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Functional characterization of allorestricted T cell receptors (TCRs) with specificity for HER2/neu and FMNL1 *in vitro* and *in vivo* for potential clinical application

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1 Abstract

T cells can specifically recognize tumors by their surface antigen receptors, T cell receptors (TCRs). Clinical trials have shown that adoptive transfer of T cells genetically engineered with tumor-specific TCRs is feasible and effective for the treatment of cancer. This strategy may be advantageous when compared to autologeous tumor infiltrating lymphocytes (TIL) for adoptive transfer as sufficient numbers of tumor-specific T cells with high avidity may be more easily obtained. However, further assessment of potential benefits and side effects of a broad range of TCRs with specificity for diverse classes of tumor-associated antigens (TAA) is a precondition for extended clinical application.

Allorestricted T cells with specificity for a TAA may be a good source for TCRs with high anti-tumor reactivity. In a previous study, several allorestricted T cell clones with specificity for the peptide 369 derived from HER2/neu ($HER2_{369}$), a TAA overexpressed in diverse malignancies, were isolated by stimulating HLA-$A2^-$ T cells from healthy donors with peptide-pulsed HLA-$A2^+$ T2 cells. In this study, four allorestricted TCRs with specificity for $HER2_{369}$ were selected and characterized. Peripheral blood mononuclear cells (PBMC) transduced with especially one TCR (TCR HER2-1) mediated specific anti-tumor reactivity after TCR optimization suggesting that this TCR represents a potential candidate for targeting HER2/neu by TCR-transduced effector cells. Other TCRs (HER2-2, HER2-3, and HER2-4) showed either high-peptide specificity without anti-tumor reactivity or $HER2_{369}$-independent recognition. However, the TCR α-chain derived from TCR HER2-2 specifically recognized $HER2_{369}$ not only in combination with the original β-chain but also with four other β-chains of the same variable family deriving from TCRs with diverse specificities. Pairing with one β-chain derived from TCR HER2-4 potentiated the chimeric TCR and improved functional avidity with CD8 independency as well as anti-tumor reactivity. Although the frequency of such TCR single chains with dominant peptide recognition is currently unknown, they may represent interesting tools for TCR optimization resulting in enhanced functionality when paired to novel partner chains. However, undirected mispairing with novel partner chains may also result in neoreactivity and thereby enhanced cross-reactivity and self-reactivity. These results may have an important impact on the further design of strategies for adoptive transfer using TCR-transduced T cells. Moreover, preliminary data showed preferential survival of tumor-bearing mice after treatment with PBMC transduced with either TCR HER2-1 or the chimeric TCR chain combination HER2-2α/HER2-4β compared to untreated mice or animals treated with GFP-transduced PBMC, suggesting potential tumor recognition by these two TCRs *in vivo*.

Similarly, one allorestricted T cell clone SK22 with specificity for the peptide PP2 derived from Formin-like 1 (FMNL1), representing as an attractive TAA in chronic lymphatic leukemia (CLL) as well as other lymphomas, was previously isolated. The TCR derived from this clone (TCR SK22) exerted specific peptide recognition and anti-tumor reactivity against HLA-$A2^+$ lymphoblastoid cell lines (LCL) after retroviral transfer into PBMC *in vitro*. In this study, tumor-bearing mice treated with TCR SK22-transduced PBMC showed a slightly increased survival in preliminary experiments.

To monitor non-invasively TCR-transduced T cells after adoptive transfer using Positron Emission Tomography imaging in combination with computed tomography (PET/CT), the reporter gene encoding human Sodium Iodide Symporter (hNIS) for PET/CT imaging as well as TCR HER2-1 chain genes were successfully cloned into the same lentiviral vector and were expressed in recipient cells after lentiviral transduction. These experiments will help to further establish tracing of TCR-transduced T cells after adoptive transfer using PET/CT imaging.

In conclusions, this study suggests a potential for selected TCRs for clinical application and provide important information for the development of TCR-transfer strategies targeting overexpressed self antigens.

Keywords: allorestricted T cells, allorestricted TCRs, TAA, HER2/neu, FMNL1, adoptive transfer, dominant TCR chains, TCR mispairing, retroviral transduction, PET/CT imaging, lentiviral transduction

1 Zusammenfassung

T-Zellen erkennen spezifisch Tumoren mit Hilfe von Antigen-Rezeptoren, den T-Zell Rezeptoren (TZRs). Es hat sich gezeigt, dass der adoptive Transfer von T-Zellen, die genetisch mit tumorspezifischen TZRs verändert wurden, machbar und wirksam in der Behandlung von Krebs ist. Im Vergleich zum adoptiven Transfer mit autologen Tumor infiltrierenden Lymphozyten (TIL) hat diese Strategie den Vorzug, dass eine ausreichenden Menge an Tumor-spezifischen T-Zellen möglicherweise mit hoher Avidität für die klinische Anwendungen gewonnen werden können. Doch im Hinblick auf eine breitere Anwendung und Weiterentwicklung der Strategie müssen eine Reihe von TZRs mit Spezifität für verschiedene Tumor-assoziierte Antigene (TAA) charakterisiert werden, um eine valide Beurteilung des potenziellen Nutzens und der möglichen Nebenwirkungen abschätzen zu können.

Allorestringierte T-Zellen mit Spezifität für ein TAA sind eine gute Quelle für TZRs mit hoher Tumorreaktivität. In früheren Studie wurden mehrere allorestringiete T-Zell-Klone mit Spezifität für das Peptid 369, das von HER2/neu ($HER2_{369}$), einem in diversen Malignomen überexprimiert TAA, abstammt, durch die Stimulierung von HLA-$A2^-$ T-Zellen von gesunden Spenders mit Peptid-beladenen HLA-$A2^+$ T2-Zellen isoliert. In dieser Studie wurden vier allorestringierte TZRs mit Spezifität für $HER2_{369}$ ausgewählt und charakterisiert. Periphere mononukleäre Zellen des Blutes (PBMC), die insbesondere mit einem TZR (TZR HER2-1) transduziert wurden, vermittelten nach TZR-Optimierung spezifische Tumorreaktivität, was darauf hindeutet, dass dieser TZR ein potentieller Kandidat für eine klinische Anwendung gegen HER2/neu-überexperimierende Tumoren durch TZR-transduzierten Effektorzellen ist. Andere TZRs (HER2-2, HER2-3 und HER2-4) zeigten entweder hohe Peptid-Spezifität ohne Tumorreaktivität oder eine $HER2_{369}$-unspezifische Erkennung von Zielzellen. Es zeigte sich allerdings, dass die TZR α-Kette von TZR HER2-2 das Peptid $HER2_{369}$ nicht nur in Kombination mit der ursprünglichen β-Kette sondern auch mit vier anderen β-Ketten von der gleichen Familie spezifisch erkannte, die aus diversen TZRs mit unterschiedlichen Spezifitäten stammten. Die spezifische TZR Einzelketten-Verpaarung mit einer β-Kette von TZR HER2-4 verbesserte den chimären TZR in Bezug auf funktionelle Avidität mit CD8 Unabhängigkeit und Tumorreaktivität. Obwohl die Häufigkeit solcher einzelner TZR-Ketten mit dominanter Peptid-Erkennung derzeit nicht bekannt ist, können diese in Kombination mit neuen Partnerketten interessante Tools in Hinblick auf eine TZR-Optimierung repräsentieren. Allerdings kann die ungerichtete Fehlpaarung mit neuen Partner-Ketten durch zu starke Erkennung des Antigens sowie durch Neoreaktivität und damit erhöhter Kreuzreaktivität auch zu einer Selbstreaktivität führen. Diese Ergebnisse sind daher von besonderer Bedeutung für die weitere Entwicklung von Strategien für den adoptiven Transfer von TZR-transduzierten T-

Zellen. Darüber hinaus weisen vorläufige Ergebnisse darauf hin, dass das Überleben tumortragenden Mäuse bei Tieren, die mit TZR HER2-1- oder chimären TZR HER2-2α / HER2-4β-transduzierten PBMC behandelt wurden, im Vergelich zu unbehandelten Tieren, oder Mäusen, die mit GFP-transduzierten PBMC behandelt wurden, verbessert war.

