Comparison of NY-ESO-specific MHC class II-restricted T cell receptors from antigen-negative and -positive hosts

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LUCIA PONCETTE

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1. Gutachter: Prof. Dr. Thomas Blankenstein

2. Gutachter: Prof. Dr. Gerald Willimsky

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1 Summary

Adoptive transfer of T cell receptor (TCR)-engineered T cells is a promising approach in cancer therapy but needs improvement for effective treatment of solid tumours. So far clinical approaches have focussed on CD8 T cells because of their cytotoxic function. However, the importance of CD4 T cells to induce tumour regression by giving essential help to CD8 T cells or by their own means has become apparent and suggests the use of CD4 T cells in adoptive T cell therapy. Regarding the development of TCRs for use in CD4 T cells, it is unclear, whether the human CD4 T cell repertoire against shared (self) tumour antigens has been shaped by tolerance mechanisms and lacks highly functional TCRs suitable for therapy. The aim of this study was to generate optimal-affinity major histocompatibility class II (MHC II)-restricted TCRs against the tumour-associated antigen NY-ESO and investigate whether such TCRs can be generated from the human TCR repertoire. TCRs were isolated from CD4 T cells of a human donor representing an antigen-positive host as well as from transgenic mice that express a diverse human TCR repertoire with HLA-DRA/DRB1*0401 restriction and are NY-ESO-negative, thus serving as antigen-negative and therefore non-tolerant source for TCRs. NY-ESO-reactive TCRs from the mice showed superior recognition of tumour cells and higher peptide sensitivity compared to TCRs from humans. We identified a candidate TCR, TCR-3598_2, which, transduced in CD4 T cells, caused tumour regression in combination with NY-ESO-redirected CD8 T cells in a mouse model of adoptive T cell therapy. A clinical version of TCR-3598_2 was tested for functionality in preparation of a clinical trial. These data suggest that MHC II-restricted TCRs against NY-ESO from humanized non-tolerant mice are of optimal affinity unlike human-derived TCRs and that the combined use of MHC I- and II-restricted TCRs against NY-ESO can make adoptive T cell therapy more effective.

2 Zusammenfassung

Der adoptive Transfer von T-Zell-Rezeptor (TZR)-veränderten T-Zellen ist ein vielversprechender Ansatz in der Krebstherapie, muss jedoch für eine effektive Behandlung von soliden Tumoren verbessert werden. Bisher haben sich klinische Ansätze aufgrund der zytotoxischen Funktion auf CD8-T-Zellen konzentriert. Die Bedeutung von CD4-T-Zellen für die Tumorabstoßung durch T-Zellhilfe für CD8-T-Zellen sowie durch eigene Zerstörungsmechanismen ist jedoch deutlich geworden und legt den Einsatz von CD4-T-Zellen in der adoptiven T-Zelltherapie nahe. Hinsichtlich der Entwicklung von therapeutischen TZRs für CD4-T-Zellen ist unklar, ob das menschliche CD4-T-Zellrepertoire gegen tumorassoziierte (Selbst-) Antigene durch Toleranzmechanismen geprägt ist und keine hochfunktionellen Therapie-geeigneten TZRs aufweist. Ziel dieser Studie war, Haupthistokompatibilitätskomplex (MHC) II-restringierte TZRs mit optimaler Affinität gegen das tumorassoziierte Antigen NY-ESO zu generieren und zu untersuchen, ob solche TZRs aus dem humanen TZR-Repertoire generiert werden können. TZRs wurden aus humanen CD4-T-Zellkulturen sowie aus transgenen Mäusen isoliert, die ein diverses humanes TZR-Repertoire mit HLA-DRA/DRB1*0401 Restriktion exprimieren und NY-ESO-negativ sind und somit als Quelle für nicht-tolerante TZRs dienen. NY-ESO-reaktive TZRs aus dem Mausmodell zeigten eine bessere Erkennung von Tumorzellen und eine höhere Peptidempfindlichkeit als TZRs aus humanen CD4-T-Zellkulturen. Wir identifizierten einen TZR-Kandidaten, TZR-3598_2, der, transduziert in CD4-T-Zellen, in Kombination mit NY-ESO-spezifischen CD8-T-Zellen in einem Mausmodell der adoptiven T-Zelltherapie eine Tumorregression verursachte. Eine klinische Version des TZR-3598_2 wurde in Vorbereitung einer klinischen Studie auf Funktionalität getestet. Die Daten dieser Arbeit legen nahe, dass MHC II-restriktive TZRs gegen NY-ESO von humanisierten nicht-toleranten Mäusen eine optimale Affinität im Gegensatz zu humanen TZRs aufweisen und dass der kombinierte Einsatz von MHC I- und II-restringierten TZRs gegen NY-ESO die adoptive T-Zelltherapie effektiver machen kann.

3 Introduction

3.1 T cells and the immune system

The hallmark of the immune system is to distinguish between self and non-self. Through a complex interplay of effector cells and molecules, the immune system protects the host from external threats like pathogens. The vertebrate immune system can be divided into an innate and an adaptive immune system. Effector cells and molecules of the innate immune system rely on pattern recognition receptors, that are germ-line encoded and enable a rapid response to invading pathogens (Brubaker et al., 2015). In contrast, recognition by the adaptive immune system is mediated by antigen-receptors that are highly diverse through somatic recombination and recognize almost any antigen in a specific manner. T cells and B cells make up the effector cells of the adaptive immune system. Upon recognition of a pathogen, specific T cells and B cells expand, and an immune response develops in the course of several days to clear the infection.

There are different subpopulations of T cells, mainly CD4 and CD8 T cells, which are determined by their CD4 or CD8 co-receptor expression. CD8 T cells, also called cytotoxic lymphocytes, kill infected host cells and are therefore important for protection against viruses. CD4 T cells are also called T helper (Th) cells, because they act primarily on other immune cells. They help CD8 T cell and B cell immune responses to develop and are thus important for protection against most pathogens.

3.1.1 The T cell receptor

The antigen receptor of T cells is the T cell receptor (TCR), which was discovered more than 30 years ago (Hedrick et al., 1984; Yanagi et al., 1984). Each T cell expresses a unique TCR on its cell surface, which binds antigenic peptides presented on major histocompatibility (MHC) molecules on host cells. The first crystal structures of a TCR in complex with peptide-MHC showed a diagonal binding orientation of both molecules (Garboczi et al., 1996; Garcia et al., 1996). The TCR is composed of a heterodimer of two polypeptides, which in most T cells consists of an α and β chain. A minor subset of T cells expresses TCRs composed of γ and δ chains (Chien et al., 2014). γδ T cells are rather associated with the innate immune system and will not be discussed here. The polypeptide α and β chains consist of a variable region and a

constant region. The constant regions contain cysteine residues to form an interchain disulphide bond and are anchored in the membrane. As the TCR has no intracellular signalling domain, it is found in a TCR complex together with CD3 and ζ chain, which recruit signalling proteins upon TCR engagement (Call et al., 2002).

The variable regions of the TCR α and β chains are assembled during T cell development in the thymus by TCR gene rearrangement (Samelson et al., 1985). The variable region of the α chain is rearranged from many different variable (V) and joining (J) gene segments, the variable region of the β chain has a diversity (D) gene segment in addition. V, (D) and J segments are organized in the TCRα and β loci on chromosomes 14 and 7 in humans, respectively (Jones et al., 1985; Rowen et al., 1996). In addition to the combinatorial diversity by assembly of V, (D) and J genes, junctional diversity is created by addition or deletion of nucleotides at the junction when the gene segments are joined (Komori et al., 1993). This highly diverse region at the junction of V-J or V-D-J gene segments is called the complementarity determining region 3 (CDR3) and forms the centre of the antigen-binding site of the TCR (Davis and Bjorkman, 1988). Recombination-activating genes 1 and 2 (RAG-1 and RAG-2) are essential components for V(D)J recombination to take place. If one of them is not functional, T cells as well as B cells cannot develop due to the inability to properly rearrange antigen receptors and the host is devoid of lymphocytes (Mombaerts et al., 1992; Shinkai et al., 1992).

3.1.2 MHC molecules

MHC I and II genes are organized in the MHC locus together with many other genes important for antigen processing and presentation (Beck et al., 1999). There are three MHC I and three MHC II molecules encoded, HLA-A, B and C, and HLA-DR, DP and DQ, respectively. MHC I and II genes are highly polymorphic (Robinson et al., 2015). Each MHC molecule can bind a certain set of peptides that have a similar binding pattern by sharing similar anchor residues (Falk et al., 1991). In addition to the polymorphic nature of the MHC molecules, they are co-dominantly expressed. Therefore, each individual can present a large but different set of peptides (Apanius et al., 1997).

MHC I molecules consist of one α chain with three domains, α1, α2, and α3, and a smaller non-polymorphic chain, the β_2-microglobulin (β_2m) (Bjorkman et al., 1987). MHC II molecules consist of one α chain and one β chain, each forming two domains, α1 and α2 and β1 and β2, respectively (Brown et al., 1993). In MHC I molecules the peptide-binding cleft is closed and

allows binding of peptides in the range of 8-11 amino acids. In contrast, the peptide-binding cleft in MHC II molecules is open and therefore peptide length is not constrained. Nested sets of peptides sharing a core region with different peptide lengths to the sides are found when eluted from MHC II (Rammensee, 1995). Although one TCR can recognize peptides on MHC II of different lengths, changes in the peptide flanking residues can alter TCR affinity (Cole et al., 2012).

MHC I molecules are loaded with peptides from mostly cytosolic and nuclear proteins that are degraded by the proteasome and transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) (Neefjes et al., 2011). Newly synthesized MHC II molecules are bound to the invariant chain, which occupies the peptide-binding site of the MHC II molecule and targets the complex to late endosomal-lysosomal antigen-processing compartments. The invariant chain is cleaved by acidic proteases leaving a short fragment in the antigen-binding site, class II-associated invariant chain peptide (CLIP), which is exchanged for other peptides catalysed by the molecule HLA-DM (Neefjes et al., 2011). Although most peptides loaded on MHC II originate from proteins entering the antigen-binding compartment via the endocytic pathway, cytosolic or nuclear proteins can enter the antigen-binding compartment via macroautophagy (Schmid et al., 2007).

3.1.3 T cell selection in the thymus

T cell precursors migrate from the bone marrow to the thymus, where the so called thymocytes develop into mature T cells. By passing through a sequence of stages, the thymocytes rearrange their T cell receptor genes and undergo positive and negative selection to ensure restriction to the individual's MHC molecules but prevent auto-reactivity. According to the affinity model, the strength of the interaction between the TCR and self-peptide-MHC complexes determines the fate of the thymocytes (Klein et al., 2014). For positive selection, the TCR must interact weakly with self-peptide MHC complexes to be protected from death by neglect. Is the interaction too strong, negative selection induces thymocytes to go into apoptosis (Klein et al., 2014). However, CD4 T cells with TCRs of too high affinity can differentiate into T regulatory cells (Tregs) instead of being negatively selected (Hsieh et al., 2012). Tregs specifically inhibit immune responses and thereby play a major role in maintaining self-tolerance.

To enable effective negative selection of auto-reactive T cells, most proteins of the body are expressed by medullary thymic epithelial cells and presented to thymocytes (Gotter et al., 2004).

The transcription factor autoimmune regulator (Aire) is responsible to turn on peripheral genes in epithelial thymic cells and its dysfunction causes autoimmune manifestations in many organs of the body (Anderson et al., 2002; Nagamine et al., 1997). More recently, the transcription factor Fezf2 was found to turn on tissue-restricted antigens, which are not regulated by Aire (Takaba et al., 2015). Deletion of auto-reactive T cells, however, is not always complete (Su et al., 2013; Yu et al., 2015). CD8 T cells recognizing the Y chromosome-encoded H-Y antigen were found in males, but fewer numbers as compared to females (Yu et al., 2015). In addition to central tolerance created in the thymus by deleting auto-reactive thymocytes, peripheral tolerance can be induced when auto-reactive T cells have escaped negative selection in the thymus. Clonal anergy or deletion occurs when TCR engagement takes place in the absence of inflammatory signals (Mueller, 2010).

3.2 Immune responses to cancer

In a series of tumour transplantation experiments, it was shown that immunization with methylcholanthrene (MCA)-induced fibrosarcomas among inbred mice could protect from subsequent transplantation challenge using the same tumour (Prehn and Main, 1957). Immunization with normal tissues did not confer protection upon tumour challenge and mice immunized with tumour transplant did not reject skin-grafts. Therefore, anti-tumour immunity was elicited by unique rejection antigens of the tumour (Prehn and Main, 1957).

3.2.1 Immunosurveillance

While it became clear that T cells can recognize cancer cells, it is an ongoing debate, whether immune responses to naturally occurring tumours, as opposed to transplanted tumours, can be destructive (Blankenstein, 2007; Vesely and Schreiber, 2013). Immunosurveillance against virus-induced cancers can be effective as demonstrated by the elevated risk of virus-induced cancers in immunocompromised individuals following organ transplantation (Engels et al., 2011). However, clear evidence for immunosurveillance of spontaneously occurring tumours is lacking. Although immune responses are often detected in cancer patients, by the time a tumour becomes clinically evident immune responses have obviously failed to prevent its outgrowth. Whether the immune system selects for less immunogenic tumour variants is unclear. Shedding light on this question, in a mouse model of sporadic cancer expressing SV40 T antigen as rejection antigen, it was

shown that tumours do not escape under immunological pressure but instead induce a state of profound tolerance (Willimsky and Blankenstein, 2005). Although strong immune responses were induced during cancer development, they were non-functional. In contrast, immunized mice remained tumour-free throughout their life, showing that in principle T cells are capable of rejecting the tumours but not when induced in the course of tumour development (Willimsky and Blankenstein, 2005). Unresponsive T cells with specificity for tumour antigens were also found in patients explaining the lack of benefit of endogenous immune responses (Lee et al., 1999).

3.2.2 Checkpoint blockade

In the clinical setting, attempts are made to break T cell tolerance induced by the tumour and make use of the endogenous anti-tumour immune response. Unresponsive T cells upregulate inhibitory receptors like PD-1 and CTLA-4, which can be blocked to unleash tumour-reactive T cells (Hirano et al., 2005; Leach et al., 1996). However, only a fraction of patients respond to immune checkpoint blockade, and responses are often limited in time (Schachter et al., 2017). T cells recognizing self-antigens of the tumour are subject to central tolerance and are therefore of low avidity (Aleksic et al., 2012). T cells recognizing mutant neo-antigens of the tumour can be of high avidity and are thought to mediate the effect of checkpoint blockade (Gubin et al., 2014; McGranahan et al., 2016). However, peripheral tolerance induced by the tumour may be irreversible and reduce the number of available tumour-reactive T cells (Philip et al., 2017). Thus, one of the reasons for patients not responding to checkpoint blockade may be the lack of available tumour-reactive T cells of high avidity.

3.2.3 Tumour antigens

Tumour antigens are any molecules on cancer cells that can be recognized by T cells (Schreiber, 2013). Tumour antigens can be divided into two main classes, self-antigens and tumour-specific antigens. Self-antigens are non-mutated and encoded in the genomes of all cells but show higher or predominant expression in cancer cells (Offringa, 2009). Because self-antigens are not cancer-specific, they are also referred to as tumour-associated antigens. One subgroup are overexpressed proteins such as HER2/neu or Wilms tumour protein 1, which are expressed on normal tissues but to a much higher extent in cancer cells (Call et al., 1990; Coussens et al., 1985). Furthermore, differentiation antigens are expressed in specific cell lineages and consequently in tumours originating from these tissues. Examples are CD19 on B cells or several melanocyte-specific

differentiation antigens, such as gp100 and MART-1 (Bakker et al., 1994; Kawakami et al., 1994). Cancer-testis antigens are self-antigens that are highly expressed in spermatocytes in the testis and can be reactivated in cancer cells (Scanlan et al., 2002). Following the identification of the first member of this group, MAGE-A1, many more were discovered including BAGE, GAGE, NY-ESO and BORIS (Boel et al., 1995; Chen et al., 1997; Loukinov et al., 2002; Van den Eynde et al., 1995; van der Bruggen et al., 1991). A great advantage of tumour-associated antigens as immunotherapy targets is that they are often shared between tumours. However, expression on normal tissues carries the risk of on-target toxicities (Johnson et al., 2009). In contrast, tumour-specific antigens are derived from mutations in the tumour and are usually truly tumour-specific. Although mutations that give a growth advantage to the tumour (cancer-driver mutations) can be recurrently found, they are often individual-specific and thus more difficult to target than shared tumour-associated antigens (Vogelstein et al., 2013). Because of the advances made in high-throughput sequencing, tumour-specific mutations as immunotherapy targets have gained more attention in recent years (Blankenstein et al., 2015). Moreover, proteins from oncogenic viruses expressed in cancer cells are tumour-specific antigens and can serve as good targets for immunotherapy (Tashiro and Brenner, 2017).

3.3 Adoptive T cell therapy

3.3.1 Unmodified T cells

First evidence for the therapeutic effect of adoptive T cell therapy (ATT) came from allogeneic stem cell transplantation for the treatment of chronic myeloid leukaemia (Odom et al., 1978). The graft-versus-leukaemia effect, the removal of residual leukemic cells, is attributed to T cells within the donor graft, as T cell depletion in the transplant caused increased risk of relapse (Horowitz et al., 1990). Furthermore, transplants from identical twins increased the risk for relapse leading to the assumption that T cells recognize minor histocompatibility antigens presented on the leukemic cells (Goulmy, 2004; Horowitz et al., 1990). Unfortunately, the clinical benefit of allogeneic stem cell transplantation is limited by graft-versus-host disease, which is often associated with the graft-versus-leukaemia effect.

Furthermore, ATT was applied to treat viral infections, which are common in immunocompromised patients following stem cell or organ transplantation. In vitro expanded CD8 T cells specific for cytomegalovirus or Epstein-Barr virus (EBV) could prevent virus

reactivation and the EBV-associated lymphoproliferative disease (Heslop et al., 1996; Riddell et al., 1992). Because the generation of virus-specific T cell lines for each individual patient is laborious and not possible if the donor lacks viral immunity, banks of multi-virus-specific T cell lines have been generated for common HLA antigens that can be given to partially MHC-matched patients (Leen et al., 2013; Tzannou et al., 2017).

Efforts have been made to implement ATT for treating non-virus induced cancers. To mobilise endogenous tumour-reactive T cells that are muted by tolerance, tumour infiltrating lymphocytes (TILs) can be expanded ex vivo and infused back into the patient (Dudley et al., 2005). This approach has been widely applied in metastatic melanoma and achieves responses in approximately half of the patients with some complete regressions (Dudley et al., 2005). TILs from complete responders were shown to contain T cells with specificities against non-mutated self-antigens as well as mutated neo-antigens (Lu et al., 2014; Lu et al., 2013; Tran et al., 2014; Veatch et al., 2018). As T cells recognizing self-antigens are usually of low avidity and therapy responses correlate with predicted neo-antigen load (Lauss et al., 2017), neo-antigen specific T cells are thought to mediate the therapeutic effect of TILs. TILs can be expanded non-specifically or specifically by stimulation with autologous tumour cells or with cells expressing tumour-derived epitopes (Dudley et al., 2010; Dudley et al., 2005; Tran et al., 2016). Nevertheless, T cell specificities are difficult to control and depend on the presence and functionality of the accessible TILs. Therapy success is difficult to predict and cancer entities that have lower mutational burden and/or less T cell infiltration may not qualify for TIL therapy.

3.3.2 Engineered T cells

TCR gene therapy allows the production of high numbers of tumour-reactive T cells by genetic engineering of patients' T cells. Great clinical success has been achieved in treating haematological malignancies with T cells engineered with a chimeric antigen receptor (CAR) targeting CD19 (Maude et al., 2014). CARs are synthetic receptors that possess the antigen-binding domain of an antibody and an intracellular signalling domain. Similar to antibodies and unlike TCRs, CARs recognize cell surface proteins, which is advantageous regarding no requirement for a TCR-matched MHC expression in the patient. However, solid tumours usually do not have a unique surface marker and may be better targeted by TCRs that recognize intracellular tumour antigens presented on MHC.

3.3.3 CD4 T cells in adoptive T cell therapy

So far, clinical trials of ATT using TCR-engineered T cells could not achieve comparable success to the CAR studies targeting CD19 in haematological malignancies. One underlying reason may be that TCR gene therapy trials have mainly focussed on CD8 T cells. However, solid tumours may require CD4 T cell help to constitute a full immune response able to achieve major clinical effects.

