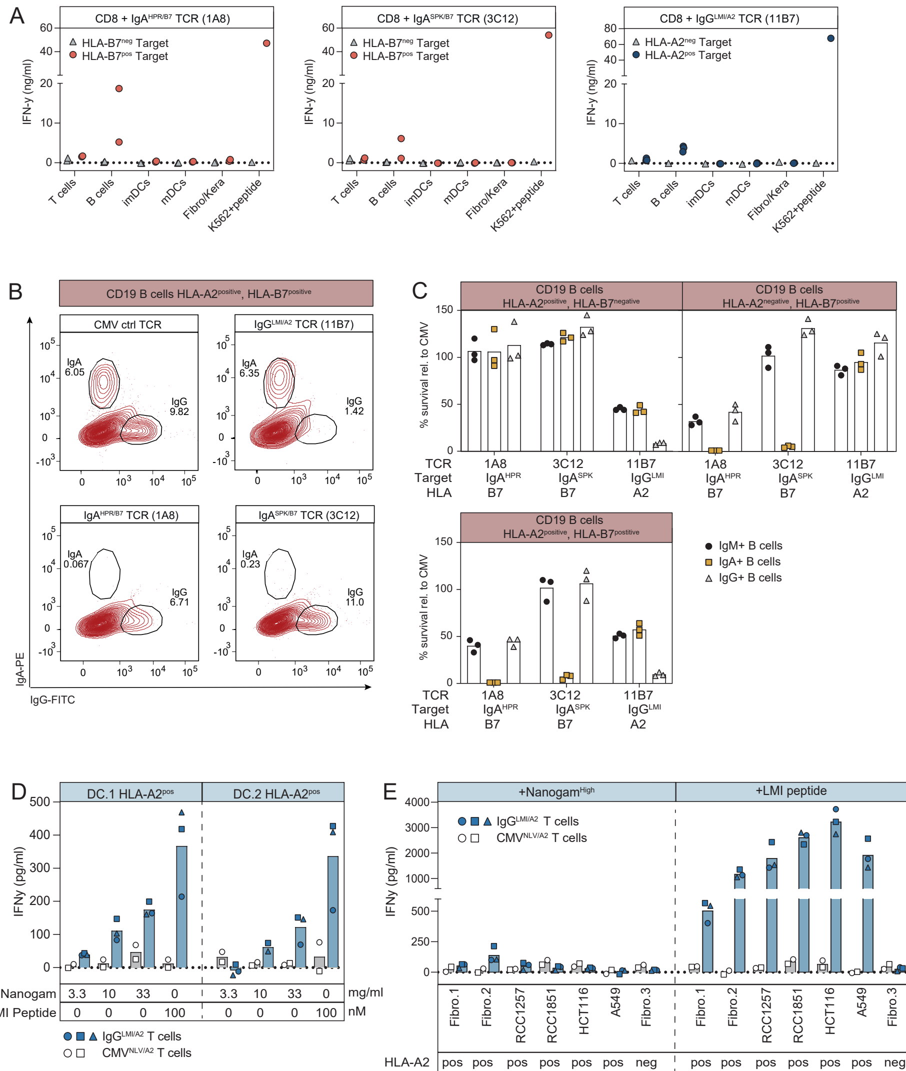


Figure 4. Recognition of healthy cell subsets by immunoglobulin-TCR T cells. A) IFN-γ production after overnight co-culture of immunoglobulin-TCR Td CD8 T cells with CD40L activated B cells, immature dendritic cells, mature dendritic cells, PHA-activated T cells and keratinocytes and fibroblasts pre-treated for 48h with 100 IU/ml IFN-γ. Each symbol represents the average value (from technical duplicate) of target cells isolated from a different donor. Target cells not expressing the relevant HLA restriction allele are depicted in grey, cells expressing the HLA restriction alleles are depicted in color. Per panel T cells with one of the immunoglobulin-TCRs are shown as indicated in the graph titles. K562 + HLA and peptide loaded K562 + HLA are included as negative and positive controls. **B)** Example of a FACS based killing experiment of CD40L activated peripheral blood B cells from and HLA-A2 and HLA-B7 positive healthy donor co-cultured overnight with CMV pp65-HLA-A2 TCR, $IgG^{LMI/A2}$ TCR (11B7), $IgA^{HPR/B7}$ TCR (1A8) or TCR $IgA^{SPK/B7}$ (3C12) CD8 T cells. Experiment was performed using an E:T ratio of 3:1. **C)** Killing assay as in B) of B cells isolated from 3 healthy donors with different HLA-A2 and HLA-B7 typing as indicated in graph titles. Survival of IgA and IgG B cells was analyzed after gating on live cells, single cells, CD3 negative and CD19 positive cells. Survival of IgM, IgA, IgG positive B cells was calculated *[Legend continues on the next page]*

4

relative to negative control CMV TCR T cells. Technical triplicates are shown. **D**) IFN-y production by Immunoglobulin-TCR (in blue) T cells from 2 donors (round and squared symbols) and the parental T-cell clone (triangular symbol) or CMV-TCR (in white) T cells from the same donors. T cells were co-cultured with monocyte-derived matured dendritic cells (DCs) from 2 HLA-A2^{positive} donors exposed to increasing concentrations of normal soluble immunoglobulins (Nanogam) or loaded with 100nM IgG LMI peptide. **E)** T cells from D) Co-cultured with fibroblasts and various adherent tumor cell lines with IFN-y exposed to the highest Nanogam concentration (33ng/ml) or IgG LMI peptide (100nM).

Immunoglobulin-TCR T cells lysed IgG or IgA positive MM cells from patients *ex vivo*

To study the implications of immunoglobulin-TCR T-cell therapy for MM, patient-derived bone marrow (BM) samples were assessed for immunoglobulin-TCR-mediated-targeting. We selected 3 BM samples from HLA-A2 and/or HLA-B7 positive patients containing MM cells expressing either *IGHG* or *IGHA* (**Figure S4**). All samples were co-cultured with immunoglobulin-TCR or control-TCR T cells and survival of MM cells was quantified using flow cytometry (**Figure 5A, 5B**). *IGHA*^{positive} MM cells from both samples MM1 and MM2 were specifically lysed by IgA-TCR T cells but not by control or IgG-TCR T cells. Conversely, *IGHG*^{positive} MM cells from MM3 were specifically lysed by IgG-TCR T cells but spared by control or IgA-TCR T cells. Together these data demonstrated Ig-subtype-specific and HLA-restricted lysis of MM cells, indicating potent *in vitro* recognition of patient MM cells.

In vivo targeting of IgA positive MM tumors by IgA-TCR T cells

Finally, we analyzed the *in vivo* tumor-targeting capacity of immunoglobulin-TCR T cells in a murine xenograft model for established MM using the MM cell line U266. We chose for the MM cell line U266, as U266 cells endogenously express *IGHA* and, readily engraft into NSG mice(21). Despite lower expression of *IGHA* compared to UM6, U266 are potently recognized by IgA TCR T cells *in vitro* (**Figure S5**). Mice were engrafted with U266 cells and treated with IgA-TCR T cells or control T cells 14 days after tumor inoculation. After T-cell injection, anti-tumor efficacy of IgA-TCR T cells was observed within a few days (**Figure 6**). 1A8-TCR T cells inhibited tumor outgrowth compared to control treated mice, which resulted in a 12-fold lower tumor burdens on day 15 after T-cell injection (**Figure 6A, Figure S6A**). 3C12-TCR T cells drastically reduced tumor burdens within 7 days after T-cell injection, resulting in near complete tumor eradication and 160-fold lower tumor burdens for 3C12-TCR treated mice compared to control mice (**Figure 6B,C, Figure S6B**). Overall, these data demonstrated the *in vivo* anti-tumor efficacy of IgA-TCR T cells, while superior results were obtained when using 3C12 TCR T cells compared to 1A8 TCR T cells.

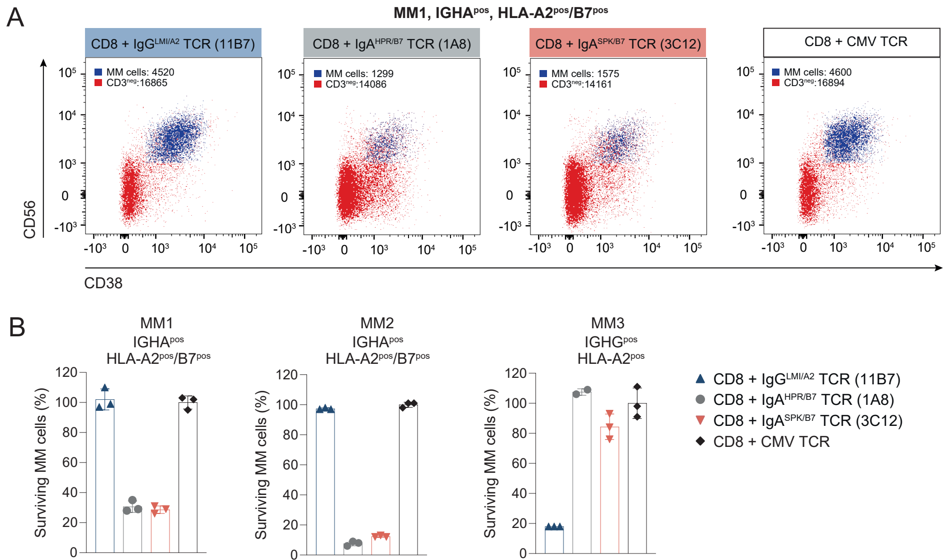


Figure 5. Killing of MM cells in patient bone marrow samples. Killing of MM cells in patient bone marrow samples was assessed in a FACS-based cytotoxicity experiment where immunoglobulin-TCR Td T cells were co-cultured with MM patient BM samples in an E:T ratio 3:1. **A)** Example of survival of an HLA-A2^{pos}/B7^{pos} patient sample after co-culture with CD8 T cells transduced with IgG$^{LMI/A2}$ TCR (11B7), TCR IgA$^{HPR/B7}$ (1A8), TCR IgA$^{SPK/B7}$ (3C12) or CMV TCR (negative control). In blue MM cells are displayed, MM cells were gated on: live cells → single cells → CD3 negative cells to exclude co-cultured T cells → CD45 negative, CD19 negative → CD56 positive, CD38 positive. MM cells (in blue) were backgated on total CD3 negative cells (in red). **B)** Percentage survival of MM cells from 3 donors after co-culture with immunoglobulin TCR T cells calculated relative to CMV TCR T cells. HLA-A2 or B7 expression of patients is indicated in graph titles. Technical triplicates are shown. Data from the same experiment as shown in A).

4

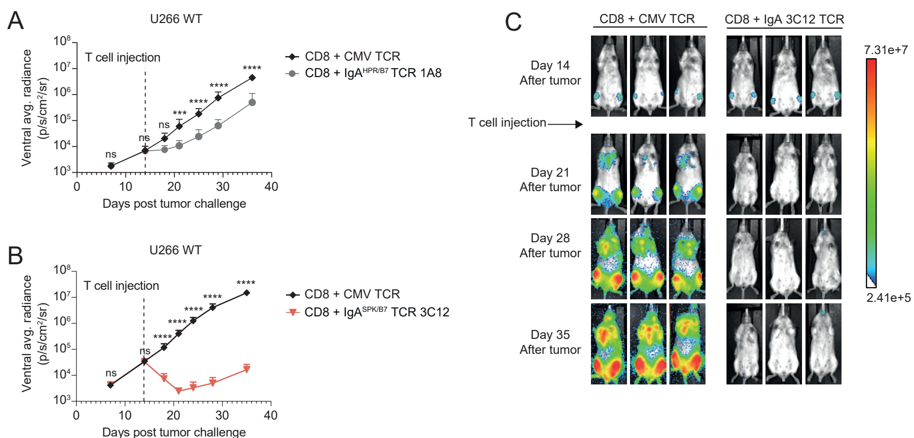


Figure 6. *In vivo* efficacy of IgA- TCR CD8 T cells. NSG mice engrafted with 2x10^{6} HLA-B7 positive U266 multiple myeloma cells transduced with *Luc2* luciferase were i.v. injected with 5x10^{6} TCR-transduced CD8 T cells after 14 days. CD8 T cells were transduced with IgA$^{HPR/B7}$ TCR 1A8, IgA$^{SPK/B7}$ TCR 3C12 or *[Legend continues on the next page]*

control CMV (pp65-NLV-HLA-A2) TCR and enriched for mTCR expression by MACS. T cells were infused 7 days after re-stimulation. Tumor outgrowth was frequently tracked by bioluminescence imaging. **A-B)** Mean and standard deviations of tumor outgrowth (average radiance) over time on the ventral side. **A)** Tumor outgrowth in CMV TCR (n=4) versus IgA$^{HPR/B7}$ TCR 1A8 (n=6) treated mice. **B)** Tumor outgrowth in CMV TCR (n=7) versus IgA$^{SPK/B7}$ TCR 3C12 (n=8) treated mice. **C)** Raw bioluminescence images of three representative CMV TCR and IgA$^{SPK/B7}$ 3C12 TCR treated mice from figure B) at different time points. Raw bioluminescence images of all mice are shown in suppl. Figure 6. Statistics depict two-way ANOVA comparing groups per timepoint with Sidak's multiple comparisons post hoc test.

DISCUSSION

Cellular therapy using redirected T cells is a promising approach for the treatment of MM. Here, we set out to broaden the landscape of therapeutically targetable antigens for MM. We hypothesized that epitopes derived from immunoglobulin constant domains pose attractive targets for TCR-mediated-targeting. Peptides for TCR-mediated-targeting of IgG and IgA immunoglobulins were identified from the HLA class-I ligandome of B-/plasma cell malignancies. pHLA-multimers were used to isolated T-cell clones from HLA-mismatched healthy donors. T-cell clones specific for the LMI peptide from the IgG constant domain presented in HLA-A2 or the SPK or HPR peptides from the IgA constant domain presented in HLA-B7 were identified. TCRs of T-cell clones that demonstrated the most potent recognition of isotype expressing MM cell lines were sequenced and transferred to third-party T cells. Immunoglobulin-TCR T cells maintained specificity and recognized as well as lysed MM cell lines. Immunoglobulin-TCR T cells did not recognize healthy cell subsets except for B cells. Recognition of B cells was mainly directed to isotype positive B cells. In the absence of immunoglobulins DCs were not recognized, but DCs co-cultured with high concentrations of immunoglobulins were recognized by IgG-TCR T cells. IgA-TCR-engineered T cells demonstrated potent anti-myeloma activity against patient MM cells *ex vivo* as well as in an *in vivo* xenograft model of IgA^{positive} MM. CAR T cells and TCR T cells have different recognition mechanisms. As CAR T cells recognize complete proteins, target proteins secreted by or cleaved from malignant cells can bind to CAR T cells and interfere with CAR T-cell functionality. This is especially of concern in BCMA-targeting CAR T-cell therapy, as MM patients often exhibit high levels of circulating soluble BCMA that render BCMA CAR T cells less sensitive(22). For TCR T cells, soluble antigen does not impair T-cell functionality, as activation of TCR T cells requires binding to protein-derived peptides presented in HLA rather than to full proteins. Instead, circulating antigen could have an indirect effect through uptake and cross-presentation by bystander cells. We demonstrated that professional APCs were indeed able to take-up soluble immunoglobulins and present immunoglobulin-derived peptides in HLA, leading to TCR T-cell recognition. The highest tested concentration corresponded to immunoglobulin concentrations found in serum of MM patients(20), and recognition was proportional to immunoglobulin concentrations. On

non-APCs, cross-presentation of immunoglobulins was absent or highly inefficient. While it is difficult to predict the precise consequences this phenomenon might elicit clinically, one could speculate that cross-presentation by APCs will lead to activation of immunoglobulin-TCR T cells *in vivo*. This may be beneficial for initial expansion and anti-myeloma activity of immunoglobulin-TCR T cells, although a potential aggravation of side effects such as cytokine release syndrome would then need to be considered. At the same time, recognition of cross-presenting APCs could lead to their depletion, which would be an undesirable effect on a long-term. However, a potential depletion of APCs should be of temporary nature, since cross-presentation of immunoglobulins will decrease when immunoglobulin-producing malignant and non-malignant cells are depleted as a result of therapy. Given the potential safety concerns, it might be desirable to limit the extent of immunoglobulin cross-presentation prior to clinical administration of immunoglobulin-TCR T cells. In this case, reducing free serum immunoglobulins using serum apheresis could be explored(23-25).

Immunoglobulin-TCR T cells could have a benefit over treatment with CD19 or BCMA CAR T cells, which deplete the entire healthy B-cell compartment compromising B-cell immunity resulting in the need for antibody substitution therapy. We demonstrate that IgA- or IgG-TCR T cells deplete peripheral blood B cells expressing the respective isotype, while B cells of the other isotypes remained. This observation likely extrapolates to plasma cells meaning that plasma cells of isotypes not targeted by the TCR T cells will survive therapy. When applying IgA-TCR T cells for therapy of MM or other IgA^{positive} malignancies, leaving IgG B and plasma cells untouched would have a major advantage because IgG is the main antibody isotype providing systemic protection against pathogens. Leaving this part of B-cell immunity intact is expected to eliminate the need for immunoglobulin substitution treatment after T-cell therapy. Overall, these patients would have better health and need less therapeutic intervention, improving their quality of life.

Finally, application immunoglobulin-TCR T-cell therapy could be extended for the treatment of other malignant and non-malignant diseases beyond MM. Immunoglobulin-TCR T-cell therapy could be applied for treatment of refractory B-cell mediated autoimmune disease. Most autoimmune diseases are associated with autoreactive B cells of an IgG immunoglobulin subtype. Recently, CAR T cells have been successfully applied for the treatment of refractory B-cell mediated autoimmune diseases including systemic lupus erythematosus and antisynthetase syndrome (26, 27). Compared to CD19 directed CAR T cells that induce general B-cell aplasia, using IgG-specific T cells would provide the unique advantage to selectively target B cells of that subtype, sparing B cells of other subtypes such as IgA and IgM.

Taken together, we have established the immunoglobulin heavy chain constant domains as targetable antigens for TCR-based treatment of MM. Immunoglobulin-TCRs induce efficient and isotype specific lysis of target cells *in vitro* and *in vivo* without inducing off-target recognition, highlighting potential for further pre-clinical and ultimately clinical development.

