DISSERTATION

Testing specificity and efficacy of T cell receptors derived from in vitro generated T cells stimulated with STAT3-mutated peptide

Untersuchung der Spezifität und Effizienz von T Zell Rezeptoren stammend aus in vitro generierten T Zellen stimuliert mit mutiertem STAT3 Peptid

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2 Abbreviations

AA	aplastic anemia
ACN	acetonitrile
ACT	adoptive cell therapy
AICD	activation-induced cell death
AITL	angioimmunoblastic T-cell lymphoma
ALCL	anaplastic large cell lymphoma
ALK+	anaplastic lymphoma kinase positive
ALK-	anaplastic lymphoma kinase negative
ANKL	aggressive NK-LGL leukemia
APC	antigen-presenting cells
ATLL	adult T cell Leukemia/ Lymphoma
ATT	adoptive T cell therapy
CAR	chimeric antigen receptor
CCR4	chemokine receptor type 4
CD	cluster of differentiation
CDR	complementarity-determining regions
CGA	cancer germline antigen
CID	collision-induced dissociation
CLPD-NK	chronic lymphoproliferative disorder of NK cells

CRR	complete response rate
CRS	cytokine release syndrome
CTA	cancer testis antigen
CTCL	cutaneous T-cell lymphoma
CTLA-4	cytotoxic T-lymphocyte-associates antigen 4
DC	dendritic cells
EATL	enteropathy associated T cell lymphoma
eGFP	enhanced-green fluorescent protein (eGFP)
ENKTL	extranodal NK/T-cell lymphoma
ER	endoplasmatic reticulum
FACS	fluorescence-activated cell scanning
HIV	human Immunodeficiency Virus
HSCT	hematopoietic stem cell transplantation
HSTL	hepatosplenic T-cell lymphoma
HPLC MS/MS	high-performance liquid chromatography-tandem mass spectrometer
KI	Knock-in
LCL	lymphoblastoid cell line
ORR	overall response rate
mAb	monoclonal antibody
MDS	myeloid dysplastic syndrome
MF	mycosis fungoides

MHC	main histocompatibility complex
MRD	minimal residual disease
NHL	Non- Hodgkin-Lymphoma
NK	natural killer
NK-LGL	large granular lymphocytic leukemia of natural killer cells
PBMC	peripheral blood mononuclear cells
PBL	peripheral blood lymphocytes
p-MHC	peptide main histocompatibility complex
PTCL	peripheral T-cell lymphoma
PTCL-NOS	peripheral T-cell lymphoma, not otherwise specified
RA	rheumatoid arthritis
SPTCL	subcutaneous panniculitis-like T cell Lymphoma
TALEN	transcription activator-like effector nuclease
T-ALL	T-cell acute lymphoblastic leukemia
T-ALLy	T cell acute lymphoblastic lymphoma
TAP	transporter associated with antigen processing
T_C	cytotoxic T cell
TCL	T cell lymphoma
TCR	T cell receptor
T_E	effector T cell
TFA	trifluoroacetic acid

T_{FH}	follicular T helper cell
T_H	T helper cell
TIL	tumor-infiltrating lymphocyte
TKI	tyrosine kinase inhibitor
T-LGL	T cell large granular lymphocytic leukemia
T_M	T memory cell
TMG	tandem minigene
T_N	naive T cell
TNF	tumor necrosis factor
T-PLL	T-cell prolymphocytic leukemia
Treg	regulatory T cell
TSA	tumor-specific antigen
SS	Sezary Syndrome
STAT	signal transducer and activator of transcription
scFv	single-chain variable fragment
γδ-PTCLs	γδ-T-cell-derived lymphomas

3 Abstract

Background: In contrast to chimeric antigen receptor (CAR) based therapy, T cell receptor (TCR) based adoptive T cell therapy relies on classical TCR recognition of processed epitopes presented in the context of MHC molecules, rather than on antibody recognition. This greatly widens the spectrum of targetable antigens, including truly cancer-specific mutant antigens, the so-called “neoantigens”. Therefore, the gene signal transducer and activator of transcription 3 (STAT3) is the ideal target, as it has a highly oncogenic potential when mutated. Constitutive activating mutations in STAT3, like D661Y, occur predominantly in difficult-to-treat T cell lymphoma. Here, we aim to generate a high-affinity TCR to specifically target HLA-A*02:01 restricted potential neoepitope IIMGYKIMYA encompassing the STAT3 D661Y mutation.

Methods: Based on isolated RNA derived from reactive T cells primed in vitro with STAT3 D661Y peptide, the most frequent variable TCR sequences were identified and cloned together with constant regions in a retroviral vector backbone for transduction into PBMCs. The generated T cell clones were used for functional T cell assays with the Hodgkin lymphoma-derived cell line HDLM-2 and K562 cells transduced with HLA-A*02:01 and a STAT3 D661Y minigene. Target peptide expression was controlled via mass spectrometry analysis for K562-A2-STAT3-D661Y minigene cells.

Results: Eight TCRs were identified and tested. Peptide titration experiments showed robust TCR T cell activation of four TCRs with high functional avidity against the 10mer peptide IIMGYKIMYA (EC50 in nM range). In the subsequent cocultures with STAT3 redirected TCR T cells and K562-A2-STAT3-D661Y minigene transduced cells, high IFN-γ secretion was detected. Surprisingly, robust CD137 upregulation and IFN-γ secretion in coculture with HDLM-2 was not observed, although it was proven that HDLM-2 cells are indeed heterozygous for the STAT3 D661Y mutation and possess an intact processing and loading machinery. Tandem mass spectrometry analysis revealed an absent target epitope on HDLM-2 cells. The TCR binding motif was identified by an Alanine scan assay and potential cross-reactivity against other HLA-A*02:01 presented peptides was excluded.

Conclusion: The data shows the identification of four STAT3 D661Y peptide-reactive TCRs. To prove whether this peptide represents a naturally presented epitope, peptide

presentation on HDLM2 and other HLA-A*02:01 positive cells endogenously expressing STAT3 D661Y cells should be evaluated by mass spectrometry and functional assays.

Abstrakt

Hintergrund: Im Gegensatz zur Therapie mit chimären Antigenrezeptoren (CAR) beruht die adoptive T-Zell-Therapie mit T-Zellrezeptoren (TCR) auf der klassischen TCR-Erkennung von prozessierten Epitopen, die im Zusammenhang mit MHC-Molekülen präsentiert werden und nicht auf Antikörpererkennung. Dadurch wird das Spektrum der angreifbaren Antigene erheblich erweitert, einschließlich krebsspezifischer mutierter Antigene, so genannter "Neoantigene". Daher ist das Gen Signal Transducer and Activator of Transcription 3 (STAT3) das ideale Ziel, da es bei Mutation ein hohes onkogenes Potenzial besitzt. Konstitutive aktivierende Mutationen in STAT3, wie D661Y, treten vor allem bei schwer zu behandelnden T-Zell-Lymphomen auf. Hier wollen wir einen hochaffinen TCR generieren, der speziell auf das auf HLA-A*02:01 beschränkte potenzielle Neoepitop IIMGYKIMYA abzielt, dass die STAT3 D661Y-Mutation umfasst.

Methoden: Auf der Grundlage von isolierter RNA aus reaktiven T-Zellen, die in vitro mit STAT3 D661Y-Peptid kultiviert wurden, wurden die häufigsten variablen TCR-Sequenzen identifiziert und zusammen mit konstanten Regionen in ein retrovirales Vektor-Backbone zur Transduktion in PBMCs kloniert. Die erzeugten T-Zell-Klone wurden für funktionelle T-Zell-Assays mit der vom Hodgkin-Lymphom stammenden Zelllinie HDLM-2 und K562-Zellen verwendet, die mit HLA-A*02:01 und einem STAT3 D661Y-Minigen transduziert wurden. Die Expression der Zielpeptide wurde mittels massenspektrometrischer Analyse für K562-A2-STAT3-D661Y-Minigenzellen kontrolliert.

Ergebnisse: Acht TCRs wurden identifiziert und getestet. Peptid-Titrationsexperimente zeigten eine robuste TCR-T-Zellaktivierung von vier TCRs mit hoher funktioneller Avidität gegenüber dem 10mer-Peptid IIMGYKIMYA (EC50 im nM-Bereich). In den anschließenden Kokulturen mit gegen STAT3 D661Y gerichteten TCR-T-Zellen und K562-A2-STAT3-D661Y-Minigen-transduzierten Zellen kann eine hohe IFN-γ-Sekretion nachgewiesen werden. Überraschenderweise wurde eine robuste CD137-Hochregulierung und IFN-γ -Sekretion in Kokultur mit HDLM-2 nicht beobachtet, obwohl nachgewiesen wurde, dass HDLM-2-Zellen tatsächlich heterozygot für die STAT3-D661Y-Mutation sind und eine intakte Prozessierungs- und Lademaschinerie besitzen. Die Tandem-Massenspektrometrie-Analyse ergab ein fehlendes Zielepitop auf HDLM-2-Zellen. Das TCR-Bindungsmotiv wurde durch einen Alanin-Scan-Assay identifiziert, und

eine mögliche Kreuzreaktivität mit anderen HLA-A*02:01-Peptiden wurde ausgeschlossen.

Schlussfolgerung: Die Daten zeigen die Identifizierung von vier STAT3 D661Y-Peptid-reaktiven TCRs. Um zu beweisen, ob dieses Peptid ein natürlich präsentiertes Epitop darstellt, sollte die Peptidpräsentation auf HDLM2 und anderen HLA-A*02:01-positiven Zellen, die endogen STAT3 D661Y-Zellen exprimieren, durch Massenspektrometrie und funktionelle Assays bewertet werden.

4 Introduction

4.1 Tumors and the immune system

4.1.1 Immune response against tumors

As early as the late 19th century, William Coley had determined a marginal tumor therapeutic effect when provoking an infection by injection of pyogenic bacteria at the tumor site (1). Decades later, world-famous scientist Paul Ehrlich investigated mouse tumor growth caused by chemical carcinogens or irradiation. Ehrlich was one of the first scientists to assume the existence of an immune response toward tumors, in the year 1909 (2). Key experiments were conducted by transplanting tumors in mice which mostly ended in the animal's death. However, it was observed that injection of attenuated tumor cells in mice can lead to the rejection of active cells of the same kind of tumor afterward.

In contrast, T-cell deficient mice could not show tumor regression, whereas the transfer of T cells showed similar protective immunity (3). Consequently, in the 1950s, the thesis of “immune surveillance” was postulated by Burnet and Lawrence (4), suggesting that immune cells can not only recognize foreign antigens but are also capable of recognizing tumors and consequently destroying them (5). According to the theory, tumors can develop because of a non-working immunosurveillance when tumors lose their immunogenicity, e.g., by selecting resistant tumor cells (6, 7). This theory is not only supported by some mouse models (overview, (8)) but also by clinical observations. For instance, patients with immune deficiency due to HIV infection or those treated with immunosuppressive drugs are more likely to develop cancer (9). Not only do tumors associated with oncogenic viruses occur often, but also lymphomas or epithelial tumors (10). The theory of immunosurveillance is being reexamined (11). However, there have been objections, as new experiments supporting the immunosurveillance theory have not been adequately conducted (12). The controversy is about immunodeficient mice modified in various ways that, according to Gunn et al., developed MCA-induced tumors more rapidly or were less able to fight off tumor transplants. However, these mice experiments were not comparable to each other, as Qin et al. discuss, especially since there were differences between the control mice and immunodeficient mice, and the alleged tumor defense could originate from a tissue repair mechanism, such as encapsulation of the MCA-induced tumor. The experiments were repeated, and the immunosurveillance theory could not be reconfirmed (12). One year later, the term

immune tolerance was coined, suggesting that sporadic tumors do not escape the control of the immune system in their growth, but do induce humoral and cellular immune responses (13). Therefore, the natural tumor development was mimicked in a mouse model, and thus the tumor's immunogenicity. Here the theory of immunosurveillance, e.g., by first elimination of tumor cells and after that by losing immunogenicity, was not confirmed. However, it was demonstrated that a CD8+ T cell-mediated response builds up, which will be further considered in this work.

4.1.2 T cells

CD8+ T cells recognize peptide fragments of endogenous antigens presented on the cell surface by major histocompatibility complex class I (MHC-I) proteins. When a CD8+ T cell binds to a peptide-MHC-I complex using the T cell receptor (TCR), and the presented epitope is recognized as foreign, activation of the T cell is initiated. The peptides, called epitopes, are generated by the proteasome as well as extra proteasomal proteases in the cytosol and transported into the endoplasmic reticulum (ER) by antigen peptide transporters (TAPs, transporters associated with antigen presentation). There, they bind to MHC-I molecules. These complexes are then transported to the cell surface (14). Using their TCR, T_H cells recognize peptides presented by MHC class II (MHC-II) molecules on professional antigen-presenting cells (APCs) such as B cells, macrophages, or dendritic cells (DCs) (15). After endocytosis, antigens are degraded via the lysosomal pathway and are loaded on the MHC class II molecules (14).

$CD4^+$ T_H-cells are divided into T_H1, T_H2, and regulatory T cells. Other subtypes are T_H17 cells, follicular T helper cells (T_{FH}) and T_H9 cells, and T_H22 cells are partially described (16). After interaction with the MHC-II complex, CD4+ T cells differentiate into distinct subsets according to the cytokine milieu (15, 16). In short, $T_H1$cells are essential for cellular immunity against intracellular microorganisms (16). They activate macrophages via IFNγ and stimulate B cells to produce IgG antibodies to opsonize extracellular pathogens for uptake by phagocytic cells (15, 17). After activation, T_H2 cells produce various cytokines and play an important role in humoral immunity and the control of extracellular pathogens or helminths (18, 19).

CD8+ T cells can also differentiate into T_C1, T_C2, and T_C17 (T_C-cells) with the characteristic cytokine profiles analogous to CD4+ T cells (20).

According to their functional status, T cells can be divided into naïve T cells (T_N), effector T cells (T_E), and memory T cells (T_M). After activation by contact with the MHC complex, T_N cells rapidly differentiate into T_E cells, which can be distinguished according to their cytokine profile (15). In contrast to T_N cells, they no longer require costimulatory molecules. After effective control of the pathogen, the majority of T_E cells die by activation-induced cell death (AICD) (21). T cells that survive the contraction phase develop into T_M cells (22) and mediate long-term immunity. In some tumors, detecting intra-tumoral T_M cells is associated with better survival and less metastasis (23, 24).

4.1.3 T cell-mediated anti-tumor response

Many tumors exhibit infiltration by a heterogeneous population of immune cells, including macrophages, B cells, NK cells, and T cells. Tumor-infiltrating CD4+ and CD8+ T cells have been detected in various solid tumors with positive prognostic significance (25-28). Both CD8+ cytotoxic T cells (25, 26) and CD4+ T helper cells (T_H-cells) are suspected of playing essential roles in the anti-tumor immune response, as they can specifically recognize tumor antigens, self-replicate, and consequently kill tumor cells (29). T_M cells mediate long-term immunity; however, in some tumors, the detection of intra-tumoral T_M cells is associated with better survival and less metastasis (23, 24). These properties make them advantageous against cancer and they can be exploited therapeutically.

4.1.4 Antigen processing and presentation

MHC class I and II molecules are part of both adoptive and innate immunity. MHC class I molecules present their peptides derived from pathogens, mostly viruses, to cytotoxic CD8+ T cells. Since viruses mainly affect nucleus-containing cells, these cells express MHC class I molecules on their cell surface. In humans, the MHC is also referred to by the gene name human leukocyte antigen (HLA). MHC molecules consist of two polypeptide chains: a larger α chain, which can be further divided into $\alpha1$, $\alpha2$, and $\alpha3$ subunits, and the $\beta2$ microglobulin subunit. Significantly, the $\alpha1$ and $\alpha2$ subunits form the peptide binding site of the HLA molecule and are therefore highly variable (30). X-ray crystal structure analyses show that, on the one hand, MHC molecules bind peptides with a very high affinity in their binding site (31). At the same time, however, a binding site can bind many different peptide sequences (32). The tight binding is essential because peptides could be easily exchanged on the surface of a cell. This process also stabilizes the MHC molecule at the cell surface, thus ensuring permanent peptide presentation.

In addition, the surface protein CD8 plays an essential role in recognizing the presented peptide. It is a dimer and consists of an alpha and beta chain. Binding CD8αβ to the α3-domain of MHC class I molecules increases the sensitivity of T cells to antigens presented by MHC-I by about a hundredfold. It is suspected that the degree of glycosylation of CD8 is related to the strength of binding to the MHC molecule (33, 34). Most class I epitopes are generated by the proteasome and extra proteasomal proteases in the cytosol and transported into the ER by TAP transporters. They bind to MHC-I molecules and are then transported to the cell surface (14). The 26S proteasome is a multicatalytic protease complex composed of the 20S catalytic core and the 19S regulatory subunit. Proteolytic activity is localized in the 20S core. Three enzymatically active subunits are known. IFNγ-inducible subunits can replace these subunits, and subsequently, they form the immunoproteasome (35, 36). The immunoproteasome is constitutively expressed in professional APCs such as DCs. Due to the expression of different catalytic subunits, the immunoproteasome differs from the standard proteasome quantitatively and qualitatively in the processing and proteolytic digestion of peptides (37-39). The cutting preferences of proteasomes are determined by the amino acid immediately present at the cleavage site and depend on the amino acid sequence surrounding it (40-42).

In addition, there are two intermediate proteasome types, which have subunits from the immunoproteasome and the standard proteasome (43). Due to the different proteolytic activity, some epitopes cannot be processed by all proteasome types, and possibly only by one type (44, 45). Only a tiny proportion (5%) of peptides processed by the proteasome can bind directly to MHC, the majority of peptide fragments generated are too short, and approximately 20% are too long for binding to MHC-I molecules (46, 47). Aminopeptidases in the cytosol and ER can also trim the N-terminus to the correct length, and the proteasome usually determines the C-terminus. However, some epitopes also require extra proteasomal proteases to generate the correct C-terminus or are processed entirely proteasome-independently (47).

4.1.5 Diversity and architecture of the T cell receptor

Among all human receptors, the T cell receptor is one of the most complex. It consists of α and β chains, and each has a variable (V) and a constant (C) region and therefore builds up similarly to immunoglobulins. The α gene locus has a variable (V) and joining (J) gene segment, the β gene locus has an additional diversity (D) segment. To enable a

diverse TCR repertoire during T cell development the V, (D), and J gene segments are rearranged in the thymus. Furthermore, diversity can be expanded by non-accurate V(D)J gene rearrangement and inclusion of P- and N- nucleotides in the junctions.

In contrast to the various α and β variable TCR chains, constant chains are homologous. There is only one α constant chain and only two β constant chains known. The structure of the receptor aims to recognize peptides bound to MHC molecules. Comparably to immunoglobulins, there is a three-dimensional antigen recognition site to which the presented peptide binds. In detail, the TCR recognizes the peptide-MHC (p-MHC) via the complementarity-determining regions (CDR) responsible for recognizing the given peptide. The hypervariable loop of the so-called CDR3 region is noteworthy, which originates from the D and J gene segments of the TCR α and β locus and interacts directly with the center of the peptide (48-50). The CDR3 region is flanked on both sides by the CDR1 and CDR2 regions in the V gene segments of the α and β locus, respectively. CDR1 and CDR2 mediate the binding with the MHC molecule by binding to the amino and carboxyl termini of the eight to ten amino acid long peptides (51, 52). Specifically, the diversity of the CDR3 variable region arises from the high number of possible recombinations of the J gene segments, whereas the TCR α locus has 61 of those segments. A minority of T cells consist of γ and δ chains instead of α and β chains. The function of T cells carrying these chains has yet to be conclusively determined. However, it is known that some of them can also recognize peptides like antibodies without MHC molecules (53).

4.1.6 Immunological synapsis: T cell recognition and activation

The interaction between the TCR and the presented antigen is crucial in cell-mediated adaptive immunity. Two signals are therefore required. The first one derives from the TCR bound to its specific peptide presented by the MHC molecule. The peptide itself is bound to the α1 and α2 domains of the MHC molecule and stabilizes it, and also interacts with the variable regions of the TCR (50, 52). The second signal, also referred to as the “costimulatory” signal, depends on the interaction of two molecules: CD28, constitutively expressed on the surface of $CD4^+$ and $CD8^+$ T cells; and B7-1 (CD80) or B7-2 (CD86) on the surface of the antigen-presenting cells (54). After activation, T cells express a second receptor named CTLA4, similar to CD28, that also binds to the B7 family of molecules (55).

In detail, the TCR recognizes the peptide-MHC (p-MHC) via the complementarity-determining regions (CDR). Subsequently, the TCR molecule conformation changes, especially at the CDR3 loop (50), which also has the highest diversity in its sequence. Consequently, CDR3 plays a crucial role in the specificity of the TCR (56). T cell activation does not only rely on TCR and p-MHC interaction, as different peptides can also provoke a strong or a weak activation. This phenomenon is not due to the conformation of the peptide or the binding angle (57, 58). Many analysis are suggesting that the dissociation rates of the TCR (half-life) correlate with the potency of the p-MHC complex (59-61). Others suggest that the ligand's heat capacity influences the grade of T cell activation (62). Upon productive recognition of the p-MHC, the αβTCR transmits signals to the CD3 complex by phosphorylation of the CD3 ζ chains, which subsequently triggers the activation of multiple intracellular signaling pathways in the T cell (63, 64).