Experiments with mouse models showed the importance of CD4 T cells for anti-tumour immunity already long ago (Greenberg et al., 1981). In an adoptive chemoimmunotherapy study CD8-depleted T cells eliminated MHC II-negative leukaemia in mice without the need for cytolytic activity (Greenberg et al., 1981; Greenberg et al., 1985). Rejection of MHC II-negative tumours by CD4 T cells was also confirmed in other murine tumour models and shown to be mediated by interferon (IFN)γ functioning on host cells and not cancer cells (Mumberg et al., 1999; Perez-Diez et al., 2007; Qin and Blankenstein, 2000). In a mouse model of transplantable melanoma, tumour rejection by CD4 T cells was IFNγ-dependent as well, but was mediated by direct lysis of MHC II-positive cancer cells (Muranski et al., 2008; Quezada et al., 2010). Furthermore, CD4 T cells exert helper function to support CD8 T cells and several cancer models require both CD4 and CD8 T cells for improved efficacy of ATT (Arina et al., 2017; Bos and Sherman, 2010; Li et al., 2017). T cell help was shown in a mouse model of insulinoma, in which CD4 T cells were necessary to recruit CD8 T cells to the tumour site and enhance their cytolytic function (Bos and Sherman, 2010). In a mouse model of ATT of established large tumours, CD4 T cells could prevent or reverse antigen-positive tumour relapse that could not be controlled by CD8 T cells alone (Arina et al., 2017). Furthermore, the cooperation of CD4 and CD8 T cells can achieve bystander elimination of antigen-loss variants, which are a common problem in the clinic (Rapoport et al., 2015; Schietinger et al., 2010). The different mechanisms by which CD4 T cells exert tumoricidal effects may reflect the versatility of CD4 T cells, which can differentiate into different subpopulations to integrate different types of immune responses (Geginat et al., 2014).

Interestingly, intratumoural CD4 T cells recognizing neo-antigens were found in several patients (Linnemann et al., 2015). Concordantly, two cases of successful TIL therapy were attributed to neo-antigen-specific CD4 T cells within the TIL product (Tran et al., 2014; Veatch et al., 2018). In another case study, a patient with metastatic melanoma experienced a durable clinical response following treatment with expanded NY-ESO-specific CD4 T cell clones, which could however not be reproduced in more patients (Hunder et al., 2008; Muranski and Restifo, 2009). Together,

these clinical data, as well as the referenced mouse models encourage further application of CD4 T cells in treatment of cancer.

However, it has to be noted that CD4 T cells can also negatively influence immune responses. CD4 T cells differentiated into Tregs are important to control exaggerated immune responses and prevent autoimmunity (Suri-Payer et al., 1998). Because of this immune regulatory property, Tregs can hinder anti-tumour immunity. This was shown in a mouse model of transplantable melanoma, in which only adoptive transfer of tumour-reactive CD8 T cells in conjunction with Treg-depleted CD4 T cells but not undepleted CD4 T cells resulted in cancer regression (Antony et al., 2005). Infiltration of Tregs are frequently found in tumour tissue and are implicated in suppression of T cell activation (Curiel et al., 2004; Woo et al., 2002).

3.3.4 Clinical studies using engineered T cells

First TCR gene therapy trials have targeted the melanocyte differentiation antigen MART-1 (Johnson et al., 2009; Morgan et al., 2006). In the first trial, a low-affinity TCR DMF4 was used to treat metastatic melanoma, but only marginal responses were seen in two of 17 patients (Morgan et al., 2006). In a next clinical trial, TCRs recognizing MART-1 (DMF5) or gp100 with higher affinity were used. Although objective responses of 19% and 30%, respectively, were seen, both TCRs recognized normal melanocytic cells resulting in toxicities in the skin, eye and ear (Johnson et al., 2009). On-target off-tumour toxicity was also seen when three patients were treated with a high-affinity TCR targeting carcinoembryonic antigen (Parkhurst et al., 2011). This shows that appropriate TCR affinity is crucial to achieve anti-tumour effects but at the same time results in on-target off-tumour toxicity, if the target antigen is not exclusively expressed by the tumour. In two clinical trials targeting the cancer-testis antigen MAGE-A3, off-target toxicities to cardiac and brain tissue, respectively, caused fatal toxicities (Linette et al., 2013; Morgan et al., 2013). Both TCRs were affinity-matured to increase their potency, suggesting that in vitro maturation increases the risk for off-target toxicity by circumventing negative selection in the thymus. In two clinical trials, the cancer-testis antigen NY-ESO was targeted in melanoma or synovial sarcoma and multiple myeloma (Rapoport et al., 2015; Robbins et al., 2015). Although an affinity-matured TCR was used, no toxicities were observed but objective responses were seen in 61%, 55% and 80% in sarcoma, melanoma and myeloma patients, respectively (Rapoport et al., 2015; Robbins et al., 2015; Robbins et al., 2008). Lack of toxicity is in line with the absence of NY-ESO expression in normal tissues aside from germ cells and thymus (Chen et al., 1997;

Gotter et al., 2004). However, one fatal severe adverse event caused by bone marrow failure was reported following TCR gene therapy using the same affinity-matured TCR against NY-ESO (Mackall et al., 2017). Moreover, it has to be noted that in the myeloma study, patients received high-dose chemotherapy and autologous stem cell therapy prior to the engineered T cells, making interpretation on efficacy of the engineered T cells difficult. However, an inverse correlation of NY-ESO expression (or LAGE-1, containing the targeted epitope) and engineered T cell persistence suggests efficacy of the T cell therapy (Rapoport et al., 2015).

3.3.5 Critical factors for T cell gene therapy

Several studies have shown that anti-tumour efficacy and on-target off-tumour toxicity cannot be separated if the antigen is expressed in normal tissues (Johnson et al., 2009; Morgan et al., 2006; Parkhurst et al., 2011). This was confirmed in a mouse model of ATT, in which TCR affinities against $gp100_{209\text{-}217}$ correlated equally with anti-tumour and autoimmune activity (Zhong et al., 2013). Therefore, selection of a target antigen whose expression is restricted to cancer cells is of utmost importance to prevent toxicity.

Furthermore, human and mouse studies have shown that only TCRs of higher affinity can reach anti-tumour efficacy (Johnson et al., 2009; Morgan et al., 2006; Obenaus et al., 2015; Zhong et al., 2013). It was suggested that TCR affinity should be at least K_d=10 µM (Zhong et al., 2013). We defined optimal-affinity TCRs as TCRs that can be found in immune responses to foreign antigens, such as viral antigens (Obenaus et al., 2015). Virus-specific TCRs have an affinity around 10 µM (Aleksic et al., 2012). However, T cell avidity does not only depend on TCR affinity but also on TCR density on the T cell surface, why it is difficult to draw a strict line of required TCR affinity. Moreover, the interaction of the TCR and the peptide-MHC complex is not only dependent on TCR affinity but also on the affinity of the peptide to MHC. It was shown that only high-affinity peptides serve as rejection epitopes leading to relapse-free tumour regression (Engels et al., 2013). Further, mouse studies showed that tumour rejection or relapse following ATT can be dependent on the strength of antigen expression (Leisegang et al., 2016a; Spiotto et al., 2004). Only high antigen expression caused tumour rejection, which in one mouse model correlated with antigen cross-presentation by tumour stroma (Spiotto et al., 2004).

Together, TCR affinity to peptide-MHC complex, peptide affinity to MHC and abundance of the target antigen are important factors for success of ATT. As the ideal situation, high TCR and peptide-MHC affinities and homogenously high antigen expression, may only rarely be fulfilled,

combination therapies such as targeting more than one antigen and/or combined use of CD4 and CD8 T cells may be required for tumour rejection.

3.4 Aims of this doctoral thesis

Based on the importance of CD4 T cells in anti-tumour immune responses, the aim of the present project was to generate MHC II-restricted TCRs for the use in ATT. As target antigen we employed the shared tumour antigen NY-ESO to allow for broad application of potential therapeutic TCRs (Chen et al., 1997). To raise optimal-affinity TCRs against NY-ESO, human TCR gene loci/chimeric MHC II transgenic (ABabDR4) mice were employed (Ito et al., 1996; Li et al., 2010). Moreover, we asked, whether the human repertoire can be used as a source for MHC II-restricted TCRs against NY-ESO.

3.4.1 NY-ESO as target for ATT

NY-ESO is a tumour-associated antigen that was found by serological expression cloning (SEREX) from an oesophageal cancer (Chen et al., 1997). In addition to oesophageal cancer, NY-ESO expression was found in a variety of cancers including melanoma, breast cancer and prostate cancer. Moreover, NY-ESO is expressed highly in male germ cells and to some extent in ovary and/or placenta (Chen et al., 1997). Besides expression in the thymus, trace amounts of NY-ESO mRNA have been found in somatic tissues including liver and pancreas, but the relevance of this finding is unclear (Gotter et al., 2004; Sato et al., 2005). Absence of NY-ESO expression in normal tissues is suggested by lack of toxicity in two clinical studies in which NY-ESO was targeted by ATT (Rapoport et al., 2015; Robbins et al., 2015).

The expression of NY-ESO in medullary thymic epithelial cells may lead to deletional tolerance of high-avidity T cells against NY-ESO (Gotter et al., 2004). This could be one of the reasons why spontaneous anti-NY-ESO immunoglobulin (Ig)G antibody and CD4 responses in cancer patients do not lead to a clinical benefit and why NY-ESO immunization strategies as cancer treatment failed (Fourcade et al., 2014; Gnjatic et al., 2003; Takeoka et al., 2017; Zeng et al., 2000). Furthermore, the treatment success achieved in one patient with expanded NY-ESO-specific CD4 T cell clones could not be reproduced in eight other patients (Hunder et al., 2008; Muranski and Restifo, 2009). Thus, it proves probable that the intrinsic immune response to NY-

ESO cannot be harnessed for cancer therapy. However, due to its restricted expression pattern, NY-ESO may be a good target for adoptive therapy using high-avidity T cells.

3.4.2 ABabDR4 mouse model

As highly functional TCRs may not be found in the human repertoire due to NY-ESO expression in the thymus and concomitant tolerance induction (Gotter et al., 2004), ABabDR4 mice were used for TCR generation (Chen et al., 2017; Ito et al., 1996; Li and Blankenstein, 2013; Li et al., 2010). ABabDR4 mice were generated by crossing HLA-DRA-IE/HLA-DRB1*0401-IE (HLA-DR4-IE)-chimeric MHC II-transgenic mice (DR4 mice) with human TCR gene loci-transgenic mice (ABab mice) (Ito et al., 1996; Li et al., 2010). DR4 mice were engineered to express a chimeric MHC II molecule which consists of the antigen-binding domains from the human HLA-DR4 and the membrane-proximal domains from the murine I-E^d to enable murine CD4-binding (Ito et al., 1996). DR4 mice mounted a specific CD4 T cell response to heamagglutinin$_{307-319}$ upon immunization and develop experimental allergic encephalomyelitis symptoms when immunized with HLA-DR4 epitopes from myelin basic protein or proteolipid protein (Ito et al., 1996). ABabDR4 mice were shown by deep sequencing to rearrange a diverse repertoire of human TCRs (Chen et al., 2017). Interestingly, TCR diversity of CD4 T cells from ABabDR4 mice, in which both TCRs and MHC II are human, was higher than from ABabHHD mice, in which TCRs are human but MHC II from mouse. This suggests that human TCR genes and human MHC co-evolved (Chen et al., 2017). Importantly, ABabDR4 mice express a TCR repertoire which was not influenced by NY-ESO-specific tolerance, as mice do not express homologous sequences to the HLA-DR4-restricted NY-ESO epitope used in this project. In ABabDR4 mice, NY-ESO is a foreign antigen.

3.4.3 Steps to be taken

First, the ABabDR4 mouse model was characterized in terms of MHC II expression and the ability to mount CD4 T cell responses. Next, immunogenicity of the chosen HLA-DR4-restricted NY-ESO epitope in the ABabDR4 mice was shown. TCRs isolated from immunized ABabDR4 mice were compared in functional assays to TCRs isolated from in vitro primings of human CD4 T cells to investigate which source is appropriate to derive optimal-affinity TCRs against NY-ESO. To analyse whether clinical application of the chosen TCR candidate can be safe, it was tested for off-target toxicity. Finally, in vivo functionality was tested in a mouse model of ATT

and a clinical version of the chosen TCR candidate was analysed for comparability to the preclinical version used in the development phase.

4 Materials & Methods

4.1 Cell lines

The human melanoma cell lines FM-82, FM-56 (both NY-ESO$^+$, HLA-DR4$^+$), FM-3 (NY-ESO$^-$, HLA-DR4$^+$) and FM-6 (NY-ESO$^+$, HLA-DR4$^-$) were provided by the European Searchable Tumour Cell Bank and Database (ESTDAB). Jurkat 76/CD4 cells are TCR-deficient and were generated by introducing human CD4 into the Jurkat 76 clone (Heemskerk et al., 2003). The murine cell line T.54ζ17 is TCR-deficient and expresses human CD4 and murine ζ chain (58/CD4 cells) (Kieback et al., 2016). The cell line K562/DR4 was generated by transducing K562 cells with HLA-DRA and HLA-DRB1*0401. All cell lines were cultured in RPMI supplemented with 10% fetal calf serum (FCS; PAN Biotech) and 1x antibiotic-antimycotic. The retroviral packaging cell lines 293GP-GLV (GALV cells) and Plat-E (producing amphotropic and ecotropic retroviral vectors, respectively) were cultured in DMEM supplemented with 10% FCS (Ghani et al., 2009; Morita et al., 2000). The panel of EBV–transformed lymphoblastoid B cell lines (LCLs) were cultured in RPMI supplemented with 10% FCS, 1x antibiotic-antimycotic, 1 mM sodium pyruvate and 1x non-essential amino acids. All cell culture reagents were purchased from Life Technologies unless otherwise indicated.

4.2 Mouse strains

All animal experiments were performed according to national guidelines and were approved by the responsible national institute (Landesamt für Gesundheit und Soziales, Berlin, Germany). All mouse strains were housed at the animal facility of the Max Delbrück Centrum for Molecular Medicine under specific-pathogen-free conditions. DR4 mice express a mouse/human chimeric MHC II molecule, HLA-DR4-IE, and are deficient for mouse MHC II molecules (Ito et al., 1996). They were purchased from Taconic. ABabDR4 mice are transgenic for the entire human TCRα and β loci and a mouse/human chimeric MHC II molecule, HLA-DR4-IE, while neither mouse TCRs nor mouse MHC II molecules are expressed (Chen et al., 2017). They were generated by crossing human TCR loci-transgenic mice (ABab) and DR4 mice (Ito et al., 1996; Li et al., 2010). DR4xRag$^{-/-}$ mice were generated by crossing DR4 mice with Rag$^{-/-}$ mice.

Table 1

Primers	Gene/product	Product size	PCR program	Forward/reverse primer sequences 5'→ 3'
hTCRa	Human TRAV10	221 bp	standard	atggcaaaaaccaagtggag/ tttgctttgtgtctgcatcc
hTCRb	Human TRBV19	691 bp	standard	cacattaggccaggagaagc/ cctgcttagtggctgagtgg
mTCRa WT	Mouse TCRα constant region	164 bp	standard	actgtgctggacatgaaagc/ ccatagatttgagccaggagg
mTCRb WT	Mouse TCRβ constant region	229 bp	standard	tgagccatcaaaagcagaga/ gaagtggttgcaaggattgt
mTCRa KO	Knockout in mouse TCRα constant region	180 bp	standard	taccggtggatgtggaatgt/ actgtgctggacatgaaagc
mTCRb KO	Knockout in mouse TCRβ constant region	248 bp	standard	taccggtggatgtggaatgt/ ttctagacccccacctagagc
DR4A	DRA-IE transgene	1276 bp	elongation 1 min 30 sec	gggaagcaggggactatgac/ ttagggcaatgacttcgtagg
DR4B	DRB1*0401-IE transgene	419 bp	standard	tgaaagcggtgcgtgctgtttaa/ cacccgctccgtcccgttgaa
I-A^{neo} (N752S/AbBE2R2)	Knockout in I-A β-chain	~ 940 bp	standard	gatggattgcacgcaggttct/ tctgcacaccgtgtccagct
I-Ab	Knockout in I-A β-chain (primers flanking neomycin cassette)	226 bp in WT (haplotype b) 1376 bp in I-Ab^{neo}	elongation 1 min 30 sec	tttcgtgtaccagttcatgg/ gtagttgtgtctgcacaccg
EaDel2	I-E α chain with or without disrupting deletion	1035 bp in B6 ~ 1720 in Balb/c	elongation 1 min 43 sec annealing at 62°C	ctagcccactgcaaaaggag/ gatgagggcttctgtgttgtc

Table 1. Genotyping of ABabDR4 mice. Displayed are PCR primers used to detect the transgenes human TCRα and TCRβ loci (hTCRa, hTCRb), HLA-DRA-IE (DR4A), HLA-DRB1*0401-IE (DR4B), as well as knockouts of mouse TCRα and TCRβ constant regions (mTCRa, mTCRb) and I-A β-chain (I-Ab).

OTIIxRag$^{+/-}$ and P14xRag$^{-/-}$ mice express transgenic TCRs with specificity for ovalbumin$_{323-339}$ presented on I-Ab and lymphocytic choriomeningitis virus glycoprotein$_{33-41}$ presented on H2-D^{b}, respectively (Barnden et al., 1998; Pircher et al., 1989). Mice were genotyped by polymerase chain reaction (PCR) using DNA isolated from ear biopsies by alkaline lysis. 300 µl 0.05 M NaOH were added to the ear biopsy and incubated at 95°C for one hour. For neutralization, 30 µl of stopping solution were added containing 1 M Tris-Cl and 10 mM EDTA at pH 8.0. PCR reactions were performed in a total volume of 10 µl, using 1 µl lysed DNA, 0.1 µl Taq DNA polymerase (FastStart, Roche), 0.2 mM dNTPs and 0.5 µM primers. Thermocycling conditions for standard PCR were as follows: 1) Initial denaturation step at 95°C for 5 min, 2) denaturation at 95°C for 30 sec, 3) annealing at 63°C for 40 sec, 4) elongation at 72°C for 1 min 10 sec, 5) 35 cycles steps 2)-4), 6) final elongation at 72°C for 10 min. Table 1 lists PCR primers used for genotyping of ABabDR4 mice.

4.3 Immunization of mice

ABaBDR4, DR4 and C57BL/6 mice were taken for immunization at 6-16 weeks of age. For peptide immunization, 100 µg of the NY-ESO peptide 116-135 (LPVPGVLLKEFTVSGNILTI, NY-ESO$_{116}$, GenScript) or Pan-DR binding peptide (AK(X)VAAWTLKAA, (X) = cyclohexylalanine; Padre, Biomatik; Alexander et al., 1994) and 50 µg CpG (CpG 1826, MOLBIOL) were prepared in 200 µl 1:1 emulsion of incomplete Freund's adjuvant and phosphate-buffered saline (PBS) and injected s.c. into the flank of the mouse. Immunizations were repeated with at least 4-week intervals. For DNA immunizations, the Helium Gene Gun system from Biorad was used. A mixture of pcDNA3.1 vectors containing NY-ESO cDNA or granulocyte macrophage colony-stimulating factor cDNA were precipitated on gold microcarriers of 1 µm diameter at a DNA loading rate of 2 µg/mg gold. For cartridge preparation, the DNA/microcarrier suspension in 0.05 mg/ml polyvinylpyrrolidone in Ethanol (both Sigma) was loaded into Gold-Coat tubing using a Tubing Prep Station (Biorad). Using Helium pressure, DNA from two cartridges was delivered into the shaved skin at the abdomen of the mouse.

4.4 Evaluation of immune response

CD4 T cell responses were analysed in peripheral blood or cells from spleen and draining lymph nodes one week after immunizations. 200 µl blood or cells from spleen and draining lymph nodes

were lysed by 1 ml or 3 ml ammonium-chloride-potassium lysing buffer (ACK buffer; 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 2 min followed by two washes with PBS containing 2% FCS. Cells were resuspended with mouse T cell medium (mTCM; RPMI supplemented with 10% FCS, 1x antibiotic-antimycotic, 1 mM sodium pyruvate, 1x non-essential amino acids, 1 mM HEPES, 50 µM β-mercaptoethanol) and cultured with 1 µg/ml NY-ESO_{116} peptide, Padre or a peptide from nonstructural protein 3 (NS3) from hepatitis C virus (YAAQGYKVLVLNPSVAAT) as negative control or $4x10^5$ dynabeads T activator CD3/28 (Invitrogen) as positive control overnight. One third of the blood sample or $1x10^6$ splenocytes were applied per well of a 96-well plate. After 2 hrs of incubation, Brefeldin A (Becton Dickinson (BD) GolgiPlug) was added to the cultures and intracellular IFNγ staining was carried out for analysis (see flow cytometry).