MATERIALS AND METHODS

IgG- and IgA-constant-domain-derived peptide identification using HLA class-I ligandome data

To identify target peptides for TCR-based-targeting of *IGHG* or *IGHA* expressing cells, previously established HLA class-I peptidome data of patient-derived B-cell malignancy samples, EBV-LCL cell lines and the MM cell line U266 was used(16-18). To generate this HLA-I ligandome data, >10*10^9 cells were lysed and peptide HLA class-I complexes were isolated by immunoaffinity using an anti HLA class-I (Clone W6/32) antibody. Peptides were eluted from the HLA molecules and identified by mass spectrometry. Ligandomes were investigated for peptides originating from IgG (IGHG) or IgA (IGHA) protein sequences according the Uniprot *Homo sapiens* database. Peptides were assigned to most likely HLA of origin, by overlapping HLA alleles expressed by materials with predicted HLA binding according to netMHC4.0. Peptides from commonly expressed HLA alleles: HLA-A*02:01 or HLA-B*07:02 were selected. Synthetic peptides were generated in house using standard Fmoc chemistry. MS/MS spectra of synthetic peptides were acquired and compared to spectra of eluted peptides to validate correct sequence identification. Peptide-HLA monomers were generated for the selected peptide-HLA combinations and monomers were subsequently used to produce PE-labelled peptide-HLA (pHLA-) tetramers as described (28).

pHLA-tetramer T-cell isolations

PBMCs were isolated from complete buffy coats obtained from HLA-typed healthy donors (Sanquin). Per experiment donors were selected to be negative for the HLA alleles of interest. pHLA-tetramers were used to isolate immunoglobulin-specific T-cell clones as previously described with minor modification (17). In short, PBMCs were incubated with pooled PE-labelled pHLA-tetramers (2 ug/ml) for 15 minutes at 37 degrees after which PE-bound cells were magnetic activated cell sorting (MACS-) enriched using anti-PE microbeads (Miltenyi Biotec). Next, enriched fractions were single-cell sorted for CD8^{positive} pHLA-tetramer^{positive} cells in 96-well round bottom plates containing feeder mix. For single-cell T-cell sorts, feeder mix contained 0.5*10^6 35Gy irradiated PBMCs and 0.5*10^5 50Gy irradiated EBV-LCL JY cells per ml in T-cell medium (TCM) with 0.8 mg/ml phytohemagglutinin (PHA; Oxoid Microbiology Products, Thermo Fisher Scientific).

After expansion T-cell clones were screened for target peptide recognition as previously described and peptide-specific T-cell clones were restimulated(17).

T-cell culture

T cells were cultured in TCM consisting of IMDM (Lonza), 1% Penicillin/Streptomycin (Pen/Strep; Lonza), 1.5% glutamine (Lonza), 100 IU/ml IL-2 (Proleukin; Novartis Pharma), 5% fetal bovine serum (FBS; Gibco, Life Technologies) and 5% human serum. T-cell clones were functionally tested between day 10-15 after (re-)stimulation. T-cell clones were restimulated every 10-15 days or cryopreserved until further use. For restimulation 0.3*10^6 T cells per 24 well were stimulated with 1 ml feeder mix containing 1*10^6 35Gy irradiated PBMCs, 1*10^5 50Gy irradiated EBV-LCL JY cells and 0.8 mg/ml PHA.

Target cell generation and culture conditions

Standard culture medium for cell lines consisted of IMDM (Lonza), 1% Pen/Strep (Lonza), 1.5% Glutamine (Lonza) and 10% FBS (Gibco, Life Technologies). For MM cell lines UM3 and UM6, standard culture medium was supplemented with 10ng/ml IL-6. ALL cell lines were cultured as previously described(29). Based on HLA typing, and target gene expression target cells were retrovirally transduced as previously described where indicated(30). Target genes were expressed in MP71 retroviral backbone vectors containing CD34, mouse CD19 or truncated nerve growth factor receptor (NGF-R) as a marker gene. Transduced cells were enriched for marker gene expression by MACS or fluorescence-activated cell sorting (FACS). When indicated adherent cells were pre-treated with 100 IU/ml IFN-γ for 24-48h to mimic inflammatory conditions and increase HLA expression prior to recognition assays. For safety screening various healthy subsets were isolated from PBMCs and cultured as previously described(21). For cross-presentation experiments adherent target cells were pre-treated with IFN-γ after which cells were cultured overnight with various concentrations of normal human immunoglobulins (Nanogam 100mg/ml, Sanquin) prior to experiments. Monocyte-derived mature DCs were used as professional APCs. To allow optimal cross-presentation Nanogam was added 16h before maturation cytokines were added. Prior to recognition assays, target cells were washed to remove IFN-γ and Nanogam. For analysis of patient-derived MM cell lysis, patient BM samples were thawed and rested overnight in medium containing 10% human serum before use in cytotoxicity experiments.

T-cell recognition experiments

T-cell recognition assays were performed in 384-well flat bottom plates. 5,000 T cells were co-cultured overnight with 30,000 stimulator cells. For adherent cells 10,000 stimulator cells were used instead. After overnight culture, IFN-γ concentrations in the supernatant was measured by ELISA (R&D systems). For peptide titration experiments target cells were loaded with serial dilutions of peptide starting at 1uM. When peptide

loaded targets were included as positive control a concentration of 100nM was used.

TCR sequencing and transfer to CD4 and CD8 T cells and pHLA-tetramer staining

TCRα and TCRβ sequences of selected T-cell clones were identified as previously described(17). Codon optimized TCRα and TCRβ variable chains were inserted in MP71-TCR-flex retroviral vectors containing codon optimized and cysteine modified murine TCR (mTCR) constant domain sequences. For TCR transduction CD8 or CD4 T cells were isolated from healthy donor PBMCs using anti-CD8 or-CD4 microbeads (Miltenyi Biotec) and MACS. T cells were activated with irradiated autologous PBMCs at 1.0*10^6 cell per ml TCM and PHA. For 0.3*10^6 T cells 1 ml feeder mix was used. T cells were retrovirally transduced on day 2 after activation. mTCR transgenic T cells were enriched using an mTCR-APC antibody (BD Pharmingen) and anti-APC MACS on day 7 after activation and included in functional experiments on day 10-14. To confirm specific TCR expression TCR-T cells were stained with mTCR-APC and pHLA-tetramers (2 ug/ml) and analyzed by flowcytometry (LSRII, BD Bioscience).

Cytotoxicity experiments

TCR-mediated lysis of various cell lines, patient BM samples or PBMC-derived CD40L activated B cells was analyzed using 6hr ^{51}Cr-release experiments or flowcytometry-based killing assays. Chromium release experiments were performed as described using E:T ratios of 10:1 and 1:1(17). Flowcytometry-based killing experiments were performed by overnight co-culture of 50,000 target cells in an E:T ratio of 3:1. B-cell co-cultures were stained using anti-CD3 (Alexa Fluor 700, BD pharmingen), anti-CD19 (PE-Cy7, BD pharmingen), anti-IgA (PE, Miltenyi Biotec), anti-IgG (FITC, DAKO) and anti-IgM (APC, BD). MM patient BM were co-cultured with TCR T cells and stained using anti-CD3, anti-CD19 (APC, BD pharmingen), anti-CD45 (FITC, BD), anti-CD38 (PE-Texas Red, Invitrogen) and anti-CD56 (PE-Cy7, BD pharmingen). MM cells were defined as CD3^{neg}, CD19^{neg}, CD45$^{int/neg}$, CD38^{pos}, CD56^{pos}. Sample were acquired by flowcytomtry (Fortessa, BD Bioscience) using fixed time and flow rates. Target cell survival was calculated relative to negative control TCR T cells.

qPCR for immunoglobulin expression

IGHG and *IGHA* expression was measured by quantitative real-time polymerase chain reaction (qPCR) as previously described(17). *IGHG* forward primer: 5'AACTCACACATGCCCACCG 3'and reverse primer: 5' GATCATGAGGGTGTCCTTGGG 3'. *IGHA* forward primer: 5' CCTTCACCTGGACGCCC 3' and reverse primer: 5' GGCAGGACACTGGACACG 3'. *GUSB*, *VPS29* and *PSMB4* were included as housekeeping genes (HKGs). Gene expression was calculated relative to HKGs.

In vivo MM targeting by IgA-TCR T cells

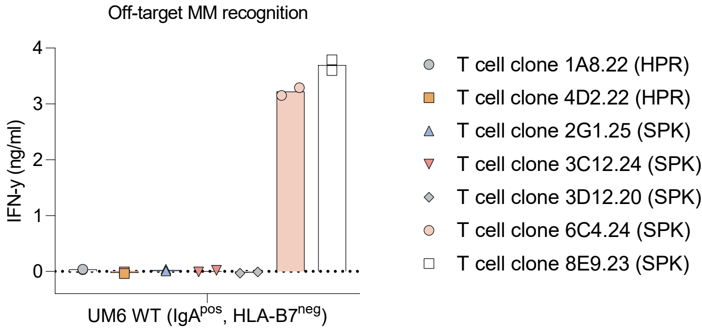
U266 cells were transduced with and enriched for Luciferase-tdTomato Red. NOD-scid-IL2Rgamma^{null} (NSG) mice (The Jackson Laboratory) were intravenously (i.v.) injected with 2*10^6 U266 cells. After 14 days, mice were i.v. injected with 5*10^6 purified IgA-TCR CD8 T cells or CMV-TCR T cells as a negative control. Tumor outgrowth was measured regularly after subcutaneous (s.c.) injection of 150 µL 7.5 mM D-luciferine (Cayman Chemical) using a CCD camera (IVIS Spectrum, PerkinElmer). Experiments were terminated when control mice reached an average luminescence of 1*10^7 p/s/cm^2/sr. The study was approved by the national Ethical Committee for Animal Research (AVD116002017891) and performed in accordance with Dutch laws for animal experiments.

REFERENCES

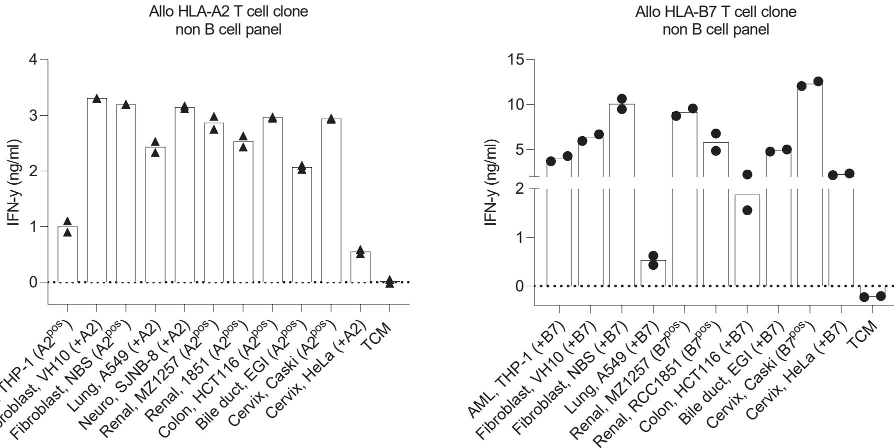
1. Cowan AJ, Green DJ, Kwok M, Lee S, Coffey DG, Holmberg LA, et al. Diagnosis and Management of Multiple Myeloma: A Review. JAMA. 2022;327(5):464-77.
2. Mateos MV, Nooka AK, Larson SM. Moving Toward a Cure for Myeloma. Am Soc Clin Oncol Educ Book. 2022;42(42):1-12.
3. Corre J, Munshi NC, Avet-Loiseau H. Risk factors in multiple myeloma: is it time for a revision? Blood. 2021;137(1):16-9.
4. Greil C, Engelhardt M, Finke J, Wäsch R. Allogeneic Stem Cell Transplantation in Multiple Myeloma. Cancers (Basel). 2021;14(1).
5. Martin T, Usmani SZ, Berdeja JG, Agha M, Cohen AD, Hari P, et al. Ciltacabtagene Autoleucel, an Anti-B-cell Maturation Antigen Chimeric Antigen Receptor T-Cell Therapy, for Relapsed/Refractory Multiple Myeloma: CARTITUDE-1 2-Year Follow-Up. J Clin Oncol. 2023;41(6):1265-74.
6. Munshi NC, Anderson LD, Jr., Shah N, Madduri D, Berdeja J, Lonial S, et al. Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. N Engl J Med. 2021;384(8):705-16.
7. Rodriguez-Otero P, Ailawadhi S, Arnulf B, Patel K, Cavo M, Nooka AK, et al. Ide-cel or Standard Regimens in Relapsed and Refractory Multiple Myeloma. The New England journal of medicine. 2023;388(11):1002-14.
8. Brudno JN, Maric I, Hartman SD, Rose JJ, Wang M, Lam N, et al. T Cells Genetically Modified to Express an Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor Cause Remissions of Poor-Prognosis Relapsed Multiple Myeloma. J Clin Oncol. 2018;36(22):2267-80.
9. Samur MK, Fulciniti M, Aktas Samur A, Bazarbachi AH, Tai YT, Prabhala R, et al. Biallelic loss of BCMA as a resistance mechanism to CAR T cell therapy in a patient with multiple myeloma. Nat Commun. 2021;12(1):868.
10. Da Via MC, Dietrich O, Truger M, Arampatzi P, Duell J, Heidemeier A, et al. Homozygous BCMA gene deletion in response to anti-BCMA CAR T cells in a patient with multiple myeloma. Nat Med. 2021;27(4):616-9.
11. Teoh PJ, Chng WJ. CAR T-cell therapy in multiple myeloma: more room for improvement. Blood Cancer J. 2021;11(4):84.
12. van der Schans JJ, van de Donk NWCJ, Mutis T. Dual Targeting to Overcome Current Challenges in Multiple Myeloma CAR T-Cell Treatment. 2020;10.
13. Rajkumar SV, Dimopoulos MA, Palumbo A, Blade J, Merlini G, Mateos MV, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. 2014;15(12):e538-48.
14. Nair B, Waheed S, Szymonifka J, Shaughnessy JD, Jr., Crowley J, Barlogie B. Immunoglobulin isotypes in multiple myeloma: laboratory correlates and prognostic implications in total therapy protocols. Br J Haematol. 2009;145(1):134-7.
15. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc. 2003;78(1):21-33.
16. Hassan C, Kester MG, de Ru AH, Hombrink P, Drijfhout JW, Nijveen H, et al. The human leukocyte antigen-presented ligandome of B lymphocytes. Mol Cell Proteomics. 2013;12(7):1829-43.
17. Meeuwsen MH, Wouters AK, Jahn L, Hagedoorn RS, Kester MGD, Remst DFG, et al. A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy. Mol Ther. 2022;30(2):564-78.
18. Meeuwsen MH, Wouters AK, Wachsmann TLA, Hagedoorn RS, Kester MGD, Remst DFG, et al. Broadly applicable TCR-based therapy for multiple myeloma targeting the immunoglobulin J chain. J Hematol Oncol. 2023;16(1):16.
19. Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD, et al. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin Cancer Res. 2011;17(17):5615-25.
20. Yadav P, Merz M, Mai EK, Försti A, Jauch A, Goldschmidt H, et al. Cytogenetic aberrations in multiple myeloma are associated with shifts in serum immunoglobulin isotypes distribution and levels. Haematologica. 2018;103(4):e162-e4.
21. Jahn L, Hombrink P, Hagedoorn RS, Kester MG, van der Steen DM, Rodriguez T, et al. TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1. Blood. 2017;129(10):1284-95.
22. Pont MJ, Hill T, Cole GO, Abbott JJ, Kelliher J, Salter AI, et al. γ-Secretase inhibition increases efficacy of BCMA-specific chimeric antigen receptor T cells in multiple myeloma. Blood.

2019;134(19):1585-97.
23. Kleeberg L, Morgera S, Jakob C, Hocher B, Schneider M, Peters H, et al. Novel renal replacement strategies for the elimination of serum free light chains in patients with kappa light chain nephropathy. Eur J Med Res. 2009;14(2):47-54.
24. Leung N, Gertz MA, Zeldenrust SR, Rajkumar SV, Dispenzieri A, Fervenza FC, et al. Improvement of cast nephropathy with plasma exchange depends on the diagnosis and on reduction of serum free light chains. Kidney Int. 2008;73(11):1282-8.
25. Burnette BL, Leung N, Rajkumar SV. Renal improvement in myeloma with bortezomib plus plasma exchange. N Engl J Med. 2011;364(24):2365-6.
26. Müller F, Boeltz S, Knitza J, Aigner M, Völkl S, Kharboutli S, et al. CD19-targeted CAR T cells in refractory antisynthetase syndrome. Lancet. 2023;401(10379):815-8.
27. Mackensen A, Müller F, Mougiakakos D, Böltz S, Wilhelm A, Aigner M, et al. Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus. Nature Medicine. 2022;28(10):2124-32.
28. Burrows SR, Kienzle N, Winterhalter A, Bharadwaj M, Altman JD, Brooks A. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. J Immunol. 2000;165(11):6229-34.
29. Nijmeijer BA, Szuhai K, Goselink HM, van Schie ML, van der Burg M, de Jong D, et al. Long-term culture of primary human lymphoblastic leukemia cells in the absence of serum or hematopoietic growth factors. Exp Hematol. 2009;37(3):376-85.
30. Jahn L, Hombrink P, Hassan C, Kester MG, van der Steen DM, Hagedoorn RS, et al. Therapeutic targeting of the BCR-associated protein CD79b in a TCR-based approach is hampered by aberrant expression of CD79b. Blood. 2015;125(6):949-58.

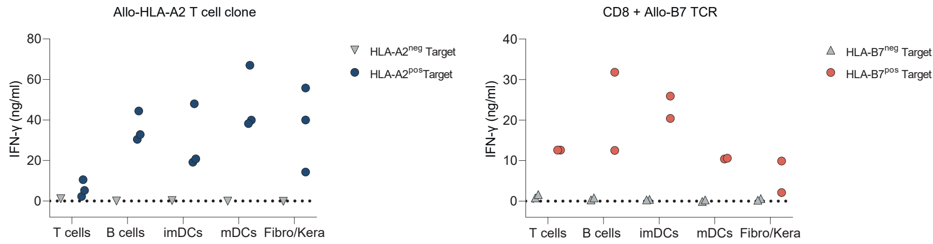
SUPPLEMENTARY MATERIAL



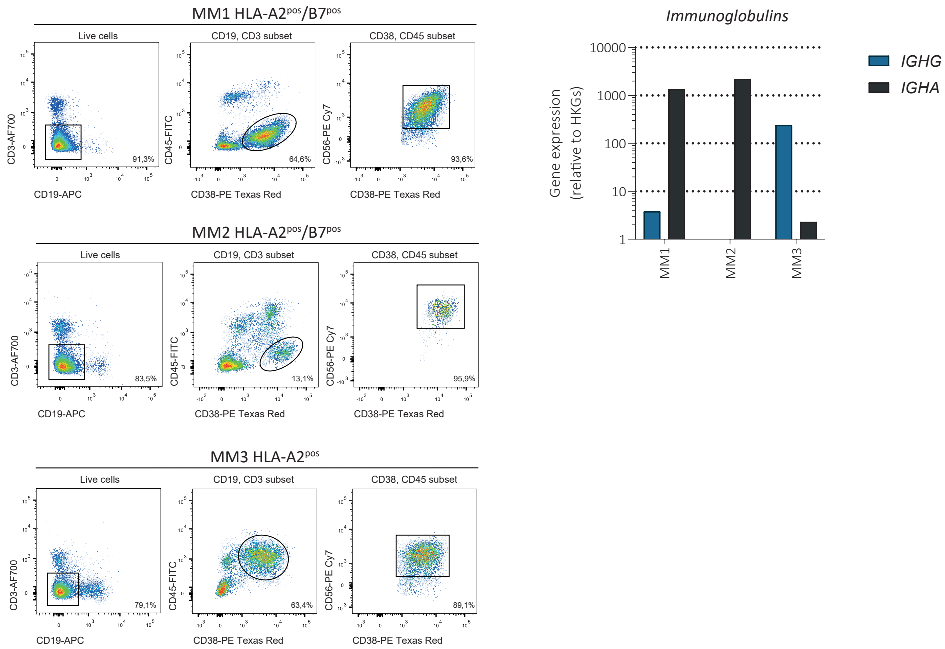
Suppl. Figure 1. IFN-γ secretion of IgM HLA-B7 specific T cell clones after overnight incubation with WT IgA^{pos} HLA-B7^{neg} multiple myeloma cell line UM6.