In vivo-Versuche wurden auch mit einem weiteren TZR, der von dem T-Zellklon SK22 isoliert worden war, durchgeführt. T-Zellklon SK22 erkennt spezifisch das Peptid PP2, das von Formin-like 1 (FMNL1), einem attraktiven TAA bei der chronischen lymphatischen Leukämie (CLL) sowie anderen Lymphome, abstammt. Der TZR dieses T-Zellenklons (TZR SK22) hatte zuvor spezifische Peptid-erkennung und Tumorreaktivität nach retroviralen Transfer in PBMC *in vitro* gezeigt. In dieser Studie zeigten Mäuse, die mit TZR SK22-transduzierten PBMC behandelt wurden, ein leicht erhöhtes Überleben.

Um nicht-invasiv TZR-transduzierten T-Zellen nach dem Transfer mittels Positronen-Emissions-Tomographie in Kombination mit der Computertomographie (PET/CT) *in vivo* zu verfolgen und funktionell zu untersuchen, wurde der humane Natriumiodide Symporter (hNIS) als Reportergen für PET/CT-Bildgebung in Kombination mit den Genen des TZR HER2-1 in einen lentiviralen Vektor kloniert und in Empfänger-Zellen transduziert. Diese Experimente sind möglicherweiser hilfreich für die weitere Untersuchung zur Nachverfolgung TZR-transduzierter T-Zellen *in vivo* nach adoptiven Transfer mittels PET/CT-Bildgebung.

Zusammenfassend wurden in dieser Studie ausgewählte TZRs als potenzielle Kandidaten für die klinische Anwendungen charakterisiert und wichtige Besonderheiten von TZRs in Hinblick auf die Entwicklung von Strategien zum Transfer von TZRs, die gegen überexprimierte körpereigene Antigene erkennen, identifiziert.

Stichwörter: allorestringierte T-Zellen, allorestringierte TZRs, TAA, HER2/neu, FMNL1, adoptiver Transfer, dominante TZR-Kette, TZR-Fehlpaarung, retrovirale Transduktion, PET / CT-Bildgebung, lentivirale Transduktion

2 Introduction

2.1 Tumor immunology

Today cancer is one of the leading causes of death all around the world. Thanks to the development of medical and surgery techniques, the life-span of cancer-patients has increased. However, an effective therapeutic approach to cure the disease is still missing in many cancer entities. Cancers generally arise from progressive growth of the progeny of a single transformed cell, whereas different cancers show unique characteristics regarding the tissue of origin, metastasis potential and tumor microenvironment. The underlying mechanisms of the failure of the host immune response to eradicate the transformed cells and inhibit tumor growth are still not fully clarified. One key question in this context has been whether the tumor is defined by immune system as ‘self’ rather than ‘non-self’ tissue.

2.1.1 Cancer immune surveillance

In the 1950s F.M. Burnet firstly proposed the hypothesis of ‘immune surveillance’ and postulated that the host immune system has the ability to detect tumor cells and destroy them. This hypothesis has been supported later on by experimental mouse studies. For example, mice with deficiencies in adaptive and innate immune system, which were generated by knocking out the essential gene for antigen receptor rearrangement and/or a crucial gene for IFNγ-signalling, showed a higher incidence of gut epithelial and mammary carcinoma than their normal counterparts (Shankaran et al. 2001). Furthermore, mice lacking perforin, which is a key component of the killing mechanism used by cytotoxic T cells and natural killer (NK) cells, more frequently developed lymphoma than their normal counterparts (Smyth et al. 2000). These studies provided evidence that immune surveillance mediated by both, the innate and adoptive immune system, plays a role in the control of the development of certain types of tumors. However, in humans, the common, non-virus-associated cancers (e.g. breast and lung) developed similarly in people with deficiency in immune system as in those with a fully functional immune system, revealing that some tumors are treated more like ‘self’ and therefore not controlled by the immune system (Groopman 1987). The true relationship of tumor and immune system might lie between the two views (Pardoll 2001). Hence, the model of the ‘immune surveillance’ hypothesis has been further modified and considered as the first phase, namely the ‘elimination phase’. This phase is followed by the ‘equilibrium phase’ in case the elimination of tumor cells is not successful or the tumor undergoes mutations or changes. When the tumor cells have accumulated sufficient

mutations to evade the attention of the immune system, the tumor cells enter the 'escape phase' and are able to grow without control and become a clinically detectable tumor. The whole process containing these three phases is known as 'immunoediting', which refers to the immunologic sculpting of surviving tumor cells (Shankaran et al. 2001; Dunn et al. 2004). Although the precise process of tumor development in immunocompetent hosts is not totally understood, it is certain that the relationship between tumor and host immune system is complicated and highly diverse in different tumor entities.

2.1.2 Tumor escape mechanisms from immune recognition

To date accumulating data demonstrate that tumors might use diverse mechanisms to avoid the evoking of an immune response or escape it: (1). Tumors may exhibit low immunogenicity by down-regulation of peptide presentation or MHC molecules or co-stimulatory molecules, which are all indispensable for the recognition by the adaptive immune system (Ikeda et al. 1997; Koopman et al. 2000; Ochsenbein et al. 2001). (2). Tumors may further induce immune suppression by secreting inhibitory molecules such as TGF-β and IL-10 or recruiting regulatory T cells (T_{reg}) which can suppress the anti-tumor function of immune cells (Tada et al. 1991; Wang et al. 2004). (3). Tumors can also establish a physical barrier by secreting molecules such as collagen and fibrin to prevent the access of lymphocytes (Murphy et al. 2008). Actually the understanding of the escape mechanisms exploited by the different tumors gives us hints to develop efficient therapeutic approaches to treat cancer. For example, considering that NK cells recognize the MHC down-regulated targets cells, the potential of NK cells for cancer therapy is investigated (Zamai et al. 2007; Sutlu and Alici 2009). Moreover, anti-CD25 monoclonal antibodies (mAb) leading to depletion of the $CD4^+$ $CD25^+$ T_{reg} cells to abolish the immune suppression have been tested for the tumor treatment (Fecci et al. 2006; Goforth et al. 2009).

2.2 Cancer immunotherapy

With the understanding of the mechanisms by which the adaptive or innate immune system mediate cancer immune surveillance and by which the tumor cells are ignored by the immune system, cancer immunotherapy, namely utilizing the immune system to attack tumors, has emerged as a promising approach to treat cancer. Compared to conventional chemotherapy and radiotherapy for cancer, tumor antigen-specific immunotherapy has a higher potential in successfully treating cancer because there is the possibility of specific tumor-targeting. Even induction of an immune memory against tumor recurrence might occur, since the adaptive immune system has the unique capability of generating an immunological memory to the same antigen.