4.5 In vitro expansion of NY-ESO-reactive CD4 T cells from ABabDR4 mice

For in vitro expansion of NY-ESO-reactive CD4 T cells, cells from spleen and draining lymph nodes from mice 10-14 days after the last immunization were prepared and meshed through a sieve to yield a single cell suspension. Without prior lysis of erythrocytes, but counting only peripheral blood mononuclear cells (PBMC), $1,5x10^7$ cells per well were cultured in a 24-well plate in mTCM (supplemented with 20 U/ml IL-2) and in presence of 10^{-8} M or indicated concentration of NY-ESO_{116} or dynabeads T activator CD3/CD28 at a ratio of 1:1. After one week, expansion of NY-ESO-reactive CD4 T cells was analysed by peptide restimulation and intracellular IFNγ staining as described. The next day, cells were stained by tetramer and sorted by flow cytometry.

4.6 Mouse IFNγ secretion assay

For T cell isolation, spleen and draining lymph nodes were prepared from mice 10-14 days after the last immunization as single cell suspension. Following lysis of erythrocytes by ACK buffer, $1x10^6$ cells per well were cultured 8 hrs in a 96-well plate in mTCM in presence of 0.5 µM NY-ESO_{116} or Padre or $1x10^6$ dynabeads T activator CD3/28 (Invitrogen) as negative and positive control, respectively. The responding cells were labelled by Mouse IFNγ Secretion Assay (Milteny) as follows. Cultured cells were harvested and washed twice with magnetic-activated

cell sorting (MACS) buffer (PBS, 0.5%, bovine serum albumin, 2 mM EDTA). Cells were stained by IFNγ Catch Reagent for 5 min, then diluted with warm mTCM and incubated 45 min on a rotator at 37°C. After two washes with MACS buffer, cells were stained by IFNγ Detection Antibody and anti-mCD4 and anti-mCD3. Prior to cell sorting by flow cytometry, 7-AAD (BD) was added to the sample for exclusion of dead cells.

4.7 In vitro priming of human CD4 T cells

To expand human NY-ESO-reactive CD4 T cells, peripheral blood leukocytes (PBLs) from a HLA-DR4^{+} donor were enriched for CD4 by magnetic separation using a negative selection kit (Milteny). Subsequently, the enriched CD4 T cells were stained with TRBV2-PE antibody (Beckman Coulter, clone IMMU 546) and enriched by anti-PE microbeads by magnetic separation over a LS column yielding 68% TRBV2^{+} CD4 T cells. 1,9x10^{6} TRBV2-enriched CD4 T cells were cultured with 2,5x10^{6} irradiated (30 Gy) autologous CD4^{-} cells as feeder cells and in presence of 2 µM NY-ESO$_{116}$ in human T cell medium (hTCM; RPMI supplemented with 10% FCS, 1x antibiotic-antimycotic, HEPES 1 mM, 10 U/ml IL-2). After 14 days, NY-ESO-reactive CD4 T cells were stained by tetramer and sorted by flow cytometry.

4.8 TCR isolation

From all T cell sorts (yielding between 500-10,000 cells), total RNA was extracted (RNeasy Micro Kit, Qiagen) and 5'rapid amplification of cDNA ends (RACE) PCR was performed using the SMARTer RACE cDNA Amplification Kit (Clontech). Following cDNA synthesis performed according to the Clontech user manual, 5'RACE PCR was performed in a 50 µl volume, with 0.5 µl Phusion High-Fidelity DNA polymerase (New England Biolabs), 10 mM dNTPs, 1x universal primer mix (Clontech) and 0.5 µM reverse primer. As reverse primers, 5'cggccactttcaggaggaggattcggaac3' (for TCRα chain) and 5'ccgtagaactggacttgacagcggaagtgg3' (for TCRβ chain) were used. Thermocycling conditions applied were as follows: 1) initial denaturation step at 98°C 2 min, 2) 5 cycles containing a denaturation step at 98°C 30 sec and an annealing/elongation step at 72°C for 45 sec, 3) 5 cycles containing denaturation at 98°C 30 sec, annealing at 70°C 20 sec, elongation at 72°C 45 sec, 4) 25 cycles containing denaturation at 98°C 20 sec, annealing at 68°C 20 sec, elongation at 72°C 45 sec, 5) final elongation at 72°C 5 min.

The RACE PCR products were purified from an electrophoresis gel and cloned into TOPO vectors (Zero Blunt TOPO PCR cloning Kit) to transform competent E. coli. Depending on clonality, 6-32 clones per TCR chain were analysed and the most frequent TCRαβ chain pairs were tested for function.

4.9 Generation of pMP71 vectors for TCR expression

If the isolated TCR chains were not clonal, they were cloned as single TCR chains into the retroviral vector pMP71-PRE (pMP71) for combinatorial expression to identify functional TCRαβ pairs (Engels et al., 2003). For this, the variable TCR region was amplified from a TOPO vector and equipped by overlapping extension PCR with the murine constant region amplified from the codon-optimized TCR-3600. The generated product was purified, digested and ligated into the pMP71 vector. Table 2 lists the primers used for cloning of single TCR chains.
Functional TCRαβ pairs were synthesized as expression cassettes containing codon-optimized TCRβ and TCRα chains linked by the porcine teschovirus-1-derived self-cleaving peptide P2A (Leisegang et al., 2008). Constant regions were murinized (Cohen et al., 2006). The synthesized expression cassette (GeneArt) was cloned into pMP71. TCR-5712 differs from TCR-3600 only in one amino acid and was generated from TCR-3600 by PCR-induced mutagenesis. A clinical version of TCR-3598_2 was synthesized containing human constant regions with amino acid exchanges at 9 positions corresponding to the murine constant regions and an additional disulphide bond (Cohen et al., 2007; Sommermeyer and Uckert, 2010).

4.10 Retroviral transduction

4.10.1 Human T cells

Virus supernatant for retroviral transduction of human cells was produced by transfecting GALV cells (Ghani et al., 2009) with the retroviral vector pMP71 (Engels et al., 2003) containing a TCR expression cassette or a single TCR chain using Lipofectamine2000 transfection reagent (Thermo Fisher Scientific). Viral supernatant was harvested after 48 and 72 hrs following transfection and used directly for transducing target cells or were stored at -80°C for later use. PBMCs were isolated from blood using Pancoll Separating Medium (PAN Biotech) and were depleted of CD8 T cells by magnetic separation over a LS column using CD8 microbeads (Milteny). $1x10^6$ CD8-

Table 2

Primers for amplification of variable regions	
Name	**Sequence 5'→ 3'**
TRAV9-2_Not1 (fwd)	gcattgcggccgccatgaactattctccaggcttagtatc
TRAV12-3_Not1 (fwd)	gcattgcggccgccatgatgaaatccttgagagttttac
TRAV13-1_Not1 (fwd)	attgcggccgccatgacatccattcgagctg
TRAV20_Not1 (fwd)	attgcggccgccatggagaaaatgttggagtgtgc
TRAV21_Not1 (fwd)	attgcggccgccatggagaccctcttgggcc
TRAV36_Not1 (fwd)	attgcggccgccatgatgaagtgtccacaggc
TRAV38_Not1 (fwd)	attgcggccgccatgacacgagttagcttgctg
TRBV2_Not1 (fwd)	gcattgcggccgccatggatacctggctcgtatgc
TRBV_15_Not1 (fwd)	gcattgcggccgccatgggtcctgggcttctc
V(D)J_alpha_rvs	agctggtacacggcaggctcggggttctg
V(D)J_beta_rvs (if TRAC2)	accttgggggggtcacgtttctcagatcc
J1-1_ms_beta_rvs (if TRAC1)	ggtcacgtttctcagatcctctacaactgtgagtctggtgcc
J1-2_ms_beta_rvs (if TRAC1)	ggtcacgtttctcagatcctctacaacggttaacctggtcc
J1-3_ms_beta_rvs (if TRAC1)	ggtcacgtttctcagatcctctacaacagtgagccaacttccc
J1-5_ms_beta_rvs (if TRAC1)	ggtcacgtttctcagatcctctaggatggagagtcgagtcc
J1-6_ms_beta_rvs (if TRAC1)	ggtcacgtttctcagatcctctgtcacagtgagcctggtcc
Primers for amplification of mouse constant regions	
Name	**Sequence 5'→ 3'**
alpha_ms_fwd	atatccagaaccccgagcctgccgtgtacc
beta_ms_fwd	ggatctgagaaacgtgacccccccccaaggt
TCR3600_beta_EcoR1_rvs	actgaattctcagctgttcttcttcttgaccatgg
huTCR3600_rvs	caggaattctcatcagctggaccac
huTCR3600_Mfe1_rvs	agtgcaattgtcagctggaccacagc

Table 2. Primers used for cloning of single TCR chains. Displayed are PCR primers used to generate single TCR chains equipped with murine constant regions by overlap extension PCR.

depleted PBMCs per well were activated on anti-CD3 (OKT3)/anti-CD28 (CD28.2; both BD Pharmingen)-coated 24-well plates in 1 ml hTCM (supplemented with 300 U/ml IL-2). The first transduction was performed 2 days after T cell activation by spinoculating the T cells with 1 ml virus supernatant for 90 min at 32°C and 800 g. The second transduction was performed the following day on virus-pre-loaded retronectin (Takara)-coated plates. Non-tissue-treated 6-well plates were coated with 1 ml retronectin (25 µg/ml in PBS) per well at 4°C overnight, blocked 30 min with 2% bovine serum albumin (Sigma-Aldrich) in PBS at 37°C and spinoculated with 3 ml virus supernatant per well for 2 hrs at 32°C and 2000g. Following removal of the virus supernatant, T cells were transferred and spinoculated for 30 min at 800g. After expanding the T cells for one week, they were cultured in hTCM (supplemented with 30 U/ml IL-2) for 3 days before using them in in vitro experiments or freezing them for later use. Jurkat 76/CD4 cells were transduced twice on two subsequent days by spinoculating $5x10^5$ cells with 1 ml virus supernatant per well in a 24-well plate for 90 min. For transduction of combinations of single TCR chains, virus supernatants were mixed.

4.10.2 Mouse T cells

Virus supernatant for retroviral transduction of mouse cells was produced by transfecting Plat-E cells as described for the GALV cells. TCR-transgenic mice were used as T cell donors. For CD4 T cells, spleen and lymph nodes from OTIIxRag$^{+/-}$ mice were prepared and subjected to magnetic separation of CD4 T cells by negative selection (Milteny). $1x10^6$ isolated CD4 T cells per well were activated on an anti-CD3/anti-CD28-coated 24-well plate in 1 ml mTCM (supplemented with 30 U/ml IL-2). The first transduction was performed 48 hrs after T cell activation by spinoculating the T cells with 1 ml virus supernatant for 90 min at 32°C and 800g. The following day the transduction was repeated. After the second transduction CD4 T cells were cultured in mTCM supplemented with IL-15 and IL-27 (each 50 ng/ml). For CD8 T cells, spleens from P14xRag$^{-/-}$ were prepared and activated by soluble anti-CD3/anti-CD28 (1 µg/ml/0.1 µg/ml) in mTCM. One day after T cell activation, the first transduction was performed on virus-pre-loaded retronectin-coated 24-well plates. The coating procedure was as described for transduction of human T cells but using 1 ml virus supernatant per well. $1x10^6$ activated T cells with $4x10^5$ dynabeads T activator CD3/CD28 per well were spinoculated for 30 min at 32°C and 800g. A second transduction was performed by adding 1 ml virus supernatant and spinoculating the cells under the same conditions but 90 min. Afterwards, CD8 T cells were cultured in mTCM

supplemented with IL-15 (50 ng/ml). One day (CD4 T cells) or 2 days (CD8 T cells) following the second transduction, surface staining was carried out to determine transduction efficiency and the T cells were used for ATT or frozen for later use in in vitro assays. 58/CD4 cells were transduced twice on two subsequent days by spinoculating $5x10^4$ cells with 100 µl virus supernatant per well in a 96-well plate for 90 min.

4.11 Co-culture experiments

All co-culture experiments with human cells were performed by incubating $1x10^4$ transduced CD4 T cells with $5x10^4$ target cells in round bottom 96-well plates for 16-18 hrs. Transduction efficiencies were in the range of 30-70% and T cells were calculated accordingly. IFNγ or IL-2 was measured in the supernatant by enzyme-linked immunosorbent assay (ELISA; BD OptEIA) according to the user manual but applying half volumes throughout the protocol. 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 5 µg/ml Ionomycin were added to T cells alone as positive control. NY-ESO peptides (LPVPGVLLKEFTVSGNILTI, $NY\text{-}ESO_{116\text{-}135}$ or PGVLLKEFTVSGNIL, $NY\text{-}ESO_{119\text{-}133}$) were added at 10^{-6} M or at indicated concentrations. Alanine-exchanged $NY\text{-}ESO_{119}$ peptides (all Genscript, >95% purity) were added at 10^{-7} M. Peptides containing the TCR 3598_2 recognition motif -L-K-E-F- (JPT Peptide Technologies, unpurified) were added to the co-culture at 10^{-6} or 10^{-7} M as indicated. HLA-DR and HLA-ABC blocking antibodies (L243, W6/32; Biolegend) were added to the target cells at 20 µg/ml and incubated at least 1 hr prior to adding the T cells. Statistic tests and bar charts were made with GraphPad Prism (version 7).

4.12 Flow cytometry

The following antibodies were used for staining at 1:100 ratio and purchased from BioLegend unless otherwise indicated: anti-I-Ek-FITC (17-3-3), anti-I-Ad-PE (AMS-32.1), anti-I-Ab-FITC (AF6-120.1), anti-mCD4-FITC (RM4-5), anti-mCD4-BV421 (RM4-5), anti-mCD8-PECy7 (53-6.7, Beckton Dickinson (BD)), anti-mCD3-PE (145-2C11; 1:200), anti-mIL-2-APC (JES6-5H4), anti-mIFNγ-BV421 (XMG1.2), anti-mTCRβ chain-APC (H57-597), anti-hCD4-PE/Cy7 (OKT4), anti-hCD3-APC (SK7), anti-hTRBV2-FITC (IMMU 546, 1:20, Beckman Coulter), anti-HLA-DR-APC (L243).

Immune responses of immunized ABabDR4 mice were analyzed by intracellular IFNγ staining of peptide-restimulated blood samples in a 50 µl volume. After Fc blocking for 15 min at 4°C (anti-mCD16/32, clone 93, Biolegend), cells were washed with PBS and stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (1:1000 in PBS, Life technologies) for 30 min at 4°C. After two washes cells were fixed (BD Cytofix/Cytoperm), washed twice (BD Perm/Wash buffer) and stained with antibodies for 30 min at 4°C. Before measuring, cells were washed with BD Perm/Wash buffer and resuspended in PBS.

For tetramer staining, cells were incubated in hTCM containing 10 µg/ml DR4/NY-ESO$_{116}$ tetramer or DR4/CLIP$_{87\text{-}101}$ control tetramer for 1 hr at 37°C. Subsequently, antibodies were directly added and the cells were incubated 30 min on ice. After two washes with PBS, cells were analysed. PE-labelled DR4/NY-ESO$_{116}$ and DR4/CLIP$_{87\text{-}101}$ tetramers were obtained through the NIH Tetramer Facility.

Adoptively transferred T cells were analysed in 50 or 100 µl peripheral blood. Antibodies were added into the blood sample and after 20 min at room temperature, 500 µl of erythrocyte lysing solution (BD) was added. After 2 minutes the cells were washed with PBS and the pellet was resuspended with 150 µl PBS. The entire sample was analysed by flow cytometry and the number of T cells were calculated according to 50 or 100 µl peripheral blood. Significance testing (t test) was performed with GraphPad Prism (version 7).

FACSAriaTM II was used for sorting of cells and FACSCanto II or LSRFortessa were used to acquire cells for analysis (all BD). Flow cytometry data were analysed with FloJo version 10.1.

4.13 Western blot

$5x10^6$-$1x10^7$ cells were used for protein extraction with cell lysis buffer (Mammalian Cell Lysis Kit, Sigma). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 50 ug protein per sample, which was heated before at 70°C for 10 min in lithium dodecyl sulfate sample buffer and sample reducing agent. Samples were run in a 10% polyacrylamide gel (bis-tris) in 2-morpholinoethanesulfonic acid SDS running buffer at 140 V for 1 hr. Proteins were blotted onto a methanol-activated polyvinylidene difluoride (PVDF) membrane in transfer buffer for 45 min at 20 V (all buffer and components from Novex, Life Technologies). Subsequently, the membrane was incubated in blocking buffer (PBS, 5% milk powder, 0,05% Tween20) for 1 hr at room temperature and then incubated with anti-NY-ESO

antibody 1:750 (E978, Sigma) at 4°C overnight. After washing the membrane 3 times in PBST (PBS, 0,05% Tween20), it was incubated with goat anti-mouse IgG conjugated to horse radish peroxidase (HRP; Santa Cruz Biotechnology) 1:5000 in blocking buffer for 1 hr. Following 3 washes with PBST, the membrane was analysed by applying Western blotting Luminol Reagent (Santa Cruz) and chemiluminescence was detected by Lumi-Imager F1 (Roche). For loading control, actin was analysed after incubation of the membrane with anti-actin-HPR (AC-15, Sigma) 1:30000 for 1 hr at room temperature and 3 PBST washes.

4.14 Mouse model of adoptive T cell therapy of cancer

A fibroblast line originating from a $TRE^{loxP}stop^{loxP}$TagLuc transgenic mouse (Tet-TagLuc cells, Anders et al., 2011) was retrovirally transduced with HLA-A2 harbouring the murine H-$2D^b$ α3 domain and fused to human β_2m (HHD, Pascolo et al., 1997) and full-length NY-ESO with mCherry reporter expression from IRES to create Tet-TagLuc-NY-ESO-HHD cells. A cell clone (Tet-TagLuc-NY-ESO-HHD clone 1) was used for subcutaneous tumour challenge of DR4xRag$2^{-/-}$ recipient mice. After 30 days, tumours were palpable and the mice were treated with $1x10^6$ CD4 T cells from OTIIxRag$^{+/-}$ mice transduced with TCR-3598_2 and/or $1x10^6$ CD8 T cells from P14xRag$1^{-/-}$ mice transduced with TCR-ESO (Obenaus et al., 2015). As controls, CD4 and CD8 T cells were transduced with TCR-1367 recognizing MAGE-A1 on HLA-A2 (Obenaus et al., 2015). Tumour volumes were determined two or three times per week and were calculated by multiplying the three dimensions of the tumour divided by two.

To isolate $CD11b^+$ stromal cells, tumour pieces were incubated on a rotator at 37°C in RPMI containing 1 mg/ml collagenase II (Gibco), 1 mg/ml dispase II and 10 µg/ml DNase I (Roche). After one hour, 1x trypsin-EDTA (Gibco) was added for further incubation of 30 min. The filtered cell suspension was stained with anti-CD11b-PE (M1/70, BioLegend) and isolated following the user manual for anti-PE microbeads (Milteny).

Co-culture of TCR 3598_2-transduced CD4 T cells and $CD11b^+$ stromal cells was performed with $1x10^5$ cells each. $2x10^5$ dynabeads T activator CD3/CD28 or $NY\text{-}ESO_{116}$ peptide were added as positive control. IFNγ was measured in the supernatant by ELISA (BD OptEIA) after 16 hrs of incubation.

5 Results

5.1 ABabDR4 mice expressed HLA-DR4 and mounted specific CD4 T cell responses

First, ABabDR4 mice were analysed for expression of MHC II molecules to confirm expression of the transgene HLA-DR4-IE and absence of any murine MHC II molecules. HLA-DR4-IE was expressed on B cells from ABabDR4 mice to a similar extent as compared to DR4 mice (Figure 1A). The intrinsic I-E^b β chain, which does not come to the surface because of the non-functional I-E^b α chain in mice with C57BL/6 genetic background, could theoretically bind to HLA-DRA-IE (Lawrance et al., 1989). However, as published for the DR4 mice, no I-E was detected above background on B cells from ABabDR4 or DR4 mice (Figure 1A; Ito et al., 1996). AKR/J mice, which express I-E molecules of the k haplotype, served as a positive control, as the antibody binds to both k and b haplotype (Figure 1A). Moreover, absence of the murine MHC II molecule I-A was confirmed by surface staining of B cells and blood samples from C57BL/6 and Balb/c mice were taken as positive controls for b and d haplotype, respectively (Figure 1A). Finally, absence of the functional I-E α chain was confirmed by PCR, showing a shorter band harbouring the destructive deletion as for C57BL/6 mice, while the longer band from the intact chain was seen for Balb/c mice (Figure 1B).

