

Targeting the hematopoietic stem cell antigen FLT3 by high-affinity T cell receptor for the treatment of high-risk acute myeloid leukemia

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NEŞE ÇAKMAK-GÖRÜR

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1st reviewer: Prof. Dr. med Antonio Pezzutto

2nd reviewer: Prof. Dr. Thomas Blankenstein

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1. Introduction

1.1. T cells

The immune system has evolved as a host defense system against invading pathogens. There are many biological structures and different type of cells as a part of this system. It is divided into two categories as innate and adaptive immune system. Innate immune system provides non-specific recognition of pathogens via detection of pathogen-associated molecular patterns (PAMPs). It is a fast, first-line response against infections. Adaptive immune system, on the other hand, is a more complex, antigen-specific defense mechanism. The versatile nature of the adaptive immune system enables the generation of effective and highly specific immune components against each infectious agent. In addition, it provides long-lasting protection to an infection with the same pathogen by forming immunological memory.¹

The T cells are the major players of the adaptive immune system and respond mainly to pathogens. They are classified into two main subsets as CD8⁺ T cells and CD4⁺ T cells based on their distinct functions and co-receptor expression. CD8⁺ T cells, also known as cytotoxic T cells, have the ability to recognize and kill virus-infected cells as well as foreign cells. They also might be able to recognize transformed cells such as cancer cells. CD4⁺ T cells are termed helper T cells, they assist the immune response exerted by other immune cells like B cells. In this thesis, we will focus primarily on CD8⁺ T cells, their biology and function as potential tools for immunotherapy of leukemias and lymphomas.²

1.1.1. The T cell receptor

Each T cell in the body expresses a unique T cell receptor (TCR) which recognizes epitopes presented in the context of major histocompatibility complexes (MHCs) on the host cell surface. The TCR is a heterodimeric receptor made of two polypeptide pairs either as $\alpha\beta$ or $\gamma\delta$ chains. The T cell subset with $\gamma\delta$ TCRs, which makes up a minority of peripheral T lymphocytes, link the adaptive and innate immune system and will not be further addressed in this study.³

The α and β chains of a TCR consists of a variable and a constant region. The constant region facilitates the heterodimerisation of the two chains and provides the transmembrane domain that anchors the TCR to plasma membrane. As the constant

region does not have signaling domains, it is noncovalently linked to the CD3 complex and a ζ chain dimer that carry out signal transduction via their immunoreceptor-based tyrosine activation motifs (ITAMs).⁴ The variable region binds to the peptide-MHC (pMHC) complexes on target cell surface and thereby confers specificity of the TCR. The α chain is encoded by the combination of a variable (V) and a joining (J) gene segment, while the β chain contains an additional diversity (D) gene segment. The genes encoding for the human TCR α and TCR β variable chains are found in a germline configuration on chromosomes 14 and 7, respectively.⁵ There are 70 V and at least 61J genes in the TCR α locus and 67 V, 2 D and 13 J genes in the TCR β locus.⁶ The combinatorial diversity of TCR variable regions is achieved by combining various segments through a process called V(D)J recombination which is carried out by the RAG1 and RAG2 recombinases during T cell development in the thymus.⁷⁻⁹ In addition to this, junctional diversity is introduced by addition and removal of nucleotides at the junction of different V-J or V-D-J segments. These hypervariable loops are called complementary determining region 3 (CDR3) and form the center of antigen-binding site of a TCR responsible for recognition of pMHC complexes.^{4,10}

1.1.2. Major histocompatibility complex and antigen presentation

Major histocompatibility complex (MHC) molecules are cell surface glycoproteins that present antigenic peptides derived from proteins to T cells. These heterodimeric molecules are divided into two classes as Class I and II molecules depending on the chains they are made of, the nature of the peptides they present and the type of T cells they interact with. The MHC Class I (MHC-I) molecules are made of a variant α chain and a non-polymorphic polypeptide chain β -microglobulin (β 2M).¹¹ They are expressed on the surface of all nucleated cells and present peptides derived from all cellular proteins to CD8⁺ T cells. The MHC Class II (MHC-II) molecules are composed of a variant α and a variant β chain.¹² They are expressed explicitly by antigen presenting cells (APCs) such as dendritic cells (DCs) and B cells and present peptides to CD4⁺ T cells. In addition to the peptides derived from cellular proteins, the MHC-II can present peptides derived from exogenous antigens internalized by endocytosis.

The MHC locus is organized on chromosome 6 in humans and contain around 200 genes that are involved in pathways essential for the adaptive immune system. In humans, the genes encoding for MHC-I and II belong to the human leukocyte antigen (HLA) complex.¹³ There are three genes coding for Class I α chain named HLA-A, -B and -C and three pairs of genes coding for the “classical” Class II α and β chains called HLA-DR, -DP,

and -DQ. Other “non-classical” MHC-II molecules are called HLA-DM and HLA-DO. The MHC Class I and II genes are co-dominantly expressed and highly polymorphic. Therefore, each individual can express up to six different Class I and ten different Class II molecules including the non-classical MHC-II. As each MHC allele has different sequence preference for peptide binding, co-dominance and polymorphism of MHC molecules provides a capacity to present a very broad range of peptides.¹⁴

The MHC-I molecules are made of a variant α chain and a non-polymorphic polypeptide chain β 2M. The α chain is composed of three main domains namely α_1 , α_2 and α_3 . The highly polymorphic α_1 and α_2 domains form the peptide binding groove of the MHC-I, while α_3 facilitates heterodimerization via α_3 - β 2M interaction, which is essential for surface expression and stability of the complex.¹⁵ The peptide binding groove of MHC-I can only bind to short peptides with an average length of 8-11 amino acids.¹⁶ The source of the peptides is typically proteasomal degradation products of cellular and nuclear proteins including pathogen-derived peptides accumulating intracellularly in case of viral infections. Once the degradation takes place, the short peptides are transported by the transporter associated with antigen presentation (TAP) complex to the endoplasmic reticulum (ER) where MHC-I assembly is carried out by multiple chaperon proteins.¹⁷ Next, the assembled MHC-I is stabilized by the binding to a peptide. The binding occurs when the groove interacts with the peptide via one residue at the carboxyl terminus and one or two residues at the amino terminus of the peptide, called the anchor residues.⁴ Finally, peptide-loaded MHC-I complexes are transported to the cell surface through the Golgi apparatus.¹⁸ The polymorphism of α_1 and α_2 domains of each MHC allele allows binding to different anchor residues increasing the range of peptides displayed on the surface, thus enhancing the protein sampling capacity. In addition to that, those sites serve as interaction points with TCRs restricting a unique TCR to a single pMHC combination.¹⁹

1.1.3. T cell development and selection in the thymus

All T cells originate from the hematopoietic stem cells (HSCs) that reside in the bone marrow and complete their differentiation and maturation in the thymus. HSCs first differentiate into multipotent progenitors (MPPs) that are the common ancestor of all myeloid and lymphoid blood cells which then differentiate into common lymphoid progenitors (CLPs). Some CLPs can migrate to the thymus, where the interaction with thymic stroma initiates a series of proliferation and differentiation events. Initially, T cell progenitors do not express a CD3-TCR complex and CD4 or CD8 co-receptors. At this stage, they are called double-negative (DN) due to the lack of co-receptor expression. Those DN precursors go through a series of phases called DN1 to DN4, then start

expressing CD4 and CD8 becoming double positive (DP). Finally, they are positively and negatively selected to become fully functional, mature CD4 or CD8 T cells.²⁰

TCR rearrangement starts at the DN3 phase with the rearrangement of the β chain. The upregulation of RAG1 and RAG2 recombinases initiates the V(D)J recombination from one of the alleles at the TCR β locus in an attempt to create a functional variable region linked to a constant region. The rearranged β chain pairs with an invariant α chain (pre-T α) forming a preliminary form of the TCR called pre-TCR. Production of a functional pre-TCR prevents the recombination of the other β allele, known as allelic exclusion, whereas a thymocyte with a nonfunctional pair can rearrange a new β chain from the other allele. The pre-TCR triggers proliferation and cells become double positive (DP) expressing both CD4 and CD8 co-receptors. At this DP stage, the thymocytes start rearranging the TCR α chain. Unlike the β chain, the rearrangement at the TCR α locus does not stop until positive selection takes place.²¹

In the thymic cortex, DP cells with a rearranged TCR $\alpha\beta$ pair interact with self-antigen-presenting-MHC-I or MHC-II molecules on the surface of thymic APCs. Only the T cell clones that can recognize self-antigen-MHC complexes above a certain affinity threshold receive survival signals and are positively selected while the rest dies by “death by neglect”.²² The positive selection is essential because this step ensures that all the surviving cells bear a TCR capable of interacting with an MHC allele. Additionally, the fate of a DP T cell is determined at this step. Cells that interact with an MHC-I molecule lose CD4 and remain CD8⁺ while the ones interacting with an MHC-II molecule lose CD8 and become CD4⁺. A portion of the positively selected cells can bind to self-antigen-MHC complexes with a high affinity, which could potentially result in auto-reactivity. These cells are removed by a process called negative selection which takes place in thymic medulla. T cell clones that recognize self-antigens with a high affinity above a certain threshold receive proapoptotic signals and are clonally deleted before they reach maturation, while some can differentiate into regulatory T cells.²³ Negative selection contributes drastically to “central tolerance”, which is a mechanism to prevent autoimmunity by deleting the self-antigen reactive T cell clones. In summary, the naïve T cells that leave the thymus are restricted to a single MHC allele and tolerant to the self-antigens thanks to the differentiation and selection processes.

1.2. Cancer Immunotherapy

1.2.1. Immune response to cancer

The observation that the immune system can recognize entities on tumor cells came from a series of tumor transplantation experiments of methylcholanthrene (MCA)-induced tumors in inbred mice. Mice immunized with MCA-induced fibrosarcoma were protected when challenged with the same tumor but not with a spontaneous tumor. In addition, immunization with normal tissue did not protect against MCA-induced tumor transplant showing an entity of MCA-induced tumor is responsible for anti-tumor immunity.²⁴ The ability of the immune system to recognize and destroy arising tumor cells by the T cells is a concept called immunosurveillance. It was formulated by Burnet in 1970. He hypothesized that tumors with neo-antigens can induce immune response resulting in tumor regression and control.²⁵ Immunosurveillance is protective against virus-induced tumors;²⁶ however, there is controversial views on whether it applies to spontaneous tumors.²⁷⁻²⁹ According to the immunosurveillance theory, a tumor can grow out when a tumor cell escapes T cell recognition by losing its immunogenicity. However, in a mouse model of sporadic cancer expressing SV40 T oncogene as tumor antigen, it was shown that the tumor evades T cell response not by losing the immunogenicity but inducing tolerance.³⁰

Cancer immunotherapy consists of the use of immune system components such as cytokines, therapeutic antibodies and T cells to recognize and eliminate cancer cells. While cytokines, such as IL-2, unspecifically induce an upregulation of all T cell responses and is antigen-independent, antibodies and T cells are directed towards selected antigens, thus possibly being antigen-specific.³¹ In the following subsections, we will summarize the tumor antigens and antigen-specific immunotherapy approaches.

1.2.2. Tumor antigens

The tumor antigens fall into two classes as tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs). TAAs are non-mutated self-antigens that are overexpressed on tumor cells, and are also present on some normal cells to a certain extent. They could be tissue differentiation antigens such as CD19 on B cells, MART-1 and gp100 on melanocytes and Willm's tumor 1 (WT1) on chronic myelogenous cells in some leukemias and HSCs.³² Another class of TAAs are the cancer-testis antigens which are expressed normally on germ cells in the testis or ovaries but are reactivated in some tumor cells.³³ The MAGE family genes and NY-ESO-1 that are expressed on melanomas and other cancers fall into this category. The advantage of TAAs is that they are shared

between tumors and individuals increasing the range of patients that could benefit from TAA-specific therapies. Yet, targeting TAAs can cause on-target toxicity in normal tissues because they are usually shared between tumor and normal cells.³³

TSAs, also termed neoantigens, are derived from somatic mutations in the tumor and are truly tumor specific. The point mutations in the protein-coding regions of genes can cause amino acid alterations in the protein sequence changing a peptide sequence at the anchor residues enabling them to bind to MHC molecules or create a new epitope that can be recognized by T cells.³⁴ Mutations in cancer-driver genes are very valuable neoantigens to target with TCRs since they are required for the oncogenic transformation and tumors cannot evade an immune response by growing variants that lack the corresponding mutation. For example, MHC-I-restricted antigenic neoepitopes from such genes like *KRAS* and *TP53* have already been described,^{35,36} although only a minority of the many different *KRAS* and *TP53* mutations found in human cancers are truly immunogenic: the peptides encompassing the mutation do not fit into the grooves of the most frequent MHC-I molecules or they only bind with a very weak affinity. Although there are examples of shared mutation leading to potentially targetable neoantigens, most of them are patient-specific. Thus, use of TCRs targeting individual neoantigens is frequently restricted to small number of patients and often require development of personalized immunotherapy.

1.2.3. Antibody-based immunotherapy

Monoclonal antibodies targeting TAAs on tumor cells have been used in the clinic since a long time. They bind to surface antigens and act on tumor cells by directly inducing apoptosis, activating the complement system or initiating antibody-dependent cell mediated toxicity.³¹ To date, there is a broad range of solid and hematological cancers that can be targeted by antibodies. For instance, an anti-HER2/*neu* antibody, Trastuzumab, is in use for HER2-positive breast cancers. In addition, Rituximab targets the CD20 molecules on B cells and used against non-Hodgkin B cell lymphomas and B cell leukemias. Other examples of antibody targets are CD52, vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR).³⁷ In addition, antibodies can be used as targeting agent to deliver anti-cancer agents to the tumor site and in this case, they are called antibody-drug conjugates. Bi-specific antibodies make another group of antibody-based therapeutics used in cancer therapy. They are artificial antibodies with two different antigen specificities that can recognize two different epitopes or antigens allowing dual targeting. The bi-specific antibodies can be developed to recognize a TAA and CD3 molecule on T cells. This serves the purpose of activating and engaging a nonspecific T cells to a tumor cell to induce tumor cell lysis.³⁸ In recent years, bio-engineered small

antibody fragments are used rather than whole antibody molecules for this purpose. Furthermore, the use of immune checkpoint inhibitors can be categorized into this class. CTLA4 and PD-1 are expressed on the surface of activated T cells which attenuate T cell activity at different stages of T cell response by different means. They keep T cell response in a physiological range during response to pathogens and maintain peripheral tolerance which protects the host from autoimmunity. Tumors can dysregulate those regulatory mechanism to evade the immune response. Use of antibodies against CTLA4 and PD-1 blocks the inhibitory signals and unleashes the T cell response against tumor.³⁹ While the response to immune checkpoint inhibitors depend on the mutational load of the tumor,^{40,41} the search for predictive biomarkers is still ongoing as the majority of the patients do not respond.⁴²

1.2.4. Adoptive T cell transfer

1.2.4.1. Adoptive transfer of non-modified T cells

The therapeutic effect of graft-versus-leukemia (GvL) reaction observed after an allogeneic hematopoietic stem cell transplant (HSCT) could be seen as one of the earliest examples of adoptive transfer of non-modified T cells.^{43,44} The evidence that this therapeutic effect is mainly orchestrated by the donor T cells comes from the increased rate of relapse in the patients who receive lymphocyte-depleted HSCs.⁴⁵ This is further supported by lower relapse rates associated with donor lymphocyte infusion followed by HSCT.⁴⁶

Efforts have been implemented on treating tumors using autologous tumor-reactive T cells that reside in the tumors and are called tumor infiltrating lymphocytes (TILs). The first use of TILs on patients with metastatic melanoma was published by Rosenberg in 1988,⁴⁷ which was followed by a trial with a higher number of patients.⁴⁸ In the second report, they showed that transfer of *ex vivo* expanded autologous TILs in combination with high dose IL-2 can induce objective response in 29 of 86 patients.⁴⁸ The *ex vivo* expanded cellular product is a heterogeneous population of CD4⁺ and CD8⁺ T cells with unknown antigen specificity and MHC restriction. Strategies were improved identify and expand tumor-reactive TIL clones to generate more uniform cellular products. The antigenic targets of identified TILs include MART-1⁴⁹, gp100⁵⁰ and some neoantigens⁵¹. The TIL isolation and production were extended to other solid tumors such as renal cell carcinoma, breast cancer, cervical cancer, neuroblastoma but the highest success rate remains in melanoma patients.⁵²

1.2.4.2. Adoptive transfer of engineered T cells

The need to use T cell products with a defined antigen specificity and T cell subtype drove the development of techniques aimed to transfer specific antigen receptors to a large number of normal T cells to be used in adoptive cell therapy (ACT). These receptors can be artificial chimeric antigen receptors (CARs) or natural TCRs. The transfer of the transgenes can be done via viral delivery using retroviral or lentiviral vectors or non-viral delivery such as “sleeping beauty” transposon/transposase⁵³ and CRISPR/Cas9 systems⁵⁴.

