Generation of TGFβR2(-1) neoantigen-specific human T cell receptors from transgenic mice

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TABLE OF CONTENTS

TO MY MOM

ZUSAMMENFASSUNG

Die adoptive T-Zelltherapie (ATT) ist im Kampf gegen Krebs breit einsetzbar geworden. Bei dieser Therapie wird durch die Expression von T-Zell-Rezeptoren (TCRs) in T-Zellen von Patienten eine neue Antigenspezifität übertragen. Sobald die T-Zellen auf diese Weise "bewaffnet" sind, werden die T-Zellen zurück in den Blutkreislauf des Patienten transferiert, wo sie ihr Ziel auf Tumorzellen finden und den Tumor zerstören. Die kritischsten Punkte sind die Wahl des Zielantigens und die Frage, ob das Antigen prozessiert wird und mit hoher Affinität an Haupthistokompatibilitätskomplex-Molekülen (MHC) bindet. In dieser Arbeit wurden zwei TCRs generiert, die eine Mutation des Transforming Growth Factor β Rezeptor 2 (TGFβR2(-1)) erkennen. Für die Generierung wurden humanisierte ABabDR4 Mäuse verwendet, die ein diverses menschliches TCR-Repertoire exprimieren, das auf HLA-DRA/DRB1*0401 (HLA-DR4) restringiert ist. Das TGFβR2(-1)-Neoantigen ist ein Frameshift-Peptid, das durch eine Verschiebung des Leserasters aufgrund einer Deletion eines Adenins (-1) innerhalb einer Mikrosatellitensequenz verursacht wird. Diese somatische Mutation wird häufig bei Mikrosatelliten-instabilen Kolorektalkarzinomen (MSI-CRC) und anderen Mikrosatelliten-instabilen Krebsarten gefunden, jedoch nicht in gesundem Gewebe. Daher handelt es sich um ein tumorspezifisches Antigen (Neoantigen), das darüber hinaus in Tumoren unterschiedlicher Patienten vorkommt. Wir haben gezeigt, dass in ABabDR4 Mäusen generierte TGFβR2(-1)-spezifische TCRs, die in humanen CD4+ T-Zellen exprimiert wurden, das Neoantigen TGFβR2(-1) erkennen, das endogen prozessiert und auf bestimmten MHC-II-Molekülen, HLA-DR4, präsentiert wird. Weiterhin haben wir gezeigt, dass beide TGFβR2(-1)-reaktiven TCRs (TCR1414_1 und TCR1414_2) die CRC-Zelllinie SW48 und HCT116, die die TGFβR2(-1)-Mutation natürlich exprimieren, erkennen können. Die hier vorgestellten Daten legen nahe, dass HLA-DR4-restringierte TCRs gegen das TGFβR2(-1)-Neoantigen in der adoptiven T-Zelltherapie einer beträchtlichen Anzahl von Krebspatienten eingesetzt werden könnten.

SUMMARY

Adoptive T cell therapy (ATT) has recently become broadly applicable in the fight against cancer. This therapy involves grafting of a new antigen specificity through T cell receptors (TCRs) transfer into patients' T cells. Once 'armed' in this way, the T cells are transferred back into the patient's bloodstream where they find and destroy their target. The most critical points are the choice of the target antigen and whether this antigen is processed and presented on major histocompatibility complex molecules (MHC) with high affinity. Here, we generated two TCRs against Transforming Growth Factor β Receptor 2 mutation (TGFβR2(-1)) using humanized mice ABabDR4 that express a diverse human TCR repertoire restricted to HLA-DRA/DRB1*0401(HLA-DR4). The TGFβR2(-1) neoantigen derives from a frameshift peptide caused by a shift of the open reading frame due to the deletion of one adenine (-1) within a microsatellite sequence. This somatic mutation is frequently expressed in microsatellite instable colorectal cancers (MSI-CRC) and other gastric cancer types but has not been found in healthy tissues, therefore it is a truly tumor-specific antigen (neoantigen), shared between different tumor types. We showed that ABabDR4-derived TGFβR2(-1)-specific TCRs transferred onto human $CD4^+$ T cells recognize the neoantigen TGFβR2(-1) endogenously processed and presented on a particular MHC II molecule, HLA-DR4. We also demonstrated that both TGFβR2(-1)-reactive TCRs (TCR1414_1 and TCR1414_2) can recognize CRC cell line SW48 and HCT116 naturally expressing the TGFβR2(-1) mutation. The data presented herein suggest that HLA-DR4-restricted TCRs against the TGFβR2(-1) recurrent neoantigen can be valuable candidates for adoptive T cell therapy of a sizeable number of cancer patients.

1. INTRODUCTION

1.1 T cell role in immune system

The immune system of vertebrates is a complexed network of various effector cells and proteins taking part in the defence against infectious agents and their toxins. The immunological system is divided into innate and adaptive immune system. The innate system is the pre-existing, first line defence that responds unspecifically to infections. Cells involved in innate mechanisms are macrophages, dendritic cells, natural killers, neutrophils, basophils, eosinophils and mast cells that in a non-selective nor pre-educated fashion eliminate pathogen-dependent infections. By producing cytokines, they interact with each other as well as with other cells to activate further the adaptive immune system. The adaptive immune system is an antigen-specific and highly selective response in which the antigen-specific lymphocytes, T cells and B cells, play the main role. A unique feature of the adaptive immune system is its immunological memory that allows to keep a record of every pathogen that has ever been defeated. Once encountered again, the pathogen will be recognized and eliminated, resulting in a protective immunity against it throughout an individual's lifetime (Murphy and Weaver, 2017).

1.1.1 T cell types and function

One of the major type of lymphocytes in the vertebrate's adaptive immunological system are T lymphocytes (T cells) (Miller, 2002). While circulating in the blood upon homeostatic condition, they show little functional activity (naive T cells). However, if the T cell encounters an antigen it becomes activated, proliferates and differentiates further into a fully functional T cell (effector T cell) (van den Broek et al., 2018). Activated effector T cells can be of different subtypes performing different types of activity. One of such function is immune-mediated cell death carried out by cytotoxic $CD8^+$ T cells known as "killer cells".

$CD8^+$ T cells are capable of directly killing virus-infected cells or any other intracellular pathogens that express a particular antigen. Additionally, they can produce cytokines, which can attract other immune cells to the infection area (Murphy and Weaver, 2017). Another important way of controlling and orchestrating the immune response is through helper $CD4^+$ T cell activity. $CD4^+$ T cell "indirectly" participate in eliminating cells recognized as foreign by releasing cytokines. The produced cytokines activate pathogen-killing macrophages or stimulate B cells in producing antibodies, which further "tag" the microbe for attack by another fraction of the immune system (Geginat et al., 2014). Upon antigen binding part of the T cells become regulatory T cells (Tregs), another type of unconventional T cells. Regulatory T cells help to modulate and supress the activity of the immune system, generally by downregulating the proliferation of effector T cells. Tregs also maintain the tolerance to self-antigens and prevent autoimmunity (Hsieh et al., 2012). Some activated T cells will differentiate into another type of T cells, memory T cells that are the hallmark of the adaptive immune system (Laidlaw et al., 2016). Memory T cells allow long-lasting immunity against particular antigens and allow vertebrates to respond faster to potential re-exposure to pathogens.

1.1.2 T cell receptors and their diversity

T cells recognize antigens via the T cell receptors (TCRs) present on their surface. T cells, which stand for ~95% express heterodimeric T cell receptors, consist of transmembrane glycoprotein chain TCRα and TCRβ (Murphy and Weaver, 2017). The extracellular part of each chains consists of two domains, a variable region and a constant region. The combination of the α- and β-chain variable region creates a single antigen-binding site. Both α- and β-chains are attached to each other by cysteine residues that form disulfide bonds at the stalk segment. Each chain spans further the T cell membrane and ends with a short cytoplasmic tail (Garcia et al., 1996; Murphy and Weaver, 2017).

T cell activation is highly specific to very particular antigens and depends strictly on the TCR. Every T cell clone expresses a unique TCRαβ. Highly diverse TCR

repertoire starts with somatic recombination that occurs in maturating lymphocytes in the thymus (Samelson et al., 1985). During T cell development, the stochastic rearrangement of variable (V), diversity (D), and joining (J) gene segments happens for TCRβ, and random rearrangement of V and J gene segments in the TCRα gene loci. This process is supervised by recombination activating gene 1 and 2 (RAG1 and RAG2) proteins. Next to V(D)J gene rearrangement itself, the terminal deoxynucleotidyl transferase (TdT) and exonucleases add or delete nucleotides at the V(D)J junctional regions (Komori et al., 1993). The hypervariable junction region of V-J for TCRα and V-D-J for TCRβ is called the complementarity determining region 3 (CDR3) and forms a single antigen binding site mediating recognition (Davis and Bjorkman, 1988; Jung and Alt, 2004). The CDR1 and CDR2 are found in the variable (V) region of each TCR α and β polypeptide chains.

Highly diverse TCR repertoire is created upon combinatorial and junctional diversity. Together with further random TCR α and β chain pairing the theoretical diversity of the TCRs repertoire expands up to ~5 x 10^{15} TCRαβ. From such enormous number of TCRs, there are ~2 x 10^{7} estimated experimentally TCRs found in human's periphery after thymic selection (Arstila et al., 1999; Nikolich-Žugich et al., 2000).

1.1.3 T cell development and thymic selection

Lymphoid progenitors once generated in the bone marrow migrate to the thymus to undergo T cell differentiation and antigen-depended maturation (Schwarz and Bhandoola, 2006). Once the lymphoid progenitors, which have developed from hematopoietic stem cells, reach the thymus, they lose their potential for B cell (Wilson et al., 2001) and natural killer cell (Michie et al., 2000) development. In the cortex of the thymus, early T cell precursors termed double negative (DN) thymocytes, do not already express CD4 nor CD8 costimulatory receptors. They go through DN1 to DN4 stages of T cell development: DN1 ($CD44^{+}$ $CD25^{-}$), DN2 ($CD44^{+}$ $CD25^{+}$), DN3 ($CD44^{-}$ $CD25^{+}$) and DN4 ($CD44^{-}$ $CD25^{-}$). Next, the T cell precursors start to express both $CD4^{+}$ and $CD8^{+}$ and become double positive (DP). At this stage, the double positive $CD4^{+}$ $CD8^{+}$ cells express already

rearranged TCRs with αβ or yδ chains. Immature T cells move further into the cortico-medullary junction where they will be educated through positive and negative thymic selection. At this stage, the $CD4^+$ $CD8^+$ $TCR\alpha\beta^+$ T cells interact with self-antigen presenting cortical thymic epithelial cells (cTECs) that express different MHC I and MHC II molecules loaded with self-peptide. This process allows to maintain self-tolerance and eliminates T cells that can recognize self-antigens expressed on normal cells, preventing further autoimmunological issues (Starr et al., 2003). The thymocyte fate depends on the strength of its TCR interaction with self-peptide:MHC complexes (von Boehmer et al., 1989; Klein et al., 2014). A great number of 85% double positive T cells do not interact with the self-peptide MHC on cTECs or interact very poorly so they do not sustain the intracellular viability signals and consequently undergo death by neglect (Mcdonald et al., 2015). Positive selection happens when $CD4^+CD8^+$ TCRαβ T cells weakly interact with cTECs and receive a survival signal to mature into $CD4^+$ or $CD8^+$ single positive (SP) T cells (Klein et al., 2014). The DP $CD4^+$ $CD8^+$ TCRαβ T cells that bind to self-peptide:MHC I complex become cytotoxic $CD8^+$ T cells and the ones binding to self-peptide:MHC II complex become $CD4^+$ T cells. Negative selection happens in medulla of the thymus when single positive $CD8^+$ or $CD4^+$ T cells interact too strong with medullary thymic epithelial cells (mTECs) (Mcdonald et al., 2015). Immature $CD8^+$ or $CD4^+$ T cells whose TCR's bind strongly to self-peptides on mTEC undergo apoptosis to prevent autoimmunity once they leave the thymus (Klein et al., 2014). Some of these high affinity $CD4^+$ T cells are not removed by programmed cell death but rather differentiate into Tregs whose role is to maintain self-tolerance and inhibit immune responses (Hsieh et al., 2012). From a large population of T cells only 10^{11} of selected, educated and maturated cells leave the thymus to circulate in peripheral lymphoid sites (Jenkins et al., 2010; Attaf et al., 2015).

1.1.4 MHC molecules

A remarkably diverse TCR repertoire interacts with a variety of antigens that are loaded onto major histocompatibility complex (MHC) molecules. These over

~4 Mb distributed MHC genes are highly polymorphic and are responsible for binding an antigen and presenting it to the T cells (Beck et al., 1999). MHC also plays a crucial role in transplantation outcome and determines the compatibility of donor tissue and recipient (Gorer, 1937). There are two primary classes of MHC gene family: MHC class I and MHC class II.

1.1.4.1 MHC class I molecules

In humans, MHC I molecules are encoded by human leukocyte antigen gene complexes HLA-A, HLA-B, and HLA-C and are expressed in all nucleated cells as well as in platelets. MHC class I molecules consist of two polypeptide chains: α-chain consisting of 3 α-domains (α1, α2 and α3) noncovalently associated with β_2-microglobulin (β2m). Domain α1 and α2 form a peptide-binding groove where a peptide of 8-11 amino acids can be loaded. Domain α3 does not take part in peptide binding but spans the cell membrane (Bjorkman et al., 1987).

The main function of MHC class I molecules is to display intracellular pathogens such as viruses or bacteria to cytotoxic $CD8^+$ T cells, immediately triggering a highly specific immune response. MHC class I molecules present peptides which come from cytosolic or nuclear protein degradation in the proteasome. Later, after being transported by TAP (transporter associated with antigen processing) to the endoplasmatic reticulum (ER), the peptide:MHC (pMHC) complex is presented externally on the cell surface to $CD8^+$ T cells (Neefjes et al., 2011). Encountering pMHC I turns $CD8^+$ T cells into effector T cells. Following clonal expansion, secretion of cytoplasmic granule toxins such as perforin or granzyme leads to apoptosis of the target cells in a direct manner (Adams et al., 2020).

1.1.4.2 MHC class II molecules

In humans, MHC II molecules are encoded by classical HLA class II genes HLA-DR, HLA-DP and HLA-DQ and non-classical HLA II class genes HLA-DO and HLA-DM (Kedzierska & Koutsakos, 2020). They are expressed only on

professional antigen-presenting cells (APCs) like dendritic cells (DCs), B cells, macrophages, monocytes, thymic epithelial cells (TECs) and some endothelial cells (Roche et al., 2015). The MHC class II heterodimer is comprised of one α-chain with two α1 and α2 domains and one β-chain forming two β1 and β2 domains (Brown et al., 1993). The antigen-binding groove is formed by α1 and β1 domains and has an open groove enabling longer peptides to bind to a minimum of 13 amino acids up to 30 amino acids (Attaf et al., 2015). Such peptides derive from extracellular proteins which entered APCs by endocytosis, micropinocytosis or phagocytosis (Roche et al., 2015). Newly formed MHC II binds to an invariant chain (Ii) in the ER. It is first transported to endosomes and then to antigen-processing compartments, where antigenic proteins are present. MHC class II cannot bind to peptides in a form of Ii-MHC II complex. Once the Ii-MHC II complex is proteolytically degraded leaving the remaining CLIP (class II associated invariant chain peptide) on the groove of the MHC II molecule, the enzyme HLA-DM regulated by HLA-DO enables the CLIP removal and further antigen binding to the MHC II molecule. The loaded pMHC II is transported to the cell surface via transport vesicles, where it interacts with $CD4^+$ T cells. Recognition of pMHC class II complexes by $CD4^+$ T cells activate them into different T helper cell phenotypes (Roche et al., 2015).

1.2 Adoptive T Cell Therapy

Adoptive T cell therapy (ATT) utilizes transfer of autologous or allogeneic T cells into the patient's blood stream. They can be used unmodified or engineered by grafting of a new antigen specificity. Once prepared, T cells are transferred back to the individual's bloodstream, where they find their specific target and mediate its elimination, breaking the anti-tumor tolerance.

The role of a T cell in tumor rejection has been discovered in 1953 when Nicholas Mitchison showed that transferred T cells, not antibodies (serum), induced resistance to allogeneic tumor transplant in mice (Mitchison, 1953). Over the next years, many aspects of the adaptive immune system have been studied to explore new anti-cancer treatments.

T cell-based cancer treatments are attractive for many intrinsic reasons. Firstly, T cells are highly specific, enabling a very precise action on cancer cell. With such an approach, it is possible to obtain a low toxicity highly efficient treatment that contrasts with the invasive methods currently in use: chemotherapy, radiotherapy or surgery. Secondly, T cells can be engineered to target many possible antigens of choice expressed by hematological malignacies as well as solid tumors, eliminating them directly or indirectly by bystander killing (Spiotto et al., 2004; Spiotto and Schreiber, 2005). Thirdly, once activated, T cells differentiate and clonally expand up to 1000-fold into effector T cells bearing identical TCR specificity, allowing robust attack of the target and mediating the anti-cancer immune responses.

1.2.1 Transfer of unmodified T cells

Transfer of unmodified T cells exploits autologous T cell or allogeneic T cell populations transfer to patient's bloodstream after *in vitro* expansion. Allogeneic MHC-matched T cell transfer has been demonstrated to be effective when EBV-specific cytotoxic T lymphocytes were infused to prevent or treat Epstein-Barr virus–positive lymphoproliferative disease (EBV-LPD) after hematopoietic stem cell or solid organs transplantation (Heslop et al., 2010). EBV-associated lymphomas that often develop in the immunocompromised host are highly immunogenic, characterized by expression of viral antigens like EBNA3. Transfer of allogenic EBV-reactive T cells prevents possible complication and achieved clinical success in up to 90% of patients (Barker et al., 2010; Heslop et al., 2010).

Another clinical therapeutic approach of unmodified T cells is the autologous T cell transfer isolated from patient's peripheral blood or tumour biopsy tissue. Such autologous T cells are selected for their specificity and reactivity required to mediate cancer regression, then expanded *ex vivo* in large number (up to 10^{11}) to be later returned into the patient. TILs (tumor infiltrating lymphocytes) obtained from the tumor site were used for the first time for ATT by Steven Rosenberg and co-workers at the NIH (National Institute of Health) in mice for liver and lung cancer treatment accompanied by IL-2 infusion (Rosenberg et al., 1986) and later

in metastatic melanoma patients (Rosenberg et al., 1988). TILs showed high clinical response rate for melanoma patients but, unfortunately, it has been proven that it can be difficult to expand TILs in sufficient number significantly limiting application of this solution (Rosenberg and Restifo, 2015). The issue can stem from the choice of the right T cell subset if TILs are expanded in antigen-specific manner, a low number of autologous T cells due to immune tolerance or simply insufficiency of material availability. To avoid aforementioned limitations, allogeneic T cells could serve as a solution but only when donor and recipient are MHC-matched.