To analyse whether ABabDR4 mice can mount a CD4 T cell immune response, they were immunized with a Pan-DR binding peptide (Padre), a chemically modified peptide binding to most HLA-DR and several mouse MHC II molecules including I-A^b in C57BL/6 mice (Alexander et al., 1994). Upon Padre restimulation of spleen and lymph node cells from an immunized ABabDR4 mouse, small percentages of CD4 T cells produced IFNγ and/or IL-2 (Figure 1C). This immune response was similar to the one seen for C57BL/6 mice, while the immune response of DR4 mice was smaller. Anti-CD3/CD28 activator beads and an irrelevant peptide from NS3 from hepatitis C virus served as positive and negative control, respectively (Figure 1C).

Taken together, ABabDR4 mice expressed exclusively DR4 as MHC II molecule and were able to mount a specific immune response. Consequently, ABabDR4 mice may serve as good model to raise human TCRs in a non-human host.

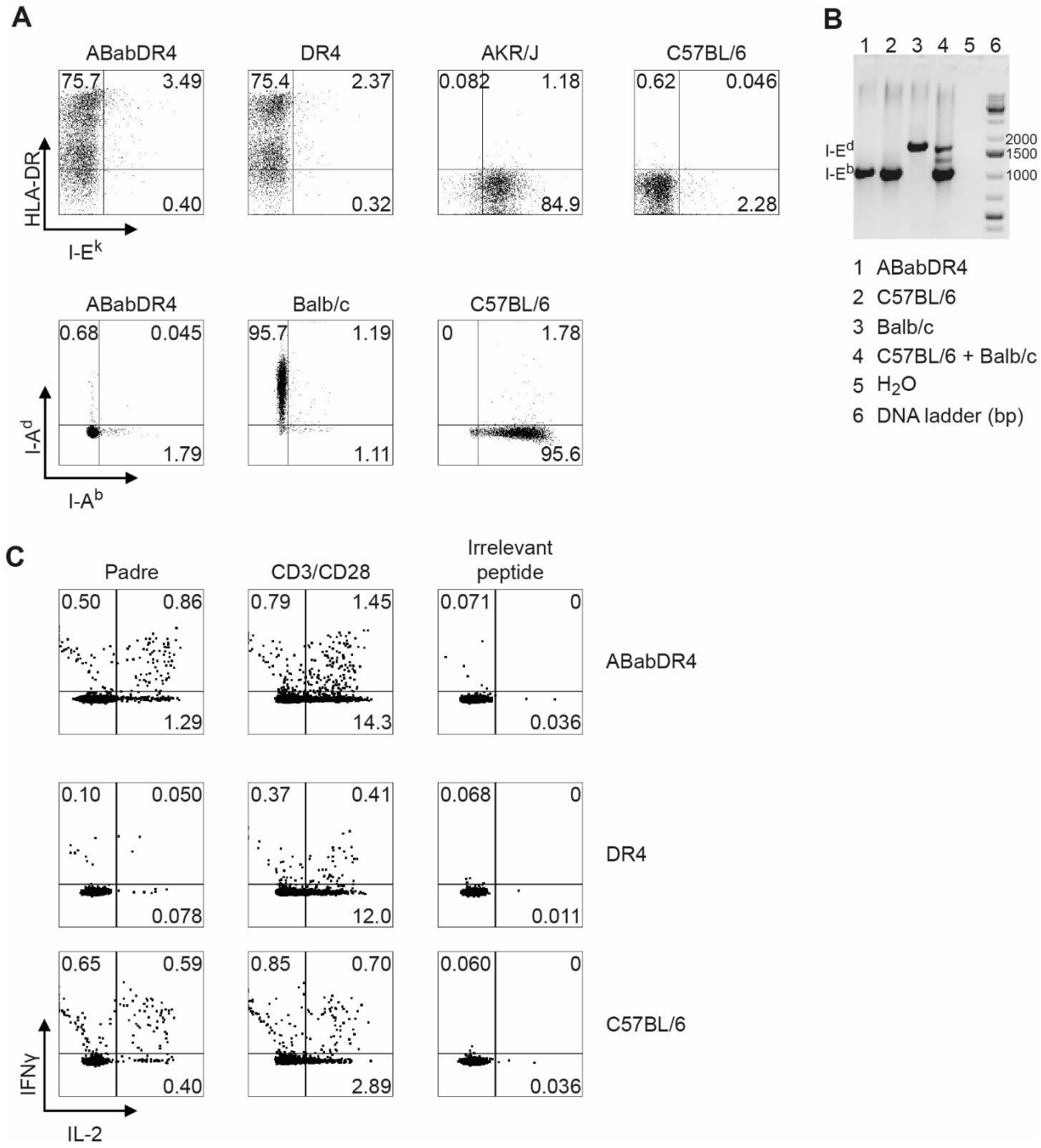


Figure 1 ABabDR4 mice expressed HLA-DR4-IE and mounted a specific CD4 T cell response. (A) Peripheral blood leukocytes from ABabDR4, DR4, AKR/J, C57BL/6 and Balb/c mice were stained for HLA-DR and I-$E^{k/b}$ and/or for I-A^d and I-A^b. Plotted cells were gated on lymphocytes and $CD19^+$ cells. (B) Tail DNA from indicated mouse strains were used for PCR with primers that flank a deletion present in the non-functional IE^b α chain that is present in C57BL/6 but not in Balb/c genetic background. (C) Splenocytes from ABabDR4, DR4 and C57BL/6 mice immunized twice with Pan-DR-binding peptide (Padre) were pulsed with Padre, anti-CD3/CD28 activator beads or irrelevant peptide (NS3 peptide) and were stained intracellularly after overnight incubation. Plotted cells were gated on lymphocytes, CD3 and CD4 positive cells. The results shown are representative of three mice.

5.2 NY-ESO$_{116-135}$ was immunogenic and naturally processed in ABabDR4 mice

To elicit T cell responses against NY-ESO, we immunized ABabDR4 mice with the 20-mer peptide NY-ESO$_{116-135}$ (NY-ESO$_{116}$), that has been described as immunogenic in a HLA-DR4 restriction setting (Zeng et al., 2000). As NY-ESO$_{116}$ has no sequence homology in the mouse, the T cell repertoire is not influenced by any tolerance mechanisms (Figure 2A). A distinct CD4 T cell response to NY-ESO$_{116}$ but not to an irrelevant peptide (Padre) appeared upon peptide restimulation of blood from immunized ABabDR4 mice (Figure 2B, top). To confirm natural processing of the NY-ESO$_{116}$ epitope, ABabDR4 mice were immunized with NY-ESO full length DNA. Similarly, CD4 T cells responded specifically to NY-ESO$_{116}$ (Figure 2B, bottom). To expand NY-ESO-reactive CD4 T cells, one-week in vitro cultures of splenocytes were prepared in presence of different peptide concentrations. Most efficient expansion of NY-ESO-reactive CD4 T cells occurred at a concentration of 10^{-8} M NY-ESO$_{116}$, which was applied in further expansion cultures (Figure 2C).

5.3 NY-ESO-reactive TCRs were isolated from ABabDR4 mice

To isolate NY-ESO-reactive TCRs, splenocytes from NY-ESO$_{116}$ peptide or NY-ESO DNA-immunized ABabDR4 mice were used. First, NY-ESO-reactive CD4 T cells were labelled by the IFNγ capture method and isolated by flow cytometry (Figure 3A). Two predominant TCRα and three predominant β chains were identified, which were matched by combinatorial expression and subsequent DR4/NY-ESO$_{116}$ tetramer staining and revealed TCR-3598 and TCR-3598_2 (Figure 3B). Three subsequent TCR isolations were conducted from DR4/NY-ESO$_{116}$ tetramer stained and sorted cells after one-week in vitro expansion and yielded one predominant TCRα and β chain combination each, TCRs 3600, 5712 and 5713 (Figure 3C). As ABabDR4 mice were boosted several times, narrowing down the T cell response to few clones that may be of optimal affinity is an expected outcome (Savage et al., 1999).

Of note, all identified TCRβ chains used the TRBV2 gene segment (Figure 4A). To confirm that the immune response to NY-ESO$_{116}$ is dominated by the TRBV2 gene segment, we stained peptide-restimulated splenocytes of an immunized ABabDR4 mouse for TRBV2 and IFNγ. Exclusively TRBV2^{+} CD4 T cells responded to NY-ESO$_{116}$ (Figure 4B). Human PBLs were retrovirally transduced with the TCRs containing mouse constant regions (Cohen et al., 2006) and stained with DR4/NY-ESO$_{116}$ tetramer (Figure 4C). In the TCR-3598_2-transduced sample not

all TCRβ$^+$ CD4 T cells bound the DR4/NY-ESO$_{116}$ tetramer. This phenomenon was also observed in transduced Jurkat/CD4 cells, that do not express endogenous TCRs (Figure 3B) and was therefore not due to formation of mixed TCR dimers composed of endogenous and transduced TCR chains but at least partly, to inefficient tetramer binding. Taken together, five different NY-ESO-reactive TCRs were identified from four immunized ABabDR4 mice.

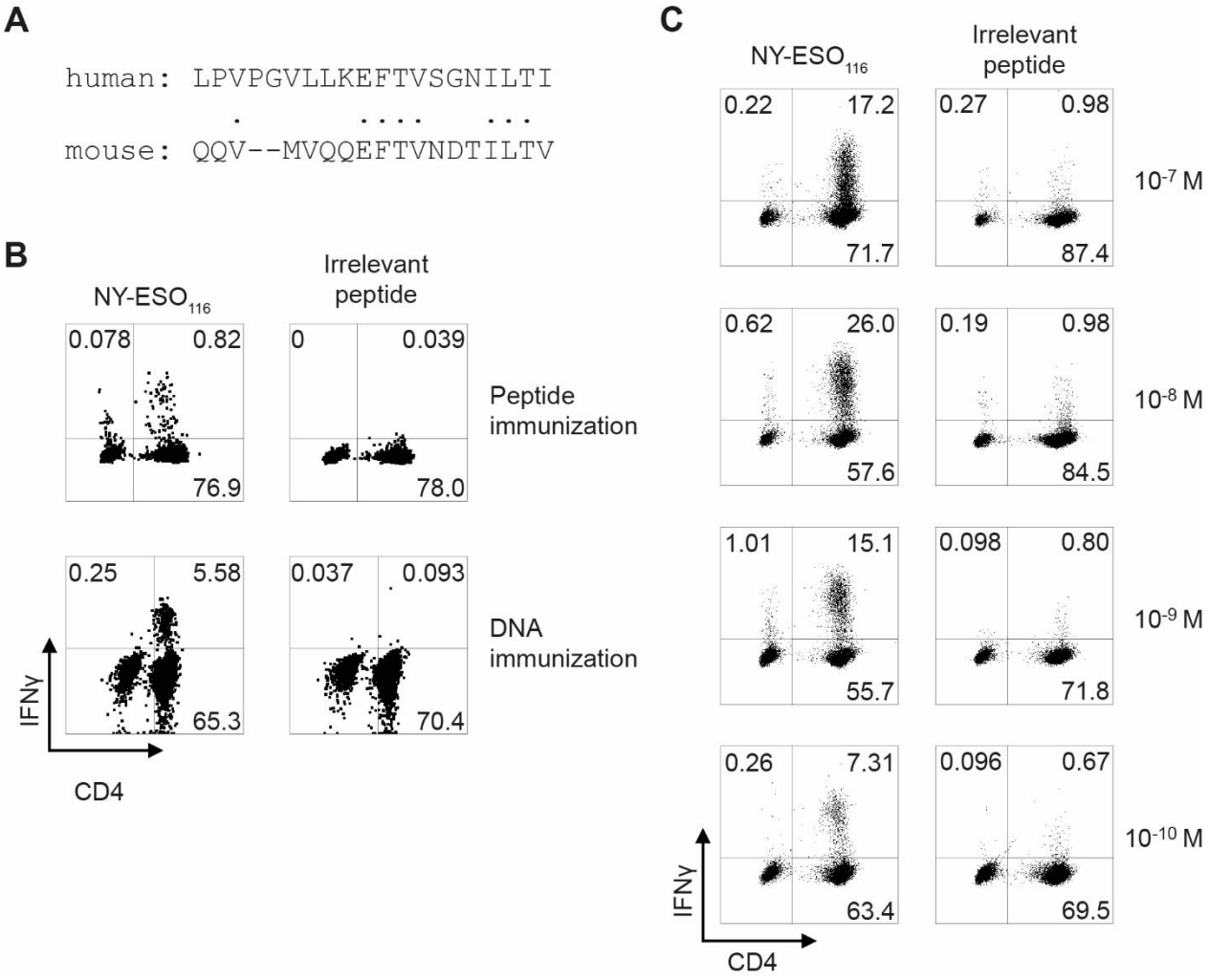


Figure 2 NY-ESO$_{116\text{-}135}$ was immunogenic and naturally processed in ABabDR4 mice. (A) The amino acid sequence of NY-ESO$_{116}$ and the closest homologous sequence in mice are displayed. **(B)** Peripheral blood leukocytes (PBLs) from an ABabDR4 mouse immunized with NY-ESO$_{116}$ peptide (upper row) or NY-ESO DNA (lower row) were pulsed with NY-ESO$_{116}$ or Padre as irrelevant peptide and were stained intracellularly after overnight incubation. The results shown are representative of >10 (upper row) and three (lower row) mice. (C) Splenocytes from an immunized ABabDR4 mouse were cultured one week in presence of different concentrations of NY-ESO$_{116}$ as indicated. Following peptide restimulation, intracellular cytokine staining was carried out. Plotted cells are gated on lymphocytes and CD3$^+$ cells.

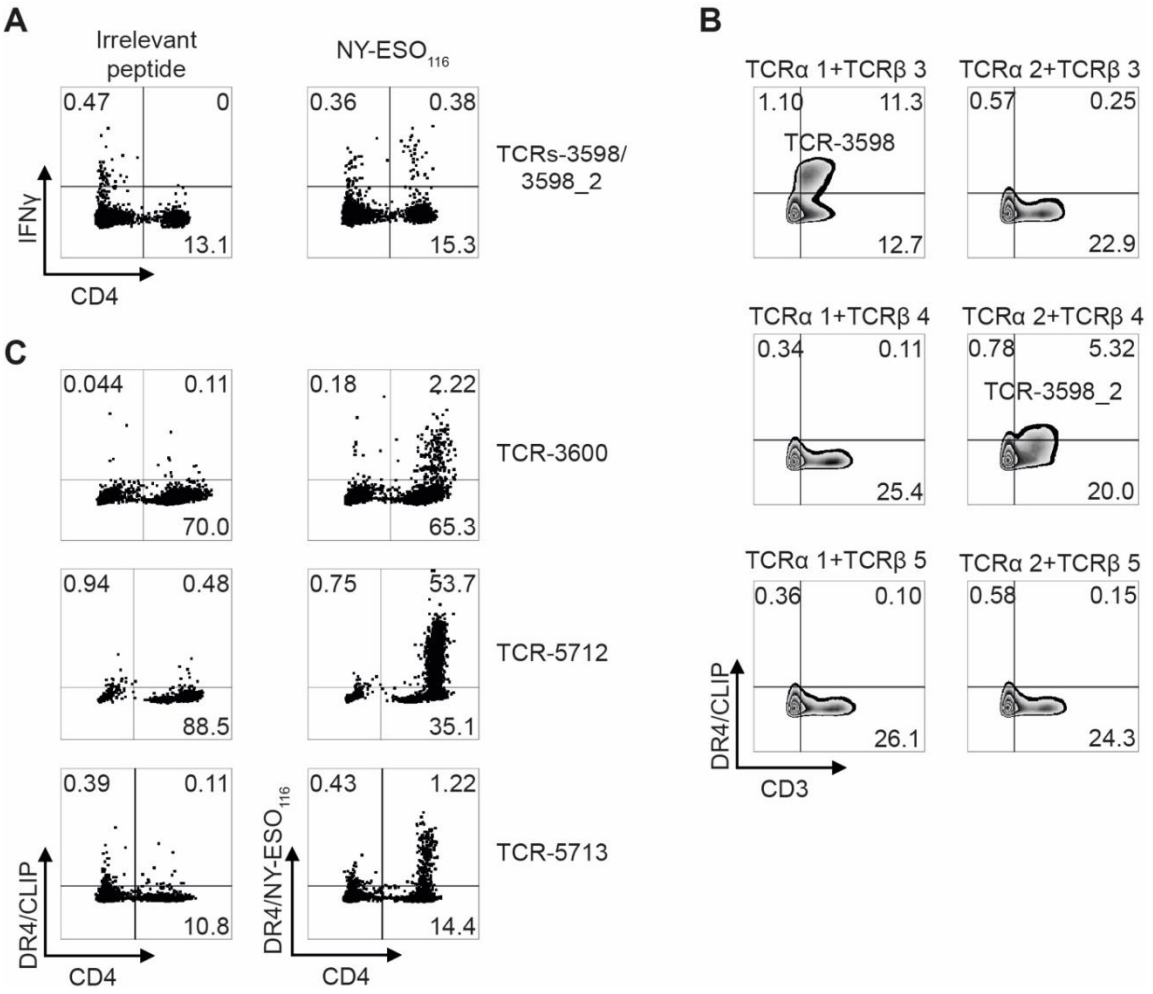


Figure 3 Labelling of NY-ESO-reactive CD4 T cells from immunized ABabDR4 mice for TCR isolation. (A) NY-ESO$_{116}$-reactive PBLs were stained for CD4 and labelled by the IFNγ capture method following NY-ESO$_{116}$ restimulation. Cells were gated on lymphocytes, CD3$^+$ cells and live cells. **(B)** Two TCRα and three TCRβ chains isolated from NY-ESO$_{116}$-reactive CD4 T cells shown in (A) were expressed in different combinations in TCR-deficient Jurkat76/CD4 cells and stained for CD3 and with DR4/NY-ESO$_{116}$ tetramer. **(C)** NY-ESO$_{116}$-reactive PBLs were stained by DR4/NY-ESO$_{116}$ tetramer following a one-week culture period in the presence of 10^{-8} M NY-ESO$_{116}$ (TCRs 3600 and 5712) or αCD3/CD28 beads (TCR-5713).

5.4 NY-ESO-reactive TCRs were isolated from human CD4 T cells

To compare the NY-ESO-reactive TCRs derived from ABabDR4 mice with TCRs from human individuals, in whom NY-ESO is a self-protein potentially leading to a skewed CD4 T cell repertoire, we isolated TCRs from human CD4 T cells. Based on the dominant TRBV2 gene segment usage of the NY-ESO-reactive CD4 T cells in ABabDR4 mice (Figure 4), we enriched human PBLs from a HLA-DR4$^+$ donor for CD4 T cells expressing TRBV2 and cultured them in the presence of NY-ESO$_{116}$ peptide. CD4 T cells depleted of TRBV2$^+$ cells were cultured alike as

control. After 2 weeks, more than two percent of the CD4 T cells of two similar in vitro cultures stained for DR4/NY-ESO$_{116}$ tetramer, while this was not the case for the TRBV2 depleted fraction (Figure 5A). From both cultures, DR4/NY-ESO$_{116}$ tetramer$^+$ CD4 T cells were sorted by flow cytometry and TCR sequences were isolated. In total, six functional TCRs were identified by combinatorial expression of single TCRα and β chains and subsequent DR4/NY-ESO$_{116}$ tetramer staining (Figure 5B, C). Single chains were considered for combinatorial expression, if they occurred at least twice in a total of more than 30 sequenced clones per TCR chain. Five TCRs, named NY1-NY5, were chosen for further analyses (Figure 6A). CD4 T cells transduced with those TCRs were stained with DR4/NY-ESO$_{116}$ tetramer (Figure 6B). Thus, human-derived NY-ESO-reactive TCRs were isolated to be compared in functional assays to the ABabDR4-derived TCRs.