4.2 Tumor antigens

The first tumor antigen was identified in 1991 (65). Since then, many tumor antigens have been identified and cloned (66). Classification varies; they can be divided into five groups (67), as follows. (a) Differentiation antigens that are tissue-specific and therefore also expressed by some normal tissues such as the melanocyte differentiation antigens Melan-A/MART-1(68, 69), tyrosinase (70, 71), or gp-100 (72, 73). (b) Antigens overexpressed by increased transcription or gene amplification such as HER-2/neu in ovarian or breast carcinoma (74), the apoptosis inhibitor survivin (75, 76), p53 (77, 78), or the transcription factor Wilms tumor 1 in leukemic cells (79-81). Since it is very likely for both antigen groups to also be expressed in normal tissues, there is a high probability of on-target off-tumor toxicity (82). (c) Cancer-testis antigens (CTAs), also called cancer-germline antigens (CGA), are predominantly expressed in tumors and in limited numbers in germ cells (testis and placenta). However, cells in the testis cannot present processed antigens on HLA-class I, reducing the risk of on-target off-tumor toxicity (82). Currently, CTAs such as MAGE family proteins (83) or NY-ESO-1(84) have high clinical significance. Interestingly, CTAs located on the X-chromosome, like NY-ESO-1, can be enhanced in their expression level by epigenetic hypomethylation (85, 86). These three groups (87) can be further summarized as Tumor-associated antigens (TAAs). Some authors define CGA also as their own class. The other two groups are: (d) neoantigens

that arise from mutations or translocations such as bcr-abl (88) or mutant p53 (89) and mutant KRAS (90); and lastly, (e) viral antigens that are expressed only in virus-infected cells, such as EBV and HPV (91). It is of advantage that viral antigens and neoantigens are only encoded in the tumor site and are therefore classified as tumor-specific antigens (92), which can avoid the induction of autoimmunity when transferring a high avidity TCR (93). In contrast, differentiation antigens, overexpressed antigens, and CTAs can be expressed, albeit at low expression levels, by normal tissue (93). When developing TCR-engineered T cells, the selection of the target antigen is very important. Through TAAs, antigen loss can occur, whether through loss of antigen expression, mutation of the antigen itself, or downregulation or failure of the antigen processing machinery of HLA molecules (94-96).

Neoantigens have the advantage of originating from mutations in the tumor genome and are therefore mutated proteins that are only expressed in a tumor-specific manner. Due to their selective expression, they are advantageous because the immune tolerance, as well as the risk of autoimmunity, is minimized (97).

Moreover, the mutation pattern in cancer is not always homogeneous, and it is unlikely that the same neoantigen will be detected in every patient. Therefore, so-called driver mutations are pursued, which provide tumors with a selective growth advantage in oncogenesis (98, 99). They are additionally called “public neoantigens” when immunogenic and restricted to common HLA subgroups (100, 101).

However, techniques to predict or determine the immunogenicity of driver mutations are also required, as it needs to be presented by MHC molecules to the TCR (102). Currently, the optimal identification of neoantigens is still in the process of research (103, 104). Unfortunately, most mutations in a tumor are not driver or recurrent mutations but individual passenger mutations (105). Consequently, for the adoptive transfer of epitope-specific T cells, they must also be developed individually, which is time- and cost-intensive, in addition to the risk of antigen loss from passenger mutation.

In summary, the ideal tumor antigen should be expressed in as many tumor entities as possible for broad application and to have a low risk of antigen loss. Furthermore, it should not have a significant expression in healthy tissues to avoid the induction of autoimmunity. Nevertheless, it is essential to preclinically test the antigen's existence in

healthy tissues, e.g., by amino acid scanning or database research (106), or by adding suicide systems like inducible apoptosis for T cell self-depletion (107), as severe side effects by self-reactive T cells have been reported (overview (93)). Apart from TSA, such as neoantigens and viral antigens, TAA, such as the CTAs or overexpressed antigens, represent potential targets.

TAA - Tumor-associated antigens		
a	Differentiation antigens	Tyrosinase, Melan-A / MART1, GP100
b	Overexpressed antigens	Her2/neu, survivin, p53, Wilms tumor1
c	Cancer germline genes	MAGE, BAGE, GAGE, LAGE/ NY-ESO1, SSX
TSA – Tumor specific antigens		
d	Neoantigens encoded by mutated genes	Mutation of bcr-abl, mut.p53, mut. KRAS, MyD88
e	Viral antigens	Antigenic peptides after e.g. HPV, EBV

Table 1. Overview of tumor antigens.

4.3 Adoptive T cell therapy (ACT)

4.3.1 Development of cancer immunotherapies

The well-established tumor therapies are chemotherapy, radiotherapy, and surgical tumor removal. However, these strategies are subject to limitations due to severe side effects, damage to healthy tissue, development of residues, or non-feasibility due to the localization of the tumor. In recent times, more specific cancer immunotherapies have been developed by better understanding the molecular mechanisms in tumor growth

(108). T cell-mediated immunotherapy can be divided into passive adoptive T cell transfer (ATT) and active tumor vaccination (109, 110). Antigen/epitope-undefined approaches or antigen/epitope-defined approaches exist for both strategies. Recently, good cancer immunotherapies have been developed that have made it to the clinic. Immune checkpoint inhibitors are antibodies that reactivate tumor defense by abrogating inhibitory signals to T cells or by promoting activating signals. For instance, Ipilimumab, an antibody against the overexpression of cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), has been approved against melanoma (111). Nivolumab and Pembrolizumab, antibodies against inhibitory receptors such as programmed cell death protein 1 (PD-1), are authorized for many tumor entities, among others, against melanomas, non-small-cell lung and renal-cell cancer, Hodgkin lymphoma, and urothelial carcinoma (111-113).

Furthermore, a promising form of cancer immunotherapy is known as adoptive cell therapy (ACT), whereby the ability of allogenic or even autologous T cells is used to promote tumor regression in vivo once they are reinjected into the patient. So far, these include an FDA approved phase 1 clinical study of tumor-infiltrating lymphocytes (TILs) (NCT05470283), as well as T cells genetically engineered with either chimeric antigen receptors (already are approved and in clinical use (114)) or T cell receptors (TCRs) (115), which are in current clinical development and will be described in the following.

4.3.2 Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs) are derived from resected tumor material, sorted and expanded ex vivo, and finally administered to the patient as a therapeutic agent. It is an elegant solution to circumvent tumor heterogeneity since matching T cells from the patient's immune response are used. TILs have the advantage that they can be used without precise knowledge of the tumor antigen repertoire and can target a variety of tumor antigens. However, their production is time-consuming and expensive, and requires sufficient tumor material, which is only sometimes available. Also, many patients fail to respond. In 1988, Rosenberg et al. showed for the first time the ability of TILs to promote regression in melanoma cancer patients (116). They were able to expand the cells by 95,652-fold and have a potent cytotoxic anti-tumor effect (116).

However, in the meantime, there have been many further developments, such as lymphodepletion before infusion of TILs (117) or sorting for PD-1 or CD137 positive tumor-reactive T cells (118, 119). Today TIL-ACT is mainly conducted in melanoma

patients. A meta-analysis of 13 clinical trials (1988-2016) receiving TILs with either a high or low-dose IL-2 regimen showed a pooled overall response rate (ORR) of 41% and a pooled complete response rate (CRR) of 12% (120). Although this suggests a promising outlook, TILs are not yet FDA-approved. They are logistically and temporally elaborated in production and cannot be administered immediately. Especially in melanoma, but also in other solid tumors, immune checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1 are, to date, the best immunotherapy. These can, when combined, achieve response rates of 58% in melanoma patients fot up to 6.5 years (107). Recently, the first randomized phase III clinical trial was published comparing Ipilimumab and TILs in advanced melanoma patients. Median progression-free survival was significantly longer in the TIL group (7.2 months) compared with patients treated with ipilimumab (3.1 months) (121).

4.3.3 Chimeric antigen receptors

Another form of ACT is the transfer of gene-modified T cells with a new specificity redirected against the tumor. For this therapy, autologous T or NK cells are usually stably or transiently transduced with a chimeric antigen receptor (CAR) (122). This synthetic receptor can recognize surface antigens primarily via a single-chain variable fragment (scFv) of a monoclonal antibody (mAb) linked to a hinge region, a transmembrane region, and an intracellular CD3ζ activation domain. Upon antigen recognition, the activation signal can be transmitted into the T cells and triggers them (123). At present, five generations of CARs exist, distinguished by additional molecules such as costimulatory domains like 4-1BB and 2B4, to induce a more robust and extended T cell response. Unlike TCRs, CARs target naturally folded surface antigens and can be used regardless of the patient's HLA haplotype. Although CARs require a high density of cell surface antigens (122), they can mediate robust T cell activation, and the cells can promote serial killing (124). The FDA granted ATT "Breakthrough Therapy Status” in 2013. Especially in CD19-positive B cell neoplasms, long-lasting remissions in more than 50% of patients in the initial clinical trials were observed (125, 126).

However, it has been observed that many patients relapse, possibly due to antigen-low cancer cells or a decrease in persistence after the initial strong response (127, 128). In addition, the severe side effect called cytokine release syndrome (CRS) has been observed, also induced by overshooting T cell activation (129).

4.3.4 T cell receptors

Furthermore, the adoptive transfer of T cells genetically engineered with antigen-specific T cell receptors (TCRs) has become another approach in ACT. Here, the principle involves the transfer of TCR α and β genes into T cells, which then allows the receptor to recognize an HLA-presented peptide. In contrast to CAR T cells, which only recognize peptides of the cell surface, the target antigen for TCR-engineered T cells can also derive from intracellular proteins (130). However, this poses the biggest hurdle in finding appropriate neoantigens for TCR development: on the one hand, it needs to be immunogenic, meaning being presented by HLA class I molecules, and on the other, if possible, exclusively expressed on the tumor site. Compared with CARs, TCRs have several structural advantages allowing them a more efficient method of T cell activation. They have more receptor subunits, greater immunoreceptor tyrosine-based activation motifs (ITAM), more coreceptors, and less dependence on antigens (131).

In contrast to CARs, TCRs need just one contact with pMHC, making them about 100-fold more sensitive (132, 133). This is explicable by looking at the interaction of three molecules that must emerge. While the affinity of pMHC interactions ranges widely due to naturally different peptide properties, the affinity of TCR to pMHC is narrow (between 1–100 µM) and, therefore, lower in contrast to the affinity of CARs to their antigens (134). However, this enables a fast on-off rate for TCR-pMHC interaction, so that one pMHC complex can activate multiple TCRs (135).

Most studies to date have developed TCRs against tumor-associated antigens or viral antigens. However, particular attention must be paid to on-target- or, when using self-antigens, to off-target toxicity (136). In the first trials with a MAGE-A3 redirected TCR, it showed cross-reactivity with titin and a similar epitope in the brain, and subsequently two patients died in each study (137, 138). However, once an ultrasensitive TCR is founf for a corresponding tumor antigen, it has therapeutic potential- clinical trials for TCR T cells in solid tumor cancer patients are conducted. For example, key studies were carried out with an HLA-A2 restricted high-affinity TCR derived from NY_ESO-1/LAGE1 peptide, 50% of synovial sarcoma patients benefitted from anti-tumor effects (139). The same TCR also showed an encouraging response rate of 80% in myeloma patients (140).

Additionally, the ability to target any protein independent of cellular localization considerably widens the spectrum of target antigens, including truly cancer-specific

mutant antigens, the so-called “neoantigens” (141). Furthermore, high-affinity neoantigen-specific TCRs can be isolated from the human repertoire, reducing the risk of both on-target and off-target toxicity. We and others have successfully isolated TCRs against individual and recurrent mutations in patients (142-146). Therefore, developing a neoantigen-specific TCR derived from a healthy donor is also the subject of this work.

4.4 Adoptive therapy for the treatment of T cell neoplasms

4.4.1 Overview of T cell neoplasms

4.4.1.1 Leukemias

Hematological neoplasms can be divided into acute leukemias, lymphomas and myeloproliferative and myelodysplastic diseases. The further classification is based on the location of degeneration during hematopoiesis. Consequently, T-cell leukemias result from disorders either in the lymphatic progenitors, such as T-cell acute lymphoblastic leukemia (T-ALL), or genetic aberrant mature T cells like large granular lymphocytic leukemia, encompassing three entities according to the current WHO classification: T-cell large granular lymphocyte leukemia (T-LGL), chronic lymphoproliferative disorder of NK cells (CLPD-NK) and aggressive NK-LGL leukemia (ANKL) (147). Furthermore, there are adult T-cell leukemia/lymphoma (ATL or ATLL) and T-cell prolymphocytic leukemia (T-PLL)(148).

Although children have a survival rate of up to 90% at present (148, 149), adult outcomes are more challenging due to higher-risk leukemia genetics, comorbidities, and lower tolerance to intensive therapies. Nevertheless, in adults, allogeneic hematopoietic stem cell transplantation (HSCT) can be an option to gain a minimal residual disease (MRD) (149, 150). In recent years, advances in CAR and antibody therapy have been made, which will be discussed further.

4.4.1.2 Lymphomas

Malignant lymphomas are neoplasms of the lymphatic system that are divided into two groups: Hodgkin lymphoma and non-Hodgkin lymphoma (NHL). The latter can be divided into the B-cell and T-cell series as well as low-grade and high-grade NHL. T-cell lymphomas (TCL) make up only 10-15 % of all NHLs (151, 152). TCLs are a

heterogeneous disease group: a distinction between mature and precursor lymphomas can be made. Precursor T cell acute lymphoblastic lymphoma (T-ALLy) is a variant from the precursor form of ALL, with manifestation in lymph nodes and thymus and less than 25% of lymphoblasts. A therapy regimen follows, therefore the one for ALL is used (153).

Mature TCL can occur as peripheral T-cell lymphomas (PTCL) and cutaneous T-cell lymphomas (CTCL) (154). PTCLs can be further described by their clinical presentation as either leukemic, nodal or extranodal. Due to the rarity of the TCL, there have only been a few clinical trials, and therefore treatment guidelines are based on expert opinion rather than on randomized trial results (155-157). However, they have a poorer prognosis than most B-lineage NHLs (158). PTCLs often have a clinically aggressive course, while CTCLs involving the skin are more indolent (155).

Common PTCL subtypes are: peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS); anaplastic large cell lymphoma (ALCL); angioimmunoblastic T-cell lymphoma (AITL); and extranodal NK/T-cell lymphoma (ENKTL) (159, 160). Interestingly, there is a strong association between extranodal ENKTL cases and EBV infection in East Asia and, to a lesser extent, in Central- and South America (161). Subtypes are further classified by proof of genetic alterations or surface markers (155). For example, next-generation sequencing studies helped to distinguish between rare subtypes in systemic ALCL, which can be further differentiated into anaplastic lymphoma kinase positive (ALK+) and anaplastic lymphoma kinase negative (ALK-) subgroups with both characteristic recurrent translocations and gene fusions. However, ALK+ anaplastic lymphoma has a better outcome than ALK- anaplastic lymphoma (162, 163). More rare PTCL subtypes are, for example, enteropathy-associated T-cell lymphoma (EATL), which makes up 5% of all PTCL and can be further distinguished into type I – celiac disease-related or type II - celiac disease independent forms (162); subcutaneous panniculitis-like T cell Lymphoma (SPTCL) or adult T cell Leukemia/Lymphoma (ATLL). ATLL is associated with the human T-cell lymphotropic virus type 1 (HTLV-1) and is a highly aggressive form (164).

Looking at CTCL, the majority of cases present as a non-aggressive disease. The most frequent CTCLs are Mycosis fungoides (MF) and the generalized form Sezary Syndrome, accounting for 70-75% of CTCLs (165, 166). MF presents with scattered epidermal lymphocytic infiltrates presenting as a few erythematous patches and progresses to more

infiltrative tumors like hemispherical nodules (167). Lymphoid cells can disseminate and spread systemically. Sezary Syndrome (SS) patients present with erythroderma, pruritus, and lymphadenopathy. It is characterized by a washout of malignant Sezary cells into the peripheral blood (168). Moreover, other CTCL entities include primary cutaneous CD30+ T-cell lymphoproliferative disorders and primary cutaneous gamma-delta T-cell lymphoma (166, 167).

Unfortunately, due to their rarity, there is no standard therapy for PTCL. The often-used CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) was derived primarily from studies of aggressive lymphomas in which few PTCL patients participated. Some patients additionally receive autologous HSCT during the first remission. However, CHOP rarely leads to a cure (169-172), while HSCT can indeed increase OS (HR 0.58, p=0.004) (173). Although the clinical outcome of specific subtypes can be improved with treatment adjustments, such as the addition of radiotherapy and asparaginase for sinonasal localized ENKTL (171, 172), there is an overall need for more effective therapy because the outcomes for most patients remain unsatisfying with low response rates and short durations of response (174).

4.4.2 Approaches to date with CAR T cells

Besides the very successful CAR T cell therapies against B cell malignancies, there are also approaches for T cell neoplasms, such as CARS against CD3, CD5, and CD7. However, the development is more complex (overview (175)). To produce CAR T cells, lymphocytes are isolated from the patient by leukapheresis and then genetically engineered. Thereby, it is almost impossible not to isolate malignant cells as well (175).

If the targeted surface markers also exist on healthy T cells, there is a risk of T cell aplasia and, consequently, immune deficiency (176, 177). Another phenomenon is the so-called CAR fratricide, which appears when the T cell target antigen is also expressed on the CAR T cells themselves (178). In some cases, it can be bypassed by using NK cells with no identical surface markers to T cells (179). Fratricide challenges have also been approached by limiting the expression of the target antigen on CAR T cells by use of gene editing methods such as CRISPR-Cas9 (180); transcription activator-like effector nuclease (TALEN) (181), an artificial sequence-specific restriction enzyme; the TET-off system or protein expression blocker, which can, in the presence of doxycycline decrease CAR expression (182); or a protein expression blocker (PEBL) system, that prevents the

transport of the target protein to the membrane (183). Despite these fundamental obstacles, there are approaches targeting pan T cell antigens against T cell neoplasms.

4.4.2.1 Pan T cell antigens

Pan T cell antigens, also called lineage T cell antigens, include, for instance, CD3, a surface antigen forming a complex with the TCR, and which is indispensable for antigen recognition and further signaling. Additionally, there is CD5, a glycoprotein with an extracellular domain, which can be found in T cells, in limited numbers in B cells (184), as well as in malignancies such as T-ALL and PTCL (185-187), rendering an attractive CAR target. Another frequent T cell and NK cell lineage antigen is CD7, a membrane-spanning glycoprotein (188). To circumvent fratricide for all three Pan T cell antigens, NK cells were used for CAR engineering instead, and demonstrated toxicity against T-ALL and PTCL cell lines as well as patient samples (179, 189, 190).

Gene editing methods to reduce cross-reactivity, like TALEN, were applied to the T cells before implementing a CD3-CAR and lysed tumor cells in PTCL and T-ALL tumor models (179, 181). Moreover, for CD5 CARs, a gene knockout in T cells by CRISPR-Cas9 or the Tet-off system was successfully demonstrated, and CAR T cells had longer in-vivo persistence (180, 182). Two groups developed a CD7 CAR in CRISPR-Cas9 modified T cells, which showed robust anti-tumor activity in mouse xenograft model (178, 191). Also, the PEBL system mentioned previously reduced the tumor burden in an aggressive T cell precursor (ETP) ALL in mice xenograft models (183). Though these were all preclinical studies, there have been clinical advances for CD7 CARs. Currently, two ongoing CD7 CAR T cell trials include refractory acute T lymphoblastic leukemia/lymphoma patients (NCT04033302 and NCT04004637). In 2015 the first CD5 CAR with a CD28 costimulatory domain could kill T-ALL and T-cell lymphoma cell lines. Although CD28 should downregulate CD5 on the own surface of CAR T cells, fratricide was observed, and tumor eradication in animal models could not be demonstrated (192). Nevertheless, there is also an ongoing clinical trial in phase I with aCD5-CD28 CAR T cells including T-ALL, T-NHL, and T-ALLy patients (NCT03081910).

4.4.2.2 Restricted T cell antigens

Furthermore, many approaches target antigens with restricted expressions in T cells, such as CD1a, an antigen only expressed on cortical T-All cells (193, 194), making it an ideal fratricide-resistant CAR target (195). Also, CD4 redirected CAR T cells and CAR

NK-92 cells were developed by Pinz et al. (196, 197), to circumvent HIV/AIDS-like syndrome. One group used alemtuzumab a CD52-specific humanized mAb, that depleted CD4 CAR T cells in mice (198). Moreover, the FDA has approved Brentuximab vedotin, a conjugated drug, consisting of an anti-CD30 mouse antibody and monomethyl auristatin E (MMAE), a microtubule-disrupting agent, among others against CTCL, PTCL, and ALCL (199). Moreover, two encouraging clinical trials are going on with CD30-CAR T cells in relapsed or refractory patients with Non-Hodgkin Lymphoma (NCT02690545, NCT02917083). Also, for CD37 and chemokine receptor type 4 (CCR4), both present in CTCL and PTCL (200-202), an antibody was developed (AGS67E - human CD37-specific mAb conjugated to MMAE (203)); (CCR4: Mogamulizumab) (204), and afterward a CAR (205, 206). Mogamulizumab is already FDA-approved and in use against relapsed CTCLs such as MF and SS (204). Another approach is based on the fact that there are only two genes (TRBC1 and TRBC2) for the T cell receptor constant beta chain, which is why both are expressed in healthy individuals. In a clonal expansion of a degenerated T cell, one of the two constant chains will be found predominantly, which is ideal for CAR T cell therapy because only one portion of the healthy T cells with the same constant beta chain would be exposed to CAR-mediated cytotoxicity. Currently, one clinical trial is recruiting patients with relapsed or refractory TRBC1-positive selected T-Non-Hodgkin Lymphoma (NCT03590574).

