Comparing the therapeutic potential of T cell receptors from tumors and healthy donors for adoptive T cell therapy in cancer

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

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Summary

The infiltration of T cells into the tumor tissue is in many cases insufficient to prevent tumor progression. In addition, tumor-infiltrating lymphocytes (TILs) in the tumor are chronically exposed to high levels of tumor antigen, which can lead to their elimination. Therefore, it is unclear how well T cells with therapeutically effective T cell receptors (TCRs) are represented in progressively growing tumors. For the successful treatment of cancer by adoptive transfer of TCR-engineered T cells (TCR-Ts), it is crucial to use TCRs of sufficiently high affinity. To determine whether the quality of tumor antigen-specific TCRs from TILs is generally inferior to that of T cells generated from antigen-negative donors, I used TCRs recognizing either of two well-characterized MHC-I-presented neoantigens. First, I analyzed a panel of TCRs directed against a neoantigen from the mutated gene cyclin-dependent kinase 4 (CDK4). These TCRs were derived from TILs, healthy donors, or from mice with human TCR gene loci. On the other hand, I isolated TCRs specific for a neoantigen derived from the mutated gene p68 (mp68) from tumor-bearing and tumor-free, immunized mice. To induce the expansion of mp68-specific T cell clones in tumor-bearing mice, I developed a cancer model in which antigen expression could be induced 3 weeks after tumor cell transplantation, thus preventing priming of mp68-specific T cells during transplantation-associated inflammation. I transferred all TCRs into donor T cells to evaluate their ability to secrete cytokines after co-culture with target cells as well as their potential to kill tumor cells in vitro. To assess the therapeutic efficacy of the TCRs, I performed adoptive T cell transfer on tumor-bearing mice. I found that the in vitro experiments that best predicted in vivo tumor control were long-term cytotoxicity assays. Furthermore, I observed that both, TIL- and immunization-derived TCRs were of variable quality which underscores the importance of testing TCRs experimentally before therapy. Most importantly, I could show that the majority of TIL-derived TCRs were therapeutically effective, and that their quality was not inferior to that of healthy donor-derived TCRs. Therefore, my results support the use of TIL-derived TCRs for adoptive transfer of TCR-Ts directed against neoantigens.

Zusammenfassung

Die Infiltration von T-Zellen in das Tumorgewebe reicht in vielen Fällen nicht aus, um das Tumorwachstum zu verhindern. Darüber hinaus sind tumorinfiltrierende Lymphozyten (TILs) in der Tumorumgebung chronisch hohen Mengen an Tumorantigen ausgesetzt, was zu ihrer Eliminierung führen kann. Es ist daher unklar, wie gut T-Zellen mit therapeutisch effektiven T-Zellrezeptoren (TCRs) in fortschreitend wachsenden Tumoren vertreten sind. Eine hohe Affinität der ausgewählten TCRs ist entscheidend für die erfolgreiche Behandlung von Krebs durch den adoptiven Transfer von TCR-manipulierten T-Zellen (TCR-Ts). Um festzustellen, ob die Qualität von Tumorantigen-spezifischen TCRs aus TILs im Allgemeinen schlechter ist als die von T-Zellen, die aus Antigen-negativen Spendern erzeugt wurden, habe ich TCRs verwendet, die eines von zwei gut charakterisierten MHC-I-präsentierten Neoantigenen erkennen. Zunächst analysierte ich eine Reihe von TCRs, die gegen ein Neoantigen des mutierten Gens Cyclin-abhängige Kinase 4 (CDK4) gerichtet sind und von TILs, gesunden Spendern oder von Mäusen mit humanen TCR-Genloci stammten. Andererseits isolierte ich TCRs, die für ein Neoantigen des mutierten Gens p68 (mp68) spezifisch sind, aus tumortragenden und tumorfreien, immunisierten Mäusen. Um die Expansion mp68-spezifischer T-Zellklone in tumortragenden Mäusen zu induzieren, habe ich ein Tumormodell entwickelt, in dem die Antigenexpression drei Wochen nach der Tumorzelltransplantation induziert werden konnte, wodurch das Priming von mp68-spezifischen T-Zellen während einer transplantationsassoziierten Entzündung verhindert wurde. Ich habe alle TCRs in Spender T-Zellen übertragen, um deren Fähigkeit zur Sekretion von Zytokinen nach der Co-Kultur mit Zielzellen sowie ihr Potenzial zur Abtötung von Tumorzellen in vitro zu bewerten. Um die therapeutische Wirksamkeit der TCRs zu beurteilen, führte ich T-Zelltherapien an tumortragenden Mäusen durch. Zunächst beobachtete ich, dass die Tumorkontrolle am besten durch Langzeit-Zytotoxizitätstests vorhergesagt wurde und dass die Qualität der TCRs sowohl aus TILs als auch aus tumorfreien Spendern variierte, was die Bedeutung der Testung aller TCRs vor der Therapie unterstreicht. Endlich zeigte ich, dass die Mehrzahl der TCRs aus TILs therapeutisch wirksam war und dass ihre Qualität dieser aus gesunden Spendern nicht unterlegen war. Daher unterstützen meine Ergebnisse die Verwendung von TCRs aus TILs für den adoptiven Transfer von TCR-Ts, die gegen Neoantigene gerichtet sind.

Introduction

The following introduction will give an overview on the shaping of the T cell receptor repertoire, introduce some basic concepts of T cell biology, give an overview on the most common immunotherapy treatments for cancer in the clinical and preclinical phases, describe the types of T cell antigens and how they are presented on target cells and lastly describe in more detail the three main sources currently used for tumor-specific TCR sequences. These sources will be the basis of the presented thesis since TCRs obtained from all three will be compared to each other. This aims to answer the question whether high-affinity TCRs can be found among TILs despite their long-term exposure to their antigen. I hypothesize that indeed, high-affinity TCRs can be among TILs. This question has relevance for the field of TCR engineered adoptive T cell therapy as the selection of the correct source and sequence of TCRs is crucial for therapy success.

The T cell receptor repertoire

The following paragraphs describe textbook knowledge on T cell biology that can be found for example in the textbook "Immunology" (Murphy, Travers, and Walport 2009).

The complete set of different T cell receptors (TCR) present at a given time in an individual is referred to as the TCR repertoire. It contains about $1x10^8$ different TCR specificities that were generated by VDJ recombination during T cell development (Murphy, Travers, and Walport 2009). Within an individual's repertoire a specific TCR can be present on only few or on many T cells. The latter is usually a result of clonal expansion which takes place after antigen encounter in the periphery. The TCR repertoire is generated by random TCR gene rearrangements and is therefore independent of present antigens. However, antigen encounter can shape the prevalence of certain clonotypes due to clonal expansion. An expanded clone can therefore give information on past infections. However, the details of the relationship between clone size and prior antigen encounter are not clear. For example, a subdominant antigen may have been present at an earlier time point but may not have induced clonal expansion.

V(D)J recombination

A TCR is a heterodimer of an α and β TCR chain. Each of these consists of an N-terminal variable and a C-terminal constant region. The variable region is created by

somatic recombination from a set of different V and J gene segments. The TCR β locus additionally contains two D gene segments that are located between the V and the J segments. The somatic recombination is dependent on the enzymes Rag1 and Rag2. A second layer of receptor diversity is created by the addition or removal of nucleotides between the gene segments with an enzyme called terminal deoxynucleotidyl transferase (TdT). These nucleotides are called N nucleotides. In summary, a TCR's specificity is defined by the identity of its α and β V and J segment and its N nucleotides. The TCRs' ligands on target cells are major histocompatibility complexes (MHC) class one or two (MHC-I and MHC-II) bound to short peptide sequences, the antigens of T cells. The most relevant regions for the interaction with the peptide-MHC (pMHC) complex are three hypervariable regions called complementarity-determining regions (CDR) 1, 2 and 3. CDR1 and 2 are located within the V segment and interact with the MHC complex whereas CDR3 lies between V and J segment. CDR3 is central for TCR specificity since it is the main interaction partner of the antigen. In fact, attempts to predict a TCR's target antigen by analyzing its CDR3 sequence in silico have been made (Chiou et al. 2021; Glanville et al. 2017).

Thymic selection

Somatic rearrangement and N nucleotide insertions create a large variety of TCRs. However, these TCRs are not necessarily functional. Therefore, T cells have to pass several selection steps in the thymus before they can be released to the periphery. After TCR β chain rearrangements are concluded, T cells continue to produce different TCR α chains until a productive TCR α β pair is produced. CD4 CD8 double positive thymocytes then go through positive and negative selection. Depending on whether they bind to MHC-I or MHC-II, they will become CD8 or CD4 single positive lymphocytes. Positive selection eliminates T cells that fail to bind to a self-peptide-MHC and are therefore not functional. It is generally assumed that negative selection, also termed central tolerance, eliminates T cells that show too high avidity to self-peptide on MHC and would therefore cause autoimmune reactions in the periphery. An exception are regulatory T cells (Tregs), that recognize self-peptide with high avidity but function by inducing peripheral tolerance in other lymphocytes (Hsieh et al. 2006; Pacholczyk et al. 2007; Moran et al. 2011). Furthermore, recent evidence suggests that self-reactive $CD8^+$ T cells are also not deleted in the thymus but evicted from the thymus prematurely (Badr et al. 2023).

The TCR repertoire in the periphery

The thymus is not the last instance that modifies the TCR repertoire. Also in the periphery, the TCR repertoire can still change because TCR clones can disappear due to clonal deletion at their first antigen encounter or later after activation. On the other hand, T cells may also become dysfunctional instead of being deleted. Although a dysfunctional TCR clone cannot react to its antigen and is therefore de facto functionally absent, the respective T cell and TCR gene sequence are still present and can be detected and characterized experimentally. Therefore, dysfunctional T cells are still part of the peripheral TCR repertoire. On the other hand, clones that are deleted in the periphery are lost to the TCR repertoire and their TCR sequence cannot be identified anymore.

T cell biology

T cells can be divided into cytotoxic T cells, helper T cells and regulatory T cells identified by the markers CD8, CD4 and CD4 with FOXP3, respectively. $CD8^+$ T cells recognize antigen presented on MHC-I and kill the cell that presents foreign antigen on MHC-I. $CD4^+$ T cells recognize antigen on MHC-II which is only expressed by antigen presenting cells. Helper T cells activate macrophages and B cells and induce class switching in B cells. Regulatory T cells suppress other T cells as described above.

Priming of $CD8^+$ T cells

Priming of naïve T cells is the necessary first step to turn them into effector T cells and in the case of $CD8^+$ T cells into cytotoxic T cells. Priming of $CD8^+$ T cells is realized either by dendritic cells (DCs) (cross-priming) (Bevan 1976; Steinman and Witmer 1978; Joffre et al. 2012) or by tumor cells (direct priming) (Wolkers et al. 2001; Christopher C Norbury and Sigal 2003; Heath and Carbone 2001; Ochsenbein et al. 2001). Direct priming by tumor cells seems to depend on the expression of CD80 (B7-1) by the tumor cells (Schoenberger et al. 1998). Antigen expression levels, tissue type and tissue damage are suspected to be relevant for the type and outcome of priming(Heath and Carbone 2001). Cross-priming by DCs is the more studied of the two mechanisms. It requires three consecutive signals that are only provided if antigen was taken up by an activated mature DC (Kurts, Robinson, and Knolle 2010). The first signal is provided by the binding of the TCR and the CD3 and CD8 coreceptors to the pMHC complex. This requires the presence of the antigen in question and its uptake by DCs. Furthermore, the DC must be able to process the exogenous antigen and load

it onto its MHC-I molecules which are normally reserved for endogenous antigens. It has been demonstrated that there can be a transfer of tumor antigens from migratory DCs to resident DCs in the lymph nodes that is facilitated by inflammation (Heath and Carbone 2009). According to current research only type one conventional DCs either migratory or resident (expressing CD103 or CD8α and lacking CD11b in mice) can successfully cross-prime $CD8^+$ T cells in the lymph node whereas type two conventional DCs prime $CD4^+$ T cells (Ruhland et al. 2020; Roberts et al. 2016; Kurts, Robinson, and Knolle 2010). Ideally, the DCs must have been licensed by danger signals binding, for example, to their toll-like receptors (TLRs) or by T helper cells through CD40-CD40L interaction. This leads to the maturation of DCs and upregulation of the B7 molecules CD80 or CD86 which bind to CD28 on T cells. This interaction represents the co-stimulation during priming and is also called signal two. It amplifies and sustains TCR signaling and induces the expression of further co-stimulatory molecules such as 4-1BB. TCR and CD28 stimulation induce IL-2 production which induces cell proliferation in an autocrine manner. Cross-priming is reviewed in Kurts et al (Kurts, Robinson, and Knolle 2010). The third signal consists of cytokines such as type one interferons (IFN α and β) and IL-12 (Curtsinger, Lins, and Mescher 2003; Curtsinger and Mescher 2010). IL-2 has also been shown to be able to replace costimulatory signals from the innate immune system (Mueller, Jenkins, and Schwartz 1989; Boussiotis et al. 1994). Lack of signals two or three can lead to reduced functionality of primed T cells. For example, it has been shown that without IL-12, CD8 T cells still undergo clonal expansion but fail to develop effector functions (Curtsinger, Lins, and Mescher 2003). More specifically, IL-12 during priming induces Tbet expression in $CD8^+$ T cells which leads to differentiation into short-lived effector cells whereas the absence of IL-12 creates memory precursor effector cells (Joshi et al. 2007). IFNα has been shown to improve the anti-tumor response of $CD8^+$ T cells after peptide vaccination (Sikora et al. 2009).

Overall, T cell priming is a highly complex and finely regulated process. It can however be summarized in the concept that effective priming requires a cascade of immune cell interactions and cytokine stimuli that originate from the danger signals and inflammation produced for example by an infection (Matzinger 2002). In the case of tumors, such signals are often lacking, therefore preventing DC activation and productive priming of T cells (Cuenca et al. 2003; Staveley-O'Carroll et al. 1998; Willimsky and Blankenstein 2005).

Peripheral tolerance

Additional layers of tolerance induction in the periphery complement the central tolerance because some self-reactive T cells may still leave the thymus. It is crucial for tolerating the mucosal microflora and food antigens as well as for preventing inflammation-induced autoimmunity. However, in the context of tumors, peripheral tolerance can prevent tumor rejection and in contrast to central tolerance, it can apply not only to self but also foreign antigens such as tumor neoantigens. Tolerance induction can happen at all stages of T cell differentiation and results in unresponsiveness or deletion of the T cell. Before priming, naïve T cells are actively maintained in a state of quiescence in which they are unresponsive to tonic TCR stimulation. This can be described as the first instance of peripheral tolerance. Quiescence applies to T cells across all antigen specificities and is controlled by several transcription factors. Their expression is required for the prevention of autoimmunity (ElTanbouly and Noelle 2021). On the other hand, self-specific T cells can also remain tolerant due to ignorance. This type of tolerance is caused by low TCR affinity, low antigen density or the expression of the antigen in immune-privileged sites (ElTanbouly and Noelle 2021). The most important type of peripheral tolerance concerning tumor immunology happens during T cell priming. Immunogenic tumors induce the expansion of tumor-specific unresponsive T cells that were likely rendered anergic during tolerogenic priming (Willimsky et al. 2008; Willimsky and Blankenstein 2005). If T cells are primed by immature DCs without receiving co-stimulation, this can result in deletion or anergy. The TCR stimulation without co-stimulation leads to the transcriptional activity of NFAT1 but not AP-1 whose localization to the nucleus is only induced after co-stimulation. NFAT1 without AP-1 activates the transcription of a distinct set of target genes from those targeted by NFAT1 and AP-1 together and ultimately leads to an anergic phenotype. NFAT is therefore a regulator of T cell dysfunction and exhaustion (Macián et al. 2002; Macian 2005). Anergic cells produce less cytokines such as IL-2, IFNγ and TNF in response to antigen but the anergic state can be reversed by removal of the antigenic stimulus (ElTanbouly and Noelle 2021). On the other hand, tolerogenic priming can also lead to deletion (Rocha and Von Boehmer 1991). The proapoptotic factor BIM is responsible for inducing apoptosis in response to priming without signal two, whereas signal two upregulates the anti-apoptotic factor BCL-X_L (Davey et al. 2002; Boise et al. 1995). However, when exactly tolerogenic priming leads to anergy and when to apoptosis is still unclear. Finally, the prolonged exposure to antigen during chronic infections or in tumors can lead to the

exhaustion of effector T cells or to activation-induced cell death (AICD) (Schietinger and Greenberg 2014; Scott et al. 2019). In this case, apoptosis is induced by the extrinsic pathway via FAS receptor (Dhein et al. 1995), TNFR1, TRAILR1 and TRAILR2 (Peter H. Krammer 2000; Krammer, Arnold, and Lavrik 2007). T cells are sensitive to those signals when they were exposed to IL-2 but not if they have been co-stimulated through CD28 (Goodnow 1996). Antigen persistence for more than 10 days have been shown to have inhibitory effects on T cells (Corse, Gottschalk, and Allison 2011). Similarly, when HY-antigen specific T cells were transferred into male mice, they were rendered dysfunctional by day 9 despite initial expansion (Rocha and Von Boehmer 1991).The different types of intrinsic peripheral tolerance are reviewed in (ElTanbouly and Noelle 2021).

Apart from the described above, Tregs can also induce peripheral tolerance in conventional T cells. Their defining lineage marker is FOXP3 (Fontenot, Gavin, and Rudensky 2003). Tregs are often present in tumors in high abundance and in many cancers, a high Treg to Teff ratio has been shown to correlate to poor prognosis (Gooden et al. 2011; Oleinika et al. 2013). They induce tolerance by consuming IL-2, killing cytotoxic T cells and releasing anti-inflammatory cytokines (Plitas and Rudensky 2020). Additionally, Tregs express CTLA-4 which binds to CD80 and CD86 on APCs and induces their transendocytosis. This leads to lower density of the co-stimulatory molecules CD80 and CD86 on APCs (Borst et al. 2021). The Treg lineage can be induced in the thymus after high-avidity self recognition (tTregs) or in the periphery after certain tolerogenic priming conditions (pTregs). Whereas the former would induce tolerance to self-antigens, the latter would do so for foreign antigens, both being of relevance for anti-tumor immunity (reviewed by (Plitas and Rudensky 2020)). Tregs are absolutely required for host survival which has been demonstrated in FOXP3 loss-of-function mice and humans with FOXP3 mutations (Fontenot, Gavin, and Rudensky 2003; Khattri et al. 2003; Bennett et al. 2001). The FOXP3 lacking individuals develop lethal autoimmunity showing that central tolerance alone is by no means sufficient for the prevention of autoimmunity.

Overall, a fine balance between T cell activation and tolerization is maintained in the periphery by T cell intrinsic and extrinsic mechanisms that are still not entirely understood.

TCR affinity, avidity and functional avidity

In T cell biology, different terms are used to refer to the binding strength of the TCR; these are affinity, avidity, and functional avidity. TCR affinity is measured by plasmon resonance, represented by the K_D value, and describes the binding strength of a single TCR binding to a single pMHC molecule (Campillo-Davo, Flumens, and Lion 2020; This et al. 2021; Zhong et al. 2013). TCR avidity, on the other hand, is measured by multimer binding to TCRs expressed on T cells (Campillo-Davo, Flumens, and Lion 2020; This et al. 2021; Zhong et al. 2013). Multimers are reagents that label T cells with TCRs of defined specificities for FACS analysis. Apart from the TCR affinity, the parameter of avidity is influenced by the TCR density on a T cell as well as the engagement of CD8 as a coreceptor for multimer binding. Thirdly, functional avidity is measured by T cell effector functions such as cytokine release or target cell killing (Campillo-Davo, Flumens, and Lion 2020; This et al. 2021; Zhong et al. 2013). Functional avidity results from a combination of TCR affinity, coreceptor engagement, TCR and antigen density as well as T cell activation status and phenotype. Therefore, although TCR affinity is the only of the three parameters that is independent of TCR coreceptors, T cell phenotype and antigen density, it is rarely measured since most experiments of TCR quality use T cell reactivity as a read-out. The three parameters described above are defined in two reviews by Campillo-Davo and This and the differences between them are evaluated experimentally by Zhong and colleagues (Campillo-Davo, Flumens, and Lion 2020; This et al. 2021; Zhong et al. 2013). Zhong et al compared a set of gp100-specific TCRs whose affinity ranged between a K_D of 1 to 100 μM and showed that the relationship between affinity and avidity was not always linear. At affinities below 10 μM, both avidity and functional avidity reached a plateau, meaning diminishing returns with TCRs of very high affinity. On the other hand, the relationship between avidity and functional avidity was linear in the case of cytokine and calcium release but plateaued with high-avidity TCRs when cytotoxicity was measured in vitro and in vivo (Zhong et al. 2013). These observations highlight that there is a limit to the effect of high-affinity TCRs on functional avidity and especially cytotoxicity and that affinity and avidity are not equivalent, especially for very high-affinity TCRs.

Importantly these definitions are impractical for this thesis because I focus particularly on the quality of TCRs when they are removed from the context of a specific T cell, i.e. when they are transduced into homogenously activated T cells. Therefore, although I have not measured K_D values, I have decided to use the term affinity when describing

the quality of the TCRs in the case that all TCRs were introduced into the same donor T cells. Since most likely the TCR affinity is the only parameter that differs in T cells transduced with different TCRs, I deduce a higher TCR affinity from improved functional avidity of the TCR-Ts. Importantly though, functional avidity of TCR-Ts may change over time, for example during in vivo experiments where T cells circulate inside of recipient mice for weeks. Furthermore, when describing the quality of TCRs derived from different backgrounds such as tumor tissue, I will also describe them in terms of affinity because their functional avidity is clearly impaired by the T cell dysfunction. Since the focus of my thesis is on the TCR quality removed from the context of its (dysfunctional) T cell, I will use the term affinity despite not having measured K_D values. Finally, I will use the terms avidity and functional avidity interchangeably, mostly meaning functional avidity since I did not assay the mean fluorescence intensity (MFI) of tetramer staining.

T cell receptor affinity and peripheral tolerance

The T cell receptor affinity is relevant for the degree of reactivity that a cytotoxic T cell shows towards an antigen. It has direct impact on the cytotoxic response, the degree of sensitivity to different antigen concentrations on a target cell, the proliferation and the exhaustion of a T cell (Zeh et al. 1999). Furthermore, there has been some research on the role of TCR and antigen affinity in tolerance induction in the periphery. The group of Linda Sherman showed that high-affinity ligands and high-affinity T cell clones are advantageous for the survival of T cells in a tolerogenic setting in vivo because they are rendered anergic at the first antigen contact and do not respond to restimulation (Redmond, Marincek, and Sherman 2005; Smith et al. 2017, 2014). Furthermore, they showed that high-affinity TCRs can better home to tumors, survive in the tumor microenvironment and eradicate tumors than low-affinity TCRs (Bos et al. 2012). On the other hand, affinity-matured TCRs with higher than physiological affinities have been shown to be deleted through AICD (Engels et al. 2012). The TCR repertoire can be modulated by factors such as faster expansion of high-affinity clones, loss of weak clones that fail to become activated and possible deletion of very high-affinity clones. Secondary immune responses have on average higher affinity TCRs than primary responses. This argues for the survival or positive selection of high-affinity TCRs (D. H. Busch and Pamer 1999; Savage, Jay Boniface, and Davis 1999). Similarly, although weak TCR antigen interactions have been shown to be sufficient for the generation of effector and memory T cells, only strong TCR signaling (strong

interaction with the antigen) could sustain T cell expansion (Zehn, Lee, and Bevan 2009). More specifically, the weaker interaction allowed similar levels of initial proliferation but the T cells reached a plateau and started contracting sooner (Zehn, Lee, and Bevan 2009). TCR affinity has been shown to be inversely correlated to pMHC affinity probably in order to obtain the optimal TCR-pMHC binding strength for each antigen (Anderton et al. 2001). T cell exhaustion (the expression of TOX) as a result of sustained TCR signaling protects against deletion (Scott et al. 2019). This also argues in favor of the survival of high-affinity T cell clones in the tumor environment (Scott et al. 2019). Since TOX induces the expression of PD-1 and other inhibitory receptors on T cells, it is not surprising that restricted TCR signaling by PD-1 expression is required for survival of high-affinity TCR clones. This hypothesis was supported by the observation that PD-1 negative T cells were shown to only harbor low-affinity TCRs in cancer patients (Simon et al. 2016). In mice, PD-1 was shown to preferentially suppress low-affinity TCR clones and in turn, PD-1 $^{-/-}$ mice had lower affinity TCRs than WT mice after immunization with tumor cells (Shimizu et al. 2021). This might be another explanation for the observation of Simon et al in humans, namely that PD-1 is required for the survival of high-affinity clones.

Cytotoxic effector mechanisms

Cytotoxic T cells can kill their target cells by the release of cytotoxic molecules (degranulation). The most important ones are perforin which can create pores in the cell membrane and granzymes which are proteases that will enter the target cells through the created pores and induce apoptosis (Russell and Ley 2002). Granzymes can also kill intracellular bacteria and inhibit viral replication (Russell and Ley 2002). Additionally, cytotoxic T cells express FAS ligand, also called CD95 ligand or APO-1 ligand, which is a membrane-bound protein. It binds to FAS receptors on target cells and induces apoptosis (Peter H. Krammer 2000). The release of IFNγ is another effector mechanism that has been shown to be especially relevant for tumor destruction by T cells (Schietinger et al. 2013). One study using intravital imaging found that adoptively transferred T cells did not require perforin for tumor destruction but instead used IFNγ which was released upon antigen-specific stable interactions with stroma cells (Schietinger et al. 2013).

Regulation of T cell activity

Since cytotoxic T cells can mount an explosive response to their antigen it is crucial for their survival to finely regulate their responsiveness. Therefore, TCR stimulation is closely followed by negative feedback mechanisms. There are several co-inhibitory receptors that a T cell can express to regulate its responsiveness. The most studied are PD-1, CTLA-4, TIM3 and LAG3. T cells start expressing PD-1 within 24 hours after initial TCR stimulation and when this molecule binds to its ligand PDL-1 or PD-L2, it hinders continued TCR signaling by inducing dephosphorylation of CD28 (Yokosuka et al. 2012; Hui et al. 2017). It can prevent effective priming as well as effector functions of T cells. PD-1 is both an activation and exhaustion marker and is used to identify tumor specific high-affinity TCRs in TIL populations (Gros et al. 2016, 2014; Inozume et al. 2010; Simon et al. 2016). Another negative regulation of TCR signaling is the internalization and degradation of engaged TCRs. This can happen in an antigen dose-dependent manner and preferentially at the immunological synapse (Monjas, Alcover, and Alarcón 2004; Dietrich et al. 1994; Martínez-Martín et al. 2011; Čemerski et al. 2008). Thirdly, IL-2 also has a dual role in T cell regulation. Initially, it is crucial for T cell proliferation and differentiation into effector T cells. Upon activation, T cells upregulate the expression of the IL-2 receptor subunit α (CD25) which strongly increases IL-2 sensitivity. IL-2 receptor signaling induces T cell differentiation into effector T cells but on the other hand leads to the expression of FAS and FAS ligand on cytotoxic T cells, preparing them for their own AICD (Kalia et al. 2010). Weaker IL-2R signaling due to lower IL-2 concentrations or lack of expression of the high-affinity IL-2R subunit α in turn lead to differentiation into memory T cells and no FAS and FASL expression (Kalia et al. 2010). IL-2 deficient mice show an increase in T cell proliferation, which underscores its role in T cell control (Sadlack et al. 1994).

Tumors as targets for cytotoxic T cells

The immune system has evolved to fight pathogens and its role in tumor surveillance is still debated (Qin and Blankenstein 2004; Klein and Klein 2005; Chen and Mellman 2013). Regardless, immunotherapy has shown remarkable results in cancer treatments with many different types of therapy that are described below. This shows that the immune system can specifically recognize and eliminate tumors. Fueled by immunotherapy's success and limitations, research on immunogenicity of tumors is ample and shows that the immunogenicity and susceptibility of tumors to immunotherapy varies depending on the tumor.

Immunogenicity of tumors

The Self-Nonself model explains T cell reactivity and tolerance based on the origin of the antigen as foreign or self (Burnet 1959). This model is supported by T cell selection in the thymus which deletes high-affinity self-reactive T cell clones or converts them into Tregs. However, this model cannot explain tolerance to commensal bacteria and autoimmunity. Therefore, it has been complemented by the danger model (Matzinger 2002). The danger model states that T cells can distinguish between dangerous and non-dangerous antigens based on the presence of danger signals which activate APCs for productive T cell priming. Danger signals can be released by cells that were injured as a result of infection, mechanical damage or toxins. These then bind to TLRs on DCs and activate them (Matzinger 2002). Tumors constitute a special case because they are neither infected nor healthy tissue. They are genetically self, except for their mutations, they lack microbial danger signals, but they are often accompanied by DNA damage, protein misfolding and hypoxia which can also induce danger signals (Matzinger 2002). The distinct inflammation status of tumors warrants caution when translating studies from T cell tolerization in healthy tissue to tumors. Instead, tumors have been compared to chronic wounds because of their constant tissue remodeling and angiogenesis as well as their attraction of fibroblasts and reparative macrophages (Balkwill and Mantovani 2001; Schäfer and Werner 2008). The cytokines that are predominant in wound healing are TGFβ and VEGF which inhibit immunogenic antigen presentation (Motz and Coukos 2011; Franklin et al. 2014). The infiltrating cells, the dominant cytokines, large amounts of dead cells, tissue remodeling and angiogenesis are all parallels between wounds and tumors that can explain the immunosuppressive tumor microenvironment. Additional suppression of T cell function comes from tumor-associated macrophages (TAMs), myeloid-derived suppressor cells and Tregs, all of which are often enriched in tumors (De Visser, Eichten, and Coussens 2006; Coffelt, Wellenstein, and De Visser 2016). Furthermore, the production of indoleamine 2,3- dioxygenase (IDO), a tryptophan catabolizing enzyme by either tumor cells or their microenvironment has tolerogenic effects on T cells. In healthy physiological processes, IDO is relevant for the prevention of immunity against apoptotic cells that can otherwise result in autoimmunity (Ravishankar et al. 2012), and for tolerance in mucosa and to the fetus during pregnancy (Munn and Mellor 2013). Furthermore, IDO is upregulated as negative feedback to inflammatory responses (Spranger et al. 2013). The mechanisms of tumor-

related immunosuppression are reviewed by Munn and Regents (David H. Munn and Regents 2016). The interplay between different cells of the innate and adaptive immune system that can result in either tumor-promoting or tumor-preventing outcomes is highly complex and varies between tumor types and animal models. Overall, T cell function is strongly inhibited by most tumors at the priming stage and beyond. Studies that have directly focused on T cell tolerization or defective priming in tumors have found that T cells are usually rendered tolerant even in nascent tumors that contain only small amounts of antigen (Willimsky et al. 2008; Westcott et al. 2021; Staveley-O'Carroll et al. 1998).

Another debated topic is the immunogenicity of dying tumor cells. The phenomenon of immunogenic cell death (ICD) has been described as a result of the treatment with some specific chemotherapeutic agents (doxorubicin, mitoxantrone, oxaliplatin, and cyclophosphamide), as well as specific types of radiation therapy and photodynamic therapy and is thought to be related to ER stress and the exposure of calreticulin. However, in general, dying tumor cells are immunosuppressive (Ma et al. 2013; Bezu et al. 2015; David H. Munn and Regents 2016; Poon et al. 2014). Increasing the immunogenicity of tumors is beneficial for the disease outcome and is therefore an additional factor that may determine efficacy of chemotherapeutics. In mouse experiments, chemotherapy (gemcitabine) and radiation have been shown to allow complete rejection of established tumors by adoptively transferred T cells despite low antigen levels. The authors identified the loading of the tumor stroma with tumor antigens and its subsequent recognition by cytotoxic T cells as the cause for this synergistic effect (B. Zhang et al. 2007). A second study found that ICD induced by mitoxantrone led to the infiltration and local maturation of dendritic cells that could then take up tumor antigen and prime adoptively transferred T cells for tumor destruction. The infiltration as well as the differentiation into $CD11c^+$ DCs were shown to be dependent on the release of ATP by dying cells and the priming was shown to be independent of draining lymph nodes, suggesting local T cell priming within the tumor (Ma et al. 2013).

Antigen processing and presentation

There are three different pathways for antigen processing and loading. Endogenous proteins are loaded on MHC-I (endogenous pathway), exogenous proteins are loaded on MHC-II (exogenous pathway) and sometimes exogenous proteins are loaded on MHC-I (cross-presentation). The general aspects of these pathways are described in

immunology textbooks, for example "Immunology" (Murphy, Travers, and Walport 2009).

