Characterization of mice with humanized T cell recognition system and isolation of optimal affinity TCR against tumor antigen NY-ESO-1

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Table of Contents

Summary

The efficacy of T cell therapies for cancer is determined in part by the T cell receptors (TCRs) affinities to their target antigens. Since most of the human cancer antigens are self-antigens, humans are tolerant and T cells with high affinity TCRs are difficult to identify in humans. Utilizing human antigen-negative human MHC transgenic mice is a solution, but concerns are raised due to the potential interspecies incompatibility between murine TCRs and human major histocompatibility complexes (MHCs). To solve the problem, we generated transgenic mice with complete human TCR α and β gene loci and a single human MHC class I (MHC I) or MHC class II (MHC II) molecule. The mice generated diverse human TCR repertoires and the peripheral T cells were functional upon antigen stimulations. To provide evidence for the existence of TCR-MHC coevolution, we compared the TCR repertoire of CD4 T cells selected by a single mouse or human MHC II in the transgenic mice. Our data showed that human MHC II yielded higher thymic output and a more diverse TCR repertoire. We hypothesized that the complementarity determining region 3 (CDR3) length adjusted for different inherent V segment affinity to MHC II to ensure thymic selection of most V genes. We also showed that humans evolved for higher non-template encoded CDR3 diversity compared to mice. Our findings demonstrate that TCRs and MHCs further co-evolved after the divergence between humans and rodents ~70 million years ago and justify the rational of generating TCR-MHC species-compatible transgenic mice.

With the established transgenic mice with human MHC I, we investigated CD8 T cell immune response against a cancer-testis antigen, NY-ESO-1. NY-ESO-1 immunization evoked robust CD8 T cell response in the mice, one of the specific TCRs showed higher affinity compared to the human-derived TCR-1G4. The mouse-derived TCR transduced human T cells also showed higher functionality than TCR-1G4 transduced T cells. Our data suggest central tolerance against NY-ESO-1 in humans and proved that the transgenic mice with the complete humanized T cell antigen recognition system are a valid tool to isolate optimal affinity TCRs against human antigens.

Zusammenfassung

Die Effektivität tumorspezifischer T-Zell-Therapien wird maßgeblich durch die Affinität der verwendeten T-Zell-Rezeptoren (TCRs) zu ihren jeweiligen Zielantigenen bestimmt. Da es sich bei den meisten humanen Tumorantigenen um Selbstantigene handelt, ist das humane Immunsystem diesen gegenüber tolerant und T-Zellen mit hochaffinen TCRs sind in Menschen nur schwierig nachzuweisen. Die Verwendung transgener Mäuse mit humanem MHC, die die entsprechenden humanen Antigene nicht besitzen, stellt eine mögliche Lösung dar, jedoch bestehen hier potentiell Inkompatibilitäten zwischen den murinen T-Zell-Rezeptoren und humanen Haupthistokompatibilitätskomplex (MHC) Antigenen. Zur Lösung dieses Problems generierten wir transgene Mäuse mit den vollständigen humanen TCR α und β Loci sowie je einem einzelnen humanen MHC Klasse I oder Klasse II Gen. Diese Mäuse generierten ein polyklonales TCR Repertoire mit T-Zellen, die funktional auf Antigenstimulation reagierten. Die humanen TCR Transgene waren in der Lage, wieder ein auf humanes MHC I restringiertes CD8 T Zell Kompartiment herzustellen. Zum Nachweis der Existenz einer TCR-MHC-Koevolution verglichen wir TCR Repertoires von CD4 T Zellen, die in diesen transgenen Mäusen durch ein einziges murines oder humanes MHC II selektioniert wurden. Humanes MHC II führte sowohl zu einer höheren (thymischen) T-Zell-Produktion, als auch zu einem stärker diversifizierten TCR Repertoire. Wir stellten die Hypothese auf, dass Veränderungen der Länge der CDR3-Regionen Unterschiede in der intrinsischen Affinität verschiedener V-Segmente für MHC II kompensieren, um die Positivselektion der meisten V Gene im Thymus sicherzustellen. Es ließ sich außerdem zeigen, dass das humane Immunsystem, verglichen mit Mäusen, evolutionär eine höhere, nicht genomisch kodierte, CDR3-Diversität entwickelte. Zusammenfassend zeigt sich, dass nach der phylogenetischen Trennung von Menschen und Nagetieren vor etwa 70 Millionen Jahren eine weitere Koevolution zwischen TCRs und MHCs stattfand, wodurch sich eine Rationale für die Generierung von spezieskompatiblen TCR und MHC transgenen Mäusen ergibt.

Nach Etablierung der human MHC I transgenen Mäuse nutzten wird diese, um die Immunantwort gegen ein „Cancer/Testis“-Antigen, NY-ESO-1, zu untersuchen. Immunisierung mit NY-ESO-1 führte zu einer stabilen CD8 T Zell Antwort in den Mäusen, wobei einer der spezifischen TCRs eine höhere Antigenaffinität zeigte als der humane NY-ESO-1 spezifische TCR 1G4. Humane T-Zellen, die mit dem in der transgenen Maus generierten TCR transduziert wurden, zeigten eine bessere funktionelle Antwort als solche, die mit TCR 1G4 transduziert wurden. Dies gibt einen starken Hinweis für die Existenz zentraler Toleranz gegen NY-ESO-1 im Menschen und zeigt gleichzeitig die Nützlichkeit von transgenen Mäusen mit einem vollständig humanen T-Zell-Antigenerkennungssystem als Werkzeug für die Generierung von TCRS mit optimaler Affinität gegen humane Antigene.

Introduction

1.1 αβ T cells

αβ T cells constitute around 90-95% of all T cells in mice and humans and are part of the adaptive immune system. They express T cell receptors (TCRs) which are heterodimers composed of one α and one β chain. According to their co-receptors, αβ T cells can be further divided into CD4 and CD8 T cells.

αβ T cells exert functions such as harmonizing immune responses to infections, or direct destruction of infected cells.

1.1.1 αβ T cell receptor

αβ T cells recognize their targets by binding of the TCRs to antigens in the form of short peptides presented by major histocompatibility complexes (MHCs) on the surface of antigen presenting cells. Each T cell clone expresses a unique TCRαβ.

To accommodate the vast amount of antigens presented by different MHC molecules, T cells must generate a diverse αβ TCR repertoire. T cells achieve the task by recombination of each one of multiple germline encoded Variable (V), (Diversity (D)), Joining (J) gene segments by recombination activating gene encoded proteins (RAGs); non-template addition/deletion of nucleotides in the V(D)J junctional region using terminal deoxynucleotidyl transferase (TdT) and exonuclease; together with random α and β chain pairing (Figure1) (Davis and Bjorkman, 1988; Jung and Alt, 2004). Upon encountering antigens, TCRs also undergo conformational adjustments, a so-called 'induced-fit' binding to ensure specific recognition of respective peptide-MHCs (pMHCs) (Krogsgaard and Davis, 2005).

Theoretically, αβ T cells can generate more than 10^{15} different TCRs (Davis and Bjorkman, 1988). Yet due to thymic selection, size of the T cell compartment, etc., the actual TCR diversity in an individual is remarkably reduced. Mice are estimated to have $2x10^6$ distinct αβ chains in the periphery and for humans it is estimated to be $2.5x10^7$ (lower bound) (Figure 1) (Casrouge et al., 2000; Arstila et al., 1999). Hence, the size of each T cell clone would be ~ 20 – 50 cells in mice and ~1,000 – 4,000 in humans. It is suggested that the T cell precursor frequency of a particular specificity is approximately 1 – 100 cells/million CD4 or CD8 T cells (Jenkins and Moon, 2013; Su et al., 2013). This proportion does not change with the size of an individual (Vrisekoop N et al., 2014).

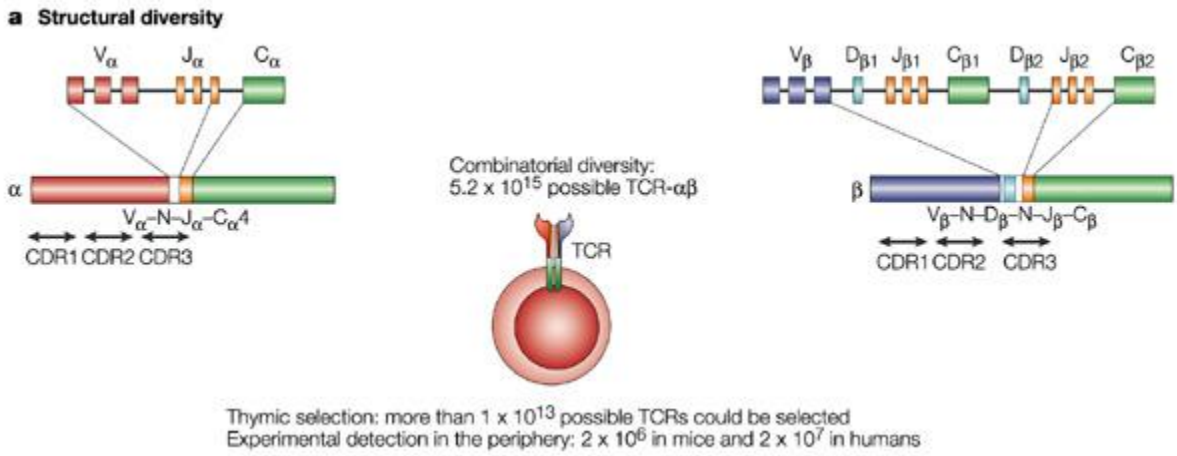

Figure 1: Schematic illustration of αβ TCRs generation. Each one of the multiple TCRα/β V, (D) and J gene segments randomly recombined together by recombination activating gene 1 and 2 (RAG1/2) to form a TCRα/β chain. The TCR diversity is further increased by deletion or addition of nucleotides at the V(D)J junction by nuclease and terminal deoxynucleotidyl transferase (TdT). Finally, one TCRα and one TCRβ chain come together and generate a TCR heterodimer. The figure is taken from (Nikolich-Zugich et al., 2004).