4.5 Oncogenic role of STAT3 in T cell neoplasia

4.5.1 The biological function of STAT3

Signal transducer and activator of transcription (STAT) is a gene family consisting of 7 members, one of which is STAT3. It can function either as a cytoplasmatic signaling molecule or as a transcription factor (207). When interleukins, cytokines, or growth factors bind to their receptors, activation receptor-associated tyrosine kinases, often Janus Kinases (106), auto phosphorylate (208). This provides STAT3 with a binding site for its conserved SH2 domain, leading to phosphorylation, dimerization, and constitutively activating STAT3 (209, 210). Phosphorylated STAT3 can directly bind to the DNA and can even recruit co-activators to promote further transcriptional activity (211, 212). Moreover, STAT3 can regulate gene expression via epigenetic modifications such as DNA methylation (213). It is involved in cell proliferation, survival, differentiation,

migration, angiogenesis, and inflammatory, autoimmune, and dysregulated neoplastic processes (214, 215).

4.5.2 STAT3 mutations in T cell neoplasia

Oncogenic mutations in STAT3, compared to the other STAT members, can not only be found predominantly in B- and T-cell lymphoma, in T-LGL, in large granular lymphocytic leukemia of natural killer cells (NK-LGL) but also in solid tumors. For the cancer entity distribution of the single STAT family members, see Figure 1 (214).

With mutation rates of up to 40%, STAT3 is one of the most frequent mutations in T-LGL (216), and it has rates of 30% in NK-LGL. Most mutations are located in the SH2 domain of the STAT3 gene, like the two most common mutations Y640F and D661Y (217). In vitro, functional studies have shown that Y640F and D661Y increase the transcriptional activity of STAT3, which increases the risk of degeneration and autoimmune diseases, and immune-mediated cytopenia in patients with these mutations (216).

Apart from in LGL, lower mutation frequencies have been identified in PTCL-NOS (3.8%), ALK+ and ALK- ALCL (both 3.7%), EATL (12.1%), and ENKTL and its subtype γδ-T-cell-derived lymphoma (γδ-PTCLs) (21%) (87, 218-221). However, mutation frequencies vary highly between studies. In the pathogenesis of ENKTL, a constitutive active JAK-STAT3 pathway plays a significant role. Still, mutation frequencies vary in the literature for specific genetic alterations like Y640F, located at the SH2 domain, i.e., from 1.5% up to 26% (220, 222-224). Frequency of STAT3 mutations in some T-cell lymphomas is shown in Figure 2.

However, so far, only the JAK-STAT3 pathway has been approached by multiple blockers like cytokines or antibodies, such as PD-L1 inhibitors (87), or JAK- or STAT-inhibitors like Stattic or tofacitinib (222, 225). Furthermore, frequent mutations in the SH2 domain, like D661Y, are promising targets for treating STAT3 mutated T cell neoplasms. STAT3 D661Y was therefore chosen as the target antigen in this study.

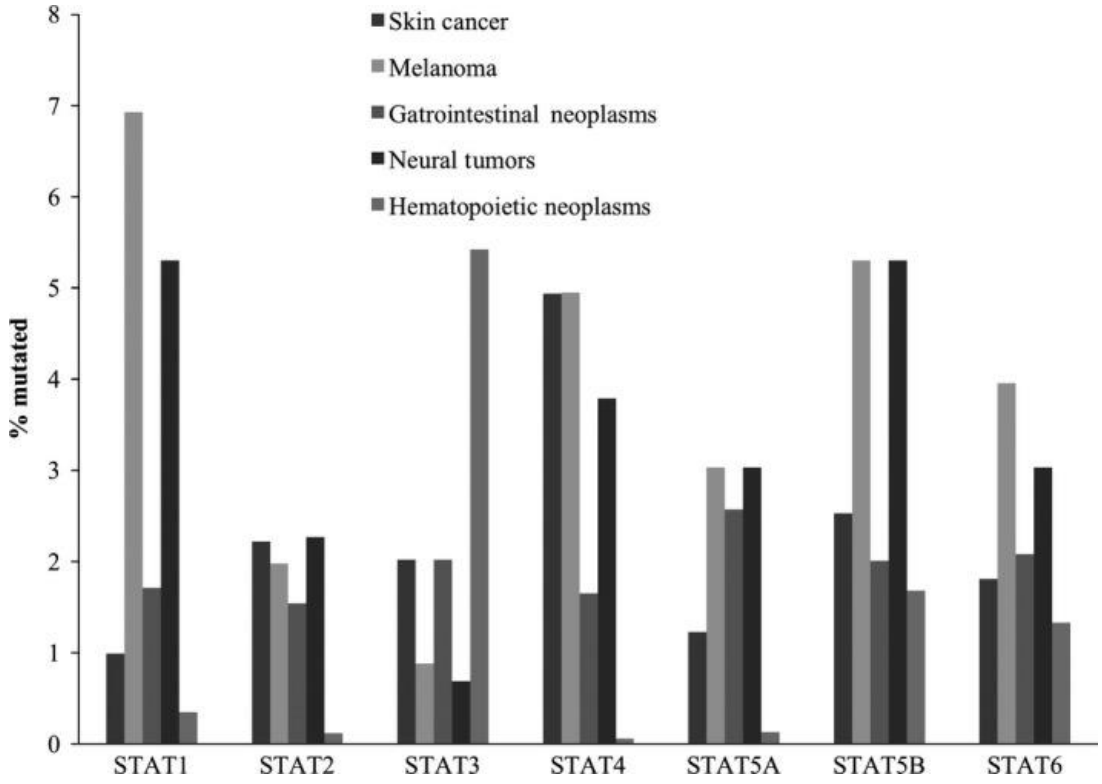


Figure 1. Mutational frequencies in the STAT gene family in different tumor entities. Data were obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC). Stat3 is, as expected, the most frequent gene mutated in hematopoietic malignancies. Figure adapted from Shahmarvand et al., Mutations in the signal transducer and activator of transcription family of genes in cancer.

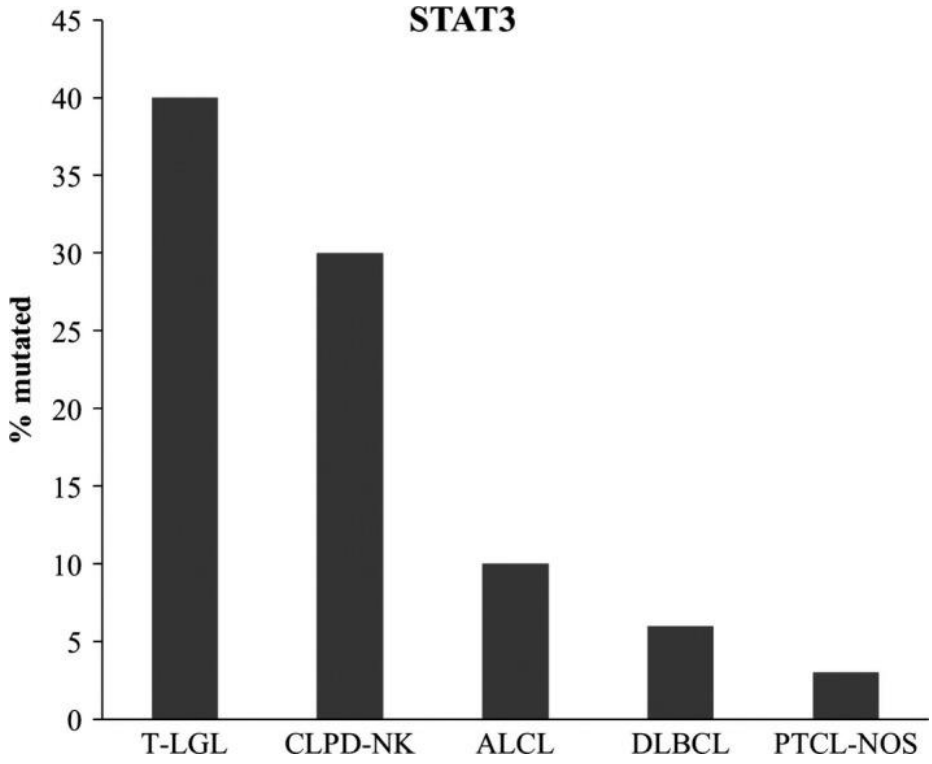


Figure 2. Frequency of STAT3 mutations in T cell large granular lymphocytic leukemia (T-LGL), chronic NK lymphoproliferative disorder (CLPD-NK) or NK large granular lymphocytic leukemia (NK-LGL), anaplastic large T cell lymphoma (ALCL), diffuse large B cell lymphoma (DLBCL) and peripheral T cell lymphoma – not otherwise specified (PTCL-NOS). Figure adapted from Shahmarvand et al., Mutations in the signal transducer and activator of transcription family of genes in cancer.

5 Aim of the thesis

Based on the information from the first chapter, the following question arises for my dissertation:

Is it possible to isolate high-avidity STAT3 D661Y mutation-specific T cells from the human system and to subsequently characterize them concerning their specificity, efficacy, and safety in vitro?

This included in detail:

- Cloning of a tandem minigene encompassing the STAT3 D661Y mutation into the retroviral vector pMP71 and transduction into K562 cells for mass spectrometry analysis concerning the presence of a STAT3 D661Y peptide - HLA-A*02:01 complex
- Identification of TCRs from mRNA of peptide-reactive T cells generated after in vitro priming of T cells with peptide-loaded autologous dendritic cells
- Transduction of these TCRs into CD8+ T cells and testing for reactivity against peptide-loaded cells as well as selective recognition of the mutant STAT3 D661Y peptide in contrast to the wild-type peptide
- Determination of functional avidity of the identified TCRs by a peptide titration assay
- Testing of reactivity of the TCRs against target cells endogenously expressing STAT3 D661Y and HLA-A*02:01 allele
- Exclusion of cross-reactivity with an Alanine scan assay

6 Material and Methods

6.1 Identification of the HLA-A*02:01-restricted neoepitopes

6.1.1 In silico prediction of antigen processing of STAT3 mutated peptides

Common mutations in the STAT3 gene were screened to see if they could be processed by the proteasome and presented via HLA-A*02:01. Selection of potential neoepitopes was influenced by predicted high binding affinities of the peptides to the HLA-A*02:01 complex. Potential immuno-peptidomic peptides were thus scanned via HLA Ligand Atlas and iedb.org. Literature research on the elution of the peptide from HLA-A*02:01 was performed in the Pubmed database (last update 30.08.2022). For the prediction of binding affinity, NetMHC V.4.0 was used. Subsequently, NetMHCpan V.4.1 was applied to predict the processing via proteasome and binding affinity to MHC for the peptide. The selected 10mer peptide of STAT3 D661Y (IIMGYKIMYA) and 9mer peptide of STAT3 D661Y (IMGYKIMYA) were ordered and synthesized as short peptides by GenScript.

6.1.2 Identification of peptide target candidates by mass spectrometry

To determine if a peptide spanning the STAT3 D661Y mutation is presented on the HLA-A*02:01 of target cells, a mass spectrometry analysis was performed by Dr. med. Martin Gunther Klatt, Charité. Therefore, this Methods section is more of a content summary of what was done. Exact details of the protocol can be found here: Klatt et al., Solving an MHC allele-specific bias in the reported immunopeptidome (226).

Isolation of HLA ligands from the K562 cell line transduced with HLA-A*02:01 and STAT3 D661Y minigene was performed utilizing immunoprecipitation and ultracentrifugation. The cells were pelleted and lysed, and the membrane proteins were solubilized using a non-ionic solvent 1% CHAPS, dissolved in PBS supplemented with a protease inhibitor. After cell lysis, lysates were spun down, and supernatant fluids were isolated.

The supernatant of the cell lysate, containing all proteins, was applied to an affinity column. With the help of the W6/32 antibody directed against HLA-A, -B, and -C (227), HLA complexes and their peptide ligands were bound to the column. Using peristaltic pumps, the supernatants of the cell lysates are running overnight at four °C through the affinity columns. To release the antibody-coupled HLA-peptide complexes from the Sepharose columns, they were exposed to 1% Trifluoroacetic acid (TFA); the acid causes

the HLA-A complexes to denature and detach from the column so that they can be eluted together with the peptide.

For the final elution of the peptide, the HLA complexes and the peptides were loaded onto a C18 column, where the MHC complexes remained suspended due to hydrophobic interactions, allowing the peptides to be eluted with the solvent 30-50% acetonitrile (ACN) due to their more hydrophilic properties. Thus, only peptides were eluted in the second column and could be subsequently added to the high-performance liquid chromatography-tandem mass spectrometer (HPLC MS/MS).

In HLPC, peptides were separated using direct loading onto a C18 column with a slow flow rate at increasing gradients of ACN, which sorted the peptides depending on their hydrophobicity. The peptides were then ionized, and the mass-to-charge ratio (m/z) was determined in the gas phase. The most abundant ion (M) and its ions with one or two additional isotopic masses (M+1, M+2) were analyzed concerning their full/unfragmented MS spectrum and the MS spectrum after collision-induced dissociation fragmentation. The same HPLC MS/MS analysis was performed with synthetic 10mer and 9mer peptides of STAT3 D661Y. Conclusions about the peptides bound to HLA could be drawn by analyzing the different CID fragments and their masses with the MS/MS software algorithm.

6.2 Molecular cloning

6.2.1 STAT3 D661Y minigene cloning into pMP71 vector

The chosen neoepitope peptide was ordered as a fragment (GeneArt, Thermo Fisher Scientific). It was designed as Tandem minigene (TMG) to contain three times the peptide sequence spanning the D661Y mutation, flanked by three nucleotides at the 5’ end and 24 additional nucleotides at the 3’ end (Figure 3b). An AAY linker sequence separates the three STAT3 D661Y sequences to ensure proteasomal cleavage was performed in vivo (228). The ordered fragment was cloned into the Vector pMP71, a retroviral vector designed for enhanced transgene expression in T-lymphocytes (229). pMP71 (Figure 3a) also contains the genes to express an enhanced-green fluorescent protein (eGFP), which allowed the controlling of transgene expression efficiently via fluorescence-activated cell scanning (FACS). The cloning was performed as follows: 1.25 ug of plasmid DNA was

digested with the restriction enzymes NotI/EcoRI (Fast Digest, Thermo Scientific) according to manufacturer's directions: the digested plasmid was separated on a 1% agarose gel, the appropriate bands were cut, and the DNA was purified (for protocol, see Invitrogen DNA gel extraction kits, Thermo Fisher Scientific). The TMG fragment (30ng) was ligated to the pMP71 vector (50 ng) with T4 DNA ligase (for protocol, see T4 DNA Ligase, Thermo Fisher) and transformed to Stellar competent cells (Takara). STAT3 D661 minigene insertion into the vector backbone was confirmed by Sanger sequencing. K562+HLA-A2 cells were transduced with the vector pMP71 carrying the STAT3 D661Y minigene cassette, as described in Section 3.3.2.

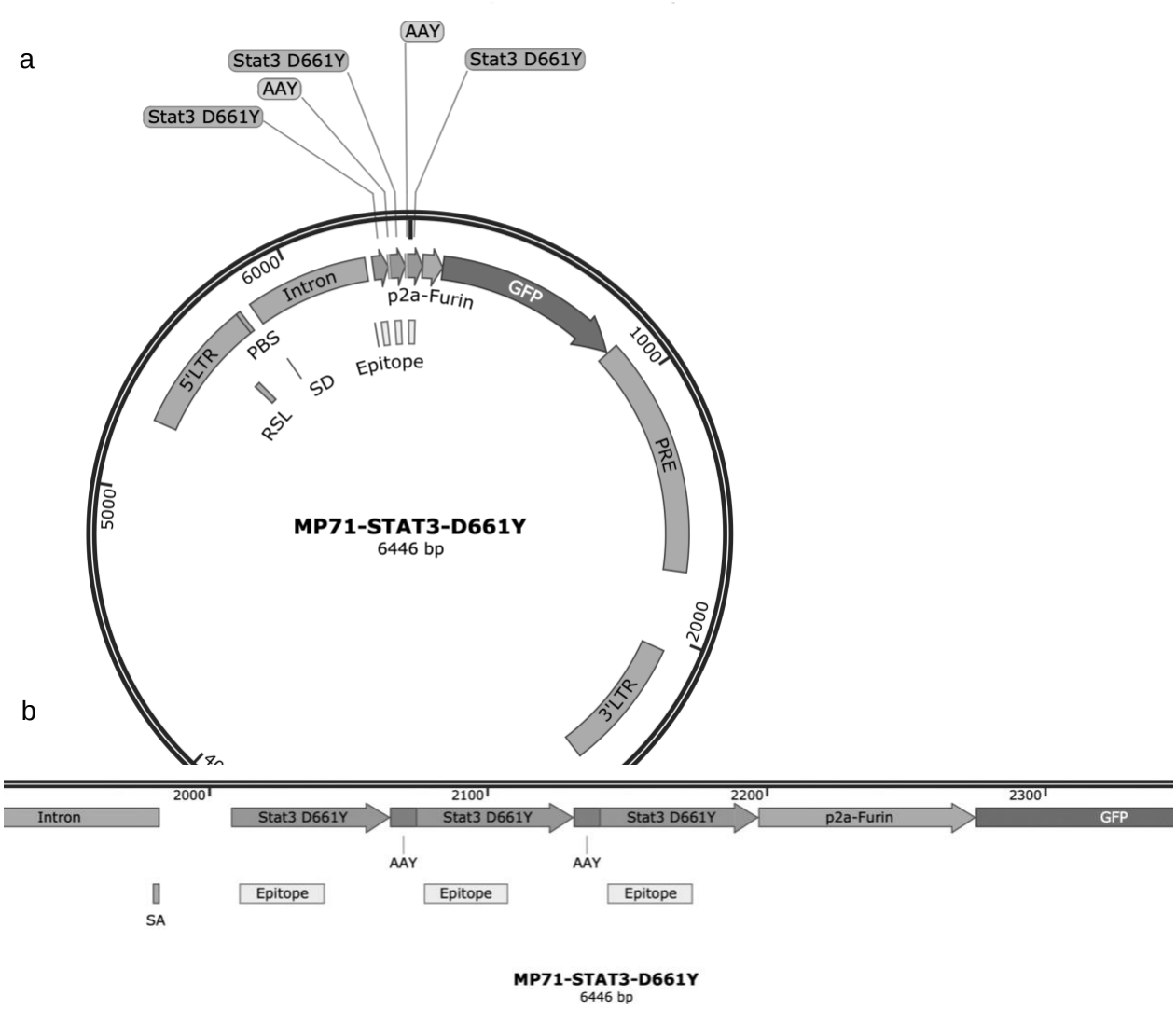


Figure 3. Retroviral vector MP71 with STAT3 D661Y minigene. (a) From 5' long terminal repeat (LTR) to 3' LTR: RSL – R region stem-loop; PBS – primer binding site; SD – splice donor; SA – splice acceptor, both flanking an intron with a packaging signal; 3x STAT3 D661 minigene with additional nucleotides at both ends, p2A- peptide 2A – cleavable peptide linker, GFP - green fluorescent protein; PRE - woodchuck hepatitis virus posttranscriptional regulatory element, gene enhancer. (b) zoom into STAT3 D661Y minigene. 10mer peptide sequence is marked. Figures were generated using Geneious V 9.1.8 and snap gene.

6.2.2 TCR cloning and TCRα/β Chain Identification

The starting point for cloning was isolated mRNA from peptide reactive T cells, which I kindly received from Dr. Özcan Çınar. Here I only summarize very briefly how he generated the mRNA.

T cell stimulation with autologous dendritic cells pulsed with antigen peptide and in vitro enrichment of peptide reactive T cells, cell sorting by fluorescence-activated cell sorting (FACS), and isolation of mRNA from viable CD8+ peptide-specific T cells all from the same donor was done by Dr. Özcan Çınar. The mRNA samples were stored at -80°C and numbered in the following way 20, 21, 45, 48, 59, 60, 63, 66, and 70; the numbers were kept the same in this study. A protocol describing the generation of mutation-specific T cells can be found in Çınar et al., High-affinity T-cell receptor specific for MyD88 L265P mutation for adoptive T-cell therapy of B-cell malignancies (143).

The kindly provided isolated mRNA from several sorted T cells was used in 5' rapid amplification of cDNA ends - polymerase chain reaction (RACE-PCR) (230), (protocol: SMARTerRACE5'/3' Kit, Takara Clontech Laboratories). In the first step, isolated T cell mRNA was converted into a first strand cDNA by use of retroviral transcriptase, followed by rapid amplification of cDNA ends (230); two RACE-PCRs according to the protocol were set up to amplify the DNA of the α and β chains of the T cell receptor. InFusion primer α was used: TCRA 5′-CGGCCACTTTCAGGAGGAGGATTCGGACC-3′ and TCRB: 5′-CCGTAGAACTGGACTTGACAGCGGAAGTGG-3′. The thermocycler was set up for 35 cycles: initial denaturation at 94°C for 2 minutes and denaturation at 94°C for 30 seconds. In the first five cycles, there was annealing for 3 min at 72°C; in the subsequent five cycles, annealing was at 70°C for 30 seconds, and in the last 20 cycles it was at 68°C. Elongation was always performed at 72°C for 3 minutes. RACE-PCR products were separated on a 2% agarose gel, and bands were observed at approximately 950 bp. PCR products at the expected size were extracted from the gel and purified (for protocol, see Invitrogen DNA gel extraction kits, Thermo Fisher Scientific). Next, a RACE-InFusion cloning was performed with the gel-purified RACE-PCR products, with transformation into E.coli Stellar competent cells using heat-shock transformation. For each transformant, a plasmid of at least 20 colonies was picked to isolate plasmid DNA using a NuceloSpin Plasmid Easy Pure miniprep kit (Marchery Nagel). A control digest was conducted with EcoRI and HindIII, and samples were

separated on 1% agarose gel. Plasmids that released an insert at approximately 700-800bp size were sequenced by Sanger sequencing. Rearranged TCR α and β chains were identified using the IMGT.org V quest tool. The most dominant rearranged α and β chains were paired to construct a TCR cassette with constant murine regions. The TCR cassette was cloned by Gibson Assembly (Gibson Assembly Protocol, New England BioLabs). The assembled TCR cassette was cloned into the pMP71 vector by restriction enzyme cloning (NotI/EcoRI). The cloning of the expression cassette into the vector backbone was confirmed by Sanger sequencing.