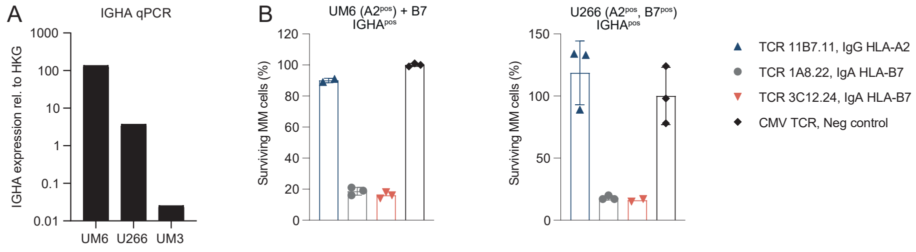
Suppl. Figure 2. Validation of stimulatory capacity of cell lines used for safety testing of Ig specific T cell clones. Indicated target cells were incubated with allo HLA T cell clones recognizing peptides derived from ubiquitously expressed genes presented in HLA-A2 (left) or HLA-B7 (right).



Suppl. Figure 3. Validation of stimulatory capacity of healthy target cells from hematopoietic origin used for safety testing of Ig specific TCR T cells. Indicated target cells were incubated with allo HLA T cell clones recognizing peptides derived from ubiquitously expressed genes presented in HLA-A2 (left) or HLA-B7 (right) and cytokine secretion after overnight coculture was measured by ELISA. Individual dots represent target cells derived from individual donors (n=3).

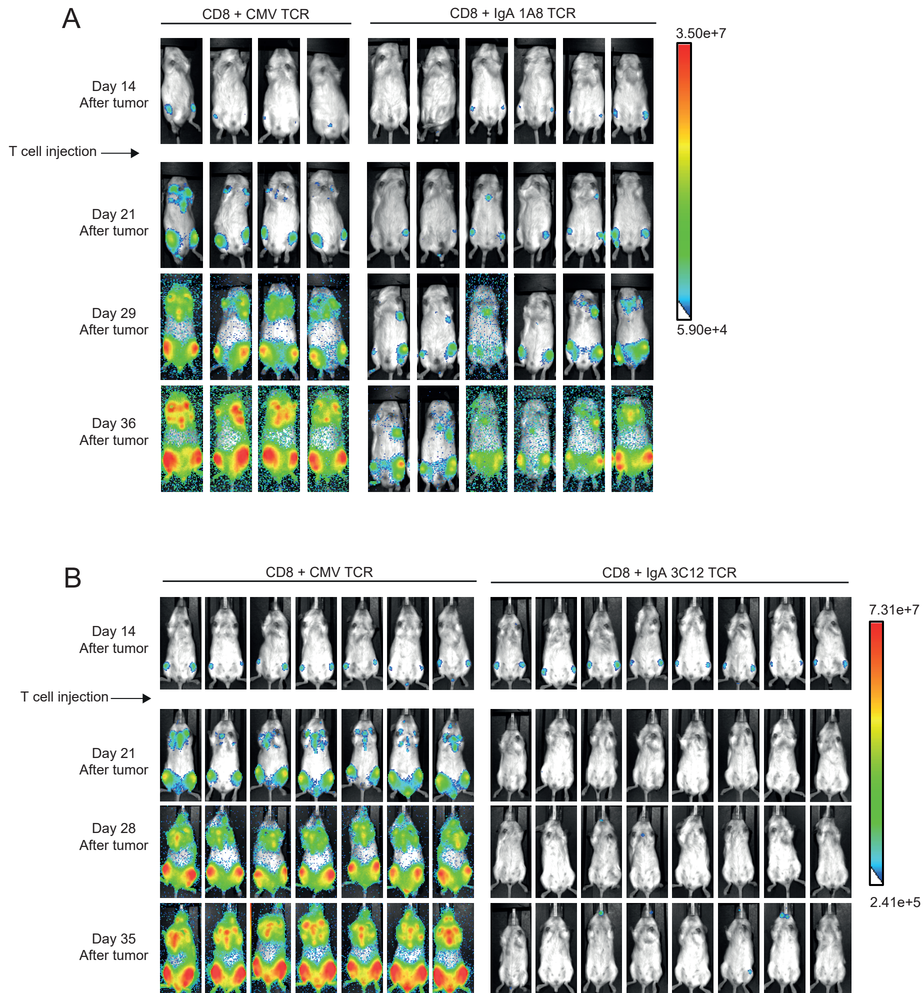


Suppl. Figure 4. Flow cytometry gating strategy used for quantification of killing of primary multiple myeloma cells from patient bone marrow samples (left). MM cells were gated on: live cells → single cells → CD3 negative cells to exclude co-cultured T cells → CD45 negative, CD19 negative → CD56 positive, CD38 positive. Quantification of IGHG (blue) or IGHA (black) expression as measured by qPCR in MM cells sorted from patient bone marrow samples (right).



Suppl. Figure 5. A) Quantification of *IGHA* expression in indicated cell lines as measured by qPCR. Expression values were normalized to expression of housekeeping genes (HKGs). **B)** Indicated TCR transduced T cells were incubated overnight with UM6 (left) or U266 (right) multiple myeloma cell lines and specific target cell lysis was quantified using flow cytometry.

4



Suppl. Figure 6. *In vivo* efficacy of IgA-B7 TCR CD8 T cells. NSG mice engrafted with $2x10^6$ HLA-B7 positive U266 multiple myeloma cells transduced with *Luc2* luciferase were i.v. injected with $5x10^6$ TCR transduced CD8 T cells after 14 days. CD8 T cells were transduced with $IgA^{HPR/B7}$ TCR 1A8, $IgA^{SPK/B7}$ TCR 3C12 or control CMV (pp65-NLV-HLA-A2) TCR and enriched for mTCR expression by MACS. T cells were infused 7 days after re-stimulation. Tumor outgrowth was frequently tracked by bioluminescence imaging. **A)** Raw bioluminescence images of CMV TCR (n=4) and $IgA^{HPR/B7}$ TCR 1A8 (n=6) treated mice. **B)** Raw bioluminescence images of CMV TCR (n=7) and $IgA^{SPK/B7}$ TCR 3C12 (n=8) treated mice.

Table S1. Overview of HLA typing of EBV-LCLs used for safety assessment

EBV-LCL	HLA-A	HLA-B	HLA-C
6023	03:01/03:03/03:04-11:01/11:02/11:03	40:02/40:35/40:37- 56:01	01:02/01:06/01:07-02:02/02:04/02:08
10299	02:01- 11:01	44:05- 51:01	02:02:02- 14:02
5857	30:04- 68:02	38:01- 55:01	03:03- 12:03
5866	02:01- 11:01	35:01- 51:01	04:01- 14:02
3829	01:01- 68:01	44:02- 44:02	05:01- 07:04
5283	24:02/24:09/24:11- 66:01/66:04	14:02- 39:06	07:02/07:10- 08:02/08:07
6978	02:01- 02:05	15:01- 45:01	01:02- 06:02
5769	02:01- 68:01	35:03- 37:01	04:01- 06:02
6461	02:01	40:02:00	02:02
6463	02:01	57:01:00	06:02
5696	02:05	58:01:00	unknown
4991	26:01/26:08/26:02-31:01/31:02/31:06	14:01- 49:01	07:01/07:05/07:06- 08:02/08:07
8425	23:01- 02:01	41:01- 40:01	17:01- 03:04
9210	02:01- 02:01	15:01- 51:01	03:03- 15:02
9271	33:01/33:03/33:05-66:01/66:04/66:08	58:01/58:02/58:11 -58:02/58:25/58:11	03:02/03:33/03:36-06:02/06:07/06:12
1854	02:01- 30:02	15:01- 39:01	03:03- 12:03
6268	02:01- 24:02	35:02- 44:02	04:01- 05:01
5702	32:01- 68:01	35:03- 52:01	12:02- 12:03
7394	01:01- 32:01	35:08- 35:08	04:01- 04:01
5171	02:01- 66:01/66:04	40:01/40:11/40:14- 41:02	03:04/03:08/03:09- 17
5524	02:01/02:07/02:09-31:01/31:02/31:06	B*15:01/15:33/15:34+-B*15:17	C*03:04/03:08/03:09-C*07:01/07:05/07:06
7007	02:05- 29:02	27:05- 44:03	01:02- 16:01:01
3480	26:01- 01:01	38:01- 18:01	12:03- 07:01/07:06
9877	01:01- 23:01/23:17	08:01- 41:02	07:01- 17:03
6945	03:01- 25:01	18:01- 35:01	04:01- 12:03
8466	23:01- 23:01	51:01- 41:01	17:01- 15:02
8463	11:01- 01:01	51:01- 50:01	15:02- 06:02
8428	11:01- 01:01	57:01- 35:01	06:02- 04:01
7358	03:01- 25:01	07:02- 44:02	05:01/05:11- 07:02/07:37
5439	03:01- 25:01	15:17- 18:01/18:03/18:05	07:01/07:05/07:06- 12:03/12:06
55	68/69- 01	53- 08	04-jul
9946	03:01/03:63/03:127-11:01/11:12/11:20	35:01/35:42- 40:01	03:04- 04:01
7929	01:01- 01:01	35:02/35:81- 52:01/52:11	04:01/04:15- 12:02/12:10

4

Table S1. Continued.

EBV-LCL	HLA-A	HLA-B	HLA-C
3281	01:01- 32:01	08:01- 45:01	06:02- 07:01/07:06/07:07
5998	24:02- 68:02	14:02- 38:01	08:02- 12:03
9619	01:01- 33:03	44:03:02- 51:01	07:06/07:18- 14:02
6316	29:02- 30:01	13:02- 44:03	06:02- 16:01
7613	01:01- 24:02	15:01- 37:01	03:03- 06:02

CHAPTER 5

Cutting Edge: Unconventional CD8+ T-cell recognition of a naturally occurring HLA-A*02:01-restricted 20mer epitope

Miranda H. Meeuwsen[*]
Anne K. Wouters[*]
Renate S. Hagedoorn[*]
Michel G.D. Kester[*]
Dennis F.G. Remst[*]
Dirk M. van der Steen[*]
Arnoud de Ru[†]
Peter A. van Veelen[†]
Jamie Rossjohn[‡,§,¶]
Stephanie Gras[‡,§,||]
J.H. Frederik Falkenburg[*]
Mirjam H.M. Heemskerk[*]

[*]Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands. [†]Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands. [‡]Department of Biochemistry and Molecular Biology & Infection and Immunity Program, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia. [§]Australian Research Council Centre of Excellence for Advanced Molecular Imaging, Monash University, Clayton, VIC, Australia. [¶]Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK. [||]Present address: Department of Biochemistry and Chemistry, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, VIC, Australia.

The Journal of Immunology 2022
Vol. 208, Issue 8, Pages 1851-1856

ABSTRACT

Unconventional HLA class I-restricted CD8+ T-cell epitopes, longer than 10 amino acids, have been implicated to play a role in human immunity against viruses and cancer. T-cell recognition of long peptides, centrally bulging from the HLA cleft, has previously been described. Alternatively, long peptides can contain a linear HLA-bound core peptide, with a N or C-terminal peptide 'tail' extending from the HLA peptide binding groove. The role of such a peptide 'tail' in CD8+ T-cell recognition remains unclear. Here, we identified a 20mer peptide (FLP) derived from the IL27RA gene restricted to HLA-A*02:01, for which we solved the crystal structure and demonstrated a long C-terminal 'tail' extension. FLP specific T-cell clones demonstrated various recognition modes, some T cells recognized the FLP core peptide, while for other T cells the peptide tail was essential for recognition. These results demonstrate a crucial role for a C-terminal peptide tail in immunogenicity.

INTRODUCTION

HLA class I (HLA-I) presented peptide epitopes are key components of CD8+ T-cell responses to pathogens and in cancer through recognition of neo-epitopes. HLA-I complexes present peptides derived from cellular proteins on the cell surface. HLA-I peptide complexes are formed in the endoplasmic reticulum (ER), where HLA-I single chains and β2-microglobulin (β2m) assemble and incorporate peptides present in the ER. The ER peptide reservoir is mainly composed of peptides derived from cytosolic protein degradation by the proteasome, which are subsequently transported into the ER through peptide transporter TAP, which preferentially binds peptides of 8-16 amino acids in length (1). Besides this classical pathway, peptides can end up in the ER, independent of TAP transport, through alternative peptide processing pathways (2). For example, the C-terminal tail of type-II transmembrane proteins, which is located inside the ER lumen, can be cleaved by signal peptide peptidase releasing C-terminal peptides into the ER (3). After localization into the ER, either through TAP or via alternative pathways, peptides are available for binding to HLA. Once binding to HLA has occurred, peptide-HLA (pHLA) complexes are transported to the cell surface where recognition by T cells can take place. The HLA-I peptide binding groove contains six pockets (A-F), of which the B and F pocket typically bind the N and C terminally located anchor residues of peptides respectively (4). Conventional peptides bind the B pocket through the amino acid residue at position 2 (p2) and the F pocket with the last residue pΩ. The HLA-I peptide binding groove is flanked by closed A and F pockets, generally limiting the length of binding peptides and resulting in preferential presentation of canonical peptides of 8-11 amino acids in length. Longer peptides can fit in HLA through binding of both anchor residues while bulging out of the groove in the center of the peptide or adapting a zig-zag conformation (5). Additionally, the C-terminal part of unconventional long peptides can protrude through the closed F pocket, resulting in a conventionally bound core peptide with a solvent exposed C-terminal 'tail' hanging from the groove (6, 7). Moreover, N-terminal peptide tails extending from the A pocket have been described (8). Mass spectrometry-based studies revealed that unconventional long peptides compose around 5-10% of the HLA-I peptide repertoire (9-12). In addition to their relatively high abundance, T-cell recognition of multiple long peptides has been described to play a role in viral as well as anti-tumor T-cell immunity (13-16).

Previously, a large set of TCR-pHLA crystal structures was reviewed and demonstrated the general consensus of TCR docking on canonical pHLA complexes (17, 18). In the reported TCR-pHLA-I complexes the TCRs were oriented in a 37-90° angle over the peptide binding groove, with the variable domain of the TCRα chain generally located over the α2 helix and the TCRβ chain over the α1 of the HLA molecule. The three loops located at the membrane distal end of both TCR chains contain CDR1, CDR2 and CDR3.

CDR1 and CDR2 (encoded by the germline variable (V) gene) interact primarily with the HLA molecule, while the hypervariable CDR3 domain binds to the peptide and HLA molecule.

Longer peptides usually bulge centrally from the peptide binding cleft (5). Bulged peptides were described to be recognized through varying TCR docking mechanisms. Rigid peptide bulges maintain their original conformation upon TCR ligation while more motile bulges can be flattened upon TCR interaction (16, 19-21). T cells recognizing bulged long peptides were shown to engage with the core of the peptide bulging out of the HLA groove. In contrast, it is unknown how *N or C-terminal* peptide tails extending from HLA cleft, impact T-cell recognition. To gain insight into the role of the peptide tail in the interaction with CD8+ T cells, we investigated HLA binding of a naturally occurring 20mer peptide FLPTPEELGLLGPPRPQVLA (referred to as FLP) derived from the IL27RA protein presented in HLA-A*02:01 (HLA-A2). We solved the crystal structure of the HLA-A2-FLP complex and revealed that only the first 11 amino acids of the peptide bound in the HLA-A2 binding groove (FLPTPEELGLL), while a proline-rich solvent exposed C-terminal tail of 9 residues (GPPRPQVLA) extended from the F pocket of the HLA-A2 molecule. T cells recognizing the FLP peptide revealed that part of the C-terminal 'tail' can be critical for T-cell recognition, providing new insight in the diversity of T cells recognizing unconventional long peptides in HLA-I.

RESULTS AND DISCUSSION

5

Identification of an HLA-A2 binding 20mer peptide

In a previous study, we mapped the HLA-I peptidome of LCLs by peptide elution followed by mass spectrometry (9, 22). HLA-I peptides were eluted using W6/32 antibody followed by selection of peptides containing anchor residues for HLA-I. This study revealed that HLA-I presented peptide longer than 11 amino acids occur more frequently than previously anticipated. Here, we investigated HLA binding and T-cell recognition of 20mer peptide FLPTPEELGLLGPPRPQVLA (FLP) identified by Hassan *et al.*, which originated from the C-terminal end of the interleukin-27 receptor α (IL27RA) protein. The FLP peptide was eluted from HLA-A2+ LCL-HHC but not from HLA-A2+ LCL-JYW (**Figure 1**). The FLP peptide contains multiple potential anchor residues that suggest binding to HLA-A2. For example, the leucine at position 2 could act as the p2 anchor residue for binding to HLA-A2. Alternatively, in case of an unconventional N-terminal peptide tail extending from the HLA groove, leucine at p8, p10 or p11 could act as the p2 anchor residue. Additionally, the C-terminal pΩ anchor residue could be the leucine at p10, p11 or p19 as well as the valine at p18. Stable binding of FLP to HLA-A2 was confirmed by successful refolding and crystallization of the HLA-A2-FLP complex.

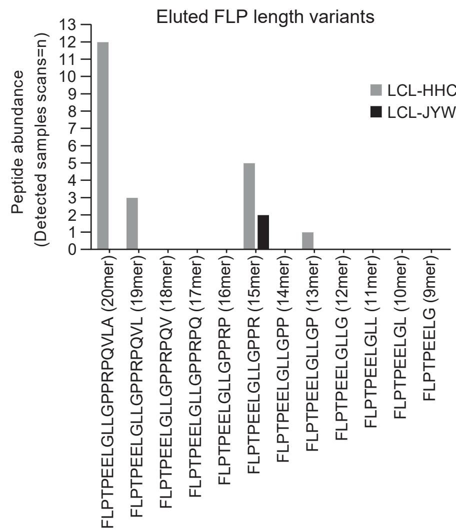


Figure 1. Identification of IL27RA derived length variants of FLP peptide from HLA-A2+ LCLs. HLA class I presented peptides were eluted from 40x10^9 HLA-A2+ LCLs and measured by mass spectrometry. Length variants of FLP peptides derived from the IL27RA protein were identified. Shown are frequencies of detection, determined by number of detected sample scans, of FLP length variant from LCL-HHC (in grey) and LCL-JYW (in black).

Crystal structure of HLA-A2-FLP revealed the presence of a long C-terminal 'tail'

To investigate how the FLP peptide was presented by the HLA-A2 molecule, we solved the crystal structure of the HLA-A2-FLP complex at 1.7 Å resolution (**Table S1**). The crystal structure contains two molecules in the asymmetric unit. The superposition of the two pHLA complexes revealed that they both adopted the same conformation, with a root mean square of 0.23 Å for the antigen binding cleft and 0.34 Å for the peptide (**Figure 2A**). Therefore, the structural analysis was performed with one of the complexes. The HLA-A2-FLP structure revealed that amino acids at p1-p11 bind in the HLA-A2 peptide binding groove in the same manner as conventional HLA class-I presented peptides, with p11-L as the pΩ anchor residue (**Figure 2B**). Amino acids p12-p20 extend from the F-pocket and form a solvent exposed C-terminal 'tail'. Part of the peptide tail (p12-p17) was clearly defined in the electron density (**Figure S1A-B**), while the electron density was weak for C-terminal residues (p18-p20) indicating high flexibility of this part. The proline-rich nature of the p12-p17 extended region presumably added rigidity to this section of the peptide, thereby enabling it to be readily visualized (19).