A typical αβ TCR contacts the pMHC with their complementarity-determining region 1, 2 and 3 (CDR1, 2 and 3) (Nikolich-Zugich et al., 2004). The CDR1 and 2 are located on Vα and β genes and predominantly interact with the MHC molecules. CDR3 represents the most diverse region of a TCR chain. It is located in the V(D)J junction and has an extensive role in engaging the peptides (Davis and Bjorkman, 1988). Most of the solved TCR-pMHC tertiary structures indicate that the TCR binds the pMHC in a conserved diagonal docking mode: the TCRα chain lies mainly over the α2 helix (MHC I) or β helix (MHC II) and the N-terminal end of the peptide, and the TCRβ over the α1 helix (MHC I) or α helix (MHC II) and the carboxyl-terminal end of the peptide (Garcia et al., 2009; Rossjohn et al., 2015).

1.1.2 T cell development

Once generated in the bone marrow, the lymphoid progenitors which emigrate through blood stream into the thymus become T cell precursors (Schwarz and Bhandoola, 2006). The thymus is a specialized lymphoid organ where T cells differentiate and mature.

Early committed T cells lack the expression of TCRs and CD4/CD8 co-receptors and thus are termed double negative (DN) thymocytes. During this stage, the cells start to rearrange their TCRβ chains. Functional TCRβ chains pair with an invariant pre-α chain to form pre-TCRs. Cells with successfully paired pre-TCR chains receive the signals to proliferate (β selection) and silence the

re-arrangement of the other TCRβ allele (allelic exclusion) (Mostoslavsky et al., 2004). Double positive (DP) lymphocytes replace the pre-α chain with a newly rearranged TCRα chain. At this stage, the lymphocytes are $CD4^+$, $CD8^+$ and TCR^+ (Germain, 2002). Subsequently, T cells undergo thymic selection before they can enter the peripheral T cell pool (Figure 2).

Thymic selection

TCR rearrangement is a stochastic event, together with the random pairing of TCRα and β chains it generates a vast spectrum of different TCRs. But it is known that there are only ~10^8 T cells in a mouse periphery and ~10^{11} T cells in humans. Most of the T cell selection events happen in the thymus, where only those T cells (3 – 5% of DP cells) with TCR having low affinities to self-peptide-MHCs can pass the selection criteria and survive (Starr et al., 2003; Surh and Sprent, 1994). These processes strongly limit the T cell diversity.

Positive selection. Despite the large number of DP T cell candidates, only ~15% of them receive a survival signal and mature into CD4 or CD8 single positive (SP) T cells (Merkenschlager et al., 1997; Mcdonald et al., 2015). This process is called positive selection. Positive selection happen mainly in the thymic cortex, with self-antigen presenting cortical thymic epithelial cells (cTECs) as the antigen-presenting cells (Figure 2) (Starr et al., 2003). DP cells with TCRs that bind to self-pMHC I complex become CD8 T cells and the ones binding to self-pMHC II complex become CD4 T cells.

A favored explanation of positive selection is to impose self MHC restriction to the T cells (Bevan, 1977; Zinkernagel et al., 1978; Davis and Bjorkman, 1988). Yet experiments suggest that there are other possible roles for positive selection. For instance, the survival of only those T cells with subthreshold self-pMHC ensures T cell sensitivity to foreign antigens and the naïve T cell maintenance in the periphery (Purbhoo et al., 2013; Surh and Sprent, 2008; Mandl et al., 2013).

Negative selection. A crucial step in thymic selection is to eliminate T cells which react to self-pMHC ligands, since they can potentially cause autoimmune diseases. Negative selection takes place mainly in the medulla of the thymus, where lineage-determined (CD4 or CD8) SP T cells get in contact with hematopoietic cells, particularly dendritic cells. Too strong signaling due to high affinities to self-pMHC ligands leads to apoptosis (Starr et al., 2003). Around 50% of the SP T cells are negatively selected (Merkenschlager et al., 1997; Mcdonald et al., 2015).

1.1.3 CD4 and CD8 T cells

CD4 and CD8 T cells are distinguished by their CD4/CD8 co-receptor expression.

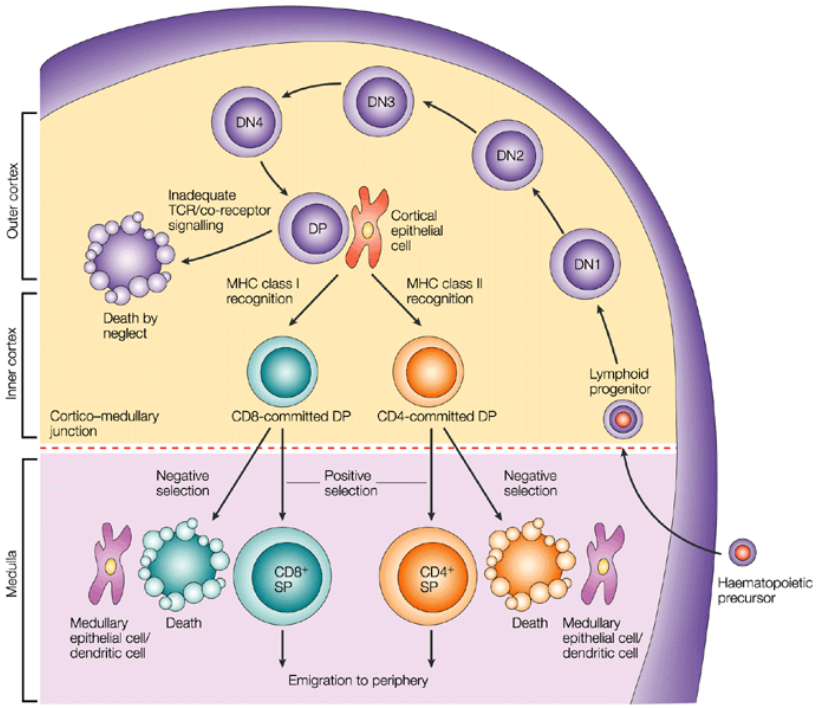

Figure 2: T cell development in the thymus. T cell precursors have to functionally rearrange their TCRβ and α chains in the DN and DP stages, respectively, to receive a survival signal. The cells with unsuccessful rearrangement events die by neglect. Only T cells with TCRs that have weak affinities to self-peptide presenting MHC-s will receive a signal to survive, a process called positive selection. Cells that have too high affinity to self-peptide-MHC-s will undergo apoptosis, a process called negative selection. Positive selection happens mainly in the cTEC of the thymus where negative selection happens in the mTEC. Figure taken from (Germain, 2002).

CD4 T cells play a crucial role in orchestrating immune responses. They are often referred to as T helper cells, since they exert their function mainly by indirectly acting on other immune cells. They exert such functions mostly by secretion of chemokines and cytokines. CD4 T cells recognize ligands which are usually 13-17 amino acids long presented by on MHC II molecules.

CD4 T cells can be subdivided into at least 4 groups: Th1, Th2, Th17 and Treg cells. The first three are also known as conventional CD4 T cells (Tcon): Th1 cells mediate immune responses against intracellular pathogens; whereas Th2 cells respond mostly to extracellular parasites and Th17 cells to extracellular bacteria and fungi (Mosmann and Coffman, 1989; Paul and Seder, 1994; Weaver et al., 2006). The fates of Tcon depend on the signal patterns they receive during their initial activation (Zhu and Paul, 2009; Nishizuka and Sakakura, 1969). Treg cells express FoxP3, they are important in maintaining self-tolerance and regulate immune responses (Sakaguchi, 2004). The fate of natural Treg cells are decided during thymic selection, that those CD4 T

thymocytes with high affinities to self-peptide-MHC develop into Treg cells (Hsieh et al., 2012). Tcon and Treg cells express distinct but both diverse TCR repertoires (Fujishima et al., 2005; Kasow et al., 2004).

CD8 T cells, also known as cytotoxic T lymphocytes (CTLs), are the major immune cell type that mediates direct killing of infected cells or cancer cells. They recognize antigens (8-11mers) presented by MHC I molecules. MHC I molecules are expressed by all nucleated cells in the body. Upon activation, CD8 T cells turn into effector T cells and secret perforin, granzymes and cytokines within the immunological synapses form between the T cell and its target cell, which lead to apoptosis of the target cells. Activated CD8 T cells also respond to Interleukin-2 (IL-2) secreted by i.e. CD4 T cells and undergo clonal expansion. Depending on the strength of the TCR signaling and the cytokine environment, naïve CD8 T cells can further develop into central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) (Restifo et al., 2012; Klebanoff et al., 2006).

Both CD4/CD8 T cell activation is initiated by the T cell binding to its cognate pMHC with its TCRs. It is shown that CD8 (Luescher et al., 1995; Wooldridge et al., 2005), but not CD4 co-receptor (Hamad et al., 1998; Huppa et al., 2010; Crawford et al., 1998), help to stabilize the interaction between TCRs and the pMHCs. But both co-receptors augment the sensitivities of the respective T cells (Huppa et al., 2010; Holler and Kranz, 2003), mainly by delivery of co-receptor bound Lck to the relevant TCR-CD3 complex (Stepanek et al., 2014; Artyomov et al., 2010).

1.2 TCR-MHC co-evolution

Ever since T cells and their targets were identified, a question was raised on how T cells with such TCR diversity of up to billions react almost exclusively in a MHC-restricted fashion. Also, T cells can react to almost any MHC molecule, considering the large polymorphism of MHC genes (i.e. in humans there are around 15,000 variants) (Robinson et al., 2003).