In the endogenous pathway, the MHC-I α chain is translated into the endoplasmic reticulum (ER) where it is folded and stabilized with the help of Calnexin. After folding, it binds to β-microglobulin to assemble the complete MHC-I complex. Calnexin is then replaced by Calreticulin and ERP-57 which further stabilize the complex and assist with disulfide bond formation. In the cytoplasm, defective self and viral proteins are labelled with ubiquitin for degradation and are cleaved by the proteasome into short peptides of about 15 amino acids in length (Pamer and Cresswell 1998). These peptides are shuttled from the cytoplasm to the ER via transmembrane proteins called Transporter associated with antigen processing (TAP) 1 and 2, and trimmed at the N-terminus to a length of usually 8-10 amino acids by endoplasmic reticulum aminopeptidases (ERAP) 1 and 2 (Pamer and Cresswell 1998). These antigens can then bind to the peptide binding grove between domains α1 and α2 of the MHC-I complex and the assembled complex is shuttled via the Golgi apparatus to the plasma membrane (Pamer and Cresswell 1998). When cells present self-antigen on MHC-I, they are not killed by $CD8^+$ T cells, whereas the presentation of viral antigens can trigger a $CD8^+$ T cell response that leads to apoptosis of the infected cell. The binding of peptides to MHC-I is improved by hydrophobic residues in position two and at the carboxyl terminus (Bouvier and Wiley 1994) which are so called anchor positions. The generation of peptides with hydrophobic carboxyl termini is incremented after IFNγ signaling leading to an increase in peptides with high affinity to MHC-I. Specifically, IFNγ signaling induces the formation of the immunoproteasome which is the proteasome with IFNγ inducible subunits LMP2 and LMP7 (Pamer and Cresswell 1998).

The exogenous pathway loads peptides of endocytosed pathogens onto MHC-II, which is only expressed by professional APCs such as B cells, macrophages and DCs. In the exogenous pathway, pathogens are taken up by phagocytosis and pathogenic proteins are degraded in phago-lysosomes by acid hydrolases (R. Busch and Mellins 1996). The phagolysosomes then fuse with endosomes containing MHC-II complexes. The MHC-II complex consist of two protein chains, α and β with two domains each and is stabilized before peptide binding by a protein called CD74 (R. Busch and Mellins 1996). This complex is trafficked to the endosome where cathepsin S degrades CD74 except for a fragment called CLIP that continues to protect the peptide binding grove (R. Busch and Mellins 1996). When the endosome fuses with the phagolysosome,

a chaperone called HLA-DM helps to replace CLIP with the pathogen's peptides (R. Busch and Mellins 1996).

Cross-presentation requires the loading of exogenous peptides on MHC-I which can be achieved by different pathways which are still under investigation. Cross-presentation is indispensable for the priming of $CD8^+$ T cells by DCs and is often studied in tumor immunology since DCs can take up tumor antigens and elicit anti-tumor responses of $CD8^+$ T cells. The two main pathways for cross-presentation are the vacuolar and the endosome-to-cytosol pathway. In the latter, proteins are shuttled from the endosome into the cytosol where they are degraded in the proteasome and trafficked via TAP to either the ER or back to endosomes (Embgenbroich and Burgdorf 2018). The vacuolar pathway resembles the processing of peptides for presentation on MHC-II as proteins are degraded into peptides in the lysosome and loaded on MHC-I also in lysosomes (Embgenbroich and Burgdorf 2018). MHC-I complexes can reach the endosomes either via membrane recycling or as newly synthesized proteins from the ER. Additionally, DCs can also receive assembled MHC-I-peptide complexes from the plasma membrane of other cells by direct membrane contact, a phenomenon called cross-dressing. Cross-presentation is reviewed by Embgenbroich and Burgdorf (Embgenbroich and Burgdorf 2018).

Neoantigens as targets for adoptive T cell transfer

The safest target option for T cell therapy are neoantigens, which are created by somatic mutations. Neoantigens can arise from amino acid exchange or insertion, by frame shifts or by fusion proteins. However, only a minority of the mutated proteins can become suitable neoantigens. For this to happen, the mutation must be adequately processed in the proteasome and bind to an MHC complex with at least moderate affinity (Schumacher and Schreiber 2015). The likelihood to find a suitable neoantigen correlates to the mutational burden of the tumor and not all tumor types have a sufficiently high mutation rate to create suitable neoantigens (Schumacher and Schreiber 2015). Carcinogens such as UV-light or chemicals lead to a higher number of mutations and the cancer type with the highest mutational burden is melanoma which is also the most studied cancer in immunotherapies (Schumacher and Schreiber 2015). Generally, neoantigens vary from patient to patient but recurrent neoantigens also exist. Examples are the BCR-ABL fusion protein or cancer driving mutations in KRAS (Tran et al. 2016; Vaughn et al. 2011). Another group of neoantigens are viral proteins in virus-induced cancers such as cervical cancer. An ideal neoantigen would be

recurrent, cancer driving, abundantly expressed and have a high affinity to an MHC complex. Antigen identification can be realized by whole exome sequencing (deep sequencing of coding regions of the genome) or mass spectrometry-based identification of HLA-bound peptides after immunoprecipitation (Bassani-Sternberg et al. 2016; Okada, Shimizu, and Fujii 2022). The latter has the advantage that only antigens that are in fact expressed and presented on the patient's MHC are identified whereas in the case of whole exome sequencing, HLA binding is initially only predicted in silico and must still be corroborated experimentally in a second step. However, mass spectrometry usually requires large amounts of tumor sample. The identification of neoantigens is still an important bottle neck for neoantigen-targeted T cell therapy.

Mutated CDK4

CDK4 is a Serine/Threonine kinase that by phosphorylating retinoblastoma proteins (pRB), induces cell cycle progression from G1 to S phase. It is regulated by type D cyclins and $p16^{INK4a}$ and dysregulation of the CDK4 pathway has been shown to be associated to tumorigenesis (Yu et al. 2006; Barghi et al. 2022). Therefore, targeted therapies using CDK4 inhibitors such as Ribociclib have been approved for the treatment of estrogen negative/HER2 positive advanced breast cancer (Sobhani et al. 2019). Also in melanoma, the CDK4 pathway is dysregulated in many patients (Curtin et al. 2005). In fact, a $CDK4_{R24C}$ mutation in a Melanoma patient was the first described public neoantigen in human cancer that was recognized by autologous T cells in 1995 (Wölfel et al. 1995). $CDK4_{R24C}$ as well as $CDK4_{R24L}$ and $CDK4_{R24H}$ create HLA-A2-restricted T cell epitopes with varying affinities. These factors, being a tumor driver, a public neoantigen and of high affinity to MHC-I, make mutated CDK4 an attractive target for TCR-Ts (Wölfel et al. 1995; Lennerz et al. 2005; Leisegang, Kammertoens, et al. 2016). TCRs that target $CDK4_{R24C}$ have been shown to be cross-reactive to $CDK4_{R24L}$ (Leisegang, Kammertoens, et al. 2016) and when combining TCRs specific for these two point-mutations, several different published TCRs can be gathered and compared. TCRs against mutated CDK4 have been isolated from TILs (Kvistborg et al. 2012), healthy donors (Strønen et al. 2016a) and from a cured patient (SK29, AV) (Livingston et al. 1979) that had carried the $CDK4_{R24C}$ mutation in the resected tumor (Wölfel et al. 1995). Mutated CDK4 is therefore an ideal model antigen to study neoantigen-specific TCR responses because of its tumor-driving function as well as the amount of available research and different TCR sequences.

Mutated p68

DEAD-box RNA helicase p68 (also called DDX5) is also an ATPase and co-activator to several transcription factors including NF- κb, β-catenin and estrogen receptor α (Dai et al. 2014). Similar to CDK4, its dysregulation was also linked to tumorigenesis and it has been studied as a potential target for targeted therapy (Dai et al. 2014; Hashemi et al. 2019). It was the first described $CD8^+$ T cell neoantigen in mouse tumors (Dubey et al. 1997) (mL9 being the first $CD4^+$ T cell neoantigen (Monach et al. 1995)). In contrast to the tumor antigens described in humans (Wölfel et al. 1995; Robbins et al. 1996; Coulie et al. 1995) it was experimentally shown to be the rejection antigen of an experimental tumor. The mutation of p68 in position 551 from Serine to Phenylalanine creates the amino acid sequence SNFVFAGI which is a strong binder to MHC-I H-$2k^b$. The one TCR that has been described to recognize this antigen so far is called 1D9. It was isolated from an immunized mouse, in vitro expanded, and selected based on its reactivity to mp68 (Leisegang, Engels, et al. 2016).

Immunotherapy in cancer

The fact that the immune system can be harnessed for cancer treatment has become increasingly clear. Approaches using cytokines, antibodies and cell therapies are approved for clinical use.

Cytokines

Different cytokines are approved for treatment and many others are still in clinical trials or have been proven unsuccessful. IFNα and IL-2 are approved as adjuvant treatment for resected melanoma, metastatic renal cell carcinoma and others (Conlon, Miljkovic, and Waldmann 2019). IFNγ and IL-7 have not shown clinical benefit in cancer patients, and IL-12 and IL-21 have not been continued in clinical studies due to high toxicity (Conlon, Miljkovic, and Waldmann 2019). All cytokine treatments are usually accompanied by high, dose-limiting toxicities. The different cytokine-based immunotherapies are reviewed in (Conlon, Miljkovic, and Waldmann 2019).

Cancer Vaccines

Preventive cancer vaccines that protect against viruses such as human papilloma virus (HPV) and hepatitis B virus (HBV) are successfully used in the clinic and conceptually indistinct from other vaccines against infectious diseases. They will not be discussed

in this section. Therapeutic cancer vaccines, on the other hand, face more obstacles. They aim at stimulating an immune response against tumor antigens when a tumor has already developed. There are currently two therapeutic cancer vaccines approved. Sipuleucel-T can be used in castration-resistant prostate cancer. It infuses autologous peripheral blood mononuclear cells (PBMCs), including APCs, that were activated with an antigen from prostatic acid phosphatase (PA2024) fused to granulocyte-macrophage colony stimulating factor (GM-CSF) (Kantoff et al. 2010; Saxena et al. 2021). Bacillus Calmette-Guérin (BCG) is a bacterium that is used for the treatment of bladder cancer as well as other non-malignant diseases such as tuberculosis and endometriosis. In this case, no tumor antigen is included in the vaccine. It was developed in the early 20th century and was applied for the treatment of bladder cancer already in 1976 (Morales, Eidinger, and Bruce 1976; Larsen et al. 2020). Apart from these, there are many therapeutic cancer vaccines in clinical trials targeting different tumor associated or tumor specific antigens and using different technical approaches such as autologous dendritic cells, mRNA nanoparticles or peptides. Cancer vaccines are reviewed in (Saxena et al. 2021). Importantly, cancer vaccines are usually unable to induce tumor regression. An analysis of past NCI vaccine trials showed that only 2.6% of patients had achieved a response according to RECIST criteria when using different types of cancer vaccines (Rosenberg, Yang, and Restifo 2004). Cancer vaccines are therefore now generally used either in an adjuvant setting or in combination with cell therapy or checkpoint inhibition. As an example, an ongoing phase 2 trial comparing mRNA vaccine plus anti-PD-1 checkpoint inhibitor versus anti-PD-1 alone in resected high risk melanoma found a statistically and clinically significant prolonged distant metastasis-free survival (Khattak et al. 2023). This suggests that once the tumor is already resected, T cells that were activated by the adjuvant treatment can eliminate surging micro metastases. Importantly, this effect is different to immunosurveillance because effective priming of T cell specific to the tumor antigens was achieved through the mRNA vaccine. In fact, it has been suggested that complete resection is a necessary requirement for the success of cancer vaccines because their contact to remaining tumor cells would render the T cells tolerant (K. Schreiber et al. 2006).

Immune checkpoint blockade

Immune checkpoint blockade (ICB) therapies refer to the blocking of inhibitory receptors on T cells by specific blocking antibodies leading to tumor destruction by a

patient's immune cells. PD-1, PDL-1, CTLA-4, TIM3 (Y. Wolf, Anderson, and Kuchroo 2020) are all targeted in checkpoint inhibitor therapy. The first approved checkpoint inhibitor therapy was ipilimumab, an antibody that targets CTLA-4. Currently, several anti-PD-1 and anti-PDL-1 antibodies are also approved for therapy in different types of cancer. CTLA-4 competes with CD28 for B7 binding and can thereby prevent effective priming of T cells (Fares et al. 2019). Therefore, the benefit of anti-CTLA-4 is most likely based on the priming of new T cells (Fares et al. 2019). PD-1 blockade may reinvigorate pre-dysfunctional T cells residing in the tumor or improve priming of new T cells (Fares et al. 2019; Borst et al. 2021). Whether reinvigoration of existing clones or infiltration of new clones is the more relevant consequence of anti-PD-1 treatment is still a matter of research (Yost et al. 2019; Kuehm et al. 2019). PDL-1 can be expressed on many different cell types including tumor cells and PD-L2 on activated DCs and macrophages. Only a small percentage of patients benefit from ICB and therefore a lot of research into predictive markers for ICB response is being carried out. Beneficial factors that have been identified so far are high tumor mutational burden, high PDL-1 expression and lymphocyte infiltration in the tumor (Fares et al. 2019; Borst et al. 2021).

Adoptive T cell transfer

The rationale of adoptive T cell transfer (ATT) is that naturally occurring tumor-specific T cells are insufficient in number and activity to control tumor growth. Therefore, T cells are ex vivo activated, expanded and possibly genetically modified and then retransferred to the patient in high numbers (Figure 1). To remove regulatory T cells and to create conditions for homeostatic expansion of the transferred T cells, patients usually receive lymphodepleting chemotherapy prior to the T cell infusion. Additionally, IL-2 is administered to the patients to induce T cell proliferation after the T cell infusion (Rohaan et al. 2018).

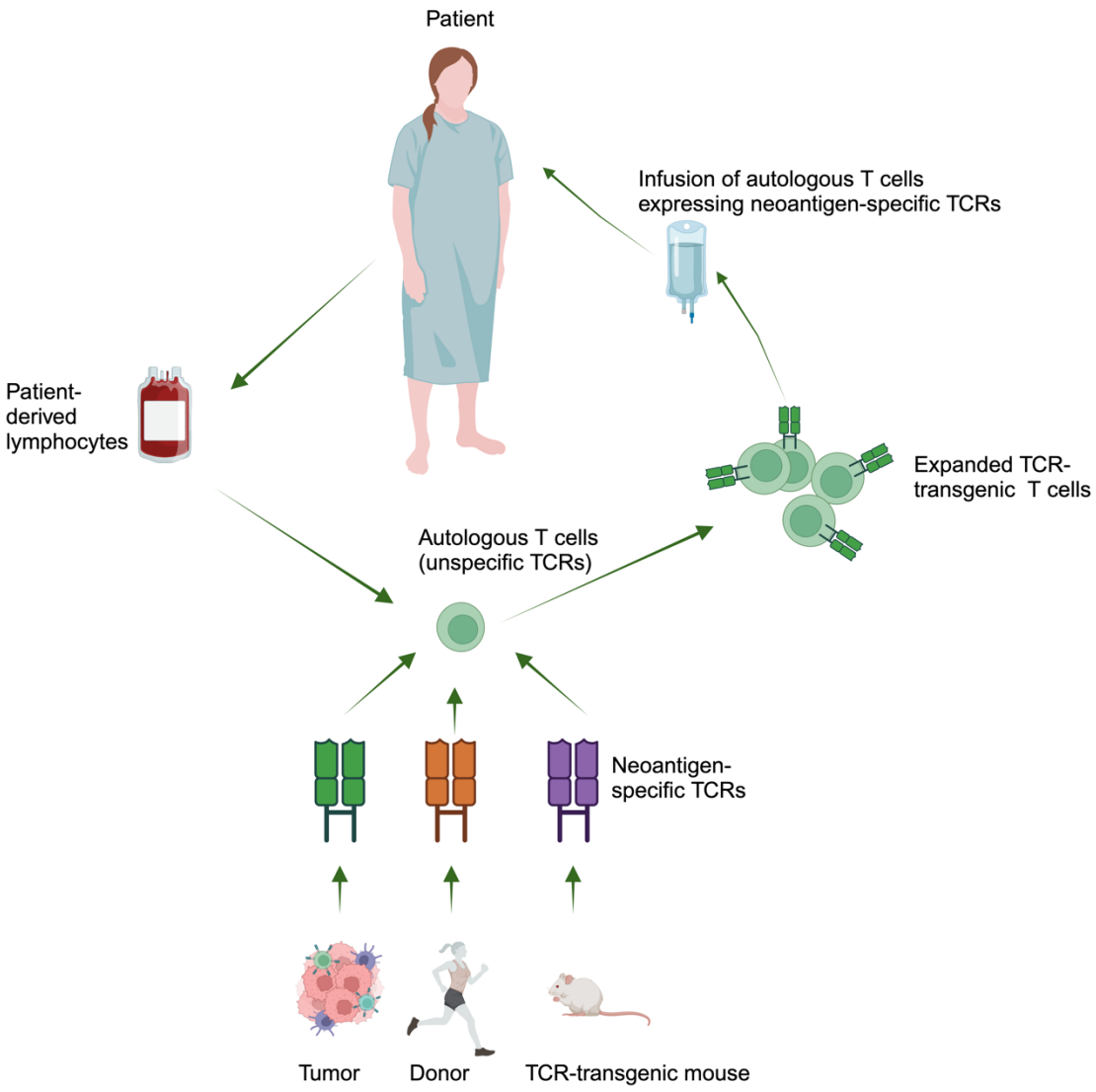

Figure 1: Adoptive transfer of TCR-transduced T cells.

The patient's peripheral blood is used as a source of T cells that will receive transgenic TCRs that are specific for the tumor's neoantigens. The TCR sequences can be derived from TILs either from the same or a different patient's tumor. Alternatively, they can be obtained from in vitro stimulated healthy donor T cells or mice transgenic for the human TCR loci and human MHC. The TCRs are transduced into the patient's T cells which are then expanded and re-infused into the patient.

TIL therapy

One approach for ATT is to stimulate and expand TILs from a patient's resected tumor without analyzing or modifying the TCR specificities and affinities. This means that the reintroduced T cells do not necessarily recognize the tumor cells but has the advantage of a possibly broad set of targets. This type of therapy has been pioneered by Steven Rosenberg already in the early 1990s (Rosenberg et al. 1994) and is constantly being refined. The most successfully treated cancer is metastatic melanoma with objective response rates of around 50% (Morgan et al. 2006; Sim et al. 2020; Kvistborg et al 2012; Rosenberg and Restifo 2015; Dudley et al. 2005; Rohaan et al. 2022;

Besser et al. 2010; Rosenberg and Dudley 2009). Recently, a stage 3 clinical trial showed that in metastatic melanoma TIL therapy allowed significantly longer progression-free survival than the anti CTLA-4 therapy ipilimumab in patients that were resistant to anti PD-1 therapy (7 versus 3 months) (Rohaan et al. 2022). It has become clear that in many cases, neoantigen-specific TCRs are the most important TCRs for a clinical response (Van Den Berg et al. 2020) and efforts to identify and enrich for tumor-specific T cells by FACS have been made. Examples are PD-1 and CD39 expression on TILs or PD-1 expression in peripheral blood T cells (Gros et al. 2016, 2014; Purcarea et al. 2022; Inozume et al. 2010; Duhen et al. 2018). A case study of a patient with metastatic colorectal cancer showed regression of lung metastases after infusion of ex vivo expanded, pre-selected TILs that were shown to recognize the hot-spot mutation K-ras$_{G12D}$. Tumor progression occurred after loss of the antigen-presenting HLA allele, HLA-C*08 (Tran et al. 2016).

TCR-Ts

More directed T cell therapy requires the identification of a target antigen that is exclusively expressed on tumor cells and a TCR that can recognize the antigen on the patient's MHC molecules. Antigens can be tumor associated self-antigens (TAAs) or tumor specific neoantigens (TSAs). Whereas TAAs are generally shared among a larger group of patients than TSAs, they pose an increased safety risk since TAAs may be expressed by healthy tissue which would then also be attacked by the transferred T cells. This has happened for example in the case of the antigen MAGE-A3 (Morgan et al. 2013). A TCR that is specific for a selected antigen is introduced by viral gene transfer or CRISPR/Cas9 gene editing into the genome of autologous donor T cells. The use of CRISPR/Cas9 to knock-in the TCR in the endogenous TCR locus is thought to be advantageous because it disrupts expression of the endogenous TCR and puts the transgenic TCR under control of the physiological TCR promoter (Eyquem et al. 2017; Müller et al. 2021; Foy et al. 2022; Ruggiero et al. 2022). When retroviral gene transfer is used, the problem of endogenous TCR expression is solved by using a minimally murinized and modified constant region in the transgenic TCR that allows preferential pairing and surface expression of the transgenic over the endogenous TCR (Cohen et al. 2006; Haga-Friedman, Horovitz-Fried, and Cohen 2012; Sommermeyer and Uckert 2010). A recent ambitious clinical trial used patient-derived, neoantigen-specific TCRs that were knocked into the TCR gene locus by CRISPR/Cas9. Up to three different TCRs were transferred into the patients and nevertheless, the study

showed disappointing results (progressive disease in almost all patients) (Foy et al. 2022). Despite the low antitumor activity, this study provided evidence for the technical feasibility of personalized TCR-engineered ATT. The authors also discussed possible limitations such as T cell dosage and too long pipeline which led to delays in patient treatment and to lack of time for extensive testing of TCRs. Furthermore, they retrospectively found that in more than one case, tumors where already lacking the HLA gene required for the presentation of the chosen neoantigen. For the authors, this hints to immunoediting prior to therapy. Another limitation of the study is that they used only MHC-I restricted TCRs. A combination with MHC-II restricted TCRs in CD4 T cells might yield better results (Tran et al. 2014; Arina et al. 2017; Quezada et al. 2010; Bos and Sherman 2010; Bos et al. 2012). In another clinical study, one patient suffering from breast cancer was treated with a $p53^{R157H}$-specific TCR derived from another patient and showed an objective response for 6 months before progressing due to HLA-A2 loss of heterozygosity (Kim et al. 2022). Despite harboring the $p53^{R175H}$ mutation, this tumor had not contained $p53^{R175H}$-specific TILs. The response of this patient with TCR-transduced T-cells was superior to the responses of patients treated directly with TILs even though they also contained mutated p53-specific TCRs of confirmed quality (Kim et al. 2022). This case study suggests that the use of TCR-Ts can induce superior clinical responses to ex vivo stimulated TILs that had already been dysfunctional. In summary, TCR-T cell therapy has not yet shown much clinical benefit with the exceptions of some isolated cases. To further explore the potential of this type of therapy, limitations to T cell fitness need to be overcome (see below), combination with CD4 T cells should be explored and criteria for TCR quality should be standardized. Most importantly, larger numbers of patients that are treated in controlled settings are required to understand the real benefit of TCR-Ts in cancer treatment.

CAR T cells

Chimeric antigen receptor (CAR) T cells have been the first receptor-modified T cells approved for therapy. CARs recognize their antigen like antibodies. Therefore, they circumvent the need for antigen processing and presentation on MHC molecules and thus, the problem of large HLA diversity in the human population. On the other hand, they are limited to surface expressed antigens. CARs consist of four components that combine characteristics of antibodies and TCRs. Antigen recognition is realized by a single chain variable fragment of an antibody. This fragment is followed by a hinge

region, a transmembrane region and finally intracellular signal transmission domains (endodomains). Second generation CARs combine a CD3ζ chain with a co-stimulatory signaling domain (CD28 or 4-1BB (CD137)) for intracellular TCR signaling. The different CAR approaches are reviewed by Sterner et al (Sterner and Sterner 2021). CAR T cell therapy has proven very successful against B cell malignancies and is approved for B cell acute lymphoblastic leukemia, B cell non-Hodgkin lymphoma, mantle cell lymphoma, follicular lymphoma (with CD19 as target antigen) and multiple myeloma (with BCMA as target antigen). CAR T cell therapy is less effective in solid tumors because of limited CAR T cell trafficking and infiltration as well as lack of suitable surface antigens (Baulu et al. 2023). Fourth generation CAR T cells combine normal T cell effector mechanisms triggered by CAR stimulation with transgenic cytokine production (Allen et al. 2022; Tokarew et al. 2019). This enhanced cytokine production can overcome issues of solid tumors such as immunosuppressive tumor environments, infiltration, and poor survival of T cells inside solid tumors.

Limitations of T cells in cancer therapy

Although T cell therapy is a very powerful cancer treatment, tumors unfortunately often relapse in this therapy as well. The success of T cell therapy in cancer is limited by T cell intrinsic and tumor intrinsic characteristics. On the tumor side, heterogeneity and mutations lead to antigen negative or MHC negative escape variants (Tran et al. 2016). Lack of antigen presentation may be due to loss of heterozygosity, downregulation but also defects in the antigen presentation machinery such as TAP deficiency (Khong and Restifo 2002). In the tumor microenvironment, Tregs can inhibit T cells through various mechanisms, for example, PD-L1 expression, IL-2 depletion, or expression of TGFβ (Anderson, Stromnes, and Greenberg 2017). Other immune and non-immune cells of the tumor stroma can similarly suppress T cell function or prevent T cell infiltration. Furthermore, limited availability of metabolites, for example glucose or amino acids, in the tumor microenvironment limits T cell effector functions (Anderson, Stromnes, and Greenberg 2017). The challenges posed to T cells by the tumor microenvironment are reviewed by Anderson et al (Anderson, Stromnes, and Greenberg 2017). On the T cell side, prolonged antigen exposure often leads to T cell exhaustion and upregulation of inhibitory receptors (Anderson, Stromnes, and Greenberg 2017; Schietinger and Greenberg 2014). This is why a lot of effort and research is focused on improving the fitness of transferred T cells (Schmitt et al. 2015). Chimeric receptors that activate T cells when binding to a ligand that normally would

suppress T cell function can improve T cell function. Examples are the FAS-receptor fused to the 4-1BB costimulatory domain (Anderson et al. 2022) and PD-1 fused to CD28 (Ankri et al. 2013; Schlenker et al. 2017). Vectors for enhanced cytokine production can also improve T cell survival. For example, synthetic expression constructs with NFAT-controlled expression of IL-12 can improve anti-tumor effects of T cells (L. Zhang et al. 2011) and synthetic Notch receptor-induced IL-2 production improves CAR-T cell infiltration (Allen et al. 2022). Disruption of inhibitory pathways, for example TGFβ, can delay T cell exhaustion (Chou et al. 2012).

Sources of T cell receptors for adoptive transfer

In the case of TCR gene therapy, a TCR with known specificity and characterized quality is cloned and transduced into autologous blood-derived T cells to obtain a large clonal population. TCR sequences with tumor specificity can be obtained from different sources. Neoantigen-specific TCRs may be enriched in tumors but can also be found at low frequencies in antigen-naïve people (Strønen et al. 2016b). In the case of self-antigens, it is in theory less likely to find high-affinity TCRs in patients or HLA-matched donors since self-reactive TCRs are generally eliminated in the thymus. Nevertheless, TCRs against tumor-associated self-antigens MART-1 and NY-ESO have been found in TILs and have been used for therapy successfully, in the case of NY-ESO after affinity maturation (L. A. Johnson et al. 2006; Robbins et al. 2015; L. A. Johnson et al. 2009; Morgan et al. 2006). Additionally, TCRs can be obtained from TCR-transgenic mice (Li et al. 2010a; Moore et al. 2021).

TILs

A natural source for TCR sequences are autologous TILs. Since autologous TCRs were selected on the patient's own HLA repertoire, the risk for alloreactivity is low (H. Schreiber 2008). The identification of T cells directed against neoantigens has been dominated by Steve Rosenberg and his group and was successful in many cases of Melanoma (Kvistborg et al. 2012; Lennerz et al. 2005; Cohen et al. 2015). Apart from Melanoma, they also found neoantigen-specific T cells in bile duct cancer (Tran et al. 2014), colon cancer (Tran et al. 2015, 2016) and breast cancer (Zacharakis et al. 2018). In the study of Kvistborg et al, TCRs directed against an HLA-A2 restricted epitope from neoantigen $CDK4_{R24L}$ and HLA-A2 restricted epitopes of MART-1, gp100, MAGE-A10 and other tumor associated self-antigens were identified from Melanoma biopsies (Kvistborg et al. 2012; Strønen et al. 2016). Surprisingly, the tumor specific T cells were

found only at low frequencies in this case whereas they were very abundant in the colon cancer and bile duct biopsies of the above-mentioned studies. In some cases, virus-specific T cells may be more abundant in tumors than tumor-specific T cells (Scheper et al. 2019; Oliveira et al. 2021) although this was not the case in the described study of Kvistborg et al (Kvistborg et al. 2012). Traditionally, the identification of tumor specific TCRs from TILs required establishing a stable culture of the patient's tumor cells or re-expression of neoantigen candidates from tandem minigenes in autologous APCs (Tran et al. 2016). The tumor cells or APCs were then co-cultured with TILs and specifically activated T cells were identified by activation markers such as IFNγ and CD137. Alternatively, TILs can now be sorted directly from tumor digest using multimer staining if the antigens of a tumor are known (Kvistborg et al. 2012; Rodenko et al. 2006). Multimers, for example the tetramers used in this work, contain several MHC molecules linked to the antigen of interest and to a fluorophore. The multimers imitate an MHC complex presenting the epitope in question and can bind to antigen-specific TCRs. In this way, specific T cells can be labeled with fluorophores and analyzed and sorted by FACS. The sorted T cells are sequenced which is usually done by single cell sequencing. An important limitation of TILs as a source for TCR sequences is that not all tumors contain tumor-specific T cells that can be used for TCR cloning (Scheper et al. 2019; Strønen et al. 2016b). The identification of tumor specific TCRs from TILs can be limited by the tumor type and mutational burden. Additionally, the tumor biopsy also is required in sufficient quality and size to obtain TCRs. Overall, there are many instances in which TCR sequences cannot be obtained from autologous TILs. Otherwise, TCRs can also be used from TILs of other patients as was done in the patient mentioned above (Kim et al. 2022). Whether it is likely that a tumor that does not contain tumor-specific TILs still has good enough neoantigens to be targeted with TCR gene therapy remains to be tested.

In vitro priming

HLA-matched healthy donors may have naïve neoantigen-specific T cells in their blood at low frequencies that can be expanded by in vitro priming with APCs presenting the antigen of interest (D'ippolito, Wagner, and Busch 2020). For this purpose, autologous APCs (matured monocytes) can be either transfected with mRNA minigenes (Strønen et al. 2016b; Ali et al. 2019) or loaded with peptide (Wölfl and Greenberg 2014; Grunert et al. 2022) and are then co-cultured with naïve (CD57 and CD45RO depleted) T cells for 10 to 15 days. Afterwards, antigen-specific T cells can be

identified by multimer staining or activation markers and single cell sorted as described above. This is an attractive alternative source for TCR sequences for several reasons. First, it circumvents the requirement to obtain TILs at high numbers which may be limited by biopsy quality and size and TIL infiltration into the tumor as well as immunogenicity of the tumor in question (D'ippolito, Wagner, and Busch 2020). Second, it allows the characterization of TCRs independent of patient sample and the TCRs may be used for several patients if their HLA type and neoantigen match. Third, it may broaden the spectrum of TCR sequences and possible targets since TILs do normally not include reactivities against all possible neoepitopes of a patient (Strønen et al. 2016b; D'ippolito, Wagner, and Busch 2020). Lastly, the neoantigen-specific TCR repertoire of antigen- naïve donors has not been modified by antigen exposure. Whether this is an advantage regarding the receptor affinity or not is studied in the presented doctoral thesis.

Immunization of humanized mice

Especially for tumor associated self-antigens such as MAGE-A1 or NY-ESO, the two above-described sources may not yield high-affinity TCRs because of central tolerance, specifically because of deletion of high-affinity self-directed TCRs. These antigens can nevertheless be attractive for T cell therapy especially because they are shared among larger patient cohorts. Therefore, Thomas Blankenstein and colleagues developed a transgenic mouse line that carries the complete human TCR α and β gene loci and chimeric HLA-A2 (HHD) instead of the murine TCR α β loci and murine MHC-I genes (Li et al. 2010b). Recently, a second mouse strain called VelociT was developed for the purpose of generating human TCRs in mice (Moore et al. 2021). It carries the human TCR loci as well as human CD8, CD4, one human MHC-I gene (HLA-A2) and one MHC-II gene (HLA-DR2), all of which were inserted in their endogenous loci (Moore et al. 2021).Both mouse strains can produce functional human TCRs that can be used for T cell therapy. To induce clonal expansion of specific clones, mice undergo a prime and boost immunization regimen using peptide or DNA vaccination (Grunert et al. 2022). After sacrificing the mice, splenocytes are stimulated with peptide in vitro and sorted based on activation markers (IFNγ or CD137) or multimer binding.

Mouse models to study anti-tumor immune responses

There are different mouse models to study whether T cells generate an anti-tumor response during tumor development. They can be separated in autochthonous and transplantation models.