As the name implies, CARs are chimeric antigen receptors with an extracellular single chain variable fragment (scFv) antibody domain linked to an intracellular TCR signaling domain. The scFv provides antibody-like recognition of the cancer surface antigens, while the intracellular domain drives the signaling pathways that lead to T cell activation. CARs provide non-MHC-restricted recognition of target cells; therefore, the recognition is independent of the MHC alleles of the patients and the antigen processing machinery of the tumor cells.⁵⁵ The antigenic targets of CARs are usually TAAs. The first CAR was developed to target CD19 molecule expressed on B cell-derived malignancies and normal B cells. It showed great success in the clinic with up to 90% response rates in patients with relapsed B cell acute lymphoblastic leukemia revolutionizing cancer immunotherapy.⁵⁶ Additional B cell surface markers have been subsequently targeted such as CD20, CD22 and BCMA.^{57–59} Further lineage-specific antigens are being evaluated for other kinds of tumors. For instance, CD5- and CD7-specific CARs were developed to target T cell malignancies.^{60,61} The examples of targets can be extended to HER2 for some solid tumors, carbonic anhydrase IX (CAIX) for renal cell carcinoma, GD2 for neuroblastoma and mesothelin and CEA for other solid tumor types.⁵²

In TCR gene therapy, T cells are equipped with a high-affinity TCR against a TAA or TSA that is present in the tumor to be targeted. It represents a refinement and a further development of TIL-based immunotherapy. Delivery of a high-affinity TCR to a defined T cell population enables generation of cell products with desired antigen specificity and MHC restriction. One major drawback of TCR gene therapy is that it can be applied only to a fraction of patients that carry both the MHC allele and the tumor antigen. While CAR-modified T cells are MHC-independent, and therefore can be applied to a broader range of patients, they can target only surface antigens on target cells limiting the number of targetable TAAs. Unlike CARs, TCRs can recognize epitopes derived from all proteins independent of their cellular localization. Therefore, the use TCR-modified T cells can expand the repertoire of TAA antigens. For example, intracellular TAAs, such as tissues-

specific transcription factors can be targets for TCR gene therapy.⁶² The biggest advantage of TCR-modified T cells is that they can recognize tumor specific neoantigens allowing development of T cell therapy with minimum or no toxicity.

1.2.5. Engineered CD8⁺ T cells in ACT

The first study showing that TCR gene transfer can redirect human T cells to a tumor antigen was published in 1999. A MART-1-specific TCR cloned from the TILs of a melanoma patient had cytotoxic effect against HLA-A2⁺ melanoma cells *in vitro*.⁶³ Experiments in mice showed that adoptive transfer of WT-1 specific TCR can eliminate leukemia.⁶⁴ The encouraging results from those studies paved the way to clinical studies on ACT of engineered CD8⁺ T cells. The first clinical trial was done in 2006 by the group of Rosenberg on melanoma patients who were refractory to previous IL-2 treatment. They engineered the autologous PBLs of the patients with an HLA-A2-restricted MART-1 TCR isolated from the TILs of a melanoma patient. The persistence of transduced cells were low after 1 month of the infusion and objective clinical response was observed in 13% of the patients.⁶⁵ Although the response rate was lower than the autologous TIL therapy, this study has been a pioneering work for new trials targeting several other TAAs. Since then, targeting an HLA-A2-restricted epitope of NY-ESO-1 antigen showed some success in melanoma, synovial sarcoma and multiple myeloma patients.⁶⁶⁻⁶⁸ A carcinoembryonic antigen (CEA)-specific murine TCR was tested on three patients with colorectal cancer, however, all patients relapsed after showing 17%-50% reduction in their tumors size and developed severe transient inflammatory colitis.⁶⁹ Nine patients with melanoma and synovial sarcoma were treated with T cells transduced to express affinity-enhanced MAGE-A3-specific TCR isolated from HLA-A2-transgenic mouse. Regression was achieved in 5/9 patients, however, 3 developed serious neurological toxicity.⁷⁰ They could not pinpoint the antigenic source of off-target recognition but proposed the TCR recognized another member of the MAGE family, MAGE-A12, which is expressed on some neuronal cells. Another MAGE-A3 TCR restricted to HLA-A1 allele was linked to cardiovascular toxicity and eventual death of the two patients involved.⁷¹ This was due to unexpected cross-reactivity of the TCR against titin, a large protein expressed in the heart. These extreme cases demonstrated the power of TCR gene therapy, while drawing the attention to the importance of selecting safe TAAs and careful testing normal tissues for potential toxicity by the TCR.

1.2.6. AB_{ab}DII mice

In theory, there are no high-affinity TCRs in the repertoire of an individual against TAAs because of the negative selection events and central tolerance. However, cell populations

with high-affinity TCRs against certain melanocyte differentiation antigens such as MART-1 and cancer testis antigens such as MAGE-A1 have been found among TILs and circulating lymphocytes of melanoma patients.^{49,72} There are two major reasons why such high-affinity TCRs against self-epitopes exist. First, in rare events, high-affinity TCRs targeting self-antigens can escape negative selection. Second, the epitope (as in the case of MART-1) or the antigen (e.g. MAGE-A1) is not expressed in the thymus, hence, negative selection does not apply.^{73,74} Yet, such antigens are rare and in order to broaden the repertoire of antigen and MHC restriction that is targetable in a variety of cancer types, it is possible to generate TCRs with desired specificity and avidity by avoiding negative selection.

Several approaches have been developed to circumvent the tolerance. First, T cells from an individual negative for a particular HLA-allele, who does not have tolerance against peptides presented by that HLA allele, can be primed *in vitro* with peptide-loaded T2 cells⁷⁵ or autologous DCs electroporated with the HLA allele that present the epitopes of a given TAA.⁷⁶ So, as an example, TCRs against a HLA-A2-restricted peptide of a given antigen can be selected from an HLA-A2 negative donor whose T cells never met before the peptide in the context of HLA-A2. This leads to the generation of a TCR of allogeneic nature. Because the T cells do not undergo negative selection for that HLA allele, the TCRs bear the risk of recognizing the MHC-I molecules of the host independent of the peptide presented on it. Selection of truly peptide-specific TCRs is therefore difficult and must be screened against a possibly largest number of HLA-alleles and peptides to exclude reactivity. A second approach is the use of HLA transgenic mice, which lack tolerance to many human self-antigens. However, this method comes with its limitations. In this mouse, the murine TCRs are selected on human MHC, hence, they often have low avidity. They require affinity maturation by *in vitro* mutagenesis to reach high-avidity for successful tumor rejection in the clinic.⁷⁷ Another drawback is that the rearranged TCRs are of mouse origin and the variable regions might induce immune reaction in humans limiting their applicability.⁷⁸ To overcome the limitations of both of those approaches, ABAbDII mice were raised in the lab: another mouse model, which is transgenic for both the human HLA-A2 molecule and the entire TCR α and β gene loci. These mice can be immunized with HLA-A2 restricted human epitopes to raise high affinity class I TCRs.⁷⁹ The use of ABAbDII mice provides solutions to the drawbacks of the first two approaches. The risk of HLA directed allogeneic toxicity is lower because the ABAbDII-derived TCRs are negatively selected on human HLA-A2. Next, unlike the mouse-derived TCRs, the rearranged novel TCRs from ABAbDII mice should not induce immune response because it uses human TCR genes. Furthermore, the ABAbDII mice select human TCRs on human

MHC enhancing the isolation of TCRs with high-affinity against TAAs.⁸⁰ Taken together ABabDII mouse present itself as a valuable tool for identification of therapeutic TCRs against tumor antigens.

1.3. Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is a hematological malignancy characterized by uncontrolled growth and differentiation of myeloid cells. The leukemic cells originate from immature cells of the bone marrow, which are mostly myeloid progenitor cells incapable of further differentiation, and in rare cases, truly stem cells capable of both myeloid and lymphoid differentiation (mixed lineage leukemias, MLL). Immature cells accumulate in the bone marrow, and depending on the presence of adhesion molecules or chemokine receptors, they may invade the peripheral blood and sometimes even organs, particularly skin and mucosa, liver, spleen, lymph nodes, bone, and central nervous system.⁸¹

AML can occur at any age, but is increasingly frequent in the older population median age of diagnosis of 70 years.⁸² The general incidence is around 4 cases in 100.000 people, but with an occurrence of around 100 cases among 100.000 individuals aged over 70 years.⁸³ Current treatments cure 35-40% of the patients younger than 60 years of age (will be referred as younger patients) and 5-15% of the patients older than 60 years of age (will be referred as older patients).⁸⁴ The mortality rate of patients older than 65 years old still remains 70% within 1 year of diagnosis.⁸⁵

The disease prognostic factors can be divided into two groups as patient-associated factors and disease-related factors. Patient-associated factors such as age and co-morbidity are important for prediction of treatment-associated early death, while disease-related factors such as white blood cell count and particularly the genetic landscape of the leukemic cells predict the response to treatment.⁸⁶ The combination of both factors is essential for deciding a particular therapeutic approach such as standard or intensive therapy (including aggressive approaches such as allogeneic stem cell transplantation) as well as the integration of novel therapeutics that are increasingly being developed as selective targets of particular genetic alterations in cellular pathways that govern growth and differentiation of hematopoietic cells.⁸⁶

Until recently, AML was classified in 8 different subtypes, M0-M7, on the basis of the differentiation pattern of the cells.⁸⁷ According to a new classification announced in 2016 by the World Health Organization (WHO), AML is divided into 7 different types based on morphology, immunophenotype and clinical parameters combined with genetic factors. Those types are (i) AML with recurrent genetic abnormalities (including 11 subtypes), (ii)

AML with myelodysplasia-related changes, (iii) Therapy-related myeloid neoplasms, (iv) AML not otherwise specified (including subtypes analog to the FAB-subtypes), (v) Myeloid sarcoma, (vi) Myeloid proliferations related to Down syndrome and (vii) Leukemias of ambiguous lineage. An eighth entity consists of (viii) neoplastic proliferation of plasmacytoid dendritic cells.⁸⁸ With improved use of cytogenetic testing, leukemia classification is becoming more complicated, with the occurrence of particular genetic alteration determining the response of the disease to therapy and therefore the prognosis of the disease. More than a hundred different recurrent genetic alterations have been defined. Currently, some genetic alterations with prognostic relevance are found with higher frequency in AML patients and therefore have clinical relevance. WHO defined 11 subgroups of AML based on the chromosomal translocations and recurrent mutations with prognostic relevance.^{88,89} The subgroups of AML defined by recurrent genetic alterations is continuously increasing and there is a realistic hope that with improved and deeper genetic analysis of AML, the pathogenesis and ultimately the therapy of the disease will be better understood leading to better, individualized treatment options. Additionally, European LeukemiaNet (ELN), a European network with the goal of leukemia research, diagnosis, and treatment, categorised AML as favorable, intermediate and adverse based on the cytogenetic profile of the neoplastic cells for better diagnosis and management in the clinic.⁹⁰

1.3.1. Standard treatment

The main objective of AML therapy is to induce remission and prevent disease relapse. The treatment consists of two phases: induction therapy, which has the goal of eliminating as many leukemic cells as possible, and consolidation therapy, which has the goal of destroying any potentially residual leukemic cell which may have survived the induction phase, and of preventing disease recurrence.⁸⁶

The intensity of induction therapy depends on patient-associated prognostic factors. Most of the younger patients and older patients that are considered to be “biologically fit” receive intensive induction therapy consisting of a combination of the chemotherapeutic agent cytarabine (Ara-C), most frequently at a very high dosage, combined with an anthracycline drug such as daunorubicin or idarubicin. This combination is usually referred to as 7+3 scheme (7 days of cytarabine, 3 days of anthracycline).⁹¹ Two to three weeks after chemotherapy, a bone marrow evaluation is performed. Complete remission (CR) is assumed when the number of immature cells (which possibly include residual leukemic cells) is below 5%.⁹⁰ If CR is not achieved, a further round of induction therapy, sometimes at a higher dosage or including additional drugs, is administered. If then the disease

persists (more than 5% leukemic cells in the bone marrow), the leukemia is considered to be refractory to the therapy and the prognosis is usually grim.

Patients in CR receive consolidation or post-remission therapy, which may differ according to age, morbidity, complications arise during induction therapy, and the quality of response. Post-remission therapy may include one or more cycles of chemotherapy, frequently including higher dosage cytarabine (HDAC), or allogeneic stem cell transplantation.⁹² The latter is used usually in younger patients considered to have a very high risk of relapse (see next chapter) or in patients who experienced a relapse and after a novel chemotherapy round could achieve a “second remission”.

1.3.2. Hematopoietic stem cell transplantation (HSCT)

The biggest challenge in the management of AML is the high relapse rate which occurs in 10-40% of the patient population.⁹³ Allogeneic-HSCT from an HLA-matched donor is the most efficient treatment method for high-risk or relapsed AML to date.⁹⁴ The HSC donor is usually an HLA-identical sibling donor, and sometimes an HLA-matched unrelated donor who might belong to the extended family of the patient or selected from a worldwide bone marrow databank. The donor is selected on the basis of HLA matching between the donor and the patient. Different centers have different matching criteria. The donor can be a 10/10 match meaning HLA-A, -B, -C, -DRB1 and DQB1 alleles are identical with the patient, and an 8/8 match when HLA-A, -B, -C and -DRB1 compatibility is sought.⁹⁵

The high cure rate of HSCT; which is around 15-20% in AML and can be as high as 80% in chronic myeloid leukemia (CML),⁹⁶ depends on two factors. First, the patient receives higher-dose chemotherapy than induction therapy that has high toxicity both against the leukemic cells and the HSCs of the patient.⁹⁷ Survival after high-dose chemotherapy is possible only because the bone marrow function is completely reconstituted by the HSCs of the donor, which is administered 24 hours after the wash-out of the chemotherapy from the body. Second, the donor lymphocytes in the transferred HSCs, although usually matched for most of the HLA-alleles, can still recognize hematopoietic cell-specific antigens of the host that differ slightly in their amino acid sequence from the donor mainly due to single nucleotide polymorphisms (SNPs) in the coding regions of the genes (so-called minor histocompatibility antigens).⁹⁸ Immunological recognition of such antigens leads to the elimination of the leukemic cells bearing them. This effect that is usually referred to as the Graft-versus-Leukemia (GvL) effect, and in reality, in most cases, it is an “anti-recipient-hematopoiesis” effect.⁹⁶ As a drawback, however, immune recognition can also be directed against normal cells of the patient in

other tissues such as skin, liver, gut, and lungs causing severe, sometimes lethal Graft-versus-Host Disease (GvHD). Despite many efforts for years, it is impossible so far to clearly separate the GvL from the GvHD by manipulating either the transplant, the “conditioning regimen” used to enable engraftment of the donor stem cells, or the patient by administering medications after infusion of the donor cells. Initial evidence on GvL in humans came in 1979 with the observation that patients who do not develop GvHD had 2.5 times higher relapse rate.⁴⁴ There is now high level of agreement that the most significant contribution of allogeneic-HSCT to cure is mediated by this immune recognition.

1.3.3. Small molecules and antibody-based therapeutics

The standard “7+3” regimen has been the backbone of AML treatment for the past 40 years now. Only in the last few years a number of novel drugs have entered the arena of AML therapy, mostly as a result of better understanding of the molecular pathways involved in leukemogenesis and of extensive molecular genetic characterization of AML patients in large study cohorts.⁹⁹ Those novel therapeutics can broadly be categorized as tyrosine kinase inhibitors, cell cycle and signaling inhibitors, epigenetic modulators, new cytotoxic agents, nuclear export inhibitors and antibody-based therapeutics targeting AML-specific antigens.⁸⁶ Research on tyrosine kinase inhibitors that target wild type and mutated forms of FLT3, which is involved in differentiation and are have received the most attention due to prognostic value of FLT3 in AML.¹⁰⁰ Other tyrosine kinase inhibitors have been used in clinical studies, but have not been approved for treatment, a number of other small molecule inhibitors are being developed and will enter therapy in the next year(s). Another popular class of drugs is epigenetic modulators that particularly target mutated isocitrate dehydrogenase (IDH)1 and IDH2 in AML. Although these drugs represent a welcome addition to the spectrum of AML therapeutics, so far, it appears that these drugs alone will not be able to cure the large majority of patients, indicating the need for further improvement. Considering that the majority of the patient population is aged, immunotherapy might represent a most desirable approach for AML.