In 1970s, Niels Jerne proposed that T cells had evolved to interact with MHC alleles, thymic selection eliminated T cells that were reactive to self-MHC-s but retained the function to interact with foreign MHCs, which is now known as TCR-MHC co-evolution hypothesis (Jerne, 1971). The theory was disfavored after the discovery of positive selection, since positive selection sustains a peripheral T cell pool that can interact with self-MHCs and it seems like there is no need for inherent affinity between TCRs and MHCs (Zinkernagel et al., 1978; Fink and Bevan, 1978). But several lines of evidences still point to the existence of TCR-MHC co-evolution. i.e. the relatively high proportion of MHC-reactive T cells in the pre-selection pool (approximately 5-20%); or the fact that ~10% of the peripheral T cells are MHC alloreactive all indicate the TCR germline bias for MHCs (Blackman et al., 1986; Blattman, 2002; Suchin et al., 2001; Zerrahn et al., 1997).

Namely, the germline-encoded complementarity determining region (CDR) 1 and CDR2 of the Vα and Vβ segments are evolutionarily conserved to react with MHC molecules, which was termed TCR germline bias (Garcia et al., 2009; Huseby et al., 2005; Marrack et al., 2008).

However, not all V gene segments share conserved residues in CDR1 and CDR2. Therefore, it was suggested that each V segment engages to its cognate MHC through a menu of structurally coded recognition motifs that have evolutionarily arisen (Feng et al., 2007; Garcia et al., 2009; Marrack et al., 2008), a comprehensive hypothesis, but is difficult to experimentally address.

1.2.1 Evidence support /against existence of TCR-MHC inherent affinity

Compelling evidence for this hypothesis resulted from structural and mutational analysis, showing that single amino acid substitutions in a mouse Vβ CDR2, e.g. Tyr48, Tyr50 and Glu54, decreased positive selection in a TCR transgenic mouse model (Dai et al., 2008; Scott-Browne et al., 2009). Furthermore, some Vβ genes of jawed vertebrates (frog, shark, trout, lizard), which diverged from mammals ~400 million years ago, share sequences in the CDR2 region of mouse Vβ8.2, while otherwise exhibiting little homology. T cells with chimeric TCRs containing such Vβ genes, e.g. derived from frogs, were positively selected in mice (Scott-Browne et al., 2011). Further evidence is mounting from the growing database of TCR-pMHC ternary crystallographic structures (Rossjohn et al., 2015). With few exceptions (Beringer et al., 2015; Rossjohn et al., 2015), many of the solved TCR-pMHC structures to date adapt a diagonal docking topology atop the pMHC complex (Adams et al., 2016; Rossjohn et al., 2015). Evidence also comes from study of TCR-MHC binding mechanism (Wu et al., 2002) or the observation of the preferential MHC I or MHC II by certain murine TCR Vα genes (Sim et al., 1996).

On the other hand, several studies showed results that might not be in favor of the co-evolution theory. For instance, one of the studies described the development of ‘antibody-like’ T cells in co-receptor and MHC-deficient mice (Van Laethem et al., 2007). The specific target of one of these T cells is the native form of CD155 (Van Laethem et al., 2013). Furthermore, some structural analyses of TCR-pMHC complexes show the lack of a clear motif for TCR-MHC binding (Beringer et al., 2015; Gras et al., 2010; Sethi et al., 2011; Tikhonova et al., 2012; Tynan et al., 2005). Hence, it is not generally accepted that TCR and MHC coevolved. This may not be surprising given the complex and flexible interactions that TCR and MHC can undergo.

1.3 Adoptive T cell therapy of cancer

T cells have revealed their role in tumor rejection in as early as 1953. In his experiments, Mitchison showed that T cells, rather than antibodies, accounted for the accelerated tumor rejection in the

mouse models (Mitchison, 1953). Thereafter, several animal and human experiments showed that T cells could control tumor growth (Southam et al., 1966; Klein et al., 1960; Leclerc et al., 1972). Yet, it remained unclear until 1990s, with the discovery of tumor-associated/specific antigens (TAAs/TSAs) that T cells can distinguish cancerous cells from normal cells (Coulie et al., 1995; Monach et al., 1995; Wölfel et al., 1995; van der Bruggen et al., 1991).

Adaptive immunity possesses several properties which makes it potent for cancer treatment (Perica et al., 2015): 1) T cell responses are specific for their targets, which makes them possible to distinguish heathy and pathological tissues (low toxicity); 2) T cells are effective in responding to their target. Upon activation, T cells can undergo up to 1,000 fold clonal expansion; 3) T cells have the potential to destruct established solid tumor through destruction of cancer stroma (bystander killing) (Spiotto et al., 2004; Spiotto and Schreiber, 2005; Zhang et al., 2008a; Gerbitz et al., 2012; Zhang et al., 2008b); 4) Therapeutic effect with T cell therapy could last for years (T cell memory).

There are three main strategies in manipulating antitumor capacities of T cells in use for cancer therapy: active immunization by therapeutic cancer vaccines (Finn, 2008), adoptive transfer of T cells into tumor-bearing hosts (June, 2007b), and more recently administration of checkpoint inhibitors (Lesokhin et al., 2015). While cancer vaccination often resulted in unsatisfactory outcomes in the clinic likely due to central/peripheral tolerance (Rosenberg et al., 2004), the latter two approaches show promise in eradicating advanced cancers.

Checkpoint Inhibitors. The rationale of checkpoint blockade in cancer treatment is to overcome immunosuppressive pathways in immune cells and enhance their antitumor responses and duration (Lesokhin et al., 2015). For example, administration of antibodies, i.e. ipilimumab, against cytotoxic T-lymphocyte antigen 4 (Anti-CTLA-4 treatment) in treatment of melanoma showed prolonged survival in patients (Hodi et al., 2010; Robert et al., 2011); Therapy with nivolumab, an antibody blocking the programmed death 1 receptor (PD-1), yielded objective and durable responses in patients with melanoma, renal-cell cancer and non-small-cell lung cancer (Topalian et al., 2004). Studies revealed that there is a subset of cancers that are susceptible to checkpoint inhibitor therapy. Although markers like PD-1 ligand (PD-L1), RNA expression signatures, mutational burden have been evaluated, it is still unclear what the molecular determinants for this type of cancers (Le et al., 2017; Snyder et al., 2014; Boussiotis, 2015). It should be noted that treatments with CTLA-4 and PD-1 antagonists are quite often associated with severe autoimmunity, especially upon combined checkpoint blockade (McDermott et al., 2015; Topalian et al., 2014; Gettinger et al., 2015; Wolchok et al., 2013).

Adoptive T cell therapy. Adoptive T cell therapy (ATT) is a thriving field in combat against cancer. In ATTs, the specificities of the T cells are manipulated *ex vivo* and infused into cancer patients to break immune tolerance. Tumor regression was seen in trials employing chimeric antigen receptors (CARs) against CD19 for acute or chronic leukemia; or TCRs against NY-ESO-1 for melanoma and synovial cell sarcoma (Hinrichs and Rosenberg, 2014). Compared to other T cell therapies, ATT creates, rather than unleashes, an anti-tumor immune response (Perica et al., 2015).

1.3.1 Transfer of unmodified T cells

The T cells used for transfer into cancer patients come mainly from two sources: allogenic donor T cells or autologous T cells that are selected for their antitumor specificities *in vitro*.

Allogenic hematopoietic stem cell transplantation (HSCT) in treating hematological malignancies makes use of the graft-versus-leukemia (GVL) effect mediated by immunocompetent donor leukocytes (Kolb, 2011; Porter and Antin, 1999). The evidence that allogenic T cells mediate GVL lies in several mouse experiments and clinical observations (Barnes and Loutit, 1957; Truitt and Atasoylu, 1991; OKunewick et al., 1994; Weiss et al., 1990; Higano et al., 1990; Horowitz et al., 1990). Donor leukocyte infusion (DLI) for treatment of relapsed chronic myelogenous leukemia (CML) after HSCT usually achieve 70 - 79% complete remission (Kolb et al., 1995; Collins et al., 1997). Another example of the effective use of DLI is for treatment of Epstein-Barr virus (EBV) – related B cell post-transplantation lymphoproliferative disorders (PTLD) after allogeneic bone marrow transplantation, with a remission rate of up to 90% (Papadopoulos et al., 1994). The antigens that the donor T cells target are most likely minor histocompatibility antigens that differ between the host and the donor (Kloosterman et al., 1994, 1995; Horowitz et al., 1990), viral antigens or, perhaps, tumor specific antigens. The success of applying allogenic immunity to treat cancer is limited, mainly due to the difficulties of finding MHC-matched donors and complications such as the graft-versus-host disease (GvHD), which is very often coupled with the GVL effect induced by donor T cells or marrow aplasia (Porter et al., 2000). Besides, allogenic immunotherapies are less effective in treating cancers except leukemia.

On the other hand, the efficacy of autologous T cells transfer is hindered by immune tolerance. Established methods to break immune tolerance and increase the efficacy of this approach include *ex vivo* stimulation and expansion of tumor-specific T cell clones targeting tumor-antigens originated from e.g. peripheral blood or infiltrated T lymphocytes (TILs) of fresh tumor biopsies, and/or pre-conditioning of the patients with chemotherapy or total-body irradiation induced lymphodepletion (June, 2007b; Hinrichs and Rosenberg, 2014; Rosenberg and Restifo, 2015).

The power of this therapeutic approach is mostly demonstrated in treatment of metastatic melanoma, where around 50% of the treated patients showed objective clinical responses (Yee et al., 2000, 2002; Mackensen et al., 2006; Rosenberg et al., 2011; Radvanyi et al., 2012; Itzhaki et al., 2011; Ellebaek et al., 2012; Hunder et al., 2008). However, a case report of TILs therapy targeting mutant KRAS G12D restricted on HLA-C*08:02 was described recently from a patient with metastatic colorectal cancer. Regression was observed after the TIL infusion, although 9 month later one of the lesions progressed due to the loss of HLA-C*0802 expression, suggesting a possible therapeutic role of TIL therapy in treating solid tumors other than melanoma (Tran et al., 2016).