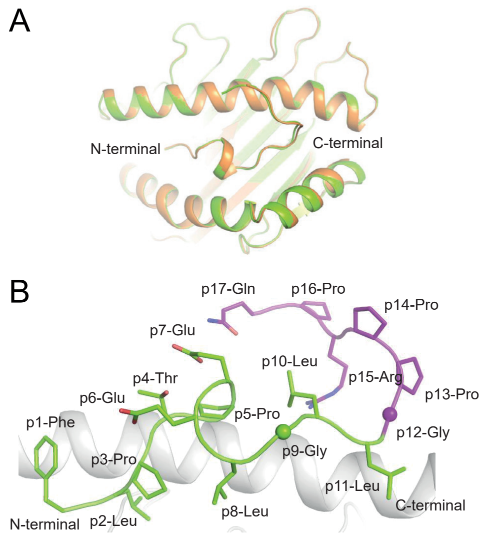


Figure 2. Crystal structure of the HLA-A2-FLP complex. A) Superposition of the two HLA-A2-FLP complexes present in the asymmetric unit represented as cartoon in green and orange. **B)** Structure of the FLP-20mer peptide (cartoon and stick) in the cleft of HLA-A2 (white cartoon). The first 11 residues of the FLP-20mer peptide colored in green are bound to the antigen binding cleft, while the C-terminal tail (purple) is coming out of the cleft.

Identification of FLP peptide specific CD8+ T cells revealed immunogenicity

To assess the immunogenicity of FLP peptide, T cells were isolated from HLA-A2 negative healthy donors to circumvent immunogenic tolerance for this non-mutated self-peptide (23, 24). Using HLA-A2-FLP-tetramers we successfully isolated CD8+ T-cell clones (**Figure 3A**). These T-cell clones demonstrated functional recognition of FLP peptide upon peptide titration using HLA-A2 Td Raji cells (**Figure 3B**). Unloaded *IL27RA* negative Raji cells were not recognized, showing FLP peptide specificity (**Figure 3B, Figure S1C**).

Recognition of endogenously processed and presented FLP peptide was observed by T cells stimulated with *IL27RA* expressing T2 cells (**Figure 3C, Figure S1C**), indicating that the identified T-cell clones were potent enough to recognize target cells naturally expressing the *IL27RA* gene. Additionally, recognition of T2 cells was enhanced by retroviral overexpression of the *IL27RA* gene (**Figure 3C**). Similarly, Td of IL27RA into Raji cells induced recognition, validating the requirement for *IL27RA* expression for target cell recognition. Recognition of TAP deficient T2 cells by FLP specific T-cell clones suggests that FLP peptide, which is located at the C-terminal end of the IL27RA protein, is processed and presented independent of TAP transport through an alternative pathway remaining to be identified. This observation is in accordance with the overrepresentation of C-terminal peptides in the ligandome of TAP deficient cells (25).

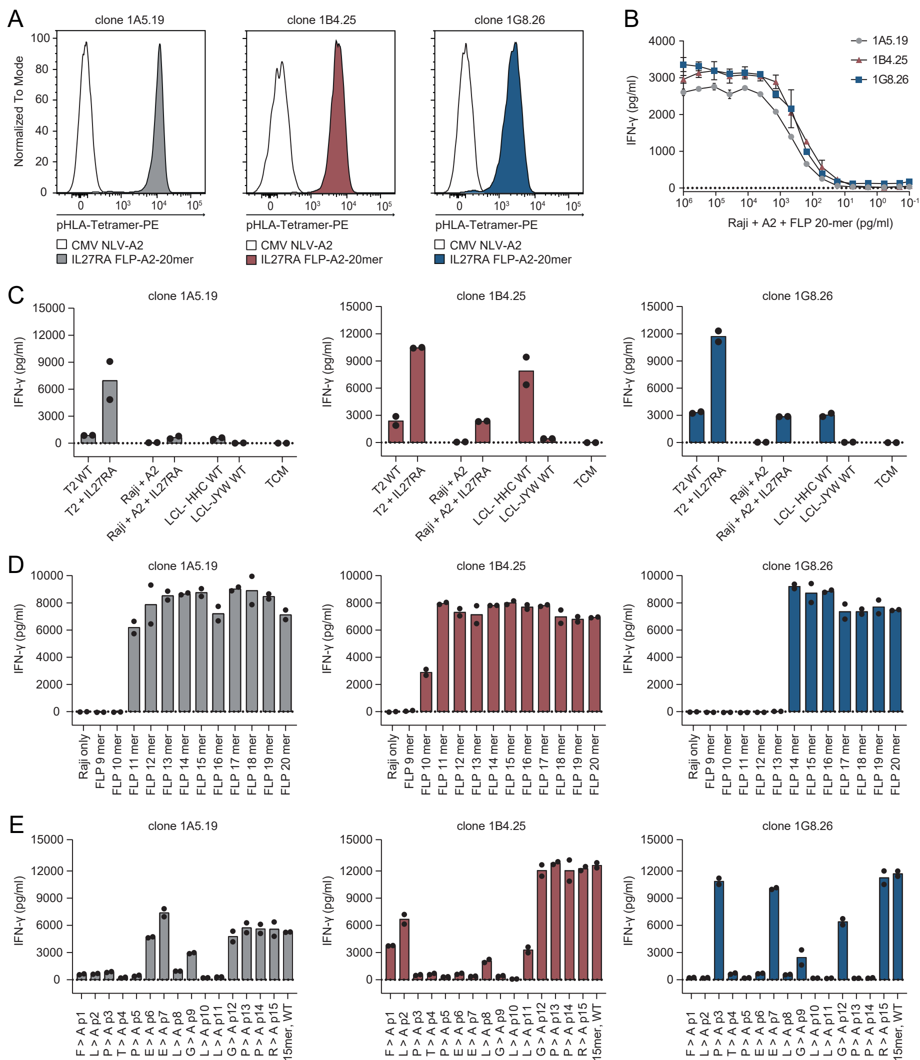


Figure 3. Reactivity of FLP specific T-cell clones. A) T cell clone 1A5.19, 1B4.25 and 1G8.26 stained with IL27RA FLP pHLA-tetramer-PE (grey red or blue) or control CMV NLV HLA-A2 tetramer (white) gated on live cells. **B-E)** IFN-y production after overnight co-culture. Values represent mean of technical duplicates, data are representative examples of three experiments. **B)** T-cell clones co-cultured with Raji cells Td with HLA-A2 loaded with decreasing FLP peptide concentrations. **C)** T-cell clones co-cultured with Raji cells Td with HLA-A2 (Raji +A2), Raji + A2 additionally Td with the IL27RA gene (+IL27RA), HLA-A2+ wild type (WT) T2 cells, T2 cells Td with the IL27RA gene (+IL27RA), HLA-A2+ WT LCLs from donor HHC, HLA-A2+ WT LCLs from donor JYW and medium (TCM) only. **D)** Raji +A2 loaded with 100nM c-terminal length variants of FLP peptide. **E)** Raji +A2 loaded with 100nM Ala scan of FLP 15mer peptide.

Finally, LCLs from donors HHC and JYW were co-cultured with the T-cell clones, despite similar natural *IL27RA* expression levels (**Figure S1C**), LCLs from donor HHC were recognized while LCLs from donor JYW were not recognized (**Figure 3C**). This corresponded with the difference in abundance of FLP length variants in peptide elution data of the two LCLs (**Figure 1**), which demonstrated that FLP length variants, including the 20mer peptide, were abundantly present on LCL-HHC cells, whereas no 20mer peptide, and only one length variant could be detected in the peptide elution data of LCL-JYW (**Figure 1**). In total, length variants of FLP were identified 21 times from LCL-HHC while only 2 times from LCL-JYW. The substantial difference in detection of FLP length variants indicated differential involvement of the pathway underlying presentation of FLP peptides in LCL-HHC and JYW.

Solvent exposed C-terminal 'tail' of FLP peptide plays a role in T-cell recognition

To investigate if the solvent exposed 'tail' of FLP peptide plays a role in T-cell recognition, C-terminal length variants of FLP were loaded on HLA-A2 Td Raji cells. Recognition by T-cell clone 1A5.19 was located in the core between p1-p11 of FLP peptide, as recognition was not influenced by length variations of the tail (p12-p20) (**Figure 3D**). Recognition was lost upon stimulation with FLP-10mer peptide, demonstrating the requirement for the p11-L anchor residue to be present that would likely conserve the same core structure as the 20mer peptide. T-cell clone 1B4.25 demonstrated a comparable recognition pattern but additionally recognized FLP-10mer peptide at a reduced level (**Figure 3D**), indicating that p10-L could act as an alternative, but less efficient anchor residue. Finally, T-cell clone 1G8.26 recognized FLP 20-14mer peptides to the same extend as FLP-20mer peptide, but recognition was abrogated upon stimulation with FLP-13mer or shorter peptides (**Figure 3D**). Therefore, the proline at p14 played an essential role in recognition by this T-cell clone, demonstrating that the proximal part of the C-terminal peptide 'tail' was involved in T-cell recognition. For all T-cell clones no differences were observed between recognition of FLP-20mer and FLP-15mer indicating that p15-p20 did not influence T-cell recognition. To confirm that amino acids in the proximal part of the 'tail' are important for recognition by T-cell clone 1G8.26, an Ala scan of FLP-15mer was generated and loaded onto HLA-A2 Td Raji cells. For T-cell clones 1A5.19 and 1B4.25 Ala substitutions between p12 and p15 did not influence recognition of the FLP peptide, whereas recognition by T-cell clone 1G8.26 was abrogated when p13 and p14 were replaced by Ala, confirming the previously observed role of the proximal part of the peptide tail for recognition by this clone (**Figure 3E**). In addition, Ala substitutions between p1 and p11 demonstrated that all three clones recognized the core region of the peptide but exhibited differential recognition patterns of the FLP pHLA-A2 complex. Thus, T-cell clone 1G8.26 recognized the core as well as the tail region of the FLP peptide, while clones 1A5.19 and 1B4.25 recognized the core peptide alone. The combined recognition of the core region as well as proximal part of the tail by clone 1G8.26 suggested that upon TCR binding, the

peptide tail is oriented away from the peptide binding groove rather than being folded back over the linear peptide core, thereby allowing simultaneous TCR contact with the core as well as the proximal part of the peptide tail.

Remesh *et al* previously reported that longer peptides extend at the C terminus by opening the F-pocket with a charged amino acid not more than 3 residues after the pΩ anchor (7). In our study, the crystal structure of the HLA-A2-FLP complex does not reveal opening of the F-pocket, and in addition the first charged amino acid at p15 was found to be irrelevant for recognition (**Figure 3D,E**). Demonstrating that a C-terminal peptide tail can extend from the HLA cleft without opening of the F-pocket by a charged amino acid.

To conclude, these results demonstrated immunogenicity of a 20mer peptide composed of a conventional core peptide and a C-terminal peptide tail coming out the F-pocket presented in HLA-A2. One of three identified T-cell clones required the peptide tail to allow peptide recognition, highlighting that the proximal part of the C-terminal peptide tail can be a crucial component of such an unconventional T-cell epitope. These results contribute to the knowledge about the dynamic range in functional T-cell epitopes that exist beyond canonical peptides. The variable recognition patterns observed between T-cell clones would indicate that the TCRs bind FLP through different docking modes, and further study will be required to gain a detailed understanding of the interaction between TCRs and this unconventional peptide.

MATERIALS AND METHODS

Peptide identification

HLA-I peptidomes of HLA-A2+ EBV transformed B lymphoblastic cell lines (LCLs) LCL-HHC and LCL-JYW were previously determined by peptide elution and mass spectrometry analysis (22). Peptide length variation was assessed for peptides containing anchor residues for binding to expressed HLA-I alleles (9). In this study pHLA monomers containing 20-mer peptide FLPTPEELGLLGPPRPQVLA (FLP) derived from the Interleukin-27 receptor subunit alpha (IL27RA) in HLA-A2 were folded and used to generate pHLA-tetramers as previously described by Hassan *et al* (9). Peptides were synthesized by standard Fmoc chemistry and dissolved in DMSO.

Protein expression, purification, crystallization and structure determination

DNA plasmids encoding HLA-A2 α-chain (1-275) and human β2m were transformed separately into a BL21 strain of *E. coli*. Recombinant proteins were expressed insoluble as inclusion bodies, extracted and purified. Soluble pHLA complex was produced by

refolding 30 mg HLA-A2 α-chain, 5 mg β2m and 5 mg FLP peptide in a 0.5L buffer of 3M Urea, 0.5 M L-Arginine, 0.1 M Tris-HCl pH 8.0, 2.5 mM EDTA pH 8.0, 5 mM glutathione (reduced), 1.25 mM glutathione (oxidised). The mixture was stirred at 4°C for 3h and dialysed against 10L of 10 mM Tris-HCl pH 8.0 three times over a 24h period (26). Soluble pHLA was purified using anion exchange chromatography using a HiTrapQ column. The HLA-A2-FLP complex was crystallised using vapor diffusion method with a 1:1 ratio of protein:mother liquor. The protein was 5 mg/ml in 0.1M Tris-HCl pH8 and 0.15M NaCl, and the mother liquor was composed of a mixture of 0.12M Monosaccharides (D-Glucose; D-Mannose; D-Galactose; L-Fucose; D-Xylose; N-Acetyl-D-Glucosamine), 0.1M of Na-HEPES and MOPS buffer at pH 7.5, 20% $^{w}/_{v}$ Glycerol and 10% $^{w}/_{v}$ PEG 4000. The crystals were flash frozen in liquid nitrogen and data were collected on the MX1 beamline at the Australian Synchrotron, part of ANSTO, Australia (27). The data were processed using XDS (28) and the structure was determined by molecular replacement using the PHASER program (29) from the CCP4 suite (30) with a model of HLA-A2 without peptide (derived from PDB ID: 3GSO **DOI:** 10.4049/jimmunol.0900556). Manual model building was conducted using COOT (31) followed by refinement with BUSTER (32). The final model has been validated and deposited using the wwPDB OneDep System with accession code: 7T5M. The final refinement statistics are summarised in supplemental Table 1. All molecular graphics representations were created using PyMOL.

T cell isolation

PBMCs were isolated from buffy coats obtained from HLA-A2 negative healthy donors after written informed consent according to the Declaration of Helsinki. pHLA-tetramer+ CD8+ T cells were enriched, single-cell sorted and clonally expanded as previously described (9). FLP-A2 specificity was determined by pHLA-tetramer stain.

Target cell generation

T2 and Raji cells were transduced (Td) with IL27RA in a MP71-IRES-NGFR vector. Td cells were purified for transgene expression by MACS before use in experiments. Raji cells were additionally Td with HLA-A2 in a PLZRS-markerless vector and purified.

T-cell assay

T-cell stimulation assays were performed by overnight incubation of 5,000 T cells with 30,000 target cells in 384 well plates. For peptide recognition target cells were loaded with 100nM peptide or decreasing concentrations starting at 1μM in peptide titration experiments. IFN- γ production was measured by ELISA. C-terminal length variants of FLP peptide were generated. Purity of all length variants was confirmed by HPLC and mass spectrometry. FLP 15-mer peptide was used for alanine (Ala) scanning, all amino acids were separately substituted by an Ala.

RT-qPCR

IL27RA expression was measured by quantitative RT-PCR (qRT-PCR) as previously described (24). Gene expression was calculated relative to the average expression of housekeeping genes: *GUSB*, *PSMB4* and *VPS29*.

5

REFERENCES

1. van Endert PM, Tampé R, Meyer TH, Tisch R, Bach JF, McDevitt HO. A sequential model for peptide binding and transport by the transporters associated with antigen processing. Immunity. 1994;1(6):491-500.
2. Oliveira CC, van Hall T. Alternative Antigen Processing for MHC Class I: Multiple Roads Lead to Rome. Front Immunol. 2015;6:298.
3. Oliveira CC, Querido B, Sluijter M, de Groot AF, van der Zee R, Rabelink MJ, et al. New role of signal peptide peptidase to liberate C-terminal peptides for MHC class I presentation. J Immunol. 2013;191(8):4020-8.
4. Nguyen AT, Szeto C, Gras S. The pockets guide to HLA class I molecules. Biochem Soc Trans. 2021;49(5):2319-31.
5. Josephs TM, Grant EJ, Gras S. Molecular challenges imposed by MHC-I restricted long epitopes on T cell immunity. Biol Chem. 2017;398(9):1027-36.
6. Collins EJ, Garboczi DN, Wiley DC. Three-dimensional structure of a peptide extending from one end of a class I MHC binding site. Nature. 1994;371(6498):626-9.
7. Remesh SG, Andreatta M, Ying G, Kaever T, Nielsen M, McMurtrey C, et al. Unconventional Peptide Presentation by Major Histocompatibility Complex (MHC) Class I Allele HLA-A*02:01: BREAKING CONFINEMENT. J Biol Chem. 2017;292(13):5262-70.
8. Pymm P, Illing PT, Ramarathinam SH, O'Connor GM, Hughes VA, Hitchen C, et al. MHC-I peptides get out of the groove and enable a novel mechanism of HIV-1 escape. Nature Structural & Molecular Biology. 2017;24(4):387-94.
9. Hassan C, Chabrol E, Jahn L, Kester MG, de Ru AH, Drijfhout JW, et al. Naturally processed non-canonical HLA-A*02:01 presented peptides. The Journal of biological chemistry. 2015;290(5):2593-603.
10. Hickman HD, Luis AD, Buchli R, Few SR, Sathiamurthy M, VanGundy RS, et al. Toward a definition of self: proteomic evaluation of the class I peptide repertoire. Journal of immunology (Baltimore, Md : 1950). 2004;172(5):2944-52.
11. Mommen GP, Frese CK, Meiring HD, van Gaans-van den Brink J, de Jong AP, van Els CA, et al. Expanding the detectable HLA peptide repertoire using electron-transfer/higher-energy collision dissociation (EThcD). Proceedings of the National Academy of Sciences of the United States of America. 2014;111(12):4507-12.
12. Burrows SR, Rossjohn J, McCluskey J. Have we cut ourselves too short in mapping CTL epitopes? Trends Immunol. 2006;27(1):11-6.
13. Pudney VA, Leese AM, Rickinson AB, Hislop AD. CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. J Exp Med. 2005;201(3):349-60.
14. Tynan FE, Borg NA, Miles JJ, Beddoe T, El-Hassen D, Silins SL, et al. High resolution structures of highly bulged viral epitopes bound to major histocompatibility complex class I. Implications for T-cell receptor engagement and T-cell immunodominance. The Journal of biological chemistry. 2005;280(25):23900-9.
15. Probst-Kepper M, Hecht HJ, Herrmann H, Janke V, Ocklenburg F, Klempnauer J, et al. Conformational restraints and flexibility of 14-meric peptides in complex with HLA-B*3501. Journal of immunology (Baltimore, Md : 1950). 2004;173(9):5610-6.
16. Chan KF, Gully BS, Gras S, Beringer DX, Kjer-Nielsen L, Cebon J, et al. Divergent T-cell receptor recognition modes of a HLA-I restricted extended tumour-associated peptide. Nat Commun. 2018;9(1):1026.
17. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. Annu Rev Immunol. 2015;33:169-200.
18. La Gruta NL, Gras S, Daley SR, Thomas PG, Rossjohn J. Understanding the drivers of MHC restriction of T cell receptors. Nat Rev Immunol. 2018;18(7):467-78.
19. Tynan FE, Burrows SR, Buckle AM, Clements CS, Borg NA, Miles JJ, et al. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. Nat Immunol. 2005;6(11):1114-22.
20. Tynan FE, Reid HH, Kjer-Nielsen L, Miles JJ, Wilce MC, Kostenko L, et al. A T cell receptor flattens a bulged antigenic peptide presented by a major histocompatibility complex class I molecule. Nat Immunol. 2007;8(3):268-76.
21. Liu YC, Miles JJ, Neller MA, Gostick E, Price DA, Purcell AW, et al. Highly divergent T-cell receptor binding modes underlie specific recognition of a bulged viral peptide bound to a human leukocyte antigen class I molecule. The Journal of biological chemistry. 2013;288(22):15442-54.