Antibody therapy for AML includes the use of monoclonal antibodies targeting AML-specific antigens as well as antibody-drug conjugates. Gentuzumab Ozogmicin, an antibody-drug conjugate targeting CD33 to deliver aDNA-damaging agent to AML cells, is the first of its class to be approved by FDA in 2000.¹⁰¹ Several antibodies targeting other AML antigens such as CD123, CLL-1 and FLT3 have been developed. Additionally, there are bi-specific antibodies that target CD3 molecules on T cells and CD33, CD123 or CLL-1 on AMLs with the aim to engage uncommitted T cells to AML cells.¹⁰²

1.3.4. Adoptive T cell therapy for AML

The limited efficacy and high toxicity of current treatments drove the urgent need to develop more effective therapeutics for refractory AML. The growing attention on ACT after the success of CD19-CAR in B-cell malignancies has led to the development of AML-specific CARs and TCRs. AML-specific antibody targets were chosen as TAAs to develop CAR-T cells. CD123 was the first antigen that was targeted with a CD123-CAR T cells. Despite CD123-CAR induced rejection of primary AML *in vivo*, there was significant toxicity to hematopoiesis in a xenograft model, so this approach never made it to clinical application.¹⁰³ Later, a CD33-CAR was also shown to reject primary AML in xenografts, yet caused anticipated hematopoietic toxicity.¹⁰⁴ Most of the AML-specific surface antigens are present on myeloid cells and HSCs, hence causing the CAR-related toxicity. Toxicity was addressed by generating a CD33-CAR resistant hematopoietic system by autologous transfer of CD33 knock-out HSCs followed by CAR-T cell infusion.¹⁰⁵

Similar efforts were put on the generation of AML-specific TCRs, which extended the range of the targetable antigens from surface molecules to cellular proteins. For instance, WT1, a transcription factor predominantly overexpressed in AML, is the most attractive AML-specific target for TCR gene therapy. A T cell clone recognizing an HLA-A24*02 restricted epitope of WT1 was identified from the peripheral blood of one out of three HLA-A24*02 positive healthy individuals.¹⁰⁶ The T cell clone efficiently lysed leukemic cells isolated from AML and ALL patients, and did not show any toxicity to HLA-A24*02 positive HSCs *in vitro*. In a Phase I trial, eight patients received T cells transduced with the TCR of the mentioned T cell clone. Although no toxicity was observed in the patients, the success rate was low. Only two patients had decrease of blasts in bone marrow, one had stable disease, while five of them had progressive disease.¹⁰⁷ Furthermore, an HLA-A2 restricted epitope (WT1₁₂₆) was targeted by multiple groups with the aim to generate WT1 specific T cell response. A T cell clone of allogeneic origin against WT1₁₂₆ was described by Gao and his colleagues. This clone was shown to be effective against WT1⁺ cell lines and safe against healthy CD34⁺ *in vitro*.¹⁰⁸ Same epitope was used to develop a peptide-based vaccine which was tested on eight patients with myeloid malignancies. A single dose was shown to induce a short-lived CD8⁺ T cell response detected by tetramer staining of the peripheral blood cells. Although the cytotoxicity assessment of the tetramer-positive CD8⁺ T cells was not performed, this approach was claimed to be safe and effective based on reduction of *WT1* mRNA detected from the patient blood.¹⁰⁹ The most recent study on 12 AML patients showed that a WT-1 TCR isolated from the periphery of an healthy HLA-A2⁺ individual targeting the WT1₁₂₆ can prevent relapse in all patients involved without causing any GvDH or toxicity.¹¹⁰

Additionally, other TCRs targeting AML-specific TAAs have been identified and evaluated such as HLA-A2 and HLA-A*24:02-restricted TERT-specific TCRs¹¹¹ and HLA-B7 restricted MPO-1-specific TCR¹¹².

1.3.5. FLT3 as an AML-specific antigen

Fsm-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor that is expressed by HSCs and is crucial for normal hematopoiesis. Activation of FLT3 by binding to FLT3L triggers the phosphatidylinositol 3-kinase (PI3K) and RAS signal transduction pathways which regulate the proliferation and differentiation of HSCs.¹¹³ It is expressed at very high levels in 70-90% of the AML patients and enhances proliferation and survival of leukemic cells. Additionally, several different mutations of FLT3 have been identified to contribute to the pathogenesis of the disease. For instance, FLT3 internal tandem duplication (FLT3-ITD) has been linked to poor prognosis of AML.¹¹⁴ FLT3 has been an attractive therapeutic target due to its significant role on leukemic cell survival and abundant expression in AML. A lot of work has been put on developing FLT3-specific tyrosine kinase inhibitors with reduced toxicity.¹¹⁵

We selected FLT3 as a target antigen because it is overexpressed in (i) AML and (ii) HSCs in the bone marrow. In our envisaged approach, HLA-A2 restricted FLT3-specific T cells expanded from an HLA-A2⁻ donor would eradicate the circulating and marrow resident FLT3⁺ AML cells as well as the hematopoietic stem cells of an HLA-A2⁺ patient. The bone marrow should be reconstituted with stem cells derived from the same HLA-A2⁻ donor: This approach could be done in the context of a haploidentical transplantation from a family related donor where at least 50% of the HLA-alleles are matched, or in the context of a so-called 9/10, single HLA-mismatch transplantation, whereby the only different HLA allele between host and donor would be HLA-A2. Lacking the appropriate peptide-embedding MHC moiety, the newly introduced bone marrow would not be recognized by the FLT3-specific TCRs: indeed, the elimination of the host residual hematopoiesis along with the host-hematopoiesis-derived leukemic cells should be facilitated, allowing for a more efficient engraftment as compared to the standard approach, where transplant rejection and loss of chimerism is associated with leukemia relapse in some patients.¹¹⁶ Adoptively transferred TCR-transduced T cells would be expected to form memory T cells in the patient and provide long term persistence in the periphery, thus prevent any potential relapse by keeping the proliferation of the patient's bone marrow cells under control. The therapeutic TCRs would need to have a high-affinity for the target antigen for efficient tumor eradication. In order to circumvent the tolerance that is present against FLT3 as a self-antigen, and based on the advantages of ABabDII mice mentioned in section 1.2.6,

we decided to employ this model for identification and selection of high-affinity TCRs against FLT3.

2. Aim of the thesis

Based on the information given in the previous chapter, in this thesis we aim answering the questions below.

- Can we induce T cell response in ABabDII mouse against HLA-A2 restricted epitopes of human FLT3?
- Are the *in silico* predicted epitopes naturally processed and presented?
- Do the identified epitopes serve as clinically relevant tumor-rejection epitopes?
- Is FLT3 a safe target for TCR gene therapy for AML in the context of the planned approach?

3. Materials and Methods

3.1. Cell lines

The human AML cell line THP1 (FLT3⁺/HLA-A2⁺) was purchased from ATCC. The human B cell precursor leukemia cell line SEM and the human AML cell line MV;4-11 (both FLT3⁺/HLA-A2⁻) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. THP1 cells were cultivated in RPMI 1640 medium supplemented with 10% FCS and 50 μ M β -mercaptoethanol. The human CML cell lines K562 and MV-4;11 were maintained in RPMI medium supplemented with 10% FBS. The SEM cell line was kept in IMDM medium supplemented with 10% FBS. TAP-deficient EBV-transformed B cell line T2 were maintained in RPMI medium supplemented with 10% FBS. K562, SEM and MV-4;11 cell lines expressing HLA-A2 were generated by γ -retroviral transduction with pMP71 vector carrying HLA-A2 molecule which was a gift from Uckert Lab, MDC. K562 cells with FLT3 and THP1 cells overexpressing FLT3 were generated by transducing the pMP71 vector carrying wild type FLT3. FLT3 and HLA-A2 surface expression of cell lines were assessed by FACS staining with antibodies against human FLT3-Alexa Fluor 647 (BD Biosciences, San Jose, CA, USA) and human HLA-A2-PE (Clone BB7.2, BD Biosciences, San Jose, CA, USA). FACS analysis was done with BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and data were analyzed with FlowJo version 10.0.8 (Tree Star, Inc., Ashland, OR, USA).

3.2. Selection of epitopes

The full length human FLT3 protein sequence was obtained from NCBI database (Reference number: NP_004110.2). The sequence was submitted to NetMHC V3.4 for prediction of binding to HLA-A2 allele. Epitope length was defined as 9-mers. Two of the predicted epitopes with binding affinities higher than 60 nM and different than the mouse FLT3 were selected for immunization.

3.3. Immunization of ABAbDII mice

Predicted peptides were dissolved in DMSO solvent as indicated by the supplier to a concentration of 2 mg/ml. Mice were immunized on day 0 and boosted on day 21 with 150 μ g of peptide in a 1:1 solution of incomplete Freund's adjuvant (IFA) and 50 μ g CpG1826 by subcutaneous injection. Blood was collected 7 days after each boost and blood cells

were cultured with 10^{-6} M peptide overnight. To analyze peripheral response, cells were first treated with Fc block (BD Biosciences, San Jose, CA, USA), stained with antibody against mouse CD8-PerCP (BD Biosciences, San Jose, CA, USA), then fixed and stained with antibody against mouse IFN- γ -APC (BD Biosciences, San Jose, CA, USA). Animals were boosted in 21-day intervals until peripheral response was observed.

Mice with IFN γ -secreting CD8⁺ T cells in the periphery were sacrificed. Spleen and inguinal lymph nodes of reactive mice were collected. CD4⁺ T cells were depleted by CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). 1×10^6 splenocytes were seeded per well of a 24-well plate and expanded for 10 days in RPMI 1640 medium supplemented with 10% FBS gold, HEPES, NEAA, Sodium Pyruvate, 50 μ M β -mercaptoethanol, 20 IU/ml human IL-2 and 10^{-8} M peptide. Splenocytes were stimulated with 10^{-6} M peptide for 4 h before mouse IFN γ secretion assay (Miltenyi Biotech, Bergisch Gladbach, Germany). The cells were treated with Fc Block, stained with antibodies against mouse CD3-APC and mouse CD8-PerCP (BD Biosciences, San Jose, CA, USA). IFN γ secreting CD8⁺ T cells were sorted with BD FACS Aria III (BD Biosciences, San Jose, CA, USA) to RTL lysis buffer for RNA isolation with RNeasy Micro Kit (Qiagen, Hilden, Germany).

3.4. Identification and cloning of TCRs

5'RACE-ready cDNA was synthesized with SMARTer RACE kit (Clontech, CA, USA) according to instructions of the manufacturer. cDNA was diluted 1:3 prior to use. TCRA and TCRB variable chains were amplified by 5'RACE-PCR in a 50 μ L reaction mix of 5 μ L diluted cDNA, 2X Q5 Hot Start High-Fidelity master mix (New England Biosciences, Ipswich, MA, USA), 5 μ L forward primer from the SMARTer RACE kit (10X Universal Primer A Mix (UPM)) and 0.5 μ M reverse primers for TCRA: 5'-CGGCCACTTTCAGGAGGAGGATTCGGACC-3' or TCRB:5'-CCGTAGAACTGGACTTGACAGCGGAAGTGG-3'. Initial denaturation was done at 98°C for 2 min seconds followed by 30 cycles of denaturation at 98°C for 30 s, annealing at 72°C for 30 s and elongation at 72°C for 45 s. Annealing temperature was decreased by 2°C at every 5 cycles for the first 10 cycles. Reaction was carried out for total 35 cycles. Final elongation was done at 72°C for 5 min.

PCR products were separated on a 2% agarose gel. Bands corresponding to the correct size were eluted from the gel and cloned using Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and sequenced with SP6 primer. Dominant TCR- α/β chains were selected and paired. The TCR constant regions were replaced with mouse counterparts. Paired TCR-

α/β chains were linked with a p2A element. TCR cassette was codon optimized, synthesized by GeneArt (Thermo Fisher Scientific, Waltham, MA, USA) and cloned into pMP71 by restriction site cloning using the NotI and EcoRI cut sites.

3.5. Virus production

HEKT-GALV-g/p cells were transfected with 18 μg pMP71 vector carrying the TCR cassette. The virus supernatant was collected 48 h and 72 h after transfection. 1×10^6 human PBMCs were stimulated on anti-CD3/anti-CD28 coated plates in RPMI 1640 medium supplemented with 10% FBS, HEPES, 100 IU/ml IL-2 and transduced with virus supernatant at 48 h and 72 h after stimulation. Transduction efficiency was determined by FACS staining for human CD8-APC (BD Biosciences, San Jose, CA, USA) and mouse TRBC-PE (Biolegend, San Diego, CA, USA). TCR-transduced-hPBMCs were expanded in T cell medium supplemented with 100 IU/ml IL-2 for 10 days and kept in 10 IU/ml IL-2 supplemented medium for 2 days before the co-culture.

3.6. Functional assays

T2 cells were loaded with serial dilutions of peptides at 10^{-5} M to 10^{-12} M for peptide titration experiments. Target cells were selected based on their HLA-A2 and FLT3 expression and labeled with 1 μM CFSE (ab113853, Abcam, Cambridge, UK) prior to seeding. 2×10^4 target cells and 2×10^4 TCR-transduced cells were seeded in 200 μL final volume in a 96-well format to reach 1:1 effector to target ratio. Cell-free supernatant was collected after overnight incubation to detect IFN- γ secretion by ELISA. Cells were collected for further analysis and stained with antibodies against human CD137-PE (BD Biosciences, San Jose, CA, USA), human CD8-APC-H7 (BD Biosciences, San Jose, CA, USA), mouse TRBC-APC (Biolegend, San Diego, CA, USA) and run on BD FACSCanto II Flow cytometer. Data was analyzed with FlowJo version 10.0.8.

3.7. FLT3 cloning

Total RNA was isolated from THP1 cell line with RNeasy mini RNA isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500 ng total RNA with Superscript II (Invitrogen) using random hexamer primers according to manufacturer's instructions. Full length FLT3 was amplified with the primers F:5'-TATGGCGCCGCGCCACCATGCCGCGT-3' and R:5'-CAGGCTCAGGTCGAAGATTCGTAA-3'. Empty MP71 vector backbone with Not1 sticky end at 5' end and blunt end at 3' was generated by digesting with EcoRI (Thermo Fisher Scientific, Waltham, MA, USA), filling in the vector with Klenow fragment (Thermo Fisher

Scientific, Waltham, MA, USA) to generate a blunt end followed by NotI (Thermo Fisher Scientific, Waltham, MA, USA) digestion and subsequent gel isolation. PCR product was cloned into pMP71 by restriction site cloning using NotI sticky end on 5' end and blunt 3' end.

3.8. FLT3 expression profiling

TissueScan™ human normal cDNA array and human brain cDNA array were purchased from OriGene. Primers were designed to detect 236 bp amplicon of *FLT3* transcript as F: 5'- CTGAATTGCCAGCCACATTTTG- 3' and R: 5'- GGAACGCTCTCAGATATGCAG- 3'. PCR was performed in a total reaction volume of 25 µL using 2X Q5 Polymerase Master Mix (New England Biosciences, Ipswich, MA, USA) with 0.5 µM of each primer. Initial denaturation was done at 98°C for 30 s followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 66°C for 30 s and elongation at 72°C for 30 s. Final elongation was done at 72°C for 2 min. *GAPDH* was amplified with primers F: 5'- AGAAGGCTGGGGCTCATTTG-3' and R: 5'- AGGGGCCATCCACAGTCTTC-3' as internal control. PCR products were visualized on 2% agarose gel.

Snap-frozen healthy brain sections from autopsy samples were provided by the Department of Neuropathology (Charité Universitätsmedizin Berlin, Germany) in accordance with ethical statement. Cryo-sectioned tissue weighing between 30 mg and 40 mg was used for RNA isolation. Total RNA was isolated using the RNeasy Lipid Tissue mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 500 ng total RNA with Superscript IV (Invitrogen) using random hexamer primers. *FLT3* and *GAPDH* amplicons were amplified with the primers listed and cycle conditions stated above.

3.9. Western blot analysis

Total protein was extracted from 100 mg to 120 mg pieces of brain sections and *FLT3* positive cell lines using N-PER neuronal protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Total protein was quantified with BCA assay. 100 µg total protein from brain sections and 40 µg total protein from control cell lines were mixed with 4X Laemmli sample buffer (Biorad, Hercules, CA, USA) in 30 µL final volume, cooked 95°C for 5 min. The samples were resolved by SDS polyacrylamide gel electrophoresis (PAGE) using pre-casted polyacrylamide gels (Mini-PROTEAN TGX Precast Gel, 4-20%, Biorad, Hercules, CA, USA), transferred to nitrocellulose membrane, probed with a polyclonal rabbit anti-human *FLT3* antibody (1:500 dilution, clone 8F2, Cell Signaling Technology, Danvers, MA, USA) and then with HPR-

conjugated goat anti-rabbit IgG (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA). Bands were visualized by chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech, Little Chalfont, UK) detection using Chemidoc MP Imaging System (Biorad, Hercules, CA, USA). Images were analyzed with Image Lab software (Biorad, Hercules, CA, USA). Membrane was stripped for re-probing with monoclonal mouse anti-human β -actin antibody (1:10000 dilution, Sigma-Aldrich, St. Louis, MO, USA) followed by HRP-conjugated anti-mouse IgG (1:25000 dilution, Sigma-Aldrich, St. Louis, MO, USA) for loading control.

3.10. Immunohistochemical and immunofluorescence procedures

Immunofluorescence and immunohistochemical staining was performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections (4 μ m-thick) according to standard procedures. The following primary and secondary antibodies were used: polyclonal rabbit anti-FLT3 (1:50, LS-A7148, Lifespan Biosciences inc., Seattle, WA, USA), monoclonal rabbit anti-Calbindin (1:100, clone C26D12, Cell Signaling, Frankfurt, Germany), monoclonal mouse anti-CD56 (1:100, clone ERIC-1, Bio-Rad, Puchheim, Germany), Alexa Fluor[®] 488-conjugated goat anti-mouse IgG (1:500, Dianova, Hamburg, Germany), Cy3-conjugated goat anti-rabbit IgG (1:500, Dianova, Hamburg, Germany). The immunofluorescence counterstaining was performed with VECTASHIELD[®] Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Immunohistochemical staining of FFPE tissue sections (4 μ m-thick) was performed on a VENTANA Benchmark XT automated staining instrument according to the manufacturer's instructions. Slides were de-paraffinized using EZ prep solution (Ventana Medical Systems, Tucson, AZ) for 30 minutes at 75 °C. Antigen retrieval was accomplished on the automated stainer using CC1 solution (Ventana Medical Systems, Tucson, AZ) for 60 minutes at 95 °C. Briefly, primary antibodies were applied and developed using the iVIEW DAB Detection Kit (Ventana Medical Systems) and the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems). All slides were counterstained with hematoxylin for 4 minutes. Omission of primary antibodies as control for nonspecific binding of the secondary antibody resulted in absence of any labeling. To validate our immunohistochemical and immunofluorescence staining we used different positive control tissues fixed and processed in similar manner to the test sections and known to contain the target molecule, e.g. tonsil tissue.