Autochthonous tumor models

In the autochthonous models, tumor initiation can either be triggered by repeated exposure to carcinogens or by the conditional expression of oncogenes in transgenic mouse lines. Both have the advantage that tumorigenesis is initiated by a single or few transformed cells, that the tumor growth is slow, and a more realistic microenvironment can form within the solid tumor (DuPage and Jacks 2013). In addition, there is no transplantation event that coincides with the malignant transformation of the cell. Tumor induction using DNA-damaging agents such as UV radiation or methylcholanthrene (MCA) is especially useful for the generation of multiple, individual neoantigens. It also has the advantage of a controlled site for tumor growth and a slow, natural tumorigenesis and outgrowth. A disadvantage is that the mutations differ between tumors and mice so that no specific target antigen can be studied in several mice (DuPage and Jacks 2013). In genetically modified mouse models (GEM), tumor initiation is dependent on the expression of oncogenes, especially K-ras$_{G12D}$ together with p53 knock-out in a spontaneous or conditional fashion (L. Johnson et al. 2001; Fisher et al. 2001). These tumors do not carry mutation antigens other than the ones required for oncogenic transformation, usually K-ras$_{G12D}$, but they can be designed to express specific antigens of interest. In the model with spontaneous oncogene activation, time for onset and site of tumor development cannot be controlled. When a conditional expression of the oncogene is used, it can be initiated for example by tissue-specific promotors to control the organ in which the tumor develops, by ligand, e.g. doxycycline, or by lentivirus. If specific antigens are studied, their expression can be engineered to be linked to tumor development (DuPage and Jacks 2013). However, since the antigen also is necessarily germline encoded, it may be present in the thymus and hence be treated as self-antigen by circulating T cells (Cheung et al. 2008; DuPage and Jacks 2013). This would be a disadvantage for my research question which specifically focusses on neoantigens to which no central tolerance of T cells exists. Another caveat in the use of GEM for this project would be the use of lentivirus because it is immunogenic and may provide danger signals that favor a T cell response against the induced antigens. GEMs in the

context of tumor immunology are reviewed by DuPage and Tyler Jacks (DuPage and Jacks 2013). Other practical considerations of the autochthonous models are the lack of control and synchronization of tumor onset and the smaller maximal tumor size in inner organs.

Transplantable tumor models

Transplantable models are often criticized because they introduce a large number of fully transformed, aggressively growing tumor cells (DuPage and Jacks 2013; Ngiow et al. 2016). In subcutaneous, transplantable tumor models, the formation of a realistic microenvironment is less likely because of the site and speed of tumor growth (DuPage and Jacks 2013; Ngiow et al. 2016). However, in large, established transplanted tumors, their microenvironment is indistinguishable from clinical tumors (K. Schreiber et al. 2006; Wen et al. 2012). Human tumors are usually heterogenous, meaning that not all tumor cells carry the same number of mutations. This factor is not well represented in transplantation models where a previously cultured cell line with homogenous gene expression is injected (Ngiow et al. 2016). If the injected cell population is not pure, e.g. with regard to a studied antigen, this may partially mimic the heterogeneity of a natural tumor. The fact that the immune system is exposed to the introduced tumor cells directly after cell injection creates unrealistic encounters between T cells, DCs and the tumor antigens (DuPage and Jacks 2013). Many tumor cells die after injection and their antigens can be taken up by macrophages (K. Schreiber et al. 2006). Also, acute inflammation at the injection site can improve DC activation and T cell priming (Prehn and Prehn 2013; DuPage and Jacks 2013; Ngiow et al. 2016; Spiotto et al. 2002). To overcome this issue which can directly impact the T cell response to neoantigens, I used the conditional expression of one dominant neoantigen. Because I studied only the T cell response to this antigen, I could be sure that none of the T cells specific to this antigen had been primed during the acute inflammation but only under resting conditions (K. Schreiber et al. 2006; Spiotto et al. 2002). The use of transplantable tumors also allows the faster adaptation to other antigens or expression patterns as it only requires the transduction of a cell line and not the creation of a new mouse line. Therefore, it is a more versatile model that is suitable to test tumor specific T cell responses in different settings.

Transplantable tumor models to study the therapeutic effect of TCR-Ts

To study therapeutic effects, transplantable tumor models are generally preferred over autochthonous tumor models (Chulpanova et al. 2020). They allow for a homogenous group of tumors with synchronized tumor onset and size and controlled tumor characteristics (Ngiow et al. 2016). There are different types of transplantable tumor models that can be grouped into xenograft models and syngeneic models.

Xenograft models use human tumor cells and to prevent their rejection, severely immunocompromised mouse strains are employed. The use of human tumor cells is especially advantageous when testing targeted therapies that mostly affect tumor cell intrinsic molecules. However, unless these mice receive a human $CD34^+$ stem cell transplant that gives rise to the human immune cells, they do not have a functional immune system (Chulpanova et al. 2020). Furthermore, since the tumor stroma is of mouse origin, it cannot be targeted with human specific agents, for example human TCRs.

For immunotherapy, syngeneic tumor models are therefore generally preferable. They use murine tumor cells stemming from the identical genetic background of the tumor receiving mice. For this reason, tumor cell rejection is less likely, allowing for the use of immunocompetent or less immunocompromised mice. Another advantage of using syngeneic tumor models is that tumor and tumor stroma express the same MHC-I and MHC-II alleles which allows for cross-presentation of tumor antigens by the tumor stroma. Therefore, in the case of TCR-T cell therapy, T cells can target tumor cells as well as cross-presenting stroma cells (Spiotto et al. 2002).

In order to test human TCRs, mouse tumor models with murine MHC genes are unsuitable because the murine pMHC complex cannot be recognized by human TCRs. In this case, MHC-chimeric mouse strains can be used. In my thesis, I made use of HHD mice (Pascolo et al. 1997) which do not express murine MHC-I but instead a chimeric MHC-I complex consisting of human HLA-A2 α1 and α2 domains and murine H-$2D^b$ α3-transmembrane and intracytoplasmic domains. This mouse strain is therefore able to present human HLA-A2-restricted antigens to human TCRs on its stroma as well as on syngeneic tumor lines. It has previously been used to test human TCRs in mice successfully and a syngeneic tumor line was created using MCA for tumor induction (Leisegang, Kammertoens, et al. 2016).

Two other important factors of transplantable tumor models are tumor size at treatment initiation and time between tumor induction and treatment (S. P. Wolf, Wen, and Schreiber 2022; Wen et al. 2012). Only if tumors are large and established for

weeks, the benefit of a therapeutic approach can actually be assessed, inflammation has subsided and a viable tumor with realistic microenvironment has formed (Wen et al. 2012; K. Schreiber et al. 2006). Therefore, all T cell transfer experiments performed in this work were done on large, established, syngeneic tumors.

Importance of targeting true tumor antigens

A lot of groundbreaking research in immunology has been done using a handful of well-established model antigens such as ovalbumin (OVA), HY-antigen and SV40 largeT antigens (TAg). However, when specifically studying TCR-Ts for tumor treatment, it is preferable to use tumor antigens that arose from somatic mutations during tumorigenesis. These antigens may be less immunogenic than viral antigens since they distinguish from the host's proteome only by small point mutations but are more realistic targets (Wells, Buuren, et al. 2020). Additionally, only by investigating true neoantigens, we can increase our understanding on the relevant parameters for target selection in patients.

Aims

Even though TIL-derived TCRs are already used for TCR-T cell therapy, it has never been investigated whether TILs are a reliable source for high quality TCRs. If TIL-derived TCRs were inherently inferior to those from other sources, this would be of great relevance for the design of future clinical studies using TCR-Ts.

Problem and significance

Fueled by the success of CAR T cell therapy in hematological malignancies and the limited success in generating TIL therapies for entities other than melanoma, TCR-Ts targeting tumor associated antigens, hotspot mutation neoantigens and personal neoantigens are being explored in preclinical and clinical research (Tran et al. 2016; Kim et al. 2022; Foy et al. 2022). Since the quality of the TCRs used for TCR-Ts is crucial for the therapy's success, it is vital to make the selection of any TCR used for clinical trials as informed and transparent as possible (Bos et al. 2012). General rules that predict the quality of isolated TCRs are required and most importantly, it is unclear whether TIL-derived TCRs should be used for TCR-Ts at all or whether they are inherently of lower quality than TCRs from antigen-naïve repertoires (healthy donors). There is only small number of examples in which TIL-derived TCRs have been used to produce TCR-engineered T cells for clinical use. In those examples, the TCRs had been affinity-matured to yield better outcomes (Robbins et al. 2015) or had been preselected in vitro and targeted oncogenic driver mutations, increasing their probability of success (Tran et al. 2016, 2014). Therefore, there is currently no evidence supporting the general use of TIL-derived TCRs for TCR-T cell therapy. My thesis is providing an empirical basis by characterizing and directly comparing the therapeutic efficacy of TIL-derived and healthy donor-derived TCRs when treating large, established tumors in mice. This aims at providing evidence for the use or avoidance of TIL-derived TCRs in future TCR-T cell therapies.

Hypothesis

I hypothesized that TIL-derived, neoantigen specific TCRs are of at least equal tumor reactivity compared to healthy-donor or immunization-derived TCRs when transduced into activated donor T cells.

Specific Aims

First, I aimed to test several human $CDK4_{R24L}$-specific TCRs from different publications side-by-side to compare the quality of a TIL-derived TCR to TCRs from antigen-naïve backgrounds. Second, I aimed to generate TCRs from experimental mice to compare the quality of a larger number of TIL-derived and immunization-derived TCRs that were only tetramer-sorted but not selected based on in vitro reactivity.

Aim 1: Compare human $CDK4_{R24L}$-specific TCRs.

1.1: Validate $CDK4_{R24L}$-specific TCRs in vitro and select TCRs for in vivo experiments.

By testing cytokine release, activation markers and in vitro cytotoxicity, I aimed at interrogating the differences in quality between TCRs from different donor backgrounds.

1.2: Compare $CDK4_{R24L}$-specific TCRs in HHD mouse tumor models.

Using an HHD-based tumor line and HHD mice, I aimed at comparing the therapeutic potential of $CDK4_{R24L}$-specific TCRs representative for different TCR donor backgrounds.

Aim 2: Study murine mp68-specific TCRs.

Since the set of human $CDK4_{R24L}$-specific TCRs contained only one TIL-derived TCR, I aimed at complementing them with a set of experimentally generated murine TCRs targeting mp68. This would create a larger number of TIL-derived as well as immunization-derived TCRs for my studies.

2.1: Generate mp68-specific TCRs.

By using a tumor line with inducible antigen-expression, I aimed to isolate TIL-derived and immunization-derived TCRs specific for the murine neoantigen mp68.

2.2: Validate mp68-specifc TCRs in vitro.

After re-expressing the newly obtained TCR sequences in donor T cells, I aimed to confirm their specificity for mp68 in cytokine release assays.

2.3: Determine in vitro parameters that predict in vivo efficacy.

After validating the TCRs' specificity, I aimed to establish in vitro assays that would be predictive for the in vivo performance of mp68-specific TCRs.

2.4: Compare mp68-specific TCRs in mouse tumor models.

I aimed at comparing the functional mp68-TCRs regarding their tumor-eradicating potential in large, established tumors overexpressing mp68.

Methods

Molecular Biology

TCR expression constructs

Mouse TCRs: TCR illumina sequencing results for the mp68 TCRs (except 1D9 and 874-44) were processed by Leo Hansmann who provided tables containing well identification, nucleotide sequence, CDR3 amino acid sequence, variable chain identification, and the number of reads for α and β TCR chains. These nucleotide sequences covered the 3′ half of the variable region, the CDR3 and J region and the beginning of the constant region. To complete the nucleotide sequence with the 5′ sequence of the variable region and the end of the constant region, the complete nucleotide sequence of the variable chains and the constant were obtained from IMGT (https://www.imgt.org/IMGTrepertoire/LocusGenes/#F). The TCR including the signaling peptide was translated in Snapgene and the TCR β chain sequence was linked via GSG and P2A element (ATNFSLLKQAGDVEENPGP) to the TCR α chain. Importantly, the β chain was always in front of the α chain. The complete amino acid sequence was used to order the gene synthesis from GeneArt (Thermo Fisher), restriction sites for NotI at the 5′ and for EcoRI at the 3′ end of the construct were added, and the nucleotide sequence was codon optimized for mus musculus. The TCR constructs were cloned into pMP71-PRE plasmids for retroviral transduction of T cells (Engels et al. 2003). TCR expression was monitored using antibodies against mouse TCR β variable regions (vβ) (Biolegend) in B6 T cells.

Human TCRs: Human TCRs were modified to contain a minimally murinized constant region because this improves surface expression and correct pairing (Cohen et al. 2006; Haga-Friedman, Horovitz-Fried, and Cohen 2012). Mutant CDK4-specific TCRs were assembled based on published variable and CDR3 regions (Kvistborg, et al. 2012; Strønen et al. 2016b; Wölfel et al. 1995) and codon optimized and assembled into β-P2A-α constructs as described above and in the literature (Figure 2) (Wilde et al. 2009; Schambach et al. 2000). For in vivo experiments, they were used in the arrangement β-P2A-α-IRES-GFP. In this case, GFP was used to quantify transduction efficiency. TCR expression was otherwise monitored by staining the mouse TCR β constant region (mCb) (Biolegend) in human T cells and human TCR β variable region in HHD mouse T cells when the corresponding antibody was available (see Table 3). A schematic representation of expression constructs with and without IRES-GFP is shown in Figure 2.

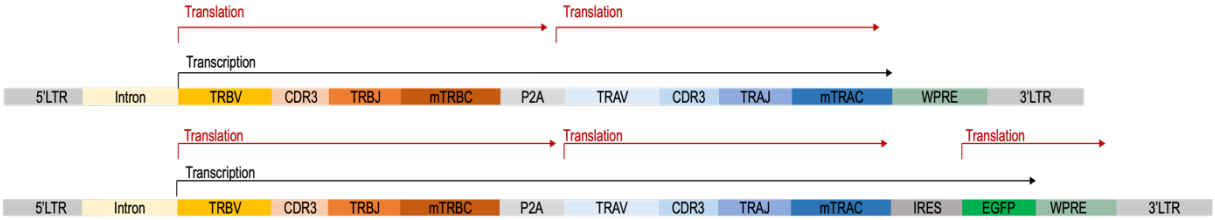

Figure 2: Schematic overview of TCR transgene expression vectors.

Mouse and human TCRs were expressed in MP71-PRE vectors, the TCR β chain was followed by a P2A element (self-cleaving peptide) and by the TCR α chain. Also for human TCRs, mouse constant regions were used. For in vivo experiments using HHD T cells, IRES GFP was used to monitor TCR expression. Black arrows indicate transcripts and red arrows polypeptide chains after translation.

PCR for TCR sequencing

The method and primers for TCR sequencing were adapted from the laboratory of Leo Hansmann (Saligrama et al. 2019; Han A, Glanville J, Hansmann L 2014). TCR mRNA from single cells was converted into cDNA using Qiagen One-Step RT-PCR kit and in the same reaction amplified with a first set of TCR primers (phase 1 primers). Phase two and three PCRs were performed each with a different set of (nested) TCR primers using Qiagen HotStarTaq. For phase three, column- and plate+row barcode primers were added to trace each sequence to the corresponding well in the 96-well plate. A schematic overview of the PCR reactions is shown in Figure 3. Detailed PCR conditions are listed in Table 1, primers are listed in Table 5. After the phase 3 PCR, the wells and plates were pooled using 5 µl of each well and the solution was purified on an agarose gel for subsequent illumina sequencing. Remaining sample from phase 3 was loaded on an agarose gel to verify amplification and correct band size (approx. 300 bp).

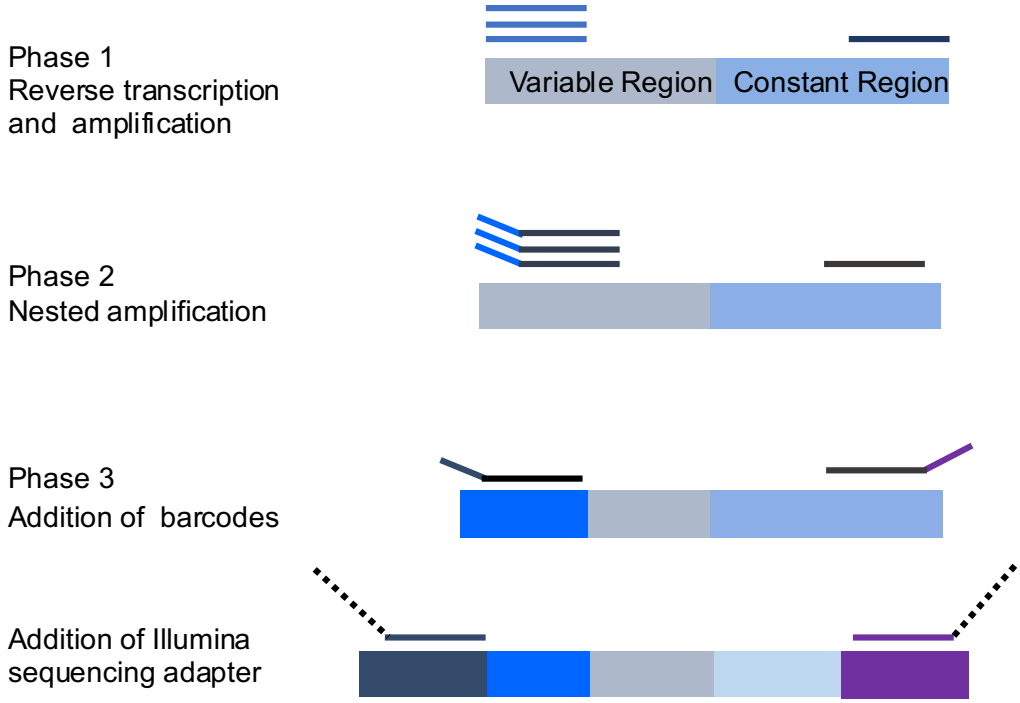

Figure 3: PCR strategy to amplify TCR transcripts from single T cells.

Phase 1 combines a reverse transcription of TCR mRNA with a first PCR for sequence amplification. Phase 2 uses nested primers that enhance the specificity, amplify the transcripts of phase 1 and add adapters to the 5' end of the transcript that are the binding site for phase 3 primers. Phase 3 adds barcodes in 5' and 3' to the transcripts that allow to trace DNA sequences to their original well positions. Additionally, in phase 3 illumina sequencing adapter are added to the transcripts.

Table 1: SINGLE CELL PCR PROTOCOL FOR TCR SEQUENCING

Phase 1, Qiagen One-Step	*Phase 2, Qiagen HotStar Taq*	*Phase 3, Qiagen HotStar Taq*
5x buffer (0.8 µl), dNTPs 10 mM (0.64 µl), TCR phase 1 primer mix (0.8 µl), enzyme mix (0.64 µl), RNase-free water (0.72 µl). 4 µl/well added to thawed 12 µl of sorting product.	*10x buffer (1.2 µl), dNTPs 10 mM (0.24 µl), TCR phase 2 primer mix (0.6 µl), water (8.89 µl), Taq polymerase (0.07 µl). 11 µl per well were added into a fresh plate together with 1 µl of PCR product from phase 1.*	*10x buffer (1.4 µl), dNTPs 10 mM (0.3 µl), PE primer 1 and 2 (0.7 µl each), water (5.09 µl), Taq polymerase (0.07 µl). 9 µl per well were added into a fresh plate together with 2 µl column barcode primers, 2 µl plate+row barcode primers and 1 µl PCR product from phase 2.*
50°C/36 min, 95°C/15 min, (94°C/30 s, 62°C/60 s, 72°C/60 s) x25, 72°C/10 min	*95°C/15 min, (94°C/30s, 64°C/60s, 72°C/60 s) x25, 72°C/7min*	*95°C/15 min, (94°C/30s, 66°C/30s, 72°C/60 s) x36, 72°C/5min*

Cell culture

Cell culture was performed under sterile conditions, cells were incubated at 37°C with 5% CO_2. Mycoplasma testing by PCR was performed regularly and infected cells were treated with plasmocin until they were mycoplasma negative. Complete medium for cell lines was RPMI 1640 GlutaMAX (Gibco) with 100 U/ml penicillin/streptomycin

(1% P/S) and 5% heat-inactivated fetal calf serum (FCS) (Pan Biotech). T cells were cultured in the same medium with 10% FCS and additional 1 mM sodium pyruvate (Gibco), 100 μM non-essential amino acids (Gibco) and 50 μM 2-mercaptoethanol (Gibco).

Viral transduction

Virus containing supernatant was produced using PlatE cells (Cell Biolabs) for mouse cells and RD114 cells for human cells. PlatE cells are derived from HEK293 cells and express gag-pol and env genes of the murine leukemia virus (MLV) required for the assembly of virus particles. PlatE cells were culture in DMEM medium supplemented with 10% FCS and 1% P/S and, except for the three days prior to infection, cultured in the presence of 10 µg/ml blasticidin and 1 µg/ml puromycin (Sigma-Aldrich). Both packaging cell lines were seeded into 6-well plates and transfected with DNA plasmids containing the transgene of interest to produce viral particles. Transfection was performed either with lipofectamine 2000 (Thermo Fisher) or with calcium chloride. In the case of lipofectamine, 3 μg DNA was used to transfect one well of PlatE or RD114 cells. The DNA was mixed with 300 μl DMEM without additives and mixed with 5 µl lipofectamine diluted in 295 μl DMEM. For calcium chloride transfection, 18 μg DNA per well was used which was mixed with $CaCl_2$ (250 mM final concentration) and 150 μl transfection buffer (1.6 μg NaCl, 74 mg KCl, 50 mg Na2HPO4, 1 g HEPES, add 100 ml H2O, pH 6.76) in a final volume of 300 μl adjusted with water.

T cell expansion

Human PBMCs: Non tissue culture-treated 24-well plates were coated with 5 μg/ml anti-CD3 (clone OKT3) and 1 μg/ml anti-CD28 (clone CD28.2) in 1 ml PBS over night at 4°C (Monday), then blocked for 30 min at 37°C with 2% BSA and washed twice with PBS (Tuesday). Human PBMCs that were purified from buffy coats and stored in liquid nitrogen were thawed and seeded at a density of $1.5x10^6$ cells/well in 1 ml T cell medium containing 400 IU/ml IL-2 (1:1,000) (Chiron). T cells were expanded in the presence of IL-2 at the same concentration for a total of 13 days and maintained a concentration of approximately $2x10^6$ cells/ml by adding fresh T cell medium. On day 13 (Monday) T cells were rested for two days at a ten times lower concentration of IL-2 (1:10,000) in 50% of the prior volume. On day 15, T cells were frozen or used for experiments.

Mouse splenocytes: For TCR transductions, splenocytes were freshly isolated each time. Donor mouse spleens were passed through a cell strainer (40 μm), washed, erythrocytes were lysed in 5 ml cold ACK lysis buffer for 5 min, cells were washed with medium again, passed through a second cell strainer and counted. The splenocytes were seeded at a concentration of $2x10^6$ cells/ml in T cell medium with anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51) and IL-2 40 IU/ml (1:10,000) (Wednesday). Approximately half of the cells could be recovered on the following day to be seeded for transduction. $1.5x10^6$ cells/well were seeded in 0.5 ml on previously centrifuged (90 min, maximum speed, 4°C) virus supernatant together with protamine sulfate, 10 μl/well mouse T cell activator beads (anti-CD3/CD28 Dynabeads, Gibco) and additional IL-2 to maintain a concentration of 40 IU/ml. The T cells together with the virus supernatant were centrifuged for 30 min at 32°C and 800 G. On the next day (Friday) fresh virus supernatant was added to the T cells after removing 1 ml of the supernatant and the cells were again centrifuged for 90 min at 32°C and 800 G. Approximately six hours later, T cells were diluted approximately 1:5 with T cell medium and received IL-15 1:1,000 (50 ng/ml) instead of IL-2. T cells were expanded for nine more days on IL-15 1:1,000 at a cell density of approximately $2x10^6$ cells/ml and frozen (Monday) or used for in vitro assays. For in vivo transfer, T cells were already used on day 4 after transduction (Monday) after removal of the T cell activator beads using EasySep magnets (Stem Cell).

Cell lines

Target cells in co-cultures were for mp68 ($p68_{S551F}$) TCRs: 8101 lines derived from an UV-irradiated C57BL/6 mouse (Dubey et al. 1997): 8101PRO (progressor variant lacking the antigen mp68), 8101RE (regressor variant with endogenous expression of the antigen mp68) and 8101PRO-mp68 (8101PRO transduced with pMP71-PRE expressing the triple epitope $(SNFVFAGI\text{-}AAY)_{3x}$-Thy1.1), this cell line was also used for in vivo adoptive T cell transfer experiments.

For $CDK4_{R24L}$ TCRs: in the HHD mouse setting: MC703-45 (clone of MC703) (Leisegang, Kammertoens, et al. 2016), MC703-ALD (Leisegang, Kammertoens, et al. 2016) (MC703-45 transduced with the triple epitope of $CDK4_{R24L}$) $(ALDPHSGHFV\text{-}AAY)_{3x}$-GFP, MC703-R24L(Leisegang, Kammertoens, et al. 2016) (transduced with the full-length mutated $CDK4_{R24L}$); in the human T cell setting: WM-902B (naturally harboring the mutated $CDK4_{R24L}$ but lacking HLA-A2), WM-902B-A2 (WM-902B

transduced with HLA-A2 transgene)(Leisegang, Kammertoens, et al. 2016), 624-Mel-38 (expressing wild-type CDK4 and HLA-A2).The used cell lines are listed in Table 2.

Table 2: Cell lines used in this work.

Short name	Parental cell line	Transgene/antigen	Reporter	Gene linker	Clonality	Host	Experiments
4E9	8101PRO	mp68 triple epitope. (SNFVFAGI)	Thy1.1	AAY	Single cell clone	C57BL/6 Mouse	Immunization and tumor induction in immune competent mice. Generation of mp68-specific TCRs.
8101PRO					Bulk		Negative control for co-cultures.
8101 RE		Endogenous mp68 expression.					Positive control for co-cultures.
8101PRO-mp68	8101PRO	mp68 triple epitope.	Thy1.1	AAY			Mouse experiments with ATT and co-cultures.
8101-12 GFP	8101-12	Endogenous mp68 expression.	GFP		Bulk (GFP) of single cell clone		Incucyte.
MC703-45	MC703				Single cell clone	HHD mouse	Negative control for co-cultures
MC703-ALD	MC703-45	CDK4 R24L-triple epitope. (ALDPHSGHFV)	GFP	AAY	Bulk		Mouse experiments with T cell transfer and co-cultures
MC703-ACD	MC703-45	CDK4-R24C triple epitope.		AAY			Co-cultures.
MC703-R24L	MC703-45	CDK4 R24L full length.		IRES	Single cell clone		Co-cultures.
MC703-R24C	MC703-45	CDK4 R24C full length.		IRES			Co-cultures.
WM-902B		Endogenous CDK4-R24L mutation.			Bulk	Human	Negative control for co-cultures.
WM-902B-A2	WM-902B	HLA-A2	GFP	IRES			Co-cultures.
SKMel-29		Endogenous CDK4-R24C mutation.					Co-cultures.
624MEL-38							Co-cultures negative control.

Functional Assays

Co-culture for IFNγ ELISA

For cytokine release assays, $5x10^4$ TCR-transduced and activated T cells (thawed) were co-cultured with the same number of target cells for 16-24 h in U-bottom 96-well plates with a final volume of 200 µl T cell medium. Untransduced T cells were used as a negative control. Target cells were either tumor cell lines (cultured for at least 2 days prior) or peptide-loaded irradiated (63 Gy) mouse splenocytes (thawed) or T2 cells (cultured for at least 2 days prior). T2 cells were incubated with peptide for 2 h and then washed before co-culture. T cells without target cells (labelled Min) and with 1 µM ionomycin (Calbiochem) and 5 ng/ml phorbol-12-myristate-13 acetate (PMA) (Promega) (labelled Max or PMA/Iono) for TCR-independent cytokine release were used as negative and positive control, respectively. Peptide serial dilutions were performed in the range of $1x10^{-6}$ to $1x10^{-12}$ M. After co-culture, 50 µl of the cell supernatant was used for IFNγ ELISA (BD) which was performed according to the manufacturer's instruction but using half of the suggested volumes. Peptides were ordered from JPT in a purity >95% purified by HPLC. The co-cultures were performed in technical duplicates and repeated with different T cell transductions.

Co-culture for CD137 upregulation and TCR internalization

The upregulation of CD137 and internalization of the transgenic TCR after 24 h co-cultures with target cells was used as an additional read-out of TCR quality for TCR-Ts co-cultured with WM-902B-A2 tumor cells. $5x10^4$ T cells were cultured with $1x10^4$ tumor cells in 96-well U-bottom plates and T cells were retrieved from the supernatant and stained with anti-CD8, anti-CD3, anti-mouse TCR β constant region and anti-CD137 (Biolegend). T cells that were cultured with and without tumor cells were measured side-by-side for normalization purposes. The percentage of $CD137^+$ cells was normalized to the transduction rate as determined from % mCb^+ of $CD8^+$ $CD3^+$ cells in the tumor cell-free wells. TCR internalization was calculated by dividing the %mCb^+ of T cells cultured with and without tumor cells ($TCR\ internalization = 100 - \frac{\%mCb\ with\ target}{\%mCb\ without\ target} \times 100$). The gate on live lymphocytes was set based on their forward and sideward scatter and the percentage of live lymphocytes after co-culture was small. If less than 1% of the cells were still alive according to this gate, the sample was censored.

Co-culture for Incucyte killing assays

For long-term cytotoxicity assays, the Incucyte (Model SX5, Sartorius) was used which is equipped with a fluorescence microscope and a camera. It can quantify the confluence of GFP-transduced tumor cell lines. The 96-well flat-bottom plates were imaged every 2 hours with a 10x objective and four images per well were taken. These assays were performed in technical triplicates and the border rows and columns were kept empty. $2x10^3$ tumor cells (WM-902B-A2 or 8101-12-GFP) were seeded one day before the addition of $10x10^3$ TCR^+ T cells. In the case of long-term cytotoxicity assays, the total number of T cells was adjusted with untransduced T cells to obtain the same number of total T cells for the different TCRs. Every three days, additional $2x10^3$ tumor cells were seeded into the wells after removing 100 μl of the supernatant (re-challenge). No fresh T cells were added. To calculate the percent of specific killing at 152 h, the following formula was used: $\%\ specific\ killing = 100 - ((\frac{Confluence\ sample}{Confluence\ UT}) x100)$.

In vivo and ex vivo experiments

Tumor induction for adoptive T cell transfer

Tumor cells were cultured for at least 3 days before inoculation in mice. $3\text{-}5x10^6$ tumor cells were resuspended in 100 μl PBS and injected subcutaneously into the left flank of anesthetized mice that were at least 8 weeks old. Mice were bred in a specific pathogen-free environment at the animal facility of the Max-Delbrück-Center for Molecular Medicine. All animal experiments were conducted under the institutional and national guidelines and approved by LAGeSo Berlin. The tumor growth was monitored using a caliper every 2-3 days. To test the human mutant CDK4-specific TCRs, HHDxRag1$^{-/-}$ mice (Pascolo et al. 1997; Leisegang, Kammertoens, et al. 2016), HHD mouse T cells and HHD mouse derived tumor line MC703-ALD were used (Leisegang, Kammertoens, et al. 2016). HHD mice express the human HLA-A2 molecule fused to the third domain of mouse H-2D^b and to human β_2-microglobulin instead of murine MHC- I. To test mouse mp68-specific TCRs, Rag1$^{-/-}$ mice (B6.129S7-*Rag*$^{1tm1Mom=}$), T cells from heterozygous Rag1$^{+/-}$ spleens and the C57BL/6-derived tumor cell line 8101PRO (Dubey et al. 1997; Leisegang, Engels, et al. 2016) transduced with the triple epitope of mp68 linked to Thy1.1 was used (8101PRO-mp68). Mice were sacrificed at humane endpoints of tumor burden, overall health condition or at the experimental end point on day 100 post ATT.

T cell therapy of tumor bearing mice

Tumor-bearing Rag1$^{-/-}$ mice received TCR-Ts intravenously 2-4 weeks after tumor induction when the majority of tumors had a size of approximately 150 mm^3. Treatment groups were allocated with a similar average tumor size except for negative controls (untransduced T cells) which were always injected into the mice with the smallest tumors. TCR-Ts were always fresh and used on day 4 after transduction (Monday) and 1x10^6 CD8$^+$ TCR$^+$ T cells per mouse were injected intravenously resuspended in 100 µl PBS. The total cell number was not adjusted between different TCRs. Control mice received untransduced T cells (UT) or no T cells. T cell expansion was monitored in blood samples taken on days 4, 7, 14, 21 and 28 after T cell transfer. T cells in 50 µl of blood were stained after FC block (anti-CD16/CD32) incubation with anti-CD8 BV421, anti-CD3 APC and TCR; in the case of mp68 TCRs mouse TCR β variable chain according to TCR and in the case of CDK4 TCRs, TCRs were linked via IRES to GFP and GFP was used to detect TCR transduced T cells.