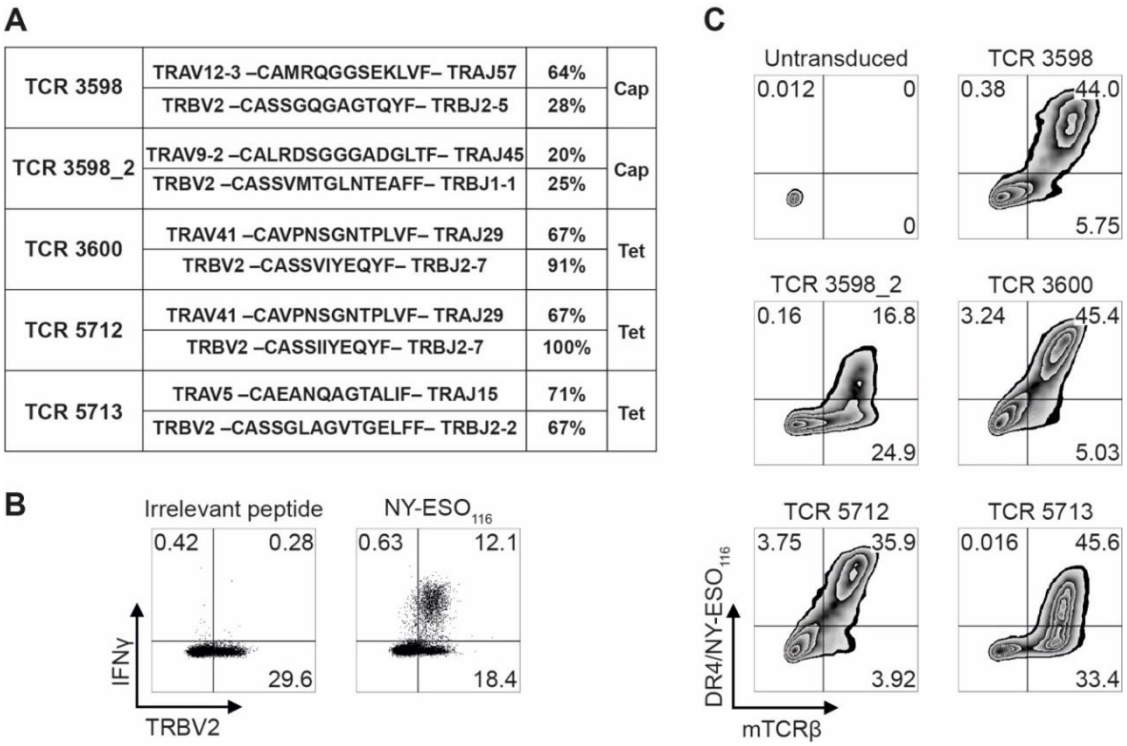
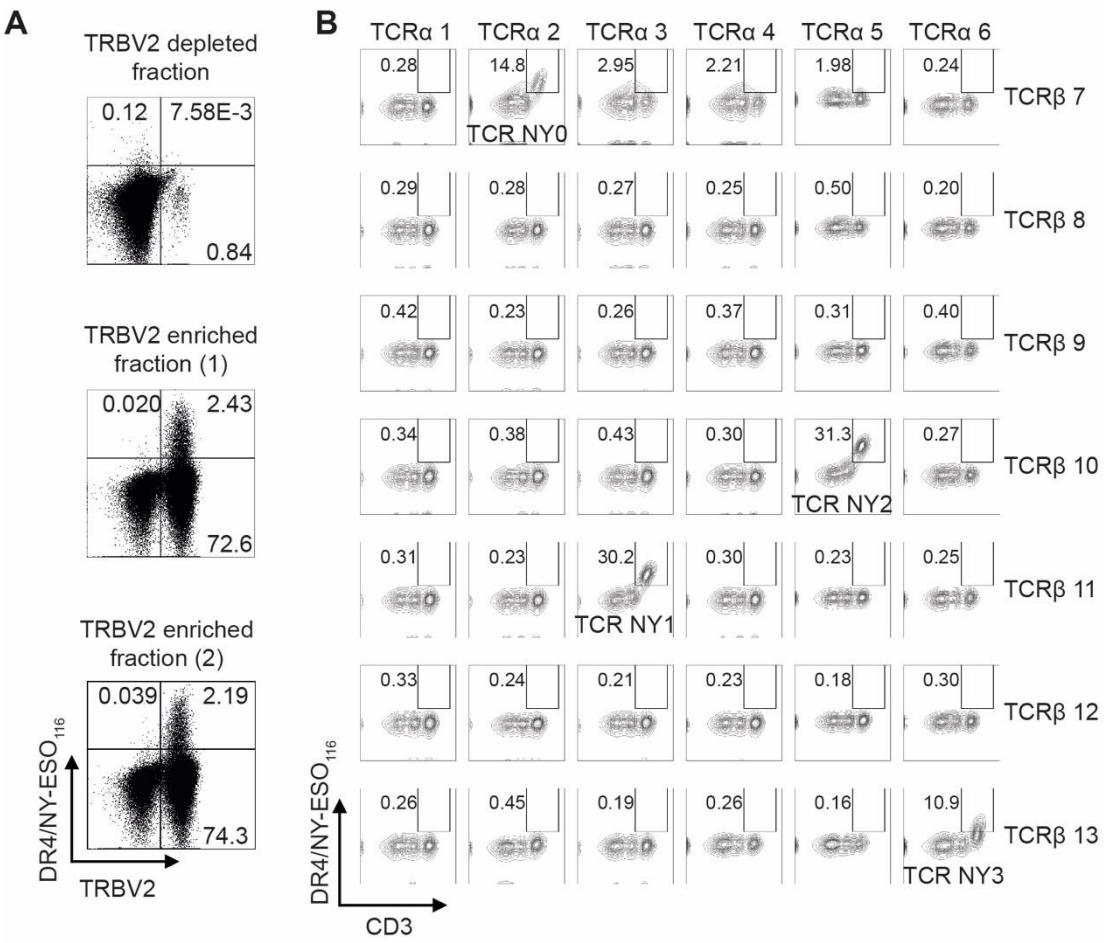


Figure 4 NY-ESO-reactive TCRs from ABabDR4 mice. (A) V and J gene segments and CDR3 regions of α and β chains of functional TCRs are shown that were isolated from the NY-ESO$_{116}$-reactive CD4 T cells labelled either by the IFNγ capture method (Cap) or by DR4/NY-ESO$_{116}$ tetramer (Tet) as shown in Figure 3. The percentages refer to the frequency in total isolated TCRα or β sequences. **(B)** Splenocytes from an ABabDR4 mouse immunized with NY-ESO DNA were pulsed with Padre as irrelevant peptide or NY-ESO$_{116}$ and stained intracellularly after 6 hours of incubation. Plotted cells were gated on lymphocytes, CD3$^+$ and CD4$^+$ and live cells. **(C)** Human CD4 T cells were transduced with the NY-ESO-reactive TCRs listed in (A) and stained with DR4/NY-ESO$_{116}$ tetramer. Plotted cells were gated on lymphocytes, live cells and CD3$^+$ cells.


A
TRBV2 depleted fraction
0.12
7.58E-3
0.84
TRBV2 enriched fraction (1)
0.020
2.43
72.6
TRBV2 enriched fraction (2)
0.039
2.19
74.3
DR4/NY-ESO116
TRBV2
B
TCRα 1
TCRα 2
TCRα 3
TCRα 4
TCRα 5
TCRα 6
TCRβ 7
TCRβ 8
TCRβ 9
TCRβ 10
TCRβ 11
TCRβ 12
TCRβ 13
0.28
14.8
2.95
2.21
1.98
0.24
TCR NY0
0.29
0.28
0.27
0.25
0.50
0.20
0.42
0.23
0.26
0.37
0.31
0.40
0.34
0.38
0.43
0.30
31.3
0.27
TCR NY2
0.31
0.23
30.2
0.30
0.23
0.25
TCR NY1
0.33
0.24
0.21
0.23
0.18
0.30
0.26
0.45
0.19
0.26
0.16
10.9
TCR NY3
DR4/NY-ESO116
CD3
C
TCRα 1
TCRα 2
TCRα 3
TCRα 4
TCRα 5
TCRα 6
TCRα 7
TCRα 8
TCRβ 9
TCRβ 10
TCRβ 11
TCRβ 12
TCRβ 13
TCRβ 14
0.45
0.51
11.7
0.43
0.28
0.39
0.40
0.37
TCR NY4
0.23
1.38
0.36
0.34
0.30
0.32
0.35
0.25
0.13
0.21
0.33
0.24
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0.27
0.33
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0.30
0.34
0.34
0.27
0.31
0.22
0.22
0.38
0.36
0.34
3.81
0.37
0.25
0.24
0.22
2.60
0.37
12.1
0.27
0.36
0.33
TCR NY5
DR4/NY-ESO116
CD3

Figure 5 In vitro expansion of NY-ESO-reactive human CD4 T cells. (A) PBLs from a HLA-DR4$^+$ donor were enriched for CD4 T cells expressing the TCRβ variable segment 2 (TRBV2) and cultured in the presence of 2 μM NY-ESO$_{116}$ and irradiated CD4$^-$ cells as feeders. The CD4$^+$ fraction depleted of TRBV2$^+$ cells was cultured alike as control. After two weeks, NY-ESO$_{116}$-reactive cells were stained with DR4/NY-ESO$_{116}$ tetramer and sorted by flow cytometry for isolation of the TCR chains. Two similar in vitro cultures are shown (1 and 2). Displayed cells were gated on lymphocytes, live, CD3$^+$ and CD4$^+$ and CD8$^-$ cells. **(B, C)** TCRα and β chains isolated from PBL in vitro cultures shown in (A) were expressed in different combinations in TCR-deficient 58 cells and stained for CD3 and with DR4/NY-ESO$_{116}$ tetramer. TCR NY0 was not considered in further experiments, because initial tetramer staining technically failed.

A

TCR-NY1	TRAV9-2 –CALSDPRGGSQGNLIF– TRAJ42	6%
	TRBV2 –CASRGEGYGYTF –TRBJ1-2	9%
TCR-NY2	TRAV38-1 –CAFMQGKSTF– TRAJ27	6%
	TRBV2 –CASGYQNQPQHF–TRBJ1-5	9%
TCR-NY3	TRAV36 –CAVIIQGAQKLVF– TRAJ54	6%
	TRBV2 –CASSTQTELPQHF– TRBJ1-5	6%
TCR-NY4	TRAV20 –CAGTQGGSEKLVF– TRAJ57	19%
	TRBV2 –CASSVMGTQYF – TRBJ2-3	23%
TCR-NY5	TRAV38-1 –CAFISTQGGSEKLVF– TRAJ57	13%
	TRBV2 –CASSPLRPSYEQYF– TRBJ2-7	6%

B

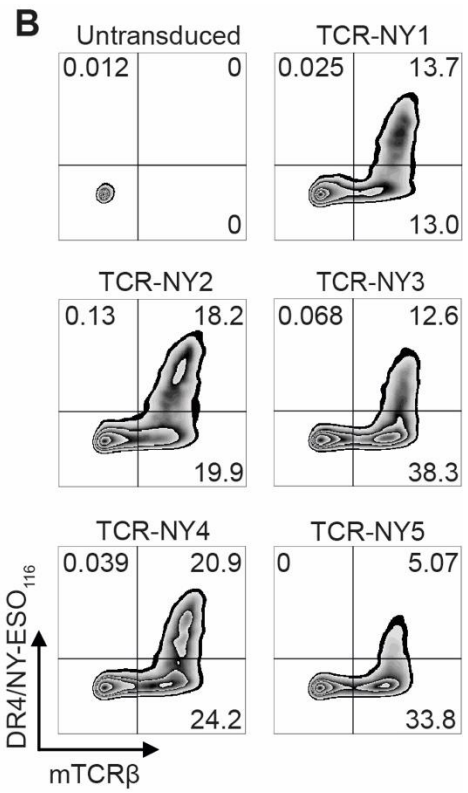


Figure 6 NY-ESO-reactive TCRs from CD4 T cells of a human donor (A) V and J gene segments and CDR3 regions of α and β chains of functional TCRs identified by combinatorial expression as shown in Figure 5 are listed. TCRs NY1-3 and TCRs NY4-5 were isolated from two similar in vitro cultures. The percentages refer to the frequency in the total isolated TCRα or β sequences. **(B)** Human CD4 T cells were transduced with the NY-ESO-reactive TCRs listed in (A) and stained with DR4/NY-ESO$_{116}$ tetramer. The untransduced sample is the same as shown in Figure 4C since experiments in Figure 6B and 4C were performed in parallel. Plotted cells were gated on lymphocytes, live cells and CD3$^+$ cells.

5.5 ABabDR4-derived TCRs recognized NY-ESO more efficiently than human-derived TCRs

First, the TCRs were tested for function by co-culturing TCR-transduced CD4 T cells with NY-ESO$_{116}$-loaded or NY-ESO-transduced cell lines. To enhance MHC II expression, melanoma cell lines were pre-treated with IFNγ, yielding HLA-DR-expression comparable to the untreated LCL BSM (Figure 7A). NY-ESO protein expression by the transduced or naturally expressing melanoma cell lines was confirmed by Western Blot (Figure 7B). All TCRs recognized the HLA-DR4$^+$ melanoma cell line FM3 loaded with NY-ESO$_{116}$ (Figure 7C, left). However, the NY-ESO-transduced lines FM3 and BSM (both HLA-DR4$^+$) were recognized by all ABabDR4-derived TCRs, but not or to a lesser extent by the human-derived TCRs (Figure 7C, right). The recognition of the NY-ESO-transduced lines was blocked by αHLA-DR antibody, confirming HLA-DR4-mediated recognition. Taken together, TCRs from both settings were able to recognize loaded NY-ESO$_{116}$, but ABabDR4-derived TCRs did better in recognizing processed NY-ESO in transduced cell lines.

Next, we sought to determine, whether the NY-ESO-reactive TCRs could recognize naturally NY-ESO-expressing melanoma cell lines. To this end we co-cultured TCR-transduced CD4 T cells with IFNγ-pre-treated melanoma cell lines that naturally express NY-ESO and/or HLA-DR4. Four of five ABabDR4-derived TCRs (3598_2, 3600, 5712, 5713) recognized the NY-ESO and HLA-DR4-expressing melanoma cell lines FM56 and FM82, while two of the human-derived TCRs (NY2 and NY3) recognized, albeit weakly, the melanoma cell line FM56 (Figure 7D). Again, αHLA-DR antibody blocked recognition. As expected, the NY-ESO-negative cell line FM3 and the HLA-DR4-negative cell line FM6 were not recognized and addition of PMA/Ionomycin elicited IFNγ secretion in all TCR-transduced CD4 T cells (Figure 7D).

To further characterize the TCRs, we tested TCR-transduced CD4 T cells in a peptide titration assay by co-culturing them with K562/HLA-DR4 cells that were loaded with decreasing amounts of NY-ESO$_{119}$. All ABabDR4-derived and two human-derived TCRs showed recognition up to 10^{-10} M, while three human-derived TCRs were less sensitive and showed recognition up to a peptide concentration of 10^{-9} M (Figure 8). ABabDR4-derived TCRs elicited higher maximal IFNγ concentrations than the human-derived TCRs. Taken together, ABabDR4-derived TCRs recognized more efficiently NY-ESO-transduced cell lines and naturally NY-ESO-expressing melanoma cell lines and were more sensitive in a peptide titration assay compared to the human-derived TCRs.

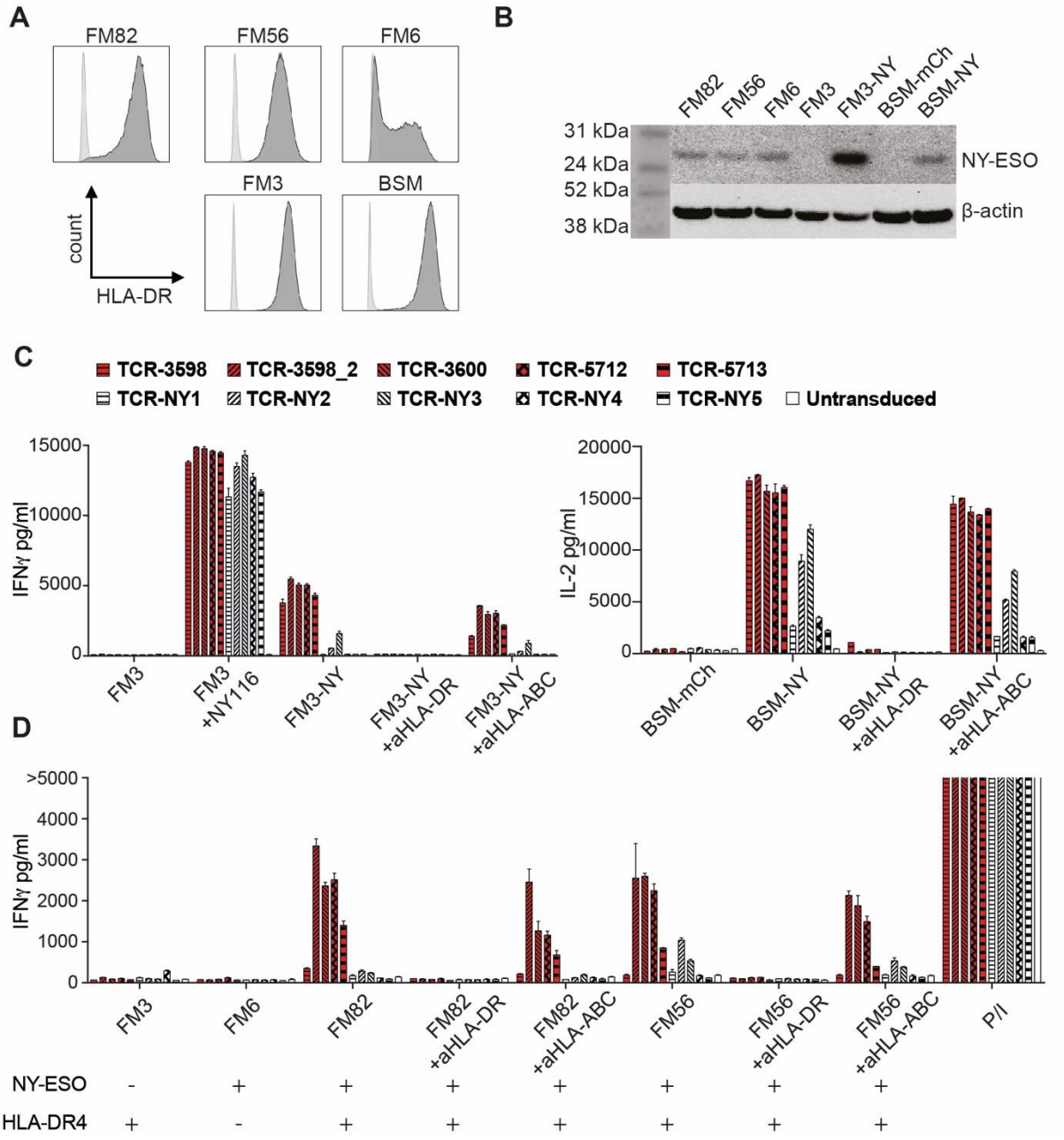


Figure 7 ABabDR4-derived TCRs recognized NY-ESO$^+$ melanoma cell lines more efficiently than human-derived TCRs. (A) Melanoma cell lines pre-treated with IFNγ and the LCL BSM were stained for HLA-DR (dark shading) or isotype control (light shading) and measured by flow cytometry. **(B)** Protein lysates from cell lines used for co-culture experiments in (C) and (D) were assessed for presence of NY-ESO protein. α-Actin was stained as protein loading control. **(C, D)** TCR-transduced CD4 T cells were co-cultured with the LCL BSM (HLA-DR4$^+$) and the melanoma cell lines FM3 (NY-ESO$^-$, HLA-DR4$^+$), FM6 (NY-ESO$^+$, HLA-DR4$^-$), FM82 and FM56 (both NY-ESO$^+$, HLA-DR4$^+$). Cell lines FM3-NY and BSM-NY were transduced to express NY-ESO, BSM-mCh was transduced with mCherry as control. NY-ESO$_{116}$ (NY116), PMA and Ionomycin (P/I) and blocking antibodies αHLA-DR or αHLA-ABC were added where indicated. After overnight incubation IFNγ or IL-2 was measured in the supernatant. Mean values of intra-assay duplicates with standard deviation are shown. The results are representative of three independent experiments performed with PBLs from different donors.