3.11. Confocal laser scanning microscopy

For confocal microscopy, a Leica TCS SP5 confocal laser scanning microscope controlled by LAS AF scan software (Leica Microsystem, Wetzlar, Germany) was used. Images were taken simultaneously and assembled to stacks.

4. Result

4.1. Identification of FLT3-specific TCRs in ABabDII mice

To generate T cell responses against FLT3₈₃₉ and FLT3₉₈₆ peptides, we immunized ABabDII mice with either of the epitopes as summarized in Table 1. FLT3₈₃₉ had two and FLT3₉₈₆ had three amino acid differences compared to the mouse homolog (Figure 1a). Mice 6782 and 6547 had IFN- γ secreting CD8⁺ T cells in the periphery after the 2nd boost while mice 6780 and 6456 did not show any response after the 10th boost (Figure 1b). Mice were sacrificed either after peripheral response was detected or due to old age. FLT3₈₃₉- or FLT3₉₈₆- reactive CD8⁺ T cells were expanded *in vitro* for 10 days, labeled with IFN- γ capture assay and isolated by FACS for subsequent identification of predominant TCR α and β chain rearrangements (Figure 1c).

Table 1 Detailed immunisation schedule of mice. Two mice were immunized for each of the two epitopes. The binding affinities were predicted by NetMHC V3.4. The numbers used to tag the mice were later used to name the TCR sequences identified from them.

| Epitope name | Epitope sequence | Predicted binding affinity (nM) | Mouse tag | Number of injections | Number of sorted cells |
|---------------------|------------------|---------------------------------|-----------|----------------------|------------------------|
| FLT3 ₈₃₉ | IMSDSNYVV | 6 | 6546 | 10 | 700 |
| | | | 6547 | 2 | 213 |
| FLT3 ₉₈₆ | GLLSPQAQV | 56 | 6780 | 10 | 2500 |
| | | | 6782 | 2 | 65300 |

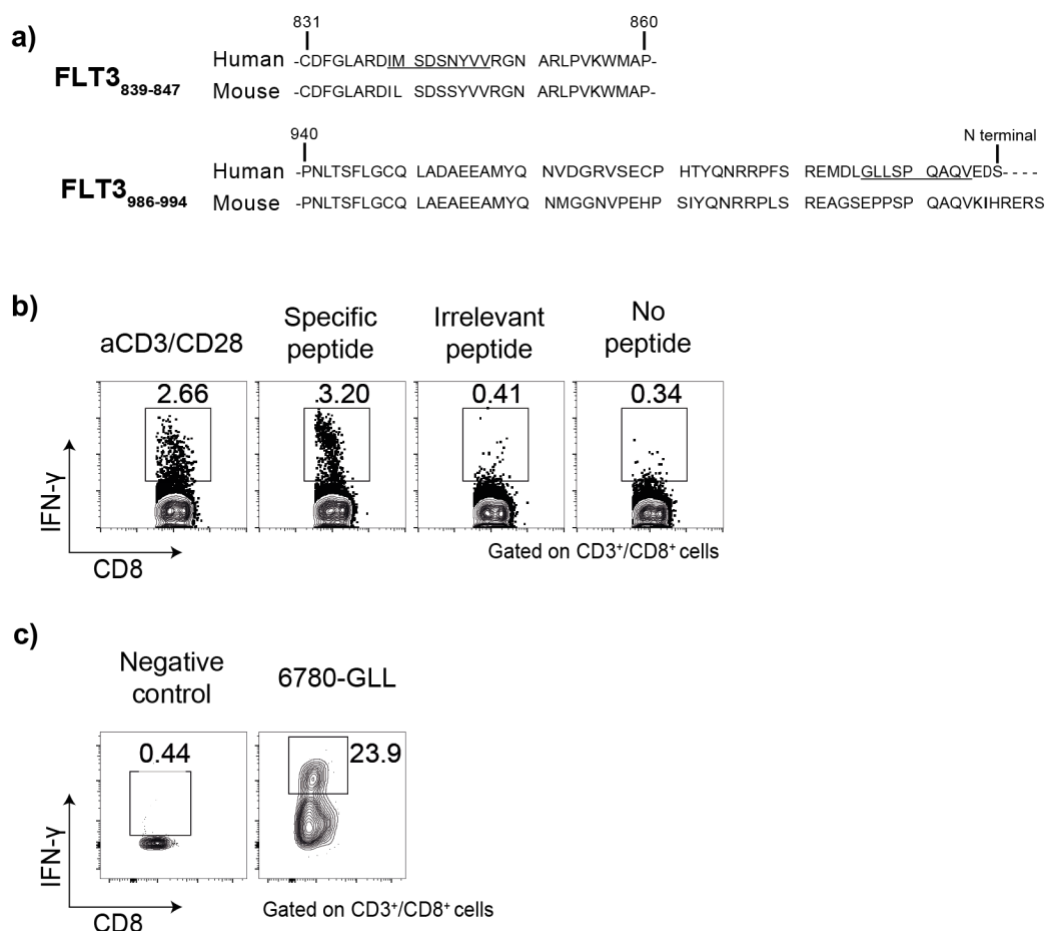


Figure 1 Identification of FLT3-specific TCRs from ABAbDII mice **a)** Homology of human and mouse FLT3 spanning the *in silico* predicted epitopes **b)** Blood were collected from mice 7 days after each injection and were cultured in the presence of 1 μ M peptide overnight as specific stimulus to detect peripheral CD8⁺ T cell response. IFN- γ ⁺-CD8⁺ T cells were detected in the periphery by performing intracellular IFN- γ staining and analyzed by FACS. Blood cells were stimulated with anti-CD3 and anti-CD28 antibodies as positive control. Cells cultured with an irrelevant peptide and without any peptide served as negative control. A representative analysis was shown from the mouse 6782. **c)** Spleen and inguinal lymph nodes were collected from the mice and cultured *in vitro* for 10 days to expand peptide reactive CD8⁺ T cells. IFN- γ ⁺/CD8⁺ population in the gate was sorted by IFN- γ capture assay for RNA isolation. A representative set of plots was shown in the figure.

Sequencing of 5'-RACE products revealed dominant TCR α and β variable chain rearrangements from mice 6780, 6782 and 6546 (Table 2). We did not observe any preferred TRV α or TRV β gene usage for the FLT3₉₈₆ epitope. We could identify the TRV β chain from mouse 6547 but not the TRV α since the 5'-RACE PCR did not work for this chain (data not shown). A TCR cassette was constructed as described in the methods section and cloned into the pMP71 vector for retroviral transduction of human PBLs.

Table 2 TCR α and β variable chain rearrangements identified from AB α DII mice immunized with either FLT3₈₃₉ (IMS) or FLT3₉₈₆ (GLL) epitopes.

| TCR | Frequency | TCR α / β V/D/J genes | | | CDR3 |
|-----------------|-----------|------------------------------------|------------|----------|----------------|
| 6780-GLL | 55% | TRAV12-1*01 | TRAJ26*01 | | CVVNMDYGQNFVF |
| | 60% | TRBV5-6*01 | TRBJ2-1*01 | TRBD2*01 | CASSLEAGYNEQFF |
| 6782-GLL | 91% | TRAV12-2*02 | TRAJ26*01 | | CAVDNYGQNFVF |
| | 94% | TRBV12-3*01 | TRBJ2-3*01 | TRBD2*02 | CASSFGRLDYQYF |
| 6546-IMS | 89% | TRAV12-2*02 | TRAJ40*01 | | CAVNRGSGTYKYIF |
| | 60% | TRBV2*01 | TRBJ2-7*01 | TRBD1*01 | CASSPGTTYEQYF |
| 6547-IMS | | TRVA chain could not be identified | | | |
| | 100% | TRBV6-2*01 | TRBJ2-1*01 | TRBD2*01 | CASSYIAGINEQFF |

4.2. Re-expression of TCRs in human PBLs

In order to verify that the identified TCR α and β pairings are correct and recognize the respective epitopes presented on HLA-A2, human PBLs were transduced with a γ -retroviral vector, pMP71, carrying the TCRs with murinized constant regions to avoid mispairing with the endogenous TCRs. Transduced cells were stained against the mouse TCR constant β chain and human CD8 to confirm re-expression of the TCRs. Transduction rate varied between 20-50% depending on the virus titer of different production batches (Figure 2, upper panel). Furthermore, cells were stained with FLT3₈₃₉-HLA-A2 (IMS) or FLT3₉₈₆-HLA-A2 (GLL) tetramers to confirm TCR-pMHC binding. Staining with the tetramer showed tetramer-positive populations demonstrating that TCR α and β pairings were correct. When gated on TCR-positive cells for analysis, we saw that 6780-GLL and 6546-IMS TCRs required presence of CD8 molecule to bind to the tetramer. However, not all 6780-GLL and 6546-IMS TCR transduced CD8⁺ T cells bound to the tetramer (Figure 2, lower panel). In case of 6782-GLL TCR, the T cells did not require the CD8 molecule for tetramer binding. The majority of the CD8⁻ T cell fraction was bound by tetramer forming 31.5% of the whole population. In addition, almost all of CD8⁺ T cells expressing the 6782-GLL TCR were bound to the tetramer (62.8% of the whole population).

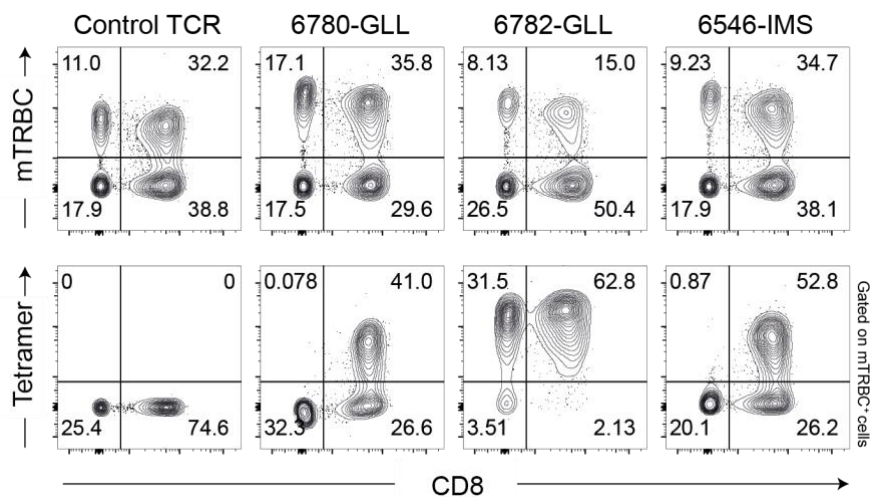


Figure 2 Re-expression of TCRs on human PBLs. Human PBLs were transduced with γ -retroviral vectors carrying the TCRs. Transduction efficiency was assessed by FACS analysis for mouse TCR β constant chain (mTRBC) and CD8 expression (upper panel). Transduced cells were further analyzed for tetramer binding (lower panel). For this analysis, cells were gated on mTRBC to exclude non-transduced cells in the population and visualized for tetramer staining and CD8 expression. All TCRs bound to their respective tetramers. A TCR recognizing a tyrosinase-derived epitope served as the control TCR. Plots are representative of multiple experiments where similar results were obtained from different PBL donors.

4.3. Evaluation of functionality of identified TCRs

Next, to assess the affinity and specificity of the identified TCRs, transduced T cells were co-cultured with T2 cells pulsed with FLT3₈₃₉ or FLT3₉₈₆ peptides from 10^{-5} M to 10^{-12} M concentration. IFN- γ release showed that all TCRs recognize their cognate peptide when loaded on HLA-A2 (Figure 3a). Among all TCRs, 6780-GLL showed the highest degree of IFN- γ release. This TCR reached half-maximum IFN- γ release at 17.91 nM peptide concentration corresponding to the lowest EC₅₀ value and the highest affinity to the pMHC complex. 6782-GLL had almost one-log lower affinity compared to 6780-GLL with the highest EC₅₀ value of 100 nM. 6546-IMS had the lowest absolute IFN- γ release but an intermediate EC₅₀ with 33.6 nM (Figure 3b). We concluded that all TCRs recognize their respective pMHC complexes when presented on the cell surface but to different extents.

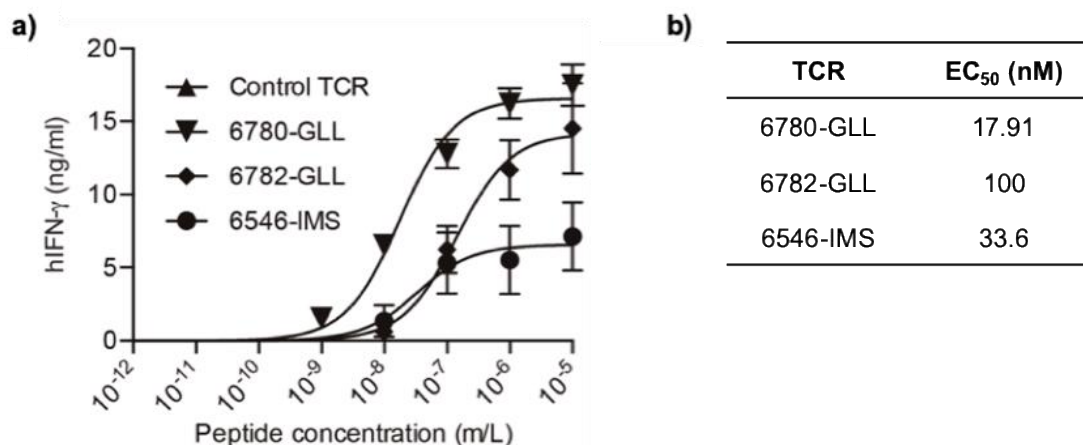


Figure 3 Peptide titration on T2 cells TCR-transduced effector T cells were co-cultured overnight with peptide pulsed T2 cells at an effector to target ratio of 1:1. **a)** IFN- γ secretion was detected from the cell-free supernatant by ELISA. All three TCRs recognized the peptide when present on T2 cells. One representative example of peptide titration out of three donors is shown. **b)** The peptide concentration required to achieve half-maximum IFN- γ release (EC₅₀) was calculated by non-linear regression to estimate affinity of TCRs.

Many studies describing the generation of therapeutic TCRs rely on *in silico* prediction of epitope processing and presentation. NetMHC, a neural network-based tool trained on big datasets of HLA-A2 bound peptides has very high accuracy in predicting potential epitopes derived from certain antigens. However, binding prediction does not necessarily mean that a given peptide is presented on the MHC complex: so, we did not have yet experimental evidence that FLT3₈₃₉ and FLT3₉₈₆ peptides are naturally processed by the proteasome and loaded on the HLA-A2 molecule. In order to investigate that, we generated a K562 cell line expressing FLT3 and HLA-A2, as a single MHC class I allele, to be used as target cells in recognition and killing experiments (Figure 4a). 6780-GLL TCR recognized K562 cells expressing both HLA-A2 and FLT3 proving that the FLT3₉₈₆ epitope is naturally processed and presented. K562 cells with HLA-A2 only but lacking FLT3 were not recognized indicating that 6780-GLL mediated target recognition is pMHC specific. We could not detect any IFN- γ release from 6782-GLL and 6546-IMS transduced effector cells (Figure 4b). Knowing that FLT3₉₈₆ is processed and presented, we can suggest 6782-GLL TCR has too low affinity to recognize pMHC level generated by proteasome. Lack of recognition by 6546-IMS, on the other hand, does not provide enough proof on natural procession and presentation of FLT3₈₃₉ epitope. It could be simply because

the 6546-IMS TCR does not have enough functional avidity to recognize the pMHC at physiological concentration.

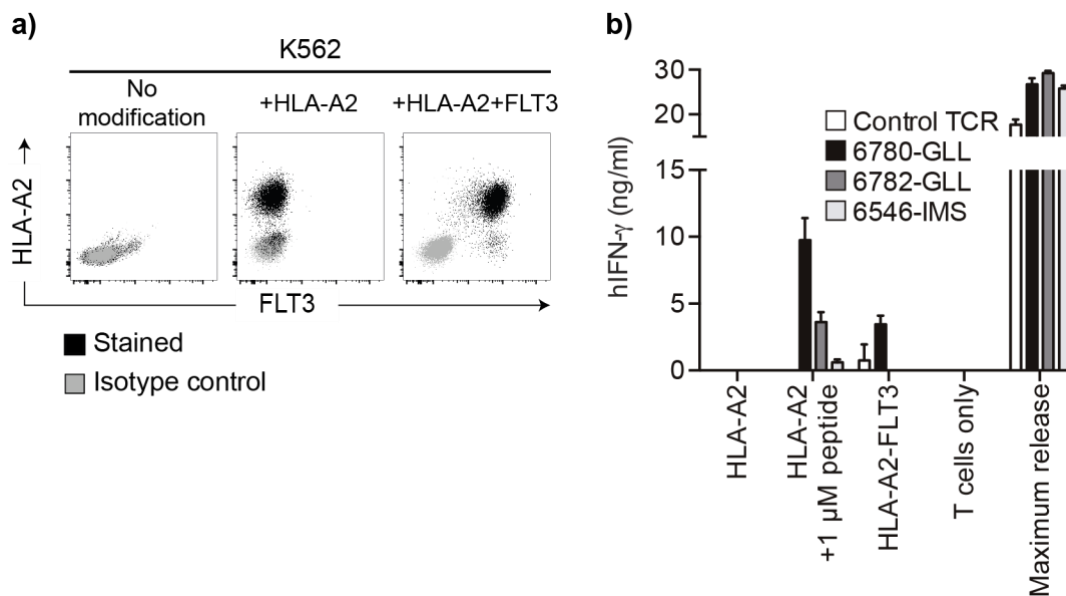


Figure 4 Co-culture with the K562 cells **a)** K562 cells were retrovirally transduced to stably express HLA-A2 molecule alone or in combination with FLT3 as shown by FACS staining. **b)** 6780-GLL and 6782-GLL TCR transduced cells released IFN- γ to peptide-pulsed K562 cells expressing HLA-A2 molecule. No IFN- γ was detected from 6546-IMS transduced cells even in the presence of the peptide. Only 6780-GLL transduced cells recognized K562 cells co-expressing HLA-A2 and FLT3.