By now diverse immunotherapeutic approaches based on different mechanisms are already applied in the clinic against cancer or under development. For example, the recombinant cytokine IL-2 has been administrated as adjuvant in many clinical trials for cancer treatment to generally stimulate the host immune system against tumors (Rosenberg et al. 1998; Shimizu et al. 1999). Another extensively investigated immunotherapy approach is based on monoclonal antibodies (mAbs), which target tumor antigens. Currently, several mAbs have been approved for clinical application in the USA as reviewed by Campoli et al. (Campoli et al. 2010). For example, MabThera™ (Rituximab), an anti-CD20 mAb, is an approved agent for the treatment of low-grade B cell non-Hodgkin's lymphoma (NHL) (Maloney et al. 1997). Several radioisotope conjugated-antibodies such as anti-CD33 (Caron et al. 1994) and anti-B1 (Kaminski et al. 1996) mAbs are under development for the treatment of radiosensitive myeloid leukemia and NHL, respectively. However, the obvious limitation of mAb-based immunotherapy is the restricted targeting of surface antigens and the difficulty in the production of humanized mAbs with low immunogenicity.

In addition to the cancer immunotherapeutic approaches described above, T cell-based cancer immunotherapy have shown great therapeutic potential against diverse virus and malignant diseases in the past decade. T cells together with B cells are the key effector cells of the adaptive immune system. T-cell mediated immune responses show two distinct features, namely, the specific recognition of target and generation of long lasting immunological memory, which renders T-cell based immunotherapy an attractive and promising approach to treat cancer.

T-cell based immunotherapy of cancer generally can be classified into (1) active immunostimulatory vaccination approaches and (2) passive adoptive transfer of tumor-specific T cells (Davis 2000). Vaccination of patients with irradiated whole tumor cells, cell lysate or polyvalent extract has been used to elicit specific immune responses against tumors (Hersey 1997; Hsueh et al. 1999). With the identification of diverse tumor-associated antigens (TAA), synthetic peptide-based vaccines (Marchand et al. 1995; Rosenberg et al. 1998) and vaccination using peptide-loaded dendritic cells (DC) (Mayordomo et al. 1995) or DC transfected with TAA-encoding DNA or RNA have been tested in clinical trials or experimental models (Paglia et al. 1998; Ying et al. 1999). Principally, vaccination stimulates the host immune system with tumor antigen and thereby evokes the immune response to tumor cells in indirect ways, whereas adoptive T-cell transfer (ACT) provides the patients directly with effector cells against the tumor. The infusion of donor lymphocytes into patients with leukemia after allogeneic stem cell transplantation is currently a routine immunotherapy in the clinic and has shown significant cure efficacy (Kolb et al. 2004). The successful isolation and expansion of tumor-specific T cells facilitate the application of ACT in therapy

for different cancers (Dudley et al. 2002). The feasibility and difficulty of ACT is addressed more in detail in the following section.

Immunologic approaches that indirectly target tumor cells might also be exploited to increase anti-tumor efficiency. Such strategies could include the use of antibodies in blocking the growth factor receptors (e.g. insulin-like growth factor I receptor) to inhibit the growth of tumor cells (Wang et al. 2006), targeting tumor stroma (Zhang 2008) or blood vessels to destroy the basement of the tumor tissue or the nutrition supply, interfering signal transduction and inducing apoptosis by targeting the apoptosis pathway or other effect effectors such as NO or histamine (Davis 2000).

2.3 Adoptive transfer of tumor-specific T cells

Obviously, the crucial point for the feasibility of adoptive T cell transfer in cancer treatment is to obtain tumor-specific T cells. To determine the source of such tumor-reactive T cells, it might be necessary to understand how T cells function and how they are naturally generated in the immune system.

2.3.1 General T cell immunology

T cells recognize their target through their surface receptor, called T cell receptor (TCR). As shown in Figure 2.1, the TCR is a heterodimer composed of disulfide-linked α- and β-chain, both of which contain variable (V) and constant (C) regions. The TCR is associated with one $\xi\xi$ homodimer and CD3 molecules (CD stands for cluster of differentiation), which is composed of one $\varepsilon\delta$- and one $\gamma\varepsilon$-heterodimer. Both CD3 molecules and $\xi\xi$ homodimer contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tail and are essential for the surface expression of TCR and the signaling transmission into the T cells. Binding of antigen with the TCR leads to the phosphorylation of ITAMs which further activates downstream proteins and thereafter initiates diverse intracellular signal cascade in T cells (Malissen and Schmitt-Verhulst 1993).

The specificity of the TCR is determined by the antigen-binding site which is conformationally constructed by the three complementary determining regions (CDRs) in the variable α and β domain, namely CDR1, CDR2 and CDR3. The TCR diversity mainly results from the stochastic formation of diverse combinations and junctions of different gene segments such as variable (V), diversity (D, only in β-chain), joining (J) and constant (C) regions for TCR α- and β-chain. In humans, the TCRβ locus contains 52 V-, 2 D-, 13 J- and 2 C-gene segments, and the TCR α locus has 70 V-, 61 J- and 1 C-gene segments. During T cell development, diverse gene segments are sequentially rearranged. In mature T cells, the TCR α-chain contains one gene segment for each V, J, and C region, while β-chain harbors one segment

for V, D, J, and C regions. The random combination of V-(D)-J-C segments for TCR α- and β-chains and of TCR α- and β-chains for a complete TCR comprise the high diversity of the TCR repertoire. In addition to combinational diversity, the junctional diversity generated from additional nucleotide inserts in the V-(D)-J combination site greatly contributes to the TCR diversity as well (Davis and Bjorkman 1988; Murphy et al. 2008). This V-(D)-J junctional region forms the center of the antigen-binding site, namely CDR3, whereas CDR1 and CDR2 are encoded within the germline V gene segments for TCR α- and β-chains (Garboczi et al. 1996; Hennecke et al. 2000).

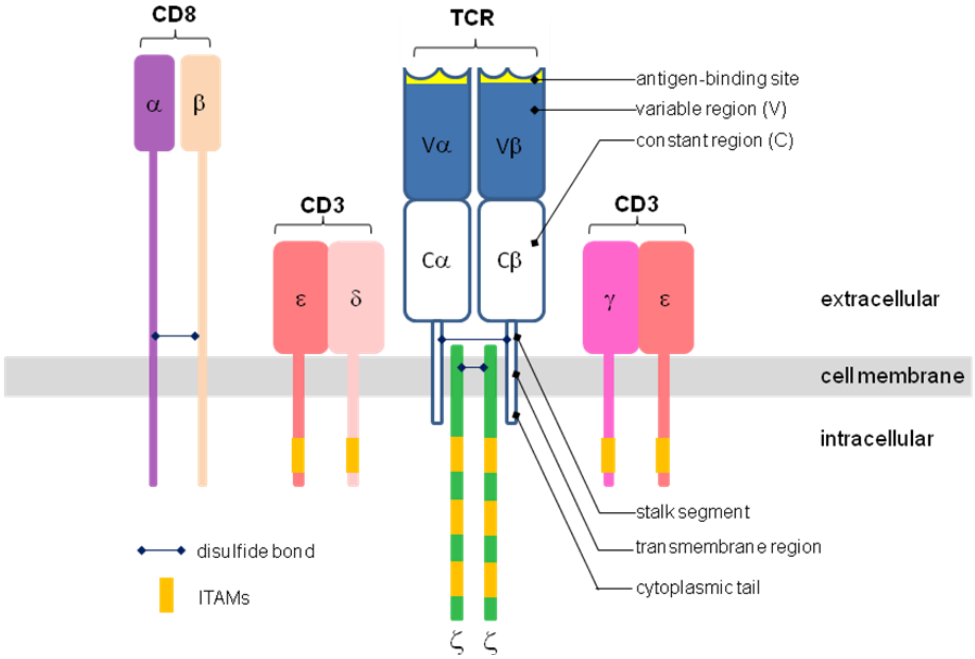

Figure 2.1 Schematic representation of the structure of the T cell receptor (TCR) complex and coreceptor on CD8$^+$ T cells.