1.3.2 Transfer of gene modified T cells

Therapies with unmodified autologous T cells are often with unsatisfactory clinical outcomes because of the inadequate functionality of the transferred T cells. One way to overcome immune tolerance, enhance T cell functionality and minimize toxicity is to utilize genetically modified T cells. For example, introduction of a constitutively expressed CD28 gene into exhausted T cells can restore their ability to produce IL-2 upon antigen stimulation (Topp et al., 2003); human telomerase reverse transcriptase (*hTERT*) gene transduced primary human $CD8^+$ T cells showed extended replicative life span *in vitro* (Rufer et al., 2012); or human CTLs transduced with GM-CSF-IL-2 receptors formed an autocrine growth loop and could proliferate without exogenous cytokines *in vivo* (Evans et al., 1999). In addition, T cells which were modified to express suicide genes, i.e. herpes simplex virus thymidine kinase (HSV-TK), showed reduced treatment-induced toxicities, both in mice and patients (Bonini et al., 1997; Kieback et al., 2008). Among all these approaches, particular efforts were made in redirecting the specificities of T cells by transferring tumor-antigen specific receptors. These receptors can be new TCRα/β chains or chimeric antigen receptors (CARs) with defined specificity (June et al., 2015; Hinrichs and Rosenberg, 2014; June, 2007b).

1.3.2.1 Transfer of TCR engineered T cells

The administration of specificity-redirected T cells with a new pair of TCRα and β chains is also called TCR gene therapy. In general, TCR gene therapy includes the identification and cloning of TCRα and β chains from tumor-antigen reactive T cell clones; re-expression of the TCRs in patient T cells; *ex vivo* expansion of TCR gene-engineered T cells and re-infusion of these T cells back into lymphodepleted cancer patients (Figure 3). Most of the current TCR gene therapy trials are performed with MHC-I restricted CD8 T cells, although recently experiments suggested a potent role of gene modified CD4 T cells in ATTs and a clinical trial targeting a MHC-II epitope of MAGE-A3 was recently conducted in cancer patients with various types of solid tumors (Lu et al., 2017;

Katsuhara et al., 2015; Lin et al., 2013; Straetemans et al., 2012; Matsuzaki et al., 2015). The primary limitation of TCR gene therapy is MHC-dependency, since the eligible patients for treatment must share the same MHC-restriction as the therapeutic TCRs.

One aspect that dictates the outcome of the therapy is the selection of the target tumor-antigens (See section “1.2.2.3 Tumor antigens”). Genetic instability, which features the cancerous cells, implies that antigens generated in cancer cells differ from normal cells both in composition and the magnitude of their expression (Pardoll, 2003). Ideal antigen targets for T cell therapies should be expressed in a tumor-restricted manner, and universally expressed in all or at least most of the cancer cells.

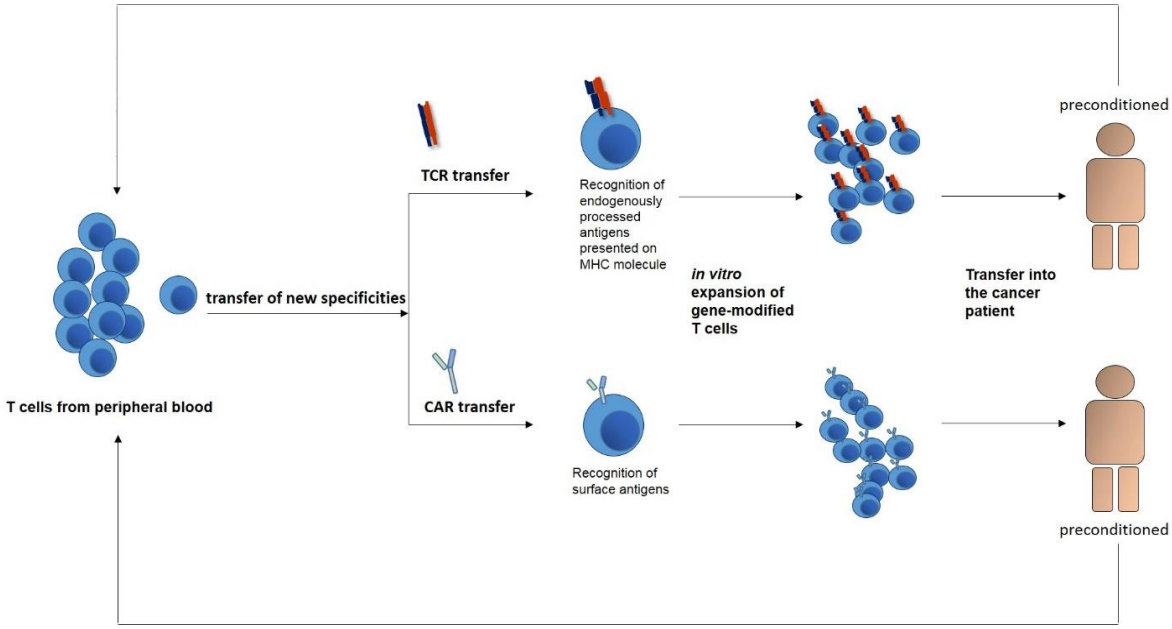

Figure 3: Schematic illustration of adoptive immunotherapy with TCR/CAR gene-modified T cells.

Another restriction factor that determines the efficacy and safety of TCR gene therapy is the selection of tumor-specific TCRs. TCRs can be isolated from tumor-reactive T cell clones either from high avidity patient TILs or from immunized human MHC transgenic mice (June et al., 2015; Hinrichs and Rosenberg, 2014). Particularly, human MHC transgenic mice represent an effective source for human-antigen-specific T cells, since mice and humans often have key differences in their protein sequences. Consequently, there is no central tolerance of murine T cells against most of the human tumor antigens. Theoretically, TCRs obtained from such source have a higher risk of *off-target* toxicities, which means that the TCRs might recognize targets expressed in normal tissues that are different from the original epitope. Hence, it requires careful assessment before

applying such TCRs in the clinic (June et al., 2015). The affinities of the TCRs can be further increased by *in vitro* mutagenesis and selection of the high-affinity clones, although the in vitro affinity matured TCRs often face the risk of losing their specificities (Holler et al., 2003; Linette et al., 2013).

Last but not least, TCR gene therapy faces several technical challenges such as:

1) Choosing the optimal T cell subset for transfer (June, 2007a). Differentiation state of the transferred T cells is crucial to the success of T cell therapies (Rosenberg et al., 2011; Gattinoni et al., 2005). Experiments revealed that T cells that were repeatedly stimulated by antigen and IL-2 had a reduced ability for proliferation and survival. Although these T cells retained their function to lyse the target cells and secret IFNγ, their antitumor efficacy declined in comparison to naïve T cells or T cells which have been stimulated limited times (Gattinoni et al., 2005, 2006; Yee et al., 2002; Wang et al., 2004; Sussman et al., 2004; Dudley et al., 2002). It is hypothesized that the differentiation state of the T cells is inversely related to their capacity to proliferate and persist (Gattinoni et al., 2006). Hence, it is proposed that naïve T cells > central memory T cells > effector memory T cells in the sense of their *in vivo* antitumor activities (Hinrichs et al., 2009, 2011; Klebanoff et al., 2004; Berger et al., 2008; Klebanoff et al., 2005; Gattinoni et al., 2011). Notably, a newly defined population of CD8 T cells, which are termed CD8 T memory stem cells (T_{SCM}), elicits special interest for their use in T cell therapies (Zhang et al., 2005). T_{SCM} phenotype has been intensely studied (Gattinoni et al., 2011; Sallusto and Lanzavecchia, 2011; Gattinoni et al., 2009), and their superior antitumor properties compared to other memory subtypes has been demonstrated in a mouse model (Gattinoni et al., 2009);

2) Identification and optimization of the therapeutic TCRs. Transferring the specificity of tumor-antigen reactive CTLs to patient T cells requires the identification of the TCRα/β chains expressed by the CTLs. Advancing technologies such as DNA/RNA deep sequencing (Linnemann et al., 2013; Howie et al., 2015), single cell PCR (Dössinger et al., 2013) etc. enable the recognition of these TCRs. The affinity of the identified TCRαβ pair can be further increased by bacteriophage display mutation and selection technology (Li et al., 2005; Robbins et al., 2008), although it might reduce the specificities of the TCRs (Varela-rohena et al., 2008).

Tumor-specific TCRs can be cloned into a retroviral or lentiviral vector and transduced into patient T cells (Engels et al., 2003; Frecha et al., 2010). Extensive attempts were made to increase the expression efficacy of the therapeutic TCRs and to reduce the chance of mispairing of the therapeutic TCRs with patient endogenous TCRs. For instance, codon-optimization of the transferred TCR genes enhanced the protein expression level of the transferred TCRs;

Substitution of the human constant regions of the TCRs by murine constant regions (murinization) improved transferred TCR expression and pairing (Cohen et al., 2006). Further researches indicated that optimal expression of the therapeutic TCRs could be achieved by just replacing a few amino acids with murine sequences in the constant region (Sommermeyer and Uckert, 2010).

1.3.2.2 Transfer of CAR engineered T cells

CARs are constructs composed of antibody single-chain variable fragments (ScFv) fused to T cell signaling domains. As demonstrated in early studies (Gross et al., 1989; Kuwana et al., 1987), CAR engineering redirects the T cells to recognize cognate cell surface antigens without the restriction of antigen processing and MHC-dependent presentation (Lim and June, 2017; Hinrichs and Rosenberg, 2014). The therapeutic benefit of this property is to prevent tumor escape by MHC downregulation (Restifo et al., 1996). On the other hand, the application of CAR T cells is limited since they cannot target intracellular antigens.