As the next step, we sought out to determine whether FLT3-specific TCRs recognize other cell lines that naturally express FLT3. This is important because cells that are genetically modified to express a given protein frequently produce much larger amounts of the protein and therefore generate more epitopes derived from that protein as compared to physiological cells. THP1 cells naturally express FLT3 and HLA-A2. Additionally, we generated FLT3-overexpressing THP1 cells by retroviral transduction (Figure 5a). SEM and MV-4;11 cells express FLT3 endogenously and were transduced stably to express HLA-A2 molecule as shown by FACS analysis (Figure 5a). We could not detect any IFN- γ release by effector cells against any of the target cells mentioned above. Neither over-expression of FLT3 on THP1 nor IFN- γ pre-treatment of THP1 cells to increase MHC-I expression elicited any IFN- γ release (data not shown).

To address whether target cells induce T cell activation at all, we analyzed effector cells for CD137 upregulation after overnight co-culture. We were able to detect CD137 upregulation in very low percentage of 6780-GLL T cells after co-culture with THP1 cells, but this might be considered as background activation. Ectopic expression of FLT3 in THP1 cells induced enhanced CD137 upregulation on 6780-GLL cells (Figure 5b). Peptide pulsed THP1 cells activated 35% of TCR-transduced 6780-GLL and 6782-GLL cells showing the TCR-modified T cells are capable of activation. In parallel, we detected CD137 upregulation on 6780-GLL cells upon co-culture with SEM and MV-4;11 cells expressing HLA-A2.

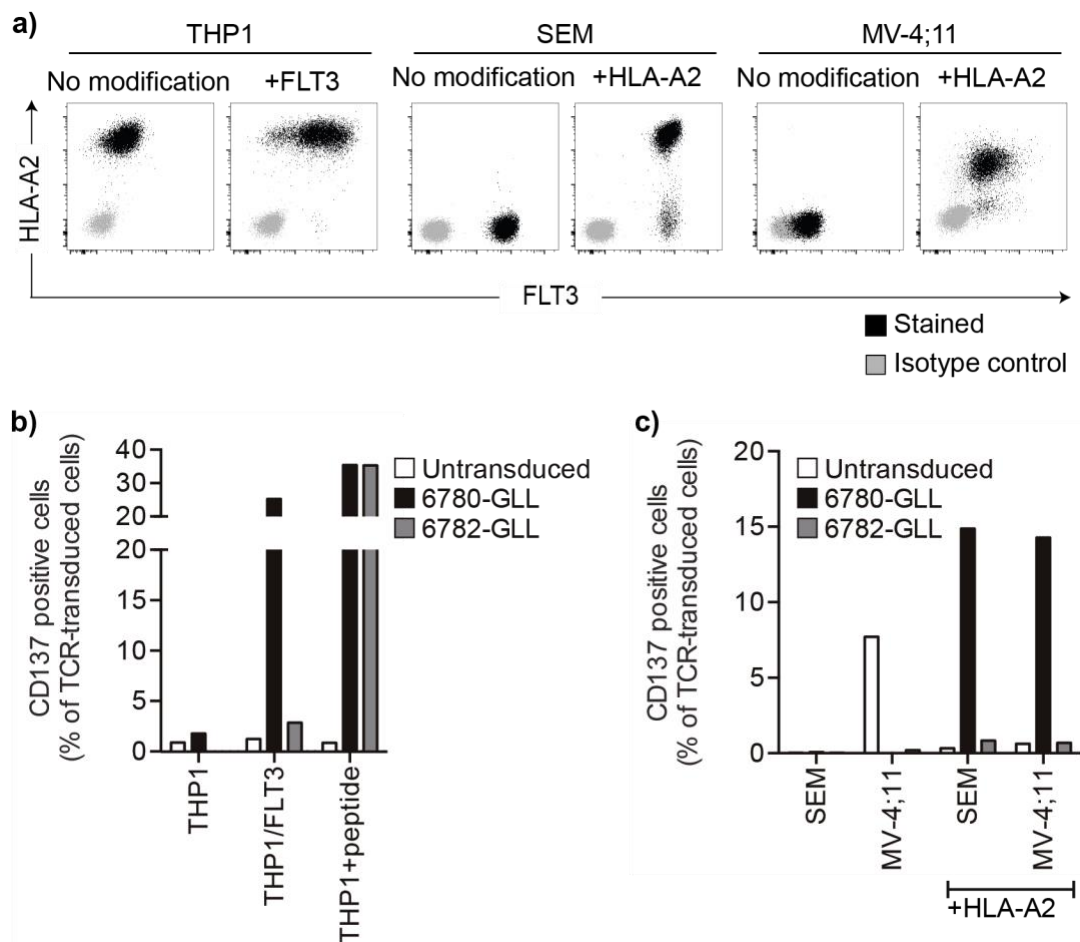


Figure 5 Co-culture with cell lines expressing FLT3 **a)** FLT3 expression level on the surface of the cell lines was assessed by FACS analysis. THP1 cell line was transduced with FLT3 to generate an FLT3-overexpressing clone. The cell lines that did not express HLA-A2 were transduced to stably express it and single cell clones with desired expression level were selected. **b)** TCR-transduced T cells were co-cultured overnight with THP1 cells and CD137 upregulation on effector cells was analyzed by FACS as an indicator of effector cell activation. THP1 cells alone did not induce considerable level of CD137 upregulation of effector cells. Overexpression of

FLT3 on THP1 cells induced activation of 6780-GLL transduced T cells. THP1 cells pulsed with FLT3₉₈₆ peptide prior to the co-culture also induced effector cell activation **c**) Only 6780-GLL TCR transduced effector cells upregulated CD137 after overnight co-culture with SEM and MV-4;11 expressing HLA-A2.

4.4. FLT3 expression profiling

4.4.1. Detection of FLT3 transcript from cDNA tissue arrays

In parallel to the experiments needed to demonstrate TCR specificity and efficacy, we conducted a number of studies in order to evaluate the assumption from the literature that FLT3 is specifically expressed only in hematopoietic cells. This is crucial in order to avoid potential on-target/off-tumor toxicity of a given TCR in case of clinical application. In order to check FLT3 expression in different healthy human tissues by RT-PCR, we first wanted to evaluate the detection limit of our PCR reaction. For this, we did serial dilution of the FLT3⁺ cell line THP1 from 10⁶ cells down to 10 cells in PBS and mixed each dilution with 10⁶ cells of the FLT3⁻ cell line K562. After 35 cycles of PCR, we could detect the 236 bp amplicon corresponding to FLT3 from as low as 10 cells. As expected, we did not detect any *FLT3* transcript from K562 cells only. We could detect *GAPDH* indicating the cDNAs were intact (Figure 6).

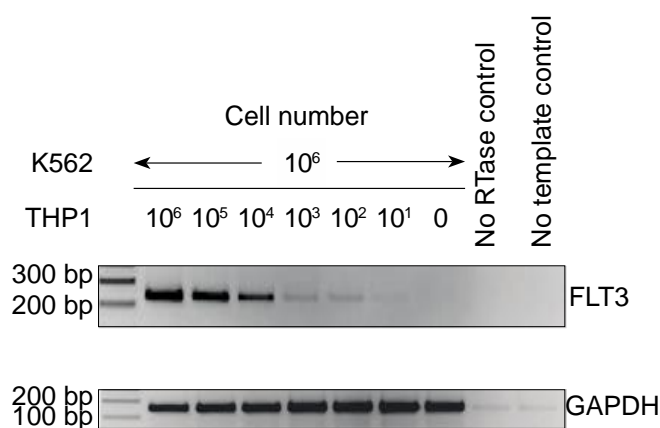


Figure 6 Optimization of FLT3 PCR THP1 (FLT3⁺) cells were titrated from 10⁶ to 10 cells and mixed with 10⁶ K562 (FLT3⁻) cells. FLT3 was amplified to estimate the lowest number of FLT3⁺ cells that can be detected in a given RNA isolate.

After showing that our PCR had high sensitivity in detecting FLT3 transcripts, we sought to determine FLT3 expression in normal human tissues. For this purpose, we did RT-PCR to commercially available cDNA array representing 48 major healthy human tissues. After 35 cycles of PCR, we detected FLT3 transcript in the lymphohematopoietic

tissues including bone marrow, lymph node, peripheral blood lymphocytes, spleen and thymus. In addition, we detected the amplicon corresponding to the FLT3 transcript from other tissues such as lung and pancreas. The presence of FLT3 in those tissues can be explained by the tissue resident activated dendritic cells in the islet Langerhans of pancreas. Very faint bands were detected from brain and spinal cord. (Figure 7).

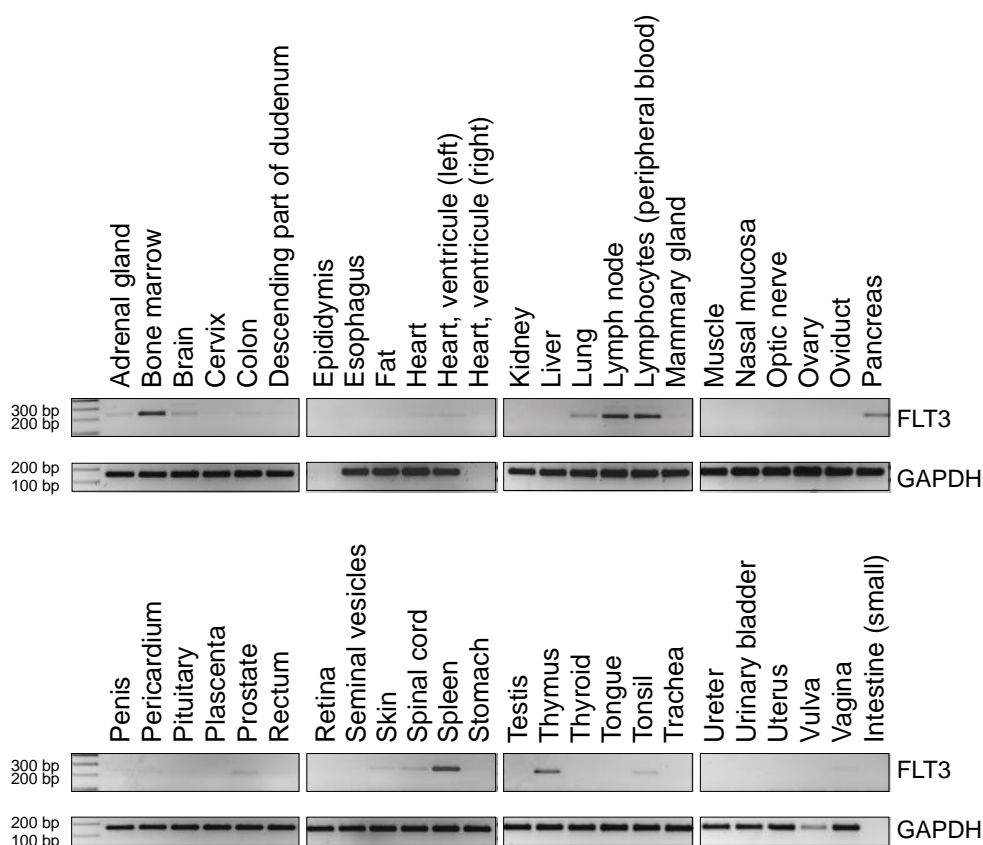
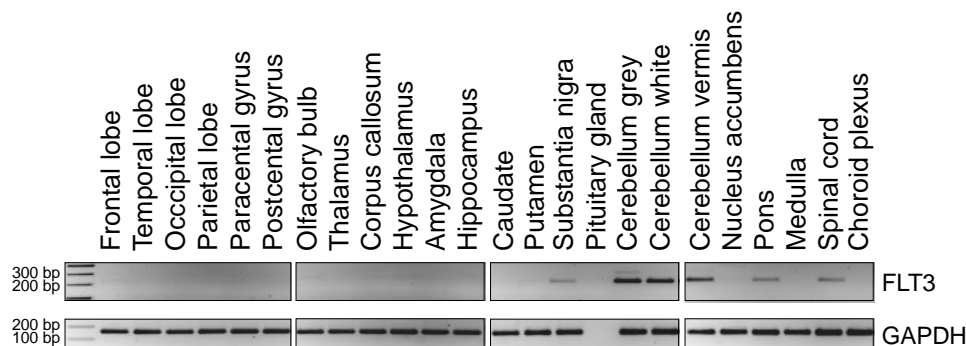


Figure 7 Investigation of FLT3 expression in human tissues FLT3 expression was analyzed by PCR using cDNA array representing 48 different healthy tissues of the human body. We detected 256 bp amplicon corresponding to the FLT3 transcript from the tissues of hematopoietic origin such as bone marrow, spleen and lymph nodes. Unexpectedly, the PCR product was detected in other tissues such as brain and pancreas revealing those tissues for potential on-target toxicity.

To investigate which brain section expresses FLT3, we did another RT-PCR with the cDNA array representing 24 different anatomical brain regions. We detected the amplicon in sites such as substantia nigra, pons and spinal cord. The strongest expression was

detected in cerebellum (Figure 8a). PCR products were sequenced to confirm amplified sequence corresponds to FLT3 amplicon (Figure 8b).

a)



b)

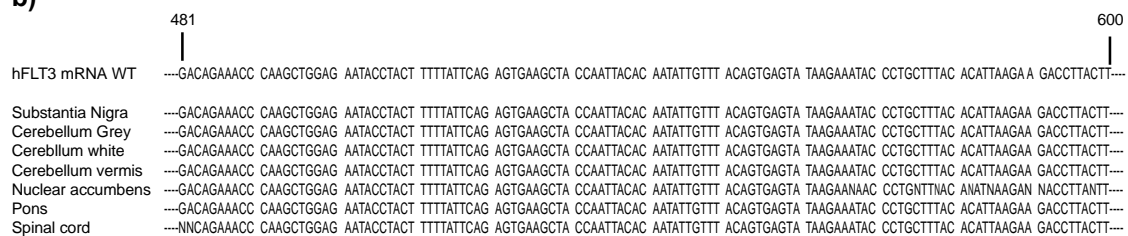


Figure 8 Detection of FLT3 from human neural tissues a) The expression of FLT3 was investigated in detail from a cDNA array representing 24 distinct anatomical sections of central nervous system by PCR. The high expression of FLT3 was detected in cerebellar sections. Lower expression was observed in S.nigra, pons and spinal cord. **b)** The detected PCR products were sanger sequenced and aligned to the wild type human *FLT3* sequence revealing the amplified region is not an unspecific product but maps to *FLT3*.

4.4.2. Detection of FLT3 in iPSC-derived human cardiomyocytes

It was reported by Pfister et al. that mouse cardiomyocytes express very high level of FLT3 on the surface.¹¹⁷ Unlike them, we detected only a very faint band from the cDNA from left ventricle of human heart (Figure 7). In order to verify our results, we obtained induced pluripotent stem cell (iPSC)-derived human cardiomyocytes from the Berlin Institute of Health (BIH) Stem cells core facility. The cardiomyocytes were thawed and RNA was collected on day 2 and day 7 after thawing. In addition, the cells were harvested on the same days for FACS analysis of both intracellular and surface FLT3 expression. The cells were stained at the same intensity with the anti-FLT3 antibody and isotype control antibody meaning the FLT3 protein could not be detected from iPSC-derived cardiomyocytes. In addition, we did not detect any difference in the mean fluorescence

intensity (MFI) either on the surface and in the cytoplasm of the cells when stained with the anti-FLT3 antibody or isotype control (Figure 9a).

In addition to the attempts to detect the FLT3 protein on iPSC-derived cardiomyocytes, we checked the level of *FLT3* transcript in the cells by RT-PCR. We detected *FLT3* amplicon from both day 2 and day 7 cardiomyocytes (Figure 9b). We observed a decrease in the level of *FLT3* expression from day 2 to 7 with similar expression level detected by the tissue array analysis.