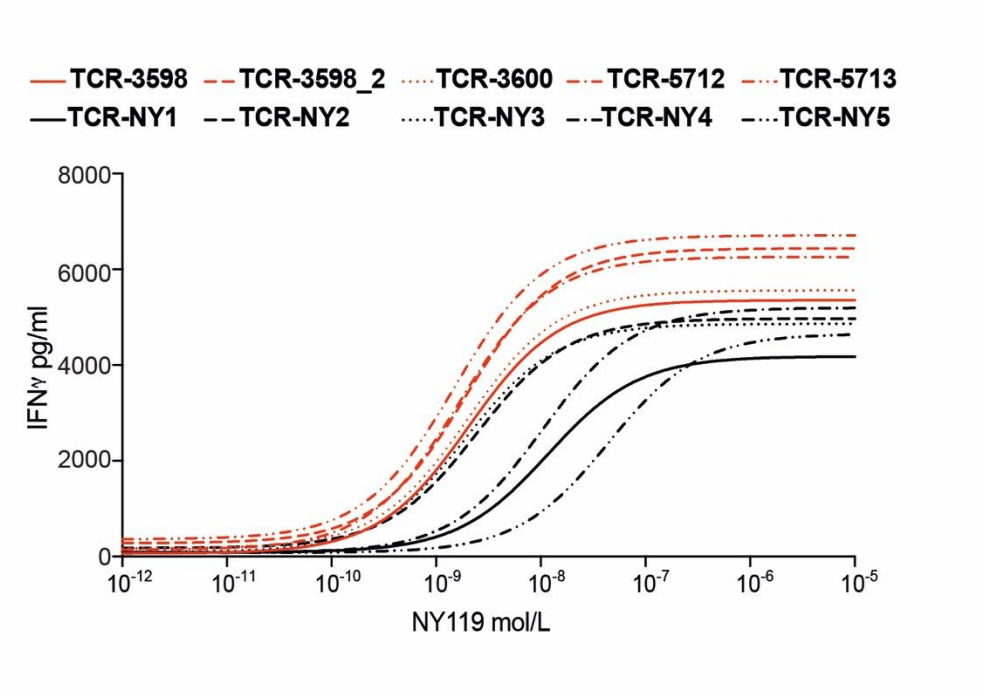
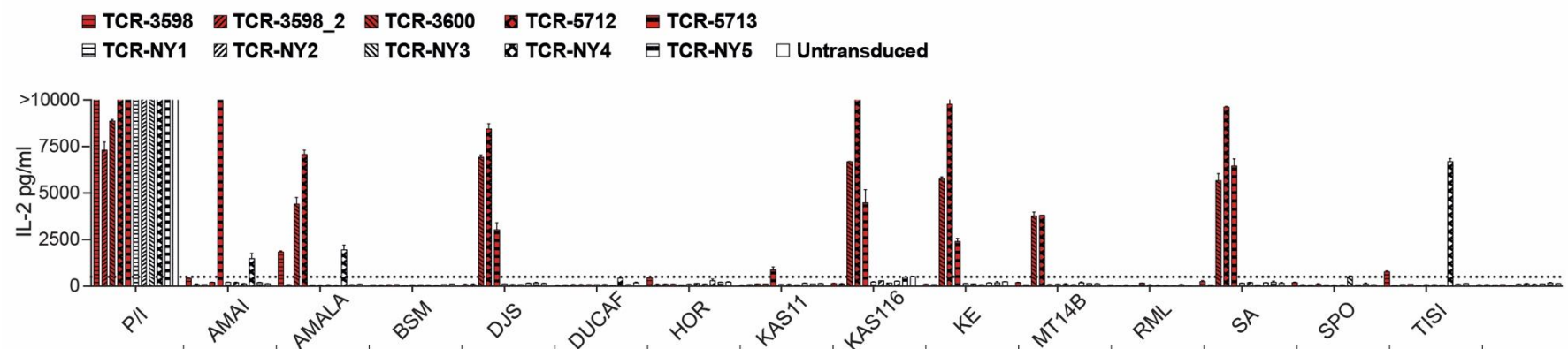


Figure 8 ABabDR4-derived TCRs were more sensitive to NY-ESO than human-derived TCRs. TCR-transduced CD4 T cells were co-cultured with K562/DR4 cells loaded with decreasing concentrations of NY-ESO$_{119}$ (NY119). After overnight incubation, IFNγ was measured in the supernatant. The data were fitted in three-parameter dose-response curves. The results are representative of three independent experiments performed with PBLs from different donors.

5.6 ABabDR4-derived TCR-3598_2 showed no alloreactivity or cross-reactivity

As in the ABabDR4 mouse only one MHC II allele is present and in the human individual only a limited number of MHC alleles, we investigated the NY-ESO-reactive TCRs for potential MHC alloreactivity. For this, TCR-transduced CD4 T cells were co-cultured with a panel of 14 LCLs expressing different MHC I and II molecules (Appendix 1). The threshold for reactivity was set to 500 pg/ml IL-2 in the supernatant, the highest background observed in this assay. Four ABabDR4-derived TCRs (TCRs 3598, 3600, 5712, 5713) and one human-derived TCR (TCR-NY4) reacted to two or more LCL (Figure 9). Which MHC molecule was recognized by the cross-reactive TCRs was not further analysed. The ABabDR4-derived TCR 3598_2 did not react to any LCL.



	P/I	AMAI	AMALA	BSM	DJS	DUCAF	HOR	KAS11	KAS116	KE	MT14B	RML	SA	SPO	TISI
TCR-3598			X												X
TCR-3598_2															
TCR-3600			X		X				X	X	X		X		
TCR-5712			X		X				X	X	X		X		
TCR-5713		X			X			X	X	X			X		
TCR-NY1															
TCR-NY2															
TCR-NY3															
TCR-NY4		X	X												X
TCR-NY5															

Figure 9 The ABabDR4-derived TCR-3598_2 showed no alloreactivity. TCR-transduced CD4 T cells were co-cultured with a panel of LCLs expressing different MHC class I and II molecules (Appendix 1). As positive control PMA and Ionomycin (P/I) were added to the T cells. After overnight incubation IL-2 was measured in the supernatant. Background is indicated by the dotted line. Mean values of intra-assay duplicates with standard deviation are shown. In the grid below, cross-reactions to LCLs are summarized. The results are representative of three independent experiments performed with PBLs from different donors.

For further analysis of potential cross-reactivity, we identified the recognition motifs of the NY-ESO-reactive TCRs. The 15-mer NY-ESO$_{119}$ was sequentially mutated to alanine at each position and recognition by the TCRs was tested at a concentration of 10^{-7} M (Figure 10A). In total, nine different recognition motifs were identified from the 10 TCRs. While all TCRs required the lysine in the 6th position, all but TCR-5713 required the glutamic acid in the 7th position and all but TCR-NY5 required the phenylalanine in the 8th position. Of note, there was no clear-cut difference in recognition by the ABabDR4-derived and the human-derived TCRs.

As in the ABabDR4 mouse, TCRs are negatively selected on mouse and not human self-peptides, peptides that are not present in the mouse are potentially cross-reactive. As TCR-3598_2 did not show alloreactivity in the LCL co-culture, it was further tested with peptides containing its recognition motif (X-X-X-X-L-K-E-F-X-X-X-X-X-X-X). Peptides were included if they had a predicted IC50 binding affinity to HLA-DR4 of below 500 nM and are present in the human but not the mouse proteome (Appendix 2). From the 50 peptides fulfilling these criteria, one peptide, X-ray radiation resistance-associated protein 1 (XRRA1)$_{729\text{-}743}$ was recognized by TCR-3598_2 at 10^{-6} M but not 10^{-7} M (Figure 10B). To exclude that this cross-reaction is relevant in a physiological setting where XRRA1 must be processed and loaded onto MHC II, TCR-3598_2-transduced CD4 T cells were co-cultured with full length XRRA1-transduced HLA-DR4^{+} BSM. XRRA1-transduced BSM were recognized only when loaded externally with NY-ESO$_{116}$ (Figure 10C). NY-ESO-transduced BSM were recognized as expected. Thus, no relevant cross-reactivity of TCR-3598_2 was detected.

5.7 NY-ESO-specific MHC I- and II-restricted TCRs synergize in tumour regression

To confirm the functionality of TCR-3598_2 in an in vivo setting, we set up a model of ATT in which tumour-bearing mice were treated with NY-ESO-specific CD4 and CD8 T cells in combination. One month after DR4xRag$^{-/-}$ mice received a subcutaneous injection of fibrosarcoma cells (Tet-TagLuc-NY-ESO-HHD clone 1), tumours were palpable and the mice were treated with TCR-3598_2-transduced CD4 T cells and CD8 T cells transduced with a TCR recognizing NY-ESO$_{157\text{-}165}$ on HLA-A2, named TCR-ESO (Obenaus et al., 2015). Thus, in this model CD8 T cells recognize only cancer cells via HHD (HLA-A2-H-2D^{b} chimeric) but not cross-presented antigen by tumour stroma cells, while CD4 T cells recognize only cross-presented antigen by stroma cells, since HHD is only present on the cancer cells and HLA-DR4 is

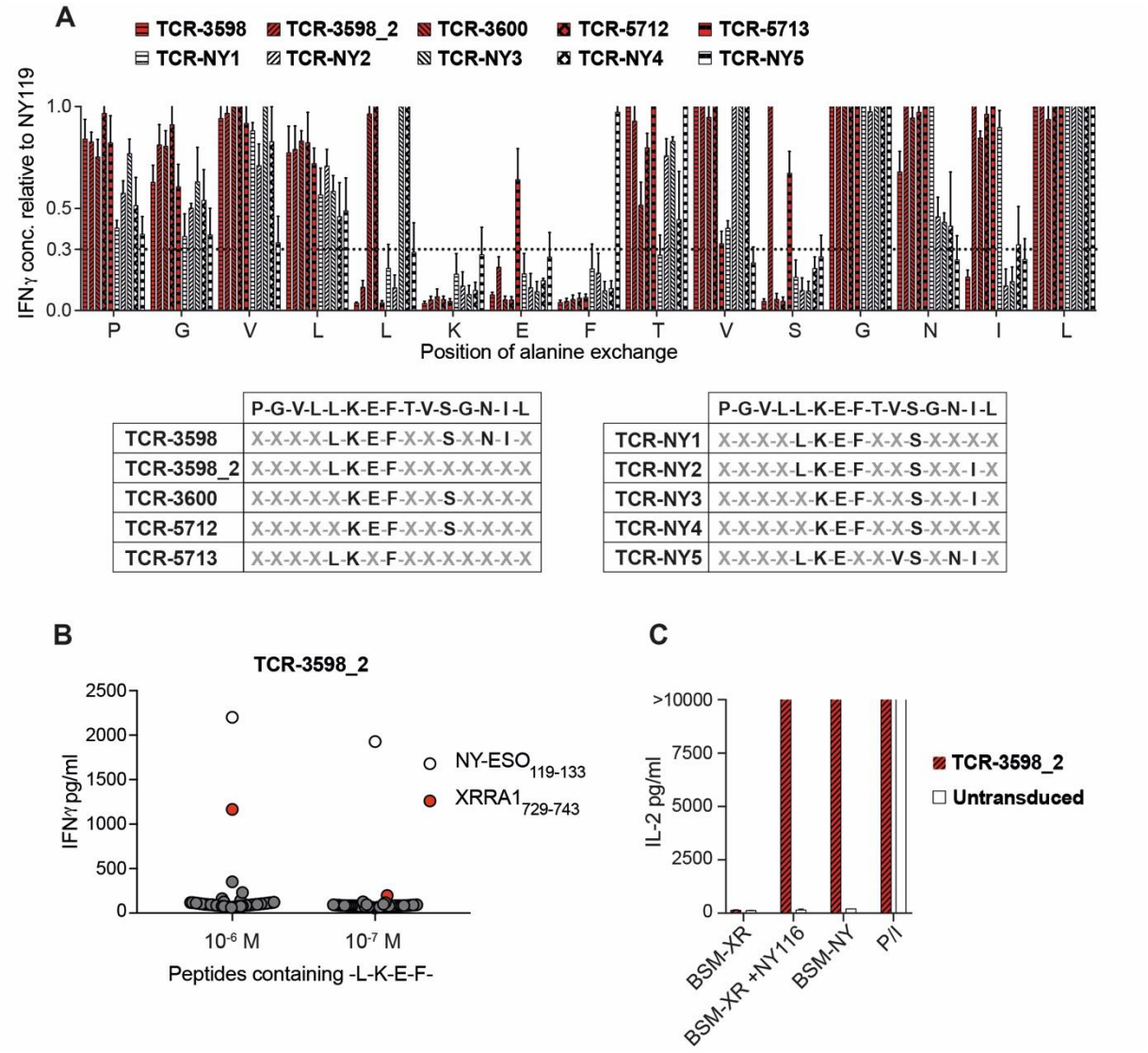


Figure 10 The ABabDR4-derived TCR-3598_2 showed no cross-reactivity. (A) TCR-transduced CD4 T cells were co-cultured with K562/DR4 cells that were loaded with NY-ESO$_{119}$ containing single alanine exchanges at 10^{-7} M. After overnight incubation, IFNγ was measured in the supernatant. An amino acid was rated as recognition site when the response to the respective alanine exchanged peptide was less than one third as compared to the unchanged NY-ESO$_{119-133}$ at a peptide concentration of 10^{-7} M. **(B)** TCR-3598_2-transduced CD4 T cells were incubated with K562/DR4 cells loaded with 50 different peptides (Appendix 2) containing the recognition motif L-K-E-F. Peptides were included if they had a predicted IC50 binding affinity to HLA-DR4 of below 500 nM and are present in the human but not the mouse proteome. After overnight incubation IFNγ was measured in the supernatant. **(C)** TCR-3598_2-transduced CD4 T cells were incubated with the HLA-DR4^{+} BSM transduced to express XRRA1 (XR) or NY-ESO (NY). NY-ESO$_{116}$ was added where indicated. Displayed are IL-2 levels in the supernatant after overnight incubation. Mean values of three independent experiments with standard deviation (A) or mean values of intra-assay duplicates with standard deviation (B, C) are shown. The results are representative of two independent experiments performed with PBLs from different donors (B, C).

only present on the host cells. As controls, NY-ESO-specific CD4 or CD8 T cells alone or CD4 and CD8 T cells expressing an irrelevant TCR were given. In group 1, which received NY-ESO-specific CD4 T cells alone, tumour growth slowed down, while in group 2, which received NY-ESO-specific CD8 T cells alone, 4 out of 10 tumours regressed (Figure 11A). In the control group 4, which received CD4 T cells transduced with an irrelevant TCR together with NY-ESO-specific CD8 T cells, 5 out of 8 tumours regressed, while all tumours grew out in the groups 5 and 6, which received a combination of both irrelevant CD4 and CD8 T cells or no T cells, respectively. Only in group 3, which received a combination of NY-ESO-specific CD4 and CD8 T cells, all tumours (10/10) regressed (Figure 11A).

Analysis of T cells in peripheral blood of the treated mice revealed a higher number of TCR-transduced CD8 T cells in group 3, which received NY-ESO-specific CD4 and CD8 T cells in combination, compared to all other groups (Figure 11B). Numbers of TCR-transduced CD4 T cells were higher in groups 1 and 3, which received TCR-3598_2-transduced CD4 T cells, compared to groups 4 and 5, which received irrelevant CD4 T cells (Figure 11B).

Finally, we analysed cross-presentation of NY-ESO by macrophages to CD4 T cells. To this end we co-cultured TCR-3598_2-transduced CD4 T cells with $CD11b^+$ stromal cells purified from a control-treated tumour (group 5). The TCR-3598_2-transduced CD4 T cells recognized the $CD11b^+$ stromal cells, while this was not the case for untransduced CD4 T cells (Figure 11C). Externally loaded NY-ESO_{116} stimulated abundant IFNγ secretion by TCR-3598_2-transduced CD4 T cells and αCD3/CD28-activating beads stimulated IFNγ secretion in the untransduced CD4 T cells as well. Thus, in vivo functionality of TCR-3598_2 was confirmed in a mouse model of ATT.

5.8 A clinical version of TCR-3598_2 displayed comparable function

For future clinical application, the fully mouse constant region (mc) of the TCR candidate TCR-3598_2 was exchanged for a human constant region containing 9 amino acids from the mouse sequence referred to as minimally murinized constant region (mmc) (Sommermeyer and Uckert, 2010). Moreover, additional cysteine bridges were included (Cohen et al., 2007). Surface expression of TCR-3598_2 mc and mmc were comparable with slightly higher mean fluorescence intensity for the TCR- 3598_2 mc as judged by vβ staining of transduced human T cells (Figure 12A). Staining of DR4/NY-ESO_{116} tetramer of both versions was only partly

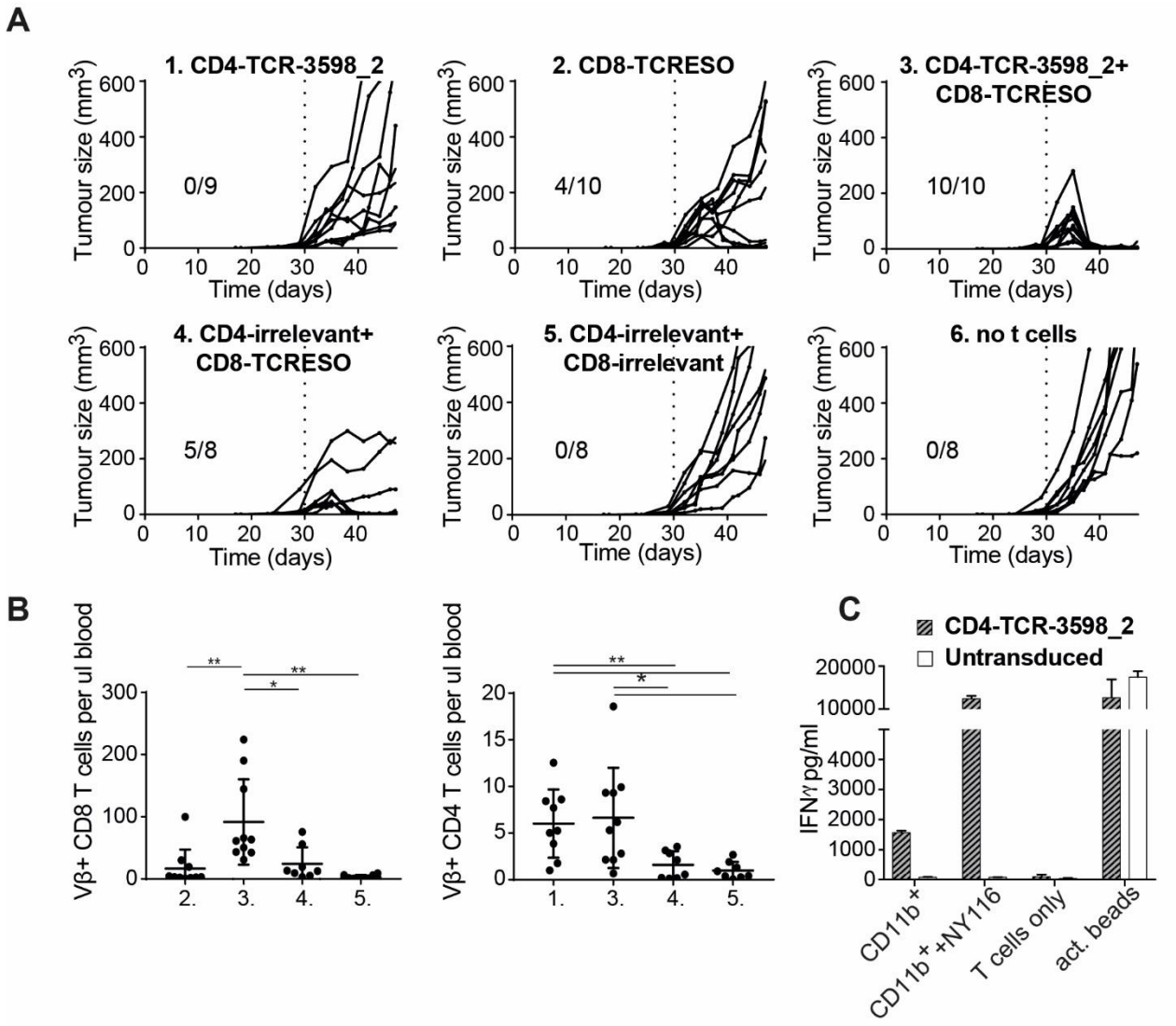


Figure 11 TCR-3598_2-transduced CD4 T cells in combination with TCR-ESO-transduced CD8 T cells caused tumour regression. (A) Tumour-bearing mice were treated with TCR-3598_2-transduced CD4 T cells and/or TCR-ESO-transduced CD8 T cells at day 30, when the transplanted tumours were palpable. TCR-1367-transduced CD4 and/or CD8 T cells were injected as controls where indicated (CD4-/CD8-irrelevant). Shown are tumour sizes days after tumour cell injection. **(B)** Adoptively transferred TCR-transduced ($V\beta^+$) CD8 and CD4 T cells were detected in blood 9 days after treatment. Group numbers refer to (A). Results from two independent experiments were combined (A, B) t test $*P< 0.05$, $**P<0.01$. **(C)** $CD11b^+$ stromal cells isolated from isolated tumour material were recognized by TCR-3598_2-transduced CD4 T cells. As positive controls, $CD11b^+$ stromal cells were loaded with $NY\text{-}ESO_{116}$ or anti-CD3/CD28 activator beads were added to the T cells. Mean values of intra-assay duplicates with standard deviation are shown. The results are representative of two independent experiments.