The scFv domains of most available anti-human CARs originated from murine monoclonal antibodies. Occasionally, human antibody and T cell responses against CARs were reported (Lamers et al., 2011; Kershaw et al., 2006; Jensen et al., 2010). To minimize the problem, transgenic mice with humanized immunoglobulin loci might represent as a solution. The first-generation CARs contained a single signaling domain usually from CD3ζ or FcRγ, but it soon became clear that it could not provide sufficient signaling for the CARs T cells to survive *in vivo* upon repeated antigen exposure (Brocker, 2000; Till et al., 2013). Based on these observations, second-generation CARs were developed with an additional costimulatory signaling domain (CD28, CD137, etc.). The CARs equipped with dual-signaling domains showed superior cell viability and were better candidates for adoptive T cell therapies (Haynes et al., 2002; Textor et al., 2014; Savoldo et al., 2011).

The most successful engineered T cell therapy up-to-date are CD19-CAR trials for B cell malignancies. Complete remission was achieved in more than 90% of the patients with relapse or refractory acute lymphoblastic leukemia (Brentjens et al., 2013; Maude et al., 2014; Turtle et al., 2016), and in two out of three patients with chronic leukemia (Kalos et al., 2011). CD19 is expressed in all B cells, yet it turned out that the B cell aplasia induced by CD19 CAR treatment is tolerable given replacement antibody therapy (Lim and June, 2017). Still, it remains a question of how likely it is to replicate the success of CD19-CAR therapy to target widely-expressed antigens with affordable toxicities (Discussed further in “1.3.2.3 Tumor antigens”).

1.3.2.3 Tumor antigens

The adaptive immune system can distinguish malignant from normal tissues by a group of antigens called tumor antigens expressed on tumor cells. In 1991, Pierre van der Bruggen et al. identified the first tumor associated antigen named melanoma-associated antigen 1 (MAGE-A1) utilizing CTL clones from a melanoma patient (van der Bruggen et al., 1991). Approaches to identify tumor antigens include serological expression analysis (SEREX), where E.coli expressing cDNA libraries from tumor cells is constructed, and reactive clones are screened with autologous IgG antibodies from patient serum or with tumor specific CTLs (Sahin et al., 1995). In the latter case, additional assays are needed to pinpoint the epitopes (short peptides) which are naturally processed and presented by MHC molecules, and are the actual targets for αβ T cells. Often, the epitopes can be eluted directly from their bound MHC I or II molecules isolated from tumor cell surface. Although it requires further improvement for the quality and reproducibility of the outcome using such method. The sequences of the eluted peptides could be identified using HPLC-coupled mass-spectrometry (Castelli et al., 1995; Cox et al., 1994).

Tumor antigens can be grouped into “over-expressed”, “differentiated”, “cancer-testis” and “tumor-specific” antigens. The first three types of tumor antigens are tumor associated-antigens, which are also expressed in normal cells (Tran et al., 2017). As a consequence of central tolerance, it is foreseeable that the autologous T cells against such antigens are deleted or have low avidity.

Over-expressed antigens. Over-expressed antigens are widely expressed in normal tissues, but their protein expression levels are elevated in cancer cells (Bright et al., 2014). Considering their wide expression among normal tissues, this group of antigens could hardly be ideal targets for ATTs. As examples, MHC-restricted fratricide was reported on T cells transduced with high avidity TCRs against survivin (Leisegang et al., 2010), an antigen expressed in various cancers but also in several normal tissues such as hematopoietic cells (Fukuda and Pelus, 2006). A clinical trial using CAR T cells against human epidermal growth factor receptor 2 (HER2) protein caused recognition of normal cells, leading to death of a patient with metastatic colon cancer (Morgan et al., 2010). A CAR trial against carbonic anhydrase IX (G250) was coupled with treatment-induced liver toxicities in patients (Lamers et al., 2013, 2006).

Differentiation antigens. The expression of differentiation antigens are restricted to certain type of tissues. The toxicities evoked by T cells targeting this group of antigens are often considered acceptable. The first TCR gene therapy trial was administrated against MART-1 in metastatic melanoma, a differentiation antigen which is expressed only in melanocytes. Two out of 15 patients experienced objective response and no autoimmune toxicities were seen in any patients (Morgan et al., 2006). In contrary, subsequent MART-1 TCR trial with a higher affinity TCR caused transient toxicities in skin, eyes and ears of the patients (Johnson et al., 2009). Other differentiation

antigens that are already therapeutic targets include gp100 (Johnson et al., 2009), the oncofetal antigen carcinoembryonic antigen (CEA) (Parkhurst et al., 2011), B cell marker CD19 as mentioned earlier, etc.

Cancer-testis antigens. Besides the normal expression in testis and fetal ovaries, epigenetic changes in tumors can trigger the expression of cancer-testis antigens. Since they do not express MHC I molecules (Fiszer D, 1998; Hotta et al., 2000), T cells cannot recognize the normal male germline cells. Additionally, cancer/testis antigens are often expressed in various types of cancers. Hence, this group of antigens represents a relatively safe and ideal target for ATTs. Gene families MAGE, GAGE and BAGE proteins are examples of cancer/testis antigens. A conflicting example is the TCR gene therapy trial against a HLA-A2 restricted MAGE-A3 epitope. Out of the nine patients treated with anti-MAGE-A3 T cells, three experienced mental status changes and two died subsequently (Morgan et al., 2014). Further investigation using real-time quantitative PCR, nanostring and deep sequencing showed that MAGE-A12, which shared the same HLA-A2 epitope with MAGE-A3, was expressed in the brain. Two patients died due to cardiogenic shock in another MAGE-A3 trial using an affinity-enhanced TCR with HLA-A1-restriction, this time caused by off-target toxicity by T cell recognition of an unrelated peptide expressed by the muscle-specific protein titin (Linette et al., 2013). The outcome of these clinical trials raises the disastrous consequence of T cell therapies with the wrong targets and calls for careful assessments, both for the antigens and the TCRs, before clinical applications.

NY-ESO-1 is one of the cancer-testis antigen. It was discovered in 1990s with SEREX detected by patient antibodies (Chen et al., 1997). Shortly thereafter, an HLA-A2 restricted epitope of NY-ESO-1 was reported using a melanoma/NY-ESO-1 reactive CTL clone and epitopes predicted by computerized algorithms (Jäger et al., 1998). This epitope, termed NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC), is shared by LAGE-1, another cancer/testis antigen. Several other NY-ESO-1 epitopes, both MHC I and MHC II restricted, have been described (Benlalam et al., 2003; Jäger et al., 2000; Yamaguchi et al., 2004; Zeng et al., 2000; Zarour et al., 2000; Jäger et al., 2002). NY-ESO-1 is widely expressed in different type of cancers, i.e. it is expressed in 10% to 50% of metastatic melanomas, breast, prostate, thyroid, and ovarian cancers, approximately 80% of synovial cell sarcomas, and 100% of round cell liposarcoma (Chen et al., 1997; Barrow et al., 2006; Jungbluth et al., 2001; Gure et al., 2005). The function of NY-ESO-1 is largely unknown.

Currently, two clinical trials targeting NY-ESO-$1_{157\text{-}165}$ have been conducted employing the same affinity-enhanced TCR derived from TCR-1G4. The wildtype TCR-1G4 was generated from a melanoma patient's CTLs (Jäger et al., 1998), and a high affinity mutant with two amino acid substitutions in the CDR3α domain was generated by bacteriophage display and identified by RNA

transfection and assay techniques (Li et al., 2005; Robbins et al., 2008). In the first trial, five out of eleven patients with metastatic melanoma and four of six patients with synovial cell sarcoma showed objective tumor responses (Robbins et al., 2015, 2011). The other trial enrolled multiple myeloma patients, and 16 out of 20 patients showed tumor responses (Rapoport et al., 2015). Both trials reported no toxicities caused by the transgenic T cells, yet the actual antitumor effect of these NY-ESO-1 T cells still need to be carefully addressed, since the high dose IL-2 (Robbins et al., 2011) or autologous hematopoietic stem cell transplantation (HSCT) (Rapoport et al., 2015) which were co-administered with the T cell transfer have been reported to show durable tumor responses alone (Klapper et al., 2008; Atkins et al., 1999; Rosenberg et al., 2011; Porrata et al., 2001).

NY-ESO-1 expression in the human thymic medullary epithelial cells (mTECs) was detected by reverse transcriptional PCR (Gotter et al., 2004). Hence, central tolerance might prevent the isolation of high affinity TCRs against NY-ESO-1 from autologous source. *In vitro* maturation of the autologous TCRs as in the case of mutant TCR-1G4 could be a solution, but whether the mutated TCRs would maintain the specificities without off-target toxicities remains a question. Another option is to utilize transgenic mice with humanized T cell recognition system, as described in Section 1.4.

Tumor-specific antigens. Tumor specific antigens include viral antigens and antigens encoded by non-synonymous mutated genes. The latter generates neoepitopes which can be targeted by T cells. The expression of tumor-specific antigens arises during the tumorigenesis in a cancer cell-restricted manner and often are crucial for tumorigenesis (driver-mutations) (Schietinger et al., 2008). In theory, T cell therapies targeting this group of antigens are tumor-specific without causing autoimmune toxicities (Blankenstein et al., 2015). Furthermore, therapies targeting driver-mutations will likely eliminate the tumor (Anders et al., 2011). Tumor associated viral antigens include those derived from EBV and human papillomavirus (HPV) (Kenter et al., 2009; Riemer et al., 2010; Papadopoulos et al., 1994). Whole exome sequencing facilitates the identification of tumor mutations. Exomic sequencing with large cohort of cancer samples of different origins indicate the different mutation rates across different cancer types, i.e. melanomas and lung cancers often contained more than 100 mutations/Mb, while pediatric cancers showed mutation frequencies as low as 0.1/Mb (Lawrence et al., 2013). It often requires further steps to recognize the naturally processed epitope containing the mutations, which is usually done by computer algorithm prediction (Yadav et al., 2014; Robbins et al., 2013; Matsushita et al., 2012; van Rooij et al., 2013; Castle et al., 2012).