6.3 Cell cultures

6.3.1 Cell lines

Profs. Mathas and Janz kindly provided the cell line HDLM-2, and the cell lines T2 and HEKT-GALV-g/p were kindly supplied by Prof. Uckert (now Professor Emeritus). Both groups were located at the Max Delbrück Center for Molecular Medicine, Berlin. The K562 leukemic cell line, T2 cell line, and HDLM-2 Hodgkin Lymphoma cell line were maintained in RPMI (with stable Glutamine, Gibco), with 12.5 mM HEPES, 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin and 50 µg/mL gentamycin. HEKT-GALV-g/p were cultured in DMEM (from Gibco) with 10% FBS, 100 U/mL penicillin, and 1 g/L sodium pyruvate. Primary human T lymphocytes were cultured in RPMI (with stable Glutamine, Gibco), with 12.5 mM HEPES, 100 U/mL penicillin/streptomycin, 50 µg/mL, but with 10% FBS. The medium was supplemented with 400U/ml IL-2 (Proleukin, Novartis) in the expansion phase (first week after transduction) and 40U/ml in the maintenance phase. In addition, T cells received IL-7 (Peprotech) and IL-15 (Peprotech) at 5ng/ml concentration.

6.3.2 Virus production and Transduction of PBLs

Packaging cell line HEKT-GALV-g/p, stably expressing MLV gag/pol and pALF-GALV, was transfected with a nine µg TCR-cassette-ppMP71 vector to produce virus particle-containing culture supernatant. Culture supernatant was collected 48 h and 72 h after transfection. From healthy donors, $1x10^6$ PBMCs were activated with soluble a-CD3 (1 ug/mL) and a-CD28 (5ug/mL) antibodies for 48h in T cell medium supplemented with 400

U/mL IL-2, 5ng/mL IL-7, and 5ng/mL IL-15, as described in 3.3.1. Cells rested in a T25 flask with supplemented Pan-T cell medium for 48 hours before transduction.

On the day of transduction, the virus supernatant was collected and filtered through a 0.45 μM filter. 2mL of the virus supernatant was spinoculated on a retronectin-coated 24-well plate at 3000g for 90 minutes at 4°C. Afterward, the virus supernatant was removed, and 1 ml of activated T cells per well was added, supplemented with 400U/IL-2 and 4 µg/mL protamine sulfate.

24h later, the cells were spinoculated a second time at 800g for 90 mins and 32 °C by adding 1.5 ml filtered virus supernatant, 400U/ml IL2, and 4µg/mL protamine sulfate to the PBMCs. Depending on the density of T cells on the plate, they were transferred to T25 flasks and T75 flasks. Every medium exchange was conducted with the replacement of cytokines, as described 3.3.1.

6.3.3 Generation of target cell lines

K562 cells were stably transduced to express the STAT3 D661Y minigene as well as the HLA-A*02:01 complex via the retroviral vector pMP71 and used as an artificial target. To generate a target cell line for the control TCR T1367, cell lines expressing the MAGE-A1 antigen were generated. T-knife kindly provided the retroviral plasmid pMP71-MAGE-A1-GFP. The MAGE A1-GFP cassette was transduced into HDML-2 cells. Both cell lines were enriched for GFP expression on the FACS Aria II device. The same transduction protocol for the transduction of PBLs was applied for both target cell lines (see 2.3.2).

6.3.4 RNA isolation and cDNA synthesis of HDML-2

To generate samples for sequencing analysis, 1-2 ml of HDML cells were freshly collected and pelleted. The RNA isolation was performed with a RNeasy Mini Kit, Qiagen, according to the manufacturer's instructions. Genomic DNA was removed by on-column RNase-free DNase I treatment using the DNase I kit from Qiagen (Cat no: 79254). Afterward, cDNA was synthesized using Superscript IV Reverse Transcriptase, Thermo Fischer. DNA was primed using random hexamers for the first cDNA strand synthesis reaction whereby the protocol of the Superscript IV Reverse Transcriptase Kit was followed. STAT3 D661Y cDNA was amplified with an accordingly designed primer pair: forward primer 5’CAGCGGTAAGACCCAGATCC 3’ and reverse primer 5’ CCTGGGTCAGCTTCAGGATG 3’. Cycling conditions were performed as follows: initial

denaturation for 30 seconds at 98°C, followed by 30 cycles of 10 seconds of denaturation at 98°C, 15 seconds of annealing at 69°C and 15 seconds of elongation at 72°C. The final extension was performed for two minutes at 72°C. Control samples were separated on 1% agarose gel and sent for Sanger sequencing.

6.4 Immunofluorescence staining and Flow cytometry analysis of T cells

Successful transduction was controlled after approximately five days by Fluorescence Activated Cell Sorting (FACS) analysis on a BD FACSCanto II instrument. Cells were collected, washed twice, and stained with diluted antibodies according to the manufacturer's information. Depending on the transduction and question regarding expression levels, different antibodies linked with a fluorescence marker were used: viability staining was done with DAPI (4′,6-diamidino-2-phenylindole) (BD Biosciences). Control of transduction efficiency for K562 HLA-A*02:01 transduced cells was done with PE-HLA-A*02:01 mouse IgG2b, κ antibody. Control of transduction efficiency of transduced PBLs was done by cell staining with PE anti-mouse TCR β chain recombinant IgG1, κ antibody, and APC mouse anti-human CD8 IgG1, κ antibody. All antibodies were purchased from BD Pharmingen. Transduced HDLM-2 cells were analyzed for GFP-reporter gene expression. Data were collected and analyzed with FlowJo software (version 10.8.1). Gates were set as followed: viable cells, lymphocytes, single cells, CD8+, and mbTCR+ subset.

6.5 Functional T cell assays

6.5.1 Peptide reactivity and cytotoxic activity against target cells

To identify the peptide-reactive rearranged TCR pairs, TCR transduced T cells were cocultured with T2 cells or K562+HLA-A2 cells loaded with STAT3 D661Y 10mer, 9mer or control peptides derived from either Tyrosinase or MAGE-A1, depending on the control TCR that was used in the particular coculture experiment. Briefly, effector cells and target cells were counted using a counting chamber. Dead cells were excluded by tryphan blue staining. $2.5x10^4$ $TCR^+/CD8^+$ effector cells and $2.5x10^4$ peptide-loaded target cells were seeded in 200 μL of medium per well of a U-bottomed 96-well plate to reach a 1:1 effector-

to-target ratio. After overnight coculture, cell-free supernatant was collected to measure IFN-γ secretion by ELISA (OptEIA Human IFN-γ ELISA Set; BD Biosciences, New Jersey, USA).

For T cell activation assays, target cells were loaded with a peptide concentration of 10 µM. For titration assays, a serial dilution of peptides from 10 µM to 1 pM was loaded on T2 cells. Peptide loading was performed as follows: peptide was added to $1x10^6$ T2 cells in 1 mL medium to reach the desired final peptide concentration and incubated for one hour at 37°C. Afterward, cells were washed twice with medium to remove excess peptide. Peptide-loaded cells were resuspended in medium at a cell density of $2.5x10^5$ cells/mL, and 100 µL cell suspension was seeded per well. Additionally, cytotoxicity against target cell lines, such as HDLM-2 or K562-A2-STAT3-D661Y minigene, or control cell lines Nalm6 and OCI-Ly3 was assessed by coculturing them with transduced TCR T cells. Each effector and target combination was always performed in duplicates.

6.5.2 Detection of CD137 upregulation on activated T cells

T cells were cocultured overnight with HDLM-2 target cells at a 1:1 effector-to-target ratio as described before. The cells were collected, washed twice, and stained with: PE-mouse anti-human CD137 Mouse BALB/c IgG1, κ antibody; Pacific Blue – anti-human CD8α Mouse IgG1, κ antibody; and APC-Cy- anti-mouse TCR β chain IgG2, λ1 antibody. Measurement was done with the BD FACSCanto instrument and analyzed with FlowJo software. The stained cells were first gated for viability; next, lymphocytes, single cells, and CD8 cells were circumscribed. The CD8+ population was chosen, and CD137+ and mouse beta TCR + cells are described here.

6.5.3 Alanine Scan Assay

For characterization of the TCR binding motif, 10mer peptides were ordered from GenScript, in which each amino acid of IIMGYKIMYA was once replaced by an Alanine, Alanine was replaced by Glycine. The peptides were reconstituted to 10mg/ml in DMSO. T2 cells were loaded with 10^5M, 10^7M, 10^9M peptide concentrations and incubated for one hour. TCR T cells and peptide loaded T2 cells were incubated in a 1:1 ratio overnight for 16 hours. Cell-free supernatant was collected to measure IFN-γ secretion by ELISA

(OptEIA Human IFN-γ ELISA Set; BD Biosciences, New Jersey, USA). The Online Software ScanProsite was used to compare the data for similar human proteoms.

6.6 Statistical analysis

Statistical analysis of data and graphical representations were performed using GraphPad Prism V.9 (GraphPad Software, Version 9.00, GraphPad Software, La Jolla, CA, USA). T cell assays with target cell lines were analyzed by two-way ANOVA using Tukey's multiple test with adjusted p values. Peptide titration analyses were baseline corrected by defining the IFN-γ signal at the highest peptide concentration as a baseline, and the concentrations (X values) were transformed in log. EC50 values, the peptide dose at which the half-maximum of T cell activity was achieved, were calculated, and the data is presented in a sigmoidal 4PL model.

7 Results

7.1 Binding affinity of STAT3 D661Y peptides to the HLA-A*02:01 complex

Among STAT3 mutations in the SH2 domain, the D661Y mutation is restricted to HLA-A*02:01, a main haplotype with 47.8% allele frequency in the Caucasian population and 16.8% in the African-American population (231). The predicted binding affinity of the WT 10mer peptide (IIMGYKIMDA) via NetMHC V.4.0 (232) was 227 nM, i.e., a weak binder. However, the mutated 10mer STAT3 D661Ypeptide (IIMGYKIMYA) is a strong binder to HLA-A*02:01 (17.7 nM) (overview, Table 3a in Section 6.7). The change in binding affinity was also an influential criterion for the target selection. An identified 9mer peptide was also screened as a WT peptide (IMGYKIMDA) and as a STAT3 mutated D661Y peptide (IMGYKIMYA). The WT peptide was predicted to be a weak binder (686.95 nM), while the mutated 9mer peptide was a strong binder (31.95 nM).

7.2 Successful generation of target cells.

K562 cells do not express any MHCI molecule on their surface. To use them as target cells for an HLA-A*02:01 restricted TCR, they had to be transduced to express this MHC-I allele. To further express the target antigen, they were also transduced with the previously described retroviral vector pMP71 that carries the STAT3 D661Y minigene. The success of the transduction was also monitored by immunofluorescence staining and FACS analysis. Transduction efficiency for the minigene expression in K562 cells was around 70% (FITC-positive cells).

Furthermore, a target cell line for the control TCR T1367 specific to the MAGE-A1 antigen was generated. It was possible to transduce the HDLM-2 cell line with the MAGE-A1-GFP antigen. Among the alive cells, the HLA-A*02:01 (PE) and MAGE-A1 GFP (233) positive subset was 29.5%.

7.3 Tandem mass spectrometry analysis of K562-A2-STAT3-D661Y minigene transduced cells

Data was provided generously by Dr. med. Martin Klatt, Charité. After generation of the K562-A2-STAT3-D661Y cells, the mutated 9mer and the 10mer STAT3 D661Y peptide

was eluted, and a peptide spectrum could be displayed. As seen in the mirror plot (Figure 4), the ionized peptide fragments correspond in mass/charge ratio to those of the synthetic 9mer or 10mer peptide. This indicates that the HLA-A2 molecules presented both the 9mer and 10mer peptides on the surface of the cells. Furthermore, when it comes to retention time during high-performance liquid chromatography, the eluted peptides, presumably the 9mer and 10mer peptides (M) as well as their isotopes (M+1 and M+2), have similar retention times and isotope peaks as their synthetic counterparts. However, the 10mer peptide was detected with 14.6 times more intensity than the 9mer Peptide (Figure 5). Therefore, the 10mer peptide was chosen as the preferred target.

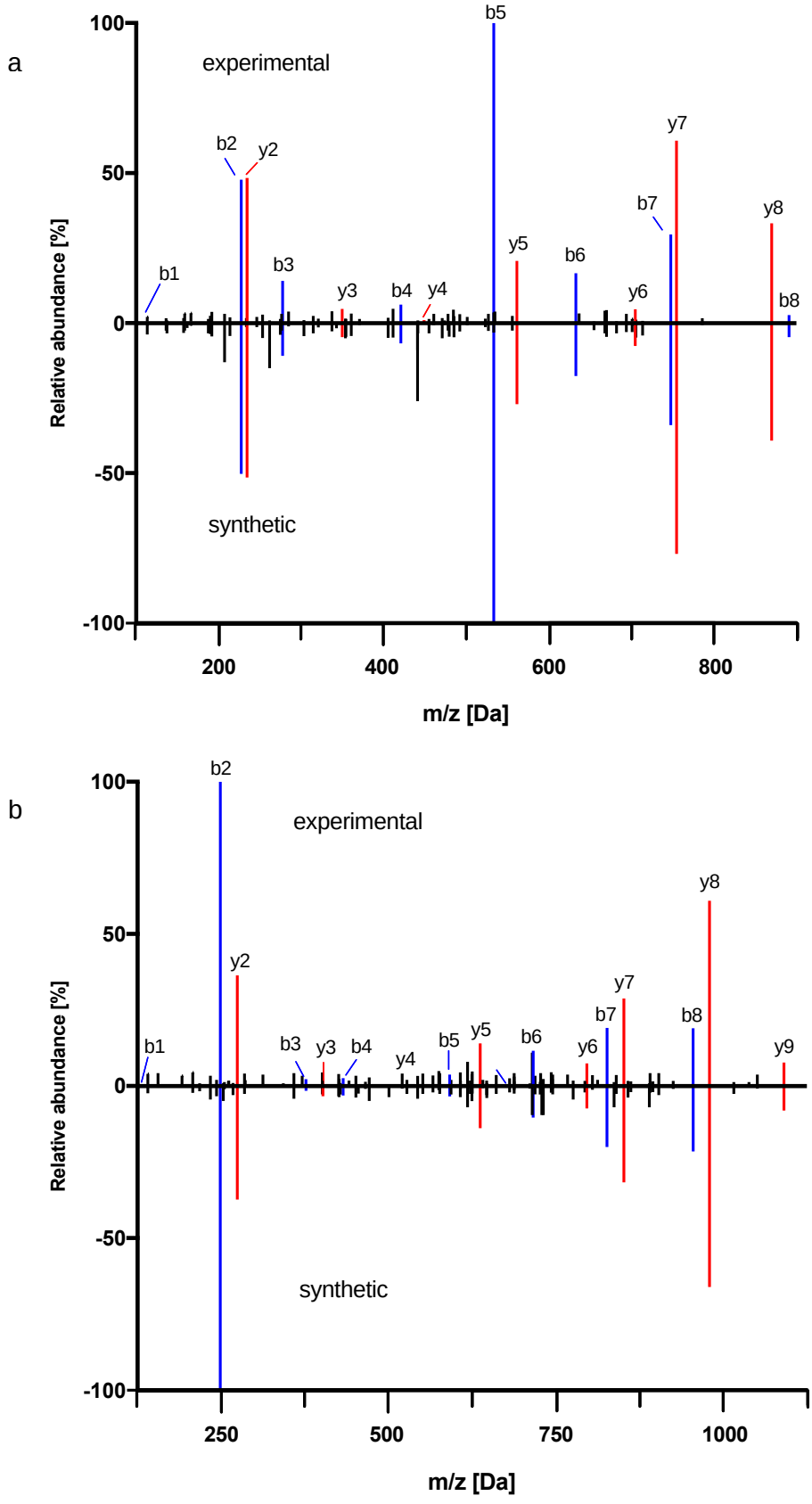


Figure 4. Mirror plot for STAT3 neoepitopes. MS/MS spectra for K562-derived HLA ligands (top) and the synthetic peptide (1). 9mer is depicted in (a), 10mer in (b). Figures were kindly provided by Dr. Martin Klatt, Charité.

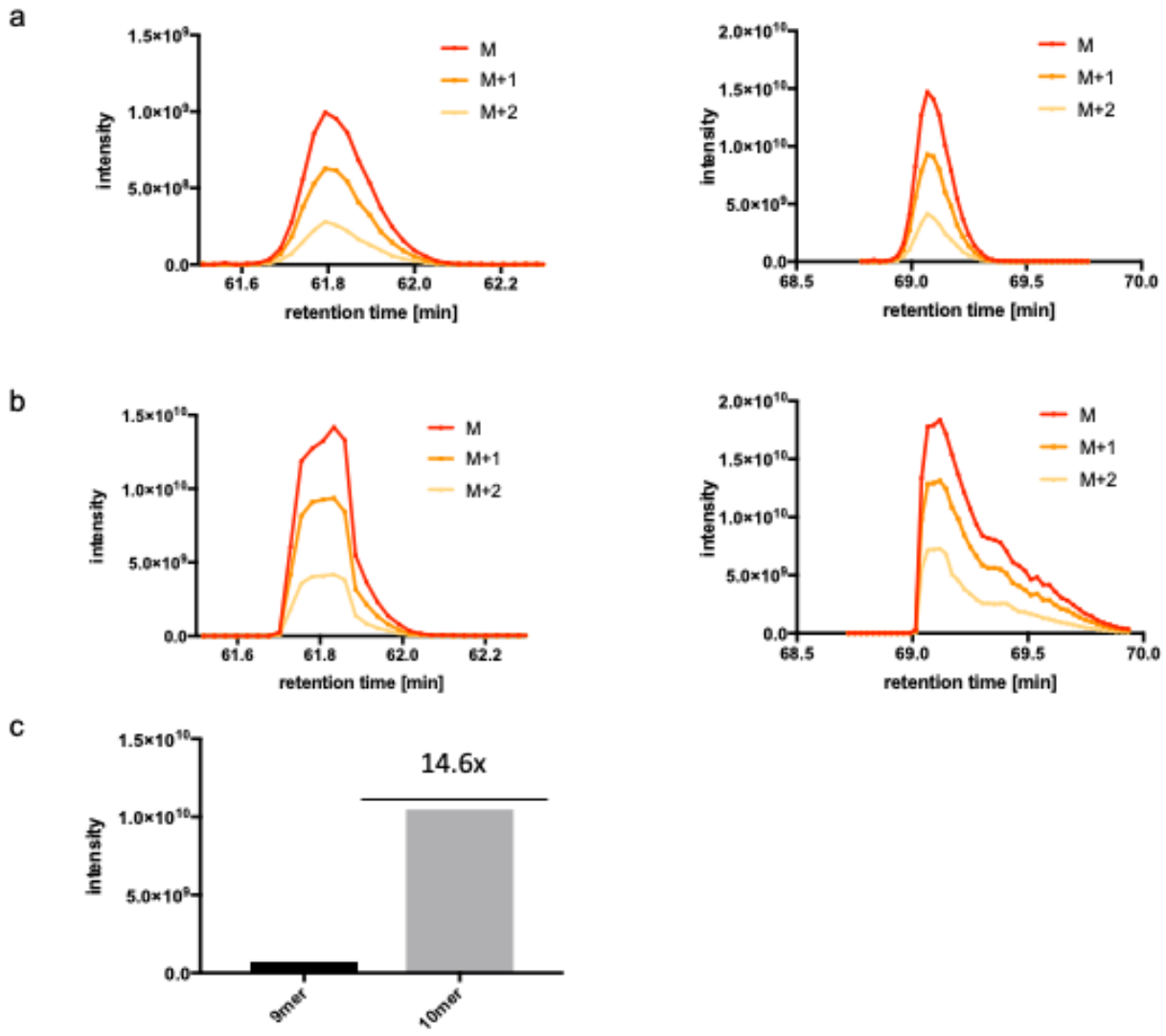


Figure 5. Key parameters for STAT3 neoepitopes. Retention times of experimentally isolated HLA ligands from K562 cells (a) and synthetic control peptides (b). The 9mer neoepitopes are shown on the left, and the 10mer on the right. (c) Relative abundance of the 99mer neoepitope compared to the 10mer epitope. Figures were kindly provided by Dr. Martin Klatt, Charité.

7.4 T cells engineered to express STAT3 D661Y mutation-specific TCR

7.4.1 4.4.1 Identification of TCR α and β chains from mRNA samples

Using RACE-PCR, cDNA was synthesized from mRNA, which was isolated from peptide-specific T cells after sorting activation marker positive cells following a coculture with peptide-loaded target cells.Subsequently, we successfully amplified DNA for the α and β chains of the T cell receptor. Step-by-step PCR products loaded on agarose gel can be seen in Figure 6. After InFusion cloning and transformation of bacteria, bacterial clones were sent to sequencing. From the sequencing results, eight TCR α/β chain rearrangements were identified. The variable α and β chains were paired together. In this doctoral thesis the sequences of the individual sequences of the alpha and beta chains are not published due to a possible patent application. Constant regions were murinized to avoid mispairing with the endogenously expressed TCR chains. TCR-expression cassettes were assembled and cloned into the retroviral vector pMP71 by ligation. Completely assembled TCRs were named 20, 21, 45, 48, 59, 60, 63, and 70 for further characterization.