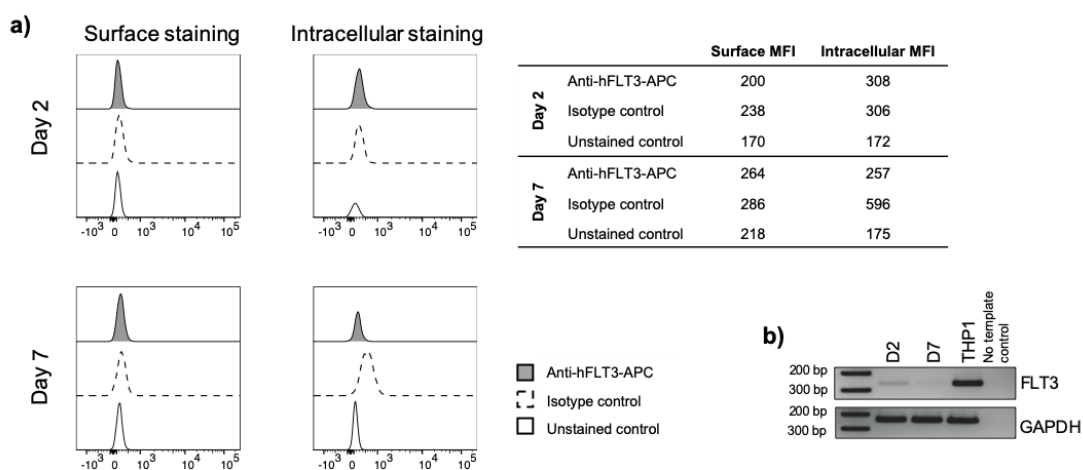


Figure 9 Detection of FLT3 expression from iPSC-derived human cardiomyocytes **a)** iPSC-derived human cardiomyocytes were thawed and stained with anti-human FLT3 antibody or with isotype control on the cell surface and intracellularly to detect FLT3 expression on day 2 and day 7 after thawing. MFI values of each sample is listed in the table. **b)** RNA was isolated from iPSC-derived cardiomyocytes on day 2 and day 7 after thawing. *FLT3* is amplified by RT-PCR to detect the mRNA. We observed a decrease in the intensity of *FLT3* expression from day 2 to day 7. THP1 cells served as a positive control for *FLT3* expression and GAPDH was amplified as internal control.

4.4.3. Detection of FLT3 in primary human brain tissues

To elucidate whether we can detect FLT3 expression in primary human cerebellum, we isolated RNA and protein from snap frozen cerebellar tissue of three different donors. In accordance with the observation we had from nerve tissue cDNA array, we detected FLT3 amplicon from cerebellar vermis of two donors and cerebellar hemisphere of all donors. We did not detect significant FLT3 expression in the frontal lobe, which served as a negative control (Figure 10a). Next, we separated total protein lysate from each sample by SDS-PAGE and immunoblotted to see whether we could detect FLT3. We observed two bands from THP1 protein lysate as expected. The upper band at 160 kDa level corresponds to glycosylated, mature FLT3. This form of FLT3 is found in the plasma

membrane where it functions as a tyrosine kinase receptor. The lower 145 kDa band is the ER-bound immature FLT3 which is retained intracellularly. We detected only 145 kDa band corresponding to immature FLT3 from cerebellar vermis and hemispheres suggesting it is found primarily intracellular (Figure 10 b).

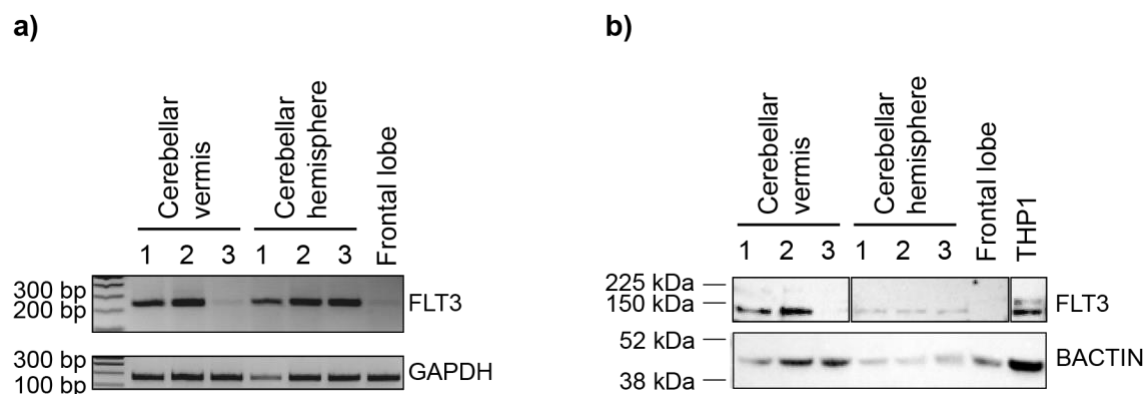


Figure 10 FLT3 expression in primary human brain tissues a) *FLT3* transcript was detected in cerebellar vermis and hemispheres. GAPDH served as the internal control for the RT-PCR reaction. **b)** Total protein lysate of the same sections were separated by SDS-PAGE. Immunoblotting revealed presence of ER-associated, intracellularly retained FLT3 in both cerebellar vermis and hemispheres. Protein lysate from THP1 cell line served as positive control where we detected both 160 kDa membrane associated FLT3 and 145 kDa ER-associated FLT3.

To investigate which cerebellar cell type expresses FLT3 and its subcellular localization, we did immunohistochemical (IHC) (Figure 11A-F) and immunofluorescence (IF) (Figure 11G-L) stainings of cryo-sectioned cerebellar tissue. In Figure 11 A-B, granular layer (GL) and molecular layer (ML) of the cerebellum can be seen. Strong expression of calbindin, an intracellular, Purkinje-specific marker, was detected showing Purkinje cells residing between those layers (arrows). Additionally, the sections were co-stained with a neuronal membrane marker, CD56 (NCAM). IHC staining with an anti-FLT3 antibody yielded strong FLT3 expression in the Purkinje neurons confirming the PCR and western blot analysis. To investigate subcellular localization of FLT3 in Purkinje cells (PCs), we analyzed the IF staining with confocal microscopy. We observed FLT3 expression (red) in the cytoplasm of the PCs. Co-localization of CD56 (green) and FLT3 on the plasma membrane was not detected indicating FLT3 expression is dominantly intracellularly-restricted (Figure 11H-L). This observation, in accordance with the western blot, suggested that FLT3 is found primarily intracellularly in PCs of human cerebellum.

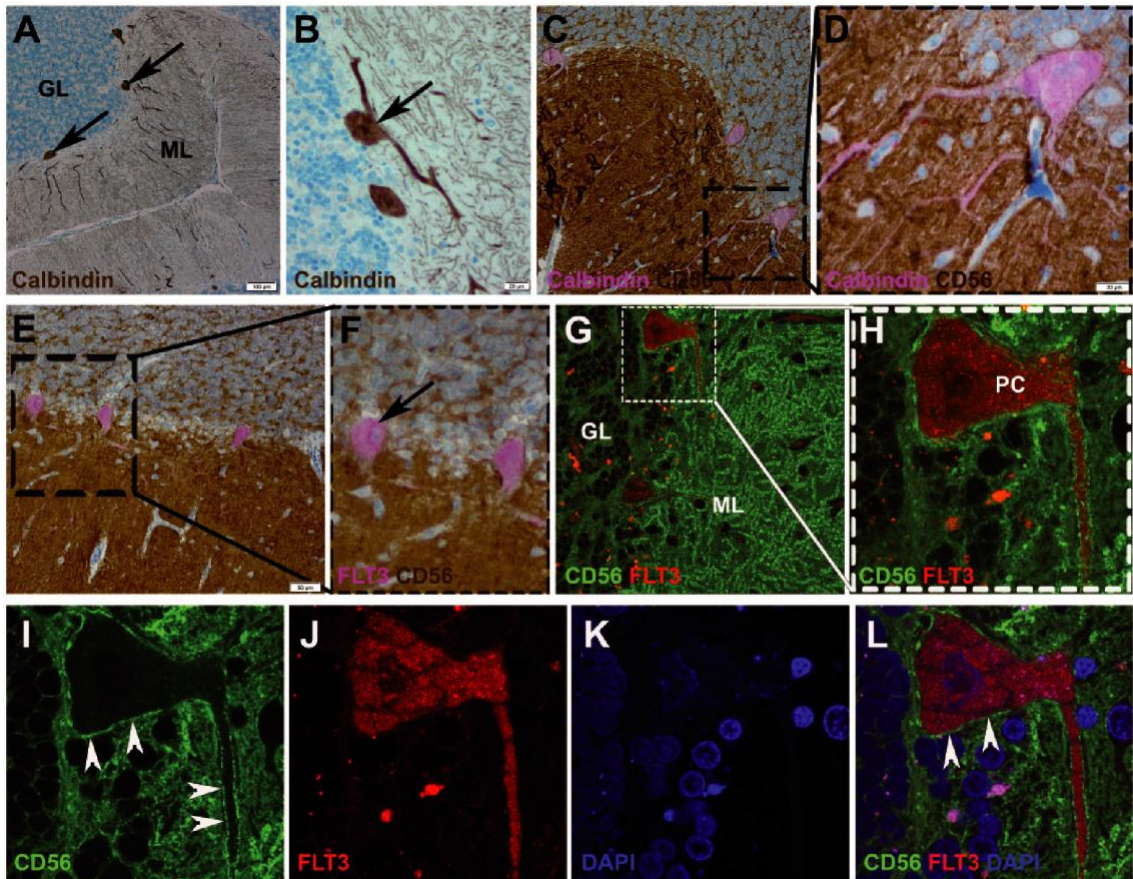


Figure 11 Immunohistochemical (IHC) (A-F) and immunofluorescence (IF) (G-H) stainings of human cerebellar sections. Purkinje cells, that were stained against Calbindin, lay in between the granular layer (GL) and molecular layer (ML) (A-B, arrows). Double IHC staining shows strong calbindin expression in the cytoplasm (C,D), membrane bound expression of CD56 (NCAM) (C-F) and intracellular expression of FLT3 (E-F, arrow). Confocal microscopy with double IF staining shows CD56 (G-L, green) is localized on the cell membrane while FLT3 (G-L, red) is present in the cytoplasm of the cell. Co-localisation of CD56 and FLT3 (L, arrows) was not detected indicating FLT3 expression is restricted to intracellular compartment of Purkinje cells

5. Discussion

In this work, TCRs against epitopes of human FLT3 were isolated from a non-tolerant host, characterized and evaluated as potential therapeutic TCRs to be used in the context of an HLA-A2 mismatch allogeneic-HSCT for HLA-A2⁺ patients with relapsed or therapy resistant AML. The ABabDII mouse model was employed to raise high-affinity TCRs as a non-tolerant host to avoid central tolerance. This murine model has helped to generate a number of high-affinity TCR against human self-antigens in the past 10 years as discussed later in this chapter. We can provide definitive evidence that one of the selected epitopes is naturally processed and presented, although the identified TCRs did not have optimal affinity for efficient target cell recognition. Most importantly, and to our surprise, we demonstrated that FLT3 expression is not restricted to the hematopoietic cell compartment; therefore, we did not proceed with the attempt to develop a higher-affinity TCR against this antigen. In our opinion, the expression of FLT3 in regions of the human brain, and particularly in the Purkinje cells of cerebellum preclude the use of a high-affinity TCR because of potential serious on-target toxicity against vital tissues.

5.1. Characterization of FLT3-specific TCRs raised in ABabDII mice

5.1.1. Identified epitopes are immunogenic in ABabDII mice

The ABabDII mouse is a good platform to generate high-affinity TCRs against human self-antigens that are not 100% homologous to the murine counterparts. This mouse model, published in 2010, is knock-out for murine TCR α and β genes, transgenic for the entire human TCR α and β gene loci and expresses a single human MHC-I allele HLA-A2 with a mouse CD8 binding domain (HHDII). These mice can rearrange human TCRs and positively select them on the human HLA-A2 allele generating a diverse T cell repertoire. As a proof of concept, ABabDII mice were immunized with HLA-A2-restricted epitopes derived from eight human TAAs which are Melan-A, tyrosinase, α -fetoprotein, gp100, melanoma-associated antigen-A1 (MAGE-A1), MAGE-A10, cancer-testis antigen NY-ESO-1 and six-transmembrane epithelial antigen of the prostate (STEAP). All mice developed CD8⁺ T cells responses against the epitopes showing the usability of the ABabDII model for the identification of CTLs against human self-peptides.⁷⁹ Furthermore, a clinically relevant, high-affinity TCR targeting MAGE-A1 was identified, which has a

higher avidity compared to TCRs identified from a tolerant human repertoire showing the value of the mouse model to pull therapeutic TCRs.⁸⁰

FLT3 has a mouse homolog, thus, to elicit a response in the ABAbDII mice, we selected epitopes with at least two amino acid differences in the peptide sequence compared to the mouse protein. The 9-mer FLT3₈₃₉ epitope (IMSDSNYVV) has two amino acid differences compared to the mouse homolog. The mouse epitope bears a Leucine instead of the Methionine at the 2nd position (ILSDSSYVV, Figure 1a). The anchor residues at the 2nd and 9th position of a 9-mer epitope facilitate binding to the HLA-A2 molecule, and therefore, has an impact on the peptide-binding affinity.¹¹⁸ There is a higher preference for an L residue at the 2nd position followed by a preference for an M residue.¹¹⁹ Thus, the murine counterpart of the sequence has similar predicted binding affinity as human FLT3₈₃₉. The amino acid change from an N to S at the 6th position should be enough for this epitope to be foreign to the mice repertoire, therefore, it is reasonable to expect an immune response. Indeed, the strong peripheral response observed in mouse 6547 immunized with FLT3₈₃₉ confirms the immunogenicity of this epitope. The other 9-mer epitope selected, FLT3₉₈₆ (GLLSPQAQV), shares 6 amino acids with murine FLT3 (EPPSPQAQV). The ABAbDII model is unlikely to have tolerance to the human epitope because the murine sequence has extreme low predicted binding affinity to HLA-A2 (38887.75 nM) making it unlikely to be presented by thymic cells to trigger any negative selection. We detected IFN- γ production by the peripheral CD8⁺ T cells of mice 6547 and 6782 immunized with either FLT3₈₃₉ or FLT3₉₈₆ peptide, respectively. Although the other two mice did not exhibit peripheral response, there were peptide reactive T cells in the spleen that could be expanded *in vitro* for subsequent TCR identification. These responses indicate that both epitopes were immunogenic in ABAbDII mice and could develop high affinity TCRs against FLT3₈₃₉ and FLT3₉₈₆.

5.1.2. FLT3₉₈₆ is naturally processed and presented by K562 cells

The TCRs that were identified from the ABAbDII mice recognize their respective epitopes when exogenously loaded on T2 cells (Figure 3). To analyze whether these epitopes are naturally processed and presented, we tested the TCRs on K562 cells that express both the FLT3 antigen and the HLA-A2 allele. K562 cells normally lack expression of class I and class II molecules on the surface. Yet, they retain the ability to present antigens to CD8⁺ T cells once transduced with an MHC-I allele because they express β 2m molecule.¹²⁰ This makes K562 cells a good test platform to detect antigen-specific CD8⁺ T cell response restricted to single allele, while avoiding background activation induced by other alleles. Human PBLs transduced with the 6780-GLL TCR recognized K562 cells

expressing HLA-A2 and FLT3 proving that the FLT3₉₈₆ epitope is processed by the cell machinery and presented by HLA-A2. However, we could not detect any IFN- γ secretion from PBLs transduced with 6782-GLL TCR (Figure 4a). This might be due to a low functional avidity of 6782-GLL TCR, as suggested by the observation that it secretes less IFN- γ compared to 6780-GLL in the peptide titration assay (Figure 3a). Yet, although there is no recognition of K562-A2-FLT3 cells by 6546-IMS TCRs, it is not possible to draw definitive conclusions on processing and presentation of the other epitope, FLT3₈₃₉. K562-A2 cells loaded with high concentrations (1 μ M) of FLT3₈₃₉ peptide were also not recognized suggesting that this could be due to a low functional avidity of the TCR rather than to the lack of peptide availability. In other words, 6546-IMS TCR seems to have such a low avidity that it wouldn't recognize the target cells even if the FLT3₈₃₉ peptide would be naturally processed and presented.

5.1.3. FLT3₉₈₆ presentation by the AML cell line THP1 is not sufficient for efficient T cell recognition

Knowing the FLT3₉₈₆ peptide is naturally processed and presented, we tested the FLT3₉₈₆-specific TCRs on three cell lines which express FLT3 endogenously. THP1 is an AML cell line that is often used in studies focusing on FLT3 function in AML and evaluating it as an AML target. It is homozygous for the HLA-A2*01 allele with high level of HLA-A2 expression on the cell surface making it a valuable and efficient model for testing the FLT3-specific TCRs. Unfortunately, we could not detect any IFN- γ release or CD137 upregulation on TCR-transduced CD8⁺ T cells after overnight co-culture with THP1 cells. It could be argued that the lack of T cell response could be due to deficient peptide processing and presentation machinery of this cell line. However, when THP1 cells overexpressing FLT3 were used as target cells, we detected CD137 upregulation on 6780-GLL TCR-transduced T cells which can be interpreted as the processing machinery of the cell line is functional.

One potential explanation for the lack of response to unmodified THP1 cells could be the sub-optimal avidity of both of our FLT3₉₈₆-specific TCRs. First, we need to make clear distinction between the terms TCR affinity and avidity. TCR affinity is defined as the binding strength of one TCR molecule to a pMHC complex. The binding kinetics of TCR-pMHC interaction is measured by surface plasmon resonance (SPR) using soluble TCRs and immobilized pMHC complexes to calculate a K_D value.¹²¹ The K_D values of high-affinity TCRs lay in the range of 1-50 μ M,¹²¹ however, this parameter cannot be used to compare our TCRs with known high-affinity TCRs because such experiments were not performed on our TCRs.