Despite the promises of targeting tumor-specific antigens as it was repeatedly shown in animal models (Schreiber et al., 2006) and indicated by the outcomes of checkpoint-inhibitor therapies (Rizvi et al., 2015; Allen et al., 2015; Snyder et al., 2014), the mutated antigens are rarely shared among individuals except for some hot-spot mutations such as mutations in KRAS. Personalized treatment represents one of the options for development of T cell therapies against tumor specific antigens (Rosenberg and Restifo, 2015; June et al., 2015).

1.4 Transgenic mice with humanized T cell recognition systems

Transgenic mice with human MHC transgenes are in use both for production of TCRs (Chinnasamy et al., 2011; Johnson et al., 2009) and identification of the epitopes (Kievits et al., 1987; Touloukian et al., 2000) of human tumor antigens. Although the TCRs that are generated with such approach often require further affinity maturation (Chinnasamy et al., 2011; Johnson et al., 2009). Human TCR transgenic mice were generated for the purpose of studying the immune tolerance mechanisms in humans, developing optimal affinity TCRs for immunotherapies, and addressing questions such as TCR-MHC coevolution, etc.

The human TCR transgenic mice were previously established in the lab. In brief, yeast cells with yeast artificial chromosomes (YACs) containing either the whole human TCRα (huTRA, ~1.4 Mb) or TCRβ (huTRB, ~0.7 Mb) gene locus were fused with 129Sv-derived embryonic stem cells to generate huTRA or huTRB transgenic mice. The single TRA/TRB transgenic mice were crossed to murine TCRα or β knocked-out mice, and subsequently crossed to each other. The T cells from the resulting mice expressed complete human αβ TCRs and lacked the expression of murine αβ TCRs. These transgenic mice were named AB*ab* mice. AB*ab* mice were then crossed to HHDII mice, which expressed a single chimeric molecule of human HLA-A*0201 linked to mouse H-2D^{b} α3 transmembrane domain and human β2m and were double knocked-out for murine H-2D^{b} and β2m (Pascolo et al., 1997), to generate AB*ab*DII mice (Li et al., 2010); or crossed to DR4 mice to generate AB*ab*DR4 mice. The DR4 mice contained a transgene which consisted of antigen-recognition regions from HLA-DRA and HLA-DRB1*0401 linked to the α2 and β2 domains from mouse I-E^{d}, and deficient in mouse MHC IIs (Ito et al., 1996) (Table 1).

1.4.1 Human TCR repertoire selected by human/mouse MHC II in studying TCR-MHC coevolution

Structural and mutational analysis of TCR-pMHC complexes depicts only few of the billions of different possible combinations. Therefore, we wished to address the problem from a different viewpoint, based on several assumptions. We reasoned that thymic selection is the most sensitive read-out to detect subtle differences in affinity between a defined MHC molecule and any given

TCR. Even though it is known that mouse TCRs can be selected on human MHC (Ito et al., 1996; Kievits et al., 1987) and human TCRs can be selected on mouse MHC, we assumed that mouse and human TCR and MHC gene loci further coevolved after their divergence ~75 million years ago (Mouse Genome Sequencing Consortium et al., 2002), resulting in changes in thymic selection of a polyclonal repertoire depending on whether TCR-pMHC interaction was inter- or intra-species specific.

Therefore, we employed AB*ab* mice with either a single human MHC II (HLA-DRB1*0401; HLA-DR4 hereafter) or a single mouse MHC II gene (I-A^b). TCR deep-sequencing of peripheral CD4 T cells from both mouse lines revealed distinct differences in their repertoire, compatible with coevolution of TCR and MHC (Chen et al., 2017).

1.4.2 AB*ab*DII mice for generation of optimal affinity human TCRs against cancer/testis antigen NY-ESO-1

One of the problems of using TCRs from human MHC transgenic mice in the clinic is the risk of developing host immune responses to the murine TCRs (Davis et al., 2010). As a result, it might compromise the persistence of the transferred T cells and the efficacy of such therapies. Moreover, the potential interspecies incompatibilities between murine TCRs and human MHCs might lower the chance of developing T cell responses in the transgenic mice. AB*ab* mice, on the other hand, have no such problem since the whole T cell recognition system is humanized.

As mentioned earlier, NY-ESO-1 is a tumor-associated antigen whose expression has been detected in human mTEC cells (Gotter et al., 2004). Central tolerance could delete high affinity CD8 T cell clones against NY-ESO-1 in humans. T cells modified with TCRs generated in AB*ab*DII mice could be a solution to circumvent the low avid human T cells and increase the therapeutic potencies (Linnemann et al., 2013; Obenaus et al., 2015).

Table 1: TCR and MHC genotypes of different mouse strains

Mouse line	Mouse TCRs	Human TCRs	Mouse MHC I	Human MHC I	Mouse MHC II	Human MHC II
AB*ab*DII	-	+	-	HLA-A2 chimeric	+	-
AB*ab*DR4	-	+	+	-	-	HLA-DR4 chimeric
HHDII	+	-	-	HLA-A2 chimeric	+	-
DR4	+	-	+	-	-	HLA-DR4 chimeric
AB*ab*	-	+	+	-	+	-

+: positive for the genotype; -: negative for the genotype

2.1 Part 1: Characterization of transgenic mice with a humanized T cell recognition system

2.1.1 Transgenic mice with a diverse human T cell antigen receptor repertoire

(Li LP, et al., *Nat. Med.* 16:1029–34. https://doi.org/10.1038/nm.2197)

Page 21- 54 are only available in the printed version

2.1.2 Human TCR-MHC coevolution after divergence from mice includes increased nontemplate-encoded CDR3 diversity

(Chen X, et al., *J. Exp. Med.* 214:3417–3433. https://doi.org/10.1084/jem.20161784)

Page 56-78 are only available in the printed version

2.2 Part 2: Identification of optimal affinity TCRs against cancer/testis antigen NY-ESO-1

2.2.1 High-throughput identification of antigen-specific TCRs by TCR gene capture (Linnemann C, et al., *Nat. Med.* 19:1534–1541. https://doi.org/10.1038/nm.3359)

2.2.2 Identification of human T-cell receptors with optimal affinity to cancer antigens using antigen-negative humanized mice (Obenaus M, et al., *Nat. Biotechnol.* 33:402–7. https://doi.org/10.1038/nbt.3147)

Page 111-136 are only available in the printed version

Discussion

The first part of the thesis characterized the transgenic mice with humanized T cell recognition system by analyzing the human TCR repertoire diversity selected on different MHCs and examining the functionalities of the T cells from the mice while encountering antigens. Also, we provided evidence supporting human TCR and human MHC II coevolution. The second part of the thesis focused on isolation of TCRs against NY-ESO-$1_{157-165}$/HLA-A*0201 from the AB*ab*DII mice. The murine derived human TCRs transduced T cells had higher affinity and functionality than TCR-1G4 which was isolated from the human tolerant system.

3.1 Part 1: Characterization of transgenic mice with humanized T cell recognition system

Similar to that in the human immunoglobulin transgenic mice (Lonberg et al., 1994; Lee et al., 2014; Green et al., 1994; Fishwild et al., 1996), the human T cell transgenic mice were capable of employing mouse rearrangement machineries to produce functional human TCRα/β chains. In addition, the transgenic T cell precursors developed normally in mouse thymus and became peripheral T cells. This required the pairing of the functional rearranged human TCRβ chains with murine pre-α chain to form pre-TCRs for β selection and the successful selection of T thymocytes by murine self-antigens presented by murine or human MHC molecules.

In the following, the human TCR repertoire selected on a single human MHC I or II will be discussed, and the evidence for TCR-MHC coevolution will be addressed mainly in MHC II settings.

3.1.1 Diverse and functional human TCR repertoire selected by a single HLA-A2 molecule in AB*ab*DII mice

Positive selection involves the contact of the germline-encoded CDR1 and 2 domains of the TCRs with self pMHCs. The inherent affinity between TCRs and MHCs has long been postulated (Marrack et al., 2008). Human HLA-A2 molecule could select a functional (Lonberg et al., 1994; Huang et al., 2013; Tan et al., 2017; Nakatsugawa et al., 2011; Ramage et al., 2004) and diverse (Firat et al., 2002) mouse TCR repertoire in HHD mice, confirming the existence of conserved amino acid interactions between the TCRs and the MHCs (Scott-Browne et al., 2011, 2009). But it was less efficient compared to the selection in AB*ab*DII mice, which was demonstrated by the elevated numbers of CD8 single positive thymocytes and peripheral CD8 T cells. This was most likely due to the inter-species incompatibility between TCRs and MHCs. Nevertheless, AB*ab*DII mice contained less peripheral CD8 T cells than AB*ab* mice, which implies that multiple MHC-Is (H-2^b and H-2^d molecules) in the AB*ab* mice compensated for the sub-optimal affinities between

human TCRs and murine MHCs. Additionally, the low HHD expression (Pascolo et al., 1997) might hinder the positive selection of T cells in AB*ab*DII mice.

A single MHC I molecule, HLA-A2, was able to select almost all human TCR α/β V and J segments. The peripheral T cell repertoire in AB*ab*DII mice was diverse, which was suggested by the TCR CDR3 length analysis of the CD3 or CD8 T cell pools.

A major concern about AB*ab*DII mice was whether TCRs with a complete human constant region could function normally in mouse T cells, since in transgenic mice with full human B cell receptor (BCR) chains, the B cells had reduced signaling by the BCRs, which limited the antibody class switching and affinity maturation (Lonberg et al., 1994; Green et al., 1994; Fishwild et al., 1996). In contrast, the human constant region was capable of producing sufficient downstream signaling for the mouse T cells, suggested by the fact that AB*ab*DII mice responded efficiently to HLA-A2 restricted antigen challenges, with the predicted IC_{50} ranging from 5 nM to more than 1000 nM. In particular, CD8 T cell responses against a HLA-A2 epitope of Mart-1 were analyzed and the specific TCRs were identified. The Mart-1 specific TCRs are rather similar, or even the same for one TCRα chain, to the ones isolated from autoimmune vitiligo or melanoma patients. It was unexpected, since Mart-1 was expressed in the thymus of humans. But later it was discovered that the respective Mart-1 epitope was secluded from the expression in the thymus (Pinto et al., 2014). The observation of closely resembled TCR usages for Mart-1 in the humans and the mice indicated that the TCR repertoire selected in AB*ab*DII mice might be functionally overlapping with the ones in humans. To sum up, AB*ab*DII mice were proven to be a valid tool to isolate suitable TCRs against human pathogens and cancer cells. They are also useful to study CD8 T cell responses in humans, such as immunohierarchy of human pathogenic antigens, public TCRs for EBVs, etc. (own unpublished observation).