22. Hassan C, Kester MG, de Ru AH, Hombrink P, Drijfhout JW, Nijveen H, et al. The human leukocyte antigen-presented ligandome of B lymphocytes. Mol Cell Proteomics. 2013;12(7):1829-43.
23. Amir AL, van der Steen DM, van Loenen MM, Hagedoorn RS, de Boer R, Kester MD, et al. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin Cancer Res. 2011;17(17):5615-25.
24. Jahn L, van der Steen DM, Hagedoorn RS, Hombrink P, Kester MG, Schoonakker MP, et al. Generation of CD20-specific TCRs for TCR gene therapy of CD20low B-cell malignancies insusceptible to CD20-targeting antibodies. Oncotarget. 2016;7(47):77021-37.
25. Martín-Galiano AJ, López D. Computational characterization of the peptidome in transporter associated with antigen processing (TAP)-deficient cells. PloS one. 2019;14(1):e0210583.
26. Chatzileontiadou DSM, Szeto C, Jayasinghe D, Gras S. Protein purification and crystallization of HLA-A*02:01 in complex with SARS-CoV-2 peptides. STAR Protoc. 2021;2(3):100635.
27. Aragão D, Aishima J, Cherukuvada H, Clarken R, Clift M, Cowieson NP, et al. MX2: a high-flux undulator microfocus beamline serving both the chemical and macromolecular crystallography communities at the Australian Synchrotron. J Synchrotron Radiat. 2018;25(Pt 3):885-91.
28. Kabsch W. XDS. Acta Crystallogr D Biol Crystallogr. 2010;66(Pt 2):125-32.
29. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. J Appl Crystallogr. 2007;40(Pt 4):658-74.
30. The CCP4 suite: programs for protein crystallography. Acta crystallographica Section D, Biological crystallography. 1994;50(Pt 5):760-3.
31. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. Acta Crystallogr D Biol Crystallogr. 2010;66(Pt 4):486-501.
32. Bricogne G. BE, Brandl M., Flensburg C., Keller P., Paciorek W.,, Roversi P SA, Smart O.S., Vonrhein C., Womack T.O. Buster version 2.10. Cambridge, United Kingdom: Global Phasing Ltd. 2011.

SUPPLEMENTARY MATERIAL

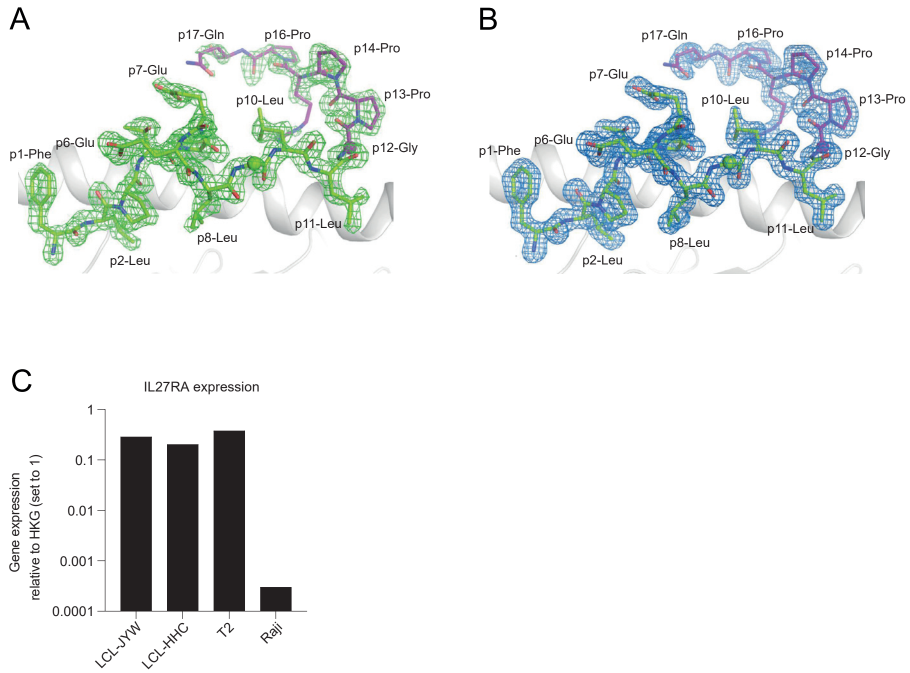


Figure S1. Crystal structures of FLP peptide and gene expression of the *IL27RA*gene. A-B) Electron density maps contoured at 3σ colored in green for the Fo-Fc map (A), and contoured at 1σ colored in blue for the 2Fo-Fc map (B). The peptide and HLA are colored as in Fig 1B. **C)** IL27RA gene expression in LCL-JYW, LCL-HHC, T2 and Raji cells measured by qPCR, depicted relative to housekeeping genes (HKG).

Table S1. Data collection and refinement statistics

Data Collection Statistics	HLA-A2-FLP
Space group	$P22_12_1$
Cell Dimensions (a,b,c) (Å)	70.18, 99.90, 135.46
Resolution (Å)	48.74 – 1.67 (0.70 – 1.67)
Total number of observations	823981 (34622)
Nb of unique observation	111273 (5154)
Multiplicity	7.4 (6.7)
Data completeness (%)	997 (94.3)
I/s_I	13.2 (2.4)
R_{pim} [a] (%)	4.1 (35.1)
$CC_{1/2}$ (%)	99.8 (72.0)
Refinement Statistics	
R_{factor} [b] (%)	18.7
R_{free} [b] (%)	20.8
rmsd from ideality	
Bond lengths (Å)	0.008
Bond angles (°)	0.98
Ramachandran plot (%)	
Favoured	99.03
Allowed	0.97
Disallowed	0

[a] $R_{p.i.m} = \sum_{hkl} [1/(N-1)]^{1/2} \sum_i | I_{hkl,i} - \langle I_{hkl} \rangle | / \sum_{hkl} \langle I_{hkl} \rangle$. [b] $R_{factor} = \sum_{hkl} | | F_o | - | F_c | | / \sum_{hkl} | F_o |$ for all data except ≈ 5% which were used for R_{free} calculation. Values in parentheses are for the highest resolution-shell.

5

CHAPTER 6

Summary and general discussion

SUMMARY

CD19- and BCMA-targeting chimeric antigen receptor (CAR) T cells have shown significant anti-tumor effects in treating B-cell malignancies and multiple myeloma (MM) (1, 2). However, relapses post-therapy highlights ongoing challenges in T-cell therapy. Antigen escape is a common mechanism behind tumor escape, occurring when tumor cells with low or no surface antigen expression survive treatment(1, 3). To counter this, we advocate for multi-antigen-targeting approaches, simultaneously targeting several antigens. Adding more specificities to cellular products can decrease the chance of antigen escape(4).

To target multiple antigens, T-cell receptor (TCR) transduced T cells would be a promising component of multi-antigen-targeting therapy. TCRs have the intrinsic ability to recognize peptides from theoretically any antigen presented in HLA molecules on the cell surface, enabling them to target antigens inaccessible to CARs. Targeting B-cell lineage antigens consistently expressed in malignant cells is promising, as evidenced by the success of CAR T cells against CD19 and BCMA.

This thesis aimed to identify targets expressed in B-cell malignancies, that are not expressed in healthy cells except for the B-cell lineage. We searched for TCRs recognizing such targets effective against B-cell or plasma cell malignancies. Our approach involved identifying TCRs specific to peptides from B-cell antigens presented in various HLA alleles. These epitopes, being self-peptides, are not immunogenic in an HLA-matched setting. To overcome this, we isolated T-cell clones across HLA-barriers using donors lacking the target HLA-alleles. We used peptide-HLA (pHLA) multimers for isolation, followed by comprehensive potency and safety tests to select T cells with precise antigen recognition.

The TCRs of selected T-cell clones were sequenced, inserted into retroviral vectors, and introduced into third-party T cells. These T cells were then evaluated for their specificity and ability to target and eliminate B-cell malignancies expressing the antigen. For certain TCRs, we demonstrated anti-tumor effectiveness using *in vivo* xenograft models.

In **Chapter 2**, we performed a broad search to identify TCRs for therapy of B-/plasma cell malignancies. We started by selecting potential antigens for TCR-based therapy of B-/plasma cell malignancies. We utilized a gene expression dataset containing samples from acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and MM patients as well as healthy cells of various origins. We selected genes that were expressed in one of these malignancies while expression in healthy cells, with the exception of B cells, was negative. This approach yielded 28 proteins which could be promising

targets for therapy of B-/plasma cell malignancies. We identified peptides derived from these genes, presented in HLA on the surface of malignant B cells by peptide elution and mass spectrometry. Our selection of target HLA alleles included HLA-A*01:01, -A*24:01, -B*08:01, and -B*35:01, thereby focusing on commonly expressed alleles that have not been extensively studied in the past. Twenty target peptides derived from one of the selected genes and presented in one of the chosen HLA molecules were identified. To bypass self-tolerance, T-cell clones were isolated from healthy donors lacking the HLA alleles of interest. Employing pHLA-multimers, we isolated 5519 T-cell clones from 13 donors. High-throughput screenings yielded 23 T-cell clones specific for one of the target peptides, which demonstrated functional reactivity to naturally processed and presented antigen. We sequenced the TCRs of six T-cell clones which were sensitive enough to recognize and target B-cell malignancies naturally presenting the antigen. Following TCR-transfer, third-party T cells effectively recognized and lysed B-cell malignancies while antigen specificity was maintained. The selected TCRs target peptides derived from FCRL5 presented in HLA-A*01:01, VPREB-3 in HLA-A*24:02, and BOB1 in HLA-B*35:01. The potential therapeutic value of each TCR varies with the type of malignancy. For instance, the FCRL5-specific TCR showed promise for CLL therapy and possibly B-cell lymphomas. VPREB-3 is expressed in ALL and CLL, while expression is lower in CLL, VPREB-3 TCR T cells recognized CLL samples and might therefore be valuable for therapy of both. BOB1, expressed in ALL, CLL, and MM, was recognized by TCR T cells, making it a candidate for treating all three malignancies. Additionally, B-cell lymphoma cells were recognized.

In **Chapter 3**, we identified TCRs recognizing peptides from the joining chain (Jchain) that can be used to treat MM. In Chapter 2, the Jchain was already identified as one of the target proteins for therapy of B-/plasma cell malignancies. In healthy plasma cells the Jchain functions as a linker molecule between monomers of IgA and IgM when secreted in multimeric forms. Expression in MM cells is irrespective of the immunoglobulin isotype that is produced and the Jchain is highly expressed in most MM samples. The Jchain could therefore be a broadly applicable target antigen for MM. The Jchain is a secreted protein and can thus not be targeted with CAR T cells, instead TCR T cells specific for Jchain derived peptides in HLA can be used to target *JCHAIN* expressing cells. Our goal in this chapter was to identify TCRs that could recognize Jchain peptides presented in various HLA-A alleles. Target peptides derived from the Jchain presented in HLA were identified from the HLA class-I peptidome of MM cells. pHLA-multimer technology as used in Chapter 2, was applied to isolate Jchain specific T-cell clones. This resulted in the discovery of four T-cell clones that effectively recognized Jchain peptides presented in HLA-A*01:01, -A*03:01, -A*11:01, or -A*24:02. These Jchain-specific T cells showed promising safety profiles. Upon equipping T cells with the Jchain-TCRs, we observed that they successfully lysed MM tumor cells from various patients but only when the *JCHAIN*

was expressed. Additionally, Jchain-TCR T cells drastically reduced tumor burdens in a pre-clinical *in vivo* model of established MM. Thus, these TCRs hold substantial potential for further development as MM therapies. Given their favorable HLA restrictions, these four TCRs collectively have the potential to treat approximately 60% of individuals with *JCHAIN*-positive MM.

In **Chapter 4**, we focused further on identification of TCRs applicable for MM therapy. MM cells are the malignant counterpart of plasma cells and therefore often secrete high amounts of immunoglobulins of a specific isotype. Most MM cells are of IgG isotype and 22% is of IgA isotype, but IgA expression is associated with an unfavorable prognosis. We therefore aimed to target these immunoglobulins, specifically peptides from the constant domains of IgG and IgA heavy chains, identified from the HLA-I peptidome of B-cell malignancies. Following the approaches as in Chapter 2 and Chapter 3, we aimed to identify T cells specific to these immunoglobulins. Extensive efforts led to the discovery of T-cell clones specific for the LMI peptide from the IgG heavy chain constant domain presented in HLA-A*02:01 or the SPK or HPR peptides from the IgA constant domain presented in HLA-B*07:02. These clones effectively recognized and lysed MM cell lines expressing the right isotype. Specificity and functionality was maintained upon TCR sequencing and transfer to third party CD8 T cells. Immunoglobulin-TCR T cells did not recognize healthy cells of hematopoietic origin other than B cells. These T cells only lysed isotype-positive B cells while sparing others, suggesting a potential clinical advantage by partially preserving B-cell immunity, especially when IgA-TCR T cells are applied. Furthermore, dendritic cells co-culture with high concentrations of IgG immunoglobulins were recognized by IgG-TCR T cells in a dose-dependent manner, most likely as of result of IgG uptake, cross-presentation, and subsequent presentation of the respective IgG peptide in HLA-A*02:01 to TCR T cells. Immunoglobulin-specific TCR-transgenic T cells specifically lysed patient-derived MM cells when the respective immunoglobulin and targeted HLA allele were expressed. Furthermore, IgA-TCR T cells effectively reduced tumor burdens in MM bearing mice.

Chapter 5 delves into an interesting aspect of the HLA class-I ligandome: the presence of unconventionally long peptides. In the method applied in Chapters 2-4, as well as by others working on T-cell epitope identification or prediction, the general assumption has been applied that HLA class-I presents short peptides of 8-11 amino acids in length. However, the HLA class-I ligandome contains a substantial fraction of longer peptides which are often dismissed as contaminants from HLA class-II. In **Chapter 5** an unconventionally long peptide was explored for its potential origin from HLA class-I. We examined a 20-amino acid (20-mer) FLP peptide from the IL27 receptor alpha, identified in HLA-A*02:01 expressing cells and investigated its binding to HLA-A*02:01. Successful refolding of FLP:HLA-A*02:01 complexes demonstrated that the FLP peptide

could indeed bind to HLA-A*02:01. Crystal structure analysis revealed that the first 11 amino acids bound HLA in a conventional linear manner, while the remaining portion formed an extended 'peptide tail'. We isolated T cells recognizing the FLP peptide, as in previous chapters, to understand how such a long peptide could be recognized. Our findings indicated that while some T cells were activated by the core peptide alone, others required the C-terminal tail, losing recognition if it was trimmed or altered. This chapter highlights the diverse way peptides can bind to HLA and shows that T cells can recognize peptides bound to HLA in unconventional manners.

GENERAL DISCUSSION

Combining CAR and TCR T cells to improve outcomes of cellular therapy

Most B-cell malignancies, unlike tumors with high mutational burdens, often lack neo-antigens that can be targeted by therapies designed to exploit mutational diversity(5). The absence of neo-antigens in B-cell malignancies limits the efficacy of treatments that rely on high mutational burdens for targeting, such as tumor infiltrating lymphocyte (TIL) therapy or immune checkpoint blockade to boost neo-antigen specific T cells. Instead, these malignancies can be treated by targeting B-cell lineage antigens, which are expressed throughout various stages of B-cell development and for which expression is often maintained after malignant transformation(6). Initially, rituximab, an antibody therapy that targets CD20 expressed on B cells, demonstrated efficacy for treatment of lymphoma, chronic lymphocytic leukemia (CLL) and other B-cell mediated diseases such as rheumatoid arthritis(7). Depletion of healthy B cells as side effect of treatment increased the risk of infections but this side effect could be overcome by treatment with immunoglobulin replacement therapy(8).

In addition to antibody mediated therapy for B-cell associated diseases, T-cell-based therapies have emerged more recently. T-cell therapies are currently based on the introduction of a chimeric antigen receptor (CAR) that targets a B-cell lineage antigen on the surface of malignant B cells like CD19 and BCMA for treatment of B-cell malignancies and multiple myeloma (MM), respectively. Despite achieving high initial response rates many patients relapse after CAR T-cell therapy(9, 10). Some patients relapse with antigen positive malignancy, often attributed to suboptimal performance of CAR T cells(11). Additionally, outgrowth of antigen low or negative tumor cells can contribute tumor escape(3). These relapses highlight the need to keep advancing cellular therapy and demonstrate that new targets should be included as part of combination therapy to mitigate the risk of antigen negative relapse. CAR T cells recognize proteins on the surface of target cells. This characteristic combined with the requirement for B-cell restricted expression limits identification of suitable CAR targets. T-cell receptor (TCR)

transgenic T cells could complement CAR T cells in one crucial aspect. TCRs possess the capability to recognize peptides derived from any protein, irrespective of its cellular localization. Therefore, TCRs can be used to target any antigen of choice if the criteria regarding a safe expression profile are met. This ability opens many avenues to explore new target proteins for treatment of B-cell malignancies. Because CARs and TCRs have a different recognition mode as well as a different composition/design, we believe that combination of TCR and CAR T cells is expected to enhance the efficacy of T-cell therapy as each modality brings unique advantages while balancing the limitations of the other and thereby improving overall outcomes following T-cell therapy. In recent years the CAR T-cell field has rapidly developed, and many clinical trials have been performed that provided new insights in the advantages and disadvantages of CAR T cells. An advantage of CAR T cells is that specific surface proteins independent of HLA are targeted, making them broadly applicable across patients independent of HLA type. CARs are artificial receptors that combine an antigen binding domain, a signaling domain and one or more costimulatory domains in a modular manner, because of this modular design CARs can easily be adapted and improved by changing an individual module. Throughout different generations of CARs, the effector function was initially enhanced by incorporation of a co-stimulatory domain and later by incorporation of alternative co-stimulatory domains(12). Additionally, CARs contain a CD3zeta signaling domain which makes CARs independent of endogenous T-cell signaling and therefore also independent of the CD8 or CD4 co-receptor. Therefore, CARs function in both cytotoxic CD8 T cells as well as CD4 helper T cells. CD4 T-cell help has been shown to be important for the effectiveness of CD19 CAR T cells. Preclinical studies have shown that CAR T cells in a defined CD8:CD4 ratio outperformed CAR T cells that were derived from CD4 or CD8 T cells alone, indicating some degree of synergy between receptor engineered CD4 and CD8 T cells(13). CD4 T cells not only 'help' other cells through specific cytokine signals or by providing costimulatory ligands but can also directly exert effector functions and anti-tumor activity(14). Furthermore, some patients demonstrated decade long persistence of CAR T cells after administration(15). Interestingly, those CAR T cells were CD4 T cells, suggesting that CD4 T cells may play a role in establishing long-term memory of engineered T cells.