1.2.2 Transfer of gene-modified T cells

Because unmodified T cells show limitations due to their insufficient quantity or functional quality, development of genetically engineered T cells has emerged to broaden the reach of ATT or cancer treatment. The gene modification may engraft new specificity but also influence the T cell phenotype and quality. It has been demonstrated to increase T cell therapeutic efficiency and minimalize their exhaustion by transducing constitutively expressed CD28 genes into exhausted cytotoxic T cells, restoring IL-2 production (Topp et al., 2003). T cell proliferative features and targets avidity could be improved by introducing iRNA that target negative regulator of lymphocyte activation (Stromnes et al., 2010).

A considerable effort has been made to improve T cell specificity by transferring additional, new antigen specificity. The techniques of introducing new anti-tumor specificity onto normal T cells rely on retroviral (Clay et al., 1999), lentiviral (Tsuji et al., 2005) or mRNA transient (Zhao et al., 2006) TCR gene delivery. There are two main ways of implementing new T cell specificity by introducing genes coding conventional TCRs or CARs (chimeric antigen receptors).

So far, the most successful, FDA approved in 2017 gene modified T cells therapy is anti-CD19 CAR-based ATT engineered in 2010 by Rosenberg Lab (Kochenderfer et al., 2010). This treatment is used nowadays in clinical application to treat advanced B cell lymphomas.

1.2.2.1 Transfer of CAR-engineered T cells

Chimeric antigen receptors pioneered by Gross in 1980 gained interest for their advantage over TCR-based technique since they can recognize surface proteins in non-MHC dependent manner (Gross et al., 1989). This impacts the tumor cells recognition since it is not dependent on antigen processing and presentation and can prevent tumor escape by MHC loss (Restifo et al., 1996). CAR-T cells combine both antigen binding and T cell function. CARs consist of an extracellular antigen recognition domain derived from antibody (single-chain variable fragment (scFv)) and the intracellular T cell-derived CD3-zeta domain responsible for activating the T cell (Gross et al., 1989). With further functionality improvement (adding costimulatory domains from receptors such as CD28, OX40, and CD137), the third generation of CAR-T became the most efficient, showing great clinical outcome to be FDA approved in 2017. The CD19-specific CAR-modified human T cells were used for patient with acute B lymphoma (ALL) or diffuse large B-cell lymphoma (DLBCL) and achieved complete remission of malignant cells in patients (Kochenderfer et al., 2010; Kalos et al., 2011; Maude et al., 2014).

Recent studies have also shown that for generation of CAR-T the CRISPR-Cas9 technology can be used to edit germline TCR gene, which could lead to improved gene transfer efficacy (Eyquem et al., 2017).

The great success of anti-CD19 CAR-T relies on high expression of CD19 in B cell malignancies. CD19 is required for normal B cell development and it is not expressed by any other cells in humans. Targeting CD19 antigen with CAR-T cells leads to B cell aplasia. B cells are responsible for the production of the all-important immunoglobulin but elimination of them upon treatment is well managed by supplementation of immunoglobulin intravenously post CD19 CAR-T therapy (van Zelm et al., 2006). Cytokine-release syndrome is a potential complication from CAR-T cell therapy. It manifests as an intensified release of inflammatory cytokines by the activated immune cells, leading to a patient's pathophysiology that can include organ failure (Lee et al., 2014; Grupp et al., 2013; June et al., 2018).

1.2.2.2 Transfer of TCR-engineered T cells

TCR-engineered T cell therapy involves the genetic modification of TCRs specificity engrafted onto normal autologous or allogeneic T cell to target defined tumor-specific or tumor-associated antigens (see chapter 1.3 Tumor antigens). Genetic modifications of TCRs may not only influence their specificity but may also improve their expression if codon optimization of the transferred genes is implemented, outcompeting the endogenous TCR repertoire by higher protein expression level (Scholten et al., 2006). Next, a modification called murinization can be implemented, where constant regions of each human-derived TCRα and TCRβ are exchanged for murine compartments. This is done to improve pairing of engineered TCR α and β chains and to avoid miss-pairing of engineered TCRs with endogenous ones (Sommermeyer et al., 2006). Another way of improving TCR pairing is the usage of additional disulphide bonds (Cohen et al., 2007).

Engineered TCR-based T cell therapy was first used in 2006 when autologous T cells transduced with retroviral vector encoding melanoma-melanocyte antigen (MART-1)-specific TCR proved their anti-tumor functionality in metastatic melanoma patients with a response rate of 2 out of 17 patients (Morgan et al., 2006). Unfortunately, severe on-target toxicity came together with further affinity improvement of therapeutic TCRs and positive clinical outcome, since the differentiation antigen MART-1 was also expressed in melanocytes of the skin, eyes and ears of cancer patients (Johnson et al., 2009). This example shows how careful eventual genetic modification should be considered.

Grafting new antigen specificity by TCR gene transfer onto patient's T cells allows broad application of this technique by targeting potentially any antigen on the tumor cells surface. Despite this advantage, as of today, there is no FDA-approved TCR-based therapy available on the market.

1.3 Tumor antigens

Tumor antigens are any molecules produced by cancer cell that can be detected by T cells or antibody. To effectively prime immune response, such antigens must

bind to specific MHC molecules with high affinity, be naturally processed and presented. Additionally, they need to successfully compete with naturally occurring peptides for the binding place on MHC molecules. Ideally, the antigen should be homogenously expressed in the majority of cancer cells (Schreiber, 2013).

Generally, we can distinguish two main classes of tumor antigens: tumor-specific antigens such as viral antigens or antigens encoded by non-synonymous mutated genes (neoantigens) and second class, tumor-associated antigens (self), which can be further divided into over-expressed, differentiation and cancer-testis antigens (Schreiber, 2013).

1.3.1 Tumor-specific antigens

Neoantigens encoded by somatic mutations can provoke a powerful immune response. In 1957, Prehn and Main discovered an induced immunity, a clear evidence for tumor-specific rejection of transplanted methylocholanthrene (MCA)-induced sarcomas (Prehn and Main, 1957). Later in 1995, it was proved that existence of tumor-specific antigens in autochthonous tumors is caused by somatic non-synonymous cancer-specific mutation (neoantigen) absent in normal cells of the host (Monach et al., 1995). Targeting the somatic mutations expressed only by tumor cells might enable tumor elimination without causing potential damage to vital healthy tissues (Blankenstein et al., 2015). Many of such somatic mutations occur in genes with functional significance (oncogenes, tumor suppressor genes) for tumorigenesis and are crucial for tumor existence (driver mutations) (Schietinger et al., 2008). Unique driver mutations are great targets to eliminate the tumor because they cannot be lost upon tumor immune selection during malignant transformation (Schreiber, 2013). Viral antigens like oncogenic human papillomavirus (HPV) are one example of driver mutation antigens causing cervical or head and neck cancers (Hung et al., 2008). Prophylactic vaccines against this oncogenic virus as well as hepatitis B have been developed to reduce occurrence of hepatocellular carcinoma and cervical cancer (Guo et al., 2013).

Because mutations occur in a random and stochastic way in the human genome, they are often very personalized. Nowadays techniques using whole exome sequencing can identify mutations specific to each patient and enable personalized T cell therapy treatment against pinpointed tumor-specific antigens (Yamamoto et al., 2019; Rosenberg and Restifo, 2015). It is also worth noting that recent studies have demonstrated some neoantigens can be shared between different cancers and even patients. Shared mutations have been found in “hot spot” genes like TP53, KRAS or suppressor gene TGFβR2 (Malekzadeh et al. 2019; Tran et al, 2016; Inderberg et al., 2017).

1.3.2 Tumor-associated antigens

Tumor-associated antigens are self-antigens, coded by non-mutated genes and are over-expressed by cancer cells but are also expressed by at least some healthy cells. They are often less immunogenic than neoantigens due to central and peripheral tolerance mechanisms which, under natural conditions, protect the human body from reacting against such self-antigens, preventing autoimmunity. As a result, autologous T cells against tumor-associated antigens will have low affinity (Schreiber, 2013). To overcome this issue, enhancement of TCR affinity has been introduced, although together with better effectiveness, it can also severely increase toxicity. Targeting tumor-associated antigen CEA (carcinoembryonic antigen) with high-affinity engineered TCRs made metastatic colorectal patients experience inflammatory colitis and colonic haemorrhage, since CEA is expressed in adults by non-malignant tissues present in organs such as colon, lung and breast (Parkhurst et al., 2011).

A better target may be cancer-testis antigens, which are widely expressed on cancer cells and in spermatocytes in the testis. Antigens in testis are not attacked by the immune system since testis are an immunologically privileged site, lacking the expression of MHC molecules. In clinical studies of gene modified $CD8^+$ TCR against HLA-A2-restricted NY-ESO-1 epitope, responses were observed in 61% patients with metastatic melanoma (2 out of 11 of patients demonstrated complete remission persisting after 1 year), 55% of metastatic synovial cell

sarcoma patients and 80% of multiple myeloma patients (Robbins et al., 2011; Rapoport et al., 2015). Unfortunately, some cancer-testis antigens are still expressed at low level on normal tissues which may lead to unwanted toxicity. Clinical trials on targeting the HLA-A2-restricted MAGE-A3 antigen led to deaths from cardiogenic shock due to titin cross-reactivity of therapeutic affinity-enhanced TCRs (Linette et al., 2013).

The fact that cancer-testis antigens are also expressed by mTEC cells in the thymus (Gotter et al., 2004) makes it hard to identify and isolate high affinity TCRs from autologous source. Consequently, antigen-negative humanized mice with a diverse human TCR repertoire restricted to human particular MHC molecules were used in several studies (Ito et al., 1996; Li et al., 2010). Two pre-clinical studies where such humanized mice were immunized with cancer specific antigen NY-ESO-1 or MAGE-A1 and high-affinity TCRs were isolated, showed no off/on-target toxicity and at the same time demonstrated effectiveness in recognizing melanoma cancers in *in vitro/in vivo* setting, respectively (Poncette et al., 2018; Obenaus et al., 2015).

1.3.3 The choice of the antigen for ATT

The biggest challenge in designing ATT is the choice of the target antigen: the ideal discovery is a truly tumor-specific antigen exclusively expressed in cancer tissues that cannot be found in healthy cells. Best if the antigen is foreign to the immune system and triggers a strong immune response, meaning the antigen is highly immunogenic. It is also advantageous if expression and presentation of the antigen is homogenous on cancer/stroma cells, shared between different cancer types, and required for the tumor viability, so it cannot be lost upon immune selection during transformation. Fulfilling all or part of those criteria is extremely difficult, requiring a lot of time and effort to create non-toxic and effective immunotherapy.

1.4 TGFβR2 pathology in cancer

Transforming growth factor β receptor II (TGFβR2) is a serine-threonine protein kinase. This transmembrane receptor binds TGFβ and forms heterodimeric complexes with other receptors and ligand effectively regulating the transcription of many signaling-related genes. TGFβ signaling pathway plays a crucial role in tissue development, cell growth and proliferation, differentiation, apoptosis and homeostasis (Jung et al., 2017). Disruption of those positively or negatively regulated pathways causes many diseases, including cancer. In advanced cancer, a “TGFβ paradox” was observed, highlighting its dual function. Although TGFβ inhibits epithelial cell growth in normal conditions, it promotes tumor cell progression, which further manifests in hallmarks of carcinogenesis like uncontrolled proliferation, epithelial-to-mesenchymal transition, loss of programmed cell death or evasion of the immune system (Principe et al., 2014).

A particular mutation in TGFβR2 caused by a mismatch repair deficiency has been discussed to potentially influence the transformation of normal colonic epithelial cells into malignant ones. Impaired TGFβR2 has been observed to cause lethal inflammatory events like polyps and colon cancer progression in the tumor microenvironment of CRC mediated by TNFα and IL-8 (Principe et al., 2016).

1.4.1 TGFβR2 frameshift neoantigens and TGFβR2(-1) in MSI-CRC

The human gene coding TGFβR2 protein has 10-adenine repeats (microsatellite) from base number 709 to 718, located in exon 3 of the gene (Markowitz et al., 1995). Somatic non-synonymous mutations in this polyadenine sequences have been observed: deletion (TGFβR2(-1)) or addition (TGFβR2(+1)) of one adenine (Saeterdal et al., 2001). Deletion or addition of one nucleotide (-1 or +1) results in a new nucleotide triplet coding new amino acids from base position 128 or 129 and, in the end, a new frameshift variant of the peptide (Figure 1). The TGFβR2(+1) frameshift gives almost an immediate termination of the peptide sequence (the stop codon comes as a fourth amino acid after the +1A mutation). The TGFβR2(-1) mutation results in a 34 amino

acids sequence that serves as a potential source of unique immunogenic neoantigens. TGFβR2(-1) neoantigen is present in ~77% of MSI-CRC patients (Tougeron et al., 2009; Schwitalle et al., 2008; Maby et al., 2015). Such high frequency of this new class, shared neoantigen (public neoantigen), allows to treat more patients with the same immunotherapy, as opposed to an expensive tailored individualized approach.

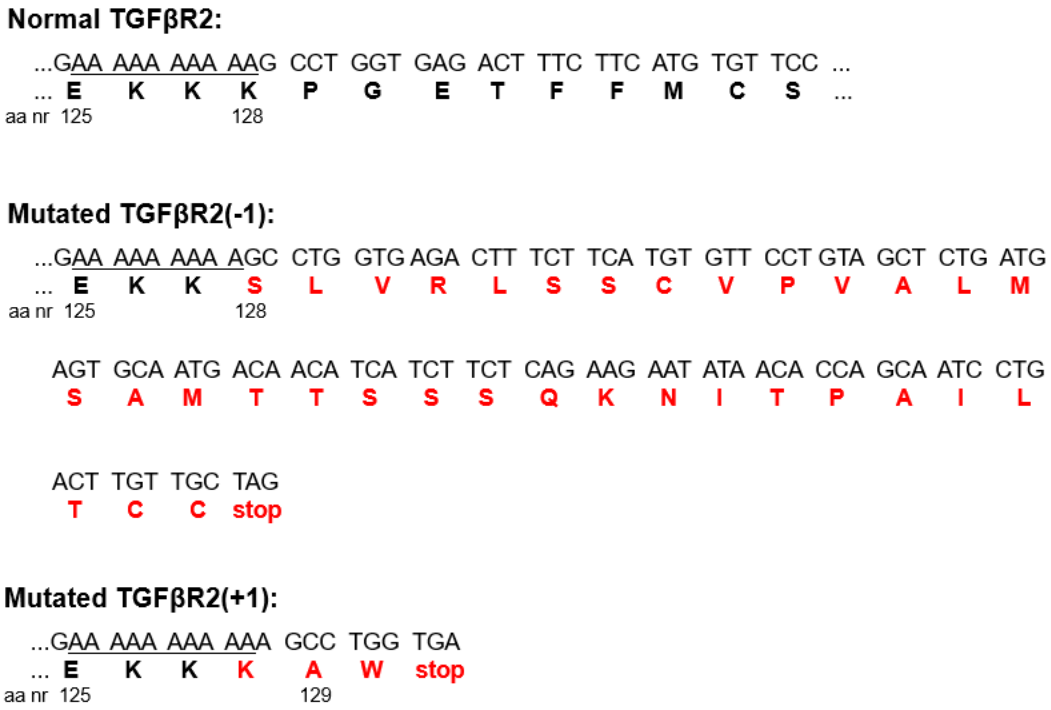


Figure 1. TGFβR2 frameshift mutations given as nucleotide and amino acids sequences. Mutated TGFβR2 deletion or addition of one nucleotide (-1 or +1) in microsatellites region (underlined) results in a new nucleotide triplet coding new amino acids from base position 128 or 129 and, in the end, a new frameshift variant of the peptide highlighted in red. The figure is adapted from Saeterdal et al., 2001.

1.5 Colorectal cancer with microsatellite instability (MSI-CRC)

The microsatellite instability (MSI) is a condition of genetic hypermutability in repeated DNA motifs (microsatellites) resulting from an impaired DNA mismatch repair system (MMR) (Ionov et al., 1993; Thibodeau et al.,1993). This MMR deficiency is caused by an epigenetic silencing (hypermethylation) of the MLH1 gene promoter or by germline mutation of DNA mismatch repair genes MLH1, MSH2, MSH6 or PMS2 (disease known as Lynch Syndrome) followed by somatic

inactivation of the second allele (Woerner et al., 2006; Buermeyer et al., 1999). The presence of MSI represents phenotypic evidence that MMR is not functioning normally and stands for 15% of all colorectal cancer (CRC) cases. Described abnormalities lead to accumulation of point mutations in DNA microsatellites and, if occurring in genes within coding repeat region, give rise to variants of mutated peptides causing the release of tumor neoantigens. Genetically instable regions of the human genome are more prone to mutations and neoantigens are more likely to occur in these locations. In CRC-MSI patients, the number of non-synonymous mutations per tumor (detected by genome-wide sequencing studies) reaches ~800 while, in other organs, this susceptibility is at 140-180 for melanoma and lung cancer, respectively (Vogelstein et al., 2013). Together with high mutational burden of immunogenic neoantigens, MSI-CRC is also often characterized by a high number of intratumoral immune cells (cytotoxic CD8 T cells, Th1, Th2, T follicular helper (Tfh) cells) within the tumor microenvironment, a positive prognostic factor for CRC-MSI patient survival (Mlecnik et al., 2016). Moreover, intratumoral immune cells often upregulate inhibitory receptors (natural breaks) such as PD-1 or CTLA-4, which in result suppress the anti-tumor response. Development of immune checkpoint inhibitors (ICI) restore immune system function and clinical studies demonstrated significant survival improvement in MSI patients with metastatic colon cancer (mCRC) after ICI treatment (Le et al., 2015). Despite pronounced responses to ICI, half of the patients still relapsed (Cohen et al., 2020). This, unfortunately, leaves many MSI-CRC patients with a minimal number of treatment options, making the herein proposed TGFβR2(-1)-specific TCR-engineered T cell immunotherapy a promising alternative on its own or used in combination with anti-PD-1 antibodies, resulting in better clinical outcomes in the future.