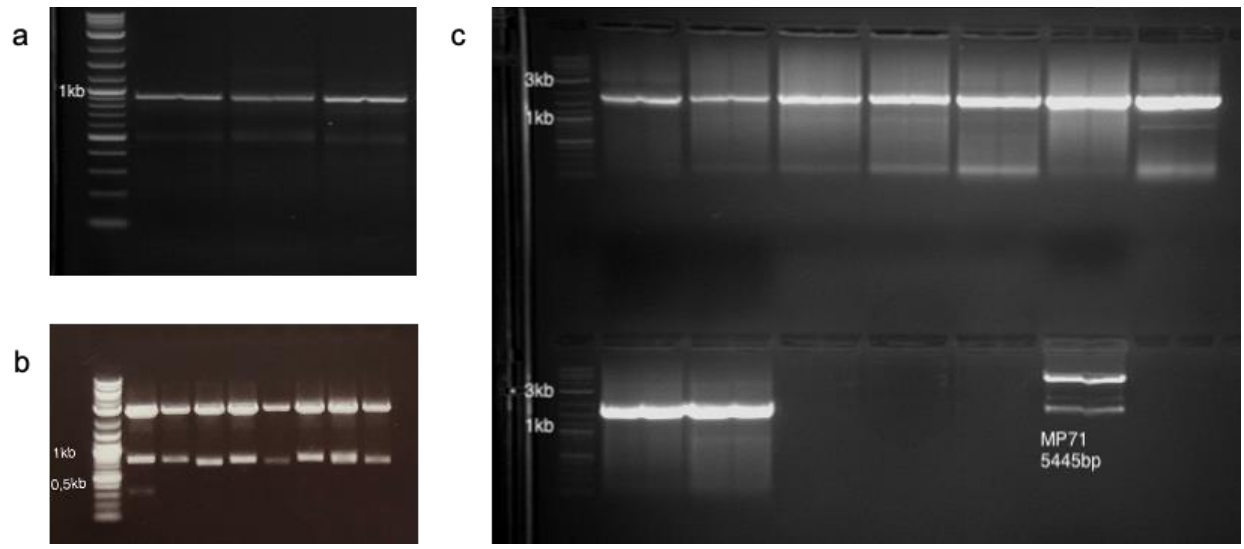


Figure 6. Overview of PCR samples separated by Agarose gel electrophoresis to identify rearranged TCR sequences from T cell clones and to construct TCR expression cassettes via Gibson assembly. (a) Rearranged TCR sequences were amplified from the RNA isolated T cell clones by RACE-PCR. The size of the PCR product of rearranged α/β variable chains was approx. 950bp. (b) Control digest of InFusion cloning vector containing beta chain 21 (approx. 750bp). (c) The variable chains Gibson Assembly PCR

product: complete TCR with variable α, β and murine constant chains (approx. 1800bp), amplified vector MP71. bp – base pairs.

7.4.2 Transduction efficiency of identified TCRs

Peripheral blood mononuclear cells (PBMCs) containing CD8+ T cells from eight healthy donors were transduced with the pMP71 vector carrying the TCR cassettes to express the mutation-specific TCRs. Yielded transduction efficiency and the survival of transduced T cells were determined by FACS using live/dead staining and antibodies against the murine β constant chain and the T cell surface marker human CD8. An overview of all transduced TCRs is shown in Figure 7. Transduction efficiency varied greatly and was between approximately 10-80% for all cells, and for CD8+ cells, a maximum of 32% was reached. Yielded transduction efficiency for one batch of transduced TCR T cells from one representative donor is shown in Table 2.

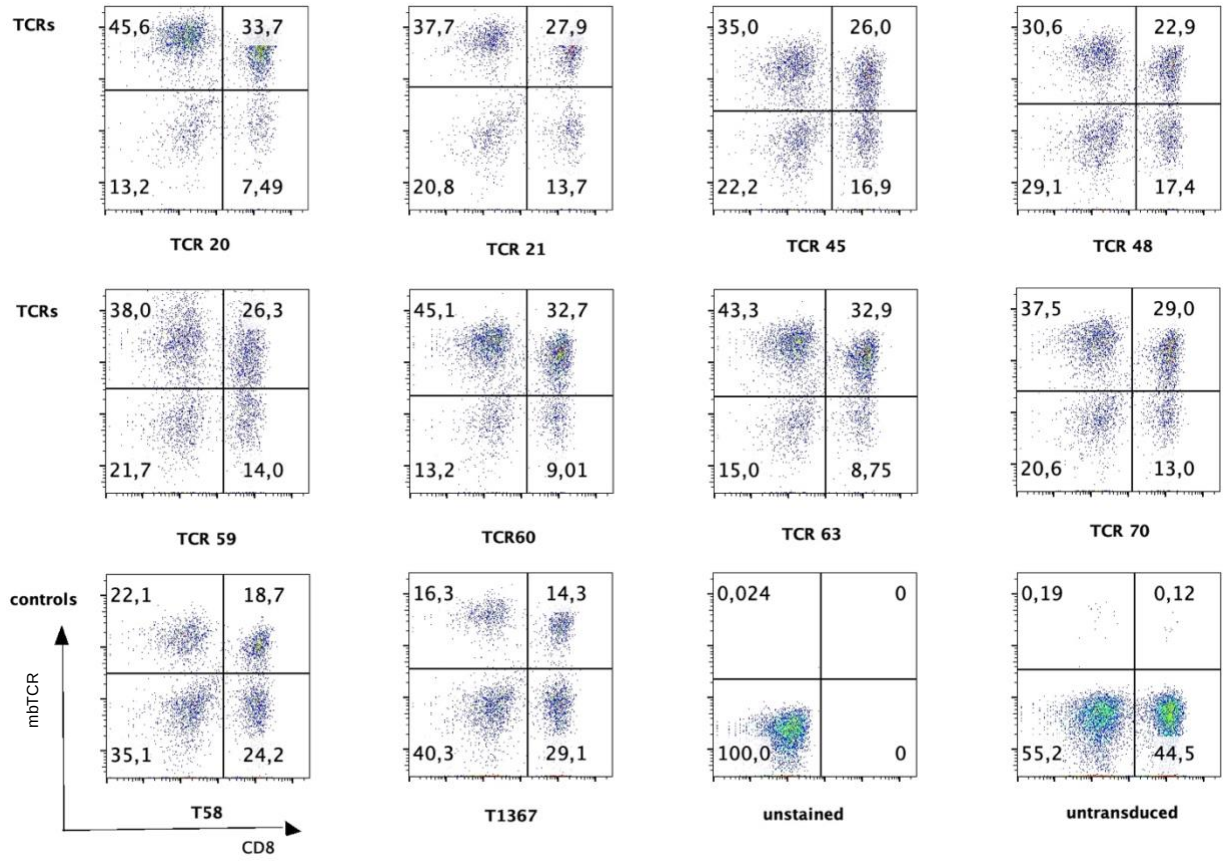


Figure 7. The transduction efficiency (CD8+ and mbTCR+ cells) of STAT3 D661Y redirected TCRs after Immunofluorescence staining. Flow cytometry analysis was performed 5 days after transduction of PBMCs from one representative donor (BC 80). Figures were created with FlowJo V. 10.8.1.

	Lymphocytes (frequency of total cells)	Single cells (frequency of lymphocytes)	alive cells (frequency of total)	Transduction efficiency (mbTCR+, CD8+)
20	96,8 %	97,5 %	74,2 %	**33,8 %**
21	21,4 %	95,5 %	19,9 %	**28,4 %**
45	62,6 %	98,6 %	60,5 %	**19,7 %**
48	36,4 %	97,1 %	34,2 %	**21,4 %**
T58	58,4 %	98,4 %	56,6 %	**15,5 %**
59	73,9 %	99,1 %	71,7 %	**21,7 %**
60	57,6 %	98,2 %	55,4 %	**28,6 %**
63	57,4 %	98,5 %	55,6 %	**27,5 %**
70	48,3 %	97,9 %	46,5 %	**25,0 %**
T1367	61,1 %	97,9 %	58,7 %	**13,8 %**
unstained	47,9 %	96,4 %	42,8 %	0 %
untransduced	57,0 %	96,3 %	56,8 %	0,11 %

Table 2. Transduction efficiency. Given are the frequencies in %, for lymphocytes, single cells, alive cells and the mbTCR+, CD8+ subset. The frequencies of alive cells are calculated based on the total cell number in the sample. The other frequencies refer to the parent population beforehand. The control TCRs are T58 and T1367, and one unstained sample and untransduced PBMCs are measured. mbTCR – mouse beta T cell receptor.

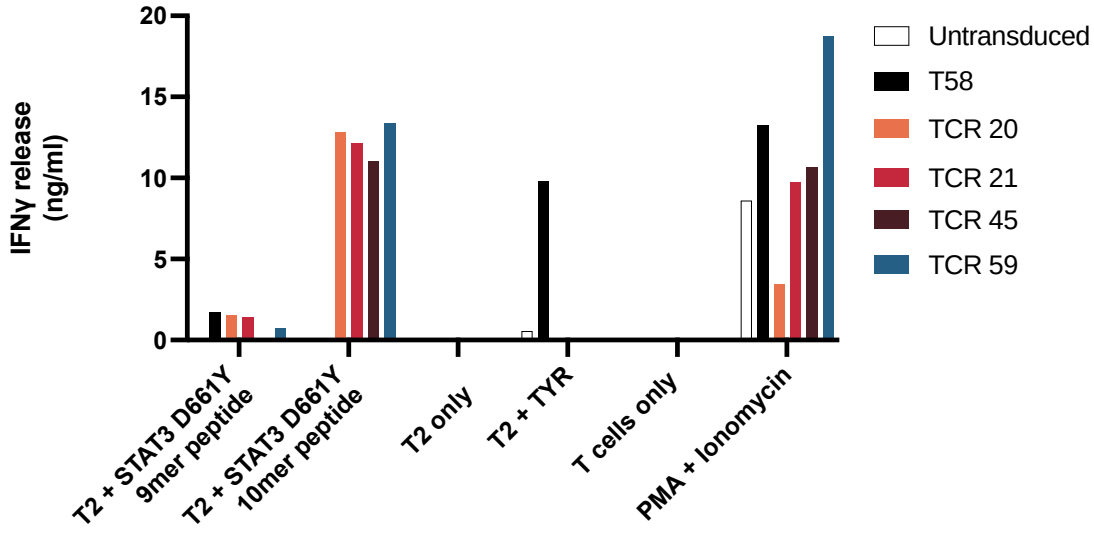
7.5 Cytotoxic activity of transduced STAT3 D661Y specific TCRs

7.5.1 Selection of isolated TCRs specific for STAT3 D661Y 10mer peptide

First, all dominant TCR receptors specific for STAT3 D661Y were cocultured with T2 cells loaded with either ten µM of STAT3 D661Y 9mer peptide or 10mer peptide to find out which peptide they recognize. The cell line T2 is deficient for the TAP protein, thus meaning it cannot transport its own processed antigens to the HLA-A*02:01 complex. This property prevents the presentation of epitopes derived from endogenously expressed proteins. Therefore, these cells can only present the peptides loaded onto them, limiting potential background T cell activation, as was done for the 9mer and 10mer STAT3 D661Y peptides. Figure 8 shows the calculated IFN-γ release. TCRs 20, 21, 45,

and 59 showed activation against the 9mer and the 10mer peptide, indicating that these TCRs can recognize STAT3 D661Y mutation (Figure 8a). The TCRs with the numbers 48, 60, 63, and 70 show no IFN-γ release against either of the peptides, indicating that these TCRs are not STAT D661Y specific, but rather recognize some unknown epitopes (Figure 8b). The TCRs that recognize the peptides, namely TCRs 20,21,45, and 59, secreted a higher amount of IFN-γ when co-cultivated with the 10mer peptide (e.g., TCR 20: 9mer 1.5ng/µl vs. 10mer 12.8 ng/µl). Therefore, in the ongoing T cell assays focus was set on those TCRs and the mutated 10mer STAT3 D661Y peptide was chosen as a target. To investigate if the TCRs 20, 21, 45, and 59 are truly specific to the mutation, the STAT3 wildtype peptide was loaded on T2 cells and cocultured together with the STAT3 D661Y mutation-specific TCR T cells. No IFN-γ release was detected (Figure 8c). Figure 9a demonstrates that the TCRs 20, 21, 45, and 59 can recognize K562 cells transduced with the HLA-A*A2:01 positive STAT3 D661Y minigene, indicating that the STAT3-D661Y epitope can be processed and presented. IFN-γ release is significantly higher when compared to K562 cells only transduced with HLA-A*A2:01. In addition, no significant IFN-γ secretion was detected after incubation with the naturally HLA-A*02:01 positive cell line Nalm6. Therefore, T cell activation seems to not be triggered by HLA-A*02:01 positivity. The detectable IFN-γ levels were similar when the TCR transduced T cells were cocultured with the HLA-A*02:01 negative cell line Oci-Ly-3, indicating that it is most likely a nonspecific background activity.

a



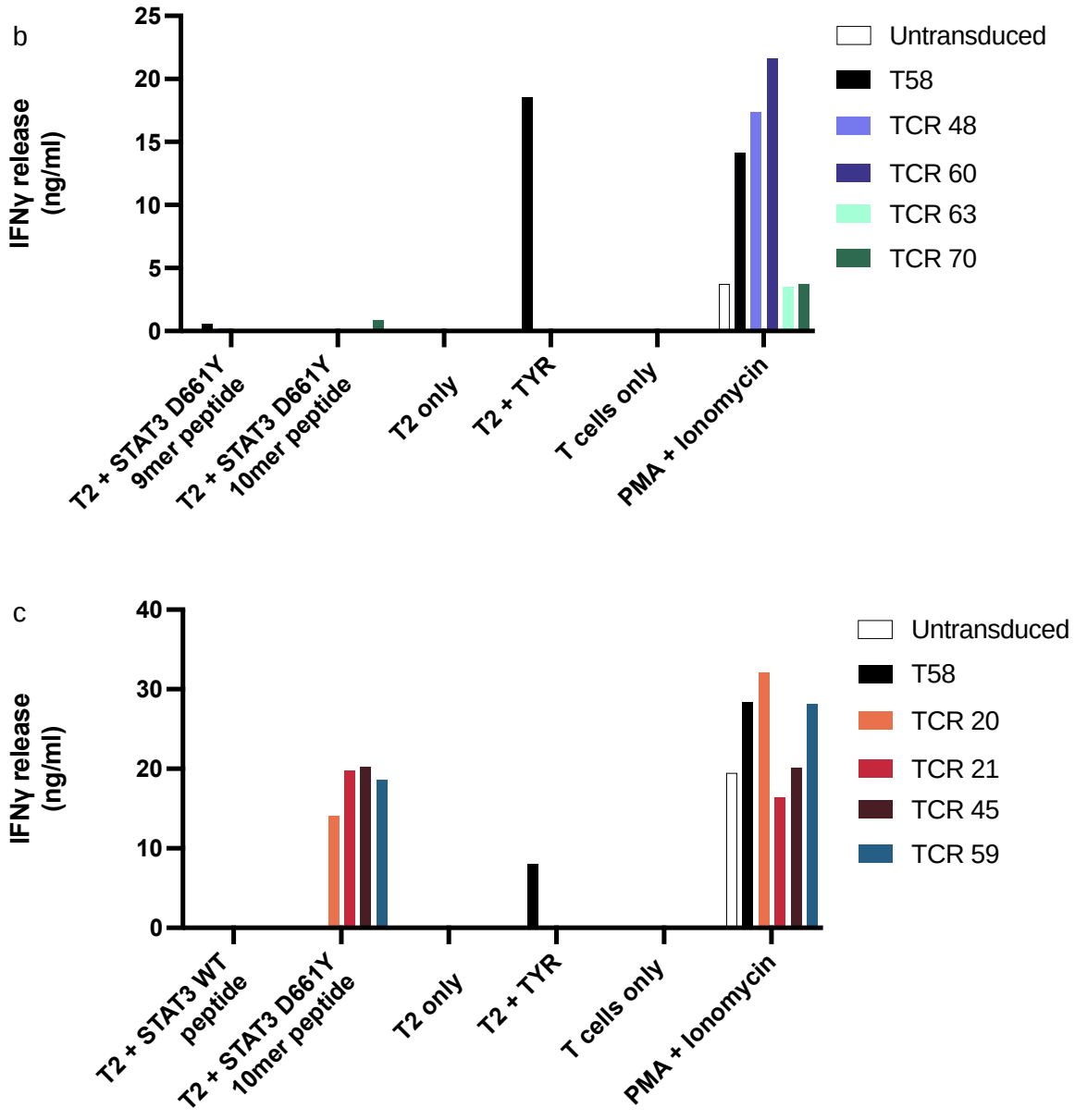


Figure 8. Comparison of STAT3 D661Y redirected TCRs by IFN-γ response measured by ELISA after overnight coculture with T2 cells loaded with STAT D661Y 9mer and 10mer peptides. (a) TCRs 20, 21, 45 and 59 recognized mutated 10mer peptide more efficiently than 9mer and 10mer peptides. (a) TCR 20, 21, 45 and 59 recognized mutated 10mer peptide more efficiently than 9mer peptide. (b) TCRs 48, 60, 63 and 70 did not recognize neither of the peptides. (c) T2 cells loaded with STAT3 wild type (139) peptide or mutated STAT3 D661Y 10mer peptide. The TCRs did not recognize the WT peptide while recognizing the mutated 10mer peptide showing specificity against mutated epitope. T58 TCR recognizing an HLA-A2 restricted tyrosinase epitope was used as control TCR. Representative data from one donor is shown, and samples were analyzed in duplicates. The experiment was conducted 3 times. IFN-γ = Interferon gamma; TYR = Tyrosine. Figures were created with GraphPad Prism V. 9.5.0.

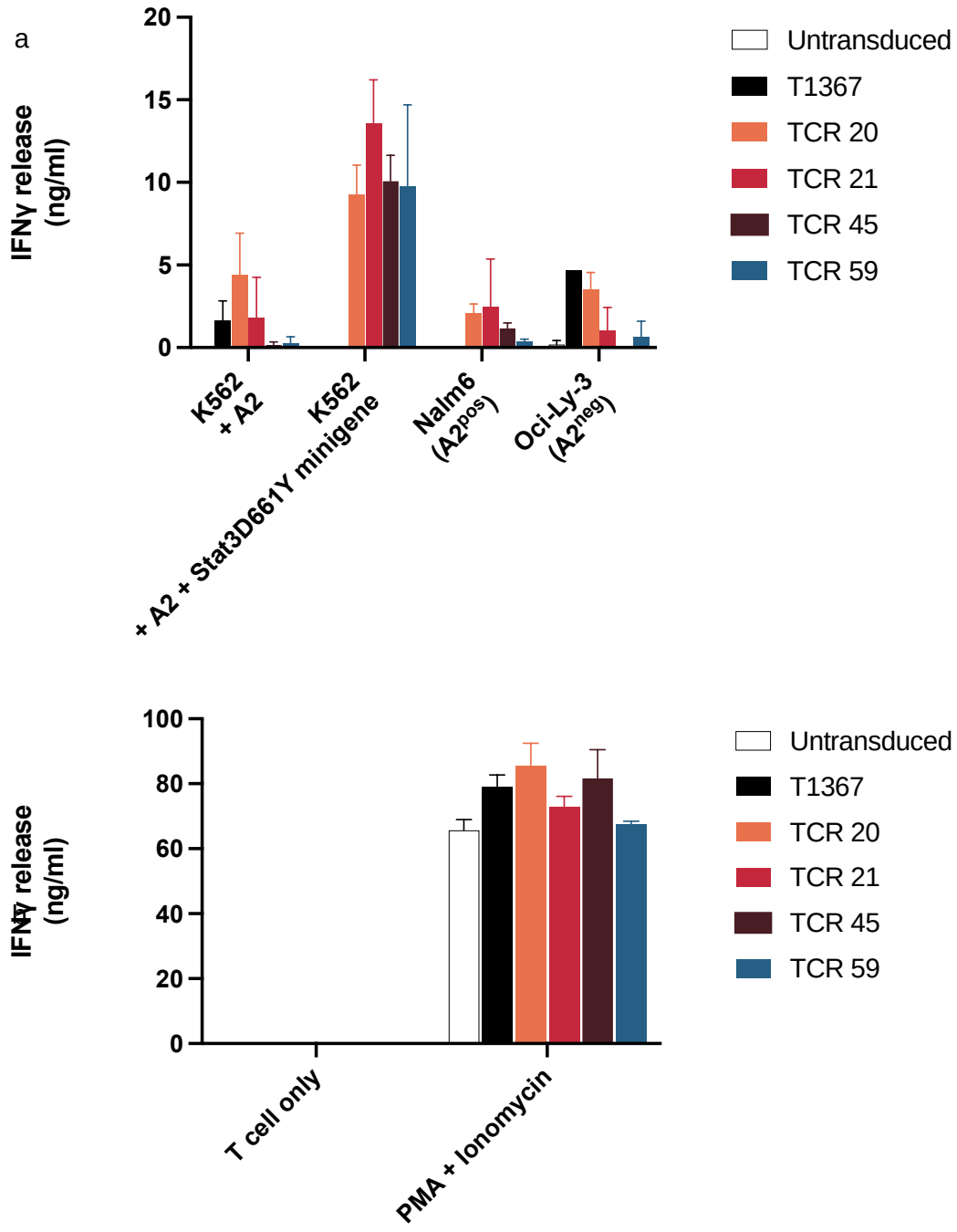
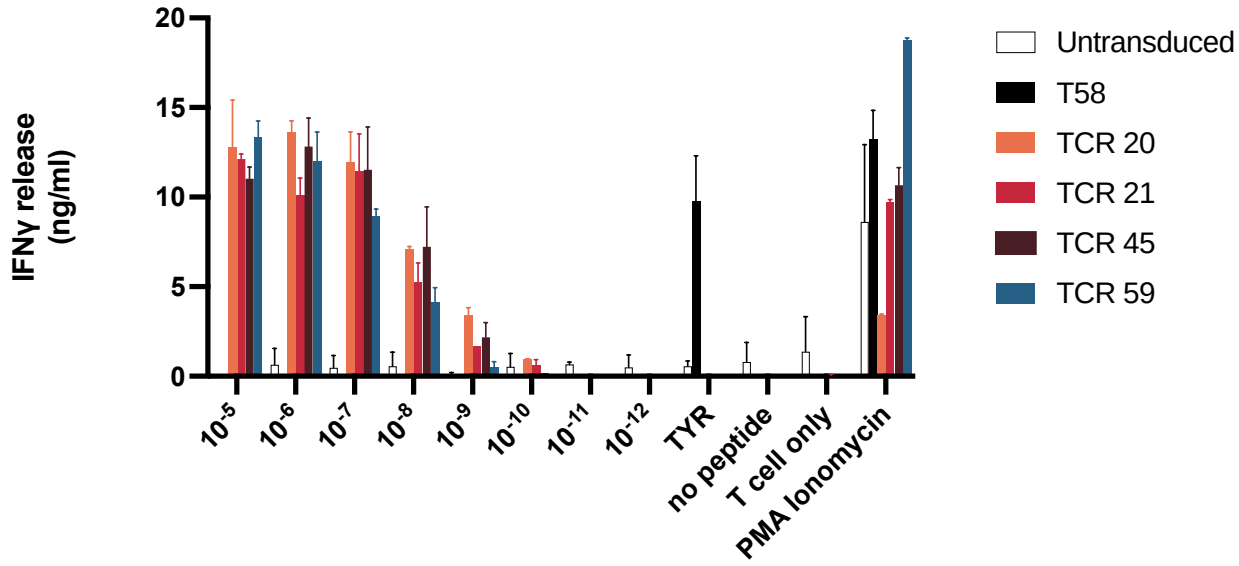