Tumor digest

Tumors were resected from sacrificed mice, homogenized using gentle MACS dissociator in MACS C tubes and single cell suspensions were generated using RPMI medium supplemented with 5% FCS, 2 mg/ml collagenase D and 10 IU/ml DNase I at 37°C for 1 h followed by 30 min with an additional 1 ml 10x trypsin-EDTA solution. Tumor digests where then washed, passed through a 40 µm cell strainer and frozen. TILs were analyzed directly from tumor digests using FACS. Tumor digests were stained for PD-1, CD3, CD8 and 7AAD to analyze the percentage of CD3$^+$ and CD8$^+$ cells within live tumor digest and to analyze GFP$^+$ T cells and their MFI in PD-1 APC. For PD-1 staining, each tumor digest was also stained with the isotype of the anti-PD-1 antibody and the MFI of the isotype was subtracted from the MFI of the anti-PD1 antibody. To calculate ΔPD-1, the MFI of PD-1 in GFP$^+$ TILs was subtracted by that of GFP$^-$ TILs. To generate tumor reisolates of pure tumor cells, the tumor digest was seeded into cell culture flasks with cell culture medium. The suspension cells were discarded, and adherent cells were passaged at least three times into fresh cell culture flasks to obtain a pure tumor cell line. These lines were called reisolates (Reis) and labelled with their respective mouse ID. Tumor reisolates were analyzed for antigen reporter gene expression (Thy1.1 or GFP) and HHD (HLA-A2) expression. The MFI of HLA-A2 APC was subtracted by the MFI of their respective isotypes. Recognition of reisolates by T cells was tested using IFNγ ELISA as described above.

Generation of mp68-specific TCRs

To obtain mp68-specific TCRs, the doxycycline-inducible tet-on system based on Heinz and colleagues (Heinz et al. 2011) was used to generate clone 4E9 from 8101PRO tumor cells. Clone 4E9 has a tightly controlled doxycycline-inducible expression of three copies of the mp68 epitope, linked with the proteasomal cleavage site AAY between each epitope and before Thy1.1 (CD90.1) as an expression marker $(SNFVFAGI\text{-}AAY)_3$ Thy1.1), similar to expression systems published previously (Engels et al. 2013) (see Table 2). For tumor induction, $Rag1^{+/-}$ (B6.129S7-*Rag*^{1tm1Mom}) immunocompetent mice that were 26 weeks old were inoculated with 3-5x10^6 4E9 cells that were cultured without doxycycline and showed a negative Thy1.1 staining prior to transplantation. After 3 weeks, all tumors were at least palpable, and mice started to receive doxycycline via their drinking water to induce mp68 expression until they were sacrificed. For immunization, 22-23 weeks old mice were also inoculated with 3-5x10^6 4E9 cells, however, these had been cultured in the presence of 500 ng/µl doxycycline for 48 h, showed a positive Thy1.1 staining and were irradiated with 20 Gy before inoculation to prevent tumor outgrowth. Immunized mice received doxycycline in their drinking water from 48 h prior to immunization onwards. The immunized mice received two boosts in the form of live 4E9 cells (cultured with doxycycline) 6 and 10 weeks later and were sacrificed ten days after their second boost. Tumors were converted into single cell suspensions as described above and were stained and sorted on the day of sacrifice. Splenocytes were frozen and then stained and sorted two days later.

Single cell sort for mp68-tetramer-binding T cells

Single cell suspensions of tumors or spleens were stained, after incubation with FC block, first with H-2K^b:mp68 tetramers (H- 2K^b: SNFVFAGI multimers, PE, MBL) for 20 min at 4°C, washed once and then stained with anti-mouse CD8 and anti-mouse CD3 for 15 min at 4°C and washed twice. Live dead staining 7AAD was added shortly before FACS measurements. Single $CD3^+$ $CD8^+$ tetramer-binding cells were directly sorted (BD Aria Fusion) into 96-well PCR plates (Sarstedt) containing 12 µl of 1x One-Step RT-PCR buffer (Qiagen) and snap frozen on dry ice. The last wells (12 E, F G, H) were left empty as a negative control on every plate. When possible, two plates were sorted for each mouse.

Immunization of a ABabDII mouse to obtain TCR M58

TCR M58 was generated by Gerald Willimsky who kindly provided it for the present comparisons. ABabDII mice were immunized with the $CDK4_{R24L}$ epitope ALDPHSGHFV three times with plasmid (1-2 μg) using gene gun and three times with peptide (100 μg) combined with CpG oligoneucelotides and incomplete Freund's adjuvant as adjuvants. The immunizations were performed 1, 5, 7, 9 and 10 months after the initial immunization. Eight days after the last immunization, the mouse was sacrificed and its splenocytes were cultured for 10 days in the presence of $1x10^{-8}$M ALD peptide and then FACS sorted after IFNγ capture assay.

Results

Human $CDK4_{R24L}$-specific TCRs

Table 3 lists the human HLA-A2 restricted $CDK4_{R24L}$ mutation-specific TCRs used in this thesis. Behind the IMGT name (Folch and Lefranc 2000) of the TRBV region, I included in brackets the corresponding v-beta name according to ARDEN (Arden et al. 1995) which is normally used to identify specific antibodies for FACS. I labeled v-beta regions for which no specific antibody was available with an asterisk. These TCRs could not be stained in mouse T cells. Therefore, for in vivo experiments, the TCRs were combined with IRES-GFP as an expression marker to identify TCR-Ts. TCR 14/35 was obtained from a patient whose melanoma carried the $CDK4_{R24C}$ mutation. Importantly, this TCR was obtained from peripheral blood after the patient was disease-free for years after tumor resection and repeated tumor cell vaccination (Livingston et al. 1979). Consequently, this TCR should not necessary be considered as patient derived. TCR 14/35 nevertheless served as a positive control for the other TCRs since it had already been extensively characterized (Leisegang, Kammertoens, et al. 2016). TCR P17 (called TCR 17 in the original publication) was the only TIL-derived TCR in the set of TCRs. It was obtained after TIL culture by tetramer staining (Kvistborg et al. 2012).

Table 3: CDK4-R24L targeting human TCRs used in this work.

Name	CDK4 mutation	Host	V Region		CDR3	Site	Reference
14/35	ACD	patient SK29(AV)	TRAV20	TRAJ58	CAVQSGTSGSRLTF	PBMCs	Wölfel et al. 1995 (1987)
			TRBV9 (vb1)	TRBJ2-1	CASSVVAGFNEQFF		
M58 (15158)	ALD	mouse	TRAV12-2	TRAJ26	CAVNMPYGQNFVF	Spleen	Not published, Gerald Willimsky
			TRBV29-1 (vb4)*	TRBJ2-3	CSVGQGDTQYF		
P17 (17)		patient	TRAV29		CAAFLQSNDMRF	Tumor	Kvistborg et al. 2012
			TRBV27 (vb14)		CASRASGREQFF		
H53 (53)		Healthy donor #3	TRAV8-2	TRAJ34	CVVSDLYNTDKLIF	PBMCs	Strønen et al. 2016c
			TRBV7-8 (vb6.2)*	TRBJ2-7	CASSQNYEQYF		
H55 (55)		Healthy donor #4	TRAV14 /DV4	TRAJ37	CAMSSLSGNTGKLIF		
			TRBV6-5 (vb13)	TRBJ2-7	CASSYSWGAGYEQYF		
H57 (57)			TRAV8-1 (va8.1)	TRAJ20	CAVILRSNDYKLSF		
			TRBV29-1 (vb4)*	TRBJ2-2	CSAGTGELFF		

*no commercial antibody available.

In vitro characterization of TCRs

The $CDK4_{R24L}$-specific TCRs were in part taken from different publications in which they had been tested and characterized separately. I expanded this data by comparing them directly side-by-side and including TCR M58 that was generated in our laboratory in an ABabDII mouse. The experiments confirmed published data and the direct comparison showed that except for TCR H55, all TCRs had similar levels of functional avidity in vitro. This was confirmed by IFNγ release, long-term killing assays using the Incucyte as well as FACS staining for activation marker CD137 and TCR internalization.

First, the TCRs were tested in HHD T cells in vitro as HHD T cells and HHD tumor cell line MC703-ALD would later be used for in vivo experiments. For in vitro experiments using HHD T cells, I used the same TCR expression vectors without reporter gene as for human T cells. Figure 4 A and B show IFNγ release by HHD T cells when co-cultured with ALD peptide-loaded HHD splenocytes. The log EC50 values in Figure 4 C are derived from B from three independent co-cultures and transductions. One can appreciate a clearly worse EC50 for TCR H55 compared to the other TCRs.

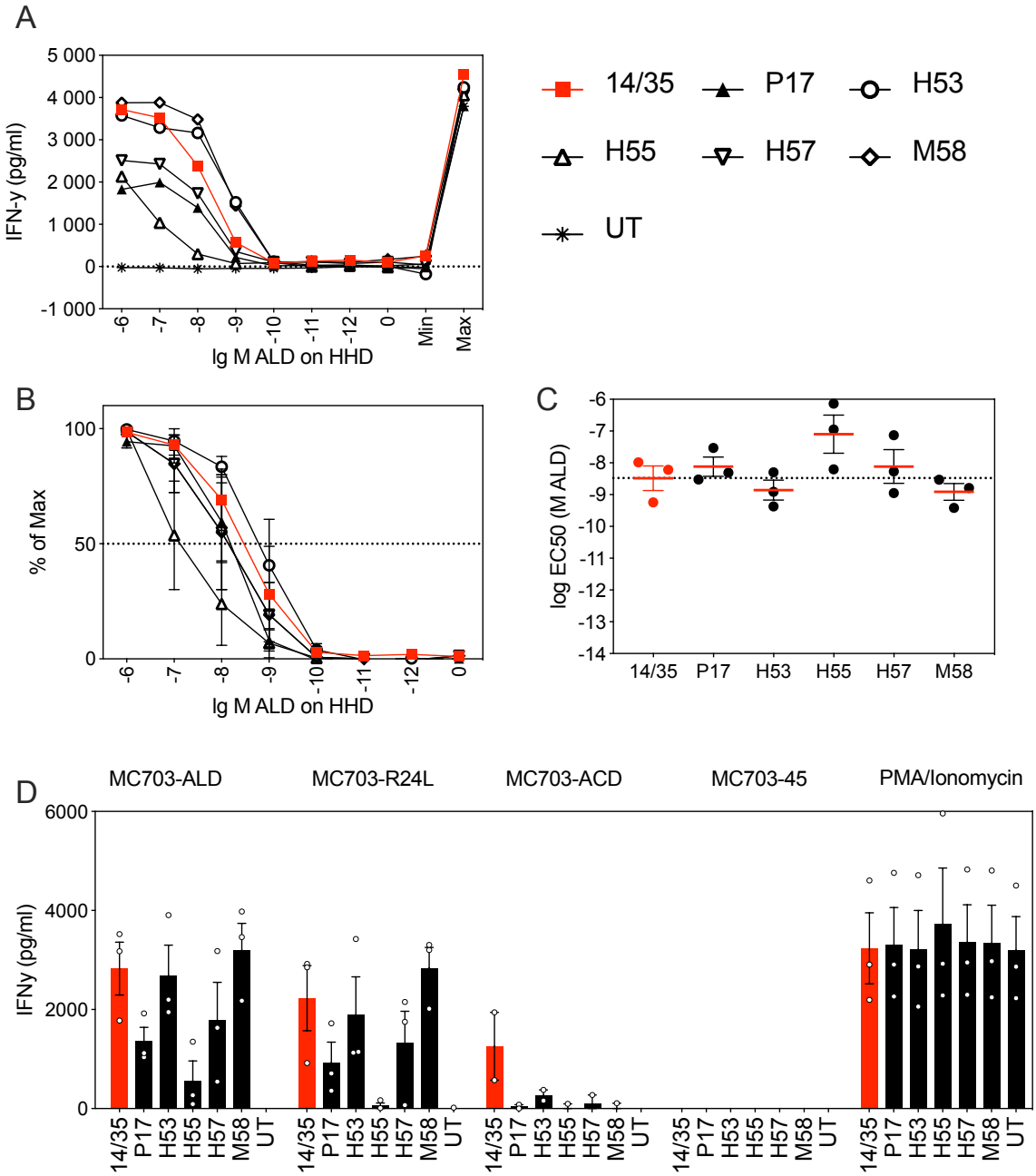

Figure 4: The function of human TCRs is recapitulated in HHD T cells.

A+B) *Graded amounts of ALD peptide were loaded on irradiated HHD splenocytes and co-cultured with TCR-transduced HHD T cells for 24 h. Absolute IFNγ concentrations from one representative co-culture* ***(A)*** *and IFNγ values normalized to the maximum of released IFNγ mean from three co-cultures* ***(B)*** *are shown.* ***C)*** *Log EC50 values derived from B. Paired t-tests: 14/35 vs. P17 p=0.269 ns, vs. H53 p=0.149 ns, vs. H55 p=0.029 *, vs. H57 p=0.299 ns, vs. M58 p=0.078 ns. P17 vs. H53 p= 0.01 **, vs. H55 p=0.1009 ns, vs. H57 p=0.9833 ns, vs. M58 p=0.0380 *.* ***D)*** *TCR-transduced HHD T cells were co-cultured for 24 h with MC703 tumor cells transduced with ALD triple epitope (ALD), full length (R24L), untransduced (45) or transduced with ACD triple epitope (ACD). The data points correspond to three independent co-cultures. Error bars correspond to standard error of the mean (SEM).*

In Figure 4 D, TCR-Ts were co-cultured with HHD mouse tumor cell line MC703, transduced with the mutated CDK4 antigen as triple epitope (ALD), as full length

(R24L), the triple epitope with the cysteine point mutation $CDK4_{R24C}$ (ACD), or no antigen (-45). $CDK4_{R24C}$ is a weaker antigen than $CDK4_{R24L}$ as it has a lower predicted affinity to HLA-A2. This explains the overall lower IFNγ values when using MC703-ACD as target cells. In fact, only TCR 14/35 shows clear recognition of $CDK4_{R24C}$ (ACD). This TCR stems from a patient whose tumor had the $CDK4_{R24C}$ mutation. Overall, the experiments in HHD T cells confirmed the functionality of the studied TCRs and their recognition of MC703-ALD.

I did further in vitro characterizations with transduced human T cells. These included co-culture with target peptide ALD loaded on T2 cells, co-culture with human melanoma lines, long-term killing assays in the Incucyte and FACS analysis of CD137 expression and TCR internalization.

Figure 5 shows the IFNγ release by TCR-Ts when co-cultured either with ALD peptide-loaded T2 cells or melanoma cell lines expressing different CDK4 mutations. Figure 5 A shows the mean of three co-cultures of TCR-Ts and T2 cells loaded with graded amounts of ALD peptide for TCRs 14/35, P17, H53, H55, H57 and M58. The IFNγ release as a percentage of the highest amount of released IFNγ (% of Max) was used to calculate the logEC50 values that are shown in Figure 5 B. One can observe a clear advantage for TCR P17 and a clear disadvantage for TCR H55. Figure 5 C shows IFNγ concentrations in supernatants after 24 h co-cultures of TCR-Ts with different human melanoma lines that naturally harbor different CDK4 mutations ($CDK4_{R24C}$ for SkMel-29 and $CDK4_{R24L}$ for WM-902B). Since WM-902B cells are HLA-A2 negative, they were stably transduced with HLA-A2-IRES-GFP to be able to present the antigen of interest to the tested TCRs. The tumor cells that lacked either HLA-A2 (WM-902B) or the CDK4 mutation (624-Mel-38) were not recognized by the T cells. Similar to MC703-ACD in Figure 4, the recognition of $CDK4_{R24C}$ was markedly lower than the recognition of $CDK4_{R24L}$. Apart from TCR 14/35, only TCR P17 recognized the $CDK4_{R24C}$ antigen in SkMel-29 cells.

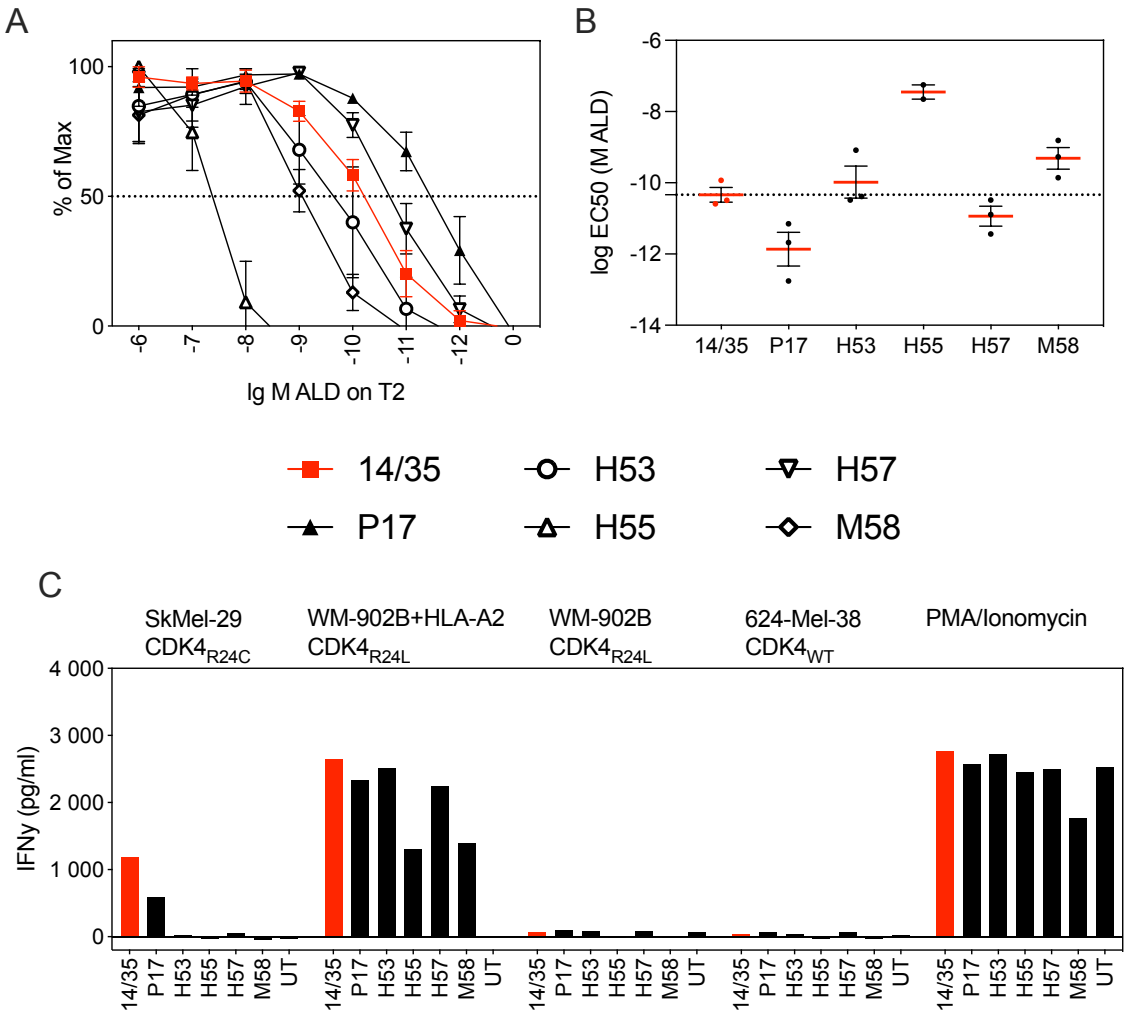

Figure 5: Functionality of all ALD TCRs was confirmed in human T cells.

A-B) *T2 cells were incubated with graded amounts of ALD peptide for 2 h, washed, and co-cultured with TCR-Ts for 24 h.* ***A)*** *IFNγ concentrations normalized to the maximum of released IFNγ within the curve are shown.* ***B)*** *Logarithmic EC50 values derived from A for three independent co-cultures. Paired t-tests: 14/35 vs. P17 p= 0.0417 *, vs. H53 p=0.2844 ns, vs. H55 p=0.0178 *, vs. H57 p=0.0454 *, vs. M58 p=0.0890 ns. P17 vs. H53 p=0.0241 *, vs. H55 p=0.0100 *, vs. H57 p= 0.0434 *, vs. M58 p=0.0168 *.* ***C)*** *IFNγ concentrations measured by ELISA after 24 h of co-culture. One representative co-culture of human TCR-Ts with different melanoma lines endogenously expressing the CDK4 mutations (as indicated on top of the graph) is shown. Error bars correspond to SEM.*

Apart from IFNγ release I also evaluated surface markers on T cells after 24 h co-cultures with target cells as shown in Figure 6. I observed a marked internalization of the stimulated TCR, i.e. fewer transgenic TCR-expressing cells after 24 h of co-culture with target cells (WM-902B-A2) as compared to cultured T cells without target cells. The same co-cultures were used to quantify the surface expression of the activation marker CD137 in T cells. The CD137 expression was normalized to the transduction rate which was the percentage of TCR^+ (mCb^+) cells in wells without tumor cells. The gating strategy is illustrated in Figure 6 A. After 24 h of co-culture, the viability of the

T cells was decreased, and I observed a trend to worse viability in T cells cultured with target cells (not shown). Viable lymphocytes were gated on $CD3^+$ and $CD8^+$ T cells to exclude $CD4^+$ T cells and contaminating tumor cells. The small population of FITC-positive $CD3^-$ cells can be attributed to GFP-positive tumor cells that had detached from the well and were therefore present in the supernatant. The gate for CD137 quantification is shown in blue and the gates for TCR surface expression are shown in red. The two FACS plots on the right-hand side of Figure 6 A correspond to T cells cultured with and without target cells and one can appreciate the difference in TCR surface expression when comparing the red gates. A strong stimulation of CD137 expression and TCR internalization was observed with all TCRs except TCR H55. Albeit lower, also TCR H55 showed a response to the target cells.

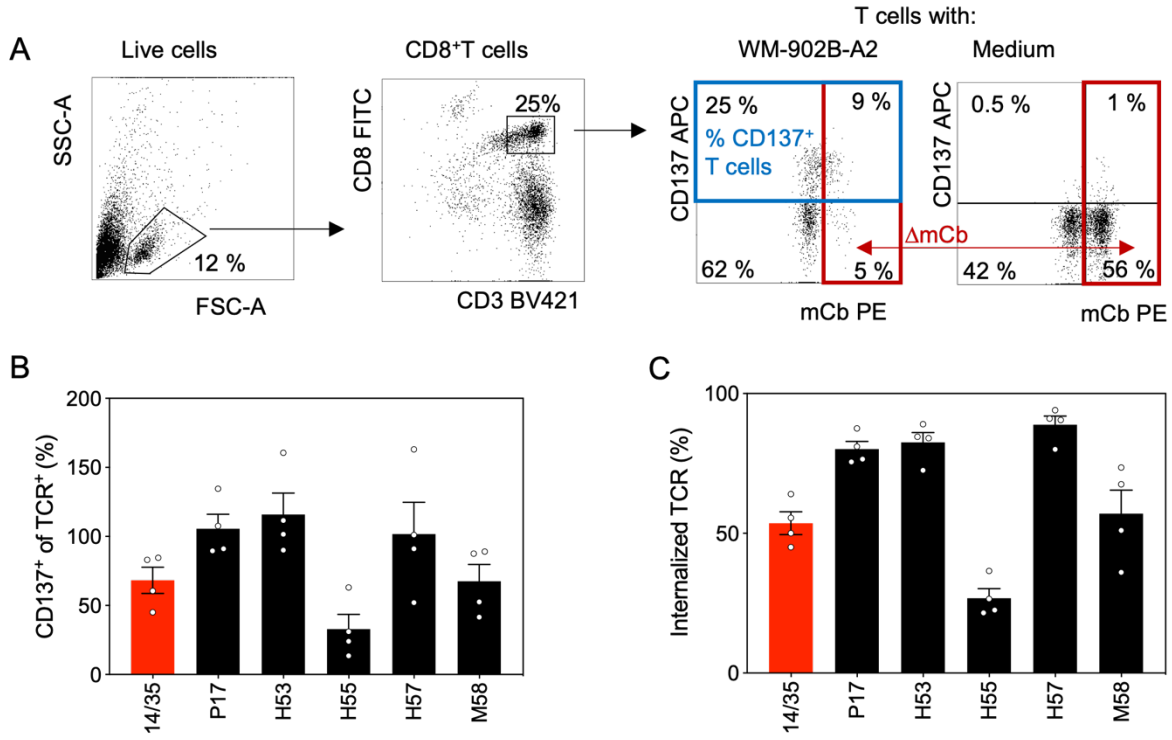

Figure 6: CD137 and TCR surface expression could complement cytokine release assays.

A) *Example of FACS plots and gating strategy for human TCR-transduced T cells from co-culture supernatant after 24 h co-culture with or without target cells WM-902B-A2. The gates used for CD137 and mouse TCR surface expression are shown in blue and red, respectively.* ***B)*** *Quantification of CD137 expression from four independent co-cultures. The percentage of $CD137^+$ T cells was normalized to the transduction rate of the respective TCR as determined by $mTCR\beta^+$ cells in T cells co-cultured without tumor cells. Paired t-tests: 14/35 vs. P17 p=0.0081 **, vs.H53 p=0.018 *, vs. H55 p=0.0637 ns, vs. H57 p=0.2425 ns, vs. M58 p=9260 ns. P17 vs. H53 p=0.1670 ns, vs. H55 p=0.0024 **, vs. H57 p=0.8443 ns, vs. M58 p=0.0125 *.* ***C)*** *The percentage of internalized TCR was obtained by comparing TCR^+ T cells in wells with and without tumor cells for each TCR. Paired t-tests: 14/35 vs. P17 p=0.018 *, vs.H53 p=0.019 *, vs. H55 p=0.015 *, vs. H57 p=0.009 **, vs. M58 p=0.727 ns. P17 vs. H53 p=0.4396 ns, vs. H55 p=0.0015 **, vs. H57 p=0.0420 *, vs. M58 p=0.0427 *. Error bars correspond to SEM.*

Cytotoxicity assays using the Incucyte as shown in Figure 7 were the only in vitro assays that could test long-term performance of the T cells. In these assays, TCR-Ts were incubated with target cells (WM-902B-A2) and the target cell confluency was assessed by automated microscopy imaging. These assays revealed a very similar long-term performance of all tested TCRs except for TCR H55 which was the only TCR that had also shown a markedly lower functional avidity in the other assays. The other TCRs could control tumor cell outgrowth for 6 days or two re-challenges with tumor cells after which outgrowth occurred slowly. I observed that the outgrowth when using fresh T cells was delayed by about one re-challenge or three days and started on day 9. Figure 7 includes experiments with fresh and frozen T cells. This assay clearly showed the insufficient avidity of TCR H55 to control tumor cell growth as can be appreciated in the grey curve that has almost the same outgrowth kinetics as the tumor cells with untransduced T cells (black curve) in Figure 7. Overall, the long-term cytotoxicity assays confirmed the in vitro experiments shown in Figure 4-6.

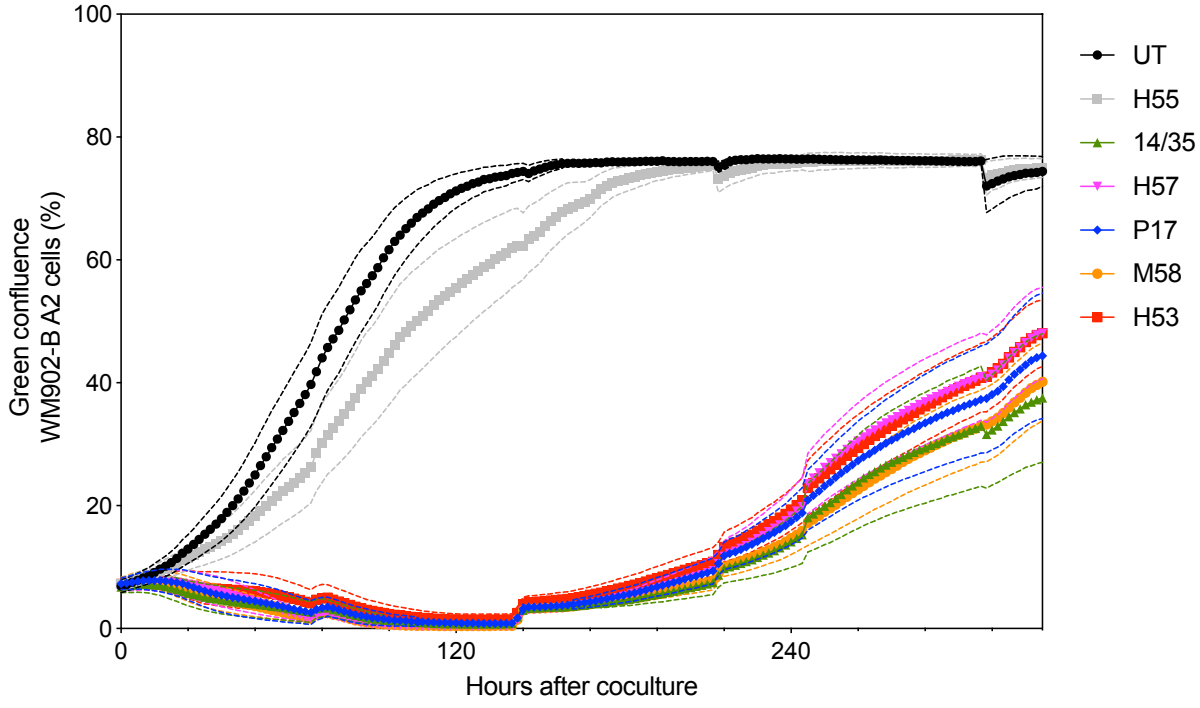

Figure 7: Cytotoxicity assays confirmed high functional avidity of mutant CDK4-specific TCR-Ts.
Cytotoxicity of TCR-Ts on WM-902B-A2 cells was measured in the Incucyte. The percent confluence of green fluorescent tumor cells was quantified from images taken every 2 hours for two weeks. The mean and SEM of five independent experiments and three different transductions is shown. TCRs are color coded: black: untransduced T cells, grey: TCR H55, green: TCR 14/35, pink: TCR H57, blue: TCR P17, orange: TCR M58 and red: TCR H53.

Adoptive T cell transfer targeting ALD on tumors in HHD mice

The best preclinical test for TCR quality is in vivo tumor treatment. I chose TCRs 14/35, P17, H53, M58 and H55 for in vivo experiments. TCR H55 had already shown a weaker

performance in vitro and was included as a negative control additionally to untransduced T cells. HHDxRag1 immunodeficient mice received subcutaneous MC703-ALD tumors, that were allowed to grow for 3-4 weeks to an average size of approximately 150 mm^3, before they were treated with a single dose ($1x10^6$ $CD8^+$ TCR^+ cells) of freshly transduced and activated HHD T cells expressing TCR-IRES-GFP. Figure 8 A shows an example of TCR-transduced HHD T cells used for in vivo experiments. GFP which was co-expressed with the transgenic TCR was plotted against CD8. The tumor growth curves separated by TCR are shown in Figure 8 C. I observed a strong regression of the tumors with all TCR-Ts except TCR H55 TCR-Ts. Some tumors treated with TCR P17 (4 tumors) and M58 (2 tumors) did not shrink but the majority did, and some mice could even reject their tumors after T cell therapy (the number of rejected and total treated tumors is shown in the tumor growth curves). The higher number of rejected tumors in 14/35-treated mice was surprising because almost no in vitro experiment had anticipated this superiority. TCR 14/35 had stood out, however, in co-cultures with the low-affinity antigen $CDK4_{R24C}$ which might hint to the TCR's higher sensitivity. Despite this superiority regarding its rejection rate, there was no statistically significant difference between TCRs 14/35, P17 and H53 in their progression-free survival (Figure 8 B). The in vivo performance of all TCRs matched their performance in vitro and confirmed that tumor-derived TCR P17 has a comparable affinity to healthy donor-derived TCRs. The weak performance of TCR H55, however, underscores the importance of careful selection of TCRs that are intended to be used in therapy. The results support the use of TIL-derived mutation-specific TCRs for therapy.