1.6 ABabDR4 humanized mice

ABabDR4 mice established in our group were engineered to express human TCR repertoire restricted to human MHC II molecule HLA-DRA/DRB1*0401 (HLA-DR4). They were made by crossing human TCR gene loci-transgenic mice

(ABab mice) with HLA-DRA-IE/HLA-DRB1*0401-IE (HLADR4-IE)-chimeric MHC II-transgenic mice (DR4 mice) (Ito et al., 1996; Li et al., 2010). As a result, the T cells from AbabDR4 mice express diverse human αβ TCRs but lack murine ones (Chen et al., 2017).

The ABabDR4 mice from which cancer-associated TCRs were isolated showed better functional activity than human-derived TCRs (Poncette et al., 2018). NY-ESO-reactive TCRs generated in ABabDR4 mice showed better recognition of NY-ESO$^+$ melanoma cell lines when comparing with those from human donor (Poncette et al., 2018). This is because ABabDR4 mice were NY-ESO negative and no NY-ESO homolog has been found in mice. It has been proven that such antigen-free host is a good source of effective TCRs since no central tolerance to human tumor antigens in TCR/MHC-humanized transgenic mice and no antigen-specific T cells deletion were observed in contrast to antigen-bearing hosts (Li et al., 2010). Another distinctive attribute of using such mouse model was reported in studies on MHC and TCR co-evolution: it was shown that if MHC and TCRs are from different species (human and mouse) the T cell development is disrupted and the size of T cell repertoire is reduced (Chen et al., 2017). This underlines the importance of aligning human TCRs and human MHCs in one host.

Isolation of DRB1*04:01-restricted TGFβR2(-1)-specific TCRs from ABabDR4 mice expressing a diverse human TCRαβ repertoire allowed us to pull out TCRs with the best functional characteristics. The TGFβR2(-1) homolog has not been found in the mouse genome, therefore, mice are non-tolerant to given TGFβR2(-1) neoantigen and serves as a great source for TCR clones with the best possible affinity features. Those mice serve as a reliable tool for testing the immunogenicity of HLA-DR4-restricted antigens as well as “best in class” source for therapeutic TCR isolation.

2. AIM OF PHD PROJECT

My PhD project aimed to evaluate if the TGFβR2(-1) neoantigen is a suitable target for immunotherapy and then to generate TGFβR2(-1)-specific TCRs for ATT. The attractiveness of the TGFβR2(-1) neoantigen is its recurrence in different types of cancers and its prevalence in different individuals, enabling application of the therapy to a broad number of cancer patients. To engineer TGFβR2(-1)-specific TCRs, we used humanized transgenic ABabDR4 mice that express a diverse human TCR repertoire with the HLA-DRA/DRB1*0401 restriction element (Ito et al., 1996; Li et al., 2010).

This PhD dissertation includes an assessment of TGFβR2(-1) neoantigen immunogenicity and neoantigen processing and presentation on HLA-DR4 (Human Leukocyte Antigen - DR4 isotype). We focused on analysing generated TGFβR2(-1)-specific TCRs (TCR1414_1 and TCR1414_2) and checked their potency, once transduced onto human $CD4^+$ T cells. Next, the safety of TCR1414_1 and TCR1414_2 was also questioned and its potency in clinical setup was discussed.

3. MATERIALS AND METHODS

3.1 Cell lines and culture

The human CRC cell line HCT116 (Brattain et al., 1981) and SW48 (Leibovltz et al., 1976) were collected from Prof Dr G. Willimsky and SW48 from Prof. U. Stein, respectively. Cell line SW48-DR4 and HCT116-DR4 were generated by retroviral transduction with HLA-DRA and HLA-DRB1*0401 (HLA-DR4) fused by the self-cleaving element P2A. Such HLA-DR4 DNA construct was introduced into MP71_IRES_GFP vector using restriction sites NotI and SalI. For generation of BSM-TGFβR2(-1) cell line (HLA-DR4^{+}) the TGFβR2(-1) minigene (Appendix 1) was first synthetized (GeneArt). Next, using restriction sites PmlI introduced via PCR, the TGFβR2(-1) minigene was cloned into the MP71_mCh vector (Engels et al., 2003). The BSM-TGFβR2(-1) cell line (HLA-DR4^{+}) was generated by retroviral transduction of the TGFβR2(-1)_MP71_mCh vector (Engels et al., 2003). The human melanoma cell line FM3 was provided by the European Searchable Tumor Cell Bank and Database (ESTDAB). All cell lines, including the LCL panel (EBV-transformed lymphoblastoid B cell lines), were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; PAN Biotech), 1x antibiotic-antimycotic, 1 mM sodium pyruvate and 100 µM non-essential amino acids (all reagents were purchased from Life Technologies). The CRC cell lines' medium was additionally supplemented with gentamicin (Life Technologies). The packaging cell line 293GP-GLV (GALV cells) (Ghani et al., 2009) producing retroviral particles was cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FCS and 1x antibiotic-antimycotic. The primary human PBL-derived T cells were kept in RPMI-1640 medium supplemented with 1 mM HEPES, 10% heat-inactivated fetal calf serum, 1x antibiotic-antimycotic, 1mM sodium pyruvate and optionally with 300 U/ml or 30 U/ml IL-2 (for details see 3.9 Transduction of primary T cells).

3.2 Mouse ABabDR4 strain

TCRs were generated in transgenic ABabDR4 mice expressing a diverse human TCRαβ repertoire restricted to human MHC class II molecule HLA-DRA/DRB1*0401 (Li et al., 2010; Chen et al., 2017). All animal experiments were approved and conducted according to national guidelines (Landesamt für Gesundheit und Soziales, Berlin, Germany). All mice were kept at the animal facility of the Max-Delbrück Center for Molecular Medicine under SPF (specific-pathogen-free) conditions.

3.3 Immunization of ABabDR4 mice

ABabDR4 mice were immunized at an age of 8 to 12 weeks using peptide immunization with TGFβR2(-1)-specific HLA-DR4-restricted peptide (VALMSAMTTSSSQKN, GenScript). The 80 µg of peptide dissolved in PBS were added to 50 µg of oligonucleotide CpG (CpG 1826, MOLBIOL) and 100 µl of incomplete Freund's adjuvant (IFA, Sigma-Aldrich). A volume of 200 µl emulsion (peptide and CpG dissolved in 100 µl PBS plus 100 µl IFA) was injected subcutaneously into mice. If necessary, immunizations were repeated at 4-week intervals.

3.4 ICS (Intracellular Staining)

One week after the last immunization, the peripheral blood of immunized ABabDR4 mice was analysed for the presence of responding $CD4^+$ T cells. The cell fraction after lysing red blood cells with ACK buffer (150 mM NH_4Cl, 1mM $KHCO_3$, 100 nM Na_2EDTA) was stimulated with TGFβR2(-1)-specific HLA-DR4-restricted peptide at 1 µM, no peptide as negative control or 20 µl containing $8*10^5$ CD3/CD28 Dynabeads T activator (Invitrogen) as positive control. After 2 hours, protein transport inhibitor Brefeldin A (BD Golgie Plug) was added and intracellular IFNy staining was performed after 13 hours of incubation in 37°C. After Fc blocking (anti-mouse CD16/32, BioLegend), cells were fixed using BD Cytofix/Cytoperm KIT (BD Bioscience) and stained with 50 µg/ml of

anti-CD3, -CD4 and -IFNy antibodies (BioLegend) for 30 min at 4°C, then analysed by flow cytometry.

3.5 IFNy secretion assay

Fourteen days after the last immunization, mice were sacrificed and splenocytes were isolated. Next, after 4 hours of restimulation with TGFβR2(-1)-specific HLA-DR4-restricted peptide at 1 µM, cells producing IFNy were stained using IFN-y Secretion Assay KIT (Miltenyi Biotec). Positive $CD4^+$ T cells producing IFNy were collected by FACS into lysate buffer RTL Plus (RNeasy Micro Kit, QIAGEN) for TCRs isolation.

3.6 TCR isolation

From positive $CD4^+$ T cells sorted by FACS during IFN-y secretion assay (Miltenyi Biotec), RNA was isolated (RNeasy Micro Kit, QIAGEN). This was followed by by synthesis of cDNA and 5' RACE PCR (SMARTer RACE cDNA Amplification Kit, Clontech) with 0.5 µM primers specific for the constant region of either TCRα (5′-CGGCCACTTTCAGGAGGAGGATTCGGAAC-3′) or TCRβ (5′-CCGTAGAACTGGACTTGACAGCGGAAGTGG-3′) chain. Each reaction was performed in volumes of 50 µl with 0.5 µl Phusion High-Fidelity DNA polymerase (New England Biolabs), 10 mM dNTPs, 1x Universal Primer Mix (10x, UPM, Clontech). Thermocycler conditions were as follows: 2 min at 98°C, 5 cycles of 98°C for 30s and 72°C for 45s, 5 cycles of 98°C for 30s, 68°C for 30s and 72°C for 45s, 25 cycles of 98°C for 20s, 68°C for 20s and 72°C for 45s, final elongation at 72°C for 5 min. The RACE PCR products were purified from an electrophoresis gel and cloned into TOPO vectors (Zero Blunt TOPO PCR cloning Kit, Invitrogen) to transform TOP10 OneShot competent E. coli (Invitrogen). Depending on clonality, around 50 clones per TCR chain were sequenced and further analyzed.

3.7 Generation of TCR transgene cassettes in MP71 vector

Isolated and amplified TCRα and TCRβ chains contain human constant regions. To reduce potential cross pairing with endogenous TCRs, we exchanged the human constant regions for the mouse counterparts (Cohen et al., 2006). To this purpose, overlapping extension PCR (OE-PCR) was performed from two PCR products. The 1st PCR murine constant region was amplified from an already murinized TCR-3600 vector (Poncette et al., 2018). Primers alpha_ms_fwd and huTCR3600_rvs were used to amplify murine alpha constant region. Primers beta_ms_fwd and TCR3600_beta_EcoR1_rvs were used to amplify murine constant beta region. The 2nd PCR included TCRα/β variable gene amplification using primers specific for each TRAV/TRBV with V(D)J_alpha_rvs and VDJ_beta_rvs, respectively (all primers listed below). The 3rd OE-PCR was the final one to combine variable regions of each TCRα/β with a constant murine region. Such full length single TCRα and TCRβ transgenes were cloned using NotI and EcoRI restriction sites into MP71 vector for combinatorial expression of different TCRα + TCRβ and to identify functional TCRαβ pairs (Engels et al., 2003). Functional TCRα + TCRβ pairs were synthesized with *Homo Sapiens* codon optimization (GeneArt) for better expression in human PBLs. Chains TCRβ with TCRα were linked with the porcine teschovirus-1-derived self-cleaving peptide P2A (Leisegang et al., 2008). Constant region remained murinized. The synthesized expression cassettes encoded two TCRs: TCR1414_1 or TCR1414_2.

alpha_ms_fwd	5′-ATATCCAGAACCCCGAGCCTGCCGTGTACC-3′
huTCR3600_rvs	5′-CAGGAATTCTCATCAGCTGGACCAC-3′
beta_ms_fwd	5′-GGATCTGAGAAACGTGACCCCCCCCAAGGT-3′
TCR3600_beta_EcoR1_rvs	5′-ACTGAATTCTCAGCTGTTCTTCTTCTTGACCATGG-3′
V(D)J_alpha_rvs	5′-AGCTGGTACACGGCAGGCTCGGGGTTCTG-3′
TRAV21_Not1	5′-ATTGCGGCCGCCATGGAGACCCTCTTGGGCC-3′
TRAV22 Not1	5′-ATTGCGGCCGCCATGAAGAGGATATTGGGAGC-3′
TRAV12-3_Not1	5′-GCATTGCGGCCGCCATGATGAAATCCTTGAGAGTTTTAC-3′
VDJ_beta_rvs	5′-ACCTTGGGGGGGGTCACGTTTCTCAGATCC-3′

TRBV28_Not1	5′-ATTGCGGCCGCCATGGGAATCAGGCTCCTCTG-3′
TRBV12-4_Not1	5′-ATTGCGGCCGCCATGGGCTCCTGGACCC-3′
TRBV18_Not1	5′-TATGCGGCCGCCATGGACACCAGAGTACTCTGC-3′

3.8 Transfection of packaging cell lines GALV

To produce amphotropic TCR-bearing retroviral particles, 293GP-GLV (GALV) cells were used. Virus supernatant was produced by transfecting GALV cells (Ghani et al., 2009) with the retroviral vector MP71 (Engels et al., 2003) containing TCR expression cassette with TCR1414_1 or TCR14141_2. For transduction of HCT116 and SW48 cell lines, GALV supernatants were prepared with vector MP71 containing HLA-DR4_GFP, GFP, TGFβR2(-1)_mCh or mCh. GALV cells seeded at $7x10^5$ per well in 6-well plates were transfected with 3 µg of plasmid DNA using either lipofectamine 2000 reagent (Thermo Fisher Scientific) or 18 µg of DNA when calcium chloride (Sigma Aldrich) method was used (Soneoka et al., 1995). 48h and 72h after transfection, 3 ml of virus supernatant were harvested, filtrated (0.45 µm pore size) and used directly for transduction or stored at -80°C.

3.9 Transduction of primary T cells

Human primary T cells were transduced with TCRs after $CD8^+$ cell depletion from PBLs using MACS anti-CD8 MicroBeads human and LD separation column (Miltenyi, cat. nr 130-045-201). One million CD8 depleted PBLs per well were seeded in 24-well plates (non-tissue culture, Thermo), previously coated with 5 µg/ml anti-CD3 (OKT3, BD Pharmingen) and 1 µg/ml anti-CD28 antibodies (CD28.2, BD Pharmingen) in 1 ml T cell medium supplemented with 200 U/ml IL-2. After 2 days, activated T cells were transduced with 1 ml of TCR1414_1-GALV or TCR1414_2-GALV supernatant supplemented with 4 µg/ml protamine sulfate (Sigma-Aldrich) and spinoculated for 90 min at 32°C and 800g. A second transduction was performed the next day on RetroNectin-coated 6-well plates (25 µg/ml in PBS), pre-coated with virus particles for 2 h at 32°C and 2000g. PBLs were transferred into virus-coated

6-well-plate and spinoculated for 30 min at 800g. Transduced PBLs were expanded for 7 days in T cell medium supplemented with 300 U/ml IL-2. For the next 3-5 days, they were transferred into low IL-2 medium (30 U/ml). After this resting phase, transduced PBLs were ready to be used for co-culture assays or were frozen and stored in liquid nitrogen. For transduction of single TCR α or β chain combinations, virus supernatants were mixed 1:1 in 1 ml volume.

3.10 Co-culture experiments

Co-culture of human T cells transduced with different non-codon optimized TCRα + TCRβ combinations were incubated with BM14 cells (HLA-DR4^{+}) as target cells at ratio 1:5, where 1x10^{4} transduced CD4^{+} T cells were seeded with 5x10^{4} BM14 target cells in round bottom 96-well plates for 16-18 hrs. Transduction efficiencies were in a range of ~30%. Co-culture of human T cells transduced with codon optimized TCR1414_1 or TCR1414_2 was incubated with target cells (BSM, BM14, SW48, HCT116 or panel of LCLs) at a ratio 1:1, where 1x10^{4} transduced CD4^{+} T cells were seeded with 1x10^{4} target cells in round bottom 96-well plates for 20-24h. Transduction efficiencies were in a range of ~60%. As positive control, 50 ng/ml of Phorbol 12-myristate 13-acetate (PMA) and 5 µg/ml Ionomycin were added to T cells alone. Negative controls were transduced T cells alone seeded in medium. TGFβR2(-1) peptide (VALMSAMTTSSSQKN, GenScript) was added at 10^{-6} M unless indicated differently. After incubation, supernatant was collected and IL-2 or IFNy concentrations were measured by enzyme-linked immunosorbent assay (ELISA; BD OptEIA).

3.11 ELISA

ELISA microwells were coated with 50 µl/well of capture antibody appropriately diluted in coating buffer and incubated overnight at 4°C. Next day, wells were washed 3 times with wash buffer. After that, plates were blocked with 100 µl/well assay diluent (10% FCS in PBS) and incubated at room temperature (RT) for 1 hour. Next, wells were washed 3 times with Wash Buffer. 50 µl of prepared standards, controls and samples were distributed into appropriate wells.

If needed, samples were diluted in assay diluent. Plates were sealed and incubated for at least 2 hours at RT. Then, wells were washed as before, but with 5 total washes. A volume of 50 µl of working detector (detection antibody + Streptavidin-Horseradish peroxidase (SAv-HRP) reagent) was added to each well and plates were incubated for 1 hour at RT. After the last extensive wash (8 times), 50 µl of Substrate Solution were added to each well and incubated for about 5 min at RT in the dark. Next 25 µl of stop solution were added to each well. Absorbance at 450 nm and 570 nm was then immediately read.

3.12 Expression profile of CRC cell lines

Cancer cell lines were lysed and RNA was isolated using RNeasy Plus Micro Kit (QIAGEN). Next, reverse transcription RT-PCR was performed using SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) to amplify cDNA. Obtained cDNA product was then used for amplification of 159-1240 bp and 60-1066 bp fragments (transcript variants 1 and 2) of TGFβR2 gene using gene specific primers TGFβR2 1k product_fwd 5'-ACTCCTGTGCAGCTTCCCTCGGC-3' and TGFBR2 1k product_rvs 5'-GGCCTTATAGACCTCAGCAAAGCGACC-3'. Amplified PCR products were visualized on 1% agarose gel, bands were cut out and DNA was purified using Zymoclean Gel DNA Recovery Kit (Zymo Research). Finally, the DNA was sequenced using Sanger sequencing technique (Eurofins).

3.13 Flow cytometry

The following antibodies (BioLegend) were used for staining at 1:100 dilutions unless stated otherwise: anti-mouse CD4-FITC (RM4-5), anti-mouse CD4-BV421 (L3T4, BD Pharmigen), anti-mouse CD3-PE (145-2C11), anti-mouse CD3-APC (145-2C11), anti-mouse IFNy-BV421 (XMG1.2), anti-mouse IFNy-PE (XMG1.2), anti-mTCRβ-chain-APC (H57-597), anti-human CD3-APC (SK7) and anti-human CD4-BV421 (OKT4). Magnetic beads anti-human CD8 (Microbeads 130-045-201) were used for CD8 depletion at 1:250 dilution (Miltenyi Biotec). FACSAria II (BD Bioscience) was used for sorting $CD4^+$ responsive T cells,

FACSAria F (BD Bioscience) was used to sort transduced cell lines. For analysis of cells, LSR-Fortessa was used (BD Bioscience).