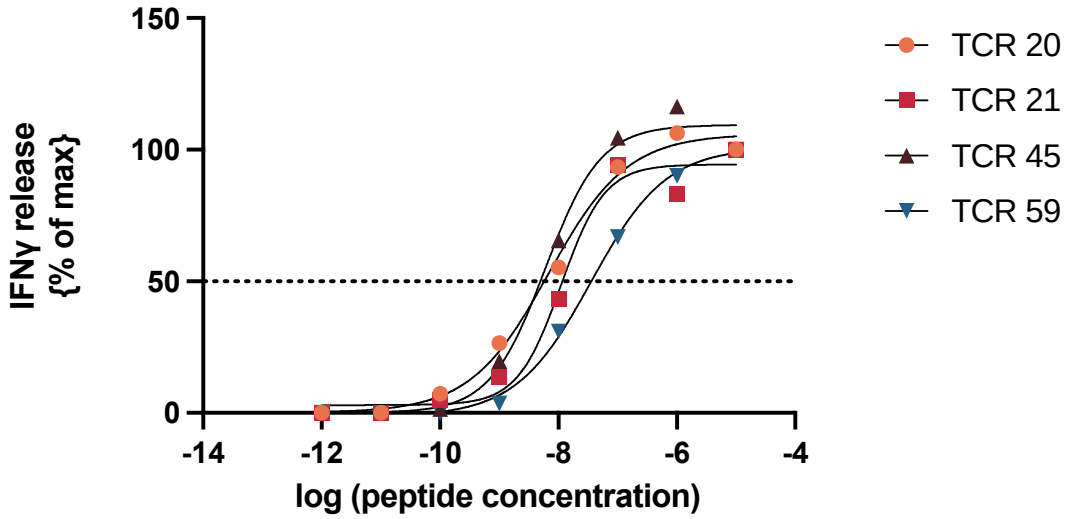
Figure 9. Mutation-specific activity of TCR-T cells. (a) Mutation-specific activation of TCR-T cells against K562 cells transduced with STAT3D661Y minigene and comparative TCR-T cell response against HLA-A*02:01 positive or negative target cells that do not bear the mutation. IFN-γ response measured by ELISA after overnight coculture with K562 cells virally transduced to express STAT3 D661Y minigene and/or HLA-A*02:01 (+A2) or endogenous HLA-A*02:01 positive (Nalm6) or negative (OciLy3) cells as indicated. (b) controls. Representative data from one donor is shown, and samples were analyzed in duplicates. The experiment was conducted three times. IFN-γ – Interferon gamma, TYR – Tyrosine. Figures were created with GraphPad Prism V. 9.5.0.

7.6 Characterization of functional avidity by a peptide titration assay

Another T cell assay was set up with T2 cells. Decreasing peptide concentrations (10 µM to 1 pM) of the mutated STAT3 D661Y 10mer peptides were loaded on T2 cells and incubated overnight with STAT3 D661Y specific TCR T cells. The respective lowest detected IFN-γ release was at 100 pM (10^{-10} M) peptide concentration for TCR 20 and TCR 21, and 1 nM (10^{-9}M) for TCR 45 and TCR 59 (Figure 10a). To analyze the data, a non-linear regression model, with a dose-response curve, was chosen. Here, the inverse correlation can be shown: the lower the peptide concentration is, to reach 50 % of the half-maximum IFN-γ release (effective concentration - EC50), the higher the avidity of the TCR (Figure 10b). Peptide titration was conducted with all four favored TCRs. On average, TCR 20 has the best avidity, with an EC50 value of 8.5nM (8.5 x 10-9M). This is the peptide concentration necessary to reach half the maximum IFN-γ release of the T cell population. As seen in the representative table for one of the three experiments, all EC 50 values range between 5.9nM-38nM.

a





	TCR 20	TCR 21	TCR 45	TCR 59
EC50	6.850e-009	1.111e-008	5.966e-009	3.546e-008

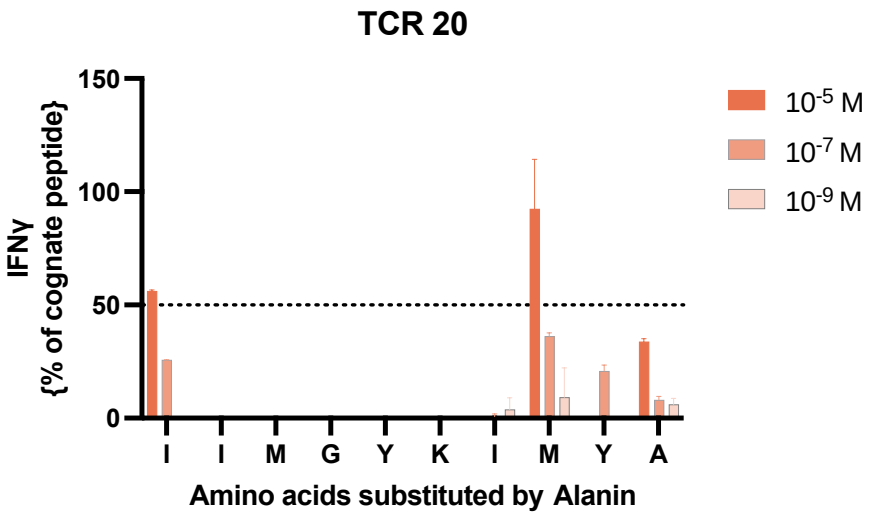
Figure 10. (a) Overview of peptide titration of STAT3 D661Y 10mer. The decreasing concentrations of the 10mer from 10 µM (10-5M) to 1 pM (10-12M) were loaded on T2 cells and cocultured overnight with STAT3 D661Y TCR-transduced T cells. (b) Non-linear r regression model, dose-response stimulation, X values in log. Line at EC50 – effective concentration of peptide that is needed to reach the half maximum IFN-γ release. Data from one representative donor is shown; the experiment was repeated 3 times. Figures were created with GraphPad Prism V. 9.5.0.

7.7 Alanine Scan Assay

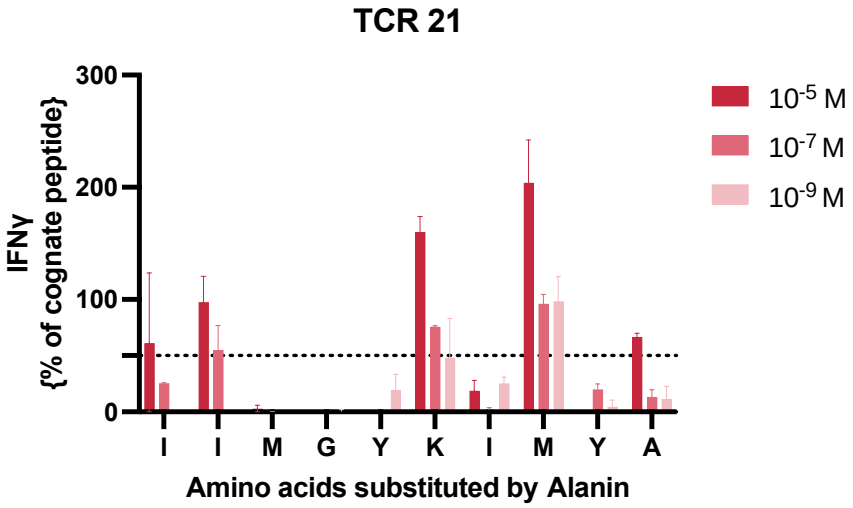
Alanine scan assays were performed to estimate the cross-reactivity (Figure 11). Conducting this experiment allows us to figure out which amino acids at which position are crucial for the recognition of the peptide. If, for example, the amino acid at position two is replaced with an Alanine and the receptor still recognizes the 10mer and releases IFN-γ, this position is not essential for motif recognition. Step by step, it is possible to infer which amino acids are necessary to trigger a recognition signal in the TCR and activate the T cell. For safety, this test is performed in several concentrations. Therefore, this experiment was conducted up to a concentration of 1 nM because the previous peptide titration had shown that IFN-detection is possible up to this concentration. To interpret the data, it must be considered that all resulting peptides can have varying binding affinities to the HLA-A*02:01 complex. Thus, if no IFN-γ release has been detected, either the position of the amino acid is essential, or the peptide binds with low affinity to the HLA

complex and is not presented sufficiently on the surface of T2 cells for the TCR T cells to bind. Therefore, a table with all peptide sequences and binding affinities to HLA-A*02:01 is included (Table 3a). For instance, the amino acids on positions 3, 4, and 5 of the 10mer seem essential for the recognition site for TCRs 20, 21, and 45 because an IFN-γ release ≥ 50% compared to the IFN-γ release of STAT3 D661Y 10mer peptide was not detected anywhere. Considering the binding affinities to HLA-A*02:01, the Stat3 D661Y Ala4 peptide is a strong binder, implicating that it should be presented sufficiently to the TCR for recognition. This makes sense, as it confirms that the central amino acids in a peptide are the most important for recognizing the p-MHC complex via the CDR3 structure of the TCR. However, for TCR 59, no IFN-γ release above 50% was detected. This raised the question of whether all ten amino acids are essential for the p-MHC interaction or if too low binding affinities interfered in the contact of p-MHC and TCR, which is very unlikely as for all four TCRs, the same Alanine containing peptides were used and IFN-γ secretion was indeed observed.

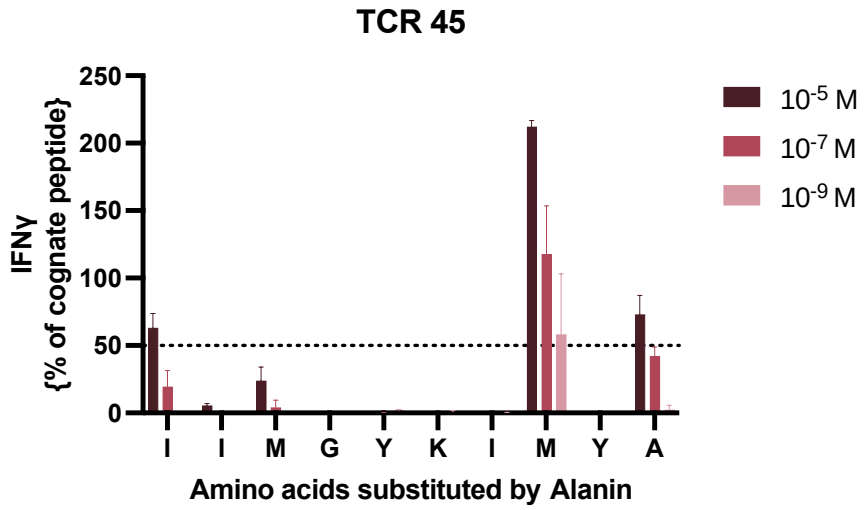
Although the point mutation D661Y is at position 9 of the mutated 10mer peptide, none of the TCRs recognizes an Alanine peptide with Tyrosine (Y) substituted to Alanine (A), interpretable as important motif position. The resulting core motifs (Table 3b) were screened in a database called ScanProsite (last time performed 20th of October 2022) to find peptides with sequence similarities concerning the critical amino acids. No similar peptides were detected with this tool.



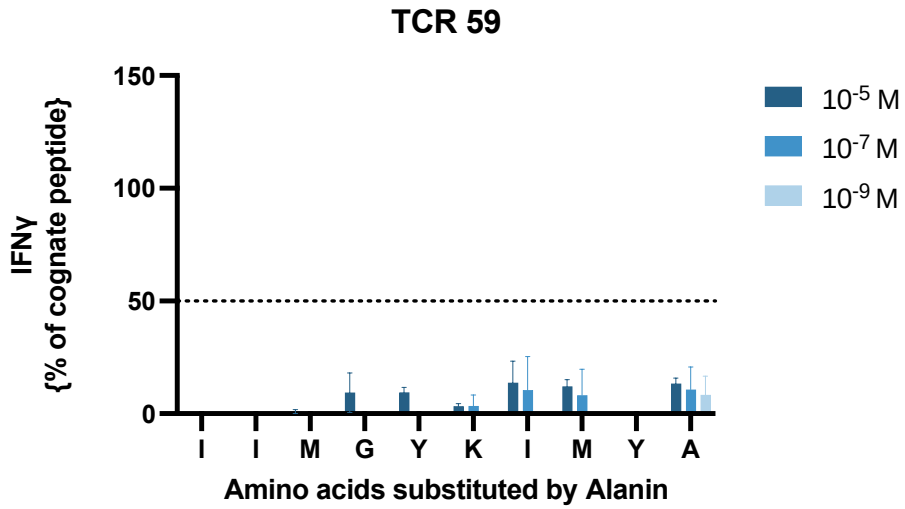
XIMGYKIXYA



XXMGYXIXYX



XIMGYKIXYX



IIMGYKIMYA

Figure 11. Alanine Scan Assay. IFN-γ response measured by ELISA after overnight coculture with T2 cells loaded with 10mer peptides in which each position was exchanged for an Alanine. The naturally occurring Alanine at position 10 was replaced by a glycine. Peptides were loaded in three different concentrations. When ≥ 50% of the IFN-γ release compared to the cognate peptide (STAT3 D661Y, IIMGYKIMYA) was detected, the position in the motif is shown by an X. Resulting motifs for the highest concentration are shown. Representative data from one donor is shown, the experiment was repeated 3 times, and samples were analyzed in duplicates. IFN-γ – Interferon gamma. Figures were created with GraphPad Prism V. 9.5.0.

a	name / position	Amino acid sequence	Binding affinity to HLA-A*02:01
	Stat3 D661Y WT	IIMGYKIMDA	277.01 nM
	Stat3 D661Y 10mer	IIMGYKIMYA	17.71 nM (SB)
	Stat3 D661Y Ala1	AIMGYKIMYA	22.43 nM (SB)
	Stat3 D661Y Ala2	IAMGYKIMYA	150.15 nM
	Stat3 D661Y Ala3	IIAGYKIMYA	81.24 nM
	Stat3 D661Y Ala4	IIMAYKIMYA	17.98 nM (SB)
	Stat3 D661Y Ala5	IIMGAKIMYA	46,86 nM
	Stat3 D661Y Ala6	IIMGYAIMYA	17.71 nM (SB)
	Stat3 D661Y Ala7	IIMGYKAMYA	61.91 nM
	Stat3 D661Y Ala8	IIMGYKIAYA	32.71 nM (SB)
	Stat3 D661Y Ala9	IIMGYKIMAA	56.77 nM
	Stat3 D661Y Gly10	IIMGYKIMYG	974.87 nM
b	Number of TCR	Amino acid motif for 10^{-5} M	Important Amino acid positions
	TCR 20	xIMGYKIxYA	2,3,4,5,6,7,9,10
	TCR 21	xxMGYxIxYA	3,4,5,7,9,10
	TCR 45	xIMGYKIxYx	2,3,4,5,6,7,9
	TCR 59	IIMGYKIMYA	all

Table 3. Alanine Scan Assay with peptides sequences and motifs. (a) peptide sequences and names indicated. Binding affinities according to NetMHC V.4.0. SB – strong binder. (b) Motif of amino acids whose substitution with Alanine was not accompanied by ≥ 50% IFN-γ release after overnight incubation compared to cognate peptide.

7.8 STAT3 D661Y TCR T cells do not kill HDLM-2

Additionally, the functional potential of all four selected TCRs was investigated under more natural expression levels for STAT3 D661Y. Therefore, the Hodgkin lymphoma-derived cell line HDLM-2, heterozygous for the STAT3 mutation D661Y, was used (COSMICv96 database, ID COSS924110) (234). However, HDLM-2 cells cocultured with the STAT3 D661Y specific TCR T cells showed no IFN-γ release (Figure 12a). As a positive control, the HDLM-2 cell line was loaded with STAT3 D661Y 10mer peptide (10 μM). As expected, the loaded peptide was recognized by the four TCR-engineered T cells. IFN-γ secretion was detectable and is significantly higher than the measured IFN-γ release of STAT3 D661Y redirected T cells cocultured with HDLM-2 alone. To investigate whether it is an issue with the processing and loading machinery of the cells, which is necessary for peptide presentation, the same cell line was transduced with the MAGE-A1-GFP antigen. TCR-T cells specific for MAGE-A1 (T1367) secreted IFN-γ at a high level when cocultured with these cells, indicating that the peptide processing and presentation machinery of the HDLM-2 cell line was intact.

7.8.1 HDLM-2 used in coculture harbor the STAT3 D661Y mutation

To ensure that the HDLM-2 cells we use in coculture are indeed heterozygous for the STAT3 D661Y mutation, we sent isolated and PCR-amplified DNA of the cells for sequencing analysis. We could determine that HDLM-2 is heterozygous as we received sequencing results with and without STAT3 D661Y mutation. However, the ratio in which WT and mutant STAT3 D661Y are expressed remains open.

7.8.2 Characterization of CD137 upregulation upon activation

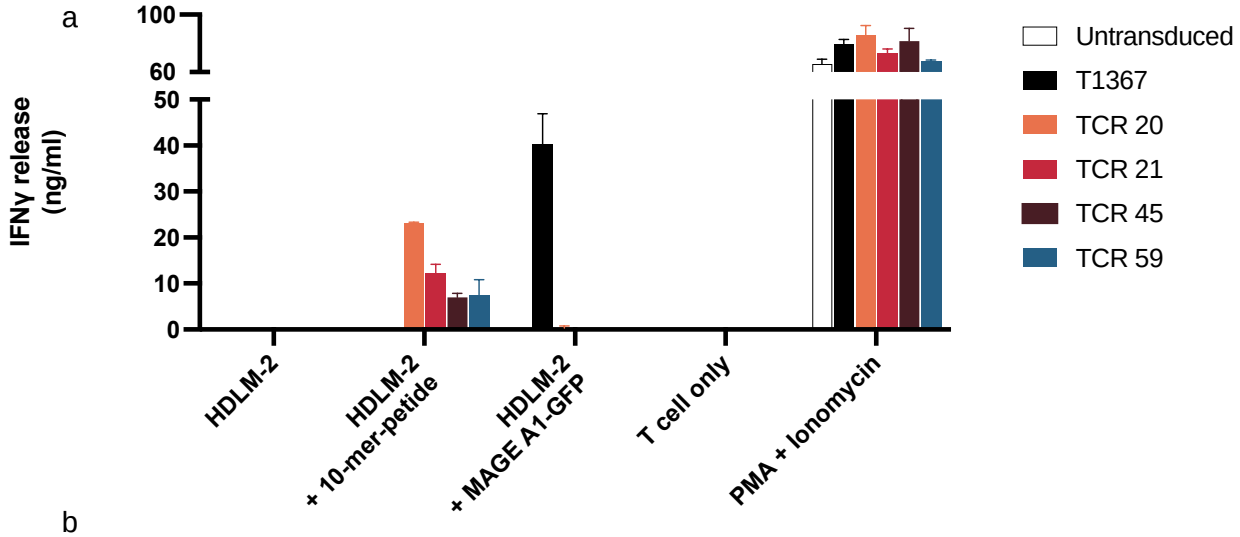
After no IFN-γ signal was detected by ELISA after cocultivation with HDLM-2 (Figure 12a), the mutation-specific TCR T cells were examined in more detail concerning their activation. After an overnight coculture with HDLM-2 cells, the TCR transduced T cells were collected and stained against CD8, mouse TCR β, and the T cell activation marker CD137. One representative FACS Plot is shown (Figure 12b). In fact, CD137(4-1BB) is a costimulatory molecule promoting survival and proliferation signals in activated T cells (235). The fluorescence signal was measured in FACS, and CD8+ T cells were gated for

mouse βTCR+ and CD137+ cells. Corresponding to the results of the ELISA with HDLM-2 and TCR T cells, almost no CD137+ TCR transduced T cells were detected (Figure 12b.1 - TCR 20 1.4 % CD137+/mβTCR+ T cells). In contrast, CD137+/mβTCR+ T cells were detected when HDLM-2 cells were loaded with the STAT3 D661Y 10mer peptide (Figure 12b.2 - TCR 20 45.5%) and when cocultured with K562-A2-STAT3-D661Y minigene transduced cells (Figure 11b.3 - TCR 20 13.9%).