TCR avidity, on the other hand, is assessed by the cytokine release upon stimulation with pMHC complexes and target cell lysis. The peptide concentration required to reach half-maximal IFN- γ release, called EC₅₀, is a good estimate of TCR avidity.¹²² The EC₅₀ of 6780-GLL TCR, which is in the nanomolar range, might be too low for recognizing target cells in physiological conditions. The ABAbDII-derived MAGE-A1 TCR has an EC₅₀ value in the picomolar range.⁸⁰ Likewise, an HLA-B7 restricted MPO1-specific TCR identified from an allogeneic repertoire has 150 pM EC₅₀.¹¹² Both those TCRs that were identified by different approaches efficiently recognize target cells. The lower EC₅₀ value indicates higher avidity meaning TCRs require lower antigen density for target cell recognition.¹²². Activation of 6780-GLL TCR, that has the highest functional avidity, probably requires a higher density of FLT3₉₈₆-HLA-A2 complexes on the surface than the THP1 cells have. In fact, overexpression of FLT3 in THP1 cells resulted in activation of 6780-GLL TCR transduced T cells as detected by CD137 upregulation on 25.33% of the TCR-transduced T cells. Based on these observations, it is likely that the avidities of 6780-GLL and 6782-GLL TCRs are not sufficient to recognize the FLT3₉₈₆-HLA-A2 complex at physiological level on an AML cell line.

5.1.4. FLT3₉₈₆-specific TCR can recognize cells with both wild type FLT3 and FLT3-ITD mutation

SEM and MV4;11 cells express a high level of FLT3. HLA allotypes of SEM and MV4;11 are A01:01, 32:01; B08:01, 15:01; C03:03 07:01 and A68:01, 03:01; B14:02, 18:01; C15:02, 08:02, respectively.¹²³ In other words, neither of the cell lines express HLA-A2 endogenously. Both cell lines were recognized by 6780-GLL TCR after viral delivery of HLA-A2 proving that the level of HLA-A2 and FLT3 expression was sufficient to reach a pMHC density on the surface that is able to interact and trigger TCR-mediated activation. MV4;11 has lower FLT3 on the cell surface compared to SEM, yet induced similar degree of T cell activation (Figure 5b). This cell line is an FLT3-ITD mutant, which interferes with FLT3 glycosylation pattern preventing its transport from the ER to the cell membrane, while SEM and THP1 cells express wild type FLT3. Although it was not experimentally shown in this work, MV4;11 cells still retains high FLT3 level in the cell due to the mutation, explaining the T cell activation. The internal tandem duplication (ITD) in the juxtamembrane domain (JM) domain of FLT3 causes constitutive activation of FLT3. This gain-of-function mutation is responsible for enhanced proliferation and survival of AML blasts, which is observed in 24% of AML cases, classifying them as high-risk AML.¹²⁴ The patients carrying this mutation are not eligible for some of the novel FLT3-specific therapeutics when these require surface FLT3 expression such as in the case of CAR-T

cells. TCRs, on the other hand, do not rely on surface expression of the target antigen, being able to recognize epitopes derived from cellular proteins. Even though 6780-GLL TCR has low avidity, it also recognized MV4;11 cells carrying FLT3-ITD mutation suggesting that FLT3-specific TCRs could be superior to CAR-T cells in eradicating FLT3-ITD mutant AMLs.

5.1.5. K562 cells are a good model for initial screening of TCRs

We detected IFN- γ release only against retrovirally modified K562 cells expressing FLT3 and HLA-A2 molecule. The other target cell lines induced some degree of T cell activation as indicated by phenotype changes, but that was not sufficient to trigger a detectable level of IFN- γ secretion. Although K562 cells that have both the antigen and the restriction element is a good model to screen TCRs initially, it does not properly mimic antigen presentation by AML cells at physiological conditions and therefore does not guarantee that the TCR will be clinically relevant. There could be multiple reasons for this. First, both transgenes were delivered with a γ -retroviral vector. It is very likely that multiple viral integration events happen upon transduction, which insert multiple copies of the transgenes in the K562 genome, and yielding much higher expression levels of the proteins than is normally observed in AML cells. Second, the strong *cis*-regulatory elements of pMP71 vector, that drive the expression of the transgenes, contributes to a high level of protein accumulation in the cell.¹²⁵ Third, K562 cells normally do not express any MHC-I molecule, whereas each nucleated cell in the body bears up to 6 different MHC-I alleles on the cell surface.¹⁴ Different MHC class I alleles compete with each other for expression on the cell surface,¹²⁶ probably through competition for β 2m,¹²⁷ the presence of other alleles may negatively affect the presentation by a particular allele. K562 cells transduced to express HLA-A2 might have only this one allele on the surface at a much higher density as compared to normal cells carrying multiple HLA-alleles. If this is the case, K562-A2 cells most likely present HLA-A2 restricted epitopes much more efficiently as compared to the other cell lines used in this study, which have the normal repertoire of several different HLA molecules. However, to support this, K562 cells carrying multiple alleles must be generated and tested for their differences in peptide presentation capacity on HLA-A2. This feature can be very useful when screening for a particular TCR reactivity, but can lead to wrong conclusions concerning the physiological density of peptide-MHC complexes as compared to normal cells or tumor cells.

5.1.6. CD8-dependence of FLT3₉₈₆-specific TCRs does not correlate with their avidities

CD8 is the co-receptor of MHC class I-restricted TCRs and expressed on cytotoxic T cells. The main function of this co-receptor is to stabilize the TCR-pMHC binding.¹²⁸ By enhancing the duration and stability of TCR-pMHC interaction and recruiting signaling elements such as Lck, CD8 potentiates T cell activation. However, it has been shown that not all class I TCRs are CD8 dependent. TCR affinity to the pMHC plays a crucial role here. Current understanding of TCR-pMHC dynamics postulates that higher affinity TCRs bind to the pMHC stable enough and may not require CD8-mediated stabilization. Therefore, CD8-independent TCRs are expected to have higher affinity and better avidity.¹²⁹ Tetramers are multivalent complexes of biotinylated soluble pMHCs and a streptavidin molecule that has four biotin binding sites.¹³⁰ Those pMHC tetramers can be conjugated to fluorophores, which enables easy detection of TCR-pMHC binding using flow cytometry. Additionally, cells can be stained simultaneously for cellular markers to deduce more information on cell type and functional characteristics of the particular antigen-specific T cell population. Co-staining of transduced PBLs with an anti-CD8 antibody and pMHC tetramer showed 6782-GLL TCR does not require CD8 molecule to bind to the tetramer. Whereas in the case of 6780-GLL and 6546-IMS, none of the CD8⁻ cells were bound to the tetramer. This suggests 6782-GLL is CD8 independent and should have highest affinity and avidity, However, the avidities of the FLT3₉₈₆-specific TCRs do not correlate with the CD8-coreceptor dependence. According to the peptide titration and target cell recognition data, 6780-GLL has the best avidity, yet, it requires CD8. We did not do further experiments to clarify the reason of contradiction with the current knowledge because the study on these TCRs were dropped due to safety profile discussed later.

5.1.7. Poor presentation of FLT3₉₈₆ by leukemic cells is likely due to low pMHC affinity and stability

It is known that TCR affinity to a cognate pMHC complex is an important parameter for efficient target cell recognition and killing. However, this is not the only parameter to consider. First, the affinity of a peptide to a certain MHC-I allele is a very important criterium.¹³¹ Early studies on peptide presentation to T cells have proven that the peptides compete with each other for MHC-I binding and cell surface presentation.^{132,133} It has been experimentally shown that the addition of a peptide with a high binding affinity can block or prevent the presentation of a peptide with lower affinity resulting in loss of recognition.^{132,133} FLT3₉₈₆ has a low predicted affinity to HLA-A2; thus, it is highly possible that it is not very efficiently presented due to competition with higher-binder epitopes

derived from other antigens even though it is processed by the cells. This can explain the lack of THP1 recognition in our experiments. It has been shown that higher gene expression and protein abundance in the cell correlates with enhanced presentation of a particular antigen.^{134,135} In line with this, we found that overexpression of FLT3 on THP1 cells can rescue target cell recognition by TCR-transduced T cells. The higher availability of FLT3 in the cell might likely have increased the density of FLT3₉₈₆-MHC-I ligands on the cell surface leading to target cell recognition despite the fact that the epitope is predicted to be a low-binder. Similarly, K562-A2-FLT3 are efficiently recognized most likely due to supra-physiological HLA-A2 ligand density on surface. Second, the affinity of a peptide to an HLA molecule alone is not sufficient to predict immunogenicity: not all peptides with high binding affinities to MHC-I are equally immunogenic, it is rather the stability of the pMHC complex that correlates better with the magnitude and strength of ICD8⁺-T cell response.¹³⁶ A tool was developed after our study design incorporating the stability of pMHC-I to the prediction of immunogenicity called NetMHCstabpan.¹³⁷ FLT3₉₈₆ was predicted to have low stability with 2.09 hours half-life meeting the minimum requirements to be immunogenic but not sufficient to evoke efficient T cell activation possibly explaining the lack of recognition at physiological levels of gene expression. Knowing that the optimal epitope binding affinity for tumor rejection and long-term control is below 10 nM, whereas intermediate binders (IC₅₀ between 50 and 500 nM) and low binders (IC₅₀ > 500 nM) are associated with relapse, we can conclude that although being processed and presented, FLT3₉₈₆ seems not to be suitable as a rejection epitope for clinical application.^{131,138} Both the low affinity of FLT3₉₈₆ to HLA-A2 molecule and the low predicted stability of FLT3₉₈₆-HLA-A2 complexes indicate that this epitope is not suitable to induce efficient target cell recognition.

Search in the IEDB database for experimentally-identified HLA-A2-restricted FLT3-restricted high-binders reveals a 9-mer (SLFEGIYTI) eluted from the immunopeptidome of BV173 cells by MS while neither of the epitopes targeted in this study were detected.¹³⁹ This peptide is predicted to have 2.93 nM affinity (NetMHC 4.0) to HLA-A2 and high stability with 11.97 h half-life (NetMHCstab) fulfilling the requirements for ideal tumor-rejection epitope. However, its 100% homology to murine FLT3 restrained us from immunizing the ABAbDII mice with it because of the likelihood of central tolerance against it. Allogeneic *in vitro* T cell priming could have been a way to avoid the tolerance against SLFEGIYTI but we did not follow that approach for the reasons discussed in the next section.

5.2. FLT3 expression in human tissues

5.2.1. FLT3 is present in non-hematopoietic tissues

We selected FLT3 as target antigen for the ACT of TCR-engineered T cells for the treatment of hematological malignancies, in particular AML, due to its high expression in hematopoietic progenitor cell and AML blasts. Several groups cloned FLT3 in the early '90s and identified it as a hematopoiesis-specific antigen specifically expressed in early stage CD34⁺-HSCs.^{140–142} In mouse, *flt3* expression was initially detected by northern blot in fetal liver, fetal brain, adult brain, and bone marrow.¹⁴⁰ Western blot analysis of several fetal and adult mouse tissues revealed the presence of the protein in adult mouse brain, cerebellum, placenta, spleen and thymus.¹⁴³ In humans, tissue distribution of *FLT3* was not studied thoroughly. It was detected in fetal liver, spleen and thymus by PCR. In adult tissues, the expression was predominantly in lympho-hematopoietic compartment such as bone marrow, spleen, monocytes, and granulocytes. The transcript was detected placenta, however, it was the only non-hematopoietic adult tissue that was analyzed.¹⁴¹ Accordingly, FLT3 was long considered to be a lineage specific marker of hematopoiesis.

Early studies on human samples indicated it could be safe to target FLT3 with a high-affinity T cell receptor due to its tissue-restricted expression. However, in the '90s, the technology to detect gene expression was limited with low sensitivity and the variety of the human tissues analyzed was not high. Therefore, to ensure the safety of targeting FLT3, in parallel to our effort to generate high-affinity TCRs, and in view of the unexpected toxicity of some TCRs thought to be tumor specific in clinical studies,^{70,71,144} we initiated an extensive analysis of *FLT3* expression in human tissues. We employed a cDNA array representing 48 major healthy human tissues. As expected, *FLT3* transcript was detected from the bone marrow, lymph node, lymphocytes, spleen, thymus and tonsil, in line with a distribution of the antigen within the hematopoietic and lymphoid compartment. We detected faint *FLT3* expression in some non-hematopoietic tissues which are the left ventricle of the heart, lung, prostate, skin and pancreas. We speculated the source of the transcript in some probes, such as the left ventricle of the heart could be due to contaminating blood cells as the tissues used to prepare the cDNA array was reported not to be washed out by NaCl perfusion by the manufacturer. However, in one study FLT3 was detected in adult mouse cardiomyocytes both at transcript level and on the surface by FACS.¹¹⁷ This led us to investigate *FLT3* expression in iPSC-derived human cardiomyocytes. Unlike Pfister *et al.*, we detected very low level of the transcript, which is consistent with the results of the cDNA array; but could not find protein expression (Figure

9). The expression in the lung and the pancreas can be attributed to the tissue resident DCs in those tissues.

The tissue array that was initially evaluated included two neuronal tissues; brain and spinal cord. We observed faint *FLT3* amplicon from both of those tissues which prompted us to investigate whether other neuronal tissues express *FLT3*. For this purpose, we amplified *FLT3* from a cDNA tissue array representing 24 different anatomical brain regions. We observed *FLT3* expression in different sections ranging from very faint in the Substantia nigra to very high in cerebellar sections. Our results were supported by the GTEx and FANTOM5 RNA sequencing datasets that pinpointed presence of significant *FLT3* mRNA in human cerebellum.¹⁴⁵ All these observations are consistent with an older report from De Lapeyriere et al.,¹⁴³ showing the presence of *Flt3* expression in adult mouse hematopoietic and nervous tissues. These authors had detected faint *Flt3* expression by *in situ* hybridization in different brain sections of adult mice but intense expression in cerebellar tissue. They reported FLT3 presence in the form of a 145 and a 160 kDa protein band in mouse lymphoid tissues, while only a 145 kDa protein band was found in brain and cerebellar tissues.¹⁴³ The authors argued that the function of FLT3 might be different in nervous tissues as compared to hematopoiesis but did not provide evidence for this.¹⁴⁶ Later, another study showed presence of *Flt3* in mouse neuronal progenitor cells, confirming the expression in some differentiated neurons in mouse brain again reaching the highest level in cerebellar Purkinje cells. The authors also showed that the mouse neurons respond to FLT3L by stopping cell division. This finding is intriguing since this behavior is completely different from the response of hematopoietic cells where FLT3L binding mediates activation and proliferation.¹⁴⁷

To investigate whether *FLT3* transcript is translated in human neuronal tissues, we set out to obtain cerebellar samples from post-mortem individuals with healthy cerebellum. We started by detecting *FLT3* amplicon from the RNA isolated from the cerebellar vermis and hemispheres to confirm the observation from the tissue array. Western blot analysis of protein lysates from the same tissue sections indicated the presence of a 145 kDa band from both regions of the cerebellum confirming the presence of the protein in this tissue. FLT3 has a molecular mass of 145 kDa in its immature form and is found intracellularly associated with the endoplasmic reticulum (ER). During maturation, it is glycosylated with N-linked carbohydrates in the Golgi apparatus. This mature form with approximately 160 kDa mass is transported to the plasma membrane and corresponds to the functional FLT3 variant.¹⁴⁸ The size of the band detected in cerebellar sections suggests that FLT3 might be present intracellularly in the cerebellar neurons. Since we did not detect the higher

molecular weight band, it could be speculated that the glycosylated, membrane associated form is not synthesized in the cerebellum.

5.2.2. Purkinje cells in human cerebellum can be a site for on-target toxicity

As a further step, and because of the important clinical implications, we examined which cell type expresses FLT3 in the human cerebellum and whether cerebellar FLT3 is present on the cell surface or possibly only intracellularly as might be speculated on the basis of western blots. IHC stainings of sections revealed that Purkinje cells in human cerebellum express FLT3 intracellularly. The cell type that expresses FLT3 overlaps with the earlier mouse studies. Nevertheless, subcellular localisation of FLT3 in Purkinje cells was not reported before and is shown for the first time in this study. Purkinje cells are the largest neurons in the human brain and belong to a class of GABAergic neurons located in the cerebellum. They integrate afferent signals mainly from the brainstem and send their axons down to the deep cerebellar nuclei.¹⁴⁹ On-target recognition of Purkinje cells by TCR-transduced T cells with consequent cellular damage may induce serious complications since Purkinje cell degeneration causes coordination deficits, such as limb ataxia, dysarthria, and oculomotor disturbances, as seen in some paraneoplastic syndromes, where autoantibodies to Purkinje cells have been demonstrated.¹⁵⁰

TCR-modified T cells recognize epitopes derived from cellular proteins presented on MHC-I molecules independent of the cellular localisation of the antigen. Having shown that Purkinje cells have intracellular FLT3, peptides derived from proteasomal digestion of the protein may be presented on MHC-I molecules of these cells to the T cells. TCRs are very potent and have been shown to recognize target cells bearing as low as 10 pMHC complexes on the surface.^{151,152} The human brain is considered to be an immune privileged site, there are some reports on the absence of MHC class I molecules in adult human brain.¹⁵³ However, a study from 2014 showed expression of β 2m in neurons in the Substantia nigra and the locus coeruleus. They detected mRNA of MHC class I molecules as well as MHC-derived peptides by mass spectrometry. Furthermore, it was shown that IFN- γ treatment induces MHC-I expression on human embryonic stem cell (hES)-derived dopaminergic neurons *in vitro*.¹⁵⁴ Even if there is contradictory evidence, it is possible that MHC-I expression, epitope presentation and T cell activation may happen simultaneously in human brain leading to CTL activity against Purkinje cells. In the light of these findings, and considering the neurological toxicity that was observed in the MAGE-A3 trial,⁷⁰ we concluded that the risk of using an FLT3-specific TCR is unacceptable in our opinion.