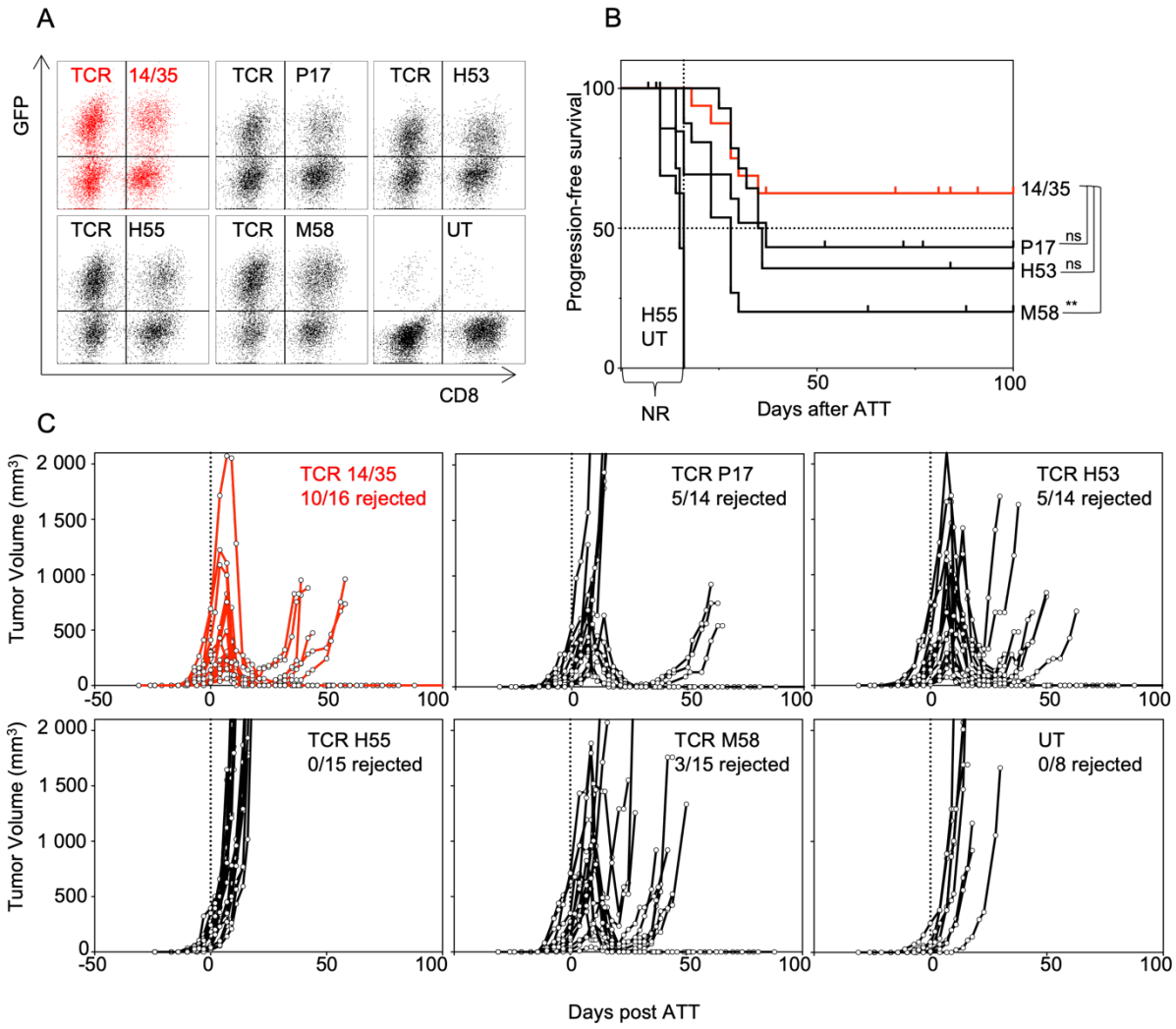

Figure 8: In vivo experiments confirmed the functionality of TCRs from all backgrounds.
A) *Representative FACS plots of TCR-Ts transferred to tumor bearing mice. The total number of T cells was adjusted to the percentage of GFP and CD8 positive cells. In this example, the transduction rates (% GFP⁺ CD8⁺) were 16.5 (14/35), 13.5 (P17), 16.6 (H53), 16.3 (H55), 23.7 (M58).* ***B)*** *Kaplan-Meier plot showing progression-free survival in mice of three independent experiments. Tumor-free mice that were sacrificed due to a poor overall health or that were found dead were censored (ticks). Log-rank (Mantel-Cox) test was performed for pairwise comparisons: TCR 14/35 vs. TCR P17 p=0.2299 ns, vs. H53 p=0.2790 ns, vs. M58 p=0.0084 **. P17 vs. H53 p=0.9045 ns, vs. M58 p=0.3262 ns.* ***C)*** *Tumor growth curves separated by TCR as labeled on top of each graph. The number of rejected tumors and total mice are indicated next to the TCR name.*

In order to understand the cause for tumor relapse, I resected all tumors when the mice reached their humane endpoints and analyzed their TILs and passaged tumor cells. Figure 9 shows the analyses of relapsed tumors. Of note in these analyses is that, naturally, rejected tumors could not be included and therefore groups with higher rejection rates may appear with a smaller difference to other groups. Figure 9 A shows the percentage of TILs (CD3⁺ CD8⁺ cells) in the total tumor digest of relapsed tumors. Unsurprisingly, tumors treated with TCR H55 TCR-Ts and untransduced T cells had

a significantly lower percentage of infiltrating T cells. Furthermore, TCR H55 TCR-Ts residing in the tumor also had a lower PD-1 expression compared to the other TCRs (Figure 9 B). Figure 9 B shows the PD-1 expression on TCR^+ TILS compared to TCR^- TILs. Apart from analyzing the TILs, I also passaged the tumor reisolates to obtain pure tumor cell cultures. Figure 9 C shows the IFNγ release of TCR 14/35 TCR-Ts after co-culture with the tumor reisolates. Almost all tumor reisolates could still be recognized by fresh 14/35 TCR-Ts, suggesting that tumor escape in vivo had not been due to antigen- or MHC-I-negative escape variants (Figure 9 C). Figure 9 D shows the MFI of HLA-A2 and GFP (the expression marker for the antigen ALD) in relationship to the parental tumor line MC03-ALD (shown in green). Almost all tumor reisolates had a lower HLA-A2 expression than the parental tumor line. When taking together all the above parameters for each tumor individually, there seem to be different causes for tumor outgrowth in different mice. Since reisolates could still be recognized by TCR-Ts in vitro, T cell exhaustion appears to be the main reason for tumor outgrowth. This explanation is supported by the persistent PD-1 expression on TILs after tumor resection shown in Figure 9 B. The explanation for the tumor outgrowth in mice with TCR H55 is that this TCR was too weak to kill tumor cells. This was already anticipated by in vitro killing assays (Figure 7).

In the case of TCRs P17 and M58, some tumors responded to the treatment, and some did not. Therefore, apart from comparing the reisolates from different TCR-Ts, I grouped the reisolates into relapsed tumors and non-responders (NR) (tumors treated with untransduced T cells were excluded). Figure 10 shows this analysis using the same parameters and data from Figure 9. By grouping the reisolates into relapsed and NR tumors, there is a visible and statistically significant difference in percentage of TILs, PD-1 expression, in vitro recognition and HLA-A2 expression. This analysis suggests that a lower T cell infiltration led to decreased selective pressure in NR tumors, resulting in higher HLA-A2 levels and better recognition by T cells. Interestingly, also when comparing relapsed to NR tumors treated with the same TCR, either P17 or M58, NR tumors tended to have lower TIL infiltration, higher HLA-A2 expression and better recognition in vitro (Figure 11). However, ΔPD-1 levels of TCR P17 and M58 TILs were comparable between relapsed and NR tumors (Figure 11 B).

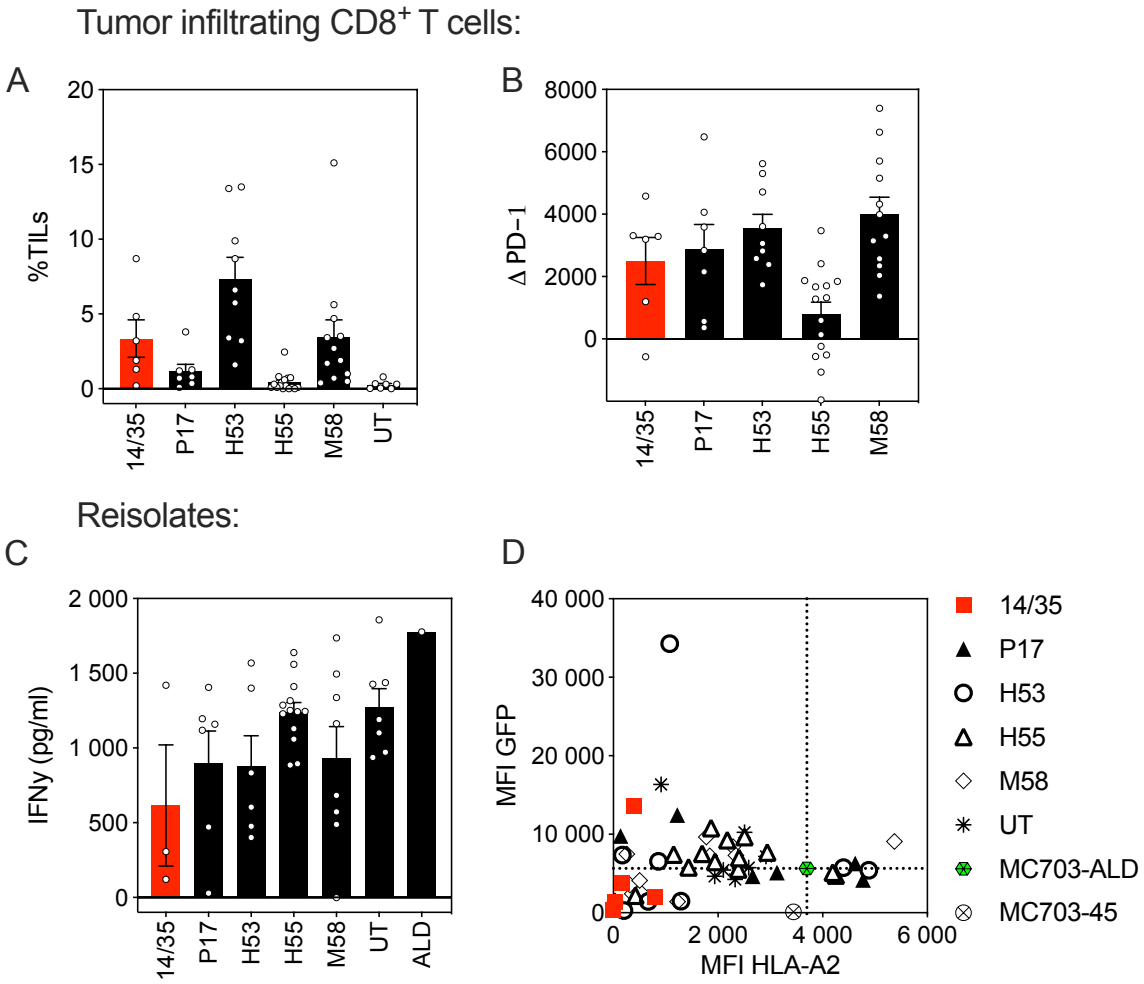

Figure 9: Analysis of relapsed tumors shows different reasons for relapse.

A+B) *Analysis of TILs from single cell suspensions of relapsed tumors.* ***A)*** *Percentage of TILs (CD8⁺ cells) in tumor digests determined by FACS. Mann-Whitney pairwise comparisons: 14/35 vs. P17 p=0.0932 ns, vs. H53 p=0.0663 ns, vs. H55 p=0.0024 **, vs. M58 p=0.9636 ns, vs. UT p=0.0023 **. P17 vs. H53 p=0.0012 **, vs. H55 p=0.0368 *, vs. M58 p=0.1045 ns.* ***B)*** *Difference in PD-1 expression (MFI geometric mean) between TCR⁺ (GFP⁺) and TCR⁻(GFP⁻) CD8⁺ TILs. Welch's tests: 14/35 vs. P17 p=0.7470 ns, vs. H53 p=0.2737 ns, vs. H55 p= 0.0812 ns, vs. M58 p=0.1397 ns. P17 vs. H53 p=0.4861 ns, vs. H55 p=0.0457 *, vs. M58 p=0.2685 ns.* ***C)*** *Co-culture of reisolates with TCR 14/35-transduced HHD T cells. Absolute IFNγ concentrations after 24 h co-culture are shown. Welch's tests: 14/35 vs. P17 p=0.5811 ns, vs. H53 p=0.5985 ns, vs. H55 p=0.2605 ns, vs. M58 p=0.5326 ns. P17 vs. H53 p=0.9579 ns, vs. H55 p=0.1764 ns, vs. M58 0.9026 ns.* ***D)*** *MFI (geometric mean) for HLA-A2 (APC) and GFP of cultured tumor reisolates. The parental tumor line MC703-ALD is labeled in green. Error bars correspond to SEM.*

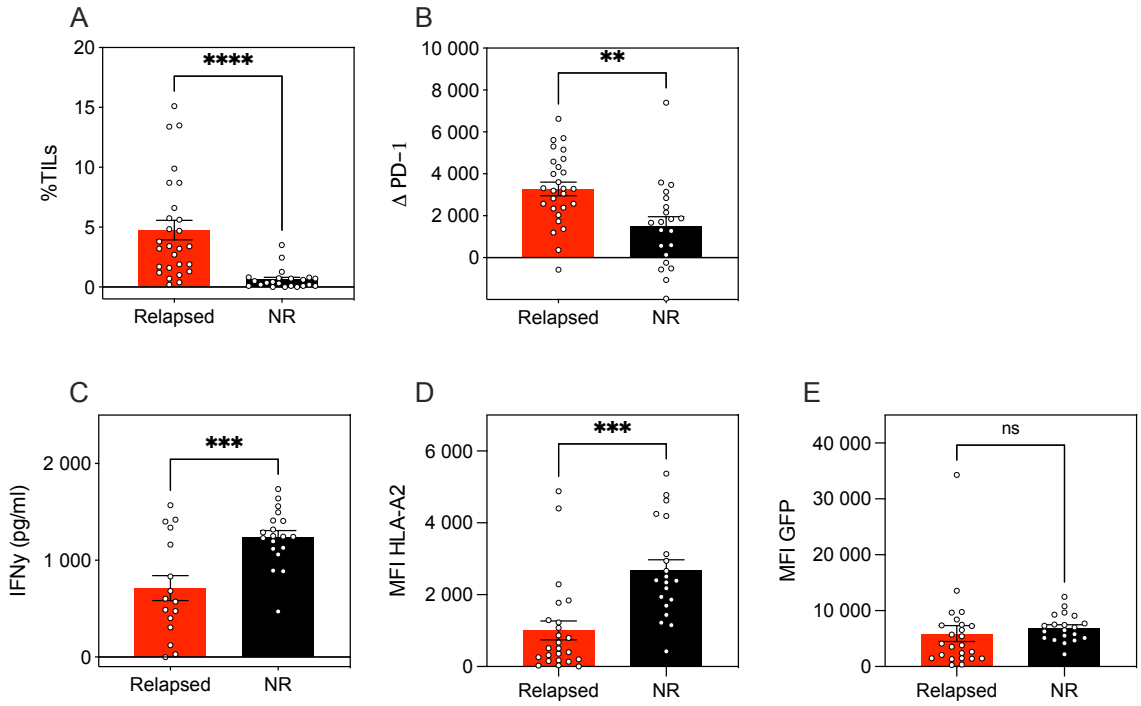

Figure 10: Relapsed tumors show reduced HHD expression compared to non-responders.

Reisolates of MC703-ALD tumors were grouped into relapsed (i.e. after regression) and non-responders (NR). The values for each parameter were taken from Figure 9 . All tumors treated with TCR 14/35 and H53 are in the relapse group whereas TCR P17 and M58-treated tumors are distributed among both groups. All TCR H55-treated tumors are in the NR group. ***A)*** *The percentage of $CD3^+$ and $CD8^+$ TILs within the tumor digest is depicted. Unpaired t-test p<0.0001 **** ***B)*** *TILs were stained for PD-1 and the difference of the MFI of PD-1 in GFP^+ and GFP^- $CD8^+$ TILs is depicted. Unpaired t-test p=0.0019 **.* ***C)*** *The concentration of IFNγ released by TCR 14/35 TCR-Ts after 24h co-culture with passaged reisolates is shown. Unpaired t-test p=0.0006 ***.* ***D-E)*** *The MFI of HLA-A2 APC* ***(D)*** *and GFP* ***(E)*** *in passaged reisolates is shown. Unpaired t-test p= 0.0001 ***(D) and p=0.5443 ns (E). Error bars correspond to SEM.*

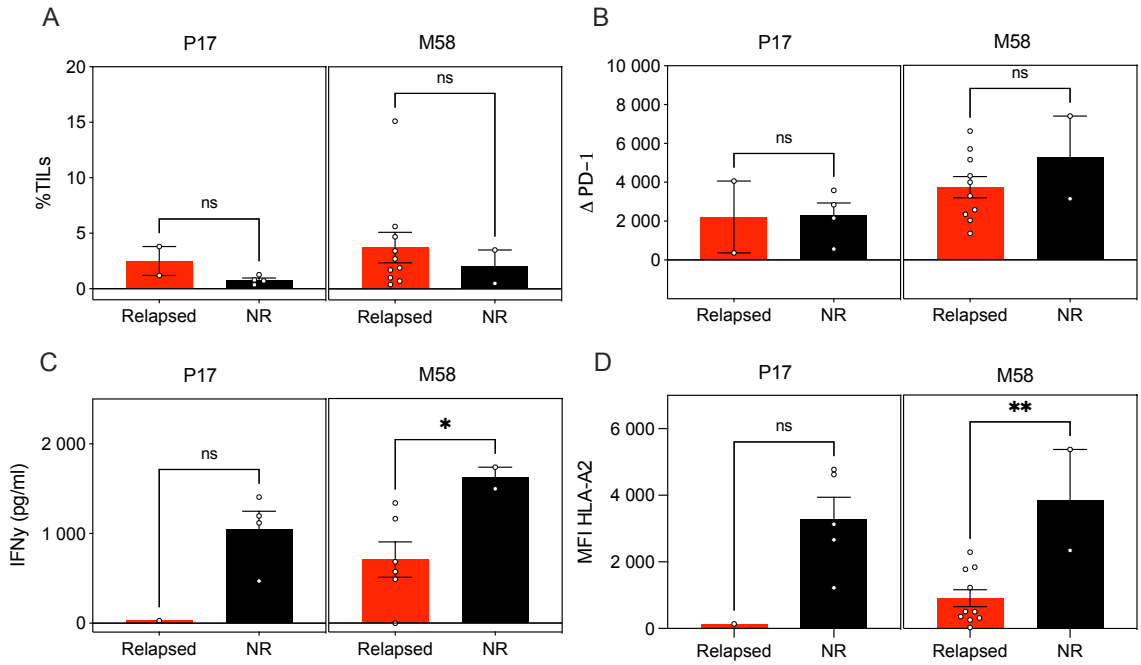

Figure 11 NR tumors treated with TCR P17 or M58 tend to have less TIL infiltration and higher HLA-A2 expression. *The data from* ***Figure 10*** *was separated by TCR.* ***A)*** *Percentage of TILs in Tumor digests.* ***B)*** *ΔPD-1 of TILs.* ***C)*** *IFNγ concentrations measured by ELISA released by TCR 14/35 TCR-Ts after co-culture with tumor reisolates.* ***D)*** *MFI of HLA-A2 APC measured on tumor reisolates. Unpaired t-tests were performed in all cases. P-values were A) 0.11 (P17), 061 (M58). B) 0.96 (P17), 0.32 (M58). C) 0.11 (P17), 0.047 (M58). D) 0.12 (P17), 0.0039 (M58). Error bars show SEM.*

Mouse mutant p68-specific TCRs

To complement the above-described comparisons of tumor- and healthy donor-derived TCRs, I developed an experimental set-up to generate mouse TCRs targeting mutant p68 (mp68). TCRs were obtained either from mp68-expressing tumors or from the spleen of immunized mice. In my introduction I described conditions and possible outcomes of optimal and suboptimal T cell priming. I mentioned that established tumors often provide suboptimal conditions for priming when they grow slowly and do not trigger acute inflammation. This is one possible reason why tumor-infiltrating T cells are often ineffective in controlling tumor growth. Based on this argument, we put emphasis in simulating these suboptimal priming conditions in experimental mice. Since we used a transplantable tumor model, it was important to avoid T cell priming during the initial phase of tumor engraftment which provides optimal priming conditions with acute inflammation and large-scale necrosis and apoptosis of tumor cells (K. Schreiber et al. 2006). We therefore used tumor cells with inducible antigen expression and waited for three weeks before induction.

Initial characterization of clone 4E9

The parental cell line of 4E9, 8101PRO, is a derivate of the UV-induced fibrosarcoma called 8101. In contrast to 8101RE, 8101PRO is characterized by its potential to form progressively growing tumors in immunocompetent mice based on its low MHC-I expression and loss of mutant p68, which has been shown to be the dominant antigen of 8101 (Dubey et al. 1997). 8101PRO was stably transduced with a vector expressing mp68 triple epitope linked to Thy1.1 as an expression marker in the presence of doxycycline. Single cell clones were generated from the Thy1.1$^-$ population of the doxycycline-naïve transduced population by limiting dilution. The clones were then tested towards their inducibility of Thy1.1 by doxycycline. Clone 4E9 was selected for further experiments as it provided the desired on-off switch of antigen and Thy1.1 expression, was recognized by mp68-specific 1D9 T cells and was not leaky, meaning not recognized when without doxycycline. Figure 12 shows that Thy1.1 expression and recognition by 1D9 T cells of clone 4E9 are dependent on doxycycline and tumor cells in the absence of doxycycline are not recognized by 1D9 T cells. This is a very important observation that forms the basis for the following in vivo experiments. It confirms that the 4E9 tumor model allows to induce tumors in mice without the presence of the antigen mp68. In turn, it suggests that mp68-specific TILs were only primed and infiltrated the tumor after the mice received doxycycline on day 21 post tumor induction (see below).

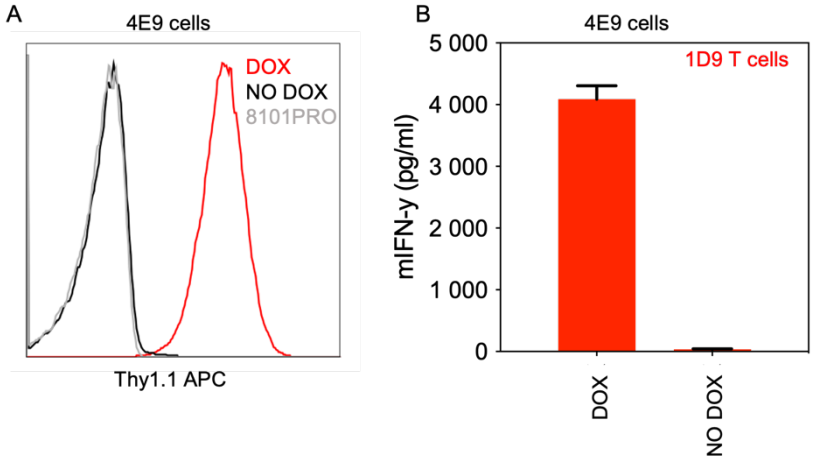

Figure 12: Clone 4E9 has a tightly controlled antigen expression conditional to doxycycline. ***A)*** *Thy1.1 expression of 4E9 with doxycycline (red) and without doxycycline (black) compared to the parental line 8101PRO (grey) determined by FACS.* ***B)*** *IFNγ release by TCR 1D9 TCR-Ts after 24 h co-culture with 4E9 cells with and without doxycycline.*

As the dominant antigen mp68 was absent in 4E9 cells without doxycycline, I expected similar growth rates in immunodeficient and immunocompetent mice. However,

when I retrospectively analyzed different 4E9 tumor growth experiments that were performed with either immunodeficient or immunocompetent mice and 4E9 cells with or without doxycycline, I found that on day 21, tumors were significantly smaller in immunocompetent mice. On the other hand, no difference between 4E9 cells with or without doxycycline was visible (Figure 13 A). Rag $1^{-/-}$ mice which do not have lymphocytes were used in the experiments on the left and immunocompetent mice that do express Rag1 (Rag$1^{+/-}$) were used in experiments on the right. In Figure 13 B, I excluded experiment 4E9-15 from the statistic because several tumors were only palpable on day 21, reducing the average tumor size. Nevertheless, the difference between immunodeficient and immunocompetent mice was significant. Interestingly, when looking at tumor growth curves of the different experiments in Figure 13 C, the difference between groups is not as obvious.

Tumor challenge and sort of mp68-specific TILs

To obtain TCRs from 4E9 tumors, I inoculated 4E9 cells that had been cultured without doxycycline subcutaneously into immunocompetent Rag$1^{+/-}$ mice. Prior to inoculation, I confirmed through FACS staining that the cells did not express Thy1.1. As expected, the tumors exhibited progressive growth in immunocompetent mice. Three weeks after inoculation, the mice began receiving doxycycline through their drinking water. At this point, all tumors were at least palpable. The slow growth of tumors in certain mice allowed for their sacrifice at various time points, ranging from three to eleven weeks post doxycycline administration. Tumor digests were subjected to staining with mp68-H-$2k^b$ tetramers, and single mp68-binding TILs were sorted into 96-well plates for subsequent amplification of TCR cDNA through PCR (as described in the methods). However, two mice (sacrificed on day 54 and 127) did not yield any tetramer-binding TILs, likely due to low amounts of material as the tumors were still small in size at the time of sacrifice. The growth curves of these specific tumors are included in Figure 14 A but lack specific labels. On day 32 after doxycycline administration, blood samples were stained, but no tetramer-binding cells could be detected (not shown). Figure 14 A shows tumor growth curves and indicates the time-point of antigen induction on day 21. Although some tumors appear to be at a size of 0 mm^3 on day 21 they were also palpable. The tumor growth curves of two independent experiments are shown in the same graph. The mouse IDs are indicated next to each growth curve if TCRs were obtained from the respective mouse.

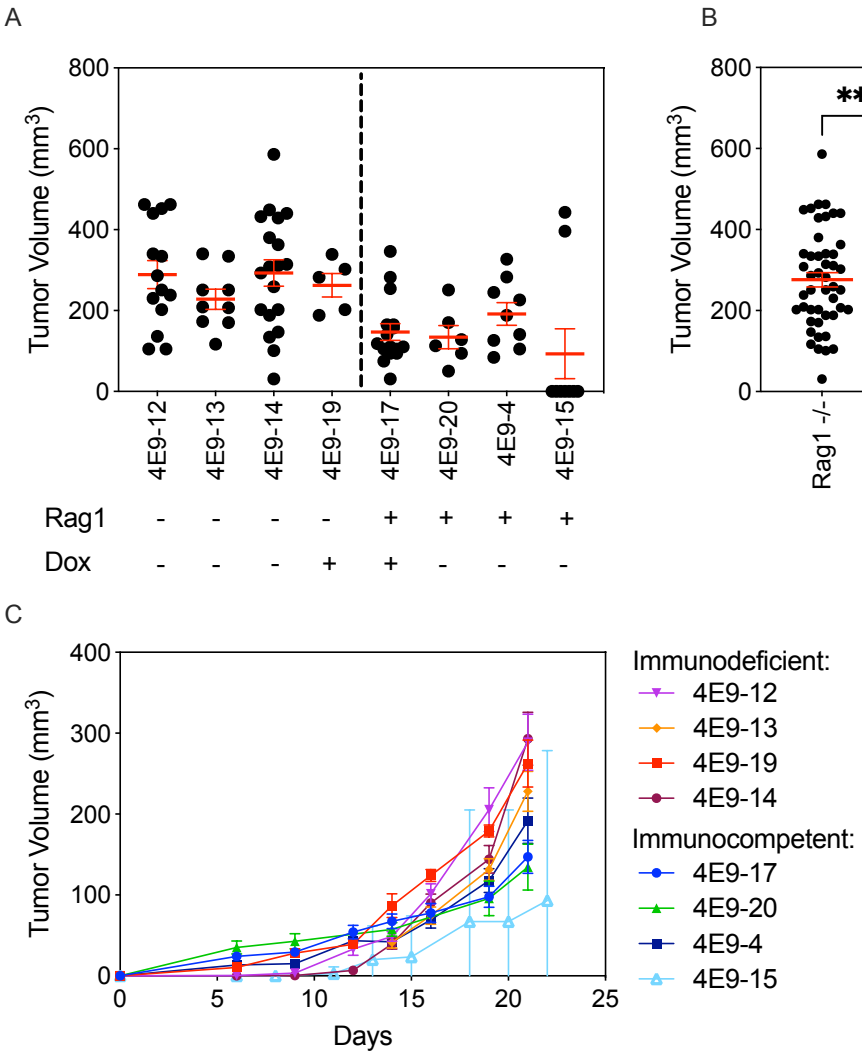

Figure 13: A delayed outgrowth of tumors in immunocompetent mice was observed independent of mp68 expression. *The shown experiments were performed with different goals and not designed to be compared to one another. The same number of 4E9 tumor cells were inoculated subcutaneously in all experiments either in immunodeficient mice (Rag1$^{-/-}$) or immunocompetent mice (Rag1 $^{+/-}$). 4E9 cells and mice were either on doxycycline prior to tumor cell inoculation or put on doxycycline on day 21 post implantation.* ***A+B)*** *Average tumor size on day 21 after tumor induction, separated by experiment* ***(A)*** *or grouped into Rag1$^{-/-}$ (lymphocyte deficient) and Rag1$^{+/-}$ (fully immunocompetent)* ***(B)****. There is a significant difference in tumor size between immunodeficient and immunocompetent mice. Welch's test p<0.0001. For B, experiment 4E9-15 was excluded because some tumors failed to grow entirely (8007 and 8011, off dox controls) and others were only palpable (6497 and 98).* ***C)*** *Tumor growth curves for the 4E9 experiments shown in A and B. 4E9-15 was the experiment that produced TIL-derived TCRs except TCR 44 and 4E9-4 produced TCRs 44 and 874-45, 874-46 from mouse 874. Mean and SEM are shown.*

Figure 14 B shows an example for a FACS plot of a 4E9 tumor digest that was gated on single, live, CD3^{+} cells and the sorted population is indicated by a black rectangle. The example plot corresponds to mouse 79. Figure 14 C shows the relative abundance of mp68 tetramer-binding T cells within all CD8^{+} TILs. We can appreciate a large variety in the relative abundance of mp68-specific T cells but a mean of 4.83 %. Mouse 98 had with 11.8% the highest abundance of mp68-specific T cells but was the mouse

with the lowest $CD3^+$ compartment within the tumor digest (18%, mean of all mice: 45%). Tumors of mice that did not receive doxycycline had no tetramer binding TILs which confirms that the used tumor clone 4E9 did not have a basal, constitutive antigen expression. Figure 14 D shows pie charts that represent the distribution of sequenced TCR clones within the sorted T cells. I detected 2-3 expanded clones per mouse. In some cases, one clone was clearly dominant whereas in others, the expanded clones had a similar frequency. The most expanded clones were synthesized, re-expressed in C57BL/6-derived T cells and tested regarding their mp68-reactivity (clones shown in red in pie charts). In many mice I observed a functional superiority of the most expanded clone (see below in TCR analysis) but this was not always the case. In all cases, however, the dominant clone was specific for mp68. The TCRs were labeled using the last two digits of the mouse ID and the clone ID, based on clone dominance. In summary, a substantial tumor size was necessary to isolate antigen-specific T cells from the tumor digest. In my experiment, I successfully obtained expanded T cell clones through tetramer sorting from TILs in all four tumors that were larger than 500 mm^3, regardless of the duration of tumor growth after antigen induction. However, despite my efforts, I was unable to detect tetramer-binding T cells in stained blood samples. Consequently, I could not compare changes in the mp68-specific repertoire over time within the same mouse.