4. RESULTS

4.1 Identification of DRB1*04:01-restricted TGFβR2(-1) peptide as a promising target

Theoretically, the TGFβR2(-1) frameshift peptide is an ideal target candidate as it arises from somatic cancer-specific mutation, providing with a truly tumor-specific neoantigen absent in normal cells of the host of tumor origin. Whether the TGFβR2(-1) neoantigen is an effective target is the question, which will be answered herein. Importantly, the TGFβR2(-1) epitope, as an effective target, must bind to the particular MHC molecules with high affinity (IC50 in nM range). Based on *in silico* pMHC binding prediction data (NetMHCIIpan 3.2 Server), the TGFβR2(-1) frameshift peptide appears to give suitable epitopes that can possibly bind to HLA-DRB1*04:01, with one of the best predicted binding affinities being 25,2 nM (VALMSAMTTSSSQKN) (Table 1). This epitope's core is located in the middle, which should give flexibility for appropriate processing. For this putative epitope, T cell receptors were generated by immunizing ABabDR4 mice with the TGFβR2(-1) peptide VALMSAMTTSSSQKN. The research was limited to the human MHC II allele expressed in our transgenic mice ABabDR4, which express a diverse human HLA-DRA/HLA-DRB1*0401-restricted TCRαβ repertoire and are non-tolerant to TGFβR2(-1) neoantigen based on nucleotide BLAST library search (Chen et al., 2017).

Frameshift peptide sequence: **SLVRLSSCVPVALMSAMTTSSSQKNITPAILTCC**				
NetMHCIIpan 3.2			NetMHCIIpan 4.0	IEDB recommended 2.22
epitope sequence	peptide core	IC50 (nM)	rank	IC50 (nM)
CVPVALMSAMTTSSS	MSAMTTSSS	47.2	21	51.0
VPVALMSAMTTSSSQ	MSAMTTSSS	33.2	8.70	50.0
PVALMSAMTTSSSQK	MSAMTTSSS	24.7	3.60	49.0
VALMSAMTTSSSQKN	**MSAMTTSSS**	**25.2**	**3.50**	**49.0**
ALMSAMTTSSSQKNI	MSAMTTSSS	30.3	9.40	67.0
LMSAMTTSSSQKNIT	MSAMTTSSS	43.4	25	153.0

Table 1. Peptide:MHC class II binding affinities of frameshift-derived epitopes for HLA-DRB1*04:01 allele predicted by prediction binding Servers NetMHCIIpan 3.2, NetMHCIIpan 4.0 and IEDB recommended 2.22. Sequence highlighted in bold letters is the one chosen for further experiments. The predicted output is given as IC50 (nM) units or as a rank. The lower the number, the higher is the affinity, the better the rank. As a rough guideline, peptides with number <50 nM are considered high affinity, <500 nM intermediate affinity and <5000 nM low affinity (Wang et al., 2008; Wang et al., 2010).

4.2 Isolation of TGFβR2(-1)-reactive TCRs from ABabDR4 mice

To support the *in silico* data about potential TGFβR2(-1) immunogenicity, we immunized ABabDR4 mice with the TGFβR2(-1) peptide VALMSAMTTSSSQKN. Significant $CD4^+$ T cell expansion was observed in immunized mice after restimulation of peripheral blood-derived T cells with TGFβR2(-1) peptide, suggesting a functional response (Figure 2A, B). To isolate TGFβR2(-1)-reactive TCRs, we collected splenocytes from the most responsive mouse nr 1414 on day 10 after 3rd immunization (Figure 2B). Following splenocytes restimulation *in vitro* for 4 hrs with TGFβR2(-1) peptide, we labelled responding T cells using Mouse IFNγ Secretion Assay KIT. Such labelled cells were quantitatively analysed and antigen-specific $CD4^+$ T cell population was collected by flow cytometry. We collected 6 000 cells which stood for 1.22% of $CD3^+$ lymphocytes (Figure 2C).

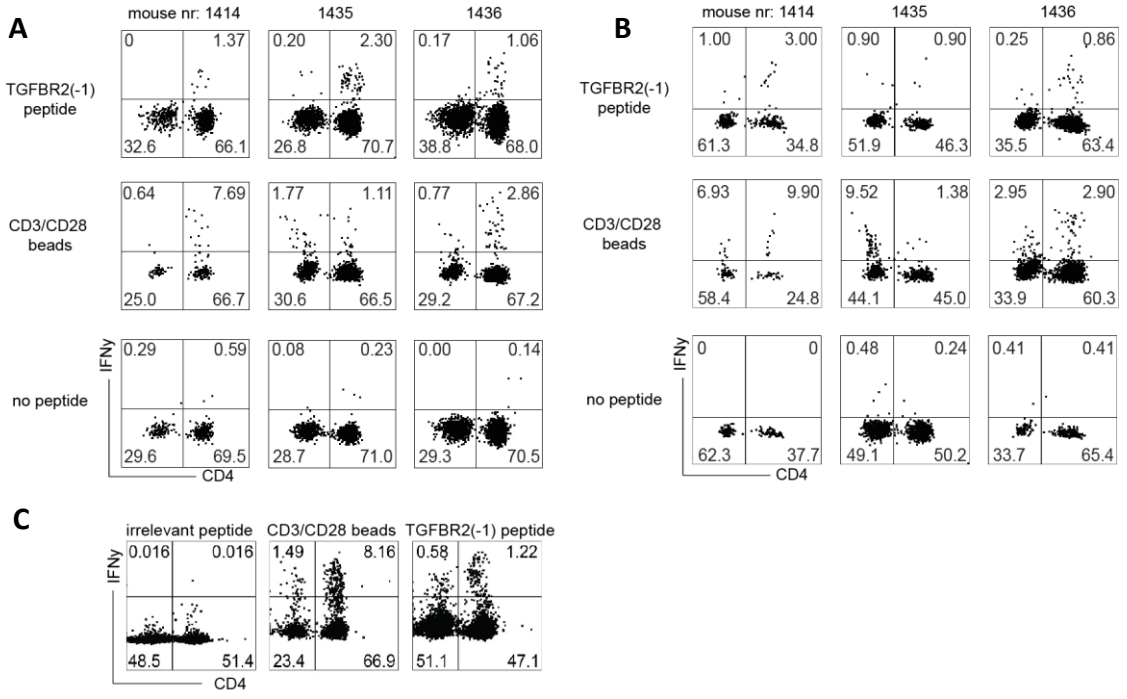


Figure 2. Generation of TGFβR2(-1)-reactive TCRs in ABabDR4 mice.

A, B. Intracellular staining (ICS) of PBLs from AbabDR4 mice 7 days after (A) 2nd or (B) 3rd TGFβR2(-1) peptide immunization. Isolated PBLs were stimulated with 10^{-6} M TGFβR2(-1) peptide, CD3/CD28 beads were used as positive control and no peptide as negative control. After 2 hrs, Brefeldin A was added to the cultures and ICS was carried out after O/N incubation. Cells are gated on single $CD3^+$ lymphocytes. Numbers in gates are population size given in percentage.

C. Sorting of TGFβR2(-1)-reactive IFNy-producing $CD4^+$ T cells from spleen of ABabDR4 mouse nr 1414 at day 10 after 3rd peptide immunization. Restimulated splenocytes *in vitro* for 4 hrs with TGFβR2(-1) peptide and CD3/CD28 beads or irrelevant peptide as controls were labelled using mouse IFN-γ Secretion Assay KIT. Labelled cells were sorted by flow cytometry. In total, 6 000 IFNy-producing $CD4^+$ TGFβR2(-1)-reactive T cells (1.22%) were collected. Cells are gated on single $CD3^+$ lymphocytes. Numbers in gates refer to population size given in percentage.

Three dominant TCRα and five dominant TCRβ chains were identified in collected antigen-specific $CD4^+$ T cells (Figure 3A). Combinatorial expression of each out of the 3 TCRα with each of the 5 TCRβ chains was performed, resulting in 15 different TCRαβ combinations that were transduced into human peripheral blood leukocytes (PBLs)-derived T cells (Figure 3B). To reduce cross pairing of identified TCRα/β with endogenous TCRα/β chains, their constant region was murinized (Sommermeyer et al., 2010). All 15 TCRαβ combinations prepared were used for co-culture with BM14 cell line (naturally expressing HLA-DRB1*04:01) to determine functional TCRαβ combinations. Functional TCRαβ combinations are the ones recognizing the epitope presented on HLA-DRB1*04:01 molecules of antigen presenting target cells BM14, resulting in IFNy production upon peptide stimulation. Functional TCR pairs 1A 1B and 2A 3B were identified as reflected by the high IFNy amount measured in the supernatant by ELISA (Figure 3C). Functional TCRαβ chain combinations were cloned together into one expression vector and linked with P2A element for equal molarity expression (Leisegang et al., 2008). Next, they were synthetized along with an amino acid codon optimization for *Homo Sapiens* species for better expression in human T cells. Functional TCR pairs TCR 1A 1B and TCR 2A 3B were called TCR1414_1 and TCR1414_2, respectively.

A

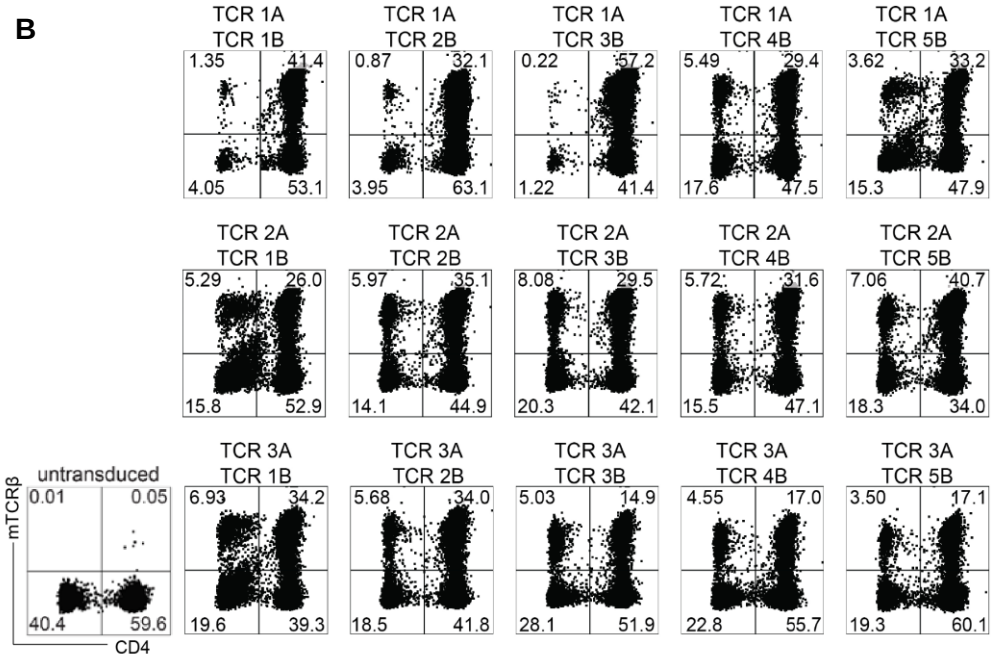
A chains:

freq.	V - CDR3 - J	TCR chain
42%	*TRAV21 - CAVKSGAGSYQLTF - TRAJ28*	TCR 1A
28%	*TRAV22 - CAAPSGNTPLVF - TRAJ29*	TCR 2A
10%	*TRAV12-3 - CAMAYSGAGSYQLTF - TRAJ28*	TCR 3A

B chains:

freq.	V - CDR3 - J	TCR chain
32%	*TRBV18 - CASSPFQETQYF - TRBJ2-5*	TCR 1B
14%	*TRBV12-4 - CASRRTGAIQYF - TRBJ2-4*	TCR 2B
10%	*TRBV28 - CASRVSGANVLTF - TRBJ2-6*	TCR 3B
10%	*TRBV28 - CASSRPGSSYEQYF - TRBJ2-7*	TCR 4B
7%	*TRBV18 - CASSQDIYEQYF - TRBJ2-7*	TCR 5B

B



C

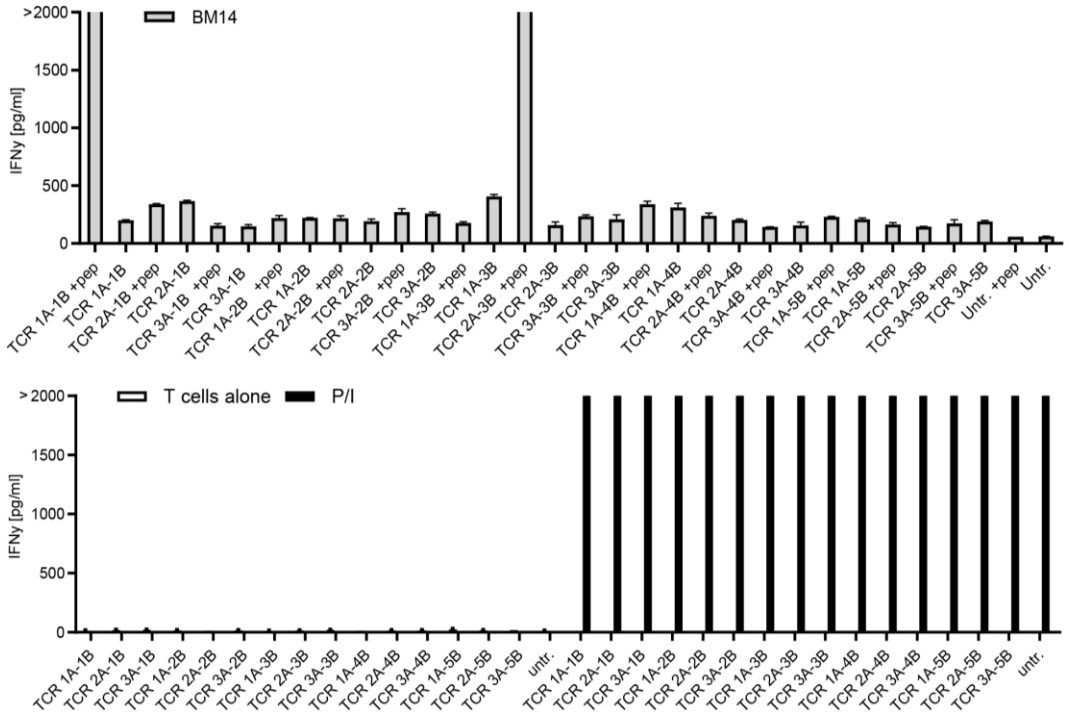


Figure 3. Analysis of ABabDR4-derived TCR combinations from ABabDR4 mouse nr 1414.
A. Frequencies of CDR3 regions and V/J gene segments of TCR α- and β- chains. After RACE-PCR from responding $CD4^+$ T cells, the amplified PCR product of each chain was separately cloned into TOPO vectors and TOP10 OneShot competent *E. coli* were transformed. Around 50 clones for each TCRα and TCRβ were analysed and their frequencies are presented in percentages.
B. Combinatorial expression of TCRα and TCRβ genes in human $CD4^+$ T cells with given transduction efficiency percentages. Human PBLs-derived $CD4^+$ T cells were retrovirally transduced with different TCRα/TCRβ gene combinations and stained with mouse TCRβ constant region (mTCRβ) antibody and human CD4 antibody. Cells are gated on $CD3^+$ lymphocytes.
C. Identification of functional pairs TCR 1A 1B and TCR 2A 3B reflected by IFNy secretion measured by ELISA in the supernatant from co-cultured T cells with target cells BM14 (HLA-$DR4^+$). Human $CD4^+$ PBLs-derived T cells were transduced with 15 ABabDR4-derived TCR combinations. As target, BM14 cells were loaded with TGFβR2(-1)-specific-HLA-DRB1*04:01-restricted peptide (+pep) at 10^{-6} M. As negative control, T cells alone in medium were used and as positive control, T cells with PMA/Ionomycin (P/I). Intra-assay duplicates with standard deviation are displayed. Similar data were obtained with two other cell lines: BSM and K562-DR4 expressing HLA-DR4 (data not shown).

The other two ABabDR4 mice nr 1435 and 1436 were immunized further as TCRs of higher average affinity for pMHC were expected to be obtained (Savage et al., 1999). From mouse nr 1435 reactive $CD4^+$ T cells were successfully isolated but we could not identify a dominant alpha/beta bearing T cell clone. From mouse nr 1436 splenocytes were taken into 7 days peptide-specific *ex vivo* expansion (data not shown). Such stimulation should let T cells with the most dominant alpha and beta outgrowth. Unfortunately, the final number of collected $CD4^+$ responsive T cells after *ex vivo* stimulation was too low to obtain further relevant data. A probable reason to that could be a low number of feeder cells in spleen-derived cell fraction resulting in insufficient peptide presentation to antigen-specific $CD4^+$ T cells and antigen availability.

4.3 Peptide sensitivity

One of the important feature of a TCR is its sensitivity to peptide concentration at which the TCR still recognizes the pMHC complex. To analyze peptide sensitivity, human $CD4^+$ T cells transduced with TCR1414_1 or TCR1414_2 were co-cultured with BSM ($HLA\text{-}DR4^+$) cells loaded with different concentrations of TGFβR2(-1) peptide. As a readout, IFNy concentration was measured by ELISA. Both investigated TCRs showed recognition up to 10^{-9} M TGFβR2(-1) peptide with IC50 values between 10 and 1 nM for TCR1414_1 and TCR1414_2, respectively (Figure 4).

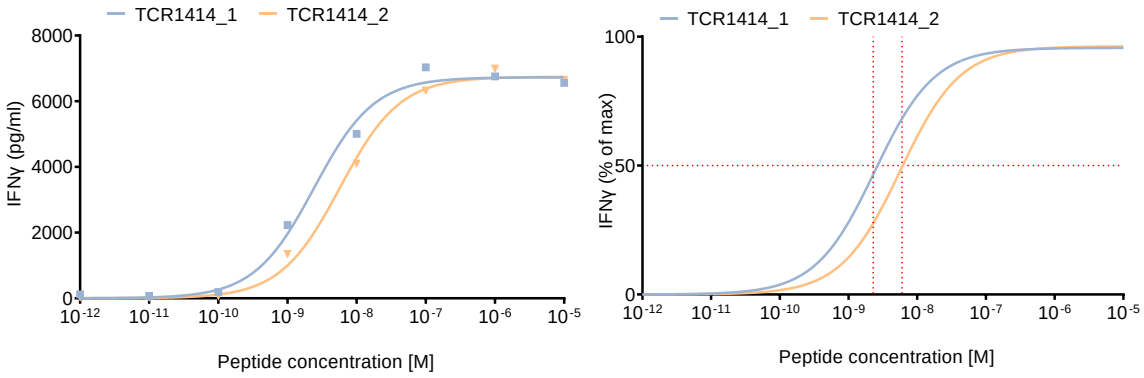


Figure 4. TCR1414_1 and TCR1414_2 sensitivity to TGFβR2(-1) peptide.