Therefore, we stopped our efforts to generate new TCRs with improved affinity and better recognition of FLT3: it is most likely that such reagents would never come to clinical use.

5.3. Evaluating FLT3 as a target for AML-specific TAA

Since the success of CAR-T cells targeting CD19 in the clinic, there has been growing interest in developing cellular therapeutics targeting AML antigens such as CD33 and CD123. Because of its high and uniform surface expression on the majority of AMLs,¹⁵⁵ FLT3 is a very attractive target for CAR-T cells. The first report on FLT3-specific CAR-T cell was published in 2016 shortly after our work had been initiated, showing that FLT3-CARs recognize AML-derived cell lines. Indeed, it was reassuring for us, that other groups were targeting the same antigen, although with a different strategy. The authors observed low-level toxicity to CD34⁺ HSCs as decreased monocyte counts in a NSGS mice model engrafted with CD34⁺ human cord blood cells.¹⁵⁶ Conversely, in 2017, Chen and coworkers reported a another FLT3-CAR with promising activity against leukemic cells and no apparent toxicity on HSCs in the same mouse model engrafted with CD34⁺ human cord blood cells.¹⁵⁷ In 2018, two more studies were published. One group followed a different approach and developed an FLT3-Ligand-based CAR. They showed their CAR-T cells recognize target cell lines with both wild type FLT3 and FLT3-ITD mutation and did not observe any reduction in the colony formation of CD34⁺ human cord blood cells upon co-culture with FLT3L-CAR T cells stating the CAR does not have toxicity against HSCs.¹⁵⁸ Last but not least, Jetani and colleagues developed another FLT3-CAR derived from an FLT3-specific antibody clone 4G8SDIEM with detectable HSC toxicity.¹⁵⁹ They showed that in a FACS based-cytotoxicity assay FLT3-CAR T cells lyse 50% of GM-CSF mobilized human CD34⁺ HSCs in 4 hours. Furthermore, they observed depletion of HSCs in a human HSCs-grafted NSG-3GS mouse model after treatment with FLT3-CAR.¹⁵⁹ Nevertheless, this antibody clone was previously evaluated both in vitro on human HSCs and on human subjects without evidence for toxicity related to recognition by the antibody.^{160,161} The same antibody clone was used to develop an FLT3xCD3 bi-specific antibody that did not have any toxicity to HSCs in an colony forming assay of HSCs obtained from three healthy donors, which was the only cell type that was tested against.¹⁶²

Case reports on application of TCR- or CAR-T cells showed the risk and seriousness of TCR- and CAR-T cell related on-target toxicities ranging from mild to fatal side effects.^{69,71,144,163–165} Despite these examples, all of the studies mentioned above focused on potential on-target toxicity on HSCs based on the current knowledge on FLT3 expression in human tissues. According to data published, none of those groups investigated FLT3 distribution on other tissues, nor did they test the antibody or CAR-T

cells for reactivity against other tissues. We detected FLT3 expression in iPSC-derived cardiomyocytes. Although the level was very low and not detectable on cell surface by FACS, testing CAR-T cells first *in vitro* on suitable models would be strongly recommended to prevent any serious complications in human subjects.

All these studies are consistent with advantages and drawbacks of antibody-based therapies such as CAR T cells compared to TCR based therapies: antibodies recognizing different epitope on a cell surface protein might have completely different biological effects, and even antibodies recognizing the same epitope might have very different effects, based on the affinity of the antibody. Paradoxically, while the most potent reagent is considered to be the best for immunotherapeutic approaches, a weaker antibody might be clinically better suitable, when the antigen is present on normal cells at a weaker extent. Possibly, such antibody would not exhibit toxicity that might instead occur when the antibody has strong activity potentially recognizing and killing even cells with a weak antigen expression. So, a weaker antibody that kills tumor might be harmless to normal tissue, while a potent, more effective antibody may induce severe toxicity to healthy tissue. In other words, it is possible to “tune” the activity of an antibody (and of all antibody-derived tools such as bispecific antibodies and CAR T cells), while this most likely might be very difficult when using TCR based strategies. Although differences in the TCR affinity might have different clinical activities, this might be much more difficult to modulate, considering that T cell activation *in vivo* and cytokine release by the microenvironment might be able to influence the level and abundance of MHC-I molecules, a critical parameter for T-cell recognition, as our own study here indicated.

While the presence of FLT3 in Purkinje cells might cause on-target toxicity when it is targeted with a TCR, in the case of FLT3-specific CAR-T cells, the cerebellar on-target toxicity may be of less concern because of the localization of FLT3 in Purkinje cells, which appear to be mainly cytoplasmic and therefore could not be available for antibody recognition. Nevertheless, in our opinion it would be strong recommended to test the CAR-T cells on neural tissues such as the cerebellum, pons and spinal cord to ensure the safety of the FLT3-specific CAR-T cells before moving to a clinical study in human subjects.

5.4. Prospects on targeting AML with TCRs

We identified three TCRs targeting two different HLA-A2-restricted epitopes derived from human FLT3. None of those TCRs had enough functional avidity to be used in the clinic leaving space for further efforts to identify better epitopes and higher-affinity TCRs. However, we restrained from further efforts as we detected FLT3-transcript in non-

hematopoietic tissues, particularly brain. It was shown that neuronal stem cells (NSCs) can differentiate into different types of blood cells when grafted to the bone marrow of irradiated Balb/C mice bringing the attention the high degree of similarity between these distinct tissues.¹⁶⁶ In other words, identification of safe, hematopoiesis-restricted TAAs could be rather challenging.

As we have learned from this study, all kind of tissues have to be examined and tested extensively before and during antigen selection and TCR identification. As an alternative to the search for AML-specific TAAs, more focus can be put on identification of truly cancer specific, shared TSAs. Two separate groups studied the mutant immunopeptidome of AML to identify targetable neoantigens. Collectively, they have identified two HLA-A2-restricted (CLAVEEVSL and a cysteinylated version C*LAVEEVSL) and an HLA-A*3:01-restricted (AVEEVSLRK) neoepitopes derived from Nucleophosmin 1 (NPM1) mutation, which is found in 27-35% of adult AML.^{167,168} Van der Lee et al. have detected CD8⁺ T cell responses against HLA-A2-restricted neoepitopes from healthy donors. They demonstrated T cells engineered to express a C*LAVEEVSL-specific TCR can lyse NPM1-mutant AML cells while sparing NPM1-WT AML *in vitro* and control leukemia *in vivo* xenograft model.¹⁶⁷ Similarly, in our group we identified HLA-B7-restricted TCRs against MyD88 L265P-derived neoantigens from the periphery of healthy individuals. MyD88 L265P is a shared mutation found in 20% of all lymphoid malignancies and can be used to target diffused B cell lymphomas (DLBCL), primary CNS lymphomas and Waldenström's macroglobulinemia as well as some cases of chronic lymphocytic leukemia that also present this mutation. The TCRs were shown to recognize mutant MyD88 DLBCL cell lines *in vitro* and the preclinical characterization is still ongoing.¹⁶⁹ These studies are encouraging examples for the search of targetable shared or private AML-specific neoantigens.

The discoveries accumulating since decades in the field of cancer immunology and T cell biology have deepened our understanding of T cell response against tumors. In the future, safer AML-specific TAAs and TSAs could be identified with the help of the advancements in the tools that are used to explore immunopeptidome, such as mass spectroscopy and prediction algorithms, as well as through the improved methods to raise potent T cell responses. Finally, it is conceivable that combining the two approaches of immunotherapy, CAR T cells and TCR based adoptive T-cell therapy might compensate the limitation of both approaches which have been shortly discussed here, including surface modulation of the CAR target and MHC downregulation of the TCR-epitope. Simultaneous or sequential administration of these different cellular products, CAR and

TCR- gene modified T cells eventually in conjunction with small molecule selectively inhibiting oncogenic pathways, might be able to finally eliminate any residual leukemic cells, possibly eradicating the disease and making chemotherapy obsolete.

6. Summary

Acute myeloid leukemia (AML) is a disease with poor prognosis. Fms-like tyrosine kinase 3 (FLT3) is a promising target because of its overexpression in AML cells. Efforts have been put to develop new therapeutics targeting FLT3 by small molecule inhibitors and most recently with chimeric antigen receptor (CAR) modified T cells. We generated HLA-A2-restricted, FLT3-specific T cell receptors (TCR) to target FLT3-positive AML and hematopoietic stem cells (HSCs) in an HLA-A2-mismatched allogeneic-HSC transplantation. In our proposed set up, FLT3-specific TCRs would eliminate AML cells as well as HLA-A2-positive HSCs of the patient allowing engraftment of a healthy, HLA-A2-negative hematopoietic system. FLT3 is a self-antigen, therefore, T cells bearing high-affinity TCRs against epitopes derived from it are deleted in the thymus during T cell development. To circumvent the tolerance, we immunized a transgenic mouse model expressing a diverse human TCR repertoire and HLA-A2 molecule (ABAbDII). The candidate epitopes for immunizations, FLT3₈₃₉ and FLT3₉₈₆, were selected among *in silico* predicted epitopes based on their binding affinity to HLA-A2 and homology to the mouse FLT3. We identified one TCR against FLT3₈₃₉ (6546-IMS) and two TCRs against FLT3₉₈₆ (6780-GLL and 6782-GLL). IFN- γ release was detected only from 6782-GLL T cells after overnight co-culture with a K562 cell line that was modified to express high levels of FLT3 and HLA-A2 proving FLT3₉₈₆ epitope is naturally processed and presented. We tested the FLT3₉₈₆-specific TCRs on three different cell lines that express FLT3 endogenously. We did not detect any CD137 upregulation by FACS or IFN- γ release by ELISA from neither of the FLT3₉₈₆-specific TCRs against an AML cell line THP1. On the other hand, co-culture with SEM and MV-4;11 cell lines that express FLT3 endogenously and were modified to express HLA-A2 molecule, and with THP1 cells modified to overexpress FLT3 induced CD137 upregulation only on 6780-GLL T cells, but did not trigger any IFN- γ secretion suggesting higher FLT3 availability might be required for target cell recognition by the 6780-GLL TCR. This could be due to i) the sub-optimal avidities of the identified TCRs to the pMHC complex ii) low binding affinity of FLT3₉₈₆ epitope to HLA-A2 molecule resulting in a poor presentation on the cell surface. In addition, recognition of MV-4;11 cells which carry the FLT3-ITD mutation suggested FLT3₉₈₆ epitope is produced from both the wild type and mutated FLT3. During the *in vitro* safety testing, we discovered high, intracellular FLT3 expression in the Purkinje cells of the human cerebellum. We have stopped our attempt to identify high-affinity FLT3-specific TCRs due to potential cerebellar toxicity. We believe FLT3 could still be a safe, valuable target for therapies other than TCR-modified T cells.

7. Zusammenfassung

Die akute myeloische Leukämie (AML) ist eine Krankheit mit schlechter Prognose. Die Fsm-ähnliche Tyrosinkinase 3 (FLT3) ist wegen ihrer Überexpression in AML-Zellen ein vielversprechendes Ziel. Es wurden Anstrengungen unternommen, neue Therapeutika zu entwickeln, die auf FLT3 durch niedermolekulare Inhibitoren und in jüngster Zeit auch durch T-Zellen modifiziert mit dem chimären Antigenrezeptor (CAR) abzielen. Wir generierten HLA-A2-restringierte, FLT3-spezifische T-Zell-Rezeptoren (TCR), um FLT3-positive AML und hämatopoetische Stammzellen (HSZ) in einer HLA-A2-inkompatiblen allogenen HSZ-Transplantation gezielt zu behandeln. In der von uns vorgeschlagenen Konstellation würden FLT3-spezifische TCRs sowohl AML-Zellen als auch HLA-A2-positive HSZ des Patienten eliminieren, so dass ein gesundes, HLA-A2-negatives hämatopoetisches System transplantiert werden könnte. FLT3 ist ein Selbst-Antigen, daher werden T-Zellen mit hochaffinen TCRs spezifisch für Epitope von FLT3, während der T-Zellenentwicklung im Thymus deletiert. Um die Toleranz zu umgehen, haben wir ein transgenes Mausmodell immunisiert, das ein breites humanes TCR-Repertoire und das HLA-A2-Molekül (ABAbDII) exprimiert. Die Epitop-Kandidaten für Immunisierungen, FLT3₈₃₉ und FLT3₉₈₆, wurden aufgrund ihrer Bindungsaffinität zu HLA-A2 und ihrer Homologie zu FLT3 der Maus unter den in silico vorhergesagten Epitopen ausgewählt. Wir identifizierten einen TCR gegen FLT3₈₃₉ (6546-IMS) und zwei TCRs gegen FLT3₉₈₆ (6780-GLL und 6782-GLL). Die IFN- γ -Freisetzung wurde nur von 6782-GLL T-Zellen nach einer Co-Kultur über Nacht mit einer K562-Zelllinie nachgewiesen, die modifiziert wurde, um hohe Konzentrationen von FLT3 und HLA-A2 zu exprimieren, was beweist, dass das FLT3₉₈₆-Epitop natürlich prozessiert und präsentiert wird. Wir testeten die FLT3₉₈₆-spezifischen TCRs an drei verschiedenen Zelllinien, die FLT3 endogen exprimieren. Bei keinem der beiden FLT3₉₈₆-spezifischen TCRs konnten wir eine CD137-Hochregulierung durch FACS oder IFN γ -Freisetzung durch ELISA gegen die AML-Zelllinie THP1 nachweisen. Andererseits induzierte eine Co-Kultur mit SEM und MV-4;11 Zelllinien, die FLT3 endogen exprimieren und so modifiziert wurden, dass sie das HLA-A2-Molekül exprimieren, und mit THP1-Zellen, die so modifiziert wurden, dass sie FLT3 überexprimieren, nur auf 6780-GLL T-Zellen eine CD137-Hochregulierung, löste aber keine IFN- γ -Sekretion aus, was darauf hindeutet, dass eine höhere FLT3-Verfügbarkeit für die Erkennung der Zielzellen durch den 6780-GLL-TCR erforderlich sein könnte. Dies könnte auf i) die suboptimale Affinität der identifizierten TCRs zum pMHC-Komplex und ii) die geringe Bindungsaffinität des FLT3₉₈₆-Epitops zum HLA-A2-Molekül, was zu einer schlechten Präsentation auf der Zelloberfläche führt, zurückzuführen sein. Darüber hinaus

wird bei der Erkennung von MV-4;11 Zellen, die die FLT3-ITD-Mutation tragen, eine Rolle spielen, dass das FLT3986-Epitop sowohl vom Wildtyp als auch vom mutierten FLT3 produziert wird. Während der In-vitro-Sicherheitstests entdeckten wir eine hohe, intrazelluläre FLT3-Expression in den Purkinje-Zellen des menschlichen Kleinhirns. Wir haben unseren Versuch, hochaffine FLT3-spezifische TCRs zu identifizieren, wegen möglicher zerebellärer Toxizität eingestellt. Wir glauben, dass FLT3 immer noch ein sicheres, wertvolles Ziel für andere Therapien als TCR-modifizierte T-Zellen sein könnte.

8. Abbreviations

| | |
|------------|---|
| ACT | Adoptive cell therapy |
| AML | Acute myeloid leukemia |
| APC | Antigen presenting cell |
| β 2m | beta-microglobulin |
| CAR | Chimeric antigen receptor |
| CLP | Common lymphoid progenitor |
| CML | Chronic myeloid leukemia |
| CR | Complete remission |
| DC | Dendritic cell |
| DLBCL | Diffused large B cell lymphoma |
| DN | Double negative |
| DP | Double positive |
| FLT3 | Fsm-like tyrosine kinase 3 |
| FLT3-ITD | FLT3 internal tandem duplication |
| FLT3L | FLT3 ligand |
| GL | Granular layer |
| GvHD | Graft-versus-host disease |
| GvL | Graft-versus-leukemia |
| HLA | Human leukocyte antigen |
| HSC | Hematopoietic stem cell |
| HSCT | Hematopoietic stem cell transplantation |
| IF | Immunoflouescence |
| IHC | Immunohistochemistry |
| iPSC | induced pluripotent stem cells |
| ITAM | Immunoreceptor-based tyrosine activation motifs |
| MCA | Methylcholanthren |
| MHC | Major histocompatibility complex |
| ML | Molecular layer |
| MLL | Mixed lineage leukemia |
| MPP | Multipotent progenitor |
| PAMP | Pathogen-associated molecular patterns |
| PC | Purkinje cell |
| pMHC | peptide-MHC |
| RACE | Rapid amplification of cDNA ends |

ABBREVIATIONS

| | |
|------|--|
| scFv | Single chain variable fragment |
| SPR | Surface Plasmon resonance |
| TAA | Tumor-associated antigen |
| TAP | Transporter associated with antigen presentation |
| TCR | T cell receptor |
| TIL | Tumor infiltrating lymphocyte |
| TSA | Tumor-specific antigen |
| WT1 | Willm's tumor 1 |

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1. Çakmak-Görür, N. *et al.* Intracellular expression of FLT3 in Purkinje cells: implications for adoptive T-cell therapies. *Leukemia* **33**, 1039–1043 (2019).

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