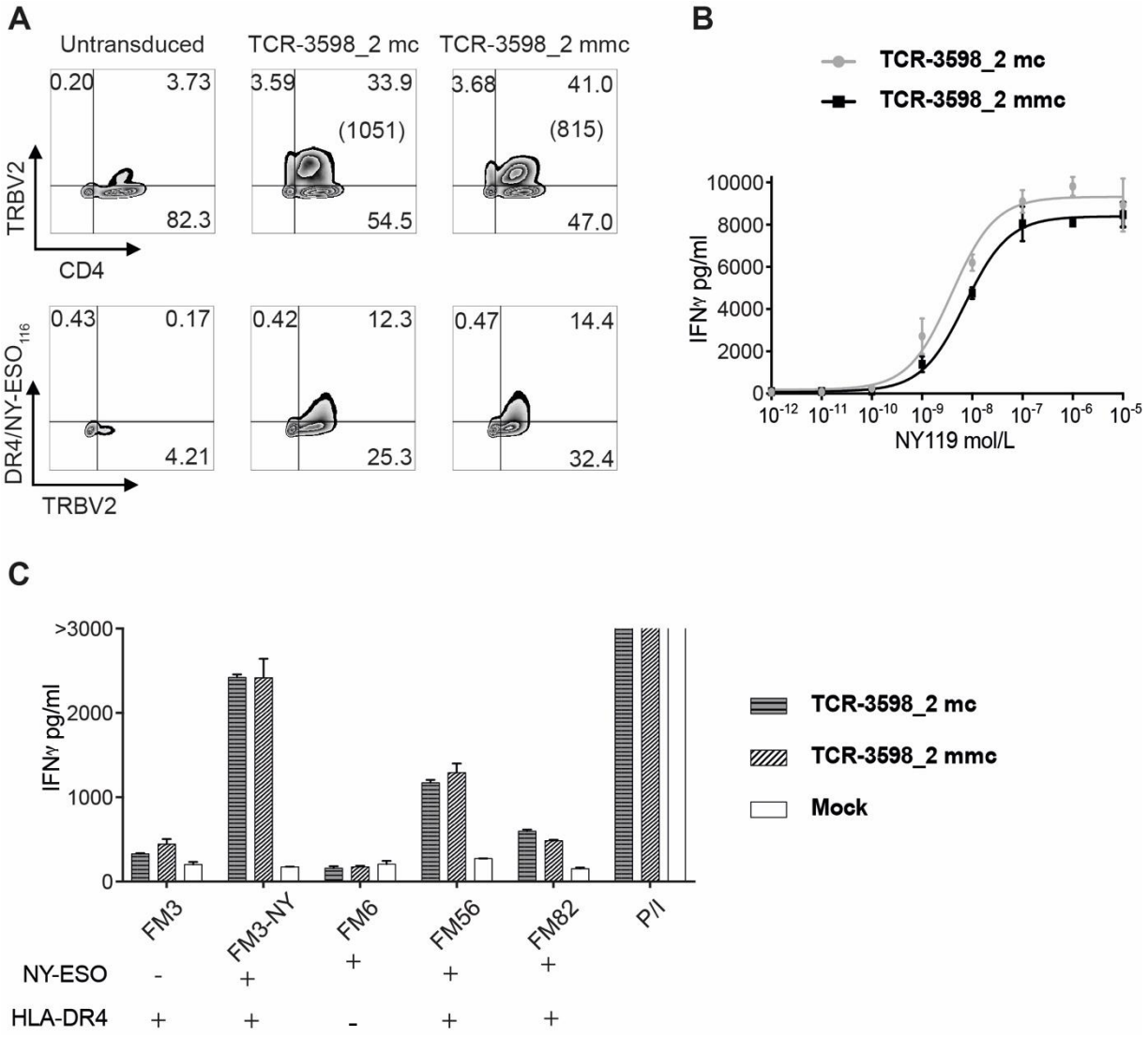


Figure 12 TCR-3598_2 mc and TCR-3598_2 mmc displayed similar functionality. (A) Human CD4 T cells were transduced with TCR-3598_2 mc or TCR-3598_2 mmc and stained for CD4, TRBV2 and DR4/NY-ESO$_{116}$ tetramer. Numbers in brackets are mean fluorescence intensity. Plotted cells were gated on lymphocytes (top) and CD4$^+$ cells (bottom) **(B)** TCR-transduced CD4 T cells were co-cultured with K562/DR4 cells loaded with decreasing concentrations of NY-ESO$_{119}$ (NY119). After overnight incubation, IFNγ was measured in the supernatant. The data were fitted in three-parameter dose-response curves. Mean values of intra-assay duplicates with standard deviation are shown. **(C)** TCR-transduced CD4 T cells were co-cultured with IFNγ-pretreated melanoma cell lines or PMA and Ionomycin (P/I) as positive control. After overnight incubation, IFNγ was measured in the supernatant. The results are representative of two experiments performed with PBLs from different donors.

positive as observed previously (Figure 12A, 3B, 4C). To compare functionality of TCR-3598_2 mc and mmc, peptide sensitivity and recognition of melanoma cell lines were tested. TCR-3598_2 mc displayed slightly higher peptide sensitivity (Figure 12B). Recognition of NY-ESO-transduced melanoma cell line FM3 and naturally NY-ESO-expressing melanoma cell lines FM-56 and FM82 was similar (Figure 12C). Overall, TCR-3598_2 mc and mmc displayed similar functionality.

7 Discussion

In this doctoral thesis, TCRs against NY-ESO were isolated from transgenic mice expressing a diverse repertoire of human TCRs as well as from human T cells. We observed a better performance of TCRs derived from the mice serving as non-tolerant host compared to TCRs derived from a human donor regarding recognition of melanoma cell lines and peptide sensitivity. The combined use of a chosen MHC II-restricted TCR candidate from the non-tolerant repertoire together with an MHC I-restricted NY-ESO-specific TCR from a non-tolerant repertoire achieved tumour regression in a mouse model of ATT.

7.1 Functionality of ABabDR4 mice

In this study, ABabDR4 mice were taken as source for HLA-DR4-restricted TCRs from a non-tolerant repertoire of T cells. ABabDR4 mice are a similar model to ABabHHD mice, which were generated for TCR isolation from CD8 T cells (Li et al., 2010; Obenaus et al., 2015). While ABabHHD mice are transgenic for the mouse/human chimeric MHC I molecule HHD (peptide binding site from HLA-A2) and do not express mouse MHC I molecules, ABabDR4 mice are transgenic for the mouse/human chimeric HLA-DR4-IE molecule and could be confirmed herein not to express any mouse MHC II molecule. Functionality of HLA-DR4-IE has previously been shown in DR4 mice by immunization and induction of autoimmune disease in case of autoantigens (Ito et al., 1996; Touloukian et al., 2000; Zeng et al., 2000). Ability of ABabDR4 mice to elicit CD4 T cell responses was shown herein by immunization with Padre and NY-ESO$_{116}$, confirming functional CD4 T cell immunity in this model. Thus, ABabDR4 mice are a useful tool to isolate human TCRs from a non-human host that can be immunized.

Although all three mouse strains, ABabDR4, DR4 and C57BL/6 mice, responded to immunization with Padre, responses of DR4 mice were smaller. This phenomenon we could observe for other HLA-DR4 epitopes as well (Chen et al., 2017). We suggested that an incompatibility of the mouse V genes to the human HLA-DR4 accounted for less efficient immune responses seen in DR4 mice compared to ABabDR4 mice (Chen et al., 2017). In ABabDR4 mice, human TCRs selected on human MHC II showed higher diversity and longer CDR3 length in the beta chain compared to ABabHHD mice, in which human TCRs are selected on mouse MHC II (Chen et al., 2017). Therefore, aside from the advantage to derive fully human

TCRs from a non-human host, matching human TCRs with human MHC II as in ABabDR4 mice gives rise to a more diverse repertoire of TCRs to isolate from.

7.2 NY-ESO-reactive TCRs from ABabDR4 mice

7.2.1 Efficiency of immunizations

Since NY-ESO has no homologue in ABabDR4 mice, they served as non-tolerant repertoire for NY-ESO-reactive TCRs. NY-ESO$_{116}$ peptide immunization was very efficient with only rare non-responders but elicited small responses in the range of 1-2% of CD4 T cells. NY-ESO-responding cells were expanded efficiently in one-week in vitro cultures in presence of the NY-ESO$_{116}$ peptide. Interestingly, 10^{-8} M peptide concentration yielded most IFNγ-secreting cells upon peptide restimulation. It can be hypothesized that higher or lower concentrations caused T cell exhaustion or inefficient stimulation, respectively. DNA immunization elicited larger immune responses after several boosts, however was not efficient leaving many non-responders. Together, eliciting anti-NY-ESO CD4 T cell responses by immunization for subsequent TCR isolation was well feasible.

7.2.2 Differences in TCR isolation technique

The NY-ESO-reactive TCRs-3598 and 3598_2 were derived from cells labelled by IFNγ capture assay from one single immunized ABabDR4 mouse without prior in vitro expansion. In contrast, from DR4/NY-ESO$_{116}$ tetramer-labelled cells in all three cases only one predominant TCRαβ pair was derived. Two of these TCRs (TCR-3600 and TCR-5712) were expanded in the presence of low concentration of NY-ESO$_{116}$, which may be one reason for deriving a monoclonal T cell repertoire. However, because DR4/NY-ESO$_{116}$ tetramer staining of non-cultured T cells failed, for the third TCR isolation, T cells were expanded non-specifically by anti-CD3/CD28 activator beads, which likewise gave rise to only one predominant TCRαβ pair. Therefore, DR4/NY-ESO$_{116}$ tetramer may not bind all NY-ESO$_{116}$-reactive T cells efficiently. In line with this, TCR-3598_2 isolated from the IFNγ-labelled T cells binds DR4/NY-ESO$_{116}$ tetramer only weakly. This TCR may have been missed if T cells were labelled by DR4/NY-ESO$_{116}$ tetramer for TCR isolation. Therefore, TCR isolation from IFNγ capture-labelled CD4 T cells may reveal more TCRs than from DR4/NY-ESO$_{116}$ tetramer^{+} CD4 T cells.

7.2.3 DR4/NY-ESO$_{116}$ tetramer binding

Failure of MHC II tetramers to bind all appropriate T cells has been reported in the literature (Sabatino et al., 2011; Van Hemelen et al., 2015). The question is, whether TCR functionality correlates with tetramer binding. In one study, tetramer-positive and tetramer-negative T cell clones showed similar proliferation in response to peptide stimulation (Van Hemelen et al., 2015). However, peptide was added at high concentration (3 µM) making conclusion regarding TCR affinity difficult. On the other side, tetramer-negative T cells have been associated with lower affinity TCRs and tetramer staining has been used as correlate for T cell avidity (Falta et al., 2005; Sabatino et al., 2011). Nevertheless, TCR-3598_2, which bound only weakly to DR4/NY-ESO$_{116}$ tetramer, showed high peptide sensitivity and recognized melanoma lines efficiently. High occurrence of mixed transgenic and endogenous TCRαβ heterodimers could be ruled out by inefficient tetramer binding in Jurkat67 cells, which do not express endogenous TCR chains (Heemskerk et al., 2003). Thus, DR4/NY-ESO$_{116}$ tetramer binding intensity cannot be taken as direct measure of TCR functionality.

It must be noted that the DR4/NY-ESO$_{116}$ tetramer used herein was fully human and not human-mouse chimeric as the HLA-DR4 molecule in the ABabDR4 mice to enable co-receptor binding of murine CD4 while preserving HLA-DR4-restricted antigen-binding. The fully human DR4/NY-ESO$_{116}$ tetramer therefore binds only to the TCR and not the murine CD4 co-receptor. Whether this is the decisive factor for inefficient tetramer binding of mouse T cells is questionable as CD4 binding to MHC is weak and is believed not to stabilize the TCR-pMHC interaction in contrast to CD8 (Wooldridge et al., 2005; Xiong et al., 2001). Apart from the tetramer staining for TCR isolation from ABabDR4 mice, human T cells or lines expressing human CD4 were stained.

Of note, TCR-3600 and TCR-5712, both derived from in vitro cultures in presence of NY-ESO$_{116}$ peptide, have a very similar β chain with only one amino acid difference and an identical α chain. On DNA level, however, CDR3 regions of the α chains were different, so that cross-contamination during the TCR isolation process can be ruled out. Rather, in vitro culturing in presence of NY-ESO$_{116}$ peptide raised very similar TCRs. Similar functionality of both TCRs could also be seen by an identical TCR binding motif, alloreactivity to the same LCLs and similar recognition capacity of NY-ESO.

7.2.4 TCR sequence characteristics

CD4 T cell responses against NY-ESO$_{116}$ in ABabDR4 mice were dominated by TRBV2 usage. As TRBV2 is not overrepresented in the ABabDR4 TCRβ repertoire, TRBV2 seems to play a predominant role in NY-ESO$_{116}$ recognition (Chen et al., 2017). Interestingly, overrepresentation of TRBV2 has also been observed for HLA-DR52/NY-ESO$_{119-143}$ tetramer positive cells from NY-ESO vaccinated patients and human PBL in vitro primings (Poli et al., 2013). Although HLA-DR52 and HLA-DR4 have 91% homology, it is unclear whether the dominant use of TRBV2 for recognition of NY-ESO on both MHC II molecules is due to similar binding of CDR2 or 3 regions of TRBV2 to the MHC. Speaking against this, TCR-3598_2 did not recognize NY-ESO$_{116}$ on HLA-DR52 and several other closely homologous HLA-DR molecules (data not shown). On the other side, recognition of a similar NY-ESO epitope, NY-ESO$_{119-134}$, on multiple HLA-DR molecules was shown for two T cell clones (Kudela et al., 2007). Thus, only less specific TCRs may recognize NY-ESO on multiple MHC molecules due to binding of CDR2 or 3 regions of the TCR to a homologous region in the MHC II molecules.

7.3 TCRs from human T cells

7.3.1 TCR sequence characteristics

Six NY-ESO-reactive TCRs were identified from human T cells of a healthy donor. In the in vitro primings, only the TRBV2^{+} fraction of CD4 T cells expanded. This suggests that dominant usage of TRBV2 by NY-ESO$_{116}$-reactive TCRs, as observed in the ABabDR4 mice, holds true for human T cells as well. As the human T cells were not primed by vaccination prior to in vitro culture, deriving a polyclonal repertoire of NY-ESO-reactive T cells was expected. Based on the conserved usage of TRBV2 of NY-ESO-reactive T cells, chain centricity could be hypothesized, in which one TCR chain confers antigen specificity with large tolerance for partner chains. However, combinatorial expression of the isolated TCR chains from the in vitro primings did not produce any conflicting results concerning TCRαβ pairing. Thus, α and β chains together confer specificity to the NY-ESO-reactive TCR without chain centricity of the β chain. Such chain centricity can be seen for example in TCRs recognizing the melanocyte differentiation antigen MART-1$_{26-35}$ on HLA-A2 (Nakatsugawa et al., 2015; Pinto et al., 2014). These TCRs predominantly use TRAV12-2, whose CDR1 and 2 play a dominant role in binding the MART-1$_{26-35}$ peptide and HLA-A2 and tolerate many different β chains as partners (Cole et al., 2009;

Nakatsugawa et al., 2015; Pinto et al., 2014). Thus, TRBV2 dominance was observed for NY-ESO$_{116}$-reactive TCRs of human origin, but α chains were not interchangeable, suggesting no strong chain centricity of the β chain.

7.3.2 NY-ESO specific CD4 T cell precursors

The six NY-ESO-reactive TCRs isolated herein were derived from two in vitro primings from human T cells from the same blood sample. Nevertheless, TCR sequences derived from both cultures did not overlap, suggesting that the expanded T cell clones grew from single precursors. Calculating from the starting number of CD4 T cells and identified TCRs, the precursor frequency of NY-ESO$_{116}$-reactive T cells can be estimated to at least 0.6 in $1x10^6$ CD4 T cells. It can be assumed that the real frequency is higher, as only well expanded clones were identified by the TCR isolation procedure used herein. CD4 T cell precursors with specificity to self or non-self were reported to range between 1 and 10 in $1x10^6$ CD4 T cells (Su et al., 2013). CD4 T cell precursor frequency with specificity for a similar NY-ESO epitope presented on HLA-DRA/DRB1*0302 was estimated to 10 in $1x10^6$ (Poli et al., 2013).

7.4 CD4 T cell tolerance to NY-ESO

The results showing better performance of the MHC II-restricted TCRs from the non-tolerant repertoire extend a finding in a similar mouse model transgenic for the human TCR gene loci and HLA-A2 (ABabHHD mice), in which we have shown that a TCR raised against the HLA-A2 epitope NY-ESO$_{157-165}$ is of higher functional activity than 1G4, a TCR obtained from a melanoma patient (Obenaus et al., 2015).

It is important to note that CD4 T cell tolerance as well as immunity has been observed for different self-antigens in mouse models. While the CD4 T cell repertoire against carcinoembryonic antigen (CEA) was shown to be tolerant, this was not the case for the CD4 T cell repertoire against p53 (Bos et al., 2005; Lauwen et al., 2008). Abundance of the antigen and availability for MHC II presentation might explain why the uniformly expressed but tightly regulated p53 does not lead to tolerance formation, while this is the case for the transgenic protein CEA, which was also shown to be expressed in the thymus (Lauwen et al., 2008). Using Cre-recombinase as an artificial tissue-restricted self-antigen, it was shown that CD4 T cell tolerance is not deletional and can be broken upon immune challenge (Legoux et al., 2015). In

this study, however, no cre-expression was detected in the thymus. As NY-ESO expression has been detected in the thymus (Gotter et al., 2004), a likely outcome is tolerance formation, which is supported by our data herein.

NY-ESO$_{116}$-reactive CD4 T cells from a human donor were expanded in vitro in presence of NY-ESO$_{116}$ peptide. Prompt expansion of NY-ESO-reactive CD4 T cells in culture was also observed in other reports and is in line with occasionally observed anti-NY-ESO IgG and CD4 T cell responses in cancer patients (Gnjatic et al., 2003; Poli et al., 2013; Wada et al., 2014). Lower functional activity of the NY-ESO$_{116}$-reactive TCRs isolated from the human PBL herein suggests that CD4 T cell tolerance to NY-ESO is present in humans and that remaining CD4 T cells are of lower avidity. Considering CD8 and CD4 T cell tolerance to NY-ESO, it is not surprising that immune responses occurring naturally or evoked by vaccination did not lead to sustained clinical success (Fourcade et al., 2014; Takeoka et al., 2017).

7.5 Recognition of melanoma lines

ABabDR4-derived TCRs recognized HLA-DR4/NY-ESO$^+$ melanoma cell lines. Presentation of endogenous antigens does not comply with the classical antigen presentation pathway in which exogenous antigens are endocytosed and loaded onto MHC II but can occur through a process termed macroautophagy (Schmid et al., 2007). However, it is unclear how relevant macroautophagy for antigen presentation on MHC II is for cancer cells, which are not professional antigen-presenting cells. NY-ESO$^+$ melanoma cell lines have been recognized by CD4 T cells in several cases (Fonteneau et al., 2016; Matsuzaki et al., 2015; Zhao et al., 2006). However, tumour recognition was either very weak (Zhao et al., 2006) or antigen processing was shown to occur in an unexpected way by intercellular antigen transfer of the melanoma cells and hence according to the classical MHC II processing pathway or by using the MHC I processing machinery for loading of a 10-mer peptide on HLA-DP4 (Fonteneau et al., 2016; Matsuzaki et al., 2014). Melanoma cell lines used herein were pre-treated with IFNγ, which in addition to upregulating MHC II on the surface can also enhance the MHC II processing machinery. By which mechanism antigen processing occurred was not object of investigation. In general, to which extent direct recognition of cancer cells by CD4 T cells plays a role for tumour rejection is uncertain as MHC II-negative tumour cells can be rejected by CD4 T cells in mouse models (Greenberg et al., 1981; Mumberg et al., 1999; Qin and Blankenstein, 2000).

7.6 Alternative approaches to isolate TCRs against tumour-associated antigens

In the case of tumour-associated antigens, which are self-antigens, T cell tolerance can be expected as observed for NY-ESO herein. To derive TCRs of higher affinity, which cannot be found in the autologous repertoire, several alternative approaches have been used.

7.6.1 HLA-transgenic mice

HLA-transgenic mice can be immunized with human tumour-antigens, which are not homologous in the mouse to derive higher affinity TCRs from responding T cells (Johnson et al., 2009; Parkhurst et al., 2009). However, the resulting TCRs are fully murine, which bears the risk of immunogenicity and consequently low half-life of the infused T cells expressing the TCR in the patient (Davis et al., 2010). Moreover, incompatibility of murine V genes and human MHC in the HLA-transgenic mice may lead to adjustment to shorter CDR3 regions of the TCRs, increasing the risk of cross-reactivity (Gavin and Bevan, 1995; Huseby et al., 2008). Therefore, isolating TCRs from mice that do not only express human HLA molecules but also the human TCRαβ loci as described herein may be superior.