The TCR is a heterodimer composed of α- and β-chain, which are linked by disulfide bonds. Both TCR α-and β-chain contain variable (V) and constant (C) regions. Three complementary determining regions (CDR), namely CDR1, CDR2 and CDR3, in the variable region of α- and β-chain conformationally construct the antigen binding site (shown in yellow). The constant region of α- and β-chain is composed of extracellular, transmembrane and cytoplasmic domain. CD3, cluster of differentiation protein 3, is a protein complex containing four transmembrane chains (two ε, one δ, and one γ). Each CD3 chain possesses a signal motif in the cytoplasmic region, called immunoreceptor tyrosine-based activation motif (ITAM, shown in orange). The TCR complex is also associated with a homodimer of the ξ-chain, which also has three ITAMs. The coreceptor of TCR complex on CD8$^+$ T cells, CD8, is shown as a heterodimer of α and β chain and involved in the TCR-antigen binding. CD8 can also be presented as αα homodimer. (Modified from Murphy et al. 2008)

The co-receptors CD8 or CD4, respectively, are also involved in the TCR-antigen recognition. The presence of CD4 and CD8 is used to classify the T cell subsets, namely $CD4^+$ or $CD8^+$ T cells, which have distinct functions. The $CD8^+$ T cells serve as cytotoxic T cells with killing function, whereas $CD4^+$ T cells function as helper T cells and are further conventionally sub-classified as T_H1 and T_H2 cells. T_H1 cells activate the differentiation and antibody-production of B cells, while T_H2 cells enhance the killing efficiency of macrophages (Abbas et al. 1996). A subtype of CD4 T cells with expression of CD25 and Foxp3 is generally defined as T_{reg} cell and suppresses the activity of other T cells to control the immune response (Sakaguchi et al. 2006).

T cells recognize antigen-derived short peptides, which are also known as epitopes and presented by major histocompatibility complex (MHC) on antigen-presenting cells (APC), for example, dendritic cells (DC) and B cells. $CD8^+$ T cells bind peptides of 8-10 amino acids presented by MHC class I molecules (Figure 2.2), whereas $CD4^+$ T cells recognize longer target peptides in the context of MHC class II molecules. The recognition of the MHC molecule by the TCR in $CD4^+$ and $CD8^+$ T cells is similar. A complex of antigenic peptide and MHC molecule (peptide-MHC) is co-recognized by the TCR (Davis et al. 1998). The TCR-peptide-MHC co-crystal structures, which have been solved to date, demonstrate that the TCR docks over the peptide-MHC complex in a diagonal to orthogonal orientation (Rudolph et al. 2006). Typically, CDR1 and CDR2 regions in the antigen binding site contribute more to the binding of the MHC molecule, whereas CDR3 mainly binds the presented peptide (Davis et al. 1998). It has, however, been previously reported that the residues in the CDR1 and CDR2 region of the β-chain derived from a human immunodominant TCR JM22 also contribute to the specific recognition of influenza peptide-HLA-A2 complex (HLA, human leukocyte antigen, MHC molecules in human), suggesting a variation in the TCR-peptide-MHC interaction (Ishizuka et al. 2008). The co-recognition of peptide-MHC by the TCR is known as MHC-restriction, which means that the TCR only recognizes the peptide restricted to a specific MHC molecule. Furthermore, binding of co-receptor CD8 or CD4 stabilizes the TCR-peptide-complex and thereby increases the sensitivity of T cells to antigenic peptide presented by MHC (Li et al. 2004).

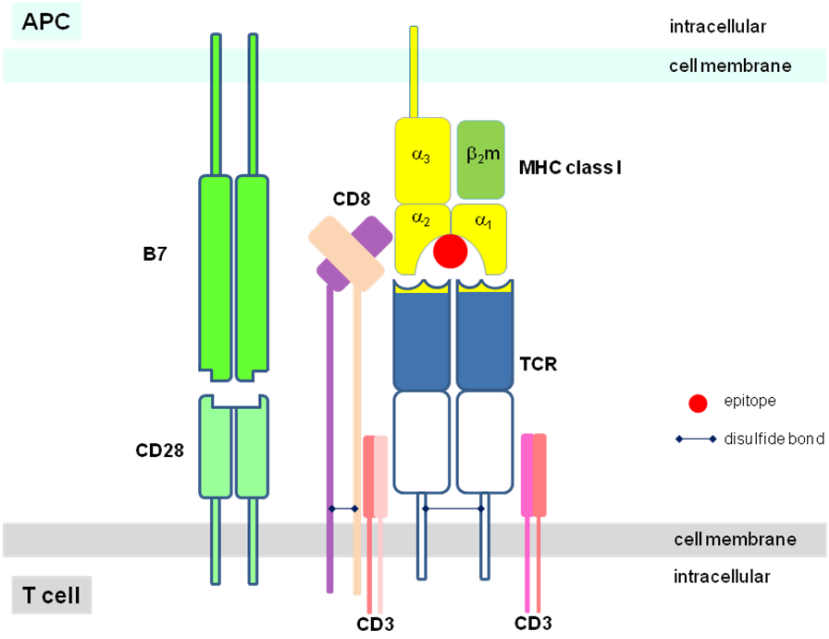

Figure 2.2 The TCR recognizes peptide presented by major histocompatibility complex (MHC) molecules on antigen presenting cells (APC).