A. ELISA results reflecting responsiveness of $CD4^+$ T cells transduced with TCR1414_1 or TCR1414_2 to varying doses of the TGFβR2(-1) peptide (VALMSAMTTSSSQKN). TCR-transduced $CD4^+$ T cells were co-cultured with BSM cells pulsed with decreasing peptide amounts. IFNy was measured in the supernatant after 16 hrs.

B. Normalized data to the maximum IFNy release. Both investigated TCRs show recognition up to 10^{-9} M TGFBR2(-1) peptide with IC50 values between 10 and 1 nM for both TCR1414_1 (blue line) and TCR1414_2 (yellow line), as indicated by the red dotted line. The results are representative of 2 independent experiments with 2 different human PBLs donors.

4.4 TGFβR2(-1) neoantigen is naturally processed and presented

Immunization of ABabDR4 mice with TGFβR2(-1) peptide VALMSAMTTSSSQKN shows its immunogenicity but does not reveal if the whole frameshift antigen is processed by endogenous endosomal–lysosomal antigen-processing compartments and presented on HLA-DRA/HLA-DRB1*04:01 molecules. To address the question whether the

TGFβR2(-1) neoantigen is processed and presented, TCR1414_1/2-transduced PBLs-derived $CD4^+$ T cells (Figure 5A) were co-cultured with antigen presenting BSM cells ($HLA\text{-}DR4^+$) transduced with a TGFβR2(-1) minigene (Appendix 1) encoding 47 amino acids containing TGFβR2(-1) frameshift peptide with HLA-DRB1*04:01-resctricted epitope of interest (Figure 5B). As a readout, IFNy and IL-2 concentrations were measured in the supernatant of the co-cultures by ELISA (Figure 5C). Results from Figure 5C show that both TCRs (TCR1414_ and TCR1414_2 recognize the BSM-TGFβR2(-1) cell line endogenously, expressing the full length TGFβR2(-1) frameshift region. This demonstrates that the given TGFβR2(-1) minigene has been processed further, generating HLA-DRB1*04:01-restricted peptide presented on BSM-TGFβR2(-1) cells surface. Control BSM-mCh cells do not express mutational TGFβR2(-1) frameshift peptide and therefore, T cells interacting with those cells do not produce IFNy or IL-2 if TGFβR2(-1) peptide is not externally loaded.

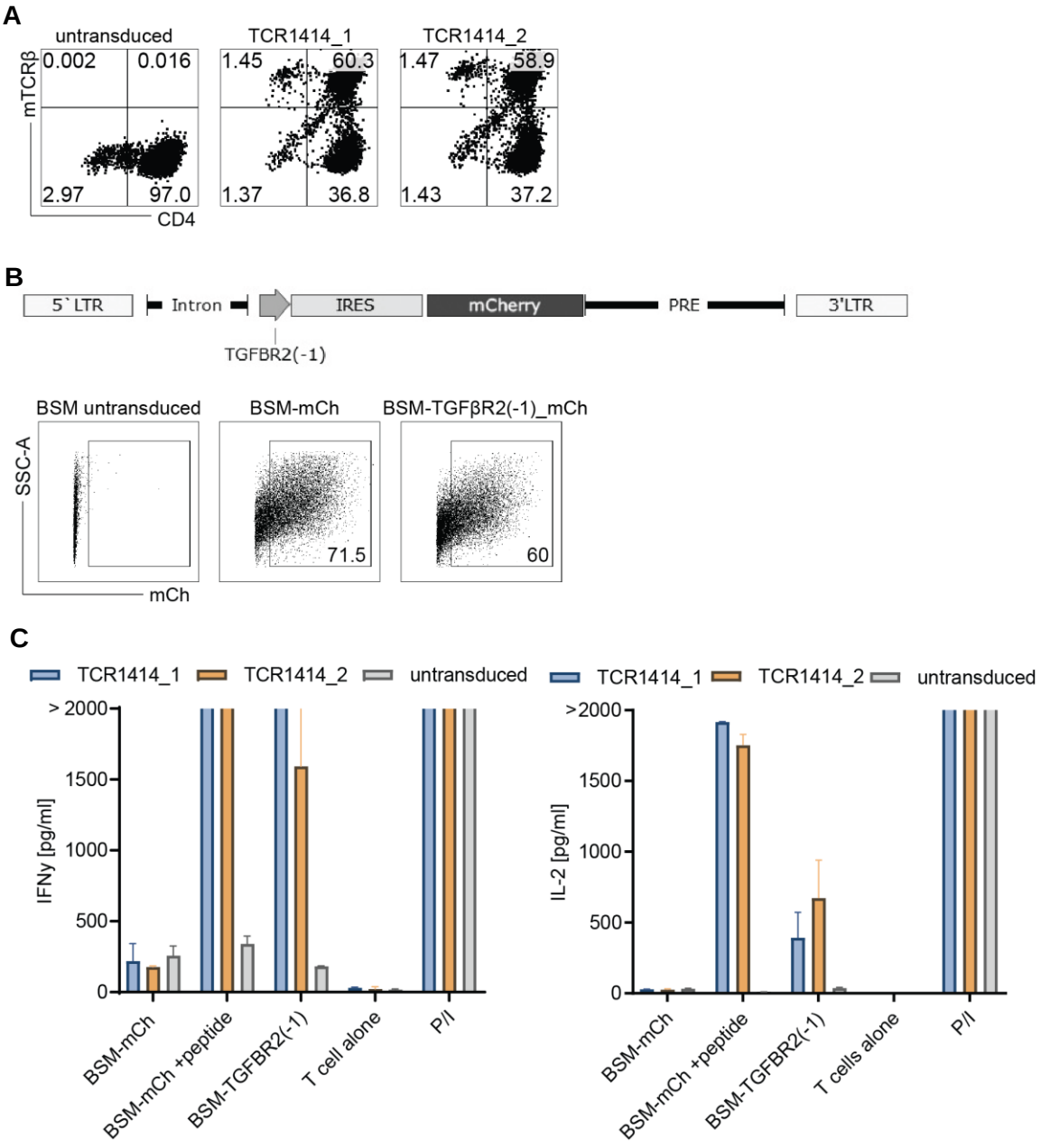


Figure 5. Recognition of naturally processed TGFβR2(-1) neoantigen by TCR1414_1 and TCR1414_2.

A. Expression of codon optimized TGFβR2(-1)-reactive TCR1414_1 and TCR1414_2 in human CD4$^+$ T cells with given transduction efficiency percentages. TCR1414_1 and TCR1414_2 were retrovirally transduced onto human PBL-derived CD4$^+$ T cells (after CD8$^+$ T cells depletion) and stained with antibody for mouse TCRβ constant region (mTCRβ) and human CD4. Cells were gated on CD3$^+$ lymphocytes. The results are representative of 3 different human PBLs donors.

B. Target cells transduction profile with TGFβR2(-1) minigene. BSM-TGFβR2(-1) was retrovirally transduced with TGFβR2(-1) minigene encoding 47 amino acids and containing HLA-DRB1*04:01-resctricted epitope of interest coupled to mCherry as reporter. BSM-mCh, was

used here as a negative control and transduced with same vector but without the minigene, only mCherry reporter gene. Numbers in gates show transduction frequencies in percentages.
C. Recognition of naturally processed TGFβR2(-1)-specific HLA-DR4-restricted epitope by TCR-transduced $CD4^+$ T cells co-cultured with BSM-TGFβR2(-1) cell line ($HLA\text{-}DR4^+$) for 16 hrs. Data reflect IFNy and IL-2 production measured by ELISA in supernatant. TGFβR2(-1) peptide at 10^{-6} M (+pep.) or PMA/Ionomycin (P/I) were added as indicated. Results are representative of 3 (for IFNy ELISA) and 2 (for IL-2 ELISA) independent experiments with different PBLs donors.

4.5 Recognition of TGFβR2(-1) neoantigen naturally expressed in CRC cell lines.

Based on seltarbase.org database, we focused on analysis of two CRC cell lines, SW48 and HCT116, reported to naturally express the TGFβR2(-1) frameshift mutation (Woerner et al., 2010). To confirm the deletion of one adenine in the TGFβR2 gene, which causes the frameshift peptide containing TGFβR2(-1) neoantigen, we isolated RNA from both CRC cell lines and performed RT-PCR, amplifying ~1 Kb fragment of the TGFβR2 gene. Electrophoresis in Figure 6A reveals two bands since PCR primers were designed to cover both splicing variants of the TGFβR2 gene. The purified ~1 Kb DNA fragments were purified from the gel and subjected to Sanger sequencing. Sequencing results showed the presence of the mutation (lack of one adenine), reflected in only 9 adenines being present in both CRC cell lines SW48 and HCT116 (Figure 6A). TGFβR2 wild type (10 adenines microsatellite) in FM3 melanoma cell line was used as a negative control in this experiment. Next, we checked if the TGFβR2(-1)-reactive TCR1414_1 and TCR1414_2 can recognize CRC cell lines SW48 and HCT116 that naturally express TGFβR2(-1) neoantigen. To do so, TCR1414_1/2-transduced human $CD4^+$ T cells were co-cultured with SW48 and HCT116 cell lines transduced with HLA-DR4, due to the absence of the particular MHC II molecule in these CRC cells (Figure 6B). As expected, SW48-DR4 and HCT116-DR4 cell lines naturally expressing TGFβR2(-1) neoantigens were recognized by both TCR1414_1/2-transduced human $CD4^+$ T cells, as reflected by IFNy release (Figure 6C).

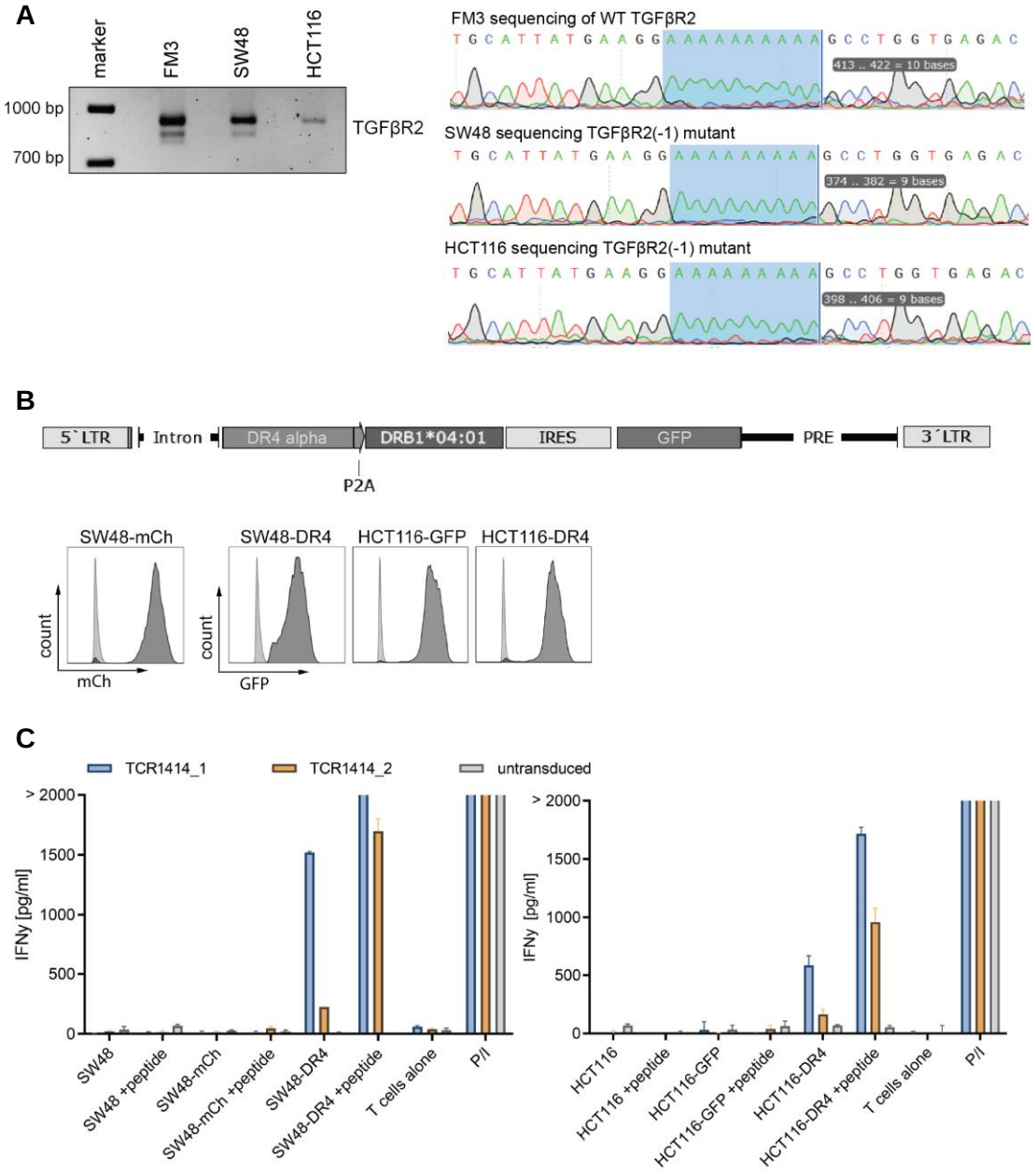


Figure 6. Recognition of naturally expressed TGFβR2(-1) neoantigen in colorectal cancer (CRC) cell lines by TCR1414_1 and TCR14141_2.

A. TGFβR2(-1) mutation detection in CRC cell lines SW48 and HCT116. Electrophoresis of amplified PCR products from cDNA shows the TGFβR2 region of interest containing the frameshift mutation. Primers for PCR were designed to cover the two splicing variants of the TGFβR2 gene. Sanger sequencing results show one adenine deletion (9A) in the 10-adenine microsatellite of TGFβR2 gene in both CRC cell lines. As control, a FM3 melanoma cell line containing the wild type (WT) TGFβR2 DNA sequence was used.

B. CRC cell lines SW48 and HCT116 (both HLA-DR*04:01$^-$) were transduced with HLA-DRA/HLA-DRB1*0401 construct, as indicated, or mock controls (mCh or GFP). Dark grey

represents transduced cells and light grey, untransduced, parental cell line. Expression of reporter genes GFP or mCh was measured by flow cytometry.

C. Recognition of naturally expressed TGFβR2(-1) neoantigen in SW48 and HCT116 cell lines by TCR1414_1 and TCR14141_2. TCR-transduced human $CD4^+$ T cells were co-cultured with CRC cell lines SW48-DR4 and HCT116-DR4 that endogenously express TGFβR2(-1) frameshift mutation. As positive controls, PMA and Ionomycin (P/I) were used, whereas for negative control, T cells alone were used. After 24h of incubation, IFNy was measured in the supernatant by ELISA. The results are representative of 4 independent experiments performed with 3 different PBLs donors.

4.6 ABabDR4-derived TGFβR2(-1)-specific TCRs showed no alloreactivity.

ABabDR4 mice used for generation of TGFβR2(-1)-specific TCRs lack more than HLA-DRA/DRB1*0401 human MHC molecules and therefore, there is a potential risk of alloreaction between these TCRs and other human HLAs. To investigate if generated TCRs are alloreactive, we transduced human $CD4^+$ T cells with TCR1414_1 or TCR1414_2 and co-cultured them with a panel of 16 Epstein-Barr virus-transformed B-LCLs expressing different MHC I and MHC II molecules (Table 2). No alloreactivity was detected (Figure 7). BSM and BM14 LCLs naturally expressing HLA-DR4 were additionally loaded with TGFβR2(-1) peptide and used here as TCR-specificity control for co-culture with TCR1414_1/2-transduced $CD4^+$ T cells.

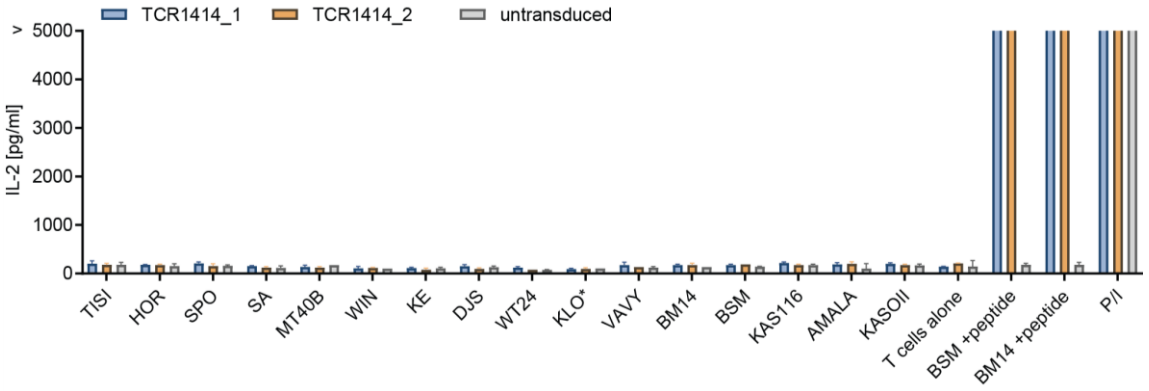


Figure 7. Alloreactivity test of ABabDR4-derived TCRs.

TCR1414_1 and TCR1414_2 transduced human $CD4^+$ T cells were co-cultured with a panel of B-LCLs expressing different HLA allotypes, including both MHC I and II molecules (full HLAs profile of each LCL in Table 2 below). As positive control, PMA/Ionomycin (P/I) and BSM, BM14 (both HLA-DR*04:01$^+$) loaded with 10^{-6} M TGFβR2(-1) peptide were used, whereas for negative control T cells alone were used. After 24h of incubation, IL-2 was measured in the supernatant by ELISA. The results are representative of 3 independent experiments performed with T cells from 3 different PBLs donors. For all experiments, all cell lines were used simultaneously except for *KLO cell line.