7.6.2 Affinity maturation

To increase the affinity of TCRs isolated from the autologous human repertoire, artificial affinity maturation has been applied (Linette et al., 2013; Morgan et al., 2013; Robbins et al., 2008). However, by circumventing negative selection physiologically taking place in the thymus, cross-reactivity of the generated TCRs is a risk. Two MAGE-A3-reactive TCRs with enhanced affinities led to fatal toxicities in clinical trials due to cross-reactivity (Linette et al., 2013; Morgan et al., 2013). Therefore, a method that allows thymic selection as by isolating TCRs from humanized mice herein presumably keeps the risk of cross-reactivity lower.

7.6.3 TCRs from T regulatory cells

In other reports, MHC II-restricted TCRs were isolated from human Tregs thereby avoiding the repertoire hole that may have been imposed by tolerance (Yao et al., 2016). In the human T cell primings herein, Tregs were not depleted. However, expansion of Tregs is unlikely under the given culture conditions including low IL-2. We cannot exclude that high-affinity TCRs against NY-ESO can be isolated from human Tregs, as CD4 T cells recognizing self-antigens can develop into Tregs instead of being negatively selected (Hsieh et al., 2012). However, the

advantage of the ABabDR4 mouse as TCR source is the natural selection for the fittest clonotypes upon repeated immunizations with sufficient long intervals, thus generating highly functional TCRs presumably of optimal affinity.

7.6.4 Allogeneic T cell primings

Another alternative approach to circumvent the problem of T cell tolerance to self-antigens is to prime T cells in an alloreactive setting, in which the MHC molecule presenting the tumour antigen is non-self (Kumari et al., 2014; Wilde et al., 2012). T cells recognizing tumour antigens on non-self MHC molecules are not deleted in the thymus by negative selection and may be a source of high-affinity TCRs. However, a drawback is the high proportion of primed T cells that recognize the MHC molecule independent of the presented peptide. Although TCRs can be analysed for cross-reactivity, decreased specificity may be a general problem of TCRs generated by alloreactive primings and may not always be detected.

7.7 Off-target toxicity of isolated TCRs

7.7.1 Alloreactivity

Several NY-ESO TCRs isolated from mouse or human in the present study showed alloreactivity towards one or more LCLs. Since the TCRs originate from individuals that bear only a limited number of MHC molecules and it has been shown that up to 10% of T cells from a naive polyclonal repertoire are alloreactive, this finding is plausible but underscores the need for thorough alloreactivity testing (Suchin et al., 2001). The presence of more MHC molecules in the thymus is thought to lead to a higher number of T cells affected by negative selection and thereby less alloreactive T cells (Ni et al., 2014). Hence, the reason for more alloreactive ABabDR4-derived TCRs in comparison to human-derived TCRs in this study may be that ABabDR4 mice express only one MHC II molecule. However, HLA-A2-restricted TCRs generated in ABabHHD mice against several antigens including MAGE-A1 and NY-ESO, did not show frequent alloreactivity, although these mice express only one MHC I (Obenaus et al., 2015 and unpublished data). Whether the frequent alloreactivity in TCRs isolated from ABabDR4 mice is a feature found primarily in MHC II-restricted TCRs or related to the NY-ESO specificity will become clear when further TCRs against different antigens will be isolated from ABabDR4 mice. It has to be noted that in principle the risk of alloreactivity applies to all TCRs that are derived

from a (partly) HLA-mismatched donor, as seen herein by the alloreactivity of one human-derived TCR against NY-ESO.

7.7.2 Cross-reactivity

The chosen TCR candidate for clinical application, TCR-3598_2, was analysed for cross-reactivity by testing epitopes present in the human but not mouse proteome containing its recognition motif as identified by alanine scan. One peptide was found to be recognized, but only at high concentration. Further analysis revealed this recognition to be irrelevant, because the epitope was not processed and presented. The relevance of cross-reactivity analysis by alanine scan was tragically exemplified by a clinical trial targeting MAGE-A3 by a patient-derived and affinity-matured TCR, in which cross-reactivity to an unrelated protein caused fatal cardiac toxicity (Linette et al., 2013). The cross-reactive epitope from the protein titin was later detected by alanine scan revealing that the epitope contained the TCR recognition motif (Cameron et al., 2013). In a recent report, however, cross-reactivity of a TCR against the minor histocompatibility antigen HA-2 was only found by a scanning approach using a combinatorial peptide library, in which 180 peptide pools are applied to test all possible amino acid combinations (Bijen et al., 2018). The cross-reactive peptide differed in 5 amino acid positions and could not be detected by alanine scan. Therefore, although more laborious, scanning with a combinatorial peptide library should be applied in the future to identify cross-reactive TCRs best possible.

7.8 Mouse model of ATT

7.8.1 Synergy of CD4 and CD8 T cells

ABabDR4-derived TCR-3598_2-transduced CD4 T cells in combination with TCR-ESO-transduced CD8 T cells caused regression of palpable tumours in a mouse model of ATT. The combined treatment was more effective than treatment with CD4 or CD8 T cells alone. Therefore, their synergistic effect was necessary to achieve efficient tumour regression and the in vivo functionality of the herein isolated TCR-3598_2 was confirmed.

Synergy of CD4 and CD8 T cell lines with specificity for NY-ESO has been shown to delay tumour growth in a xenograft mouse model (Matsuzaki et al., 2015). In this model, the CD4 T cells see an 8-9-mer peptide that is presented on MHC II of the xenografted cancer cells, but cross-presentation of NY-ESO cannot occur in a xenograft model (Matsuzaki et al., 2014;

Matsuzaki et al., 2015). However, recognition of cross-presented antigen was shown to be crucial for tumour rejection and required for bystander elimination of antigen-negative cancer cells (Schietinger et al., 2010; Spiotto et al., 2004). Therefore, improved recognition of cross-presented antigen may be one feature of CD4 and CD8 T cell synergy. TCR-3598_2-transduced CD4 T cells recognized re-isolated tumour stromal cells, showing that specific recognition of cross-presented antigen was implicated in the anti-tumour effect of the CD4 T cells in the model used herein. Synergy of CD4 and CD8 T cells with specificity for model antigens has been shown in several models (Arina et al., 2017; Bos and Sherman, 2010; Li et al., 2017; Schietinger et al., 2010). It has become apparent that CD4 T cells, to synergize with CD8 T cells, must be specific and act locally at the tumour site, because non-specific CD4 T cells that gave help to CD8 T cells only during priming did not improve the outcome (Bos and Sherman, 2010; Schietinger et al., 2010). Local activity at the tumour site of the specific CD4 T cells in the tumour model used herein can be assumed as they recognized re-isolated tumour stromal cells. Moreover, CD8 T cell numbers in the blood were higher in the group that received the combined treatment of CD4 and CD8 T cells. Therefore, CD4 T cells also induced proliferation and/or survival of the CD8 T cells.

7.8.2 Efficacy of CD4 or CD8 T cells alone

Transfer of NY-ESO-specific CD4 T cells alone delayed tumour growth but did not lead to tumour regression. Most in vivo models in which CD4 T cells alone can eradicate transplanted tumours either involve very early treatment on day 0 or 1 following tumour injection (Mumberg et al., 1999; Perez-Diez et al., 2007) or treatment in conjunction with chemotherapy 5 days after tumour injection (Greenberg et al., 1981). In one model, CD4 T cells can reject large B16 tumours, however only if treated with radiotherapy and anti-CTLA-4 or Th17-differentiation of the CD4 T cells prior to infusion (Muranski et al., 2008; Quezada et al., 2010). Following the transduction protocol for CD4 T cells used herein, it can be assumed that CD4 T cells have a Th1 phenotype, which is supported by their ability to secrete IFNγ upon stimulation with $CD11b^+$ stromal cells. It would be interesting to investigate, whether Th17 differentiation of the CD4 T cells together with tumour irradiation improves the outcome in the model used herein, in which the antigen is seen only by cross-presentation and not on the cancer cells. Together, failure to reject tumours by CD4 T cells alone was not surprising, as tumours established for one month and were not sensitized by radiotherapy or chemotherapy prior to ATT.

Transfer of NY-ESO-specific CD8 T cells alone or with irrelevant CD4 T cells caused tumour regression only in part of the mice. Although CD8 T cells have been shown to reject even large established tumours, this was the case when the target was a strong model antigen or when the target was overexpressed as trimer minigene (Anders et al., 2011; Leisegang et al., 2016b; Schreiber et al., 2012). The Tet-TagLuc cells used in the present study were engineered to express the target antigen NY-ESO as full-length protein, which resembles more the physiologic situation than a trimer minigene. Moreover, in this model CD8 T cells cannot recognize cross-presented antigen on stromal cells because of absence of HLA-A2 in the host mice. Insufficient rejection by CD8 T cells alone was therefore not unexpected.

7.8.3 Limitations of the model

Although NY-ESO-specific CD4 and CD8 T cells in combination caused tumour regression initially, antigen-negative variants started to outgrow 2-8 weeks following treatment (data not shown). Thus, recognition of cross-presented antigen by CD4 T cells in addition to CD8 T cells allowed tumour regression in the first place but did not eradicate antigen-loss variants unlike other models (Arina et al., 2017; Schietinger et al., 2010). Irradiation or chemotherapy concomitantly with ATT may increase the extent of cross-presentation to CD4 T cells thereby compensating for the lack of cross-presentation to CD8 T cells and preventing relapse of antigen-loss variants.

While the positive results of the mouse model are an indicator for efficacy of the therapy, safety cannot be studied in full, because off-target and on-target toxicity may not be comparable to humans due to species-specific protein expression. For safety, in vitro assays help to estimate the risk of toxicity.

Although a preclinical mouse model is very useful to evaluate whether a therapy is promising and should be tested in patients, it can never exactly reflect the real situation. A clinical trial will reveal how successful the developed therapy can be.

7.9 Translation into the clinic

The NY-ESO-specific TCR-3598_2 derived from the ABabDR4 mouse model showed a favourable safety profile with no detectable alloreactivity or cross-reactivity but high functional

activity towards NY-ESO, which could be confirmed in an in vivo setting. Therefore TCR-3598_2 is a promising candidate for clinical application.

7.9.1 Clinical version of TCR-3598_2

To analyse the isolated NY-ESO-reactive TCRs, they were expressed with murine constant regions to increase correct pairing of introduced α and β chains and moreover enable staining of TCR expression in human cells by anti-mTCRβ (Cohen et al., 2006). However, immunogenicity of the mouse constant regions in a human host may lead to low persistence and consequently low efficacy of the TCR-engineered T cells. Antibodies against murine variable regions were observed in patients treated with murine TCRs against gp100 or p53 (Davis et al., 2010). Therefore, for future clinical application the fully murine constant regions (mc) were exchanged for minimally murinized human constant regions (mmc) containing an additional disulphide bond to decrease immunogenicity but maintain improved pairing and expression (Cohen et al., 2007; Sommermeyer and Uckert, 2010). Both versions of TCR-3598_2, mc and mmc, showed comparable functionality with only slightly reduced surface expression and peptide sensitivity of TCR-3598_2 mmc. TCR-3598_2 mmc is therefore the version of choice for clinical application.

7.9.2 Clinical application of combined CD4 and CD8 T cells therapy

Although ATT of solid tumours has achieved objective responses in clinical trials, efficacy must be improved, because responses in the majority of patients were short-lived (Rapoport et al., 2015; Robbins et al., 2015). Low persistence of adoptive T cells and/or outgrowth of antigen-negative cancer cells, as has been observed in earlier clinical trials, may be prevented by giving antigen-specific CD4 T cells in addition to CD8 T cells to induce a more comprehensive anti-cancer response. The results of the in vivo model herein clearly demonstrated that the combination of NY-ESO-specific CD4 and CD8 T cells was more effective than treating with CD4 or CD8 T cells only. Therefore, to improve efficacy in ATT, combination of CD4 and CD8 T cells is a promising approach to test in a first clinical trial.

Eligible patients for the combined CD4 and CD8 T cell therapy developed herein need to be positive for HLA-DR4 and HLA-A2. As the allele frequencies for HLA-DR4 and HLA-A2 are approximately 8% and 30%, respectively, only approximately 5% of patients are expected to have this haplotype (Schmidt et al., 2009). To be able to recruit enough patients for a clinical study, cancer entities should be chosen that display high expression rate of NY-ESO. NY-ESO is

expressed in a variety of carcinoma entities, however only in a minor fraction of each (Kerkar et al., 2016; Park et al., 2016). In contrast, high expression rates were reported in sarcomas with 76% of synovial sarcoma and >89% of myxoid/round cell liposarcomas with mostly homogenous expression pattern of NY-ESO (Hemminger et al., 2013; Lai et al., 2012; Pollack et al., 2012). Therefore, synovial sarcoma and myxoid/round cell liposarcoma would be ideal candidates for a first clinical trial of combined NY-ESO-specific CD4 and CD8 T cell therapy.

8 Abbreviations

7-AAD	7-amino-actinomycin D
ACK	Ammonium-chloride-potassium
APC	Allophycocyanin
ATT	Adoptive T cell therapy
AIRE	Autoimmune regulator
β2m	β2-microglubulin
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HLA	Human leucocyte antigen
HHD	HLA-A2-H-2D^{b} chimeric molecule
hTCM	Human T cell medium
IC50	Half maximal inhibitory concentration
IFNγ	Interferon γ
Ig	Immunoglobulin

IL	Interleukin
LCL	Lymphoblastoid B cell line
MACS	Magnetic-activated cell sorting
MCA	Methylcholanthrene
MHC	Major histocompatibility
mTCM	Mouse T cell medium
NS3	Nonstructural protein 3
Padre	Pan-DR-binding peptide
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PBS	Phosphate-buffered saline
RAG	Recombination-activating gene
TAP	Transporter associated with antigen processing
CLIP	Class II-associated invariant chain peptide
s.c.	Subcutaneously
SEREX	Serological expression cloning
RACE	Rapid amplification of cDNA ends
TCR	T cell receptor
Th	T helper
TRAV	T cell receptor α variable region
TRBV	T cell receptor β variable region
TIL	Tumour infiltrating lymphocyte
WT	Wild type
XRRA1	X-ray radiation resistance-associated protein 1

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11 Curriculum Vitae

For reasons of data protection, the curriculum vitae is not published in the electronic version.

12 Publications

Poncette, L., Chen, X., Lorenz, K. M., Blankenstein, T. (2018). Optimal-affinity T cell receptors targeting NY-ESO via MHC II help MHC I-restricted T cell receptors in tumour regression. *Submitted.*

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

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13 Appendix

Appendix 1

HLA allotypes of the LCL panel and the blood donation used for TCR isolation from the human repertoire.

B-LCL	A*		B*		C*		DRB1*		DRB3*	DRB4*	DRB5*	DQA1*		DQB1*		DPA1*		DPB1*	
AMAI	6802		5301		0401		1503				0101	010201		0602		0301		0402	
AMALA	021701		15010101		0303		1402		0101			0501		0301		010301		0402	9401
BSM	020101		15010101		30401		040101			01030101		03		030201		01		020102	
DJS	0201	0301	3501	3702	0401	0602	0101	1601				0101	0102	0501	0502			0401	0402
DUCAF	3002		1801		0501		0301		0202			050101		0201		0103		0202	
HOR	330301		440301		1403		130201		030101			0102		0604				0401	
KAS011	010101		3701		0602		160101				0202	010202	010201	050201		020101	010301	1401	04010
KAS116	24020101		5101		1203		0101					010101		050101		0201		1301	
KE	0201	2902	4403	4405	0202	1601													
MT14B	3101		4001		0304		0404			0101		03		0302				0402	
SA	24020101		070201		0702		0101					0101		050101				0402	
SPO	0201		4402		0501		1101		0202			010202		0502		01		020102	
TISI	24020101		3508		0401		1103		0202			0505		0301		0103		0402	
WIN	0101		570101		0602		0701			0101		0201		0202	030302	0103	020102	0401	1301
Blood donor	020101	31010	440201		05010		040101			0103		0303		030101		0103		040101	

Appendix 2

Peptide sequences containing the TCR-3598_2 recognition motif X-X-X-X-L-K-E-F-X-X-X-X-X-X-X.

Protein	Peptide*	IC50 (nM)
Neuroserpin	EFSFLKEFSNMVTAK	11,40
Folliculin-interacting protein 2	CQRFLKEFTLLIEQI	20,00
Cytosol aminopeptidase	AAAFLKEFVTHPKWA	20,50
Gamma-parvin	FFLHLKEFYLTPNSP	33,50
DNA mismatch repair protein Msh3	IIKYLKEFNLEKMLS	39,50
Protein Lines homolog	RPLVLKEFDTAFSFD	68,90
Ankyrin and armadillo repeat-containing protein	NPAFLKEFQMQQTLV	73,70
Piwi-like protein 3	RHHTLKEFINTLQDN	93,90
Protein LAP2	QLSGLKEFWMDANRL	95,80
Apolipoprotein L1	RNWFLKEFPRLKSEL	101,10
Formin-like protein 2	HNTLLKEFILNNEGK	105,40
Apolipoprotein L2	RQWFLKEFPRLKREL	107,60
Formin-like protein 1	DCMVLKEFLRANSPT	109,40
Transcriptional-regulating factor 1	CSICLKEFKNLPALN	120,20
Separin	LLPALKEFLSNPPAG	134,80
Netrin-G2	SAKGLKEFFTLTDLR	139,90
Discoidin domain-containing receptor 2	EPDDLKEFLQIDLHT	149,80
Apolipoprotein L4	REWFLKEFPQIRWKI	151,30
Protein Jumonji	LYLSLKEFKNSQKRQ	153,50
Apolipoprotein L3	REWFLKEFPQVKRKI	154,10
Tripartite motif-containing protein 59	IFYLLKEFVWKIVSH	161,00
Cell cycle checkpoint control protein RAD9A	ITFCLKEFRGLLSFA	165,10
Putative E3 ubiquitin-protein ligase UNKL	HYRYLKEFRTEQCPL	180,20
NACHT, LRR and PYD domains-containing protein 1	KKEELKEFQLLLANK	181,80
Alpha-hemoglobin-stabilizing protein	ISAGLKEFSVLLNQQ	194,30
Peroxisome biogenesis factor 1	TKDGLKEFSLSIVHS	202,00
Probable small intestine urate exporter	WNETLKEFKAMSGIL	202,60
X-ray radiation resistance-associated protein 1	**AKRLLKEFQARYRQL**	**205,90**
Protein-arginine deiminase type-4	QLFKLKEFSKAEAFF	210,40
Vacuolar protein sorting-associated protein 13A	ANAFLKEFCLKCPEY	221,40
Coiled-coil domain-containing protein 127	ARLLLKEFEAVLTER	222,00
Probable small intestine urate exporter	WNETLKEFKAMAPAY	224,90
Breast cancer type 1 susceptibility protein	NTSELKEFVNPSLPR	231,80
Fibrous sheath-interacting protein 2	INSLLKEFSDAQIKV	238,40
Dystonin	FYSKLKEFSILLQKA	241,10
Ropporin-1B	LPKMLKEFAKAAIRA	261,50
Nucleotide exchange factor SIL1	SHQNLKEFALTNPEK	286,90
Sodium channel modifier 1	RQMALKEFSSVYSEE	298,70
Poly(A) RNA polymerase, mitochondrial	LNTLLKEFQLTEENT	318,90
Phosducin-like protein	GKMTLKEFAIMNEDQ	330,50
Ropporin-1A	LPKMLKEFAKAAIRV	387,30
Collagen alpha-3(VI) chain	GFPLLKEFVQRVVES	390,30
Gem-associated protein 4	PDEVLKEFVLPFLRL	392,00
Olfactory receptor 10X1	NQTILKEFILVGFSV	455,30
Spectrin alpha chain, erythrocytic 1	SEETLKEFSTIYKHF	459,00
Protein NYNRIN	FKRALKEFIFLHGKK	459,70
UPF0565 protein C2orf69	YPEVLKEFAQTGIIV	463,60
Leucine-rich repeats and immunoglobulin-like domains protein 3	LPEHLKEFQSLETLD	487,70
Epididymal-specific lipocalin-10	SFQSLKEFMDACDIL	492,40
Required for meiotic nuclear division protein 1 homolog	MLKPLKEFENTTCST	497,00

*Peptides were included if they had a predicted affinity to HLA-DR4 of below 500 nM and are present in the human but not the mouse proteome.