The TCR recognizes specific peptide, also called epitope, presented by a MHC molecule. There are two classes of MHC molecules. The TCR on $CD4^+$ T cells binds a peptide-MHC class II complex, whereas $CD8^+$ T cells recognize peptide-MHC class I complexes as shown here. A MHC I molecule is a heterodimer of a transmembrane α-chain and a noncovalently bound β_2-microglobulin (β_2m). The α-chain consists of α_1, α_2 and α_3 domain, whereby the α_1 and α_2 domain forms the peptide binding cleft. The complex of the antigenic peptide and MHC molecule is co-recognized by the TCR ('signal 1'). The co-receptor CD8 also binds the peptide-MHC I complex. The interaction between the co-stimulatory receptor CD28 on naive T cell and its ligand B7 expressed on specialized APC such as dendritic cells (DC) is required for the activation of naive T cells ('signal 2'). (Modified from Murphy et al. 2008)

Peptides presented by MHC I and MHC II are processed by different pathways in APC. MHC I molecules deliver peptides that are derived from the cytosolic antigens such as viral proteins, cytosolic bacterial proteins, old or improperly folded proteins, and also tumor antigens. These cytosolic antigens are degraded by the proteasome into small peptides. Subsequently, the peptides are actively transported by Transporters associated with Antigen Processing proteins (TAP) into the endoplasmatic reticulum (ER), where freshly synthesized MHC I molecules are retained. Upon the binding of peptide, the MHC I molecules is fully formed and delivered to the cell surface (Figure 2.3) (Pamer and Cresswell 1998). MHC II molecules mainly present peptides derived from extracellular pathogens such as most bacteria and toxins, which are internalized by phagocytosis, endocytosis, or macropinocytosis into vesicular compartments of APC and further degraded into peptides. In these vesicles, the peptides are loaded onto MHC II molecules and subsequently delivered to the cell surface (Hiltbold and Roche 2002). Peptide-loaded MHC I and MHC II can then be

recognized by $CD8^+$ and $CD4^+$ T cells, respectively. Considering the origin of the presented peptide, $CD8^+$ cytotoxic T cells are thought to kill virus or cytosolic bacteria infected cells or tumor cells, whereas $CD4^+$ T cells play an important role in eliminating most bacterial infections. However, the presentation of extracellular antigens by MHC I molecules is observed and described as 'cross-presentation', indicating the cross-talk between the two pathways (Heath and Carbone 2001).

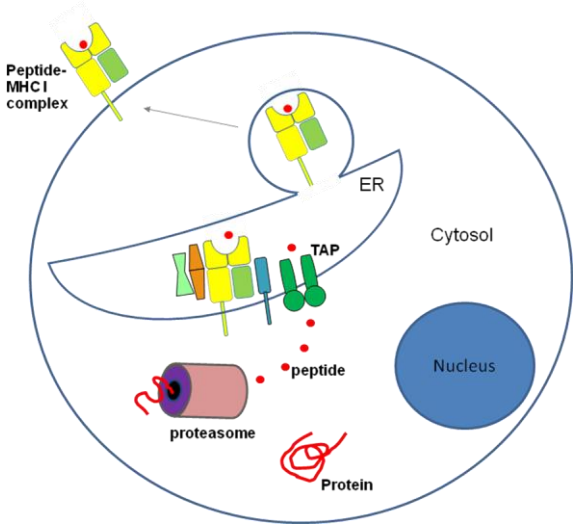

Figure 2.3 Schematic view of the antigen processing and presentation by MHC I molecules in cells.

The TCR does not directly recognize the whole antigen. The up-taken antigen is first degraded by the proteasome to small peptide fragments in the cytosol. The Transporters associated with Antigen Processing (TAP) deliver these peptide fragments into the endoplasmatic reticulum (ER). Upon binding of the transported peptide fragment, the MHC I molecule is completely folded, released from ER and exported to the cell membrane. The proteins in light green, orange, and blue surrounding the MHC I molecule are the chaperone proteins Erp57, calreticulin, and TAP-associated protein tapasin, respectively. They make sure that the partly folded MHC I molecule is retained in the ER. (Modified from Murphy et al. 2008)

T cells originate from the bone marrow and mature in the thymus, from which they got their name. During development and maturation, only T cells with adequate recognition of self MHC molecules survive. This process is known as positive selection. However, T cells strongly recognizing self-antigen in the context of self-MHC molecules are eliminated. This is called negative selection. Theoretically, only T cells with recognition of foreign antigens with self-MHC restriction fully develop after positive and negative selection (central tolerance). Finally, less than 5% T cells in the thymus survive and mature after selection and migrate into periphery (Starr et al. 2003). The few self-reactive T cells, which eventually escaped the described selection mechanisms, are controlled by peripheral immune tolerance mechanisms such as regulation by T_{reg} cells (Gallegos and Bevan 2006). Discrimination

between self and foreign by immune system therefore is maintained through those fine mechanisms to prevent self-attacking.

Following maturation in the thymus, the T cells, called naive T cells, come to the periphery, where they meet their antigens and are activated to eliminate the antigens. For the activation of naive T cells in the periphery, the interaction of TCR and peptide-MHC delivers the first signal for naive T cell activation ('signal 1'), which, however, is not sufficient to initiate the T cell activation. The interaction between costimulatory receptors (e.g. CD28) on naive T cell and their ligands (e.g. B7 molecule) expressed on APC such as DC is also required to activate the T cells and is called 'signal 2' (Figure 2.2). (Murphy et al. 2008) In the absence of this co-stimulatory signal, T cells enter into an 'anergy' state instead of activation. However, the underlying mechanism for anergy is not fully clarified. Anergic T cells are thought to undergo apoptosis or to persist in this state. This is also one of the mechanisms which maintain tolerance to self-peptide presented on tissue lacking co-stimulatory molecules (Schwartz 2003). On the other hand, some tumors seem to evade the recognition by the immune system by this mechanism (Ochsenbein et al. 2001).

Activated cytotoxic $CD8^+$ T cells can kill their target cells by inducing apoptosis. One of the mechanisms used by $CD8^+$ T cells to induce apoptosis involves Fas and Fas ligand which belong to the tumor-necrosis factor (TNF) family. The interaction between Fas and Fas ligand activates caspases, which initiate the caspase-mediated apoptosis pathway (Medana et al. 2000). $CD8^+$ T cells can also use cytotoxic proteins such as perforin and granzymes to kill their targets. Perforin plays an essential role in delivering the granzymes into the target cell through an unknown mechanism. The imported granzymes target BID and pro-caspase-3 and thereby activate different apoptosis pathways (Barry et al. 2000; Lieberman and Fan 2003). In addition, most CD8 cytotoxic T cells also release cytokines such as IFNγ and TNFα, which recruit other immune cells to enhance the immune response against targets. IFNγ additionally has shown significant anti-tumor effect in various studies (Shankaran et al. 2001; Street et al. 2001).

2.3.2 Tumor-specific T cells for adoptive transfer

To date, adoptive transfer of T cells with specificity for viruses such as cytomegalovirus (CMV) or Epstein-Barr virus (EBV) have exerted significant efficacy against virus-associated diseases in immune-suppressed patients after allogeneic stem cell or organ transplantation (Bollard et al. 2004; Cobbold et al. 2005). Since viral antigens are actually foreign antigens, virus-specific T cells are available in the natural T cell repertoire. However, most tumors consist of 'self'-cells without a 'foreign'-tag. T cells with strong self-recognition are normally deleted from the repertoire and therefore are difficult to obtain. However, the identification of diverse tumor antigens evokes our hope to obtain tumor-specific T cells for cancer therapy in

alternative ways. To date, several sources for tumor-specific T cells are under investigation for therapeutic application.

2.3.2.1 Autologous tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TIL) have been successfully isolated from melanoma patients. However, adoptive transfer of $CD8^+$ cytotoxic TIL together with IL-2 adiministration did not induce impressive anti-tumor effects (Rosenberg et al. 1988). Two modified protocols including (1) co-transfer of $CD4^+$ T helper cells and $CD8^+$ TIL and (2) previous lymphopenic conditioning of patients both substantially enhanced the clinical response in melanoma patients, suggesting that the help function of $CD4^+$ T cells and the survival of the transferred T cells are of high importance for an effective clinical response (Dudley et al. 2002). The combination of the two protocols resulted in impressive anti-melanoma effects with control of disease in 50% patients (Rosenberg and Dudley 2004). However, the difficulties in isolating TIL from most tumors and in cultivating TIL *in vitro* hampered the application of this strategy for broader treatment of several cancers.