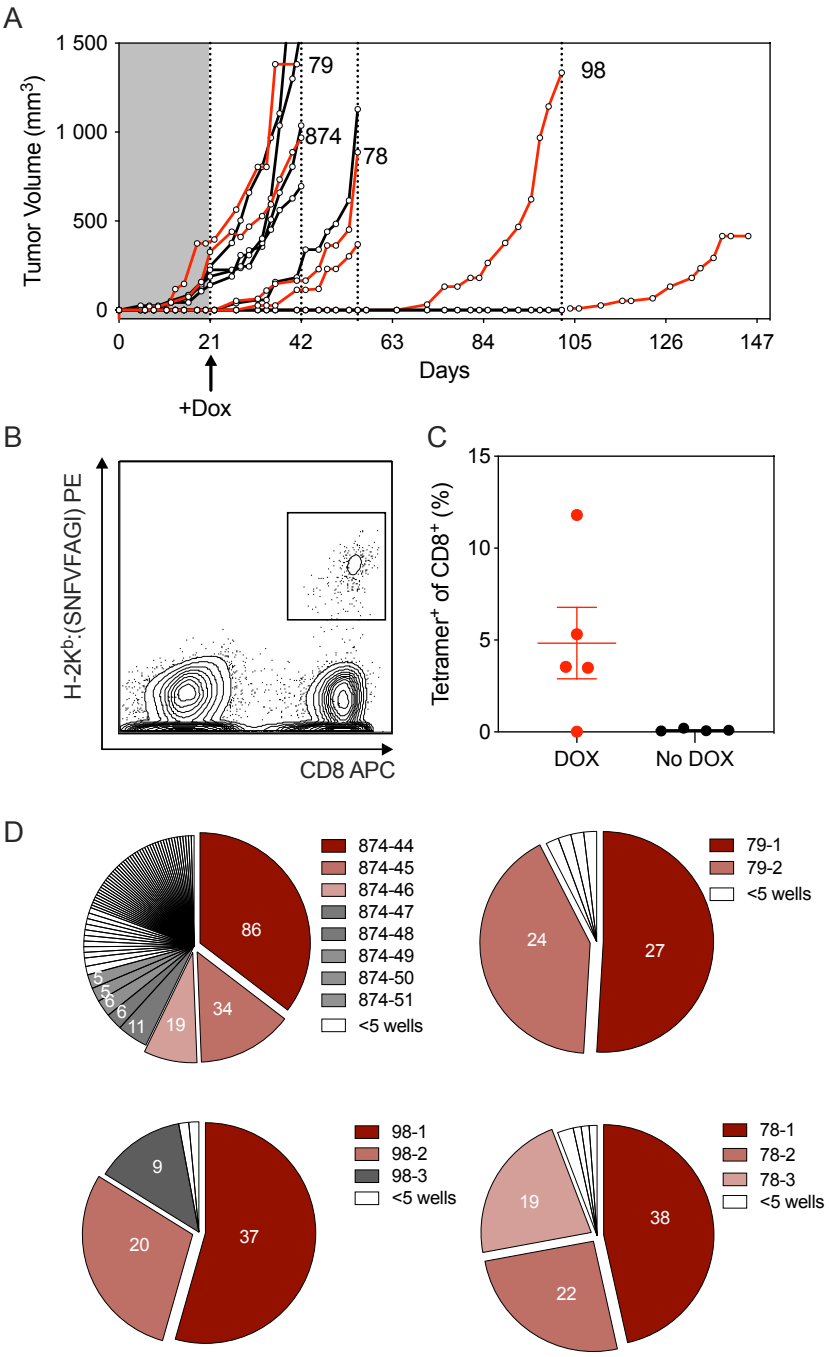

Figure 14: Mp68 specific TCRs were successfully isolated from tumor-bearing mice.

A) *Tumor growth curves from two experiments. Mice shown as red lines received doxycycline 21 days after tumor induction and mice shown as black lines did not receive doxycycline. The mouse IDs are indicated next to the tumor growth curves for mice that showed a sortable mp68-tetramer-binding T cell population in their tumor. Time points of tumor isolation are indicated by dotted lines: three, four and twelve weeks after doxycycline-mediated antigen expression.* ***B)*** *FACS plot of mouse 79, gated on CD3⁺ cells of the tumor digest. The gate used for single-cell sorting is indicated in black.* ***C)*** *Relative abundance of mp68-tetramer binding CD8⁺ T cells as a percentage of all CD3⁺ CD8⁺ cells in the tumor digest. Each dot corresponds to one mouse. No mp68-tetramer binding T cells were detected in tumors of mice that did not receive doxycycline. Mean and SEM are shown.* ***D)*** *Pie charts of identified TCR clones within tetramer sorted and sequenced TILs. Each pie chart corresponds to one mouse, the main clonotypes are labelled according to mouse ID and clone prevalence among the sequenced cells. The white numbers inside the pieces show the total number of wells in which each TCR clone was identified. Only clones indicated in red were synthesized and tested. TCR clones that were found in less than 5 wells are not labelled.*

Immunization

To obtain mp68-specific TCRs under optimal priming conditions, I immunized mice with irradiated 4E9 cells that had been cultured in the presence of doxycycline and were confirmed to express Thy1.1 before inoculation. The irradiation step was necessary to prevent tumor outgrowth. Subsequently, I performed two additional tumor challenges (boosts) 6 and 10 weeks later using live 4E9 cells on doxycycline. In contrast to tumor bearing mice, mp68-binding T cells were detectable in the blood of immunized mice and Figure 15 A illustrates their expansion. In the case of mouse 7146, I detected no significant expansion of mp68-directed T cells during the immunization regimen. This could explain why no expanded clones were sequenced from the sorted T cells of mouse 7146, as those were likely false positive events and not mp68-specific T cells. Importantly, the endpoint corresponds to the percentage of mp68-tetramer binding T cells in the spleen, not in the blood and the drop with respect to day 7 is likely attributable to this. Mice were sacrificed ten days after the second boost, spleens were frozen and were stained for sorting two days later. Single $CD3^+$ $CD8^+$ mp68-tetramer binding cells were sorted into 96-well plates for TCR sequence amplification by PCR and illumina sequencing. Figure 15 B shows as an example the FACS plot and gating of the spleen from mouse 47, gated on $CD3^+$ cells. Figure 15 C displays pie charts like those described in Figure 14 representing the number of wells in which each T cell clone was identified. The TCRs labeled in red were synthesized and subjected to testing. Two out of four immunized mice had expanded, mp68-specific TCR clones, a third mouse, 6149, had one expanded clone that did not recognize mp68 when re-expressed in activated T cells, and a fourth mouse, 7146, did not have a strong mp68-tetramer-binding T cell population and no expanded clone was identified after sequencing of the sorted T cells. Especially striking was the dominance of clone 1 in mouse 47, as it was found in 142 out of 184 wells. This clearly dominant TCR also proved to be highly functional when re-expressed in activated T cells (see below). The example of mouse 47 shows that the immunization regime could induce strong expansion of mp68-specifc TCRs although the immunization did not yield mp68-specific TCR expansion in all four mice. Unsurprisingly, when analyzing the TCR functionality (see below), a correlation between clear tetramer staining, strong clonal expansion and TCR functionality could be observed.

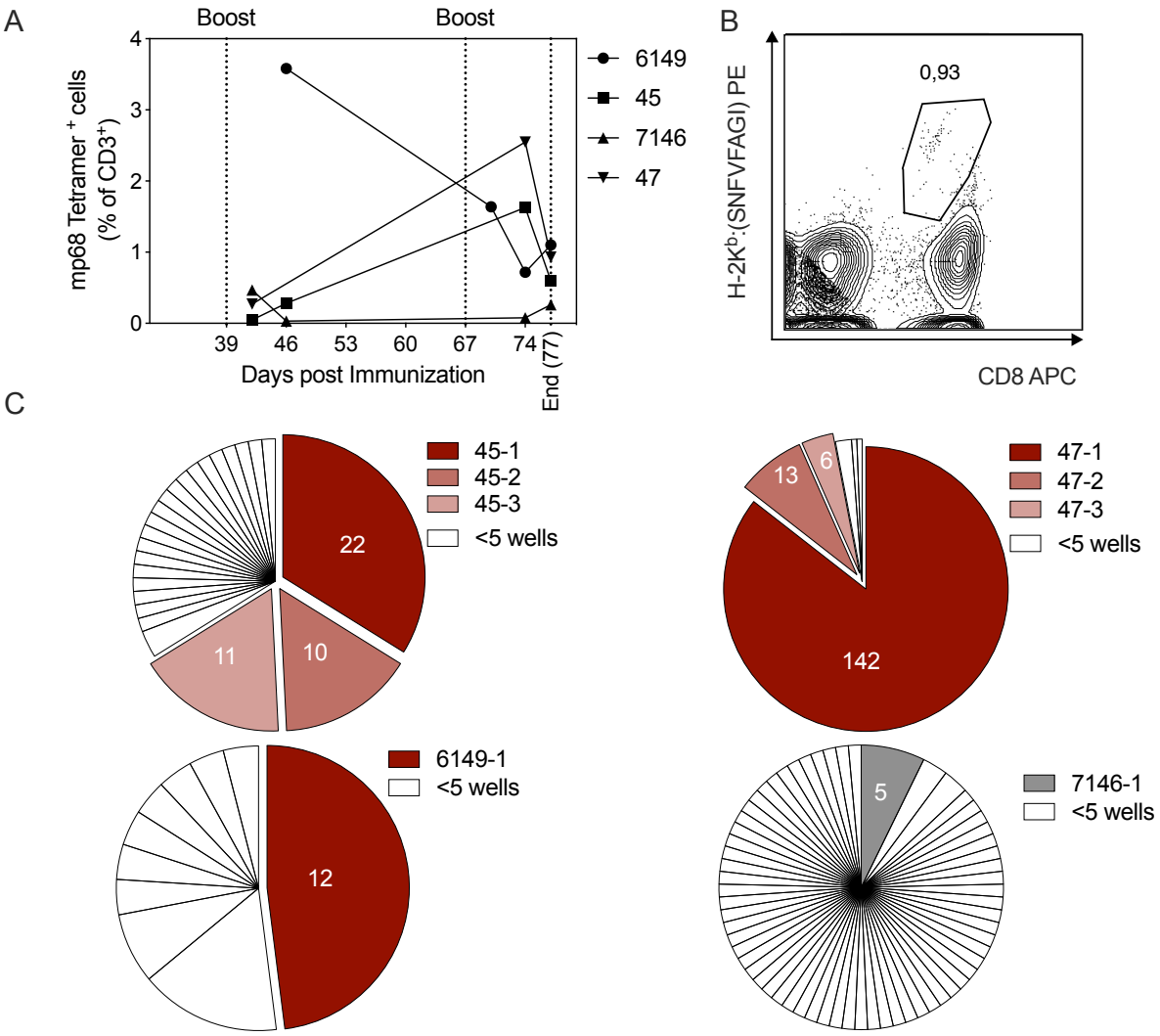

Figure 15: Mp68-specific TCRs were successfully isolated from immunized mice.

A) *Percentage of H-2kb:SNFVFAGI tetramer-binding CD8⁺ T cells within CD3⁺ cells in mouse blood or spleen (at end point, day 77). The blood was analyzed on days 3 and 7 after boost 1 and 2 (except for mouse 6149, no blood was available on day 3 post boost 1 and for mouse 47, no blood was available on day 3 post boost 2).* ***B)*** *FACS plot of spleen of mouse 47 gated on CD3⁺ cells show CD8 staining on the x-axis and mp68-tetramer staining on the y-axis. The gate used for sorting is shown in black.* ***C)*** *Pie charts illustrating the clonotype distribution within sorted and sequenced T cells. Each mouse is represented by one pie chart, the clonotypes are labelled according to mouse ID and clone prevalence among the sequenced cells. The white numbers inside the pieces indicate the total number of wells in which each TCR clone was identified. Only clones indicated in red were synthesized and tested.*

Table 4: Mutant p68 targeting mouse TCRs used in this work.

Name	V region		CDR3
1D9	TRAV1	TRAJ30	CAVRSDTNAYKVIF
	TRBV19	TRBJ2-7	CASSKRLSSYEQYF
TIL-derived:			
874-44 (44)	TRAV1	TRAJ30	CAVRADTNAYKVIF
	TRBV19	TRBJ2-7	CASSIRQGSGEQYF
874-45	TRAV12-2	TRAJ30	CALTSDTNAYKVIF
	TRBV15	TRBJ1-3	CASSRTGNTLYF
874-46	TRAV19	TRAJ40	CAAGGFNTGNYKYVF
	TRBV19	TRBJ2-7	CASSIRQGSGEQYF
79-1	TRAV9-3	TRAJ50	CAVSIASSSFSKLVF
	TRBV16	TRBJ2-1	CASRTQGNYAEQFF
79-2	TRAV16D/DV11	TRAJ53	CAMRESSGGSNYKLTF
	TRBV13-3	TRBJ2-4	CASSHRLGQNTLYF
78-1	TRAV19	TRAJ40	CAAGGVNTGNYKYVF
	TRBV13-2	TRBJ2-2	CASGEAGGVTGQLYF
78-2	TRAV9D-4	TRAJ50	CVLSAIASSSFSKLVF
	TRBV16	TRBJ1-1	CASSPQGNTEVFF
78-3	TRAV13-4/DV7	TRAJ30	CAMEHDTNAYKVIF
	TRBV12-1	TRBJ2-1	CASSLRGYAEQFF
78-3′2	TRAV19	TRAJ40	CAAGGVNTGNYKYVF
	TRBV12-1	TRBJ2-1	CASSLRGYAEQFF
98-1	TRAV16D/DV11	TRAJ37	CAMREGLTGNTGKLIF
	TRBV16	TRBJ2-4	CASSLNPGLGGSQNTLYF
98-2	TRAV19	TRAJ40	CAAGGVNTGNYKYVF
	TRBV19	TRBJ2-2	CASSILGGDTGQLYF
Immunization (Spleen)-derived:			
45-1	TRAV14D-1	TRAJ57	CAASDQGGSAKLIF
	TRBV13-2	TRBJ2-4	CASGDALGENTLYF
45-2	TRAV8D-2	TRAJ48	CATSYGNEKITF
	TRBV1	TRBJ2-3	CTCSADAGRSAETLYF
45-3	TRAV9-1	TRAJ31	CAVSASNNRIFF
	TRBV14	TRBJ1-1	CASNDRGRNTEVFF
47-1	TRAV9-1	TRAJ49	CAVKGYQNFYF
	TRBV1	TRBJ2-7	CTCSGDWGGSEQYF
47-2	TRAV13D-2	TRAJ23	CAIEALNYNQGKLIF
	TRBV12-1	TRBJ2-3	CASSPRQALGAETLYF
47-3	TRAV8-1	TRAJ38	CATEHNVGDNSKLIW
	TRBV16	TRBJ1-6	CASSRDRNSPLYF
6149-1	TRAV8D-2	TRAJ9	CATGSSNMGYKLTF
	TRBV1	TRBJ2-5	CTCSPSWGGDQDTQYF

Table 4 lists the characteristics of all tested mp68-specific TCRs from obtained TILs and immunization. I compared all identified TCRs to each other to find recurring

sequence motifs or TCR chains. I identified some similarities but no identical TCR sequence in two different mice and overall, I did not find a dominant or avidity-predicting sequence feature. The identified sequence similarities were first, that TCR 874-44, in the following called 44, is almost identical to TCR 1D9 and they differ only in a few amino acids of the CDR3 region. Second, one α chain was present in several mice: TRAV19-TRAJ40-CAAGGVNTGNYKYVF. However, it was always paired with different TCR β chains, resulting in different functional avidities of the TCRs (as shown in the next section). I also analyzed CDR3 length, CDR3 sequence alignments, number, site and type of n nucleotides, sequencing reads, TCR 3D structures predicted by alpha fold 2 (Jumper et al. 2021), and clonal convergence (different nucleotide sequences resulting in identical TCRs). None of those parameters could predict the functionality of the TCRs and I did not see clonal convergence in any TCR.

Figure 16 shows the efficiency of single cell sorting and sequencing in terms of wells with sequenced α and β TCR chains relative to the total number of sorted wells. The number of wells from which I recovered a complete TCR α and β pair was divided by the total number of sorted wells in each mouse. The recovery rate was below 50% except for mouse 47, which showed a good tetramer stain and a strongly expanded clone that was later shown to be also highly specific for mp68.

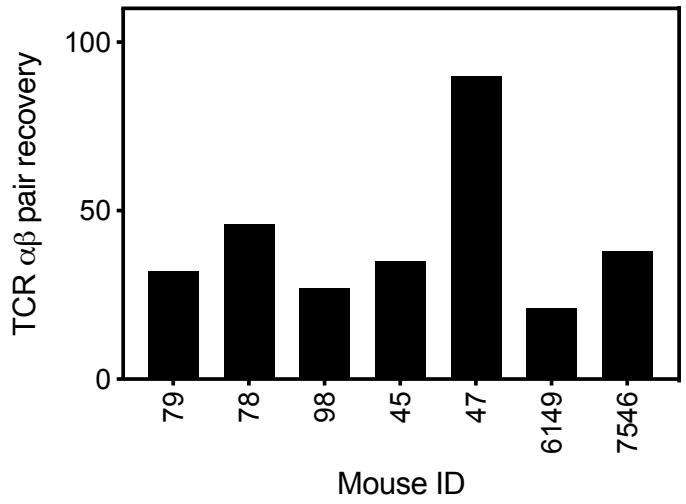

Figure 16: The average efficiency of tetramer sort and TCR sequencing was 41%.

The final recovery of TCR α β pairs (number of wells) was divided by the total number of sorted wells.

In vitro analysis of mp68-specific TCRs

In order to confirm the specificity and quality of the newly isolated TCRs from Table 4, they were transduced into activated T cells derived from Rag1$^{+/-}$ spleens. Co-cultures were done with target tumor cells 8101RE, 8101PRO-mp68 and 8101PRO as negative control. Additionally, co-cultures with irradiated splenocytes loaded with graded amounts of mp68 peptide were performed and in both cases, IFNγ release after 24 h was measured using ELISA. IFNγ release allowed to group the TCRs into three categories: strong TCRs, weak TCRs and non-functional TCRs. The non-functional TCRs were TCRs 874-45, 874-46, 78-3, 78-3'2 and 6149-1. These TCRs are not represented in the figure as they were excluded from later transductions and experiments. Figure 17 A shows the mean log EC50 values based on the peptide titrations shown in Figure 17 B. Most TCRs showed a similar log EC50 to TCR 1D9 but TCRs 79-1 and 98-2 from TILs and 45-1 and 45-2 from immunized mice had a markedly worse log EC50. Figure 17 B and C show the peptide titration curves of mp68 peptide loaded onto irradiated splenocytes in graded amounts as percent of the maximal IFNγ release (B) and absolute IFNγ concentrations (C) measured by ELISA. In Figure 17 D, the mean IFNγ concentration of five co-cultures is shown for TCR-Ts co-cultured with 8101 tumor cell lines. The levels of IFNγ release correlate with the log EC50 values in A with TCR 79-1, 98-2 and 45-2 TCR-Ts producing lower IFNγ concentrations. In summary, I could show that almost all sequenced TCRs were specific for mp68, that TIL-derived TCRs elicited similar cytokine release in TCR-Ts and that a spectrum of different functional avidities was observable in cytokine release assays depending on the TCR. These observations were promising with regard to the quality of TIL-derived TCRs for ATT.

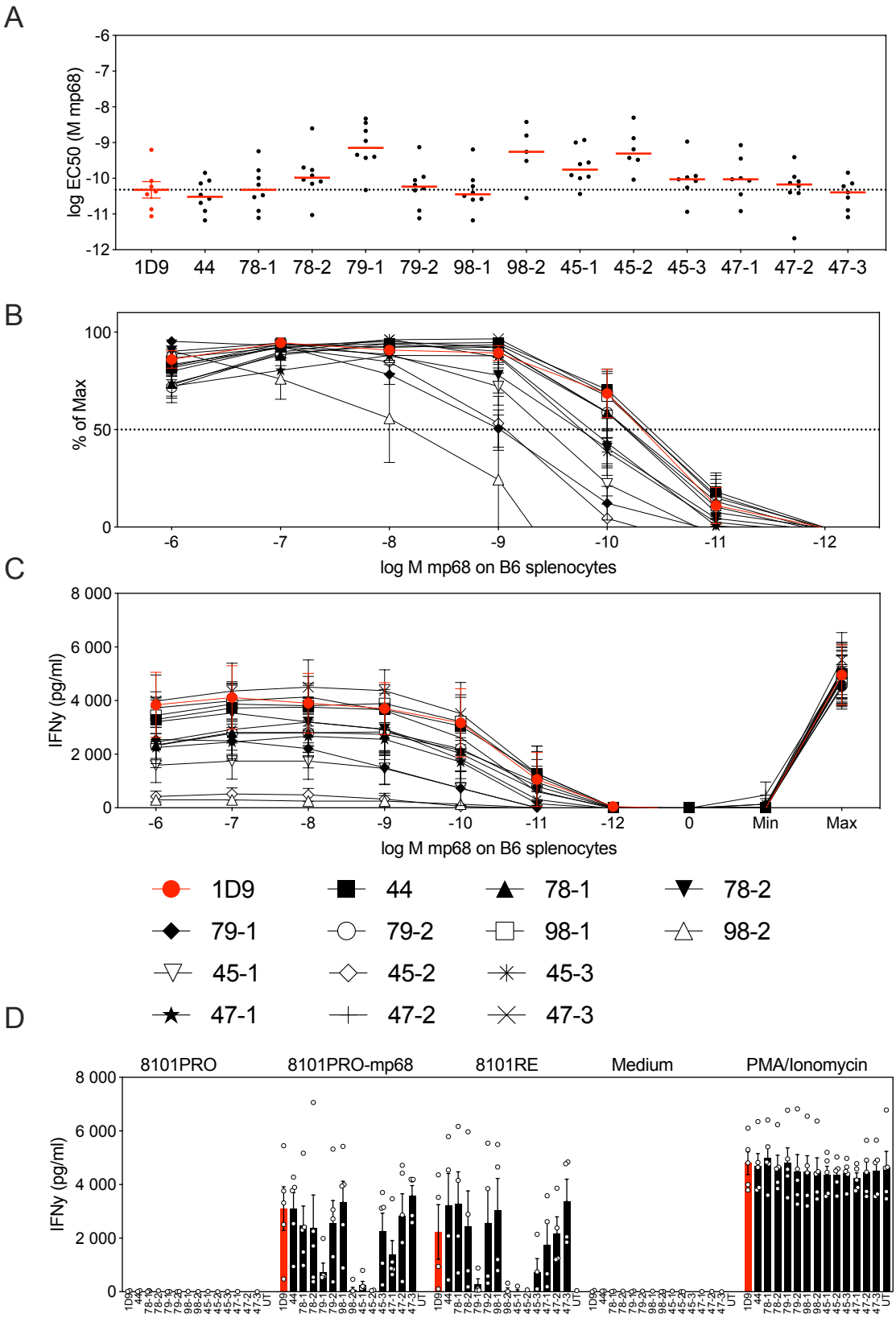

Figure 17: Mp68 TCRs displayed a range of affinities in cytokine release assays.

A) *Peptide titration: Logarithmic EC50 values of SNFVFAGI (mp68) peptide concentrations (M) based on the peptide titrations shown in B. Each dot corresponds to a different co-culture. A dotted line is included at the mean EC50 value of TCR 1D9 as an orientation.* ***B)*** *Peptide titration: Percentage of the highest IFNγ value within the peptide titration curve plotted against the peptide concentration of mp68 that was loaded on irradiated splenocytes in graded amounts. Seven independent*

experiments were combined. ***C)*** *The data of B is shown in absolute IFNγ values as determined by independent ELISAs for each co-culture. B and C share the figure legend between them.* ***D)*** *Co-culture of the TCR-transduced T cells with different 8101-derived tumor lines lacking mp68 expression (8101PRO), overexpressing mp68 as a triple epitope (8101PRO-mp68) or naturally expressing mp68 (8101RE). Additionally, T cells were co-cultured without tumor cells (Medium) or with PMA/Ionomycin. Absolute IFNγ values are shown for five independent co-cultures and ELISAs. Mean and SEM are shown in all experiments.*

In order to not only confirm the TCRs recognition of mp68 but also show their specificity towards mutated over wild-type p68, I performed co-cultures of TCR-Ts with WT p68 loaded splenocytes. Since the WT p68 has a lower predicted affinity to H-2k^b and because self-reactive TCRs are believed to be deleted in the thymus, I did not expect significant cross-reactivity of the TCRs. To my surprise, I found that nevertheless, some TCRs did recognize wild-type p68 (SNFVSAGI) when loaded on irradiated splenocytes at a concentration of 1x10^{-6} M (Figure 18). These were most dominantly TCR 47-3 and 45-2 from immunized mice. However, also TIL-derived TCRs 44 and 78-1 recognized the wildtype peptide. Whereas TCR 47-3 also shows a strong recognition of mp68, this is not the case for TCR 45-2. The TCRs' relative IFNγ levels in response to 1x10^{-6} M WT p68 compared to the same concentration of mp68 were 60% (45-2), 42% (47-3), 15% (78-1), 2% (45-1) and 6% (44). Consequently, TCR 45-2 has a similar recognition of the wildtype peptide despite it being of a lower predicted affinity to H-2k^b (2.9 nM vs 89.6 nM predicted affinity to H-2k^b according to NetMHC 4.0). Importantly, when WT p68 was titrated to lower concentrations, only TCR 47-3 consistently continued to induce IFNγ release up to a concentration of 1x10^{-7} M, and in one assay TCRs 78-1 and 45-2 recognized WT p68 up to 1x10^{-7} M and 47-3 up to 1x10^{-8} M (not shown). Otherwise, the other TCRs did not recognize the WT peptide at concentrations below 1x10^{-6} M. The fact that wildtype recognition does not correlate with functional avidity to the mutant peptide is surprising as the amino acid exchange between the peptides corresponds to an anchor residue (Dubey et al. 1997). Additionally, Dubey and colleagues, who first described the mp68 antigen in the 8101 tumor, showed that the wildtype peptide was unable to stabilize H-2k^b molecules on the surface of RMA-S cells, indicating a very low affinity of the peptide (Dubey et al. 1997). In line with the results of Dubey and colleagues we did confirm that TCR 1D9 does not bind the wildtype peptide. Whether WT p68 recognition resulted in increased toxicity in vivo was not systematically studied. Only in one in vivo experiment in which the transferred T cells seemed particularly activated, there was visible and early toxicity that resulted in sacrifice or death of mice within one week after T cell transfer. In this experiment, indeed this problem was more frequent in the TCRs that recognize

WT p68. Specifically, 2 out of 4 mice treated with TCR 45-2, 2 out of 4 with TCR 47-3 and 1 out of 2 mice with TCR 44 were the only mice in this experiment that were sacrificed within one week after ATT due to their health condition. The third mouse treated with TCR 47-3 was later also sacrificed due to overall health on day 21 post ATT after rejecting the tumor. In a second in vivo experiment, no early toxicity was observed, but two mice treated with TCR 47-3 died on days 25 and 64 post ATT unrelated to tumor burden.

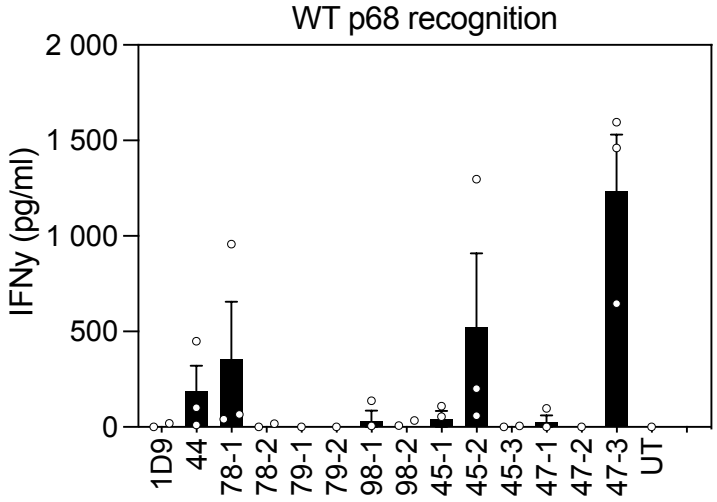

Figure 18: Wild-type p68 was recognized by TCR 47-3 and others.
A co-culture as in Figure 17 was performed with 1x10^{-6} M of wild-type p68 peptide (SNFVSAGI). Bar graphs show the mean of three independent transductions and co-cultures. Error bars correspond to SEM.

To complement the IFNγ ELISAs, long-term killing assays in the Incucyte with 8101-12-GFP, a mp68-positive clone of 8101RE, as target cells were performed. Figure 19 shows the tumor cell outgrowth in 96-well plates when co-cultured with TCR-Ts. Importantly, the killing assays required fresh T cells as opposed to cytokine release assays that could be performed with frozen T cells. The data for TCR 1D9 and untransduced T cells is included in each of the different graphs for comparisons in red and grey, respectively. The graphs were cut at 6 days because tumor cells in wells with untransduced T cells and weak TCRs reached their maximum confluence and later started detaching from the wells. However, I found that strong TCRs could control the tumor cell growth for up to three re-challenge cycles (performed every 3 days) or 10 days. These details can be appreciated in Figure 20 which shows the hours that each TCR could keep the tumor cell confluence below 10%. As long as the T cells controlled the tumor cell growth, the confluence was usually kept steady at around 2% and at 10% confluence tumor cells were in the process of quick outgrowth. The graphs in

Figure 19 illustrate how some TCRs could entirely suppress tumor growth (e.g. TCR 1D9) whereas others could only slow it down (e.g. TCR 78-2) or did not have any effect on tumor cell growth (e.g. TCR 79-1). The long-term cytotoxicity experiments therefore allowed a finer separation of the strong TCRs and could distinguish between strong and intermediate TCRs. Interestingly, only strong TCRs, according to these assays experiments, were able to control tumor growth in vivo (see next section). In Figure 20, the percentage of specific killing based on the confluence at 152 h compared to the confluence in tumor cells co-cultured with untransduced T cells is shown. This graph also allows the mentioned segregation of the TCRs. In the following section we will see that TCRs 1D9, 44, 78-1, 98-1, 47-1 and 47-3 could control tumor growth whereas TCR 79-2 could do so in some cases and the other TCRs could not. A cut-off at 50% specific killing on day 6 allows to reflect this segregation. Therefore, long-term in vitro cytotoxicity assays proved useful for the evaluation of TCR quality to predict therapy outcome.

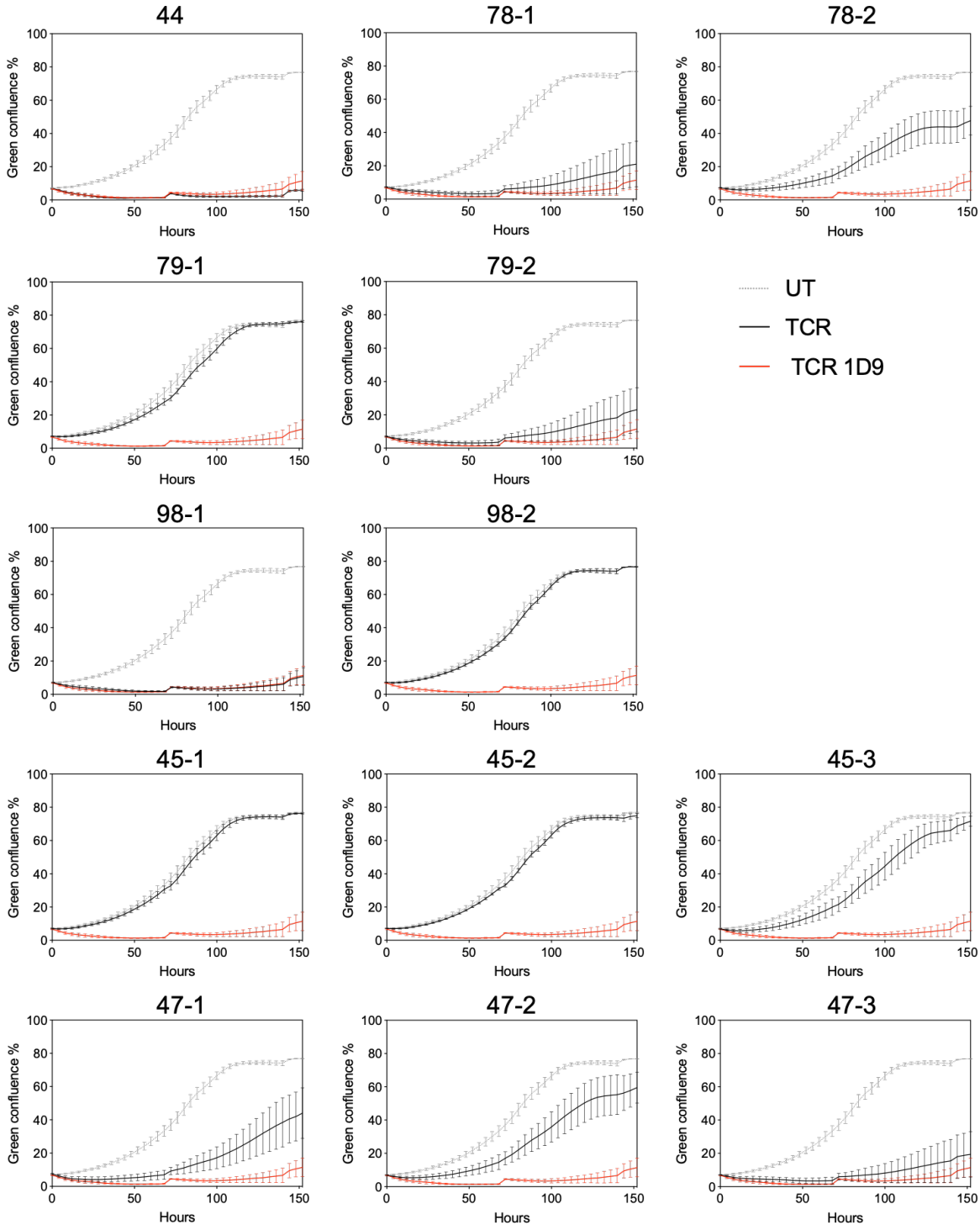

Figure 19: Cytotoxicity assays allow grouping of TCRs into high, medium and low affinity.