B-LCL	A*	B*	C*	DRA*	DRB1*	DRB3*	DRB4*	DRB5*	DQA1*	DQB1*	DPA1*	DPB1*
BSM	02:01:01:01	15:01:01:01	03:04:01:01	01:01:01	04:01:01:01, 04:01:01:02		01:03:01:01, 01:03:01:03		03:01:01	03:02:01	01:03:01:01	02:01:02:01
BM14	03:01:01:01	07:02:01:01	07:02:01:03	01:01:01	04:01:01:02		01:03:01:11		03:01:01	03:02:01	01:03:01:04	04:01:01:01
MT14B	31:01:02:01	40:01:02:01	03:04:01:01	01:01:01	04:04:01		01:03:01:01 01:03:01:03		03:01:01	03:02:01	01:03:01:05	04:02:01:02
VAVY	01:01:01:01	08:01:01:01	07:01:01:01	01:02:02	03:01:01:01	01:01:02:01, 01:01:02:02			05:01:01:02	02:01:01:01	02:01:02:02	01:01:01
KLO	01:01:01:01, 02:08	08:01:01:01, 50:01:01	06:02:01:02, 07:01:01:01		03:01:01:01, 07:01:01:01	01:01:02:01, 01:01:02:02	01:03:01:01, 01:03:01:03		02:01:01:01, 05:01:01:02	02:01:01, 02:02:01:01	01:03:01:02, 02:01:02:01	01:01:01, 104:01
KAS011	01:01:01:01	37:01:01:01	06:02:01:01	01:01:01	16:01:01			02:02	01:02:02:01	05:02:01:01	01:03:01:02, 02:01:01:02	04:01:01:01, 14:01:01:01
WIN	01:01:01:01	57:01:01:01	06:02:01:01	01:01	07:01:01:01, 07:01:01:02		01:01		02:01:01:01, 02:01:01:02	02:02:01:01, 03:03:02:01	01:03:01:02, 02:01:04	04:01:01:01, 13:01:01:01
TISI	24:02:01:01	35:08:01	04:01:01:06	01:01:01	11:03:01	02:02			05:05:01:01, 05:05:01:02	03:01:01	01:03:01:05	04:02:01:02
SPO	02:01:01:01	44:02:01:01	05:01:01:02	01:01:01	11:01:01:01	02:02:01:02			01:02:02	05:02:01	01:03:01:01	02:01:02
HOR	33:03:01:02	44:02:01:01	14:03	01:02:02	13:02:01	03:01:01			01:02:01:09	06:04:01:01	01:03:01:01	04:01:01:26
AMALA	02:17:02:01	15:01:01:01	03:03:01:01	01:02:02	14:02:01:01	01:01:02:05			05:03:01:01	03:01:01:01	01:03:01:15	04:02:01:02
KAS116	24:02:01:01	51:01:01:03	12:03:01:01	01:01:01	01:01:01				01:01:01:01	05:01:01	02:01:01:01	13:01:01:01
DJS	02:01:01:01 03:01:01:01	37:01:01, 35:01:01:02	04:01:01:01, 06:02:01:01		01:01:01, 16:01:01			02:02	01:01:01:01, 01:02:02	05:02:01, 05:01:01:03	01:03:01:02, 01:03:01:05	04:01:01:01, 04:02:01:02
WT24	02:01:01:01	27:05:02	02:02:02:01		16:01:01			02:02		05:02:01	01:03:01:03	03:01:01
KE	02:03:01, 11:01:01:01	38:02:01, 15:02:01:01	08:01:01, 07:02:01:01		04:05:01, 08:14:01		01:03:01:01		03:03:01, 01:03:01:05	04:01:01, 06:01:01	02:02:02	02:02, 05:01:01
SA	24:02:01:01	07:02:01:01	07:02:01:03	01:01:01	01:01:01				01:01:01	05:01:01	01:03:01:05	04:02:01:02

Table 2. HLA allotypes of the LCL obtained from IPD-IMGT/HLA - Human Leukocyte Antigen Sequence Database (Robinson et al., 2020).

5. DISCUSSION

In this doctoral project, we evaluated two therapeutic TCRs generated in ABabDR4 mice including a comprehensive analysis of their functional activity and safety profile. We confirmed that the TGFβR2(-1) frameshift neoantigen is immunogenic upon immunization of ABabDR4 mice with DRB1*04:01-restricted peptide VALMSAMTTSSSQKN. We showed that the TGFβR2(-1) frameshift neoantigen is processed and presented by HLA-DR4 molecules. We also showed that both ABabDR4-derived TCR1414_1 and TCR1414_2 transduced onto human $CD4^+$ T cell recognized naturally expressed TGFβR2(-1) frameshift neoantigen in HLA-DR4-transduced CRC cell lines HCT116 and SW48.

5.1 Immunogenicity of TGFβR2(-1) frameshift peptide

First, we showed that HLA-DR4-restricted peptide is immunogenic since 3 AbabDR4 mice already immunized second time with HLA-DR4-restricted peptide (Figure 2A) demonstrated expression of IFNy by $CD4^+$ T cells upon peptide restimulation, indicating a functional immune response. Both investigated TCR1414_1 and TCR1414_2 were pulled from AbabDR4 mouse nr 1414 since this mouse showed the highest IFNy expression by $CD4^+$ T cells after 3rd immunization (Figure 2B).

5.2 Sources of neoantigen-specific TCRs

There are few ways to obtain therapeutic neoantigen-specific TCRs. The safest option is to isolate TCRs directly from the cancer patient's tumor infiltrating lymphocytes (TILs) or peripheral blood T cells. These autologous T cells are screened for reactivity against certain mutations and, once identified as neoantigen-specific TCRs, are transferred into suitable T cells and introduced back to the same patient's blood stream. This autologous T cell transfer is the safest option and does not bring any off/on-target toxicity. Two clinical trials based

on expansion of autologous TILs have reported complete and durable responses in 22% and 24% metastatic melanoma patients with 20% and 51% of these responses going on beyond 3 years (Rosenberg et al., 2011, Goff et al., 2016). Many clinically meaningful responses rely on specifically expanded T cells by co-culturing them with autologous tumor cells or other cells presenting neoantigens pulsed with neoantigen peptides or transfected with tandem minigenes (TGMs) (Tran et al., 2014). Those high-throughput assays require patient's cells availability (T cells, tumor cells or APCs) and even if these cells can be obtained from tumor tissue biopsy, metastasis surgical resections or from periphery, they still need to be isolated in sufficient number for further screening. Another solution would be to use cells from a cancer patient harboring a tumor with the same neoantigen but, in this case, additional tests need to be carried out on allogeneic TCRs to avoid GvHD, extending the treatment's preparation time. Peptide vaccines targeting neoantigens could increase the number of such specific TCRs however, has been shown that patient-derived neoantigen-specific T cells might be less potent due to dysfunctional terminally differentiated state and do not display a proliferative phenotype as a consequence of continuously encouraging antigen in the host (Schietinger et al., 2016; Philip et al., 2017; Scott et al., 2019). Isolating neoantigen-specific TCRs from healthy HLA-matched donor could solve the issue with limited patient's material availability, including the low frequencies of neoepitope-specific T cells and their fitness (Strønen et al., 2016; Nielsen et al., 2017). Naïve T cell repertoire, unaffected by cancer immunosuppressive environment and primed *in vitro* with mRNA-transfected DCs with TMG containing neoepitopes demonstrated to be a great source of neoantigen-specific T cells. They recognized human tumors from different hosts, even when many neoantigens were ignored by the patient's own autologous TILs (Strønen et al., 2016). Isolating neoantigen-specific TCRs from healthy HLA-matched donor could help solve the issue of treatment's preparation time and cost, a clinical problem for many patients with metastatic malignancies (Kato et al., 2018). It is crucial, however, to HLA-match both individuals to avoid donor-derived TCRs to recognize the recipient's HLA molecules present on non-tumor tissues. Since donor-derived TCRs have not undergone the patient's

thymic selection for lack of potential self-reactivity, it may result in autoimmunity damages (Bendle et al., 2009). Although patient material appears to be the most likely to succeed as a source of therapeutic neoantigen-specific TCRs, it has some limitations that could potentially be solved by using HLA-compromised mice. Human neoantigens are foreign to murine immune system, therefore, high avidity T cell responses can be achieved while immunizing such HLA-transgenic mice with tumor-specific peptides (Theobald et al. 1995; Theobald et al., 1997; Stanislawski et al., 2001). Unfortunately, murine TCRs may be immunogenic when transferred onto patient's autologous T cells, provoking humoral response and antibody release against murine variable chain of TCRs. Consequently, cells bearing therapeutic murine antigen-specific TCRs are eliminated in patients (Davis et al., 2010). Additionally, studies on MHC and TCR co-evolution showed that if MHC and TCRs are from different species (human and mouse), the T cell development is disrupted and the size of T cell repertoire from which an antigen-specific TCR could be obtained is reduced (Chen et al., 2017). This underlines the importance of matching human TCR and human MHCs in one host, therefore, a major improvement has been achieved by generating a MHC-transgene mouse model (ABabDR4 mice) expressing the entire human TCR loci while their murine counterparts were knocked out (Ito et al., 1996; Li et al., 2010; Li and Blankenstein, 2013, Chen et al., 2017).

The usage of ABabDR4 mice to develop DRB1*04:01-restricted TGFβR2(-1)-specific TCRs allowed us to select the TCRs with the best functional characteristics and safety level from a diverse human TCRαβ. The TGFβR2(-1) homolog has not been found in mice genome. It is therefore non-tolerant to given antigen, which allowed us to pull out TCRs with the best affinity features while avoiding possible risk of neoantigen-specific T cell elimination, a potential issue in tumor bearing host.

5.3 HLA-DR4-restricted TGFβR2(-1)-specific TCRs

While other studies showed processing and presentation of the TGFβR2(-1) frameshift neoantigen in HLA-A2-dependent manner (Linnebacher et al., 2001; Inderberg et al., 2017), the HLA-DR4-restricted processing and presentation of

TGFβR2(-1) frameshift neoantigen was proved herein. Our data support TGFβR2(-1) neoantigen recognition by tumor-derived $CD4^+$ TILs suggested in previous studies but shows different MHC II-restriction elements and binding affinity (Saeterdal et al., 2001). In Saeterdal et al., isolated biopsy-derived TILs were reactive upon co-culture with peptide SLVRLSSCVPVALMSAMTTSSSQ and further examinations pointed at HLA-DRB1*1401-dependency. Their SLVRLSSCVPVALMSAMTTSSSQ peptide, once analyzed using MHC-binding affinity algorithm for HLA-DRB1*1401, shows epitope's affinity above 200nM, a value considered as a weak-binder, suggesting that they may not be successful target epitopes when used in a natural human setting. As a comparison, our DRB1*04:01-restricted peptide VALMSAMTTSSSQKN is predicted to have affinity lower than IC 50 nM, a value considered as a strong binder affinity. Our data also support TGFβR2(-1) neoantigen recognition by tumor-derived $CD4^+$ TILs suggested in previous studies. HLA-DR-dependence is also indicated but another restriction element HLA-DRB1*1401 is specified and a slightly different region of the TGFβR2(-1) frameshift peptide (SLVRLSSCVPVALMSAMTTSSSQ) is used for reactive T cell identification (Saeterdal et al., 2001).

5.4 Importance of generation CD4-based TGFβR2(-1)-specific TCRs

While the great majority of current immunotherapies focus on MHC I-restricted TCRs or non-MHC-dependent CAR technologies to engineer therapeutic $CD8^+$ T cells, several studies have shown that $CD4^+$ T cells can bear similar relevance to a successful rejection of the tumor. The importance of $CD4^+$ T cells in anti-tumor responses has been known for many years (Greenberg et al., 1981) as well as their ability to reject tumor once $CD4^+$ T cells were adaptively transferred into tumor bearing mice (Greenberg et al., 1985). Other murine models showed that $CD4^+$ T cell mediated anti-tumor immunity depends on IFNy acting on stroma cells, suggesting that tumor rejection may not entirely rely just on tumor cell elimination (Qin and Blankenstein, 2000). Later on, it was also proved that $CD4^+$ T cells can directly lyse MHC II-positive melanoma cells

mediated by IFNy (Quezada et al., 2010). The effort was exploited into clinical usage of neoantigen-specific $CD4^+$ T cell which resulted in regression for metastatic cholangiocarcinoma and melanoma patients (Tran et al., 2014; Veatch et al., 2018). Other clinical studies demonstrated complete melanoma tumor remission ongoing for 7 years post neoantigen-TILs infusion (Linnemann et al., 2015). Our study contributes to the importance of TCR-transduced $CD4^+$ T cells therapeutical approach to potentially cure TGFβR2(-1)$^+$ cancer patients in the future. It is relevant to target MHC class II-restricted antigens since many nonhematopoietic cancers with high mutational rates are instable and downregulate or lose the MHC I expression to escape immunosurveillance (Restifo et al., 1996; Patel et al., 2017; Tran et al., 2016). In such setups, $CD4^+$ T cells play a key role in recognition of antigen cross-presented by stroma cells on MHC II molecules, leading to bystander killing and cancer regression (Qin and Blakneistein, 2000; Spiotto et al., 2004; Schietinger et al., 2010; Arina et al., 2017).

5.5 Off-target toxicity of isolated TGFβR2(-1)-specific TCRs

Generation of TCRs in AbabDR4 mice limited to express only one MHC II isotype brings some potential risk of alloreactivity, meaning, the T cell could recognize self-peptides present on allogeneic MHC molecules, other than the HLA presenting the cognate antigen (HLA-DR4 in this case). Since ABabDR4 mice lack human thymic negative selection restricted to other molecules than HLA-DR4 molecules (TCRs were only selected from the same human HLA-DR4 molecules in the mouse during T cell development), T cells with cross-reactive TCRs to the rest of MHC molecules cannot be deleted and could potentially cross react with pMHC of normal tissues (Hinrichs and Restifo, 2013). We examined this issue by co-culturing TCR-transduced T cells with a panel of LCLs expressing different class of HLA molecules (Table nr 2). No recognition has been reported in our analysis, which points out, on a safety profile of TCR1414_1 and TCR1414_2 (Figure 7).

5.6 Recognition of CRC cell lines by isolated TGFβR2(-1)-specific TCRs

Our study provides two TGFβR2(-1)-specific TCRs which recognized naturally expressed neoantigen TGFβR2(-1) by CRC cell lines SW48 and HCT116. TCR1414_1 showed slightly better performance in neoantigen recognition than TCR1414_2. This correlates positively with the experiment showing TCR1414_1/2 sensitivity to HLA-DR4-restricted peptide where TCR1414_1 and TCR1414_2 have been observed to be sensitive to TGFβR2(-1) peptide at IC50 ~10 nM and 1 nM, respectively. A possible explanation may rely on the fact that TCR1414_1 was generated assembling TCRα and TCRβ from the most dominant T cell clones after isolating them from the ABabDR4 mice's spleen, which suggests that they come from the most potent T cell clone. We observed that, upon co-culture with SW48-DR4 cell line, both TCRs stimulated them more efficiently than with HCT116-DR4, which is reflected in higher IFNy production by $CD4^+$ TCR-transduced T cells. The CRC recognition difference could possibly be due to the TGFβR2 gene expression level, which seem to be better in SW48 than HCT116 cell line based on electrophoresis data, where part of the gene of interest was amplified (Figure 6A). The MHC class II processing compartment efficiency may play a role as well. The TGFβR2(-1) neoantigen was endogenously processed and bound to HLA-DR4 molecules, further transported onto cell surface and presented to $CD4^+$ T cells. The presentation of the HLA-DR4-restricted-TGFβR2(-1)-specific epitope requires the HLA-DR4 molecule presence which HCT116 and SW48 do not naturally have on their surface, therefore, HLA-DR4 molecule was introduced to those CRC cell lines retrovirally. It would be ideal to test TGFβR2(-1)$^+$ and HLA-DR4$^+$ cells. Nevertheless, to our knowledge, HLA-DR*04:01-restricted processing and presentation of TGFβR2(-1) frameshift neoantigen was proved herein for the first time.

5.7 Importance of targeting TGFβR2(-1) neoantigen

The TGFβR2(-1) neoantigen frequency is high, reaching 77% of MSI-CRC patients and almost 72% of Lynch syndrome patients (Tougeron et al., 2009; Maby et al., 2015). The frameshift has been demonstrated to be present in other cancer types such as gastric cancer at frequencies of 65-71% and endometrium at 14-17% (Myeroff et al., 1995; seltarbase.org). Such high frequencies of shared neoantigen (called public neoantigen) allow treating more patients with immunotherapy, excluding at the same time the expensive and personalized approach to look for a unique antigen based on whole-exome and transcriptome sequencing analysis. As a consequence, a shorter time is required for preparation of therapeutic TCR-transduced T cells. Importantly, being truly tumor-specific, we reduce the risk of off-target toxicity, which led to severe neurological problems and brain damages, as well as 2 patient's deaths in clinical trials while targeting tumor-associated antigen MAGE-A3 by autologous TCR-engineered $CD8^+$ T cells. In fact, T cells transduced with TCRs recognized similar epitopes within MAGE-A12, MAGE-A2 MAGE-A6, some of which were found to be expressed in the brain (Morgan et al., 2013).

5.8 Closing remarks

TCR1414_1 and TCR1414_2 are the first DRB1*04:01-restricted receptors described so far with proven functional activity and safety characteristics. In conclusion, DRB1*04:01-restricted-TGFBR2(-1)-specific TCR1414_1 and TCR1414_2 isolated from ABabDR4 mice are promising candidates for immunotherapy. Ongoing studies will determine whether TCR1414_1 and TCR1414_2 are eligible for clinical trials.

ABBREVIATIONS

7-AAD	7-amino-actinomycin D
ACK	Ammonium-chloride-potassium
APC	Allophycocyanin
ATT	Adoptive T cell therapy
AIRE	Autoimmune regulator
β2m	β2-microglubulin
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EBV	Epstein-Barr virus
EBV-LPD	Epstein-Barr virus–positive lymphoproliferative disease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HLA	Human leukocyte antigen
HHD	HLA-A2-H-2D_b chimeric molecule
hTCM	Human T cell medium
IC50	Half-maximal inhibitory concentration
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin

LCL	Lymphoblastoid B cell line
MACS	Magnetic-activated cell sorting
MAGE	melanoma-associated antigen
MHC	Major Histocompatibility Complex
mTCM	Mouse T cell medium
NIH	National Institute of Health
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PBS	Phosphate-buffered saline
RAG	Recombination-activating gene
RMPI-1640	Roswell Park Memorial Institute 1640 Medium
TAP	Transporter associated with antigen processing
CLIP	Class II-associated invariant chain peptide
RACE	Rapid amplification of cDNA ends
TCR	T cell receptor
TGFβR2	Transforming growth factor beta receptor 2
TGFβR2(-1) deletion	Transforming growth factor beta receptor 2 with one adenine
Th	T helper
TRAV	T cell receptor α variable region
TRBV	T cell receptor β variable region
TIL	Tumour infiltrating lymphocyte
WT	Wild type

REFERENCES

Adams, N. M., Grassmann, S. & Sun, J. C. Clonal expansion of innate and adaptive lymphocytes. Nat Rev Immunol 1–14 (2020).