7.8.3 Tandem mass spectrometry analysis of HDLM-2 cells

Finally, to investigate whether our target peptide is presented on HDLM-2 cells via HLA A*02:01, Dr. Martin Klatt, Charité, isolated pMHC complexes and performed a tandem mass spectrometry analysis of the eluted peptides. The analysis revealed that neither the 9mer peptide IMGYKIMYA nor the 10mer peptide IIMGYKIMYA could be eluted and fragmented during tandem mass spectrometry. This indicates that the HDLM-2 cell line, naturally expressing mutated STAT3 D661Y, is not able to process or present our target epitope (IIMGYKIMYA). The results can only be given descriptively. For exact data, please contact Dr. Martin Klatt, Charité.

a



b

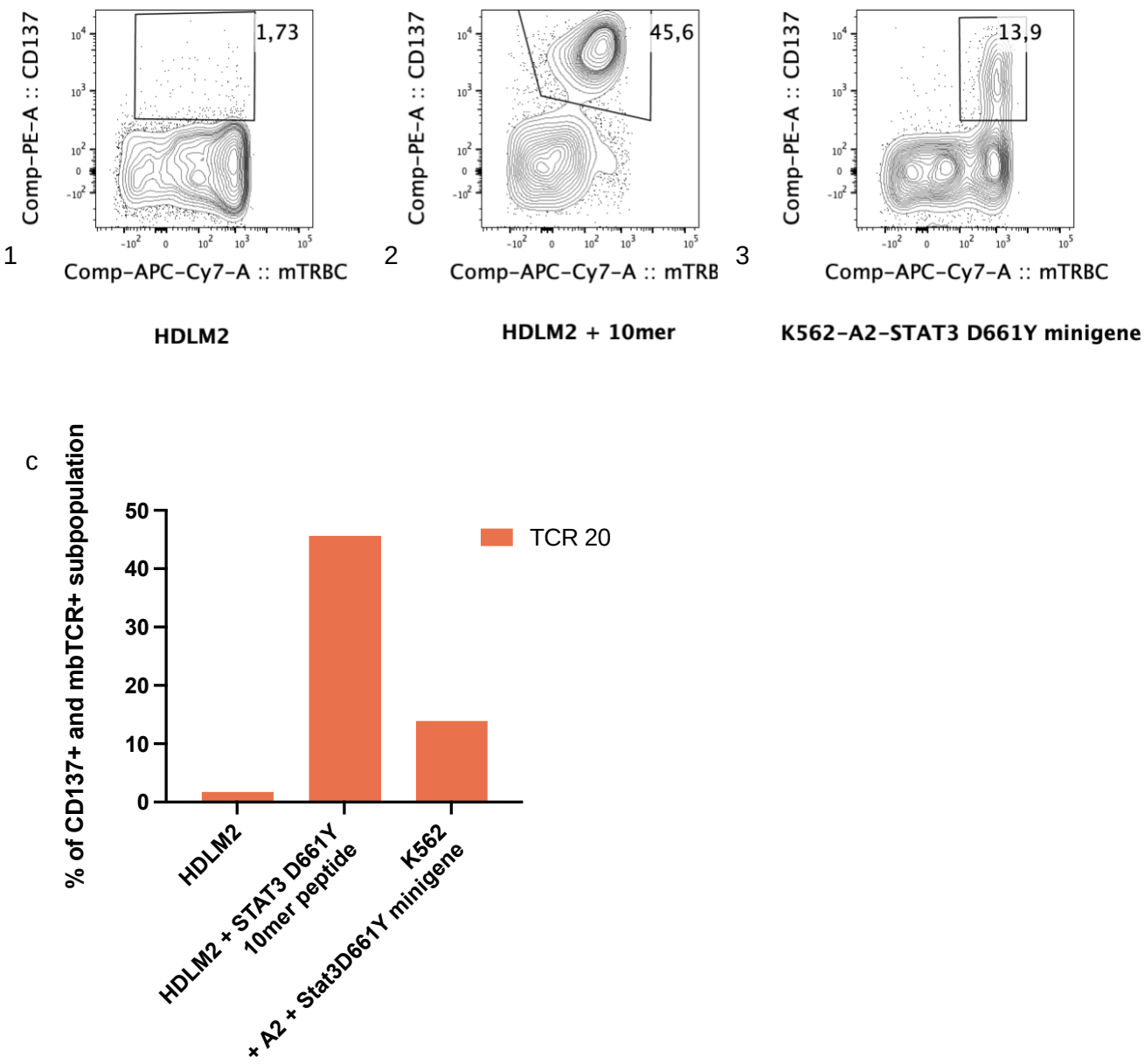


Figure 12. Coculture with HDLM-2 lines that express Stat3 D661Y mutation and HLA-A2 allele endogenously. (a) IFN-γ response measured by ELISA after overnight coculture with HDLM-2 cell line, HDLM-2 loaded with STAT3D661Y 10mer (10 µM) and HDLM-2 transduced with MAGE A1-GFP antigen and controls. Indicated pairwise comparisons by 2-way ANOVA, Tukey's multiple comparison test. Adjusted p value classification: * p ≤ = 0.05, ** p ≤ 0.01, ***p ≤ 0.005, **** p ≤ 0.0001. Representative data from one donor is shown, and samples are analyzed in duplicates. The experiment was repeated 3 times. (b) CD137 upregulation staining after overnight coculture of TCR-transduced T cells with 1) HDLM-2, 2) HDLM-2 +10mer, and 3) K562-A2-STAT3-D661Y minigene transduced cells. Representative data for TCR 20 is shown. (c) Frequency in percent of CD137 upregulation in mouse beta TCR positive cells. Figures were created with GraphPad Prism V. 9.5.0 and FlowJo V. 10.8.1.

8 Discussion

Precision cancer immunotherapy is a rapidly emerging field with several different approaches, where TCR T cell therapy aims to process epitopes presented on the MHC-I complex, which can originate from proteins of the entire cell. To take advantage of this, cancer-specific mutations creating neoepitopes are the best chance in fighting T cell neoplasms as they differ by definition from non-malignant T cells.

Although CAR T cell therapy has led to a breakthrough in CD19+ B cell neoplasms (236, 237), its successful application in T cell neoplasms has been lacking behind. One reason is the problematic isolation of autologous T cells for CAR-T products without contamination with malignant T cells (175). Additionally, the production of allogenic CAR-Ts is possible but is always accompanied by an increased risk for graft vs. host reactions if the endogenous TCR is not knocked-out, or they may lack long persistence due to elimination by the host immune system (238).

Furthermore, targeting shared surface markers among T cells poses the potential for fratricide between CAR-T cells themselves and the elimination of non-malignant T cells. Therefore, the administration can lead to decreased T cell numbers and, in the worst case, to immune deficiency (239). Additionally, as frequently observed for CARs against B lineage antigens, it can be assumed that T lineage antigens can also lead to loss of surface antigen expression while maintaining intracellular expression (240). Considering this, TCR T-cell therapy is beneficial over CAR T-cell therapy in fighting T cell neoplasms, especially since TCR T cells also only require low levels of antigen expression (241).

Generated TCRs fit perfectly into the signal transduction pathways of T cells. Therefore, no optimization of T cell activation is required. Furthermore, considering antigen loss, it is helpful to target driver mutations like mutated STAT3, as cancer immune evasion is less likely, at least for those T cell neoplasms for which mutated STAT3 has been shown to be an oncogenic driver.

In fact, STAT3 is the most frequently mutated gene in the STAT family involved in the JAK/STAT pathway. It can function either as a transcription factor or as a signaling molecule for cell survival and proliferation (213, 242). Therefore, overactivated STAT3 is considered as an oncogene and plays a decisive role in tumor development.

As a driver mutation, it is involved in tumor formation and progression, and the development of metastasis (243). As it can be found in almost all cytoplasm of mammalian cells, it is involved in many tumor entities, but remarkably often, in T cell malignancies, as is the case in about 40 % of patients with large granular lymphocytic (LGL) leukemia, encompassing T-LGL leukemia (T-LGLL) and chronic lymphoproliferative disorders of natural killer cells (CLPD-NK), with mutational hotspots at Y640F in 11–17% and at D661Y in 9–11% of patients. STAT3 mutations are also common in various other T cell malignancies, more frequently in CD30-positive T-cell lymphomas than in CD30-negative lymphomas: such as ALK-negative anaplastic large cell lymphoma (ALK- ALCL) and ALK+ ALCL, γδ-hepatosplenic lymphoma (GD-HSTCL), extranodal natural killer T-cell lymphoma (NKTCL), peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), cutaneous T-cell lymphoma (CTCL), and adult T-cell leukemia/lymphoma (ATLL) (216, 243, 244). Mutated STAT3 is therefore a suitable target, as it represents a commonality in the otherwise heterogeneous group of T cell malignancies.

Additionally, there are cases of immune-mediated myelodysplastic syndrome (MDS) and aplastic anemia (AA) in which patients also have clonal LGL-like cells with STAT3 D661Y mutation, indicating a relationship between both diseases (245). Furthermore, LGL is a rheumatoid arthritis (RA)-associated disease, as one-third of patients suffer from RA (246, 247). Moreover, one study detected STAT3 SH2 domain mutations in 42% of patientis with felty syndrome, a severe complication of RA (248). Although the Y640F hotspot mutation was seen in this study, it is likely that D661Y mutations are also present but were not detected here, especially since this study had only a narrow patient cohort.

Thus, as an alternative to long-term immunosuppressive therapy, TCR T cells targeting STAT3 D661Y would also pose an ideal off-the-shelf treatment option for T cell malignancies.

In this work, as part of my doctoral thesis, we identified four high-avidity TCRs isolated from healthy donor-derived T cells against an HLA-A2*01 restricted epitope derived from STAT3 D661Y. From a total of nine T cell clones from one donor, eight TCRs were identified, successfully re-expressed on healthy human T cells, and subsequently tested in various T cell assays in vitro. Transduced TCR T cells were not reactive to the STAT3 WT peptide but recognized the STAT3 D661Y 10mer peptide IIMGYKIMYA, and K562

transduced cells with a STAT3 D661Y minigene spanning the mutation, indicating we had indeed identified high affinity TCRs for the neoepitope IIMGYKIMYA. An Alanine Scan Assay was performed to exclude cross reactivity by determination of essential positions for peptide recognition and to subsequently scan databases for similar peptides, but none could be identified. However, transduced T cells could not kill the target cell line HDLM-2, which is heterozygous for the STAT3 D661Y mutation and expresses the HLA-A2*01 allele. Mass spectrometry of HDLM2 cell analysis of Dr. Martin Klatt, Charité, indicated nonexistent epitope presentation of our target IIMGYKIMYA. These briefly summarized results are discussed in detail below.

8.1 Repertoires for isolation of neoepitope-specific T cells

There are two delicate moments in developing an ATT against neoantigens: first, identifying a neoepitope that is processed and immunogenic, and second, successfully isolating high-affinity TCRs from a T cell repertoire. To date, there are several options for isolation, which have been compared side by side and discussed by Grunert et al. (142): First, the usage of the human repertoire, whereby a distinction can be made whether the TCRs originate from patients with the neoepitope-specific mutation or healthy donor's blood, and it is screened for neoepitope-specific TCRs. Second, using human HLA transgenic mice is possible, and T cells can be sorted from a peptide immunized murine repertoire.

Using TILs and isolating them from tumor material of patients with the specificity against neoantigens has been done successfully for melanoma patients (249, 250) but appears to be more difficult for epithelial tumors, such as, ovarian cancer or pancreatic cancer, as these are low mutational load tumor types (251, 252). However, isolation of neoepitope-specific TILs is often very challenging, as there may be little tumor material or low numbers of CD8+ tumor-reactive lymphocytes with any ability at all to recognize autologous tumor cells (114), and there are not necessarily many neoantigen-specific T cells among the TILs (253). Furthermore, the isolation and propagation of neoepitope-specific T cells appears difficult, as cells derive from a sick individual (254).

To circumvent these obstacles, groups started to screen for neoepitope-specific lymphocytes in the peripheral blood of cancer patients, e.g., by PD1 positivity and

tetramer staining (249, 255), but unfortunately, also with limited success. However, the difficulty in finding neoepitope-specific T cells may be because tumors are immunosuppressive and thus do not ensure a good immune response when priming naive T cells against the tumor antigen. This is supported by the fact that Strønen et al. could isolate neoantigen-specific TCRs from the PB of healthy donors but not from TILs (253).

Several groups have also shown that they can isolate neoantigen-specific TCRs from the T cell repertoire of HLA-A-matched healthy donors (143, 256, 257). By exome sequencing of the tumor, it was determined which mutations lead to altered protein sequences, followed by prediction of the pMHC affinity. Healthy donor dendritic cells were loaded with short peptides corresponding to the so-called neoepitope to prime autologous T cells in vitro. This approach was also applied here by Özcan Çınar, who did the preliminary work for this dissertation. T cells were primed with either STAT3 D661Y 9mer or 10mer peptide loaded on autologous dendritic cells and reactive T cell lines were expanded for ten days. Afterward, he set up an initial FACS sorting and found a total of nine T cell clones which showed reactivity towards peptide loaded target cells by secreting IFN-γ and CD137 upregulation. This is a remarkable result, considering that from the T cells of only one donor we identified nine reactive T cell clones at once. Further characterization of the reactive TCR clones in this work could determine four STAT3 D661Y 10mer peptide reactive TCRs.

Although the probability of allo- or cross-reactive T cells is higher when using a TCR repertoire other than the patient's own repertoire, the method has certain advantages, as blood sampling from healthy individuals can be ethically performed in larger quantities. Furthermore, there is no immunosuppression by a tumor, and thus, neoantigen-specific T cells can be more easily isolated from healthy individuals and then expanded. A highly neoantigen specific TCR could therefore have potential as an off-the-shelf product. In contrast, using the patient's own repertoire it is often very time consuming in production, and protocols for ATT are currently being optimized (256, 257), which contradicts the application in patients with often very advanced tumor stages needing quickly effective therapies.

My working group has also published protocols for isolating neoantigen-specific T cells from the healthy human and murine repertoire (258). Here, HLA-A*02:01-transgenic

(ABabDII) mice with humanized TCRαβ gene loci (259) are immunized with the selected mutant peptide to isolate high avidity TCRs. This method is advantageous when targeting tumor-associated antigens that are also self-antigens, such as FLT3 (260). In humans, T cells with high avidity TCRs against self-antigens are deleted as they undergo negative selection in the thymus, creating a central tolerance. Thus, it is possible to identify these T cells in the mouse, which is a non-tolerant host, as they are not selected against in the murine thymus. However, they bear the risk of cross-reactivity with human tissues. Meanwhile, this approach has also been applied to cancer-testis antigens like MAGE-A1, NY-ESO and viral antigens (261-263).

However, Grunert et al. have showed in their direct comparison of different repertoires that the success rate also depends on the donor's TCR repertoire, since they were able to isolate specific TCRs from one donor but not from the other. This may be explained by somatic re-arrangement that produced suitable high-avidity TCRs only in the reactive donor. In this present work, a similar alpha variable and joining region usage was observed, while the CDR3 region remained with varying rearrangements. Six of nine TCR sequences had the same V gene preference, and five of nine had the same J gene preference. Similar observations were made for the beta variable region. Although each human carries a hugely diverse repertoire of TCRs by somatic recombination - despite a significantly smaller number of gene loci, there is indeed a pattern in a small group of similar TCRs concerning the V, J, (D) genes in this work. This could explain that all TCRs reactive against STAT D661Y 10mer peptide, which were indeed derived from one donor only, shared similar V(D) J usage, as similarly by Grunert et al. Unfortunately, in this doctoral thesis, the individual sequences of the TCR alpha and beta chains are not published due to a possible patent application.

8.2 Characterization of STAT3 D661Y specific TCRs

8.2.1 Four high avidity TCRs against STAT3 D661Y 10mer peptide

For characterization, identified TCRs were re-expressed on PBMCs using a retroviral transduction and lymphocytes were further propagated. Eight cloned TCR constructs were re-expressed in PBLs; however, a transduction efficiency of ≥ 25% could not always be achieved to provide enough TCR T cells to perform T cell assays.

The transduction efficiency can be influenced by many factors, such as the transfection efficiency of the used packaging cell line (here HEKT-GALV-g/p with the transfer plasmid), the virus particle production, the handling of T cells in the transfection process itself, and the varying amount of CD8+ T cells in the population of PBMCs or vector DNA quality. To date, several groups are working on optimizing protocols to ensure expanding sufficient numbers of T cells for clinical use (92, 264) or to accelerate protocols from the screening of neoepitope reactive T cells to TCR-engineered cells (256, 265).

Characterization of the isolated TCRs was performed in coculture experiments of TCR transduced T cells with the 9mer and 10mer peptides which were also used to prime the T cells. In this first step, we observed robust T cell activation for four of eight identified TCRs (TCRs 20, 21, 45, and 59) after coculture with target cells loaded with the STAT3 D661Y 10mer peptide, but not with the 9mer peptide (Figure 8). Cross-reactivity to the wild-type STAT3 peptide was excluded by coculture experiments where no IFN-γ could be measured, indicating that no TCR is reactive to wildtype STAT3, which is ubiquitously expressed in human tissues.

Further characterization was focused on the STAT3 D661Y 10mer peptide and the respective peptide-reactive TCRs. A peptide titration was performed to determine the functional avidity of the four TCRs, meaning T cell activation and fitness at different concentrations of target peptide, to answer the efficiency of T cell activation. It is usually described by the EC50 concentration, the peptide dose at which the half-maximal IFN-γ-release is reached. In general, higher functional avidities, meaning lower EC50 values, are associated with better recognition of the epitope even when presented at a lower density (266). We detected IFN-γ until very low peptide concentrations (10^{-10} M), and EC50 values were in the 10^{-9} -10^{-8} M range, as displayed in the non-linear regression model (Figure 10). This indicates that the STAT3 D661Y 10mer peptide has a good antigenic potency because of a robust expansion of peptide-reactive T cell lines after T cell priming. We identifed eight TCRs from one donor, and four of eight TCRs, TCRs 20, 21, 45, 59 were sensitive to the STAT3 D661Y 10mer peptide even at low concentrations (up to 10^{-10} M). A clinically relevant TCR, the NY-ESO-1 redirected TCR, showed EC50 values of up to 10^{-10} M when derived from ABabDR4 transgenic mice, however NY-ESO-1 TCRs isolated from the human repertoire showed an EC50 range of 10^{-9} M to 10^{-8}M, comparable to the EC50 values determined in this study. Poncette et al. suggested that

there might be a tolerance mechanism of T cells derived from human PBLs decreasing their functional avidity (262). Normally, T cells promote responses to physiological epitope densities; however, tumors can circumvent TCR recognition, e.g., by MHC complex downregulation. Therefore, high-avidity TCRs with high sensitivity to the peptide are beneficial (267).

To improve T cell avidity in the sense of enhanced T cell activation, approaches have been made to increase the affinity between pMHC and TCR to the higher end of the physiological range (1-100 μM). TCRs with higher affinities and longer half-lives of TCR-pMHC binding kinetics commonly show faster T cell responses (268). However, in a comparison of NY-ESO-1 restricted TCRs with different affinities within the physiological range, it was shown that high-affinity TCRs only contribute to an improvement of avidity up to a threshold of 5 μM and not significantly beyond (269). However, clinically tested TCRs today, like the MART-1, gp100, WT1 protein, CEA or NY-ESO-1 redirected TCRs are affinity-enhanced TCRs as they showed improved efficacy in tumors with low epitope densities (overview (270))

However, when optimizing sensitivity, it must be ensured that the TCR does not lose its specificity, as became dramatically evident in the past. Typically, TCRs with a strong affinity to pMHC would physiologically undergo negative selection and would be depleted to prevent auto-reactivity. In the first clinical trials, affinity-enhanced TCRs have shown a dangerous potential for cross-reactivity against epitopes from benign tissues. The most prominent example is a high-affinity MAGE-A3 TCR against myeloma and melanoma, which has not shown any off-target antigen recognition in preclinical studies. However, even though the target epitope is a CTA that occurs only in the testis and ovaries, the TCR led to cardiac heart failure after administration into the first two patients. Afterward, an autopsy determined off-target toxicity with myocardial protein titin (137).

Moreover, an affinity-enhanced TCR redirected against MAGE-A3/A9/A12 led to neurologic toxicity, since the MAGE-A12 antigen is expressed in the human brain (138). A high-affinity TCR against CEA was administered to three patients. It induced metastatic colorectal cancer regression in one patient, but in all cases, patients had a severe colitis (271). Additionally, lethal outcome after administration was not only observed in affinity-enhanced TCRs; there is also a case report of a MART-1 specific TCR against metastatic melanoma, in which a patient died of a multi-organ failure and irreversible neurological

damage after infusion of the TCR-T cell product , but which was presumably caused by severe cytokine release syndrome and not by cross-reactions (272).

Although the STAT3 D661Y redirected TCRs tested here were not affinity-enhanced, we performed an Alanine scanning assay to assess peptide cross-reactivity, in which we generated a profile of amino acid residues of the target peptide that are important for recognition by STAT3 D661Y-specific TCRs. T cells transduced with TCR 59 were not activated in any peptide constellation with Alanine, indicating that this is a very specific TCR (Figure 11). The other TCRs recognized some peptides from the Alanine Scan panel. However, when we screened the resulting core patterns, we found no corresponding peptides from the human proteome presented on HLA-A*02:01. This gives the very first information about the potential cross-reactivity of these four TCRs. Furthermore, we could not find similar peptides to STAT3 D661Y 10mer core sequences needed for TCR recognition in the human proteome. Of course, in future experiments, further testing for cross-reactivity would need to be done before clinical trials. Sanderson et al. extended the peptide screening and substituted each amino acid residue of their target epitope with the naturally occurring amino acids and screened for cross-reactivity in vitro (273). The resulting profile was used for testing their TCRs in primary tumor material or various human cell lines and against induced pluripotent stem cells covering a representative number of human tissues. It was helpful to exclude major off-target reactivity before initiating a clinical trial with their MAGE-A4-specific TCR (274). If our four STAT3 D661Y redirected TCRs had recognized the target cell line HDLM-2, we would have conducted a so-called lymphoblastoid cell line (LCL) assay for further evaluation. This is frequently conducted to test HLA haplotype reactivity as EBV-transformed B-lymphoblastic cell lines (B-LCLs) express multiple HLA alleles and are, therefore, useful to test the alloreactivity of engineered TCR T cells against other HLA haplotypes (143, 274).