Tumor cell confluence (clone 8101-12 transduced with GFP) on six days after the addition of freshly transduced T cells. The confluence was measured and analyzed in the Incucyte. Each graph includes the identical data for TCR 1D9 (red line) and untransduced T cells (grey line) additionally to the TCR indicated on top of each graph (black line). The mean of three independent transductions and experiments is shown. Error bars correspond to SEM.

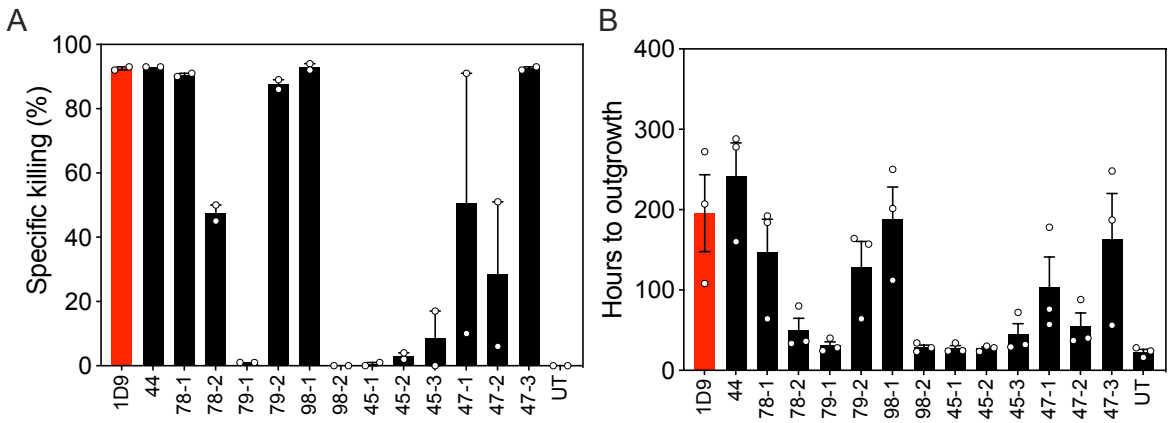

Figure 20: TCR-Ts can control tumor cell outgrowth in vitro for more than one week.

Cytotoxicity assays using the Incucyte. ***A)*** *The percentage of specific killing is shown for two independent transductions and co-cultures.* ***B)*** *The number of hours required for tumor cells to reach 10% confluence is shown for three independent transductions and co-cultures. The third experiment was excluded from A because earlier tumor cell outgrowth with all TCRs lead to confluence at day 6 for several TCRs, masking the differences between the TCRs.*

In vivo analysis of mp68-specific TCRs

To find out whether the described TCRs could control tumor growth in vivo, I transferred activated, TCR-Ts into tumor-bearing $Rag1^{-/-}$ mice. All TCRs shown in Figure 19 were also tested in vivo in at least two independent experiments each. The used tumor model was the 8101PRO line overexpressing mp68 as a triple epitope connected to Thy1.1, separated by AAY proteasomal cleavage sites. This line was enriched to about 99.8% for Thy1.1 expressing cells. Figure 21 shows the tumor growth curves of 8101PRO-mp68 tumors. The growth curves are separated by TCR as indicated in the top right corners. Additionally, the number of rejected over the total number of treated tumors is indicated. A dotted line indicates day 0 on which the T cell transfer took place. The treatment of the tumors with $1x10^6$ TCR-Ts successfully induced tumor regression in the TCRs that had also best controlled tumor cell growth in vitro (Figure 19). In these cases, one could observe a tumor shrinkage from about day 4-7 post ATT onwards that was usually followed by tumor relapse at day 20-40 post ATT. Figure 21 shows that these were TCRs 1D9 as well as 44, 78-1, 98-1 from TILs and 47- 1 and 47-3 from a tumor free mouse. Additionally, TCR 79-2 could induce regression in some tumors. The other TCRs, 78-2, 79-1, 98-2, 45-1, 45-2, 45-3 and 47-2 were not able to reduce the tumor size or select antigen negative tumors. The rejection of tumors was mostly observed with TCR 1D9 in 6 of 10 mice and in 3 out of 6 mice treated with TCR 47-1. One mouse that was treated with TCR 47-1 had a relapse that still retained some antigen and consequently was also oscillating in volume. Due to its overall health, this mouse had to be sacrificed before the possible selection of an

antigen-negative tumor. In order to follow the expansion of TCR-Ts in the mice, blood samples were stained weekly to detect TCR-Ts. In most mice, the peak of T cell expansion was observed on day 14. The blood count on day 14 shown in Figure 22 corresponds to the number of $CD3^+$ $CD8^+$ $v\beta^+$ cells in 50 μl of mouse blood as determined by FACS. The measured cell numbers varied greatly which may be due to different degrees of expansion as well as different days of peak expansion in the different mice. The largest number of TCR-Ts was observed in TCR 1D9 which was also the strongest TCR in terms of tumor rejection. All other functional TCRs did also show expansion on day 14 albeit dwarfed by the counts of 1D9 T cells. There was also visible T cell expansion with TCRs 45-1 and 47-2, both of which did not induce tumor shrinkage in any mouse. In the case of 47-2 this is consistent with Incucyte results that showed that TCR 47-2 could control the tumor cell proliferation to some extent. These results show that also the weaker TCRs responded to the tumor antigen. In general, however, to follow T cell expansion with weekly blood samples may be of limited information on TCR quality for the following reasons. First, a short peak expansion might not coincide with the day of blood sampling and might therefore be overlooked, second, tumor homing might be a competing parameter for TCR avidity that could reduce the amount of circulating T cells. Figure 22 B shows the antigen status of tumor reisolates based on Thy1.1 expression. A loss of Thy1.1 expression in the reisolates indicates that the tumor relapse was based on the approximately 0.2% of antigen-negative tumor cells that were present in the initial tumor cell suspension. Therefore, TCRs that led to Thy1.1 negative tumors had been able to kill all $mp68^+$ tumor cells. In the case of 1D9 and 47-1, rejected tumors could naturally not be included in the analysis. Except for the mentioned tumor treated with TCR 47-1, there is a very clear distinction between relapsed tumors which were $Thy1.1^-$ and unresponsive tumors which were $Thy1.1^+$. Therefore, this graph allows to clearly evaluate the TCR quality in terms of its selection of antigen negative variants. TCR 98-1 performed weaker than expected based on in vitro killing assays because of two unresponsive tumors, the same is true for TCR 79-2. Nevertheless, these TCRs were able to induce tumor regression. The tumor model only responded to strong TCRs, and it is possible that a different tumor model, for example with a higher $H\text{-}2k^b$ expression would also respond to TCRs of moderate avidity, such as 78-2 and 47-2. The results consistently show that there is no disadvantage for tumor-derived neoantigen-specific TCRs. All tumors that contained mp68-specific T cells, did contain high-affinity TCRs that were suitable for adoptive T cell transfer. In fact, this was a better outcome than what I

observed in immunized mice, where out of four mice, two had mp68-specific T cells in their spleen and only one of them had high-affinity TCRs (mouse 47).

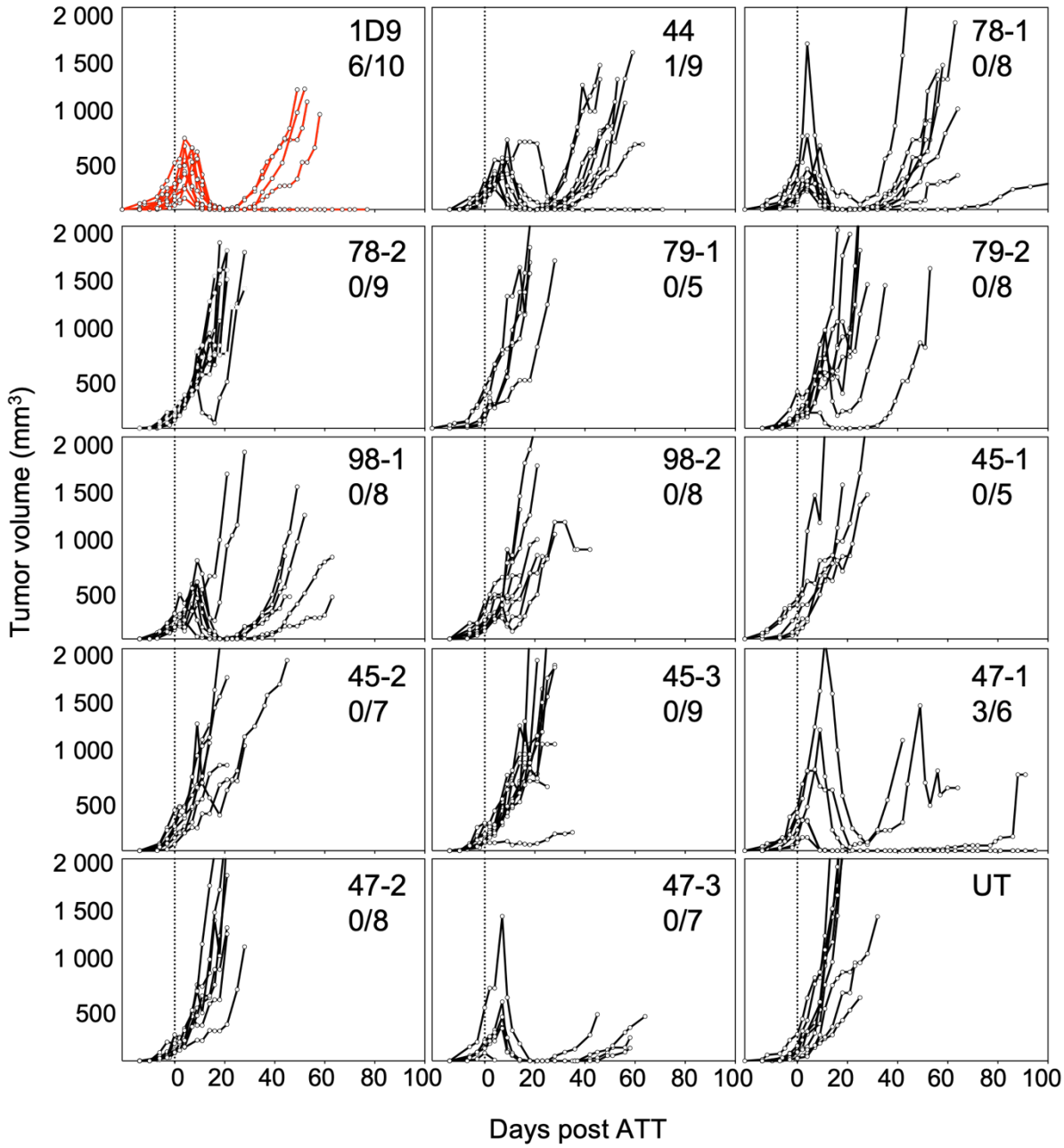

Figure 21: Only high affinity TCRs can induce regression of established tumors.

Tumor volume in mice treated with TCR-Ts as indicated above each graph. The numbers in the top right corners refer to the number of rejected and total number of treated tumors in each group. UT refers to mice treated with either untransduced T cells or no T cells.

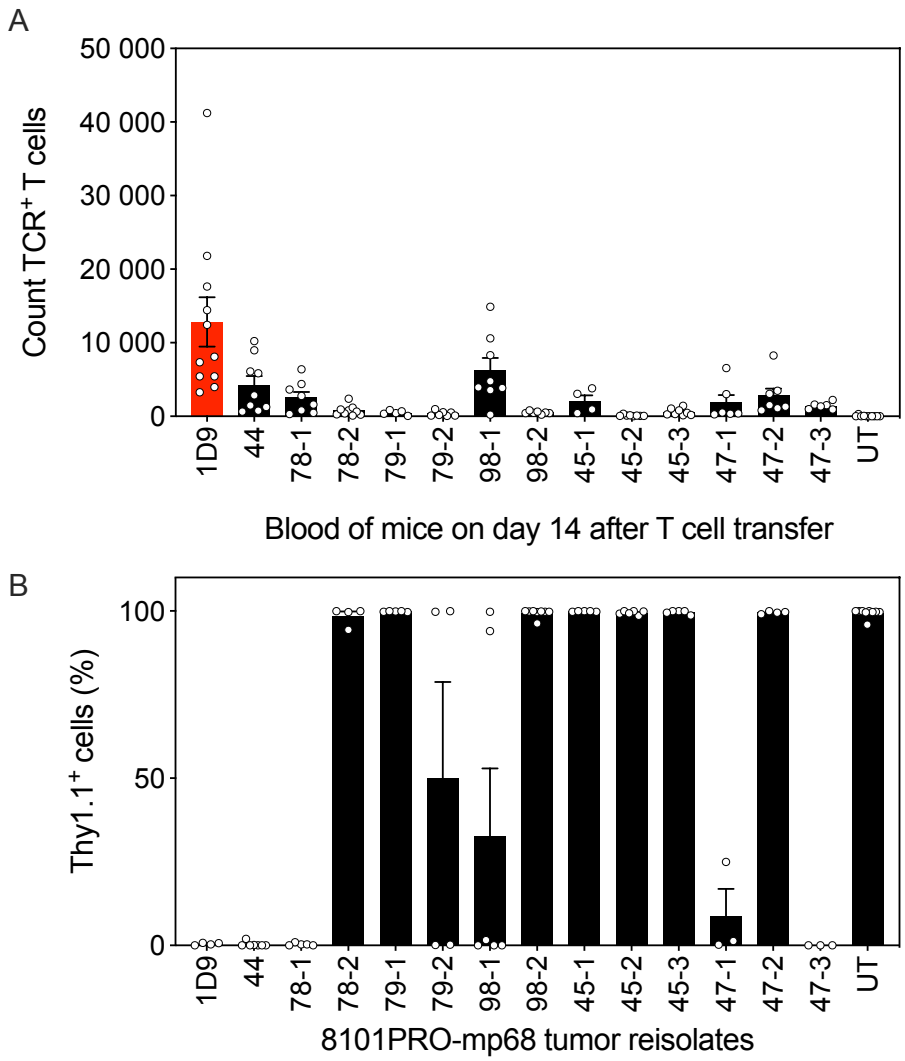

Figure 22: Tumor relapse can be attributed exclusively to outgrowth of antigen-negative tumor cells.
A) *The number of TCR⁺ and CD8⁺ T cells in the total measured blood sample of 50 µl on day 14 after T cell transfer. Day 14 corresponded to the peak expansion of T cells in most mice.* ***B)*** *The percentage of Thy1.1 positive tumor cells of total live cells in tumor reisolates from mice treated with the TCRs as indicated on the x-axis. Error bars correspond to SEM.*

Integration of TCR assays into heatmap overview

The main goal of the assays described above was to establish a functional hierarchy between the TCRs in order to analyze whether TIL-derived TCRs can be as functional as healthy mouse-derived TCRs. Due to the large number of different TCRs and assays, I decided to create a heat map that summarizes the TCR hierarchy for each assay. This heatmap is shown in Figure 23. For each assay, the respective parameter was normalized to the strongest TCR of the measurement. There were three classes of parameters: those derived from in vivo experiments (tumor rejection, Figure 21, tumor selection and count of TCR+ T cells in the blood on day 14 post ATT, both Figure 22), those from long-term cytotoxicity experiments (specific killing on day 6 and hours to

outgrowth, both Figure 20), and those from cytokine release assays after overnight co-cultures (all Figure 17). In mouse experiments, there was a split into two groups of TCRs; those that could select antigen negative escape variants and those that could not (and did also not induce tumor regression). This split is indicated by a red line. The two parameters obtained from long-term cytotoxicity assays are matching with in vivo tumor selection, validating it as an in vitro assay that is predictive of therapy outcomes. The cytokine release assays showed mp68 recognition by all TCRs. Even though TCRs 98-2 and 45-2 appear as non-functional TCRs in the heatmap, they had also responded to mp68 in cytokine release assays clearly above background. In summary, the heatmap illustrates how the therapeutic potential of some TCRs might have been overestimated by cytokine release assays. It also shows that among the 6 new TCRs that showed therapeutic efficacy in mice, 4 were TIL-derived. On the other hand, of the 7 TCRs that could not control tumor growth, 4 were immunization-derived and 3 were TIL-derived.

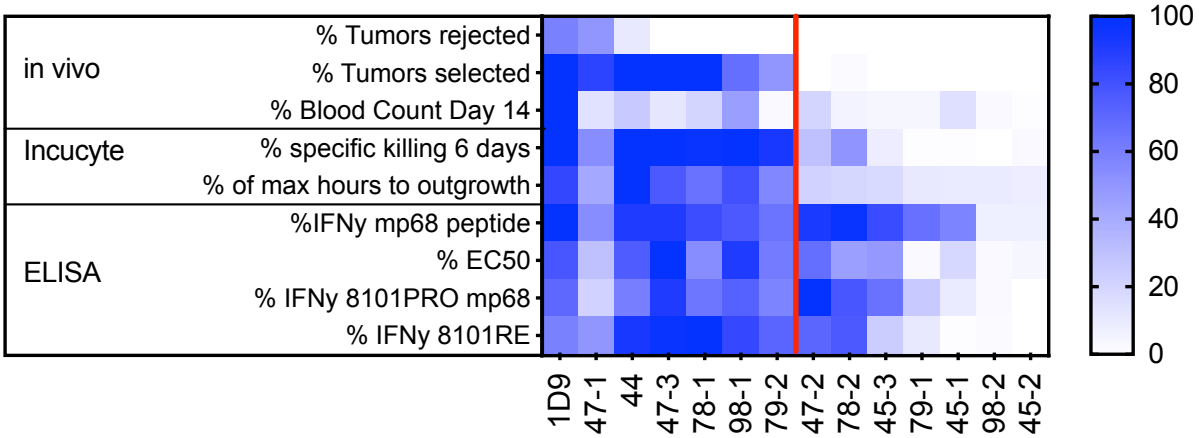

Figure 23: Only in vitro cytotoxicity assays could predict in vivo tumor control with mp68-specifc TCR-Ts.
The heatmap gives an overview of the assays shown above and a red line separates strong TCRs (left) which were able to induce tumor regression in mice from weak TCRs (right) that could not control tumor growth. For each parameter, TCRs were normalized to the strongest TCR to obtain a value between 0 and 100 percent. The data of each parameter can be found in the previous figures.

Discussion

This thesis compared the quality of several TCRs to determine whether TIL-derived neoantigen-specific TCRs are of lower quality than TCRs from tumor-free backgrounds. I could show with my experiments that high-affinity TCR clones for strong neoantigens can regularly be found among TILs and that these TCRs are suitable for adoptive T cell therapy. Therefore, my results support the use of TIL-derived TCRs.

TCR affinity and clonal deletion

As described in my introduction, there are different mechanisms that lead to peripheral tolerance and T cell dysfunction that have been shown to be relevant for tumor immunology. These include T cell anergy, exhaustion and AICD. Furthermore, experiments using altered peptide ligands showed that high-avidity interactions between TCR and ligand induce T cell anergy whereas low-avidity interactions induce T cell deletion (Smith et al. 2014; Redmond, Marincek, and Sherman 2005). My results are in line with those findings as I could obtain high-affinity TCRs from TILs. These TCRs, although of high functional avidity when introduced into activated T cells and therefore assumingly of high affinity, were unable to control tumor growth in vivo likely because the T cells were dysfunctional.

Quality of TIL-derived TCRs in current literature

The affinity of neoantigen-specific TCRs has been studied in few instances. By analyzing melanoma-derived TILs, Oliveira et al found that TCRs specific for neoantigens had higher avidities than those specific for melanoma-associated antigens but that they also displayed a more exhausted phenotype (Oliveira et al. 2021). Furthermore, some TIL-derived TCRs have been used to treat patients successfully which also argues for the quality of those TCRs. For example, MART-1 specific TCRs which were isolated from resected melanomas could induce partial responses in some patients (L. A. Johnson et al. 2009, 2006; Morgan et al. 2006). Mart-1 is not a neoantigen which makes the finding of high-affinity TCR DMF5 in TILs even more surprising. The reason may be that MART-1 is expressed as a truncated protein lacking epitope MART-$1_{26\text{-}35}$ in medullary thymic epithelial cells. This might lead to escape of high-affinity MART-1 specific T cells from negative selection in the thymus and their overrepresentation in the periphery compared to other self-specific TCRs (Pinto et al.

2014). The NY-ESO TCR 1G4 was obtained from TILs (Rubio-Godoy et al. 2001), however, it was affinity matured before its clinical application (Robbins et al. 2015). Therefore, it is unclear, whether this TCR in its original sequence was of high quality. A clinically active TCR specific for K-ras$_{G12D}$ was also identified in TILs of metastatic colorectal cancer (Tran et al. 2016). However, this TCR was selected in vitro based on its high reactivity and its clinical effect may be due to the specific target since mutated K-ras is an oncogene essential to the tumor. Furthermore, no direct comparison of this TCR has been made to test whether it is of high affinity or not. In summary, all TIL-derived TCRs that have successfully been tested so far were either preselected in vitro, affinity matured, or both.

Strønen et al who first isolated TCRs (H) 53, 55 and 57 had already compared them to TCR (P)17 in peptide titration and tumor cell recognition. Some of their assays had a low resolution that could not show the inferiority of TCR H55 but the peptide titration assay could show the disadvantage of TCR H55 and this assay also showed a similar or slightly superior reactivity of TCR P17 over TCR H53 (Strønen et al. 2016b). This was already an indication for the high quality of TCR P17. Nevertheless, the performed assays did not go into depth regarding this comparison because many different TCRs and antigens were tested in the same publication. The main goal of the work published by Strønen et al was to broaden the repertoire of available TCRs for neoantigen-directed therapy. The authors found that of 126 predicted neoepitopes, reactivity could only be found against two in the patient TILs, proving the need for alternative sources of TCR sequences. They argued that by stimulating healthy donor T cells with the patient's antigens, they could find TCRs against some of the other 126 neoepitopes and tested this hypothesis with 20 neoepitopes expressed as tandem minigenes. However, they only found a response against 5 of the 20 neoepitopes, one of which was the same neoepitope that had elicited a response in the patient as well (CDK4$_{R24L}$)(Strønen et al. 2016b). This suggests that the number of neoantigens that can be targeted is not broadened as much as expected by using donor T cells, but rather that the properties of the neoantigen are relevant for inducing a response and when they are favorable, a response can also be found in TILs. Nevertheless, they could show the feasibility of their approach which can generate additional TCRs against additional antigens and is independent of the patient's immune status. Overall, the data presented in the original publication of TCRs H53, H55 and H57 could be confirmed by my experiments which also confirmed the equal affinity of TCR P17 from TILs to TCRs H53 and H57.

The neoantigen-specific TCRs used in the clinical study by Foy et al had lower affinities than TCRs from other published clinical studies (DMF5, 1G4 NY-ESO, HPV16-E6, HPV16-E7, TP53 R175H, MAGE A4, KRAS G12D) (Foy et al. 2022). This study therefore is in line with my conclusion that careful selection and testing of TCRs is vital for therapeutic success since not all neoantigen-specific TCRs in patients are of high affinity. However, the affinity of the chosen antigen to its MHC complex plays an at least equally important role for the EC50 of a TCR. Therefore, comparing TCRs with different target antigens provides only limited information. This is another reason why I specifically focused on different TCRs for the same antigen. The TCR sequences used in the mentioned study had been obtained from peripheral blood of the cancer patients (Foy et al. 2022). The clonal composition and TCR quality of neoantigen-specific TCR clonotypes in the peripheral blood may be different to that of TILs. In fact, a paper published in 2023 by Schmidt and colleagues concluded that neoantigen-specific TCR clonotypes derived from TILs have a higher structural and functional avidity than dominant TCR clonotypes found in T cells circulating in peripheral blood in the cases of neoantigen specific T cells. On average, neoantigen specific clones had a higher avidity than TAA specific clones, although their avidity could range from low, similar to TAA-specific TCRs, to high, similar to virus specific TCRs. As expected, the highest avidity was found in TCRs that were specific for viral antigens (Schmidt et al. 2023). Importantly, the authors only tested structural avidity using a reversible tetramer staining. The large range of affinities in neoantigen-specific TCRs is in line with my own observations and shows that extensive testing is imperative for the selection of a suitable TCR for therapy. I have not compared the avidity of blood and TIL-derived TCRs in my mouse model because I could not detect tetramer-binding T cells in the 50 μl of blood that can safely be withdrawn from mice. However, I could find high quality TCRs in TILs which is in line with the study of Schmidt et al.

My results support the use of TIL-derived TCRs while underscoring the importance of selecting high-affinity clones. As mentioned above, Foy and colleagues used TCRs that were derived from the peripheral blood of their patients and did not see clinical responses in their patients. They compared their TCRs to those from other studies and found that their TCRs had worse EC50 values. The study therefore does not argue against the use of patient-derived TCRs but shows that each TCR needs to be tested in vitro to assess its quality before use in therapy. Importantly, in the case of the mp68-TCRs, I observed that log EC50 values alone when determined by 24 h co-cultures were not sufficient to distinguish TCRs of high and medium avidity because TCRs 78-

2, 45-3 and 47-2 showed an equal EC50 value to 1D9 but could not induce tumor regression. Long-term killing assays using the Incucyte where necessary to predict in vivo outcomes and distinguish between high and medium avidity TCRs. I did not determine which aspect was most relevant to long-term killing of tumor cells. It may be due to better survival, proliferation, less exhaustion, better effector functions or a combination of them.

It is important to point out that this thesis only looked at neoantigen-specific TCRs for two strong antigens. There may still be a requirement for immunization derived TCRs when targeting tumor associated self-antigens or lower affinity antigens. As discussed by Strønen et al, there are usually fewer if any neoantigen reactive TILs when compared to the total number of neoantigens of a tumor. Therefore, in vitro stimulation or immunization would be required to be able to target additional antigens on tumors.

Background of antigens and TCRs

Mutant CDK4

The first part of my thesis used human $CDK4_{R24L}$ specific TCRs. A mutation in CDK4 in position 24 can create three different antigens when replacing arginine by leucine (ALD), cysteine (ACD) or histidine (AHD). CDK4 is also of historic relevance for the T cell therapy field since TCR 14/35 was isolated from a patient that had had a CDK4 mutation (ACD) and was the first mutation-specific TCR described in humans (Wölfel et al. 1995). TCR 14/35 was used as the basis for comparison of the other mutant CDK4-specific TCRs. However, this TCR is somewhat difficult to classify as either from tumor-bearing or tumor-free background as it was isolated from a patient that had been treated with tumor resection and vaccination with autologous, irradiated melanoma cells plus BCG and was in year-long remission at the point of TCR isolation (Livingston et al. 1979). There is no evidence that this specific TCR was present in the resected tumor, it may have been expanded during the vaccination regime or it may have surged even later. In fact, it may even have been induced ex vivo during the culture with autologous melanoma cells. Important for the comparison though were the other TCRs that were from clear antigen-status: TCR P17 from TILs, TCRs H53, H55 and H57 from tumor-free donors and TCR M58 from a tumor-free mouse. This collection of published TCRs created the unique opportunity to directly compare the quality of a TIL-derived TCR and therefore test whether it is inferior. Ideally, more than one TCR from TILs would have been used but as TCR P17 turned out to function

equally well to TCR H53, this one TCR was sufficient to prove the posed hypothesis. TCR P17 came from a resected stage IV melanoma TIL product and TILs had been cultured for two weeks prior to analysis. This culturing of TILs may have induced variations in the clonal composition and may have expanded high affinity TCR clones.

Mutant p68

To obtain a larger number of TIL-derived TCRs for comparison, I generated mp68-specific TCRs in a mouse tumor model using inducible antigen expression. Similar to TCR 14/35 for CDK4, a well-studied TCR called 1D9 existed in the literature for mp68 which stems from an immunized mouse (Leisegang, Engels, et al. 2016). This TCR was used for comparison to the newly generated TCRs. Using a tumor model with inducible antigen expression, I could uncouple the mp68 expression from tumor induction. I switched on the antigen expression three weeks after tumor induction. This allowed for any injection-related inflammation to resolve and for the tumor to become a palpable, established, progressively growing tumor. On the other hand, I injected mice with already mp68-positive tumor cells. The resulting TCRs were called TIL-derived and immunization-derived, respectively.

Tumor growth despite high-affinity TCRs

Antigen induction in established tumors

My experiments confirmed that T cells cannot control tumor growth when they are first confronted with neoantigens in an established tumor, without the context of acute inflammation (K. Schreiber et al. 2006). There are different explanations for the failure of T cells to control tumor growth. These include the T cells' clonotype, more specifically their TCR, their phenotype, more specifically their activation status, and the tumor characteristics. My thesis focused on the TCR affinity as a possible explanation for tumor progression. Tolerization of T cells, AICD, exhaustion and the upregulation of inhibitory receptors are all controlled at least partially by the TCR signaling strength. Therefore, the TCR affinity is a relevant parameter for T cell survival in the tumor. It was a possibility that high-affinity TCRs would not survive in the presence of high antigen levels in the tumor microenvironment which in turn would result in a tumor with only low-affinity tumor-specific TCRs. My experiments showed that TIL-derived TCRs can be of high affinity since I could find high-affinity TCRs in all tumors that showed a positive tetramer staining. When compared side-by-side to immunization-derived TCRs, they were not inferior and showed an equal

potential to induce tumor regression and rejection when transduced into activated T cells. This was true for the human neoantigen $CDK4_{R24L}$ as well as the mouse neoantigen mp68.

Since I found that the TILs carry high-affinity TCRs with the potential to induce tumor regression, the tumor progression in 4E9 tumors (and in the patient that was suffering from melanoma and was the donor for TCR P17) was either due to T cell phenotype, tumor characteristics, or both. There are different causes for unresponsiveness of T cells as described in the introduction. Unless a tumor is associated with virus, bacteria or acute inflammation which is not the case for the transplanted 4E9 tumors, there are few danger signals such as pro-inflammatory cytokines or extracellular RNA or DNA that could activate DCs. Therefore, the lack of danger signals in the tumor most likely lead to unproductive T cell priming (Matzinger 2002). The chronic exposure to antigen can lead to T cell exhaustion which also renders them unresponsive (Blank et al. 2019; Schietinger and Greenberg 2014). Furthermore, T cells can also upregulate inhibitory receptors such as PD-1 which render them unresponsive. On the other hand, tumors can also actively inhibit T cells directly or through their immunosuppressive microenvironment as described in the introduction. In summary, tumor outgrowth was not due to lack of high-affinity TCRs among TILs.