While the design of CARs brings many benefits, CAR T cells also have some disadvantages which we previously described in the introduction of this thesis. In short: Depending on the CAR design the artificial signaling mechanism of CAR T cells can induce tonic signaling, which can lead to antigen-independent proliferation, differentiation, and upregulation of co-inhibitory molecules(16). Upon antigen-dependent activation, CAR T cells are sensitive to overactivation, which may compromise their efficacy especially in settings with high tumor burdens or in solid tumors. Another limitation of CAR T cells is the risk of antigen escape which can occur for various reasons: heterogeneous

expression levels, genetic loss of target antigen, trogocytosis or target protein cleavage which has been described in the case of BCMA(17).

We believe that TCR T cells can be used to overcome disadvantages associated with CAR T cells because TCR T cells have distinct advantages. TCRs are the natural receptors of T cells that do not contain costimulatory or signaling domains, additionally TCRs are exclusively activated upon cognate antigen encounter. TCR T cells might therefore be less sensitive to overstimulation compared to CAR T cells, increasing their ability to deal with relatively high tumor burdens and potentially leading to increased persistence *in vivo*(18). The degree of trogocytosis that occurs between a T cell and target antigen is dependent on the receptor affinity for its ligand(19). TCRs generally have lower affinity for their ligand than CARs(20), which likely underlies the limited trogocytosis by TCR T cells. Furthermore, TCR T cells have the theoretical capability to target peptides derived from any protein presented by HLA molecules, thereby expanding their targetable protein repertoire to include secreted proteins or those essential for tumor cell survival, such as certain transcription factors. Targeting antigens essential for survival makes it unlikely that tumor cells evade TCR T cells through complete loss of these vital proteins. Although TCR T cells offer several advantages some limitations should also be considered. For instance, the HLA-restricted recognition of TCR T cells restricts the applicability of TCR T cells to patients expressing the targeted HLA restriction element. In addition, HLA class-I-restricted TCRs often depend on presence of the CD8 co-receptor for optimal functionality, limiting their applicability in CD4 T cells(21). This observation is confirmed in **Chapters 2, 3 and 4** where it is demonstrated that all HLA class-I restricted TCRs are dependent on the CD8 co-receptor for optimal functionality. CD8-dependent TCRs are not functional in CD4 T cells, and CD4 T-cell help can thus not be provided by introducing the TCR alone into CD4 T cells. In a setting of combination therapy with CAR T cells, this might not be a problem as the TCR could be inserted in CD8 T cells only, while the CAR could be introduced in both CD8 and CD4 T cells. Consequently, CAR-CD4 T cells could provide help to the TCR-engineered CD8 T cells. An alternative approach is to functionalize TCR-CD4 T cells by co-introducing the CD8 molecule alongside the TCR in CD4 cells to generate functional TCR-engineered CD4 T cells(22).

While antigen escape can compromise efficacy of CAR T-cell therapy, antigen escape after TCR T-cell therapy can also occur, partially through alternative mechanisms. After TCR T-cell therapy, tumor cells can escape that do not or no longer present the targeted epitope or HLA molecule(23). Epitope negativity can occur when the protein from which the epitope is derived is not expressed or expression is lost, when problems in the peptide processing and presentation machinery hamper presentation of the targeted epitope, or when a mutation in the targeted epitope arises resulting from the genetic instability of the malignancy. In cancer, mutations in the peptide processing and presentation

machinery can lead to HLA negative tumor cells that could escape TCR-mediated recognition(24). Alternatively, full genetic loss of one of the HLA loci can occur as a result of immunological pressure. This is sometimes observed after haploidentical allogeneic stem cell transplantation for treatment of leukemia and the same might following TCR T cell therapy(25). Overall tumor cells can escape TCR-mediated recognition via multiple mechanisms, some of which may not overlap with potential escape mechanisms following CAR T cell therapy.

In summary, CAR and TCR T cells have distinct mechanisms of antigen recognition as well as various functional differences. In CAR and TCR combination therapy, one subset might be able to cope better with low tumor burdens while the other subset might cope better with high tumor burdens. Additionally, both CD4 and CD8 tumor-targeting T cells can be part of a combination product. Furthermore, the incorporation of multi-antigen-targeting through different mechanisms should ensure that tumor cells escaping recognition by one T-cell population can still be targeted by the other. Consequently, CAR and TCR therapies are likely to complement each other very well, improving the overall outcome of T-cell therapy. In this combined approach multiple TCRs and CARs could be incorporated. By including multiple receptors, the likelihood of antigen escape is further reduced. When selecting multiple TCRs, an optimal combination should target different antigens as well as different HLA alleles, thereby reducing the chance of HLA-based escape or escape through target protein loss.

TCRs targeting immunoglobulins

Targeting immunoglobulin heavy or light chains via TCR-mediated therapy might be a promising strategy for immunotherapy of multiple myeloma (MM). MM cells are the malignant counter part of plasma cells and are therefore characterized by high production and secretion of monoclonal immunoglobulins. In MM, 54% of cases produce IgG immunoglobulins and 22% produced IgA, but IgA production correlates with poorer prognosis(26). Other immunoglobulin subtypes are rare, and 20% of the MM cases MM secrete immunoglobulin light chains only(27). As B cells develop into plasma cells BCR surface expression is lost. Consequently, malignant plasma cells do not express surface BCR preventing targeting by CAR T cells. Instead, TCR mediated targeting of constant domains of immunoglobulin heavy or light chains forms an attractive approach for TCRs with widespread applicability for therapy of MM. In **Chapter 4** of this thesis, TCRs specific for peptides from IgG and IgA immunoglobulin heavy chain constant domains, presented by HLA-A*02:01 and HLA-B*07:02, respectively, were identified. MM cells isolated from patient bone marrow samples expressed IgA or IgG immunoglobulin at levels 100-1000-fold higher compared to housekeeping genes. Effective lysis of MM cells by immunoglobulin-TCR T cells combined with lack of recognition of healthy cells other than B cells, underscores the potential of immunoglobulin-specific TCR T cells as

a therapeutic strategy for MM.

Immunoglobulin-specific TCR T cells offer a theoretical benefit over CD19 or BCMA CAR therapy, as they only deplete the peripheral blood B cells expressing the targeted isotype, while leaving B cells of another isotype intact. Especially in the context of IgA-specific TCR T cells for therapy of MM or other IgA+ malignancies, preserving IgG+ B/plasma cells holds significant advantages. IgG serves as the primary antibody isotype responsible for systemic protection against pathogens. By sparing IgG+ B/plasma cells, patients would retain this crucial aspect of B-cell immunity. Consequently, this preservation could potentially alleviate the need for patients to receive soluble immunoglobulins after T-cell therapy and thereby improve health and reduce the need of additional therapeutic interventions, leading to a better quality of life.

Previously, immunoglobulin kappa light chain specific CAR T cells have been developed to treat surface BCR positive B-cell malignancies(28). However, efficacy of IgK CAR T cells has fallen behind that of CD19 CAR T cells, hampering their further application. The functionality of IgK CAR T cells was inhibited by immunoglobulin proteins present in human serum(29, 30). Additionally, CARs covered by circulating antigen lose their ability to respond to antigen positive tumor cells(17). Given these functional differences, we hypothesized that TCR T cells might be influenced differently when the target protein is present in serum. TCR T cells recognizing peptides from proteins present in circulation might indirectly recognize cells that naturally don't express the targeted protein. In **Chapter 4**, we proposed that cells proficient in endocytosis, such as antigen presenting cells (APCs), might take-up full protein and sequentially process and cross-present peptides from these proteins in HLA class-I on their cell surface. We investigated the effect of co-culturing dendritic cells (DCs) as well as various adherent cells of different tissue origins with varying concentrations of soluble IgG immunoglobulins. DCs co-cultured with varying concentrations of soluble IgG immunoglobulins stimulated IgG-TCR T cells in a dose dependent manner, while adherent cells co-cultured with high concentrations of immunoglobulins did not generally induce T-cell activation. Only limited recognition of fibroblasts from one of two donors was observed after co-culture with the highest dose of immunoglobulins. These data suggest that uptake of immunoglobulins followed by cross-presentation and recognition by IgG-TCR T cells is dose dependent and mostly restricted to cells with high endocytic capacity and high T-cell stimulatory capacity such as DCs. We hypothesize that immunoglobulin-TCR T cells *in vivo* will be boosted by cross-presenting APCs, stimulating the anti-tumor response of immunoglobulin-TCR T cells. This phenomenon is likely restricted to the initial period after start of therapy when immunoglobulin serum concentrations are high. When immunoglobulin-specific TCR T cells are effective and MM tumor burdens reduce, serum immunoglobulin concentrations will reduce simultaneously abrogating cross-

presentation of immunoglobulin peptides. While it hasn't been investigated in this study it is reasonable that as a consequence of T-cell recognition DCs cross-presenting these immunoglobulin epitopes are killed. In natural immune responses, CTLs lyse antigen expressing DCs as part of the negative feedback loop after initiation of an immune response(31). While cross-presentation by APCs is expected to benefit TCR T cells, cross-presentation by other cell types might result in undesired on-target off-tumor toxicity. Our data showed that cross-presentation of immunoglobulin peptides by adherent cells is very limited, but it might still occur under specific circumstances. It remains difficult to predict how observations made in an *in vitro* co-culture assay translate to an *in vivo* setting. If cross-presentation of immunoglobulin peptides occurs more broadly than anticipated and leads to unexpected toxicity it should be considered that recognition of cross-presented immunoglobulin peptides was dose-dependent and consequently reducing the concentrations of serum immunoglobulin will reduce cross-presentation by bystander cells and subsequent consequences.

MM is typically characterized by the secretion of monoclonal immunoglobulin(27). However, 20% of MM cases secrete immunoglobin light chains only, and some MM are classified as non-secreters(27). When considering TCR therapy targeting immunoglobulins, it becomes essential to establish whether these malignancies completely lack production of immunoglobulin heavy chains or if secretion is hampered while production continues. If secretion is impaired but immunoglobulins are still produced, peptides derived from these proteins can still be presented in HLA molecules, enabling TCR T cells to recognize these malignancies. On the other hand, if light-chain-only or non-secreting MM completely lack heavy chain production, these malignancies cannot be treated with immunoglobulin heavy chain-targeting TCR T-cell therapy.

In addition to MM, TCRs targeting IgA and IgG could be explored in the context of other B-cell mediated diseases such as autoimmune disorders. Recently, CD19-targeting CAR T cells have shown promise in treating patients with refractory systemic lupus erythematosus and other B-cell-mediated autoimmune diseases such as anti-synthetase syndrome and myasthenia gravis(32, 33). However, CD19 CAR T cells deplete all B cells irrespective of their Ig subtype. Most autoimmune disorders are thought to be associated with B cells of an IgG subtype. The subtype-specificity of IgG-targeting TCRs could therefore provide an interesting conceptual advantage by sparing healthy B cells of non-IgG subtypes such as IgA, which plays a crucial role in mucosal immunology. Additionally, IgA-targeting TCR T cells could be an interesting approach to treat patients suffering from IgA nephropathy, as it would leave IgG producing B cells intact.

Verifying epitope presentation prior to therapy

As T-cell therapy is an extremely costly treatment it will be very important to select

and treat patients for which a good response to therapy is expected. For TCR T cells to recognize malignant cells the target proteins need to be expressed, the target HLA needs to be expressed and epitopes derived from the target protein need to be processed and presented in HLA. In this thesis, expression of the target gene in the malignant population of interest was measured by qPCR. QPCR determines the average expression level of the target gene on a bulk level. In this thesis as well as in other studies we observed that in most cases, target gene expression combined with expression of the respective HLA allele as determined by genotyping can be used to predict lysis by TCR T cells. However, exceptions might exist in which HLA and gene expression do not precisely predict lysis by TCR T cells. In **Chapter 3**, an example was observed: Jchain HLA-A*24:02 specific TCR T cells did not lyse MM materials MM.J3 and MM.J4 despite high *JCHAIN* expression of 14- and 39-fold compared to housekeeping genes, respectively. An HLA-A*24:02 restricted control T-cell clone did lyse these MM materials, indicating that HLA-A*24:02 was indeed expressed. In contrast, Jchain positive, HLA-A*24:02 positive MM sample MM.J2 was lysed. These data suggest that despite expression of the target gene, the Jchain A24 epitope is not always presented by HLA-A*24:02 positive MM cells. Rather than a general lack of processing and presentation of this target peptide it seems that the targeted peptide is absent in HLA molecules from some cell populations.

Cells of the hematopoietic lineage as well as MM cell can express immunoproteasomes, which preferentially cleaves peptides with hydrophobic c-terminal amino acid(34). The Jchain A24 epitope contains such an c-terminal amino-acid and predictions suggest that the Jchain A24 epitope relies on processing and presentation by the immunoproteasome(35). Activity of the immunoproteasome varies between MM tumors (36), potentially influencing processing and presentation of the Jchain A24 epitope. To test this, immunoproteasome activity in MM samples should be determined and correlated to recognition by Jchain A24 TCR T cells. The discrepancy between gene expression and TCR T-cell recognition observed in this case underscores the inadequacy of solely relying on gene expression as a diagnostic tool to predict recognition by TCR T cells.

In addition to investigating gene or protein expression, it is essential to establish HLA expression as well as peptide presentation before initiating therapy. Ideally, peptide:HLA complexes should be detected in a fast and direct manner, for example by staining the peptide:HLA complex of interest. Target peptide:HLA complexes on malignant cells can be detected by staining with a soluble variant of the therapeutic TCR. However, sensitivity currently only allows detection of artificially overexpressed peptide:HLA complexes and further optimization would be needed to achieve sufficient sensitivity to detect natural levels of peptide:HLA complexes on cells(37). Rather than direct detection of the target peptide:HLA complex, functional analysis of TCR T-cell recognition might be a more

feasible way to assess targetability of malignancies. Potential TCR T-cell recognition of a patient sample could be swiftly assessed by using TCR transgenic T-cell reporter cell lines like the Jurkat 76-based triple parameter reporter(38). These cells allow detection of TCR triggering by flowcytometry within one day of co-culture with target cells of interest. Confirming TCR T-cell recognition of malignancies prior to therapy will be critical, especially for certain epitopes. Incorporating such diagnostics into clinical trials could be valuable regardless of the targeted epitope, as the generated data could ultimately be used for outcome predictions or to refine therapies in the future.

Expand coverage of TCRs for therapy of MM

As the field of TCR therapy advances, identifying new TCRs with alternative HLA restrictions and specificities becomes crucial to extend the patient population eligible for TCR therapy. Maximizing accessibility will require identification of TCRs targeting less frequently expressed HLA alleles. Among the various HLA types, HLA-A alleles exhibit the lowest degree of polymorphism, positioning these alleles as the most interesting restriction alleles for TCRs to efficiently achieve broad coverage within the population(39). Alternatively, HLA-C shares a similar degree of polymorphism. However, identification of HLA-C restricted TCR comes with some challenges, which became apparent when we tried to identify HLA-C restricted Jchain specific TCRs (data not shown). T cells expressing certain KIR molecules bound HLA-C pHLA-multimers via the KIR receptor instead of their TCR. Interference by KIRs could be overcome by counterstaining and exclusion of KIR+ T cells(40).

Attempts to identify TCR targeting less common HLA alleles currently face considerable obstacles, including limited research funding, publication bias, a lack of data on rarer HLA alleles, and the challenge of recruiting patients for clinical trials. Scientific data on rare HLA alleles is limited for example in peptidome databases, which mostly collected data from materials expressing common HLA alleles. As a result, peptide binding motifs for rare alleles are weakly defined, hampering identification of peptides presented by rare alleles, which is a fundamental starting point of the TCR identification pipeline. Specific attempts are needed to establish peptide binding motifs for rare HLA alleles. Approaches may involve introduction of single HLA molecules into HLA class-I negative cells followed by peptidome analysis. Alternatively, HLA positive malignant cell lines could be used, for which endogenous HLA knockout followed by introduction of a single HLA allele should be performed prior to peptidome analysis.

Furthermore, the identification of new target peptide-HLA combinations is central to extending the repertoire of available TCRs, a process that could be improved by targeted mass spectrometry. Targeted mass spectrometry involves spiking the peptide of interest, labeled with a stable epitope, into the biological sample, followed by parallel reaction

monitoring (PRM) mass spectrometry(41). Targeted mass spectrometry is a refined approach with increased sensitivity and selectivity over regular methods.

Moreover, the limited availability of patient material for pre-clinical testing underscores the necessity for comprehensive biobanking and the open exchange of diagnostic samples among institutes. This collaborative spirit is vital for the pre-clinical evaluation of therapies targeting rare HLA types.

Combination therapy with TCRs

Effective viral clearance after infection is achieved through oligo or polyclonal T-cell responses directed towards multiple antigens and multiple HLA restriction alleles(42). Similarly, effectiveness of allogeneic stem cell transplantation (allo-SCT) for hematologic malignancies has been attributed to polyclonal T-cell responses(43). In transgenic T-cell therapy, single-antigen-targeting regimens are associated with antigen escape and disease relapse. To improve efficacy, it would be ideal to include multiple receptors with different characteristics in a transgenic T-cell product to mimic the polyclonal immune responses underlying effective viral defense or cure after allo-SCT for malignancies(44). For multi-targeting TCR therapy of B-/plasma cell malignancies different types of target antigens are explored, one type of targets are tumor-associated antigens (TAAs). TAAs are proteins that are expressed by tumors of various origins while expression in healthy adult tissues is absent or restricted to immune privileged sites. TAAs like MAGE family members, PRAME and NY-ESO-1 are expressed in some MM cases. TAAs, despite heterogeneous expression, might be appealing targets as they often appear in advanced disease stages and expression inversely correlated with survival (19, 45). Many TAA-specific TCRs have already been identified but these TCRs typically target commonly expressed HLA alleles and therefore the target HLA diversity of such TCRs is limited(46). The efficacy of an HLA-A*02:01 restricted TCR targeting NY-ESO-1 has been investigated for therapy of MM. NY-ESO-1 is expressed in one-third of the MM cases (45). 70% of the patients with NY-ESO-1+ tumor had a (near) complete response after treatment with autologous stem cell transplantation followed by NY-ESO-1 TCR T-cell infusion (23). Specific depletion of NY-ESO-1+ tumor cells demonstrated on-target activity of NY-ESO-1 TCR T cells and simultaneously revealed that antigen escape variants can arise when targeting heterogenous TAAs (23, 45). To achieve full tumor clearance with TCR T cells, especially when targeting heterogeneous antigens like TAAs, combination therapy should be employed. However, for many patients TAA-targeting TCRs are not available, either because TAAs are not expressed in the patient's malignant cells or due to the limited HLA diversity of currently available TAA-targeting TCRs. Therefore, many patients would benefit from an additional set of TCRs with different HLA restriction and targeting different types of antigens, such as the B-cell antigen targeting TCRs identified in this thesis.