Arina, A., Karrison, T., Galka, E., Schreiber, K., Weichselbaum, R. R. & Schreiber, H. Transfer of allogeneic $CD4^+$ T cells rescues $CD8^+$ T cells in anti-PD-L1–resistant tumors leading to tumor eradication. Cancer Immunol Res 5, 127–136 (2017).

Arstila, T. P. A direct estimate of the human T cell receptor diversity. Science 286, 958–961 (1999).

Attaf, M., Legut, M., Cole, D. K. & Sewell, A. K. The T cell antigen receptor: the Swiss army knife of the immune system. J Clin Exp Immunol 181, 1–18 (2015).

Barker, J. N., Doubrovina, E., Sauter, C., Jaroscak, J. J., Perales, M. A., Doubrovin, M., Prockop, S. E., Koehne, G. & O'Reilly, R. J. Successful treatment of EBV-associated posttransplantation lymphoma after cord blood transplantation using third-party EBV-specific cytotoxic T lymphocytes. Blood 116, 5045–5049 (2010).

Beck, S., Geraghty, D., Inoko, H., and Rowen, L. (The MHC sequencing consortium). Complete sequence and gene map of a human major histocompatibility complex. Nature 401, 921-923 (1999).

Bendle, G. M., Haanen, J. B. & Schumacher, T. N. Preclinical development of T cell receptor gene therapy. Curr Opin Immunol 21, 209–214 (2009).

Bjorkman, P. J., Saper, M., Samraoui, B., Bennett, W. S., Strominger, J. & Wiley, D. C. Structure of the human class I histocompatibility antigen, HLA-A2. J Cancer Immunol 174, 6–19 (1987).

Blankenstein, T., Leisegang, M., Uckert, W. & Schreiber, H. Targeting cancer-specific mutations by T cell receptor gene therapy. Curr Opin Immunol 33, 112–119 (2015).

Brattain, M. G., Fine, W. D., Khaled, F. M., Thompson, J. & Brattain, D. E. Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Res 41, 1751–1756 (1981).

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. & Wiley, D. C. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364, 33–39 (1993).

Buermeyer, A., Deschenes, S., Baker, S. & Liskay, R. M. Mammalian DNA mismatch repair. Annu Rev Genet 33, 533–64 (1999).

Chen, X., Poncette, L. & Blankenstein, T. Human TCR-MHC coevolution after divergence from mice includes increased nontemplate-encoded CDR3 diversity. J Exp Med 214, 3417–3433 (2017).

Clay, T. M., Custer, M. C., Sachs, J., Hwu, P., Rosenberg, S. A. & Nishimura, M. I. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. J Immunol 163, 507–513 (1999).

Cohen, C. J., Li, Y. F., El-Gamil, M., Robbins, P. F., Rosenberg, S. A. & Morgan, R. A. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. Cancer Res 67, 3898–3903 (2007).

Cohen, C. J., Zhao, Y., Zheng, Z., Rosenberg, S. A. & Morgan, R. A. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. Cancer Res 66, 8878–8886 (2006).

Cohen, R., Rousseau, B., Vidal, J., Colle, R., Diaz, L. A. & André, T. Immune checkpoint inhibition in colorectal cancer: microsatellite instability and beyond. Targ Oncol 15, 11–24 (2020).

Davis, J. L., Theoret, M. R., Zheng, Z., Lamers, C. H. J., Rosenberg, S. A. & Morgan, R. A. Development of human anti-murine T-cell receptor antibodies in both responding and nonresponding patients enrolled in TCR gene therapy trials. Clin Cancer Res 16, 5852–5861 (2010).

Davis, M. M. & Bjorkman, P. J. T-cell antigen receptor genes and T-cell recognition. Nature 334, 395–402 (1988).

Engels, B., Cam, H., Schüler, T., Indraccolo, S., Gladow, M., Baum, C., Blankenstein, T. & Uckert, W. Retroviral vectors for high-level transgene expression in T lymphocytes. Hum Gene Ther 14, 1155–1168 (2003).

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S. J. C., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M. & Sadelain, M. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 543, 113–117 (2017).

Garcia, K. C., Degano, M., Stanfield, R. L., Brunmark, A., Jackson, M. R., Peterson, P. A., Teyton, L. & Wilson, I. A. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. Science 274, 209–219 (1996).

Geginat, J., Paroni, M., Maglie, S., Alfen, J. S., Kastirr, I., Gruarin, P., De Simone, M., Pagani, M. & Abrignani, S. Plasticity of human CD4 T cell subsets. Front Immunol 5, (2014).

Ghani, K., Wang, X., de Campos-Lima, P. O., Olszewska, M., Kamen, A., Rivière, I. & Caruso, M. Efficient human hematopoietic cell transduction using RD114- and GALV-pseudotyped retroviral vectors produced in suspension and serum-free media. Hum Gene Ther 20, 966–974 (2009).

Goff, S. L., Dudley, M. E., Citrin, D. E., Somerville, R. P., Wunderlich, J. R., Danforth, D. N., Zlott, D. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Klebanoff, C. A., Hughes, M. S., Restifo, N. P., Langhan, M. M., Shelton, T. E., Lu, L., Kwong, M. L. M., Ilyas, S., Klemen, N. D., Payabyab, E. C., Morton, K. E.,

Toomey, M. A., Steinberg, S. M., White, D. E. & Rosenberg, S. A. Randomized, prospective evaluation comparing intensity of lymphodepletion before adoptive transfer of tumor-infiltrating lymphocytes for patients with metastatic melanoma. J Clin Oncol 34, 2389-2397 (2016).

Gorer, P. A. The genetic and antigenic basis of tumour transplantation. J Pathol Bacteriol 44, 691–697 (1937).

Gotter, J., Brors, B., Hergenhahn, M. & Kyewski, B. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. J Exp Med 199, 155–166 (2004).

Greenberg, P. D., Cheever, M. A. & Fefer, A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-$1^{+}2^{-}$ lymphocytes. J Exp Med 154, 952–963 (1981).

Greenberg, P.D., Kern, D.E., and Cheever, M.A. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-$1^{+},2^{-}$ T cells. Tumor eradication does not require participation of cytotoxic T cells. J Exp Med 161, 1122-1134 (1985).

Gross, G., Waks, T. & Eshhar, Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc Natl Acad Sci U S A 86, 10024–10028 (1989).

Grupp, S. A., Kalos, M., Barrett, D., Aplenc, R., Porter, D. L., Rheingold, S. R., Teachey, D. T., Chew, A., Hauck, B., Wright, J. F., Milone, M. C., Levine, B. L. & June, C. H. Chimeric antigen receptor–modified T cells for acute lymphoid leukemia. N Engl J Med 368, 1509–1518 (2013).

Guo, C., Manjili, M. H., Subjeck, J. R., Sarkar, D., Fisher, P. B. & Wang, X.-Y. Therapeutic cancer vaccines: past, present and future. Adv Cancer Res 119, 421–475 (2013).

Heslop, H. E., Slobod, K. S., Pule, M. A., Hale, G. A., Rousseau, A., Smith, C. A., Bollard, C. M., Liu, H., Wu, M.-F., Rochester, R. J., Amrolia, P. J., Hurwitz, J. L., Brenner, M. K. & Rooney, C. M. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 115, 925-935 (2010).

Hinrichs, C. S. & Restifo, N. P. Reassessing target antigens for adoptive T-cell therapy. Nat Biotechnol 31, 999–1008 (2013).

Hung, C.-F., Wu, T. C., Monie, A. & Roden, R. Antigen-specific immunotherapy of cervical and ovarian cancer. Immunol Rev 222, 43–69 (2008).

Hsieh, C.-S., Lee, H.-M. & Lio, C.-W. J. Selection of regulatory T cells in the thymus. Nat Rev Immunol 12, 157–167 (2012).

Inderberg, E. M., Wälchli, S., Myhre, M. R., Trachsel, S., Almåsbak, H., Kvalheim, G. & Gaudernack, G. T cell therapy targeting a public neoantigen in microsatellite instable colon cancer reduces in vivo tumor growth. OncoImmunology 6, e1302631 (2017).

Ionov, Y., Peinado, M. A., Malkhosyan, S., Shibata, D. & Perucho, M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363, 558–561 (1993).

Jenkins, M. K., Chu, H. H., McLachlan, J. B. & Moon, J. J. On the Composition of the Preimmune repertoire of T cells specific for peptide–major histocompatibility complex ligands. Annu Rev of Immunol 28, 275–294 (2010).

Johnson, L. A., Morgan, R. A., Dudley, M. E., Cassard, L., Yang, J. C., Hughes, M. S., Kammula, U. S., Royal, R. E., Sherry, R. M., Wunderlich, J. R., Lee, C.-C. R., Restifo, N. P., Schwarz, S. L., Cogdill, A. P., Bishop, R. J., Kim, H., Brewer, C. C., Rudy, S. F., VanWaes, C., Davis, J. L., Mathur, A., Ripley, R. T., Nathan, D. A., Laurencot, C. M. & Rosenberg, S. A. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114, 535–546 (2009).

June, C. H., O'Connor, R. S., Kawalekar, O. U., Ghassemi, S. & Milone, M. C. CAR T cell immunotherapy for human cancer. Science 359, 1361–1365 (2018).

Jung, B., Staudacher, J. J. & Beauchamp, D. Transforming growth factor β superfamily signaling in development of colorectal cancer. Gastroenterology 152, 36–52 (2017).

Jung, D. & Alt, F. W. Unraveling V(D)J recombination: insights into gene regulation. Cell 116, 299–311 (2004).

Kalos, M., Levine, B. L., Porter, D. L., Katz, S., Grupp, S. A., Bagg, A. & June, C. H. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 3, 95ra73 (2011).

Kato, T., Matsuda, T., Ikeda, Y., Park, J.-H., Leisegang, M., Yoshimura, S., Hikichi, T., Harada, M., Zewde, M., Sato, S., Hasegawa, K., Kiyotani, K. & Nakamura, Y. Effective screening of T cells recognizing neoantigens and construction of T-cell receptor-engineered T cells. Oncotarget 9, 11009–11019 (2018).

Kedzierska, K. & Koutsakos, M. The ABC of major histocompatibility complexes and T cell receptors in health and disease. Viral Immunol 33, 160–178 (2020).

Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol 14, 377–391 (2014).

Kochenderfer, J. N., Wilson, W. H., Janik, J. E., Dudley, M. E., Stetler-Stevenson, M., Feldman, S. A., Maric, I., Raffeld, M., Nathan, D.-A. N., Lanier, B. J., Morgan, R. A. & Rosenberg, S. A. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. Blood 116, 4099–4102 (2010).

Komori, T., Okada, A., Stewart, V. & Alt, F. Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. Science 261, 1171–1175 (1993).

Laidlaw, B. J., Craft, J. E. & Kaech, S. M. The multifaceted role of $CD4^+$ T cells in $CD8^+$ T cell memory. Nat Rev Immunol 16, 102–111 (2016).

Le, D. T., Uram, J. N., Wang, H., Bartlett, B. R., Kemberling, H., Eyring, A. D., Skora, A. D., Luber, B. S., Azad, N. S., Laheru, D., Biedrzycki, B., Donehower, R. C., Zaheer, A., Fisher, G. A., Crocenzi, T. S., Lee, J. J., Duffy, S. M., Goldberg, R. M., de la Chapelle, A., Koshiji, M., Bhaijee, F., Huebner, T., Hruban, R. H., Wood, L. D., Cuka, N., Pardoll, D. M., Papadopoulos, N., Kinzler, K. W., Zhou, S., Cornish, T. C., Taube, J. M., Anders, R. A., Eshleman, J. R., Vogelstein, B. & Diaz, L. A. PD-1 blockade in tumors with mismatch-repair deficiency. N. Engl. J. Med. 372, 2509–2520 (2015).

Lee, D. W., Gardner, R., Porter, D. L., Louis, C. U., Ahmed, N., Jensen, M., Grupp, S. A. & Mackall, C. L. Current concepts in the diagnosis and management of cytokine release syndrome. Blood 124, 188–195 (2014).

Leibovltz, A., Stlnson, J. C., McCombs, W. B., McCoy, C. E., Mazur, K. C. & Mabry, N. D. Classification of human colorectal adenocarcinoma cell lines. Cancer Res 36, 4562-4569 (1976).

Leisegang, M., Engels, B., Meyerhuber, P., Kieback, E., Sommermeyer, D., Xue, S.-A., Reuß, S., Stauss, H. & Uckert, W. Enhanced functionality of T cell receptor-redirected T cells is defined by the transgene cassette. J Mol Med 86, 573–583 (2008).

Li, L.-P., Lampert, J. C., Chen, X., Leitao, C., Popović, J., Müller, W. & Blankenstein, T. Transgenic mice with a diverse human T cell antigen receptor repertoire. Nat Med 16, 1029–1034 (2010).

Li, L. & Blankenstein, T. Generation of transgenic mice with megabase-sized human yeast artificial chromosomes by yeast spheroplast–embryonic stem cell fusion. Nat Protoc 8, 1567–1582 (2013).

Linette, G. P., Stadtmauer, E. A., Maus, M. V., Rapoport, A. P., Levine, B. L., Emery, L., Litzky, L., Bagg, A., Carreno, B. M., Cimino, P. J., Binder-Scholl, G. K., Smethurst, D. P., Gerry, A. B., Pumphrey, N. J., Bennett, A. D., Brewer, J. E., Dukes, J., Harper, J., Tayton-Martin, H. K., Jakobsen, B. K., Hassan, N. J., Kalos, M. & June, C. H. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. Blood 122, 863–871 (2013).

Linnebacher, M., Gebert, J., Rudy, W., Woerner, S., Yuan, Y. P., Bork, P. & von Knebel Doeberitz, M. Frameshift peptide-derived T-cell epitopes: A source of novel tumor-specific antigens. Int. J. Cancer 93, 6–11 (2001).

Linnemann, C., van Buuren, M. M., Bies, L., Verdegaal, E. M. E., Schotte, R., Calis, J. J. A., Behjati, S., Velds, A., Hilkmann, H., Atmioui, D. el, Visser, M., Stratton, M. R., Haanen, J. B. A. G., Spits, H., van der Burg, S. H. & Schumacher, T. N. M. High-throughput epitope discovery reveals frequent recognition of neo-antigens by $CD4^+$ T cells in human melanoma. Nat Med 21, 81–85 (2015).

Maby, P., Tougeron, D., Hamieh, M., Mlecnik, B., Kora, H., Bindea, G., Angell, H. K., Fredriksen, T., Elie, N., Fauquembergue, E., Drouet, A., Leprince, J., Benichou, J., Mauillon, J., Le Pessot, F., Sesboue, R., Tuech, J.-J., Sabourin, J.-C., Michel, P., Frebourg, T., Galon, J. & Latouche, J.-B. Correlation between density of $CD8^+$ T-cell infiltrate in microsatellite unstable colorectal cancers and frameshift mutations: a rationale for personalized immunotherapy. Cancer Res 75, 3446–3455 (2015).

Malekzadeh, P., Pasetto, A., Robbins, P. F., Parkhurst, M. R., Paria, B. C., Jia, L., Gartner, J. J., Hill, V., Yu, Z., Restifo, N. P., Sachs, A., Tran, E., Lo, W., Somerville, R. P. T., Rosenberg, S. A. & Deniger, D. C. Neoantigen screening identifies broad TP53 mutant immunogenicity in patients with epithelial cancers. J Clin Invest 129, 1109–1114 (2019).

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R., Zborowska, E., Kinzler, K., Vogelstein, B. & et, al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268, 1336–1338 (1995).

Maude, S. L., Frey, N., Shaw, P. A., Aplenc, R., Barrett, D. M., Bunin, N. J., Chew, A., Gonzalez, V. E., Zheng, Z., Lacey, S. F., Mahnke, Y. D., Melenhorst, J. J., Rheingold, S. R., Shen, A., Teachey, D. T., Levine, B. L., June, C. H., Porter, D. L. & Grupp, S. A. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 371, 1507-1517 (2014).

McDonald, B. D., Bunker, J. J., Erickson, S. A., Oh-Hora, M. & Bendelac, A. Crossreactive αβ T cell receptors are the predominant targets of thymocyte negative selection. Immunity 43, 859–869 (2015).

Michie, A. M., Carlyle, J. R., Schmitt, T. M., Ljutic, B., Cho, S. K., Fong, Q. & Zúñiga-Pflücker, J. C. Clonal characterization of a bipotent T cell and NK cell progenitor in the mouse fetal thymus. J Immunol 164, 1730–1733 (2000).

Miller, J. F. A. P. The discovery of thymus function and of thymus-derived lymphocytes. Immunol Rev 185, 7–14 (2002).

Mitchison, N. A. Passive transfer of transplantation immunity. Nature 171, 267–268 (1953).

Mlecnik, B., Bindea, G., Angell, H. K., Maby, P., Angelova, M., Tougeron, D., Church, S. E., Lafontaine, L., Fischer, M., Fredriksen, T., Sasso, M., Bilocq, A. M., Kirilovsky, A., Obenauf, A. C., Hamieh, M., Berger, A., Bruneval, P., Tuech, J.-J., Sabourin, J.-C., Le Pessot, F., Mauillon, J., Rafii, A., Laurent-Puig, P., Speicher, M. R., Trajanoski, Z., Michel, P., Sesboüe, R., Frebourg, T., Pagès, F., Valge-Archer, V., Latouche, J.-B. & Galon, J. Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. Immunity 44, 698–711 (2016).

Monach, P. A., Meredith, S. C., Siegel, C. T. & Schreiber, H. A unique tumor antigen produced by a single amino acid substitution. Immunity 2, 45–59 (1995).

Morgan, R. A., Dudley, M. E., Wunderlich, J. R., Hughes, M. S., Yang, J. C., Sherry, R. M., Royal, R. E., Topalian, S. L., Kammula, U. S., Restifo, N. P., Zheng, Z., Nahvi, A., de Vries, C. R., Rogers-Freezer, L. J., Mavroukakis, S. A. & Rosenberg, S. A. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314, 126–129 (2006).

Murphy, K. and Weaver C. Janeway's immunobiology, 9th edition. New York: Garland Science. (2017).

Myeroff, L. L., Parsons, R., Kim, S.-J., Hedrick, L., Cho, K. R., Orth, K., Mathis, M., Kinzler, K. W., Lutterbaugh, J., Park, K., Bang, Y.-J., Lee, H. Y., Park, J.-G., Lynch, H. T., Roberts, A. B., Vogelstein, B. & Markowitz, S. D. A transforming growth factor β receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res 55, 5545–5547 (1995).