3.1.2 Evidence supporting human TCR-MHC II coevolution in AB*ab*DR4 mice

The TCR-MHC coevolution hypothesis represents an interesting explanation for the TCR bias for MHCs. Controversial discussions were made regarding the hypothesis, with good arguments from both sides. Although most of the TCR-pMHC crystallographic tertiary structures or CDR1 and CDR2 mutations analysis suggest the existing of inherent affinities of TCRs to MHCs (Rossjohn et al., 2015; Marrack et al., 2008), they depict only few of the millions of TCR-pMHC interactions. Meanwhile, several other studies suggested that the MHC restriction on TCRs might be imposed during thymic selection (Beringer et al., 2015; Gras et al., 2010; Sethi et al., 2011; Tynan et al., 2005; Tikhonova et al., 2012; Van Laethem et al., 2007), and it is not always easy to distinguish what is the exception and what is the rule.

Hence, we took another approach to address the question. With the observation that HLA-A2 selected higher numbers of CD8 T cells in AB*ab*DII mice than in HHD mice, we proposed that murine TCRs might have sub-optimal affinity to human MHCs and vice versa. We assumed that the differences in the inherent affinity between TCR and MHC in mice and humans are subtle due to the relatively short evolutionary distance, and that thymic selection is the most sensitive read-out to detect such differences. Using AB*ab*DII and AB*ab*DR4 mice, which expressed a single murine or human MHC II, we investigated the different capacity of a single MHC to select T cells with any possible functionally rearranged TCRs using TCR deep-sequencing. By comparing the human TCR repertoires pre- and post-selected on a single human or mouse MHC II allele, we provided evidence for TCR-MHC coevolution.

Biased TCR usage by MHC II molecules

Consistent with previous reports (Robins et al., 2010; Rubelt et al., 2016), the V, (D), J usages and V-(D)-J combinatory events were not random to generate the pre-selection TCR repertoire. This feature was seen in both humans and mice. Thymic selection with a single MHC II further biased the repertoire, where different MHC IIs had distinct imprints for selecting TCRs. The preference for different TCR V genes was determined by specific amino acids on the MHCs (Sharon et al., 2016). The imprinted choice for TCRs was the most likely reason that there were higher numbers of shared TCRα/β clonotypes within than between the two mouse lines. Even more interesting is the fact that humans shared more TCR clonotypes with human MHC II-expressing mice (11% ± 0.3% TCRα and 1.3 ± 0.1% TCRβ chains) than with AB*ab*DII mice (9.6% ± 0.2% TCRα and 1.1 ± 0.2% TCRβ). It provided another evidence for the intra-species TCR-MHC coevolution. The abundance of shared TCRα or TCRβ single chains between different species and independent of the MHC II profile suggests that αβ chain combinatorial pairing plays a larger role for creating diversity than previously thought (Arstila et al., 1999).

Human HLA-DR4 selected a more diverse human TCR repertoire than murine I-A^b

The I-A^b molecule selected almost all human TCR V, (D), J genes and V-(D)-J combinations. The peripheral CD4 T cell repertoire in AB*ab*DII mice was diverse, suggesting that most of the inherent affinity between TCRs and MHCs existed long before human and mice diverged. It agreed with the studies showing that there were conserved germline-encoded features of some TCR V regions that promote recognition of MHCs (Scott-Browne et al., 2011; Feng et al., 2007; Scott-Browne et al., 2009; Maynard et al., 2005; Lee et al., 2000; Newell et al., 2011; Sim et al., 1996; Manning et al., 1998; Dai et al., 2008). However, compared to AB*ab*DR4 mice, AB*ab*DII had a reduced thymic

output and lower peripheral TCR repertoire diversity. In fact, AB*ab*DR4 generated 30% more TCRα/β clonotypes in CD4 T cells than AB*ab*DII mice. The increased efficiency in selecting the human TCR repertoire by HLA-DR4 molecule was likely due to the slightly higher inherent affinity between many of the human TCR V genes and the HLA-DR4 molecule, likely in the CDR1 and CDR2 regions (Marrack et al., 2008). To note, HLA-DR4 selected a less diverse murine than selection of human TCR repertoire, since the CD4 T cell immune response was lower in DR4 mice than in AB*ab*DR4 mice.

The increased TCR repertoire selected by HLA-DR4 is reflected in the increased CDR3β length

Besides of having a broader range of CDR3 length distribution, AB*ab*DR4 had in average one amino acid longer CDR3β in CD4 T cells than AB*ab*DII mice. Since AB*ab*DII and AB*ab*DR4 had a similar CDR3 length distribution in the pre-selection TCR repertoire, the longer CDR3βs could only be because of the thymic selection with HLA-DR4 molecule. We know that wildtype C57BL/6, which have species-compatible TCRs and MHCs, produced the same average CDR3β length as AB*ab*DR4 mice. Therefore, the shorter CDR3β length seen in AB*ab*DII TCRs is probably due to the inter-species incompatibility between human TCRs and mouse I-A^b. The species-specific TCRs and MHCs evolved to have optimal intrinsic affinity to each other, whereas the sub-optimal TCR-MHC intrinsic affinity between mouse and human assumedly led to the positive selection of TCRs with shorter CDR3 domains (Gilfillan et al., 1995; Marten et al., 1999; Yassai et al., 2002). Since there are two recombination events in the CDR3 domains of TCRβ chains, namely, V-D and D-J rearrangement, the effect became more prominent in the CDR3βs. TCRs with shorter CDR3s have a higher risk of cross-reactivity, while longer CDR3s endow a higher specificity to the TCRs (Gavin and Bevan, 1995; Huseby et al., 2008). Accordingly, a significantly higher proportion of CD4 T cells in AB*ab*DII mice developed into Treg cells (19.9 ± 4.4%), compared to AB*ab*DR4 mice (10.8 ± 4.0%). It was interpreted that the TCRs with shorter CDR3 and having higher risk of being cross-reactive in AB*ab*DII mice developed into Treg cells. The CD4 TCRs with average shorter CDR3β selected in AB*ab*DII mice again proved the existence of inherent affinity between TCRs and MHCs within species and TCR-MHC co-evolved.

Mice generate shorter TCRβ CDR3 regions than humans. It was reflected in the pre-selection T cell pool, supposedly because of the recombination machinery involved in generating the CDR3 regions. This includes the combined effect of exonuclease and terminal deoxynucleotidyl transferase (TdT). Notably, both excising and adding of nucleotides from the enzymes increase the diversity of the TCR repertoire, which would explain the 20-fold higher TCR diversity in humans

compared to mice (Arstila et al., 1999; Casrouge et al., 2000; Nikolich-Zugich et al., 2004; Vrisekoop et al., 2014).

A group of human TCR V genes might have sub-optimal affinity to I-A^b

All the evidence stated above indicated that there might be a sub-group of human TCR V genes that have sub-optimal CDR1 and CDR2 domains for contacting mouse I-A^b molecule. We identified these human TCR V genes by comparing the individual V gene usage frequency before and after thymic selection. The pre-selection pool was approximated by out-of-frame TCRs. The "under-represented" TCR V genes, which were less frequent in the post-selection TCR pool, had in average the shortest CDR3 length in AB*ab*DII mice. In contrast, the "under-represented" V genes in AB*ab*DR4 mice did not have such feature. In fact, they had the longest CDR3 lengths among all groups. Hence, we concluded that the "under-represented" TCR V genes are most likely to have included those V genes that had sub-optimal affinities to I-A^b.

Previous works have suggested that each TCR contained multiple germline-encoded features that maximize their chance of being positively selected by different MHC molecules (Garcia et al., 2009; Marrack et al., 2008). In our study, we propose that in addition to the inherent recognition motifs for MHC, the affinities between TCRs and MHCs can be adjusted by different CDR3 lengths to ensure that TCRs with basically all V genes can be positively selected, which is supported by the fact that AB*ab*DII mice generated CD4 T cells with a diverse human TCR repertoire.

3.2 Part 2: Isolation of optimal affinity TCRs for cancer/testis antigen NY-ESO-1$_{157-165}$ in AB*ab*DII mice

NY-ESO-1 is expressed in various types of cancers. In some cases, for instance round cell liposarcoma, it is expressed in 100% of the cancer cells. In contrary, the expression of NY-ESO-1 in normal tissues is restricted to testis and placenta. Therefore, NY-ESO-1 belongs to a group of cancer antigens termed "cancer-testis antigens" (see section 1.3.2.3 for detail). Although it remains unclear whether humans are tolerant to cancer-testis antigens, evidence from different studies favors its existence. For example, the expression of several cancer-testis antigens, including NY-ESO-1, were detected at cDNA level in human mTECs (Gotter et al., 2004). In another study, Huijbers and his colleagues described the central tolerance in mice against a mouse cancer-testis antigen P1A. P1A-knockout mice, which were generated in this study, contained higher reactive T cells against a P1A epitope than in wildtype mice (Huijbers et al., 2012).