Antigen induction before tumor transplantation

Contrary to my expectations, the 4E9 tumor outgrowth was not prevented by the expression of mp68. When the two lines, the regressor and the progressor line of 8101 were first described, the absence of mp68 from 8101PRO, the progressor line, was striking and mp68 was therefore described as the rejection antigen in 8101RE (Dubey et al. 1997). However, using 8101PRO transduced with mp68 (clone 4E9), I also observed tumor outgrowth in all mice when I injected 4E9 cells on doxycycline, i.e. actively expressing mp68. In this case, mp68 was not sufficient to cause rejection. The 4E9 tumor model is a rapidly growing progressor variant with low MHC-I expression which also grew out when mp68 was expressed during the tumor cell injection. Therefore, the discussion on the reasons for tumor outgrowth should not necessarily focus on the acute inflammation compared to resting conditions. Both situations, mp68 expression from the beginning onwards and from day 21 onwards, share several important features. First, the cell line 4E9 may be able to escape T cell control regardless of the T cells' activation status and TCR quality due to low MHC-I expression and fast growth. In favor of this argument is the published observation that

the regressor line 8101RE that is characterized by higher MHC-I expression is rejected in immunocompetent mice (Dubey et al. 1997). On the other hand, in vitro activated, TCR-transduced T cells could induce tumor regression and selection of antigen negative escape variants in established 8101PRO-mp68 tumors which shows that mp68 can be presented on 8101PRO cells in sufficient amounts. A second explanation may be the large amount of antigen that the T cells are exposed to after tumor cell injection and in the established tumors. Long-term exposure to their antigen can induce exhaustion or tolerance in T cells (Schietinger and Greenberg 2014; Scott et al. 2019; Th. den Boer et al. 2001), and too high antigen levels during priming can induce tolerance or T cell deletion (Redmond, Marincek, and Sherman 2005; Critchfield et al. 1994). Furthermore, there may be insufficient danger signals during and after tumor cell inoculation. Although the tumor cell transplantation is accompanied by large numbers of dying cells which create danger signals, these may not have been sufficient for optimal DC activation. As described in the introduction, dying tumor cells are not usually immunogenic, they can in fact be immunosuppressive (Poon et al. 2014). This depends on the type of apoptosis and the eat-me or don't eat me signals associated to the cells and how they are phagocytosed. In the case of tumor cell transplantation, many of the tumor cells injected as a suspension likely undergo anoikis which is defined as a type of apoptosis that is triggered by detachment of cells from their extracellular matrix and cell-cell contacts. This type of apoptosis is not yet determined to be either be tolerogenic or immunogenic (Birmpilis et al. 2022). The large amounts of antigens that are released in this context could be beneficial for cross-priming by DCs, however, too large amounts of antigens can also result in tolerogenic priming or T cell deletion (Critchfield et al. 1994). Mutant p68 may be a weaker antigen than initially assumed based on the high predicted affinity for H-2K^{b} and the rejection of 8101RE (Dubey et al. 1997). In favor of this argument are findings from Gejman et al. They performed tests of antigenicity comparing several different peptides presented by RMA-S cells and found that C57BL/6 mice rejected RMA-S cells presenting mp68 in 5 of 20 mice only. When presenting WT p68, 1 in 10 mice rejected the cells. On the other hand, presentation of OVA (SIINFEKL) lead to 29 rejections among 30 mice (Gejman et al. 2018). These results showed that mp68 is only moderately immunogenic. In general, neoantigens are assumed to be less immunogenic than viral antigens (Luksza et al. 2017; Balachandran et al. 2017). The level of foreignness is thought to be relevant for the antigenicity. This may be explained by a coevolution of pathogens and TCR repertoires that might lead to a higher precursor frequency of

pathogen specific TCRs (Luksza et al. 2017). There have been many attempts to predict antigenicity of peptides. I want to focus on a bioinformatic study by Well and colleagues that combined "strong binding affinity, high tumor abundance, high binding stability, and peptide recognition" (Wells, van Buuren, et al. 2020). The parameter of peptide recognition considers two peptide features that are related to the negative selection of self-specific T cells in the thymus, namely agretopicity which is the difference in binding affinity between the mutated and the corresponding wild-type peptide and foreignness which is the homology of a given mutation antigen to a known pathogenic peptide. The authors found that the integration of all these features created strong predictions of immunogenic peptides. Other studies had also found that including the homology of mutation antigens to microbial peptides improved their predictive models for peptide immunogenicity (Balachandran et al. 2017; Richman, Vonderheide, and Rech 2019; Luksza et al. 2017). These data support the lower immunogenicity of mp68 as it has no homology to microbial peptides. At least, none were found when queried online at https://www.iedb.org/home_v3.php but the mouse pathogen database is likely not complete or rather, very limited. This means that a related microbial peptide from a mouse pathogen still may exist. Whether in my experiments productively primed T cells against mp68 were among TILs in this setting (mp68 expression at the time of tumor induction) was not studied. The question whether tumor outgrowth was due to a lack of activated T cells or lack of tumor cell recognition or due to fast tumor growth therefore remains unanswered at this point but may be an interesting continuation of the work. I could observe in retrospect by combining the different animal experiments with 4E9 cells that although there was no difference in outgrowth between 4E9 tumors on or off doxycycline, there was a significant difference in outgrowth between immunodeficient and immunocompetent mice (Figure 13). This suggests that despite the outgrowth of the 4E9 tumors, there was some level of lymphocyte-mediated immune response against them initially although it did not lead to tumor rejection. However, this was not investigated in a direct comparison. Importantly, boosts using live 4E9 cells were readily rejected in primed mice which confirms that the initial immunization had resulted in a productive immune response against 4E9 cells. In 4E9 cells, the expression of mp68 is dependent on doxycycline but the synthetic transactivator M2 is always expressed and may also trigger an immune response. The 4E9 cells also carry other mutations that might elicit an immune response and lead to delayed outgrowth.

Experimental techniques

In vitro assays for mouse T cells

I showed that, although co-cultures measuring IFNγ release could identify weak TCRs, they failed to distinguish between medium and high-avidity T cells. This led to the problem that some TCRs unexpectedly could not control tumor growth in vivo. Long-term cytotoxicity assays using the Incucyte resolved this issue as TCRs that did not control tumor growth in vivo also allowed faster outgrowth of tumor cells in the Incucyte. Fewer TCRs were able to mediate tumor cell killing than those that were able to trigger IFNγ release. In early TIL research, similar observations had been made when showing that of 15 TIL cultures, only 8 were cytolytic but 14 released IFNγ in response to the corresponding tumor cells (Barth et al. 1991). However, in this early work, the authors showed that IFNγ release in vitro and in vivo was required and sufficient for tumor control and therefore more predictive for in vivo success than in vitro killing. Importantly, there are several differences between the mentioned publication and my experiments. First, the group of Rosenberg studied the cytolytic capacity of cultured TILS and not of TCR-transduced donor T cells. The TCRs of those TILs were not characterized and the differences in their cytolytic capacity were most likely based on their phenotype, not on their TCR affinity. The authors state that their TIL culture conditions lead to one third of proliferative, non-lytic $CD8^+$ lymphocytes and the nonlytic TILs therefore appear to be an artefact of the culture conditions. TILs from the same culture conditions and phenotypes were used for in vivo T cell transfers. Therefore, the nonlytic phenotype of TILs was successful in vivo, most likely because the expressed TCRs were of high affinity. The authors' conclusion was that IFNγ is required for in vivo tumor control but cytolytic ability was not. Additionally, in the mentioned publication, the authors counted the number of pulmonary metastases as read-out for tumor control. This may be more reliant on cytokines than on direct killing compared to the established subcutaneous tumors I studied. Importantly, I do not conclude from my cytokine release assays and in vitro killing assays on the relative contribution or relevance of these effector mechanisms for in vivo tumor control. Furthermore, long-term in vitro killing assays may depend on direct killing as well as cytokine release. It is possible that also my tumor model requires above all IFNγ and not cytolytic activity of the transferred T cells for tumor control. This was not studied. I found that to distinguish between different TCRs, Incucyte killing assays were more stringent and in my case more useful to predict in vivo outcomes.

IRES GFP reporter constructs to monitor TCR expression in HHD T cells

For in vivo experiments, I coupled the mutant CDK4-specific TCRs with IRES-GFP in a single transcript. All TCRs were confirmed to be functional when expressed together with IRES-GFP. However, I observed, that TCR P17 was possibly negatively affected by the combination with IRES-GFP for the following reasons. I observed that when I performed in vitro experiments either in human or HHD T cells using the TCR only or TCR+IRES-GFP vectors, TCR P17 consistently performed worse in IRES-GFP constructs in terms of transduction rate, MFI of GFP, amounts of released IFNγ in co-culture assays and long-term killing in the Incucyte (in human T cells). One possible explanation for this observation is that the mRNA stability of the longer TCR+GFP mRNA is decreased specifically for the sequence of TCR P17. Although all TCRs have a similar length and identical constant regions, there might be specific residues in the P17 mRNA that change its tertiary structure. Unfortunately, to resolve this issue is beyond the scope and besides the focus of this thesis. The putatively lower TCR P17 expression or IFNγ release might have affected the outcome of in vivo experiments. There were four tumors treated with TCR P17 TCR-Ts, that grew out despite treatment. Nevertheless, the tumor control rate of TCR P17 was high and the rejection rate was identical to that of TCR H53. A possible scenario to explain the in vivo results and the tumor progression in four tumors is that initial tumor control after T cell injection depends on the TCR density and cytokine release (assumingly being lower in TCR P17 TCR-Ts than in TCR H53 and TCR 14/35 TCR-Ts) whereas long-term tumor control and rejection rate might depend more on TCR affinity (being equal or better for TCR P17 than for TCRs H53 and 14/35). In conclusion, the performance of the patient-derived TCR P17 may have been underestimated in my in vivo experiments because of the use of IRES-GFP reporters. Importantly, this observation does not change the conclusion of this thesis, namely that patient-derived neoantigen-specific TCRs are not inferior to healthy donor-derived TCRs. On the contrary, the TIL-derived TCR P17 may be of even higher quality than what was observed in experiments of adoptive T cell transfer into tumor-bearing HHD mice.

TCRs from immunized mice

The immunization of immunocompetent mice with 4E9 cells on doxycycline (expressing mp68) induced the expansion of mp68-tetramer-binding T cells in the peripheral blood of three out of four mice (Figure 15). After sequencing and re-expressing expanded, tetramer-binding T cell clonotypes in spleen-derived T cells, the

TCRs of two of the three mice proved to be specific for mp68. Despite this, all mice were immune against the 4E9 cells on doxycycline as the boosts were performed with live cells that were readily rejected. Therefore, although I could not find T cell clones for mp68, the immune system of all mice had responded to the immunization and was functional. There are different possible reasons for the small yield of high affinity mp68-specific TCRs from the immunization experiments. It is possible that the rejection antigen was not mp68 because 4E9 cells also express other mutation antigens and that consequently the immune system of the two mice without mp68-specific TCR clonotypes focused on other antigens. To clarify this question, a boost with 4E9 cells off doxycycline could be done. If these 4E9 cells had grown out, the answer would be that mp68 was the dominant antigen during priming that would be required for rejection during rechallenge. Importantly, even if 4E9 cells off doxycycline were still rejected, immunity against mp68 may still have been induced at the initial priming step but it would show that mp68 was not the exclusive rejection antigen. Other reasons may relate to technical reasons such as timing in the immunization scheme or quality of the tetramer stain.

Tetramer-based single cell sort

Since labeling of antigen-specific T cells by tetramer staining depends on the surface expression of TCR and CD8 at a certain density, it is possible to miss some antigen-specific T cells in tetramer-based FACS sorting. The result may be the skewing of the obtained TCR repertoire towards high or low affinity clones or a reduced number of identified TCR clones. For example, it has been described that high avidity T cells die from the TCR stimulation offered through tetramer binding (Schmidt et al. 2011; Guillaume et al. 2006). On the other hand, dysfunctional T cells with internalized TCRs may not bind the tetramer in the first place (Chattopadhyay et al. 2008). Using the tyrosine kinase inhibitor dasatinib enhances TCR surface expression, yielding stronger tetramer signals and might be an option to capture more TCR clones (Chattopadhyay et al. 2008).

Future challenges of TCR-gene therapy

As briefly described in my introduction, there are several obstacles for TCR-gene therapy that need to be overcome in order to achieve better clinical outcomes and benefit more patients. The obstacles are related to manufacturing time, production costs, target antigen identification, T cell quality, TCR quality and tumor escape.

Regarding manufacturing time, a timeline published by Foy an colleagues suggests that their most time-consuming step was to identify TCR clonotypes for the personal neoantigens using their impact platform with about 100 days (Foy et al. 2022). The mean time from consent to cryopreservation of the final T cell product was about one year. This is owed to the personalized approach of the therapy that does not allow for off-the-shelf products and explains why much focus is put on hotspot mutations and TAAs as targets for T cell therapy. Regarding T cell phenotype, it has been described that shorter culturing times lead to early effector T cells or T cells that still retain stemness which is advantageous for their proliferation potential in vivo (Dickinson et al. 2023; Gattinoni et al. 2005). A modified manufacturing protocol for CAR T cells called T-Charge that reduces the culturing time from 9 to 2 days was shown to improve the T cell expansion and therapy efficacy of CAR T cells (Dickinson et al. 2023). Tumor escape is often the reason for relapse after TCR gene therapy. Tumors may relapse by losing the expression of the antigen-presenting MHC molecule or by losing the expression the targeted antigen itself. The addition of $CD4^+$ T cells has repeatedly been shown to prevent tumor relapse (S. P. Wolf et al. 2024; Tran et al. 2014; Arina et al. 2017). Therefore, it should be considered in future clinical trials to use both, MHC-I and MHC-II restricted TCRs for a more effective therapy.

The TCR quality was the focus of this thesis. Since often times individual research groups use only one established source for TCR sequences in their experiments, little comparative data on the quality of TCRs from different sources (TILs, healthy donors, humanized mice) is available. However, as the clinical research on TCR gene therapy advances and TCR isolation protocols become more standardized, it is essential for the design of effective T cell therapies to choose the right source for TCR sequences as well as to choose the most predictive preclinical tests. Comparing TCRs derived from TILs and healthy donor blood of spleen of immunized mice, I could show that TIL-derived TCRs are of high quality and are therapeutically effective. This supports the use of TIL-derived TCRs for TCR gene therapy.

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Table 5: Mouse TCR sequencing primers used to obtain mp68 TCRs.

Primer name	Primer Sequence
musTRBV1Ph1	GGGACAAAGAGGTCAAATCTCTTC
musTRBV2Ph1	CCTCAAGTCGCTTCCAACCTCAAA
musTRBV3Ph1	GTCATGGAGAAGTCTAAACTGTTTAA
musTRBV4Ph1	CTCATTGTAAACGAAACAGTTCCAA
musTRBV5Ph1	CGAAATGAGACGGTGCCCAGTC
musTRBV12-1,2Ph1	CCCAGCAGATTCTCAGTCCAACA
musTRBV13-1Ph1	GGAGATGTCCCTGATGGGTACAA
musTRBV13-2,3Ph1	AGATATCCCTGATGGRTACAAGGCC
musTRBV14Ph1	GGCCTAAAGGAACTAACTCCACTC
musTRBV15Ph1	GGTGGGGCTTTCAAGGATCGATT
musTRBV16Ph1	GATGATTCAGGGATGCCCAAGGAA
musTRBV17Ph1	GGGAAGCTGACACTTTTGAGAAGT
musTRBV19Ph1	GATCTATCTGAAGGCTATGATGCGT
musTRBV20Ph1	CTGTGAACTCAGCAATCAAATATGAA
musTRBV23Ph1	GGTCAAGGAGAGATTCTCAGCTGT
musTRBV24Ph1	CAGACTTGGTCAAGAAGagattCTCA
musTRBV26Ph1	GTTCTTCAGCAAATAGACATGACTGA
musTRBV29Ph1	CGATGTTGATAGTAACAGCGAAGGA
musTRBV30Ph1	GCCACATACGAGAGTGGATTCAC
musTRBV31Ph1	GGTAGAGTCGGTGGTGCAACTGA
musTRAV21Ph1	GACTCACGGTCTACAACAAAATACAA
musTRAV19Ph1	CCGTACGCTCAAATGTGGATAAGA
musTRAV17Ph1	CGTTGTTAAAGGCACCAAGGGCTT
musTRAV16Ph1	GGTCATTATYCTCTGAACTTTCAGAAGC
musTRAV15Ph1	cgctaytctgtagtcttccagaaatca
musTRAV14Ph1	GTGTCCRATAAAAAGGAAGATGGA
musTRAV13-1/4Ph1	GTTSTACAATCCTTCTGGGACAAAGCA
musTRAV13-2/4Ph1	CAATCCTTCTGGGACAAAGCACAC
musTRAV13-3/D3/N3Ph1	GCAGAGCAGAGAGGTGGAAGACT
musTRAV13-5.01Ph1	GCCTGTCCTACATTCCTGGAATGA
musTRAV12Ph1	CGCCACTCTCCATAAGAGCAGCA
musTRAV11Ph1	GACAAAACGTCAAATGGGAGATACTC
musTRAV10Ph1	GGACAGAAAACAGAGCCAAAGACTT
musTRAV9Ph1	GGARACCCAGTGGTTCAAGGAGTGAA
musTRAV8Ph1	CGTTCAAATGAGMGAGAGAAGCGCA
musTRAV7-5Ph1	CTCTGATGGTGAAAAGGAAGAAGGCA
musTRAV7-4Ph1	GAAGGCAGATTCACAGCTCACCT
musTRAV7-6Ph1	GGCAGATTGACAGTTTACCTCAATA
musTRAV7-3Ph1	AGATTCACAATTCACCTCAATAAAGC
musTRAV7-2Ph1	GGTGAAAAGGAAGAAGGCAGATTCA
musTRAV5-4Ph1	GCAGACCCAAGGACTCATCGTTTT
musTRAV5-1.01Ph1	GAAAACAGAATCAAAGACTCACCCTT
musTRAV4Ph1	CAGGAACAAAGGAGAATGGGAGGT
musTRAV4-2Ph1	GCTCAAGGAACAAAGGAGAATGGAA
musTRAV4-4Ph1	GCTTCAGGAACAAAGGAGAATGGGA
musTRAV3-3Ph1	CGGAAATAAACGAAGGACAAGGATT
musTRAV3-1,4Ph1	GTGGACAGAAAAGAAGAACAAGGAC
musTRAV2Ph1	GGACTATGTGGTAAATGAAgtggca
musTRAV1Ph1	GAAGGACAGTGGGCATTTCTCCA

musTRBV1Ph2	CCAGGGTTTTCCCAGTCACGGGTCACTGATACGGAgctga
musTRBV2Ph2	CCAGGGTTTTCCCAGTCACCCATTTAGACCTTCAGATCACAGCT
musTRBV3Ph2	CCAGGGTTTTCCCAGTCACGGATCAGTTTTCAGTTGAAAGACCA
musTRBV4Ph2	CCAGGGTTTTCCCAGTCACCCTCAGTCTTCAGATAAAGCTCATTT
musTRBV5Ph2	CCAGGGTTTTCCCAGTCACGCCCAGACAGCTCCAAGCTACTT
musTRBV12-1,2Ph2	CCAGGGTTTTCCCAGTCACCCAACAGTTTGATGACTATCACTCT
musTRBV13-1Ph2	CCAGGGTTTTCCCAGTCACGCCACCAGAACAACGCAAGAAGA
musTRBV13-2,3Ph2	CCAGGGTTTTCCCAGTCACCAAGGCCTCCAGACCAAGCCAA
musTRBV14Ph2	CCAGGGTTTTCCCAGTCACGGCCTAAAGGAACTAACTCCACTC
musTRBV15Ph2	CCAGGGTTTTCCCAGTCACGCTGAGATGCTAAATTCATCCTTCT
musTRBV16Ph2	CCAGGGTTTTCCCAGTCACGCTCAGATGCCCAATCAGTCGCA
musTRBV17Ph2	CCAGGGTTTTCCCAGTCACCAGTCGGCCTAACAATTCTTTCT
musTRBV19Ph2	CCAGGGTTTTCCCAGTCACCGAGAGAAGAAGTCATCTTTTTCTCT
musTRBV20Ph2	CCAGGGTTTTCCCAGTCACCCATCAGTCATCCCAACTTATCCT
musTRBV23Ph2	CCAGGGTTTTCCCAGTCACCCTCCAGCTCACTCTGCAGCCT
musTRBV24Ph2	CCAGGGTTTTCCCAGTCACCAGCTAAGTGTTCCTCGAACTCaC
musTRBV26Ph2	CCAGGGTTTTCCCAGTCACGCTGAGTGTCCTTCAAACTCACCT
musTRBV29Ph2	CCAGGGTTTTCCCAGTCACGGATACAGGGTCTCACGGAAGAA
musTRBV30Ph2	CCAGGGTTTTCCCAGTCACCAAGTTTCCAATCAGCCGGCCAAA
musTRBV31Ph2	CCAGGGTTTTCCCAGTCACGCTTCCAGGCCGAAGGACGAC
musTRAV21Ph2	CCAGGGTTTTCCCAGTCACCCTGGCTATTGCCTCTGACAGAAA
musTRAV19Ph2	CCAGGGTTTTCCCAGTCACCAGTTTTCTTGAACAAAAGCGGCAAA
musTRAV17Ph2	CCAGGGTTTTCCCAGTCACgccgagtttaggaagagtaactcctct
musTRAV16Ph2	CCAGGGTTTTCCCAGTCACCAGAAGCCAAAAAGTTCCATCGGA
musTRAV15N1Ph2	CCAGGGTTTTCCCAGTCACTCAAATCCATCAGCCTTATCATTTCA
musTRAV15Ph3	CCAGGGTTTTCCCAGTCACCAARTCCATCAGCCTTgTCATTTCA
musTRAV14Ph2	CCAGGGTTTTCCCAGTCACGATTCACAATCTTCTTCAATAAAAGGGAG
musTRAV13Ph2	CCAGGGTTTTCCCAGTCACCGCRGCTCTTTGCACATTTCCTCCT
musTRAV13-5.01Ph2	CCAGGGTTTTCCCAGTCACCCTCAACAGTCACTAAGGGACGT
musTRAV12Ph2	CCAGGGTTTTCCCAGTCACCAGCTCCTTCCATCTGCAGAAGT
musTRAV11Ph2	CCAGGGTTTTCCCAGTCACCTCAGCAACTCTGGATAAAGATGCTA
musTRAV10Ph2	CCAGGGTTTTCCCAGTCACTGGATAAGAAAGCCAAACGATTCTC
musTRAV9Ph2	CCAGGGTTTTCCCAGTCACGCTTYGAGGCTGAGTTCAGCAAGAG
musTRAV8Ph2	CCAGGGTTTTCCCAGTCACGAGCCACCCTTGACACYTCCAGC
musTRAV7-5Ph2	CCAGGGTTTTCCCAGTCACTTTACAGCTCACCTCAATAGAGCCA
musTRAV7-5.02Ph2	CCAGGGTTTTCCCAGTCACCAGCTCAGGTCAATAGAGCCAGCCT
musTRAV7-4Ph2	CCAGGGTTTTCCCAGTCACCTCACCTCAATAAGGCCAGCCTG
musTRAV7-6Ph2	CCAGGGTTTTCCCAGTCACCTCAATAGAGCCAGCCTGCATGTT
musTRAV7-3Ph2	CCAGGGTTTTCCCAGTCACCTCAATAAAGCCAGTCTGCATTTCTC
musTRAV7-2Ph2	CCAGGGTTTTCCCAGTCACCCAGCCTGCATACTTCCCTGCA
musTRAV5-4Ph2	CCAGGGTTTTCCCAGTCACGGATAAGAAAGCCAAACGCTTCTC
musTRAV5-1.01Ph2	CCAGGGTTTTCCCAGTCACAGAAAACCAAACACCTTTCCCTGCA
musTRAV4Ph2	CCAGGGTTTTCCCAGTCACGGTTAAAGTCAACATTCAATTCTAAGGA
musTRAV4-4Ph2	CCAGGGTTTTCCCAGTCACCTAAAGTCAGCATTTGATTCTAAGGA
musTRAV3Ph2	CCAGGGTTTTCCCAGTCACCACTGTCYTACTGAACAAGAAAGACAA
musTRAV2Ph2	CCAGGGTTTTCCCAGTCACcatctctgtttatctctgctgaccgga
musTRAV1Ph2	CCAGGGTTTTCCCAGTCACGCCGCTCGAATGGGTACAGTTAC
musTRBCPh1	GCACACGAGGGTAGCCTTTTGTTT
musTRBCPh2	CTGCTTTTGATGGCTCAAACAAGGA
musTRACPh1	GTCAAAGTCGGTGAACAGGCAGA
musTRACPh2	CCTGAGACCGAGGATCTTTTAACTG

musBetaBC1	CTGCTGAACCGCTCTTCCGATCTatGTTCACCTTGGGTGGAGTCACATTTCT CA
musBetaBC2	CTGCTGAACCGCTCTTCCGATCTtaCAGGACCTTGGGTGGAGTCACATTTCT CA
musBetaBC3	CTGCTGAACCGCTCTTCCGATCTgaTTATACCTTGGGTGGAGTCACATTTCT CA
musBetaBC4	CTGCTGAACCGCTCTTCCGATCTcaCCTGTCCTTGGGTGGAGTCACATTTCT CA
musBetaBC5	CTGCTGAACCGCTCTTCCGATCTagACCGCCCTTGGGTGGAGTCACATTTCT CA
musBetaBC6	CTGCTGAACCGCTCTTCCGATCTtgACTTACCTTGGGTGGAGTCACATTTCT CA
musBetaBC7	CTGCTGAACCGCTCTTCCGATCTgtGCTAGCCTTGGGTGGAGTCACATTTCT CA
musBetaBC8	CTGCTGAACCGCTCTTCCGATCTctGACGTCCTTGGGTGGAGTCACATTTCT CA
musBetaBC9	CTGCTGAACCGCTCTTCCGATCTacGGCTACCTTGGGTGGAGTCACATTTCT CA
musBetaBC10	CTGCTGAACCGCTCTTCCGATCTtcGAATGCCTTGGGTGGAGTCACATTTCT CA
musBetaBC11	CTGCTGAACCGCTCTTCCGATCTgcCCAACCCTTGGGTGGAGTCACATTTCT CA
musBetaBC12	CTGCTGAACCGCTCTTCCGATCTcgGAGACCCTTGGGTGGAGTCACATTTCT CA
musAlphaBC1	CTGCTGAACCGCTCTTCCGATCTatGTTCAGTACACAGCAGGTTCTGGGTTC T
musAlphaBC2	CTGCTGAACCGCTCTTCCGATCTtaCAGGAGTACACAGCAGGTTCTGGGTT CT
musAlphaBC3	CTGCTGAACCGCTCTTCCGATCTgaTTATAGTACACAGCAGGTTCTGGGTTC T
musAlphaBC4	CTGCTGAACCGCTCTTCCGATCTcaCCTGTGTACACAGCAGGTTCTGGGTTC T
musAlphaBC5	CTGCTGAACCGCTCTTCCGATCTagACCGCGTACACAGCAGGTTCTGGGTT CT
musAlphaBC6	CTGCTGAACCGCTCTTCCGATCTtgACTTAGTACACAGCAGGTTCTGGGTTC T
musAlphaBC7	CTGCTGAACCGCTCTTCCGATCTgtGCTAGGTACACAGCAGGTTCTGGGTTC T
musAlphaBC8	CTGCTGAACCGCTCTTCCGATCTctGACGTGTACACAGCAGGTTCTGGGTTC T
musAlphaBC9	CTGCTGAACCGCTCTTCCGATCTacGGCTAGTACACAGCAGGTTCTGGGTTC T
musAlphaBC10	CTGCTGAACCGCTCTTCCGATCTtcGAATGGTACACAGCAGGTTCTGGGTTC T
musAlphaBC11	CTGCTGAACCGCTCTTCCGATCTgcCCAACGTACACAGCAGGTTCTGGGTT CT
musAlphaBC12	CTGCTGAACCGCTCTTCCGATCTcgGAGACGTACACAGCAGGTTCTGGGTT CT
PEprimer1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT
PEprimer2	AAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCT CTTCCGATCT
PlateNN1A	CCTACACGACGCTCTTCCGATCTNNgcagaGAtaagcCCAGGGTTTTCCCAGTC ACGAC
PlateNN1B	CCTACACGACGCTCTTCCGATCTNNgcagaGAtgcacCCAGGGTTTTCCCAGTC ACGAC
PlateNN1C	CCTACACGACGCTCTTCCGATCTNNgcagaGActcagCCAGGGTTTTCCCAGTC ACGAC
PlateNN1D	CCTACACGACGCTCTTCCGATCTNNgcagaGAggaatCCAGGGTTTTCCCAGTC ACGAC
PlateNN1E	CCTACACGACGCTCTTCCGATCTNNgcagaGAcgaggCCAGGGTTTTCCCAGT CACGAC
PlateNN1F	CCTACACGACGCTCTTCCGATCTNNgcagaGAaggagCCAGGGTTTTCCCAGT CACGAC
PlateNN1G	CCTACACGACGCTCTTCCGATCTNNgcagaGAtgttgCCAGGGTTTTCCCAGTC ACGAC
PlateNN1H	CCTACACGACGCTCTTCCGATCTNNgcagaGAcaactCCAGGGTTTTCCCAGTC ACGAC

PlateNN2A	CCTACACGACGCTCTTCCGATCTNNTCGAAGAtaagcCCAGGGTTTTCCCAG TCACGAC
PlateNN2B	CCTACACGACGCTCTTCCGATCTNNTCGAAGAtgcacCCAGGGTTTTCCCAG TCACGAC
PlateNN2C	CCTACACGACGCTCTTCCGATCTNNTCGAAGActcagCCAGGGTTTTCCCAG TCACGAC
PlateNN2D	CCTACACGACGCTCTTCCGATCTNNTCGAAGAggaatCCAGGGTTTTCCCAG TCACGAC
PlateNN2E	CCTACACGACGCTCTTCCGATCTNNTCGAAGAcgaggCCAGGGTTTTCCCAG TCACGAC
PlateNN2F	CCTACACGACGCTCTTCCGATCTNNTCGAAGAaggagCCAGGGTTTTCCCAG TCACGAC
PlateNN2G	CCTACACGACGCTCTTCCGATCTNNTCGAAGAtgttgCCAGGGTTTTCCCAGT CACGAC
PlateNN2H	CCTACACGACGCTCTTCCGATCTNNTCGAAGAcaactCCAGGGTTTTCCCAG TCACGAC
PlateNN3A	CCTACACGACGCTCTTCCGATCTNNAACAAGAtaagcCCAGGGTTTTCCCAG TCACGAC
PlateNN3B	CCTACACGACGCTCTTCCGATCTNNAACAAGAtgcacCCAGGGTTTTCCCAG TCACGAC
PlateNN3C	CCTACACGACGCTCTTCCGATCTNNAACAAGActcagCCAGGGTTTTCCCAG TCACGAC
PlateNN3D	CCTACACGACGCTCTTCCGATCTNNAACAAGAggaatCCAGGGTTTTCCCAG TCACGAC
PlateNN3E	CCTACACGACGCTCTTCCGATCTNNAACAAGAcgaggCCAGGGTTTTCCCA GTCACGAC
PlateNN3F	CCTACACGACGCTCTTCCGATCTNNAACAAGAaggagCCAGGGTTTTCCCA GTCACGAC
PlateNN3G	CCTACACGACGCTCTTCCGATCTNNAACAAGAtgttgCCAGGGTTTTCCCAG TCACGAC
PlateNN3H	CCTACACGACGCTCTTCCGATCTNNAACAAGAcaactCCAGGGTTTTCCCAG TCACGAC

Abbreviations

AICD	activation-induced cell death
APC	antigen presenting cell
APC	allophycocyanine
ATT	adoptive T cell transfer
BCG	Bacillus Calmette-Guérin
BCMA	B-cell maturation antigen
bp	base pairs
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDK4	cyclin-dependent kinase 4
cDNA	complementary DNA
CDR	complementarity-determining region
CpG	unmethylated Cysteine-Guanine dinucleotide motif

CRISPR	clustered regularly interspersed short palindromic repeat
CTL	cytotoxic T lymphocyte
DC	dendirtic cell
DNA	deoxyribonucleid acid
dNTP	deoxyribose nucleoside triphosphate
dox	doxycycline
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked-immunosorbent assay
ER	endoplasmic reticulum
ERAP	endoplasmic reticulum aminopeptidase
FACS	fluorescence-activated cell sorting
FC block	antibody blocking FC-receptors CD32 and CD16
FCS	fetal calf serum
FITC	fluorescing isothiocyanate
FSC	forward scatter
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
Gy	Gray
h	hour(s)
HBV	hepatitis B virus
	Human leukocyte antigen (HLA)-A2.1
HHD	transgenic mice
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HY	histocompatibility Y chromosome antigen
ICB	immune checkpoint blockade
ICD	immunogenic cell death
ID	identification number
IDO	indoleamine 2,3-dioxygenase
IFA	incomplete Freund adjuvant
IFN	interferon
IL	interleukin

Iono	Ionomycin
IRES	internal ribosomal entry site
LaGeSo	Landesamt für Gesundheit und Soziales
lg	log-10
LTR	long terminal repeats
m	mutated or murine
mCb	mouse constant beta chain (of TCR)
MFI	mean fluorescence intensity major
MHC	major histocompatibility complex
min	minute
MLV	murine leukemia virus
mp68	mutated p68 (SNFVFAGI)
mRNA	messenger RNA
NFAT	nuclear factor of activated T-cells
NK	natural killer
ns	not significant
NR	Non-responder
OVA	ovalbumine
p2a	picorna virus-derived peptide element
PBMCs	perpheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed death protein 1
PDL-1	programmed death ligand 1
PE	phycoerythrine
PMA	phorbol-12-myristate-13 acetate
pMHC	peptide-MHC
Rag1	recombination-activating gene
Reis	Tumor reisolate
RNA	ribonucleic acid
RNAse	ribonuclease
RT-PCR	reverse transcription PCR
SEM	Standard error of the mean
SSC	sideward scatter
TAA	tumor associated antigens

TAA	tumor associated antigens
TAM	tumor associated macrophage
TAP	transporter associated with antigen processing
TCR	t cell receptor
TCR-T	TCR-transduced T cell
TdT	terminal deoxynucleotidyl transferase
TGF	transforming growth factor
TILs	tumor-infiltrating lymphocytes
TLR	toll-like receptor
TMB	tumor mutational burden
TNF	tumor necrosis factor
TRAV	T cell receptor alpha variable region
TRBV	T cell receptor beta variable region
TRCB	T cell receptor constant region beta
Treg	regulatory T cell
TSA	tumor specific antigen
UT	untransduced T cells
UV	ultra violet
VDJ	variable -diversity-joining gene segments
VEGF	vascular endothelial growth factor