2.3.2.2 Allogeneic tumor-specific T cells

T cells with self recognition in the context of self-MHC molecules are eliminated mostly from the repertoire. However, alloreactive T cells, which recognize self-peptides presented by a foreign MHC molecule, have not been under negative selection and therefore represent a source of tumor-specific T cells. As a matter of fact, such alloreactive T cells exist in T cell repertoire with the frequency of up to 10% (Suchin et al. 2001). The mechanisms accounting for the allorecognition of T cells remain to be clarified. Two early models of the molecular basis of allorecognition suggest that the TCR primarily interacts either with the MHC molecule or with the peptide, namely the peptide-independent model and the peptide-specific model of allorecognition (reviewed by Whitelegg and Barber 2004). However, the accumulating findings, especially the solved crystal structures of TCR-peptide-MHC, reveal that both the bound peptide and the MHC molecule are involved in the TCR interaction in allorecognition (Weber et al. 1995; Housset and Malissen 2003; Colf et al. 2007). The relative contribution of bound peptide and the allogeneic MHC molecule to the TCR interaction is yet still unclear. Moreover, alloreactive T cells with high peptide specificity have been identified in numerous studies (Alexander-Miller et al. 1993; Pittet et al. 2006). In addition, the broadness and diversity of the TCR repertoire in alloreactive T cells have been demonstrated in studies using synthetic peptide libraries (Obst et al. 1998; Munz et al. 1999). These studies potentiate the similarity between allogeneic and autologeous TCR recognition.

Interestingly, it has been shown both in MHC I and MHC II system that some alloreactive T cells can recognize more than one but a limited number of peptide-allogeneic-MHC complexes with a high degree of specificity, suggesting the polyspecificity of allorecognition.

Actually such polyspecificity is not only observed in allogeneic T cells but also in conventional T cells. Therefore, polyspecificity seems to be a general and inherent feature of TCR recognition and may provide an explanation for the high frequency of alloreactive T cells (Felix and Allen 2007). However, this gives rise to the question whether specificity and degeneracy contradict each other in T cell recognition. In fact, it has been suggested that both specificity and degeneracy are the ability of T cells: T cells are specific because only the small fraction of all possible peptides (approximately 1 in 10^5-10^6 peptides) are recognized by T cells, but are also degenerate because the number of potential peptides is particularly large (e.g. 5 x 10^{11} potential 9-mer peptide sequences for $CD8^+$ T cells) (Wucherpfennig et al. 2007).

Clinically alloreactive T cells elicit vigorous responses, manifested as the transplant rejection and graft-versus-host disease (GVHD) after transplantation over MHC barriers. However, alloreactive T cells with specific peptide recognition, known as allorestricted T cells, show the potential for cancer therapy. It has been demonstrated that adoptive transfer of donor lymphocytes into patients with recurrent leukemia after allogeneic stem cell transplantation results in a graft-versus-leukemia (GVL) response (Kolb et al. 1995). The GVL response could be contributed to the recognition of hematopoiesis-restricted minor histocompatibility antigens (mHAgs) of the recipient by infused donor T cells (Marijt et al. 2003). This strategy was also used to treat solid tumors like renal-cell carcinoma (Childs et al. 2000). Moreover, tumor peptide-specific allorestricted T cells have been identified, isolated, and expanded *ex vivo* (Sadovnikova and Stauss 1996; Dutoit et al. 2002; Mutis and Goulmy 2002; Amrolia et al. 2003). However, the observed GVHD in leukemia patients after infusion of allogeneic donor lymphocyte (Horowitz et al. 1990; Kolb et al. 2004) and the generation of alloreactive T cells lacking peptide specificity *ex vivo* hamper the use of allorestricted T cells for adoptive transfer (Dutoit et al. 2002). Hence, more extensive characterization and investigation of allorestricted T cells are required before their clinical application.

2.3.2.3 Genetically engineered T cells with tumor-specific receptors

Antigen binding receptors, B cell receptor (BCR) or antibody and TCR, mediate the antigen-response of B cells and T cells. Therefore, it has been tested to generate tumor-specific effector cells for cancer therapy based on the assumption that introducing tumor-specific receptors into immune effector cells might redirect the recognition of recipient cells toward tumor targets. One attractive advantage of this approach is that a large number of primary T cells can be simultaneously transduced with a defined tumor-specific receptor. Subsequently, modified recipients T cells can be expanded to sufficient numbers for transfer in a relatively short period of time, allowing the rapid generation of tumor-specific T cells (Morgan et al. 2006; Schmitt et al. 2009). As shown briefly in Figure 2.4, diverse strategies have been developed to obtain genetically modified T cells specific to tumor antigens.

Chimeric antigen receptors

One strategy utilizes a chimeric antigen receptor (CAR), which contains the single-chain variable fragment (svFv) of an antibody and the transmembrane and intracellular signalling domains derived from FcRγ or ξ-chain from the TCR complex to trigger the T cell effector function upon antigen binding. Intracellular signalling domains from co-stimulatory molecules such as CD28 (Maher et al. 2002; Willemsen et al. 2005) or 4-1BB (Imai et al. 2004) have also been combined to the ξ signalling tail in order to additionally deliver co-stimulatory signals to enhance the T cell function (Figure 2.4 A). CARs targeting CD19, a surface receptor expressed on B cell lymphoma, are currently the most investigated example for this strategy. T cells equipped with CD19-specific CARs have shown B cell lymphoma killing *in vitro* (Brentjens et al. 2003; Cooper et al. 2003) as well as in experimental models (Serrano et al. 2006). Furthermore, it has been observed that the addition of co-stimulatory signalling domains enhances the anti-tumor T cell response *in vivo* (Friedmann-Morvinski et al. 2005). Clinical trials evaluating CD19 specific CARs are currently ongoing, and preliminary clinical efficacy has been observed. However, one serious adverse event in one enrolled subject has been reported recently, indicating that these CARs need further optimization for clinical application (Brentjens et al. 2010). One attractive advantage of using antibody-based CARs is that their antigen recognition is HLA-independent, while recognition by TCRs is HLA-restricted. One obvious disadvantage of CARs is that they can only target surface tumor antigens as mentioned for antibody-mediated immunotherapy, whereas TCRs can target intracellular tumor antigens by recognizing their peptides presented by MHC molecules on APC or tumor cells. This study focuses on tumor-specific TCRs.