Throughout this thesis, multiple TCRs targeting proteins expressed in MM cells are described, namely TCRs specific for peptides from BOB1 in HLA-B*35:01, Jchain in HLA-A*01:01, Jchain in HLA-A*03:01, Jchain in HLA-A*11:01, Jchain in HLA-A*24:02, IgG in HLA-A*02:01, and IgA in HLA-B*07:02 TCR. Previous work has identified a BOB1-targeting TCR recognizing a peptide presented in HLA-B*07:02(47). Collectively, these TCRs target 3 different target proteins: BOB1, the Jchain, and immunoglobulin heavy chains. Each of these target proteins presents a promising candidate for TCR T-cell therapy because of favorable characteristics. BOB1, encoded by the *POU2AF1* gene, is a transcriptional coactivator and BOB1 is expressed in at normal levels in MM. Expression of BOB1 has been shown to be essential for the survival of MM cells by us and others(unpublished data)(48). MM cells with knockout of the BOB1 protein do not survive, indicating that it is unlikely that MM cells escape BOB1-TCR therapy by loss of the target protein. All patient-derived MM samples tested to date express the BOB1 protein. While we did not analyze single cell-based gene expression profiles, the necessity of BOB1 suggests homogenous expression within MM tumors. The Jchain represents a different type of antigen, which is expressed in approximately 80% of MM samples independent of the produced immunoglobulin isotypes(49, 50). Currently, there is no evidence for an indispensable role for the Jchain in MM cells. A benefit of targeting the Jchain is the enormous amount of Jchain protein produced in MM. As established in **Chapter 3** Jchain gene expression exceeds expression of housekeeping genes by many-fold. Immunoglobulin heavy chains share characteristics with the Jchain. An essential role for immunoglobulins in MM seems unlikely, but the enormous levels of immunoglobulins produced by MM cells enables potent recognition by immunoglobulin-specific TCR T cells. Our data has demonstrated that both Jchain- and immunoglobulin-targeting TCR T cells can effectively eliminate MM cells from patient BM samples in short term co-cultures. For highly expressed target proteins, the likelihood of identifying TCRs with sufficient avidity to recognize malignant cells increases(51), which probably contributed to the identification of TCRs targeting these proteins in this thesis. BOB1, Jchain and immunoglobulin TCRs identified in this thesis have various HLA restrictions. In a combination regimen, our TCRs can be used to target different proteins, each with their own characteristics, across different HLA restriction alleles.

To explore the hypothetical MM patient coverage that could be treated with one or more of these TCRs, several factors need to be considered, including gene expression frequencies of target antigens and HLA allele frequencies in the population. The expression frequencies of the target proteins are in MM: 100% for BOB1, 80% for Jchain, 52% for IgG and IgA is expressed in 21% of cases. Calculations including gene expression and HLA alleles frequencies of the identified TCRs reveal that TCRs identified in this thesis can be used to treat approximately 67% of patients with MM. For 28% of the patients more than one TCR would be available. Together these identified TCRs can

significantly contribute to the availability of TCRs for patients with MM as well as the potential option to generate multi-targeting TCR products for therapy of patients with MM complementing TAA-targeting TCRs such as the NY-ESO-1 TCR.

REFERENCES

1. Cohen AD, Garfall AL, Stadtmauer EA, Melenhorst JJ, Lacey SF, Lancaster E, et al. B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma. J Clin Invest. 2019;129(6):2210-21.
2. Sheikh S, Migliorini D, Lang N. CAR T-Based Therapies in Lymphoma: A Review of Current Practice and Perspectives. Biomedicines. 2022;10(8).
3. Brudno JN, Maric I, Hartman SD, Rose JJ, Wang M, Lam N, et al. T Cells Genetically Modified to Express an Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor Cause Remissions of Poor-Prognosis Relapsed Multiple Myeloma. J Clin Oncol. 2018;36(22):2267-80.
4. Hamieh M, Dobrin A, Cabriolu A, van der Stegen SJC, Giavridis T, Mansilla-Soto J, et al. CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. Nature. 2019;568(7750):112-6.
5. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013;500(7463):415-21.
6. Anderson KC, Bates MP, Slaughenhoupt BL, Pinkus GS, Schlossman SF, Nadler LM. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. Blood. 1984;63(6):1424-33.
7. Salles G, Barrett M, Foà R, Maurer J, O'Brien S, Valente N, et al. Rituximab in B-Cell Hematologic Malignancies: A Review of 20 Years of Clinical Experience. Adv Ther. 2017;34(10):2232-73.
8. Casulo C, Maragulia J, Zelenetz AD. Incidence of hypogammaglobulinemia in patients receiving rituximab and the use of intravenous immunoglobulin for recurrent infections. Clin Lymphoma Myeloma Leuk. 2013;13(2):106-11.
9. Rodriguez-Otero P, Ailawadhi S, Arnulf B, Patel K, Cavo M, Nooka AK, et al. Ide-cel or Standard Regimens in Relapsed and Refractory Multiple Myeloma. N Engl J Med. 2023;388(11):1002-14.
10. Park JH, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, et al. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. N Engl J Med. 2018;378(5):449-59.
11. Deng Q, Han G, Puebla-Osorio N, Ma MCJ, Strati P, Chasen B, et al. Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. Nat Med. 2020;26(12):1878-87.
12. Zhao Z, Condomines M, van der Stegen SJC, Perna F, Kloss CC, Gunset G, et al. Structural Design of Engineered Costimulation Determines Tumor Rejection Kinetics and Persistence of CAR T Cells. Cancer Cell. 2015;28(4):415-28.
13. Sommermeyer D, Hudecek M, Kosasih PL, Gogishvili T, Maloney DG, Turtle CJ, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity in vivo. Leukemia. 2016;30(2):492-500.
14. Liadi I, Singh H, Romain G, Rey-Villamizar N, Merouane A, Adolacion JR, et al. Individual Motile CD4(+) T Cells Can Participate in Efficient Multikilling through Conjugation to Multiple Tumor Cells. Cancer Immunol Res. 2015;3(5):473-82.
15. Melenhorst JJ, Chen GM, Wang M, Porter DL, Chen C, Collins MA, et al. Decade-long leukaemia remissions with persistence of CD4(+) CAR T cells. Nature. 2022;602(7897):503-9.
16. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med. 2015;21(6):581-90.
17. Pont MJ, Hill T, Cole GO, Abbott JJ, Kelliher J, Salter AI, et al. γ-Secretase inhibition increases efficacy of BCMA-specific chimeric antigen receptor T cells in multiple myeloma. Blood. 2019;134(19):1585-97.
18. Wachsmann TLA, Wouters AK, Remst DFG, Hagedoorn RS, Meeuwsen MH, van Diest E, et al. Comparing CAR and TCR engineered T cell performance as a function of tumor cell exposure. Oncoimmunology. 2022;11(1):2033528.
19. Condomines M, Hose D, Raynaud P, Hundemer M, De Vos J, Baudard M, et al. Cancer/testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis. J Immunol. 2007;178(5):3307-15.
20. Mao R, Kong W, He Y. The affinity of antigen-binding domain on the antitumor efficacy of CAR T cells: Moderate is better. Front Immunol. 2022;13:1032403.
21. Kessels HW, Schepers K, van den Boom MD, Topham DJ, Schumacher TN. Generation of T cell help through a MHC class I-restricted TCR.

J Immunol. 2006;177(2):976-82.
22. van Loenen MM, Hagedoorn RS, de Boer R, Falkenburg JH, Heemskerk MH. Extracellular domains of CD8α and CD8ß subunits are sufficient for HLA class I restricted helper functions of TCR-engineered CD4(+) T cells. PLoS One. 2013;8(5):e65212.
23. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat Med. 2015;21(8):914-21.
24. Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy. Int J Cancer. 2010;127(2):249-56.
25. Vago L, Perna SK, Zanussi M, Mazzi B, Barlassina C, Stanghellini MT, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. N Engl J Med. 2009;361(5):478-88.
26. Nair B, Waheed S, Szymonifka J, Shaughnessy JD, Jr., Crowley J, Barlogie B. Immunoglobulin isotypes in multiple myeloma: laboratory correlates and prognostic implications in total therapy protocols. Br J Haematol. 2009;145(1):134-7.
27. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc. 2003;78(1):21-33.
28. Ramos CA, Savoldo B, Torrano V, Ballard B, Zhang H, Dakhova O, et al. Clinical responses with T lymphocytes targeting malignancy-associated κ light chains. J Clin Invest. 2016;126(7):2588-96.
29. Vera J, Savoldo B, Vigouroux S, Biagi E, Pule M, Rossig C, et al. T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. Blood. 2006;108(12):3890-7.
30. Köksal H, Dillard P, Juzeniene A, Kvalheim G, Smeland EB, Myklebust JH, et al. Combinatorial CAR design improves target restriction. J Biol Chem. 2021;296:100116.
31. Hermans IF, Ritchie DS, Yang J, Roberts JM, Ronchese F. CD8+ T cell-dependent elimination of dendritic cells in vivo limits the induction of antitumor immunity. J Immunol. 2000;164(6):3095-101.
32. Müller F, Boeltz S, Knitza J, Aigner M, Völkl S, Kharboutli S, et al. CD19-targeted CAR T cells in refractory antisynthetase syndrome. Lancet. 2023;401(10379):815-8.
33. Müller F, Taubmann J, Bucci L, Wilhelm A, Bergmann C, Völkl S, et al. CD19 CAR T-Cell Therapy in Autoimmune Disease- A Case Series with Follow-up. N Engl J Med. 2024;390(8):687-700.
34. Toes RE, Nussbaum AK, Degermann S, Schirle M, Emmerich NP, Kraft M, et al. Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products. J Exp Med. 2001;194(1):1-12.
35. Gomez-Perosanz M, Ras-Carmona A, Reche PA. PCPS: A Web Server to Predict Proteasomal Cleavage Sites. Methods Mol Biol. 2020;2131:399-406.
36. Besse A, Kraus M, Mendez-Lopez M, Maurits E, Overkleeft HS, Driessen C, et al. Immunoproteasome Activity in Chronic Lymphocytic Leukemia as a Target of the Immunoproteasome-Selective Inhibitors. Cells. 2022;11(5).
37. Walseng E, Wälchli S, Fallang LE, Yang W, Vefferstad A, Areffard A, et al. Soluble T-cell receptors produced in human cells for targeted delivery. PLoS One. 2015;10(4):e0119559.
38. Rosskopf S, Leitner J, Paster W, Morton LT, Hagedoorn RS, Steinberger P, et al. A Jurkat 76 based triple parameter reporter system to evaluate TCR functions and adoptive T cell strategies. Oncotarget. 2018;9(25):17608-19.
39. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernández-Viña MA. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. Hum Immunol. 2001;62(9):1009-30.
40. Schlott F, Steubl D, Ameres S, Moosmann A, Dreher S, Heemann U, et al. Characterization and clinical enrichment of HLA-C*07:02-restricted Cytomegalovirus-specific CD8+ T cells. PLoS One. 2018;13(2):e0193554.
41. Hassan C, Kester MG, Oudgenoeg G, de Ru AH, Janssen GM, Drijfhout JW, et al. Accurate quantitation of MHC-bound peptides by application of isotopically labeled peptide MHC complexes. J Proteomics. 2014;109:240-4.
42. Schober K, Buchholz VR, Busch DH. TCR repertoire evolution during maintenance of CMV-specific T-cell populations. Immunol Rev. 2018;283(1):113-28.
43. Van Bergen CA, Rutten CE, Van Der Meijden ED, Van Luxemburg-Heijs SA, Lurvink EG, Houwing-Duistermaat JJ, et al. High-throughput characterization of 10 new

minor histocompatibility antigens by whole genome association scanning. Cancer Res. 2010;70(22):9073-83.
44. D'Ippolito E, Schober K, Nauerth M, Busch DH. T cell engineering for adoptive T cell therapy: safety and receptor avidity. Cancer Immunol Immunother. 2019;68(10):1701-12.
45. van Rhee F, Szmania SM, Zhan F, Gupta SK, Pomtree M, Lin P, et al. NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and induces spontaneous humoral and cellular immune responses. Blood. 2005;105(10):3939-44.
46. Shafer P, Kelly LM, Hoyos V. Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects. Front Immunol. 2022;13:835762.
47. Jahn L, Hombrink P, Hagedoorn RS, Kester MG, van der Steen DM, Rodriguez T, et al. TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1. Blood. 2017;129(10):1284-95.
48. de Matos Simoes R, Shirasaki R, Downey-Kopyscinski SL, Matthews GM, Barwick BG, Gupta VA, et al. Genome-scale functional genomics identify genes preferentially essential for multiple myeloma cells compared to other neoplasias. Nature Cancer. 2023;4(5):754-73.
49. Nilssen DE, Brandtzaeg P, Frøland SS, Fausa O. Subclass composition and J-chain expression of the 'compensatory' gastrointestinal IgG cell population in selective IgA deficiency. Clin Exp Immunol. 1992;87(2):237-45.
50. Xu AQ, Barbosa RR, Calado DP. Genetic timestamping of plasma cells in vivo reveals tissue-specific homeostatic population turnover. Elife. 2020;9.
51. González PA, Carreño LJ, Coombs D, Mora JE, Palmieri E, Goldstein B, et al. T cell receptor binding kinetics required for T cell activation depend on the density of cognate ligand on the antigen-presenting cell. Proc Natl Acad Sci U S A. 2005;102(13):4824-9.

APPENDICES

Nederlandse samenvatting
List of Publications
Curriculum vitae
Dankwoord

NEDERLANDSE SAMENVATTING

Het immuunsysteem

De functie van het immuunsysteem is om ons lichaam te beschermen tegen ziekteverwekkers zoals bacteriën en virussen. Het immuunsysteem is een complex systeem bestaande uit verschillende soorten cellen die, ieder met hun eigen functie, onderling samenwerken om ziekteverwekkers onschadelijk te maken. Twee belangrijke celtypen die een rol spelen in het onderzoek beschreven in dit proefschrift zijn de B-cellen en de T-cellen. Zowel B- als T-cellen kunnen, middels receptoren die zij op hun celoppervlak dragen, onderscheid maken tussen de 'bekende' gezonde weefsels van het eigen lichaam en vreemde structuren zoals door virussen of bacteriën geïnfecteerde weefsels. B-cellen ontwikkelen zich tijdens een infectie tot plasmacellen. Deze cellen produceren antilichamen die binnengedrongen ziekteverwekkers kunnen binden. De ziekteverwekkers worden hierdoor voorzien van herkenningsstructuren waardoor andere immuuncellen de bacteriën en virussen opnemen en doden.

T-cellen kunnen geïnfecteerde cellen herkennen via de T-celreceptor (TCR), een eiwit dat zich op het oppervlak van T-cellen bevindt. Lichaamscellen presenteren continu peptiden, kleine fragmenten afkomstig van de eiwitten aanwezig in de cel, die in humaan leukocytenantigeen (HLA) op het celoppervlak komen. Wanneer een antigeen, een peptide afkomstig van een lichaamsvreemd eiwit, in HLA gepresenteerd wordt, kan dit vervolgens door T-cellen via de TCR worden herkend. T-cellen die middels hun TCR een antigeen herkennen, worden geactiveerd. Dit heeft verschillende gevolgen. Voor cytotoxische T-cellen is de belangrijkste uitkomst dat geïnfecteerde cellen worden aangevallen en gedood.

Naast het herkennen van geïnfecteerde cellen zijn T-cellen in sommige gevallen ook in staat om tumorcellen, die als gevolg van DNA-schade gemuteerde peptide presenteren, te herkennen. Dit kan echter alleen plaatsvinden wanneer een tumorcel zoveel DNA-schade heeft dat de T-cellen hem kunnen onderscheiden van gezonde lichaamscellen. Vaak is dit niet het geval waardoor tumoren niet worden aangevallen en ongecontroleerd kunnen groeien en ziekte veroorzaken. Wanneer T-cellen wel in staat zijn om tumorcellen te herkennen, kan het voorkomen dat de tumorcellen zich over tijd zo ontwikkelen dat ze T-cel-activatie kunnen remmen en daardoor niet langer worden aangevallen.

B-celmaligniteiten

Kanker kan ontstaan doordat in cellen een ophoping van DNA-mutaties heeft plaatsgevonden. Deze mutaties kunnen een combinatie zijn van spontane DNA-afwijkingen en van DNA-schade opgelopen door blootstelling aan externe invloeden. Wanneer DNA-mutaties hebben plaatsgevonden in genen die verantwoordelijk

zijn voor het reguleren van celdeling en geprogrammeerde celdood kunnen cellen ongecontroleerd gaan delen. Afhankelijk van de afkomst van de cel van origine ontstaat een bepaald type kanker. Wanneer B-cellen of plasmacellen maligne transformatie ondergaan ontstaan B-cel- of plasmacelmaligniteiten.

Genetisch gemodificeerde T-cellen als kankerbehandeling

CAR-T-celtherapie, wat staat voor Chimeric Antigen Receptor T-celtherapie, is een innovatieve vorm van immuuntherapie die kan worden gebruikt voor de behandeling van B-celmaligniteiten. CARs zijn artificiële eiwitten gebaseerd op de structuur van antistoffen. CARs worden ontworpen om te binden aan eiwitten die specifiek tot expressie komen op het oppervlak van kankercellen, in dit geval B-cel-specifieke eiwitten. Een voorbeeld van een eiwit waar CAR T-cellen tegen kunnen zijn gericht is het CD19-eiwit op het oppervlak van B-cellen.

Het proces van CAR-T-celtherapie begint met het verzamelen van de eigen T-cellen van de patiënt. Vervolgens worden deze T-cellen voorzien van de genetische informatie die codeert voor de CAR. Nadat de T-cellen genetisch zijn gemodificeerd om de CARs tot expressie te brengen, worden ze in grote aantallen in het laboratorium vermeerderd. Vervolgens worden ze teruggegeven aan de patiënt via een infusie. Wanneer deze CAR T-cellen in het lichaam een kankercel vinden die het doeleiwit draagt, bindt de CAR aan het oppervlak van de kankercel en activeert de T-cel om deze te vernietigen.

CAR-T-celtherapie heeft bijzondere successen behaald in de behandeling van B-celmaligniteiten, waaronder genezing van een aanzienlijk aantal patiënten. Echter, een belangrijke beperking is het fenomeen van antigeenverlies, waarbij tumoren veranderingen ondergaan en ze het doelantigeen verliezen, wat de effectiviteit van de therapie kan hinderen. Dit laat zien dat het ontwikkelen van nieuwe en aanvullende therapieën belangrijk is.

Dit proefschrift

Om in de toekomst meer patiënten te kunnen genezen is het belangrijk behandelingen te ontwikkelen die CAR T-cellen kunnen complementeren en in de toekomst wellicht tegelijkertijd als behandeling kunnen worden ingezet om zo het fenomeen van antigeenverlies te kunnen compenseren. Als behandeling focussen wij ons op TCR T-cellen die in staat zijn om peptiden afkomstig van B-cel-specifieke eiwitten te herkennen. Het voordeel van TCR T-cellen is dat ze peptiden afkomstig van zowel intracellulaire als oppervlakte eiwitten kunnen herkennen en daardoor een breder arsenaal hebben dan CAR T-cellen. Het doel van dit proefschrift is om zoveel mogelijk TCRs te identificeren die in de toekomst eventueel gebruikt kunnen worden voor TCR-T-celtherapie bij de behandeling van B-celmaligniteiten en multipel myeloom.