Neefjes, J., Jongsma, M. L. M., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol 11, 823–836 (2011).

Nielsen, J. S., Chang, A. R., Wick, D. A., Sedgwick, C. G., Zong, Z., Mungall, A. J., Martin, S. D., Kinloch, N. N., Ott-Langer, S., Brumme, Z. L., Treon, S. P., Connors, J. M., Gascoyne, R. D., Webb, J. R., Berry, B. R., Morin, R. D., Macpherson, N. & Nelson, B. H. Mapping the human T cell repertoire to recurrent driver mutations in MYD88 and EZH2 in lymphoma. Oncoimmunology 6, e1321184 (2017).

Nikolich-Žugich, J., Slifka, M. K. & Messaoudi, I. The many important facets of T-cell repertoire diversity. Nat Rev Immunol 4, 123–132 (2004).

Obenaus, M., Leitão, C., Leisegang, M., Chen, X., Gavvovidis, I., van der Bruggen, P., Uckert, W., Schendel, D. J. & Blankenstein, T. Identification of

human T-cell receptors with optimal affinity to cancer antigens using antigen-negative humanized mice. Nat Biotechnol 33, 402–407 (2015).

Parkhurst, M. R., Yang, J. C., Langan, R. C., Dudley, M. E., Nathan, D.-A. N., Feldman, S. A., Davis, J. L., Morgan, R. A., Merino, M. J., Sherry, R. M., Hughes, M. S., Kammula, U. S., Phan, G. Q., Lim, R. M., Wank, S. A., Restifo, N. P., Robbins, P. F., Laurencot, C. M. & Rosenberg, S. A. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol. Ther. 19, 620–626 (2011).

Patel, S. J., Sanjana, N. E., Kishton, R. J., Eidizadeh, A., Vodnala, S. K., Cam, M., Gartner, J. J., Jia, L., Steinberg, S. M., Yamamoto, T. N., Merchant, A. S., Mehta, G. U., Chichura, A., Shalem, O., Tran, E., Eil, R., Sukumar, M., Guijarro, E. P., Day, C.-P., Robbins, P., Feldman, S., Merlino, G., Zhang, F. & Restifo, N. P. Identification of essential genes for cancer immunotherapy. Nature 548, 537–542 (2017).

Philip, M., Fairchild, L., Sun, L., Horste, E. L., Camara, S., Shakiba, M., Scott, A. C., Viale, A., Lauer, P., Merghoub, T., Hellmann, M. D., Wolchok, J. D., Leslie, C. S. & Schietinger, A. Chromatin states define tumour-specific T cell dysfunction and reprogramming. Nature 545, 452–456 (2017).

Poncette, L., Chen, X., Lorenz, F. K. M. & Blankenstein, T. Effective NY-ESO-1–specific MHC II–restricted T cell receptors from antigen-negative hosts enhance tumor regression. J Clin Invest 129, 324–335 (2018).

Prehn, R.T., and Main, J.M. Immunity to methylcholanthrene-induced sarcomas. J Natl Cancer Inst 18, 769-778 (1957).

Principe, D. R., DeCant, B., Staudacher, J., Vitello, D., Mangan, R. J., Wayne, E. A., Mascariñas, E., Diaz, A. M., Bauer, J., McKinney, R. D., Khazaie, K., Pasche, B., Dawson, D. W., Munshi, H. G., Grippo, P. J. & Jung, B. Loss of TGFβ signaling promotes colon cancer progression and tumor-associated inflammation. Oncotarget 8, 3826–3839 (2016).

Principe, D. R., Doll, J. A., Bauer, J., Jung, B., Munshi, H. G., Bartholin, L., Pasche, B., Lee, C. & Grippo, P. J. TGF- : Duality of Function Between Tumor Prevention and Carcinogenesis. J Natl Cancer Inst 106, djt369–djt369 (2014).

Qin, Z. & Blankenstein, T. $CD4^+$ T cell–mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFNγ receptor expression by nonhematopoietic cells. Immunity 12, 677–686 (2000).

Quezada, S. A., Simpson, T. R., Peggs, K. S., Merghoub, T., Vider, J., Fan, X., Blasberg, R., Yagita, H., Muranski, P., Antony, P. A., Restifo, N. P. & Allison, J. P. Tumor-reactive $CD4^+$ T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med 207, 637–650 (2010).

Rapoport, A. P., Stadtmauer, E. A., Binder-Scholl, G. K., Goloubeva, O., Vogl, D. T., Lacey, S. F., Badros, A. Z., Garfall, A., Weiss, B., Finklestein, J., Kulikovskaya, I., Sinha, S. K., Kronsberg, S., Gupta, M., Bond, S., Melchiori, L., Brewer, J. E., Bennett, A. D., Gerry, A. B., Pumphrey, N. J., Williams, D., Tayton- Martin, H. K., Ribeiro, L., Holdich, T., Yanovich, S., Hardy, N., Yared, J., Kerr, N., Philip, S., Westphal, S., Siegel, D. L., Levine, B. L., Jakobsen, B. K., Kalos, M. & June, C. H. NY-ESO-1–specific TCR–engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat Med 21, 914–921 (2015).

Restifo, N. P., Marincola, F. M., Kawakami, Y., Taubenberger, J., Yannelli, J. R. & Rosenberg, S. A. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. J Natl Cancer Inst 88, 100–108 (1996).

Robbins, P. F., Morgan, R. A., Feldman, S. A., Yang, J. C., Sherry, R. M., Dudley, M. E., Wunderlich, J. R., Nahvi, A. V., Helman, L. J., Mackall, C. L., Kammula, U. S., Hughes, M. S., Restifo, N. P., Raffeld, M., Lee, C.-C. R., Levy, C. L., Li, Y. F., El-Gamil, M., Schwarz, S. L., Laurencot, C. & Rosenberg, S. A. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically

engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29, 917–924 (2011).

Robinson, J., Barker, D. J., Georgiou, X., Cooper, M. A., Flicek, P. & Marsh, S. G. E. IPD-IMGT/HLA Database. Nucleic Acids Res 48, D948–D955 (2020).

Roche, P. A. & Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. Nat Rev Immunol 15, 203–216 (2015).

Rosenberg, S. A., Spiess, P. & Lafreniere, R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science 233, 1318–1321 (1986).

Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C. & Seipp, C. A. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 319, 1676–1680 (1988).

Rosenberg, S. A. & Restifo, N. P. Adoptive cell transfer as personalized immunotherapy for human cancer. Science 348, 62–68 (2015).

Rosenberg, S. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Hughes, M. S., Phan, G. Q., Citrin, D. E., Restifo, N. P., Robbins, P. F., Wunderlich, J. R., Morton, K. E., Laurencot, C. M., Steinberg, S. M., White, D. E. & Dudley, M. E. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 17, 4550–4557 (2011).

Saeterdal, I., Bjorheim, J., Lislerud, K., Gjertsen, M. K., Bukholm, I. K., Olsen, O. C., Nesland, J. M., Eriksen, J. A., Moller, M., Lindblom, A. & Gaudernack, G. Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer. Proc Natl Acad Sci 98, 13255–13260 (2001).

Samelson, L. E., Lindsten, T., Fowlkes, B. J., van den Elsen, P., Terhorst, C., Davis, M. M., Germain, R. N. & Schwartz, R. H. Expression of genes of the T-cell antigen receptor complex in precursor thymocytes. Nature 315, 765–768 (1985).

Schietinger, A., Philip, M., Krisnawan, V. E., Chiu, E. Y., Delrow, J. J., Basom, R. S., Lauer, P., Brockstedt, D. G., Knoblaugh, S. E., Hämmerling, G. J., Schell, T. D., Garbi, N. & Greenberg, P. D. Tumor-specific T cell dysfunction is a dynamic antigen-driven differentiation program initiated early during tumorigenesis. Immunity 45, 389–401 (2016).

Schietinger, A., Philip, M., Liu, R. B., Schreiber, K. & Schreiber, H. Bystander killing of cancer requires the cooperation of $CD4^+$ and $CD8^+$ T cells during the effector phase. J Exp Med 207, 2469–2477 (2010).

Schietinger, A., Philip, M. & Schreiber, H. Specificity in cancer immunotherapy. Semin Immunol 20, 276–285 (2008).

Scholten, K. B. J., Kramer, D., Kueter, E. W. M., Graf, M., Schoedl, T., Meijer, C. J. L. M., Schreurs, M. W. J. & Hooijberg, E. Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. Clin Immunol 119, 135–145 (2006).

Schreiber, H. Cancer Immunology. In Fundamental Immunology, 7^{th} edition. Philadelphia, PA, USA: Pippincott Williams and Wilkins, a Wolters Kluwer business. (2013).

Schwarz, B. A. & Bhandoola, A. Trafficking from the bone marrow to the thymus: a prerequisite for thymopoiesis. Immunol Rev 209, 47–57 (2006).

Schwitalle, Y., Kloor, M., Eiermann, S., Linnebacher, M., Kienle, P., Knaebel, H. P., Tariverdian, M., Benner, A. & von Knebel Doeberitz, M. Immune response against frameshift-induced neopeptides in HNPCC patients and healthy HNPCC mutation carriers. Gastroenterology 134, 988–997 (2008).

Scott, A. C., Dündar, F., Zumbo, P., Chandran, S. S., Klebanoff, C. A., Shakiba, M., Trivedi, P., Menocal, L., Appleby, H., Camara, S., Zamarin, D., Walther, T., Snyder, A., Femia, M. R., Comen, E. A., Wen, H. Y., Hellmann, M. D., Anandasabapathy, N., Liu, Y., Altorki, N. K., Lauer, P., Levy, O., Glickman, M. S.,

Kaye, J., Betel, D., Philip, M. & Schietinger, A. TOX is a critical regulator of tumour-specific T cell differentiation. Nature 571, 270–274 (2019).

Sommermeyer, D., Neudorfer, J., Weinhold, M., Leisegang, M., Engels, B., Noessner, E., Heemskerk, M. H. M., Charo, J., Schendel, D. J., Blankenstein, T., Bernhard, H. & Uckert, W. Designer T cells by T cell receptor replacement. Eur J Immunol 36, 3052–3059 (2006).

Savage, P. A., Boniface, J. J. & Davis, M. M. A kinetic basis for T cell receptor repertoire selection during an immune response. Immunity 10, 485–492 (1999).

Sommermeyer, D. & Uckert, W. Minimal amino acid exchange in human TCR constant regions fosters improved function of TCR gene-modified T cells. J Immunol 184, 6223–6231 (2010).

Soneoka, Y., Cannon, P. M., Ramsdale, E. E., Griffiths, J. C., Romano, G., Kingsman, S. M. & Kingsman, A. J. A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res 23, 628–633 (1995).

Spiotto, M. T., Rowley, D. A. & Schreiber, H. Bystander elimination of antigen loss variants in established tumors. Nat Med 10, 294–298 (2004).

Spiotto, M. T. & Schreiber, H. Rapid destruction of the tumor microenvironment by CTLs recognizing cancer-specific antigens cross-presented by stromal cells. Cancer Immunol 5, (2005).

Stanislawski, T., Voss, R.-H., Lotz, C., Sadovnikova, E., Willemsen, R. A., Kuball, J., Ruppert, T., Bolhuis, R. L. H., Melief, C. J., Huber, C., Stauss, H. J. & Theobald, M. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat Immunol 2, 962–970 (2001).

Starr, T. K., Jameson, S. C. & Hogquist, K. A. Positive and negative selection of T cells. Annu Rev Immunol 21, 139–176 (2003).

Stromnes, I. M., Blattman, J. N., Tan, X., Jeevanjee, S., Gu, H. & Greenberg, P. D. Abrogating Cbl-b in effector $CD8^+$ T cells improves the efficacy of adoptive therapy of leukemia in mice. J Clin Invest (2010).

Strønen, E., Toebes, M., Kelderman, S., van Buuren, M. M., Yang, W., van Rooij, N., Donia, M., Böschen, M.-L., Lund-Johansen, F., Olweus, J. & Schumacher, T. N. Targeting of cancer neoantigens with donor-derived T cell receptor repertoires. Science 352, 1337–1341 (2016).

Theobald, M., Biggs, J., Dittmer, D., Levine, A. J. & Sherman, L. A. Targeting p53 as a general tumor antigen. Proc Natl Acad Sci U S A 92, 11993–11997 (1995).

Theobald, M., Biggs, J., Hernández, J., Lustgarten, J., Labadie, C. & Sherman, L. A. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. J Exp Med 185, 833–841 (1997).

Thibodeau, S., Bren, G. & Schaid, D. Microsatellite instability in cancer of the proximal colon. Science 260, 816–819 (1993).

Topp, M. S., Riddell, S. R., Akatsuka, Y., Jensen, M. C., Blattman, J. N. & Greenberg, P. D. Restoration of CD28 Expression in $CD28^-$ $CD8^+$ memory effector T cells reconstitutes antigen-induced IL-2 production. J Exp Med 198, 947–955 (2003).

Tougeron, D., Fauquembergue, E., Rouquette, A., Le Pessot, F., Sesboüé, R., Laurent, M., Berthet, P., Mauillon, J., Di Fiore, F., Sabourin, J.-C., Michel, P., Tosi, M., Frébourg, T. & Latouche, J.-B. Tumor-infiltrating lymphocytes in colorectal cancers with microsatellite instability are correlated with the number and spectrum of frameshift mutations. Mod Pathol 22, 1186–1195 (2009).

Tran, E., Turcotte, S., Gros, A., Robbins, P. F., Lu, Y.-C., Dudley, M. E., Wunderlich, J. R., Somerville, R. P., Hogan, K., Hinrichs, C. S., Parkhurst, M. R., Yang, J. C. & Rosenberg, S. A. Cancer immunotherapy based on mutation-specific $CD4^+$ T cells in a patient with epithelial cancer. Science 344, 641–645 (2014).

Tran, E., Robbins, P. F., Lu, Y.-C., Prickett, T. D., Gartner, J. J., Jia, L., Pasetto, A., Zheng, Z., Ray, S., Groh, E. M., Kriley, I. R. & Rosenberg, S. A. T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. N Engl J Med 375, 2255–2262 (2016).

Tsuji, T., Yasukawa, M., Matsuzaki, J., Ohkuri, T., Chamoto, K., Wakita, D., Azuma, T., Niiya, H., Miyoshi, H., Kuzushima, K., Oka, Y., Sugiyama, H., Ikeda, H. & Nishimura, T. Generation of tumor-specific, HLA class I–restricted human Th1 and Tc1 cells by cell engineering with tumor peptide–specific T-cell receptor genes. Blood 106, 470–476 (2005).

van Zelm, M. C., Reisli, I., van der Burg, M., Castaño, D., van Noesel, C. J. M., van Tol, M. J. D., Woellner, C., Grimbacher, B., Patiño, P. J., van Dongen, J. J. M. & Franco, J. L. An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med 354, 1901–1912 (2006).

Veatch, J. R., Lee, S. M., Fitzgibbon, M., Chow, I.-T., Jesernig, B., Schmitt, T., Kong, Y. Y., Kargl, J., Houghton, A. M., Thompson, J. A., McIntosh, M., Kwok, W. W. & Riddell, S. R. Tumor-infiltrating BRAFV600E-specific $CD4^+$ T cells correlated with complete clinical response in melanoma. J Clin Invest 128, 1563–1568 (2018).

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A. & Kinzler, K. W. Cancer Genome Landscapes. Science 339, 1546–1558 (2013).

van den Broek, T., Borghans, J. A. M. & van Wijk, F. The full spectrum of human naive T cells. Nat Rev Immunol 18, 363–373 (2018).

von Boehmer, H., Teh, H. S. & Kisielow, P. The thymus selects the useful, neglects the useless and destroys the harmful. Immunol Today 10, 57–61 (1989).

Wang P., Sidney J., Dow C., Mothé B., Sette A., Peters B. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol. 4, e1000048 (2008).

Wang, P., Sidney J., Kim Y., Sette A., Lund O., Nielsen M., Peters B. Peptide binding predictions for HLA DR, DP and DQ molecules. BMC Bioinformatics 11, 568 (2010).

Wilson, A., MacDonald, H. R. & Radtke, F. Notch 1–deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med 194, 1003–1012 (2001).

Woerner, S. M., Kloor, M., von Knebel Doeberitz, M. & Gebert, J. F. Microsatellite instability in the development of DNA mismatch repair deficient tumors. Cancer Biomark 2, 69–86 (2006).

Woerner, S. M., Yuan, Y. P., Benner, A., Korff, S., von Knebel Doeberitz, M. & Bork, P. SelTarbase, a database of human mononucleotide-microsatellite mutations and their potential impact to tumorigenesis and immunology. Nucleic Acids Res 38, D682–D689 (2010).

Yamamoto, T. N., Kishton, R. J. & Restifo, N. P. Developing neoantigen-targeted T cell–based treatments for solid tumors. Nat Med 25, 1488–1499 (2019).

Zhao, Y., Zheng, Z., Cohen, C. J., Gattinoni, L., Palmer, D. C., Restifo, N. P., Rosenberg, S. A. & Morgan, R. A. High-efficiency transfection of primary human and mouse T lymphocytes using RNA electroporation. Mol Ther 13, 151–159 (2006).

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PUBLICATIONS

Plewa, N., Poncette L., Blankenstein T. Generation of TGFβR2(-1) neoantigen-specific HLA-DR4-restricted T cell receptors for cancer therapy. – to be submitted

Schachtner, H., Weimershaus, M., Stache, V., **Plewa, N**., Legler, D. F., Höpken, U. E. & Maritzen, T. Loss of Gadkin Affects Dendritic Cell Migration In Vitro. PLOS ONE 10**,** e0143883 (2015).

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APPENDIX

TGFβR2(-1) minigene:

ATG GCT GCT TCT CCA AAG TGC ATT ATG AAG GAA AAA AAA AGC CTG GTG AGA CTT
M A A S P K C I M K E K K **S L V R L**

TCT TCA TGT GTT CCT GTA GCT CTG ATG AGT GCA ATG ACA ACA TCA TCT TCT CAG
S S C V P V A L M S A M T T S S S Q

AAG AAT ATA ACA CCA GCA ATC CTG ACT TGT TGC TAG
K N **I T P A I L T C C** stop

Appendix 1. Nucleotide sequence of TGFβR2(-1) minigene and encoded amino acids sequence. TGFβR2(-1) minigene encodes 47 amino acids containing TGFβR2(-1) frameshift peptide SLVRLSSCVPVALMSAMTTSSSQKNITPAILTC (highlighted in bold) with HLA-DRB1*04:01-resctricted epitope of interest VALMSAMTTSSSQKN highlighted in green.