Wild type tumor-specific TCRs

A tumor-specific TCR is obviously a good candidate for redirecting primary T cells towards the tumor (Figure 2.5 B). Many groups are exploiting the potential of this strategy for the treatment of diverse cancers. *In vitro*, TCR-modified T cells endowed recognition of a defined tumor antigen and showed similar avidity for the tumor antigen as the parental T cells from which the TCR was isolated (Morgan et al. 2003; Schaft et al. 2003). Moreover, T cells equipped with tumor-specific TCRs could eradicate tumors after adoptive transfer into mice (Kessels et al. 2001; Morris et al. 2005). The first clinical trial with TCR gene-modified T cells was carried out by the Rosenberg group. Autologous T cells were retrovirally transduced with genes encoding α- and β-chain of a TCR with specificity for MART-1, a melanoma antigen, and adoptively transferred into melanoma patients. High level engraftment of TCR-transduced T cells was observed in 15 patients. Two of the patients also showed a clinical response (Morgan et al. 2006). This pivotal study demonstrated that genetically engineered T cells can mediate anti-tumor effects in a clinical setting, indicating the feasibility and potential of this strategy (called TCR gene therapy) for cancer treatment. However, clinical efficacy of

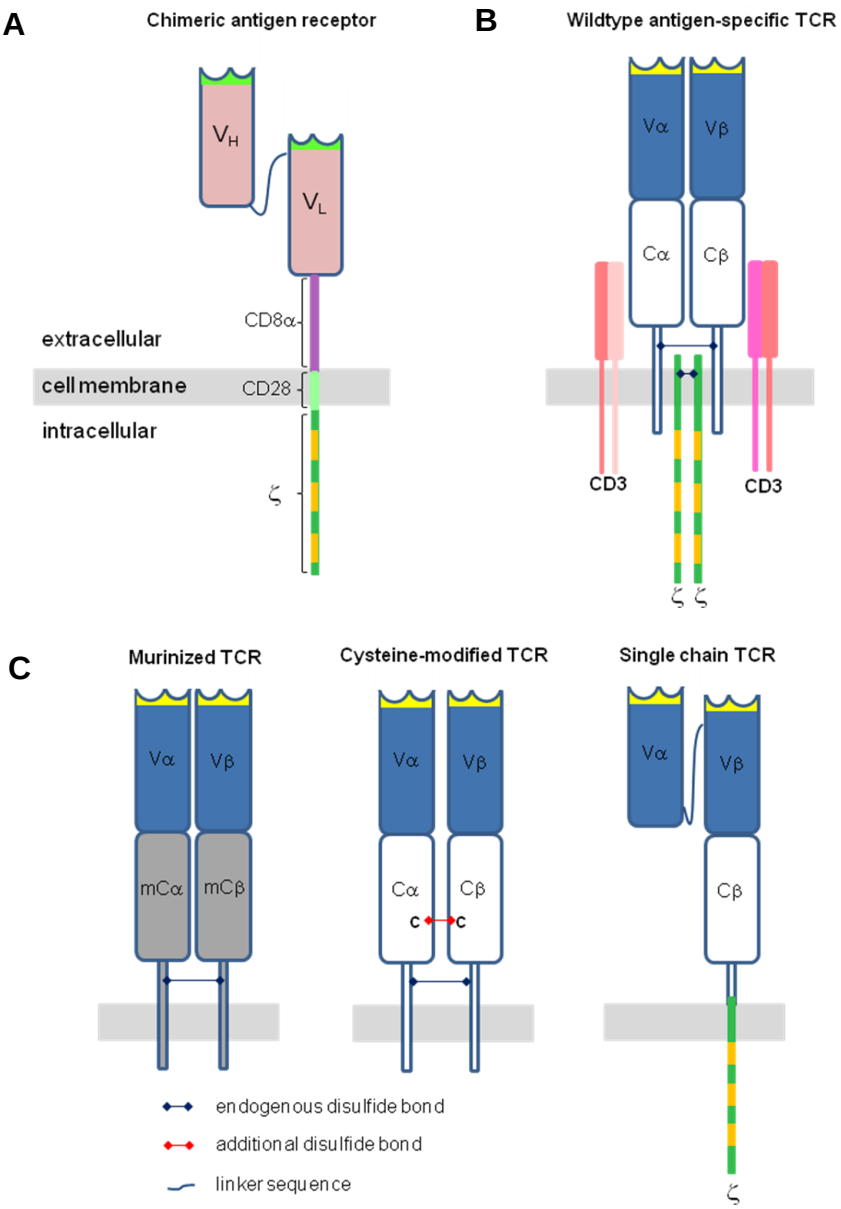

Figure 2.4 Tumor-specific antigen receptors for genetically engineered T cells for cancer therapy and genetic strategies addressing TCR pairing.

(A). The antibody-based chimeric antigen receptor is composed of a svFv fragment derived from a tumor-antigen binding antibody, signaling domains from TCR ξ-chain and co-stimulatory signaling domains such as CD28. CD8α hinge is used to link svFv fragment and cytoplasmic signaling domains (Milone et al. 2009). CARs are able to recognize tumor-associated antigens (TAA) and trigger T cell activation, and therefore are potential candidates for genetically engineered T cells for cancer therapy. (B). The wildtype TCR α- and β-chain isolated from T cells with specificity for TAA serves as a good source for TCR-modified T cells for cancer therapy (TCR gene therapy). (C) Strategies for optimizing pairing of transduced TCR chains in recipient cells including replacing the human constant α and β regions with their murine counterparts (left panel), introducing an additional disulfide bridge between TCR α- and β-chains through site-directed cysteine-mutation (middle panel) and generating single chain TCR containing variable region of α-chain and entire β-chain, which is fused to TCR ξ-chain (right panel) (Modified from Govers et al. 2010).

this study was not in the same level as that in the clinical trial utilizing melanoma-specific TIL (Rosenberg and Dudley 2004), indicating that further modification of this approach is required. The limited clinical response in this trial might be due to the relatively low avidity of the used TCR, which has been obtained from TIL. Considering the development of the T cell repertoire, it may be difficult to isolate TCRs with high avidity for self TAA from autologous T cells. However, such high avidity TCRs can be obtained from allorestricted T cells (Sadovnikova and Stauss 1996; Xue et al. 2005) or human TAA-specific murine T cells (Altmann et al. 1995). Along that line, the second clinical study by the Rosenberg group, in which peripheral blood mononuclear cells (PBMC) transduced with one human TCR or one mouse TCR with high avidity for MART1 or GP100 were used, showed improved clinical outcomes compared to previous study (Johnson et al. 2009). However, unexpected targeting of skin, eye, and ear was observed in the patients, which might be due to the natural expression of MART-1 and GP100 in these tissues. The potential recognition of low amounts of antigen expressed on normal tissues by high avidity TCRs emphasizes the choice of suitable tumor-antigens and the extensive characterization of TCR-modified T cells before transfer.