In **hoofdstuk 2** hebben we een brede zoektocht uitgevoerd om TCRs te identificeren voor de therapie van B-cel- en plasmacelmaligniteiten. We begonnen met het selecteren van potentiële doeleiwitten voor TCR-gentherapie van deze maligniteiten. Hierbij maakten we gebruik van genexpressiedata van patiënten met acute lymfatische leukemie, chronische lymfatische leukemie en multipel myeloom en van gezonde cellen. We selecteerden genen die tot expressie kwamen in een van de maligniteiten, terwijl de expressie in gezonde cellen, met uitzondering van B-cellen, afwezig was. Deze aanpak leverde 28 doeleiwitten op die veelbelovend zouden kunnen zijn voor de therapie van B-cel- en plasmacelmaligniteiten. We identificeerden peptiden afkomstig van deze eiwitten, gepresenteerd in HLA op het oppervlak van maligne B-cellen door middel van peptide-elutie en massaspectrometrie. Onze selectie van doel-HLA-allelen omvatte HLA-A*01:01, -A*24:01, -B*08:01 en -B*35:01. Twintig doelpeptiden afgeleid van een van de geselecteerde eiwitten gepresenteerd in een van de gekozen HLA-moleculen werden geïdentificeerd. Om immunologische tolerantie te omzeilen, werden T-celklonen geïsoleerd uit materiaal van gezonde donoren die de HLA-allelen van interesse niet tot expressie brachten. Door middel van peptide-HLA-multimeren isoleerden we 5519 T-celklonen. Hierna identificeerden we via screenings 23 T-celklonen specifiek voor een van de doelpeptiden, met functionele reactiviteit tegen van nature gepresenteerd antigeen. We bepaalden de sequenties van de TCRs van zes T-celklonen die gevoelig genoeg waren om B-celmaligniteiten te herkennen en aan te vallen. Na TCR-overdracht naar donor-T-cellen herkenden en doodden TCR T-cellen effectief en specifiek B-celmaligniteiten. De geselecteerde TCRs herkennen peptiden afkomstig van FCRL5, gepresenteerd in HLA-A*01:01, VPREB-3 in HLA-A*24:02 en BOB1 in HLA-B*35:01. Afhankelijk van het expressieprofiel van de doeleiwitten zijn deze TCRs waardevol voor behandeling van een of meerdere typen B-cel- en plasmacelmaligniteiten.

In **Hoofdstuk 3** hebben we TCRs geïdentificeerd die peptiden herkennen van de joining chain (Jchain), die kunnen worden gebruikt voor de behandeling van multipel myeloom. In Hoofdstuk 2 was de Jchain al geïdentificeerd als een van de doeleiwitten voor de therapie van B-cel- en plasmacelmaligniteiten. In gezonde plasmacellen fungeert de Jchain als een verbindingsmolecuul tussen monomeren van IgA en IgM wanneer ze als multimeren worden uitgescheiden. De *JCHAIN* expressie in multipel myeloomcellen is onafhankelijk van het immunoglobuline-isotype dat wordt geproduceerd, en de *JCHAIN* komt hoog tot expressie in de meeste multipel myeloom tumoren. Omdat de Jchain een eiwit is dat door cellen wordt uitgescheiden, kan het niet worden herkend door CAR T-cellen. TCR T-cellen specifiek voor Jchain-afkomstige peptiden kunnen wel worden gebruikt om *JCHAIN*-positieve cellen aan te vallen. Ons doel in dit hoofdstuk was om TCRs te identificeren die Jchain-peptiden gepresenteerd in verschillende HLA-A-allelen kunnen herkennen. Doelpeptiden afgeleid van de Jchain gepresenteerd in HLA werden geïdentificeerd uit het HLA-klasse-I-peptidoom van multipel myeloom-cellen.

Met behulp van de pHLA-multimer-technologie, zoals gebruikt in Hoofdstuk 2, werden Jchain-specifieke T-celklonen geïsoleerd. Dit resulteerde in de identificatie van vier T-celklonen die Jchain-peptiden gepresenteerd in HLA-A*01:01, -A*03:01, -A*11:01, of -A*24:02 goed herkenden. Deze Jchain-specifieke T-cellen lieten veelbelovende veiligheidsprofielen zien. Door T-cellen uit te rusten met de Jchain-TCRs, observeerden we dat ze multiple myeloom cellen van verschillende patiënten doodmaakten, en alleen wanneer de *JCHAIN* tot expressie kwam. Bovendien verminderde Jchain-TCR T-cellen drastisch de tumorgroei in een preklinisch *in vivo* model van multiple myeloom. Deze TCRs hebben dus potentie voor verdere ontwikkeling als therapie voor patiënten met multipel myeloom. Gezien hun gunstige HLA-restricties zouden met de vier TCRs gezamenlijk ongeveer 60% van de patiënten met *JCHAIN*-positieve multipel myeloom behandeld kunnen worden.

In **Hoofdstuk 4** hebben we ons specifiek gericht op het identificeren van TCRs die kunnen worden toegepast als therapie voor patiënten met multipel myeloom. Multiple myeloomcellen zijn maligne plasmacellen en scheiden daarom vaak grote hoeveelheden immunoglobulinen van een bepaald isotype uit. De meeste patiënten hebben multipel myeloom van het IgG-isotype en 22% van de patiënten heeft het IgA-isotype. Multipel myeloompatiënten met IgA-expressie hebben een ongunstige prognose. We wilden daarom deze beide immunoglobulinen aanvallen via peptiden afkomstig van de constante delen van de IgG- en IgA-zware ketens. Volgens dezelfde aanpak als in Hoofdstuk 2 en Hoofdstuk 3 hebben we geprobeerd T-cellen te identificeren die specifiek zijn voor deze immunoglobulinen. Dit resulteerde in de ontdekking van T-celklonen specifiek voor het LMI-peptide uit het constante deel van de IgG gepresenteerd in HLA-A*02:01, of de SPK- of HPR-peptiden uit het constante deel van IgA gepresenteerd in HLA-B*07:02. Deze T-cel klonen herkenden de multipel myeloomcellijnen en maakten ze dood wanneer de tumorcellen het juiste isotype tot expressie brachten. Specificiteit en functionaliteit bleven behouden na TCR-overdracht naar donor CD8 T-cellen. Immunoglobuline-TCR T-cellen herkenden geen gezonde cellen van zowel niet-hematopoietische als hematopoietische oorsprong, behalve B-cellen. Alleen de B-cellen die het specifieke isotype tot expressie brachten werden gedood, terwijl immunoglobuline-specifieke T-cellen andere B-cellen juist spaarden. Dit wijst op een potentieel klinisch voordeel door behoud van een gedeelte van de B-celimmuniteit, vooral wanneer IgA-TCR T-cellen worden gebruikt. De immunoglobuline-TCR T-cellen waren daarnaast in staat om heel effectief multipel myeloomcellen op te ruimen uit het beenmergweefsel van patiënten wanneer het respectievelijke immunoglobuline en het doel-HLA-allel tot expressie kwamen. Bovendien verminderde IgA-TCR T-cellen effectief de tumorgroei in muizen met multipel myeloom.

In **Hoofdstuk 5** wordt een specifiek aspect van het HLA-klasse-I-ligandoom onderzocht, namelijk de aanwezigheid van onconventioneel lange peptiden. In de methode die we hebben toegepast in de hoofdstukken 2-4, evenals door anderen die werken aan de identificatie of voorspelling van T-cel-epitopen, is de algemene veronderstelling dat HLA-klasse-I korte peptiden van 8-11 aminozuren lang presenteert. Echter, het HLA-klasse-I-ligandoom bevat een aanzienlijk aantal langere peptiden die vaak worden afgedaan als contaminanten uit HLA-klasse-II. In hoofdstuk 5 werd een onconventioneel lang peptide onderzocht op zijn potentiële oorsprong uit HLA-klasse-I. We onderzochten een 20-aminozuur lang (20-mer) FLP-peptide van de IL27-receptor alfa, geïdentificeerd in HLA-A*02:01-positieve cellen, en bestudeerden de binding ervan aan HLA-A*02:01. Het succesvol vouwen van FLP:HLA-A*02:01-complexen toonde aan dat het FLP-peptide inderdaad kon binden aan HLA-A*02:01. Analyse van de kristalstructuur toonde aan dat de eerste 11 aminozuren op een conventionele lineaire manier aan HLA-A*02:01 bonden, terwijl het overige gedeelte een ‘peptidestaart’ vormde die uit het HLA hing. We isoleerden T-cellen die het FLP-peptide herkenden, zoals beschreven in de eerdere hoofdstukken, om te begrijpen hoe zo’n lang peptide kon worden herkend. Onze bevindingen gaven aan dat terwijl sommige T-cellen werden geactiveerd door het kernpeptide alleen, andere de C-terminale staart nodig hadden en de herkenning verloren ging als deze werd afgeknipt of veranderd. Dit hoofdstuk benadrukt de diverse manieren waarop peptiden kunnen binden aan HLA en laat zien dat T-cellen peptiden gebonden aan HLA-klasse-I op onconventionele wijze kunnen herkennen.

Samenvattend hebben we in dit proefschrift elf nieuwe TCRs beschreven die veelbelovend zijn voor behandelingen van B-cel- en plasmacelmaligniteiten. Een groot deel van de TCRs, namelijk de BOB1, Jchain en immunoglobuline TCRs, herkent een doeleiwit dat tot expressie komt in multipel myeloom. Berekeningen waarbij we rekening houden met genexpressie- en HLA-allelfrequenties van de geïdentificeerde TCRs onthullen dat de in dit proefschrift geïdentificeerde TCRs gebruikt kunnen worden om ongeveer 67% van de patiënten met multipel myeloom te behandelen. Voor 28% van de patiënten zou meer dan één TCR beschikbaar zijn. Terwijl nog veel onderzoek nodig zal zijn om te bepalen of deze TCRs inderdaad waardevolle en veilige opties zijn voor behandeling, kunnen deze TCRs potentieel aanzienlijk bijdragen aan de beschikbaarheid van TCRs voor de behandeling van patiënten met multipel myeloom met TCR-gentherapie.

LIST OF PUBLICATIONS

- Wachsmann TLA, **Meeuwsen MH**, Remst DFG, Buchner K, Wouters AK, Hagedoorn RS, Falkenburg JHF, Heemskerk MHM. *Combining BCMA-targeting CAR T cells with TCR-engineered T-cell therapy to prevent immune escape of multiple myeloma.* Blood Adv. 2023 Oct 24;7(20):6178-6183.

- van Amerongen RA, Tuit S, Wouters AK, van de Meent M, Siekman SL, **Meeuwsen MH**, Wachsmann TLA, Remst DFG, Hagedoorn RS, van der Steen DM, de Ru AH, Verdegaal EME, van Veelen PA, Falkenburg JHF, Heemskerk MHM. *PRAME and CTCFL-reactive TCRs for the treatment of ovarian cancer.* Front Immunol. 2023 Mar 21;14:1121973.

- **Meeuwsen MH**, Wouters AK, Wachsmann TLA, Hagedoorn RS, Kester MGD, Remst DFG, van der Steen DM, de Ru AH, van Hees EP, Kremer M, Griffioen M, van Veelen PA, Falkenburg JHF, Heemskerk MHM. *Broadly applicable TCR-based therapy for multiple myeloma targeting the immunoglobulin J chain.* J Hematol Oncol. 2023 Feb 27;16(1):16.

- de Rooij MAJ, Remst DFG, van der Steen DM, Wouters AK, Hagedoorn RS, Kester MGD, **Meeuwsen MH**, Wachsmann TLA, de Ru AH, van Veelen PA, Verdegaal EME, Falkenburg JHF, Heemskerk MHM. *A library of cancer testis specific T cell receptors for T cell receptor gene therapy.* Mol Ther Oncolytics. 2022 Dec 2;28:1-14. Erratum in: Mol Ther Oncolytics. 2023 Oct 23;31:100738.

- **Meeuwsen MH**, Wouters AK, Hagedoorn RS, Kester MGD, Remst DFG, van der Steen DM, de Ru A, van Veelen PA, Rossjohn J, Gras S, Falkenburg JHF, Heemskerk MHM. *Cutting Edge: Unconventional CD8^{+} T Cell Recognition of a Naturally Occurring HLA-A*02:01-Restricted 20mer Epitope.* J Immunol. 2022 Apr 15;208(8):1851-1856.

- Morton LT, Wachsmann TLA, **Meeuwsen MH**, Wouters AK, Remst DFG, van Loenen MM, Falkenburg JHF, Heemskerk MHM. *T cell receptor engineering of primary NK cells to therapeutically target tumors and tumor immune evasion.* J Immunother Cancer. 2022 Mar;10(3):e003715.

- Harryvan TJ, Visser M, de Bruin L, Plug L, Griffioen L, Mulder A, van Veelen PA, van der Heden van Noort GJ, Jongsma ML, **Meeuwsen MH**, Wiertz EJ, Santegoets SJ, Hardwick JC, Van Hall T, Neefjes J, Van der Burg SH, Hawinkels LJ, Verdegaal EM. *Enhanced antigen cross-presentation in human colorectal cancer-associated fibroblasts through upregulation of the lysosomal protease cathepsin S.* J Immunother Cancer. 2022 Mar;10(3):e003591.

&

- Wachsmann TLA, Wouters AK, Remst DFG, Hagedoorn RS, **Meeuwsen MH**, van Diest E, Leusen J, Kuball J, Falkenburg JHF, Heemskerk MHM. *Comparing CAR and TCR engineered T cell performance as a function of tumor cell exposure.* Oncoimmunology. 2022 Feb 1;11(1):2033528.

- **Meeuwsen MH**, Wouters AK, Jahn L, Hagedoorn RS, Kester MGD, Remst DFG, Morton LT, van der Steen DM, Kweekel C, de Ru AH, Griffioen M, van Veelen PA, Falkenburg JHF, Heemskerk MHM. *A broad and systematic approach to identify B cell malignancy-targeting TCRs for multi-antigen-based T cell therapy.* Mol Ther. 2022 Feb 2;30(2):564-578.

- Jahn L, Hombrink P, Hagedoorn RS, Kester MG, van der Steen DM, Rodriguez T, Pentcheva-Hoang T, de Ru AH, Schoonakker MP, **Meeuwsen MH**, Griffioen M, van Veelen PA, Falkenburg JH, Heemskerk MH. *TCR-based therapy for multiple myeloma and other B-cell malignancies targeting intracellular transcription factor BOB1.* Blood. 2017 Mar 9;129(10):1284-1295.

&

&

CURRICULUM VITAE

Miranda Meeuwsen is geboren op 13 mei 1992 in Zwijndrecht. Daar voltooide ze in 2010 het VWO aan het DevelsteinCollege. Vervolgens heeft ze de bachelor Biomedische Wetenschappen gedaan aan de UvA. Voor haar bachelorstage kwam ze voor het eerst in het LUMC terecht, waarna ze in Leiden is gebleven om haar master te behalen. Als onderdeel van deze master liep zij stage bij HAL Allergy op het Bioscience Park in Leiden, waar ze onderzoek deed naar desensitisatietherapie voor allergieën. In de zomer tussen de twee jaren van de master liep ze een stage bij het DKFZ in Heidelberg, Duitsland. Haar laatste stage liep ze bij de afdeling Oncologie in het LUMC, waar ze onderzoek verrichtte naar de remmende effecten van melanoomcellen op T-celactivatie. In september 2015 begon ze aan haar promotieonderzoek bij de afdeling Hematologie in het LUMC onder begeleiding van prof. dr. Mirjam Heemskerk en prof. dr. Fred Falkenburg. Het doel van het promotieonderzoek was het identificeren van T-celreceptoren die gebruikt kunnen worden voor therapie van B-celmaligniteiten. Het resultaat van dit onderzoek staat beschreven in dit proefschrift. Na haar promotieonderzoek was Miranda van mei 2021 tot april 2024 werkzaam op de afdeling Hematologie als postdoc om het onderzoek naar T-celtherapie voort te zetten.

DANKWOORD

Om te beginnen wil ik iedereen van de afdeling Hematologie bedanken: stuk voor stuk lieve, leuke en behulpzame collega's. Met zijn allen zorgen jullie voor een gezellige en leuke werkomgeving. Speciaal wil ik iedereen van D2-36, C5 en de hardcores bedanken voor de bijzondere en gezellige jaren. Dankzij jullie heb ik een fantastische tijd gehad die ik voor altijd zal koesteren. Anne, wat kunnen wij goed samenwerken. Zij-aan-zij hebben wij dagenlang van 's ochtends vroeg tot 's avonds laat op het lab doorgebracht. We hebben eindeloos veel veel-te-grote proeven samen gepipetteerd en eindeloos veel ELISA's gedaan met onberispelijke uitkomsten. Renate, jij hebt eindeloos veel TCRs gesequenced en fantastisch werkende constructen gemaakt. Dennis, jij hebt eindeloos veel in vivo plaatjes gemaakt en qPCRs gedaan. Michel en Dirk, jullie hebben eindeloos veel peptide-eluties gedaan en mono- en tetrameren gebouwd. De hoeveelheid werk die is verzet door jullie allemaal is enorm, bedankt daarvoor. Zonder jullie was dit project onmogelijk geweest! Esther, Gerrie, Marian, Henk en Nelleke: op het oog wellicht een willekeurige combinatie aan collega's, maar met een grote gemene deler. Bij jullie kon ik altijd terecht voor vragen en hulp, dat was superfijn, dank daarvoor. Hanny, jij bent een drijvende kracht achter het lab: tijdens alle grote proeven die we doen, zorg jij er altijd voor dat we niks tekortkomen, dat alles op het lab spik en span is en dat de voorraden op orde zijn. Je bent een topper. Ten slotte wil ik iedereen die over de jaren onderdeel is geweest van groep Mirjam bedanken voor jullie waardevolle input op mijn project tijdens de werkbesprekingen, Fredinars op dinsdag of daarbuiten.

Dear Fugies, our friendship has been unvaluable to me, having friends that completely understand all the ups and downs that are part of a PhD-project is crucial to keeping your sanity. I will never forget all the special moments, happy or sad and can't wait to see what the future has in store for us!

My paranymphs: Rianne and Laura. The two of you are my rocks and I know it won't be any different on the day of the defense. Thank you for being by my side.

Highschool b*****s, bedankt voor jullie steun en interesse. Jullie hebben eindeloos geduld moeten hebben voor het lang beloofde promotiefeest, maar nu is het echt zover. Jullie zijn schatten.

Sander, jij laat mij zien hoe belangrijk het is om je dromen na te jagen op zoek naar geluk. Dat is een inspiratie. Papa, dankzij jouw praktische en probleemoplossende instelling ben ik nu een praktisch en probleemoplossend mens: dit zijn cruciale eigenschappen voor een jonge onderzoeker. Mama, jouw aansporing om eerst een universitaire studie af te ronden alvorens verder te fantaseren heeft me op dit punt gebracht. Nu kan het

fantaseren beginnen. Rianne, geboren worden met een beste vriendin is het beste wat me ooit is overkomen. Dat ik altijd op jou en Stefan terug kan vallen geeft mij het lef om het leven op een ietwat alternatieve manier te benaderen: van anti-kraak wonen tot verhuizen naar Amerika. Duuk en Owen, jullie zijn de allerliefsten en ik ben superblij en dankbaar dat ik jullie tante ben! Sinds ik jullie tante ben, realiseer ik me extra hoe belangrijk het is om in het hier en nu te leven. De tijd vliegt en als ik even niet oplet, zijn jullie groot.

Lieve Tassi, waar een PhD-traject vaak eenzaam en overweldigend kan zijn, was jij de afgelopen 3,5 jaar mijn steun en toeverlaat. Als ik hulp nodig had, was jij er telkens weer. Of het nu ging om een FACS midden in de nacht, samen muizen meten in het weekend, jij die mijn artikelen verbeterde of een snelle literatuurstudie deed de avond voor mijn seminar. De laatste loodjes wegen het zwaarst, maar ook die zijn nu bijna achter de rug. Terwijl opgeven nooit een optie was, weet ik niet of ik het wel gered had zonder jou. Ik ben je superdankbaar. Binnenkort beginnen we een nieuw avontuur waarin we niet meer samen zullen werken en voor het eerst zullen ervaren hoe het is om onderscheid te hebben tussen werk en privé. Wat zullen we elkaar veel te vertellen hebben aan het einde van de dag. Ik ben superbenieuwd en kijk er ontzettend naar uit.

&