Due to its shared expression among cancer cells and its restricted expression in normal tissues, NY-ESO-1 is claimed to be a good target for cancer therapies. Two clinical trials were conducted against the HLA-A*0201 restricted NY-ESO-$1_{157\text{-}165}$ employing an *in vitro* matured TCR isolated from a melanoma patient called TCR-1G4. This TCR has mutations on the CDR3α of TCR-1G4 (Jäger et al., 1998), where leucine and tyrosine substituted the original threonine and serine at position 95 and 96 of TCRα chain (Robbins et al., 2008). In both clinical trials, anti-tumor responses but no toxicities were induced by the T cell treatment (Rapoport et al., 2015; Robbins et al., 2011), suggesting that NY-ESO-1 could be a safe target for T cell therapy for cancer. To note, the specificity of the mutant TCR-1G4 and its efficiency in cancer treatment is still unclear and yet to be addressed.

ABabDII mice responded robustly to NY-ESO-$1_{157\text{-}165}$

Human MHC I transgenic mice are widely used to understand the immune responses to human viral and cancer antigens in humans (Chinnasamy et al., 2011; Johnson et al., 2009; Kievits et al., 1987; Touloukian et al., 2000; Alexander et al., 2003; Vitiello et al., 1991). Concerns for such approaches come from two aspects: 1) the antigen processing and presentation machineries may differ between humans and mice (Kotturi et al., 2009). Mainly, the murine transporter associated protein (TAP) is biased strongly to peptides with hydrophobic C-termini, while human TAP is more permissive to peptides with both hydrophobic and basic termini (Burgevin et al., 2008; Huang et al., 2016). Two endoplasmic reticulum aminopeptidases (ERAPs) are involved in peptide trimming in humans while there is only one in mice (Saveanu et al., 2005). Besides, there might be subtle differences in immunoproteasome and tapasin activities between humans and mice (Sesma et al., 2003). 2) The murine TCRs and human MHCs might be incompatible for each other and limit the immune responses in the transgenic mice (See Section 3.1).

NY-ESO-$1_{157\text{-}165}$ contains a cysteine at the 9th position, which might be the anchor position for HLA-A*0201 binding. It is predicted to be a bad binder to HLA-A*0201 (IC50: 1015 nM), yet the immunization with NY-ESO-$1_{157\text{-}165}$ peptide evoked efficient CD8 T cell responses in AB*ab*DII mice. Interestingly, *ABabDII* mice immunized with NY-ESO-1 full antigen responded robustly against NY-ESO-$1_{157\text{-}165}$, demonstrating that AB*ab*DII mice processed and presented NY-ESO-1 similarly as in humans. Moreover, it suggests that mouse CD4 T cells, which were restricted on murine I-A^b molecule presented epitopes, could provide sufficient signaling for priming of human HLA-A2 restricted NY-ESO-1 specific CD8 T cells. Hence, AB*ab*DII mice are proven to be useful tools to study the CD8 T cell responses to NY-ESO-1. More than 85% of the AB*ab*DII mice responded to the naturally processed NY-ESO-$1_{157\text{-}165}$ epitope, indicating that NY-ESO-$1_{157\text{-}165}$ is efficiently

processed and presented in mice, and probably also in human cancer cells. T cell therapies targeting this epitope are promising due to its abundancy in cancers.

ABabDII mice generated high-affinity TCRs against NY-ESO-1$_{157-165}$

An important facet in the success of ATTs using TCR-engineered T cells is to obtain optimal affinity TCRs. Wildtype TCR affinities are in the range of 1-100 μM (K_D) (Davis et al., 1998). *In vitro* mutagenesis can generate TCRs exceeding this range, but it might not improve T cell avidities, since TCR affinity and T cell functionality do not correlate linearly, but as a bell-shape curve (Schmid et al., 2010; Kalergis et al., 2001). Additionally, *in vitro* affinity matured TCRs might lose their specificity (Linette et al., 2013). HLA transgenic mice are often human antigen negative, the additional human TCR transgenes ensure optimal affinities between the TCRs and the human MHCs. Hence the mice are good sources to generate effective TCRs against human antigens.

Using a high-throughput DNA-based strategy, we were able to identify TCRs specific for NY-ESO-1$_{157-165}$ in the AB*ab*DII mice that were immunized and responded to NY-ESO-1. TCR-ESO, which is one of the TCRs isolated, showed higher affinity and functionality compared to the melanoma patient-derived TCR1G4. Our results demonstrated that optimal affinity TCRs against human cancer antigens can be isolated from AB*ab*DII mice. High affinity NY-ESO-1$_{157-165}$ specific TCRs are most likely clonally deleted in humans due to central tolerance, similar to the case for MAGE-A1 (Obenaus et al., 2015).

3.3 Final remarks

AB*ab*DII and AB*ab*DR4 mice were generated for the purpose of producing optimal affinity TCRs against human pathogens and cancers, and increasing the efficacy of adoptive T cell therapy. We showed in both MHC I and MHC II settings, that the human TCR transgenes increased the thymic output of the human MHC transgenic mice and the human TCR repertoire restricted on human MHC molecules responded better to antigen stimulation. With the human TCR gene loci transgenic mice, we were able to investigate the interactions between TCRs and MHCs. Our findings suggest, mostly in the MHC II setting, that the intrinsic affinities between TCRs and MHCs developed long before the divergence of humans and mice. Afterwards, human TCRs and MHCs co-evolved further to maintain optimal germline affinity for each other. We identified a sub-group of human TCR V genes that have sub-optimal affinities to mouse I-A^b molecule, and they adopted shorter CDR3βs in order to be positively selected. This group of V genes might be different for other MHCs, since each MHC has its own preferred and non-preferred V genes (Sharon et al., 2016).

Our data supports the TCR-MHC coevolution hypothesis from a new aspect, and gives insights into how TCRs adjust their CDR3 lengths to cope with the inherent affinity difference to MHCs, in order to be positively selected. The TCR-MHC interspecies incompatibility between humans and mice represents a potential obstacle, since human MHC transgenic mice with mouse TCR repertoire are widely used in pre-clinical studies. The TCR repertoire in these mice might be limited and the TCRs might be sub-optimal to the human antigens that are presented on human MHCs. Mice with a complete humanized T cell recognition system can overcome such problems. Hence, they are proper tools to study human T cell responses and to isolate therapeutic human TCRs.

In our study, we compared the human TCR repertoires restricted on HLA-DR4 or I-A^b molecule. In order to generalize our finding, further studies are required that include more human and mice MHCs, especially with those which are homologues to each other.

Following the characterization of AB*ab*DII *and AB*ab*DR4 mice, we utilized the AB*ab*DII mice to study CD8 T cell immune response against an HLA-A*0201 epitope, NY-ESO-$1_{157\text{-}165}$, of the cancer antigen NY-ESO-1. AB*ab*DII mice naturally processed and presented NY-ESO-$1_{157\text{-}165}$, and generated robust CD8 responses against this epitope. The TCRs isolated from the ABabDII mice showed higher affinity and functionality than the one isolated from human, suggesting that humans are tolerant to NY-ESO-1.

Due to the cysteine at the anchor position of NY-ESO-$1_{157\text{-}165}$, computer algorithms could not properly predict the IC50 of its binding to MHC. Hence, it remains an open question, and further experiments await for evaluation of the epitope as clinical target for cancer treatment. In the future, the specificities of the isolated NY-ESO-$1_{157\text{-}165}$ TCRs need to be addressed. It is also our particular interest to compare our TCRs to the therapeutic mutant TCR-1G4, both in vitro and in a mouse tumor model.

Abbreviation

ATT	Adoptive T cell therapy
BCR	B cell receptor
CARs	Chimeric antigen receptors
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CML	Chronic myelogenous leukemia
cTECs	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T-lymphocytes antigen 4
CTLs	Cytotoxic T lymphocytes
D gene	Diversity gene
DLI	Donor leukocyte infusion
DP T cells	Double positive T cells
EBV	Epstein-Barr virus
ERAPs	endoplasmic reticulum aminopeptidases
Glu	Glutamic acid
GvHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HER2	Human epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
HPV	Human papillomavirus
HSCT	Hematopoietic stem cell transplantation
IgG	Immunoglobulin G
IL-2	Interleukin 2
J gene	Joining gene
MHCs	Major histocompatibility complexes
mTECs	Medullary epithelial cells
PD-1	Programmed death 1 receptor
pMHCs	Peptide-MHCs
PTLD	Post-transplantation lymphoproliferative disorders
SEREX	Serological expression analysis
SP T cells	Single positive T cells
TAP	Transporter associated protein

T_{CM}	Central memory T cell
Tcon	Conventional T cells
TCRs	T cell receptors
TdT	Terminal deoxynucleotidyl transferase
T_{EM}	Effector memory T cell
Th cells	T helper cells
TILs	Infiltrated T lymphocytes
T_N	Naïve T cell
Treg	Regulatory T cells
T_{SCM}	T memory stem cell
Tyr	Tyrosine
V gene	Variable gene
YACs	Yeast artificial chromosomes

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Contribution to the publications

1. Li, L., et al., 2010, *Nat. Med.*: **Figures 2d, 3d** and **4**, **Supplementary Figure 10** and **Supplementary Table 1**. Designed, performed the experiments, analyzed and interpreted the data.
2. Chen, X., et al., 2017, *J. Exp. Med.*: **Figure 1-8**, **Table 1**, **supplementary Figure 1-5.** Developed the concept, performed experiments, analyzed the data, and wrote the manuscript.
3. Linnemann, C., et al., 2013, *Nat. Med*.: **Figure 3a**. Designed, performed, analyzed and interpreted experiments in AB*ab*DII mice.
4. Obenaus, M., et al., 2015, *Nat. Biotechnol.*: **Figure 4**. Planned and performed experiments, and analyzed data.

Publications and patent

Publications

Chen, X., L. Poncette, and T. Blankenstein. 2017. Human TCR-MHC coevolution after divergence from mice includes increased nontemplate-encoded CDR3 diversity. J. Exp. Med. 214:3417–3433. doi:10.1084/jem.20161784.

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Patent

EP 16 712 262.1, title "Combined T cell receptor gene therapy of cancer against MHC I and MHC II-restricted epitopes of the tumor antigen NY-ESO-1".

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Berlin, den 17.11.2017

Xiaojing Chen

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Introduction Figure 2: 4237741144621