8.2.2 Selected neoepitope is processed and presented in K562-A2-STAT3-D661Y minigene transduced cells and recognized by STAT3 D661Y specific TCRs

Another hurdle in the development of TCR T-cell therapy is the question of whether the target neoepitope is ultimately processed by the (immune)proteasome of the patient's cancer cells and whether a peptide spanning the mutation is presented on MHC complexes. First, prediction algorithms such as NetMHCpan 4.1 were used to identify

potential neopeptide candidates, but this was also investigated experimentally. For this purpose, we designed a plasmid containing a STAT3 minigene, as described in Section 3.2.1, and transduced K562+HLA-A2*01 cells retrovirally (K562-A2-STAT3-D661Y minigene). K562 is a cell line lacking MHC-I and MHC-II molecules but still expresses β2-microglobuline, which allows it to express MHC molecules when transduced. This property makes it an ideal tool for HLA ligand analyses, as there is no risk of interference from other HLA-presented peptides (275, 276). Therefore, in vitro antigen processing was examined by elution of HLA ligands from K562-A2-STAT3-D661Y minigene cells and subsequently, mass spectrometry analysis of eluted ligands revealed the presence of the 9mer and 10mer peptide, the latter to a greater extent (Figure 5).

Now, in line with the immunopeptidome analysis from our K562-A2-STAT3-D661Y minigene transduced cells, the four STAT3 D661Y specific TCRs 20, 21, 45, and 59 secreted IFN-γ in a coculture experiment. First, this demonstrates that K562 cells transduced with the STAT3 D661Y minigene process with their proteasome machinery and present the 10mer epitope on the HLA-A*02:01 molecule on their cell surface. And second, the T cell activation measured by IFN-γ secretion shows that the TCRs are high-affinity TCRs for the target peptide IIMGYKIMYA, as they are reactive in coculture with the target peptide expressing cells but not when the WT peptide is presented to them (Figures 9 and 8).

8.2.3 Stat3 D661Y redirected TCRs fail to recognize the target cell line HDLM-2

We characterized TCRs 20, 21, 45, and 59 by coculturing TCR transduced T cells with the lymphoma-derived cell line HDLM-2, a cell line heterozygous for the STAT3 D661Y mutation, and expressing HLA-A2*01 complex (234). After overnight incubation, neither IFN-γ release nor CD137 upregulation could be detected, indicating that there was no T cell activation (Figure 12). To check whether the HDLM-2 cell line we had in culture harbored the mutation, we amplified the STAT3 transcript with the amplicon spanning the mutated region for sequencing. The obtained reads corresponded to both the STAT3 D661Y mutant and wild type, confirming that the HDLM-2 is indeed heterozygous for our target neoantigen.

Considering peptide dosage, one first potential explanation was that the TCRs are not reactive against the STAT3 WT peptide, as was demonstrated in our experiments.

Possibly, a potent T cell activation is missing, because both mutated and WT peptides are processed and presented on the HLA complex of HDLM-2 cells. Unfortunately, other cell lines carrying the STAT 3 mutation for comparison, like SNK6 and SNT-8 (220, 277, 278), could not be acquired during that study period. However, predicted binding affinity by NetMHC 4.0 declares the 10mer STAT3 WT peptide as a low binder to the HLA-A*02:01 complex (277.01 nM) compared to STAT3 D661Y 10mer mutated peptide (17.71nM), and therefore should bind less to the HLA complexes. However, the peptide titration showed that IFN-γ could be detected by the STAT3 D661Y specific TCRs up to a concentration of 10^{-10} M, indicating a low concentration, and should therefore not represent any limitation. Ultimately, tandem mass spectrometry analysis of HDLM2 cells by Dr. Martin Klatt revealed that our target peptide is not presented via HLA*02:01 and therefore we could not detect TCR T cell activation. Further implications of this result are discussed below.

8.3 Immunopeptidomics of HDLM-2 cells without adequate processing and presentation of the target epitope

*8.3.1 IIMGYKIMYA is not an HLA-A*02:01 restricted epitope on HDLM-2 cells*

With the knowledge after the sequencing analysis of HDLM-2 cells that the mutation existed at the genetic level, we passed on HDLM-2 cells of our culture to Dr. Martin Klatt, Charité, to address potential epitope discrepancies of our two target cells. For further sensible use of HDLM-2 in assays as a target cell line, it had to be clarified whether IIMGYKIMYA is present as an epitope. To investigate this question further, an HLA ligand elution and tandem mass spectrometry analysis of HDLM-2 cells was performed by Dr. Martin Klatt, Charité. Unfortunately, within the scope of our preliminary work, the HDLM-2 cell line was not yet available to us. Consequently, we only checked the processing beforehand by means of bioinformatical prediction and by elution and tandem mass spectrometry analysis of STAT3 D661Y minigene transduced K562 cells in advance and not from a cell carrying the endogenous mutation.

Finally, the results provided to us show that the target epitope IIMGYKIMYA and the 9mer IMGYKIMYA could not be eluted and displayed during the mass spectrometry analysis. Hence, we did not detect TCR T cell activation in our cocultures, as no TCR-peptide

interaction could occur. Therefore, HDLM-2 cells are no longer suitable target cells to find out whether there is a broad use in STAT3 D661Y mutated cells of the high-affinity TCRs described in this work. This leads to the question of whether the availability of our target epitope is only insufficient in the HDLM-2 cells or whether our target epitope is also not presented in other endogenously STAT3 D661Y-expressing cells. Accordingly, further clarification on whether we are pursuing a suitable neoepitope could be achieved by knock-in (KI) of the full-length STAT3 D661Y construct or by transduction of the STAT3 D661Y mutant full-length plasmid into K562-A2 cells. Alternatively, the KI can be performed in any HLA-A*02:01 positive cell line that is proven not to have a defect in the peptide processing machinery. Through this method, we can compare TCR T cell activation in coculture with other endogenously mutant STAT3 D661Y expressing cells. Moreover, a peptide elution and tandem mass spectrometry analysis of epitopes of the KI cells or full plasmid mutant STAT3 D661Y cells could be conceivable here. This would also allow us to verify whether it is only a discrepancy in epitope differences between HDLM-2 and another full-length mutant STAT3 D661Y expressing cell line or if our target epitope in general is not processed and presented as was predicted.

So far, we have four high-affinity TCRs reactive against our target peptide IIMGYKIMYA. Possible reasons for the lack of activation of those TCRs in coculture with HDLM-2 in contrast to in coculture with K562-A2-STAT3-D661Y minigene transduced cells will be discussed in the following.

8.3.2 Immune escape mechanisms, Immunoproteasome processing and pMHC complex loading functions in HDLM-2 cells

Since only K562-A2-STAT3-D661Y minigene transduced target cells elicit TCR transduced T cell activation, and mass spectrometry analysis revealed the abundance of IIMGYMKIMYA on HDLM-2 cells endogenously carrying the mutation, the question arises of why the epitope is not presented.

One first hypothesis is that the lack of HDLM-2 recognition could be due to a deficit in the antigen processing and presentation mechanism of the cells. To initially exclude a general deficiency of processing and surface loading functions, we used a MAGE A1 specific TCR as a control and transduced HDLM-2 cells with MAGE A1 antigen linked to a GFP reporter gene. In coculture with the control TCR T1367, specific for the MAGE A1 antigen, a high

level of IFN-γ was detectable, indicating that the entire protein processing machinery of the HDLM-2 cells was indeed functioning.

8.3.3 Potential differences in peptide processing machinery of HDLM-2 cells compared to K562-A2-STAT3-D661Y minigene transduced cells

The fact that the proteasome is a highly dynamic cellular unit means it can adapt to the cellular environment's conditions and the resulting demands on the cell. Therefore, the proteasome's structural and conformational composition and substrate specificities are regulated by many factors, such as transcriptional regulation, the kinetics of subunit assemblies, post-transcriptional modifications, and interactions with regulatory proteasome-interacting proteins (279-281). Thus, it can be assumed that the proteasome of K562 cells does not function in a manner identical to the proteasome of HDLM-2 cells.

As K562 is a chronic myeloid leukemia cell line (282) and HDLM-2 is a Hodgkin lymphoma cell line (234), both cancer cell lines deriving from hematopoietic cells, they should both express the immunoproteasome on a constitutive level besides the constitutively expressed 20S proteasome (280). However, the expression of immunoproteasome subunits is enhanced in the presence of IFN-γ and other inflammatory cytokines. This leads to an increase in the rapidity of protein turnover, and more processed peptides are recognizable on MHC-I for CD8+ T cells (283, 284). This self-reinforcing process is likely to have in K562 cells with a high concentration of IFN-γ in the coculture supernatant but not in the coculture milieu of HDLM-2 cells. Consequently, the immunoproteasome and constitutive proteasome are probably not equally expressed in K562 and HDLM-2 cells. Moreover, in general, the composition of proteasomes among cell lines can differ (285). K562 has mostly standard proteasomes (286), where the proteasome composition of HDLM-2 cells is unclear and could possess immunoproteasome or alternative variants with further combinations of proteasomal subunits, like the single intermediate proteasome or the double intermediate proteasome(285). It is conceivable that they are not processing the epitope or cleaving in our sequence and producing other peptides. In addition, there are different opinions on whether the immunoproteasome qualitatively provides differently processed peptide spectra than the constitutive proteasome or if there are just quantitatively more processed peptides (280). Furthermore, it was detected that proteasomes can fuse already excised fragments together, thereby generating new

immunoreactive epitopes, so-called splicetopes (36, 37), which might have taken place in HDLM-2 cells.

8.3.3.1 Evidence for immunogenicity of predicted neoepitopes by in vitro digestion experiments only from full-length gene

As discussed in detail in the sections above, the immunogenic processing of a neoepitope and its actual occurrence in the target cell is tough to predict and is often not very reliable (287). Therefore, other groups also introduced a tandem minigene into target cells to verify the neoantigens processing and presentation on the HLA complex either by elution of mass spectrometry analysis or by activation of their specific TCR T cells after coculture with minigene-bearing cells (142, 288-290). This method has been used broadly by other groups and by us; however, this study shows that the processing and trimming dynamics of the STAT3 D661Y minigene in K562 cells are not transferable to the ones of HDLM-2 cells. This method was not reliable here as the minigene design mimics the expression too artificially. Although we have a high-affinity TCR reactive to the target peptide, the question was not answered of whether our target epitope is commonly expressed in HLA-A*02:01 positive STAT3 D661Y mutated cells.

A similar problematic constellation was recently faced by Immisch et al., whose H3.3K27M-specific TCR recognized the predicted neoepitope peptide sequence when loaded on T2 cells, but neither the mutation-carrying cell line nor cells over-expressing the mutation through a transgene were recognized. A mass spectrometry-based analysis finally resolved the discrepancy, showing that the epitope could not be detected even on target cells overexpressing the mutation (291). Willimsky et al. generated TCRs against $KRAS^{G12V}$- and $RAC2^{P29L}$-derived neoepitopes based on a prediction algorithm. Unfortunately, their TCR T cells also failed to recognize the target epitopes in vitro and in vivo in immunized mice expressing a $KRAS^{G12V}$ minigene. Here, however, due to peptide contaminations and hydrophobicity of the KRAS amino acid composition, in vitro proteasome processing was complicated and resulted in no definitive evidence about the existence of predicted target neoepitopes (292).

In our study, with regard to the pMP71 vector, the minigene underlies a viral promotor so that the STAT3 D661Y epitope, which is encoded three times in a row, is highly overexpressed in K562 cells. This is not the case in cancer cells like HDLM-2. In the STAT3 D661Y minigene, the peptide sequence is only prolonged about 3bp up- and 24bp

downstream. The so-called tandem minigene does not have all the genetic information compared to a full-length gene: e.g., splicing patterns, signals for gene regulations, or genes encoding for miRNA (35, 36). Since the whole STAT3 locus has a total of 75171 bp (NCBI Gene ID 6774) and is much larger than the minigene, gene length differences could alter processed mRNA in terms of translation and splicing. For example, it is conceivable, that the full-length gene has other up- and downstream cleavage sites, and consequently another peptide spanning the mutation is processed. The active site of the 20s subunit of the proteasome consists in turn of the β1-, β2-, and β5-subunits, with enzymatic activity comparable to the following enzymes: caspase, trypsin, and chymotrypsin (285). To get a rough idea of how different the processing in the STAT3 D661Y full-length gene could be, the adjacent 24 amino acids up- and downstream flanking the STAT3 D661Y IIMGYKIMYA peptide were analyzed concerning possible cleavage sites of the above-mentioned enzymes by using Expasy PeptideCutter (last performed on 19th July 2023). This resulted in 12 possible cleavage sites including four in our target peptide sequence. Accordingly, other proteasomal products are conceivable. Of course, this brief cleavage site prediction should only be understood as an illustration that other peptide fragments can arise from the full-length gene compared to the minigene.

In any case, in future studies, the setup could be optimized by using two cell lines that endogenously possess the correct HLA type and endogenously express the full-length gene of interest to mimic endogenous expression conditions. Thereby, one could exclude processing and presentation discrepancies in one cell line and target epitope expression could be furthermore confirmed by mass spectrometry analysis. As already mentioned above, we are planning to address this question by establishing a second target cell line expressing the full-length STAT3 D661Y gene by KI or full-length plasmid transduction.

*8.3.3.2 Other HLA-A*02:01 restricted epitopes could derive from the STAT3 D661Y full-length gene*

To investigate if there are other documented epitopes spanning position 661 in the STAT3 gene, we looked for HLA-A*02:01 restricted ligands in immunopeptidome databases. In the HLA Ligand Atlas, provided by the University of Tübingen (293), a 10mer peptide (KIMDATNILV) and a 9mer peptide (IMDATNILV) both deriving from STAT3 were listed.

However, the HLA Ligand Atlas data was derived from benign human tissues. Therefore, only the unmutated peptides were eluted and analyzed.
Given these newly found peptides are indeed processed and presented via MHC-I, from the full-length STAT3 gene, so we further checked the mutated and WT peptide sequences for their predicted binding affinities in silico.
Therefore, using NetMHC-4.0, we predicted the binding affinity of the newfound unmutated KIMDATNILV and the potential D661Y-mutated version (KIMYATNILV) and compared it to the 10mer target IIMGYKIMYA, which was used in the present study.

Both unmutated and mutated new peptide candidates are predicted to be strong binders to HLA-A*02:01 molecules (17.78 nM and 19.19 nM affinity, last performed on 13th of May 2023). However, NetMHC-4.0 HLA affinity prediction categorizes our target IIMGYKIMYA also as a strong binder (17.71 nM affinity). Binding prediction to the respective HLA-molecule via NetMHC4.0 is based on 81 different human MHC alleles and 41 animal alleles (232). Artificial neural network training is constantly updated with new data, and predictions can differ and do not represent reality, as was seen in this work.

It can be assumed that, the new-found peptide is indeed processed, and it is more likely to be bound to HLA-A*02:01 since it has a higher binding prediction score than the corresponding WT peptide.
It has been described that amino acid positions one or two as well as nine, or, as stated in another publication, the last amino acids, are anchor residues for stable binding to the HLA cleavage and therefore influence the pMHC affinity (294-296). Taking this into account, the characteristics of our target peptide IIMGYKIMYA with the mutation at position 9 (Tyrosine (Y) instead of Aspartic acid (D)), could explain the change in predicted binding affinity from a low to a strong binder, which was also a reason for its selection as a target epitope. Nonetheless, it was shown that predicted binding affinities were of no consequence up to this point, since the epitope is not presented on HDLM-2. Furthermore, it is also known that the variable CDR3 region of the TCR is in contact with the central amino acids of the peptide (297). Since for our original target IIMGYKIMYA, the central amino acids are the same for the WT and mutant peptide, one could have discussed whether a TCR isolation for the mutant peptide is even possible, or whether potential TCRs are subject to negative selection because of potential alloreactivity, which was not seen here. On the other hand, the change in HLA binding affinity in the mutant vs. WT peptide state means that the WT peptide presentation via MHC on healthy cells,

e.g., in the thymus, is unlikely due to lower binding affinity. Thus, negative selection in the thymus is also rather unlikely to have occurred, as the WT peptide was not presented and we indeed isolated high affinity TCRs. In contrast, the newfound peptide KIMYATNILV and its WT are both declared as strong binders. Indicating, the WT peptide could be an epitope in thymic epithelia. In the new potential target KIMYATNILV, the mutated amino acid is at the center of the peptide and, therefore, it is available for TCR recognition, and a potential reactive TCR is more likely to discriminate between WT and mutated peptide. Even if the WT peptide binds to MHC, TCRs reactive towards the WT peptide would be negatively selected against in the thymus to ensure self-tolerance (298). In contrast, TCRs against the non-naturally occurring KIMYATNILV are unlikely to be eliminated due to the lack of this altered peptide in thymic epithelia. Of course, this is only a theoretical consideration and it must be verified in vitro.

In summary, these findings indicate that there might be another promising peptide spanning the STAT3 D661Y mutation. However, confirmation could also be obtained by mass spectrometry analysis of cells with the mutated STAT3 D661Y full-length gene before isolating a new TCR for this target.

8.4 Future perspective

Based on the experiments performed in this work, we identified four high-affinity TCRs reactive to the target peptide IIMGYKIMYA. However, we demonstrated by mass spectrometry and functional assays that the Hodgkin lymphoma cell line HDLM-2, heterozygous for the STAT3 D661Y mutation, does not process and present the STAT3 D661Y 10mer epitope. It is therefore not yet possible to say whether the TCR can find a broad application in STAT3 D661Y mutation-bearing cancer cells, as the natural availability of the epitope must be confirmed in cell lines homozygous for the mutation. To address this question, and due to the circumstances, in that we have no other cell line available that carries the STAT3 D661Y mutation endogenously, we are planning a CRISPR Cas9 knock-in of the STAT3 D661Y mutation into an HLA-A 02*01 positive control cell line such as K562, or a T cell line to address the discrepancies of peptide expression between the minigene and the endogenous STAT3 gene. Alternatively, transduction of HLA-A 02*01 positive cells with a STAT3 D661Y full-length gene plasmid could be performed. In the case of confirming a sufficient expression of the target epitope

IIMGYKIMYA via mass spectrometry, we will set up a coculture with STAT3 D661Y redirected TCR T cells. The data generated from this planned experiment may provide us with information on whether strong TCR T cell activation also occurs in coculture with a cell line other than HDLM-2 that endogenously processes the mutated STAT3 D661Y gene on a natural level and does not overexpress the epitope as fin the case in a minigene. In case we detect robust T cell activation, and therefore recognition of the target epitope, further experiments will follow. First, an LCL assay should be conducted to exclude HLA haplotype reactivity. Finally, in vivo mice experiments could be planned.

8.5 Conclusion

Eight TCR sequences were identified and tested. In subsequent T cell assays, four high-affinity TCRs against the HLA-A 02*01 restricted STAT3 D661Y 10mer IIMGYKIMYA peptide were determined. However, T cells transduced with these TCRs were unable to show a potent cytotoxic response against HDLM-2, a Hodgkin lymphoma cell line, endogenously expressing the STAT3 D661Y mutation. Added MHC isolation with bound peptides and subsequent tandem mass spectrometry analysis of the cells revealed an abundance of IIMGYKIMYA as epitope on HDLM-2. Unfortunately, we relied on a minigene to predict proteasome processing and MHC peptide presentation, as opposed to examining the full-length gene. Finally, it is not yet possible to decide on the applicability of the four STAT3 D661Y redirected TCRs, as the natural presence of the target epitope must be verified in further experiments, so as to decide whether IIMGYKIMYA is an ideal target.

Nevertheless, this work gives reasons to consider neoepitope selection and TCR T cell generation as carefully as possible in advance. Here, using a minigene to overexpress STAT3 D661Y resulted in sufficiently expressed epitopes and TCR T cell activation. However, pMHC and TCR T cell interaction was lacking in coculture with HDLM-2 cells.

Therefore, we generally recommend the following workflow: insilico prediction of the HLABinding affinities for the target epitope combined with a mass spectrometry analysis of the epitopes from at least two target cell lines. Furthermore, this work shows that it is fundamental to use a cell line that endogenously has the correct HLA type and endogenously expresses the peptide to mimic natural expression conditions.

Nevertheless, we believe mutated STAT3 is a promising target for TCR T cell therapy as it is a driver mutation and frequently mutated in T cell neoplasms, which are not sufficiently targetable by CAR T cells. Still, improvement of current therapeutical options is urgently needed.

9 References

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10 Eidesstattliche Versicherung

„Ich, Paula Schmidt, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Testing specificity and efficacy of T cell receptors derived from in vitro generated T cells stimulated with STAT3-mutated peptide“ / „Untersuchung der Spezifizität und Effizienz von T Zell Rezeptoren stammend aus in vitro generierten T Zellen stimuliert mit mutiertem STAT3 Peptid“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

_____ _____

Datum Unterschrift

11 Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12 Publikationsliste

Mini-Review in Frontiers in Immunology: Engineering NK Cells for CAR Therapy-Recent Advantages in Gene Transfer Methodology, January 2021, Paula Schmidt, Martin J. Raftery, Gabriele Pecher, Medizinische Klinik für Hämatologie, Onkologie, Tumorimmunologie, CCM, Charité

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14 Nachweis einer statistischen Beratung

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Bescheinigung

Hiermit bescheinige ich, dass Frau Schmidt innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: 10.11.2022

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Keine Tests durchführen, bei Einzelmessungen. In diesen Fällen das Ergebnis der jeweiligen Rezeptoren tabellarisch oder graphisch aufzeigen.
- Nicht-lineare Regression mit Baseline-Korrektur ist geeignet, um EC50 Wert zu bestimmen.

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

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