Modified tumor-specific TCRs

In addition to the choice of a suitable target antigen and an effective TCR, sufficient expression of the transduced TCR in recipient cells is essential for exploiting TCR-modified T cells for adoptive therapy. The endogenous TCRs can interfere with the expression of the transduced TCR by competition for surface CD3 complexes or by mispairing of endogenous TCR α- (β-) chain and transduced TCR β- (α-) chain. Moreover, the mispaired TCRs can harbour new specificity and induce self-recognition (Stanislawski et al. 2001; Schumacher 2002). Hence, several strategies are exploited to modify the TCR constructs to reduce the mispairing (Figure 2.4 C): (1). Exchange of TCR α and β constant domains with corresponding domains from murine TCR results in enhanced correct pairing and expression of the transduced TCR. This is most likely achieved by preferential pairing of murine constant domain and improved TCR/CD3 stability. Along this line, human T cells transduced with murinized TCRs demonstrated enhanced anti-tumor reactivity (Cohen et al. 2006). (2). Another strategy is the introduction of an additional disulfide bridge between TCR α- and β-chain. Site-directed cysteine-mutation in the constant region of TCR α- (48 threonine→cysteine) and β-chain (57 serine→cysteine) resulted in a second disulfide bond linking the TCR α- and β-chain (Figure 2.4 C) (Cohen et al. 2007; Kuball et al. 2007). Transduction of cysteine-modified WT1-specific TCRs into human T cells improved TCR expression as well as T cell cytotoxicity and IFNγ secretion against cells which presented the specific peptide (Kuball et al. 2007). (3). The transduction with single chain TCR (scTCR) constructs is an alternative strategy to overcome the mispairing. scTCR comprise the TCR α-

chain variable domain (Vα) and the entire TCR β-chain as well as the ξ-chain (Figure 2.4 C). The TCR Vα region is connected with the entire TCR β-chain by a linker sequence. Additionally, the ξ-chain is fused to the TCR β-chain to transmit downstream signals for T cell activation (Chung et al. 1994; Willemsen et al. 2000). It has been shown that the expression of scTCR with specificity for MA1 is increased compared to its double chain counterpart after transduction in T cells (Willemsen et al. 2000). However the anti-tumor reactivity was compromised, revealing the limitation of this strategy and that further optimization of this strategy is required.

To date, introduction of TCR genes into recipient cells is mediated mostly by the gamma-retroviral vectors. These retroviral vectors comprise proviral sequences, which allow the incorporation of the vector into the DNA of the target cell, the gene(s) of interest (i.e. the TCR α and/or β gene(s)), and viral and cellular gene promoters (e.g. CMV promoter) to enhance the expression of the introduced gene (Kurian et al. 2000). For TCR transduction, firstly, the TCR chain encoding retroviruses have to be generated. For this purpose, the TCR gene bearing retroviral vector is transported into packaging cell lines by non-viral transfection protocol using electroporation or calcium-phosphate. In addition to the retroviral vector, vectors containing the retroviral genes *gag*, *pol*, and *env* are co-transfected, which encode indispensable proteins for virus assembly. Retroviruses containing the TCR gene are assembled within the packaging cells, released into cell culture medium and can then be used to infect the TCR recipient cells, where the retroviral vector integrates into the genome of target cells. Using this co-transfection strategy, newly produced virus is not able to replicate, since it does not contain genes encoding the essential viral structural proteins env, gag, and pol. Several retroviral vectors have been currently used for TCR gene transfer and showed different transduction efficiencies. For example, the mouse myeloproliferative sarcoma virus (MPSV)-based retroviral vector pMP71 has demonstrated high level transduction of TCR genes in T cells in several studies (Engels et al. 2003; Leisegang et al. 2008; Schub et al. 2009). Lentiviral vectors, which are based on a lentivirus such as human immunodeficiency virus (HIV), have also been exploited to deliver TCR genes into T cells. An advantage of lentiviral vector-based transduction is that lentiviruses may also infect non-dividing cells, while gamma-retroviruses only transduce dividing cells. Therefore, T cells may be modified by lentivirus at a less differentiated stage, which might be important for clinical application (Yang et al. 2008).

With the integration of the MHC-peptide multimer technology, expression of the transduced TCR with specificity for a known epitope can be quantitatively evaluated by flow cytometry. This technology is based on the specific interaction within TCR-peptide-MHC complexes (Figure 2.5). Monomeric MHC-peptide complexes are unstable because of the low affinity of TCR-peptide-MHC interactions (Matsui et al. 1994). However, the multimerization of the

MHC-peptide complexes improves their relative-binding avidity to the TCRs on T cells, which allows the stable binding of fluorescence-labelled MHC-peptide multimers to T cells for their identification (Altman et al. 1996). The MHC-peptide multimer technology is widely applied to phenotypically detect, characterize, and quantify antigen-specific T cells in both mouse and human models (reviewed by Klenerman et al. 2002).

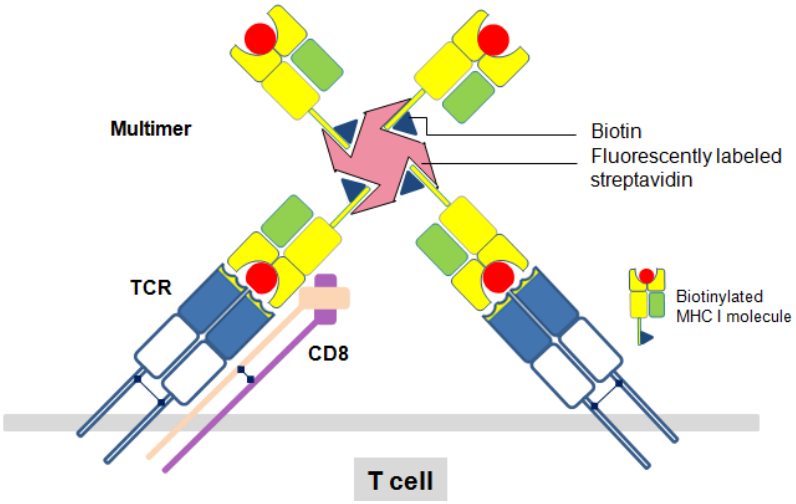

Figure 2.5 Schematic representation of the structure of a fluorescence-labeled MHC I-peptide multimer and interaction between MHC I-peptide multimer and CD8$^+$ T cells.

The MHC I-peptide multimer consists of multiple biotinylated MHC I-peptide complexes, which are multimerized using streptavidin. Multimeric MHC I-peptide complexes are able to bind more than one TCR on T cells to enhance the stability of the interaction between MHC I-peptide multimer and CD8$^+$ T cells. This strong interaction allows the detection of T cells expressing the TCR of interest by staining with the respective fluorescence-labeled MHC-peptide multimer and subsequent flow cytometric analysis (Modified from Klenerman et al. 2002).

2.3.3 *In vivo* monitoring of tumor specific T cells after adoptive transfer

Although accumulating data demonstrate that T cells genetically modified with tumor-specific receptors like CAR and TCR display specific antigen recognition and anti-tumor reactivity in *in vitro* and *in vivo* models (Hughes et al. 2005; Johnson et al. 2006; Zhao et al. 2009), clinical responses of cancer patients treated with TCR-modified effector T cells were disappointing (Johnson et al. 2009; Morgan et al. 2010). Several properties of the adoptively transferred tumor-specific T cells *in vivo* such as persistence and migration capability into tumor sites have been shown to correlate with their clinical efficacy (Pockaj et al. 1994; Dudley et al. 2002; Robbins et al. 2004). Therefore, long-term monitoring of survival and spatial distribution of TCR-modified T cells is important to understand the impact of genetically engineered T cells on the clinical outcome and to improve protocols for TCR gene therapy (Dobrenkov et al. 2008). Recently, diverse monitoring systems have been

investigated to trace immune-derived cells *in vivo*. Bioluminescence imaging (BLI) has been used to detect T cells marked with reporter-genes (e.g. luciferase) in mouse models (Prins et al. 2008). However, BLI methodology can later not be easily transferred to humans because of limitations of light penetration emitted by the bioluminescent reporter-gene system (Su et al. 2006).

Radioactive tracer-mediated non-invasive molecule imaging system positron emission tomography (PET) provides imaging with high resolution and sensitivity and has emerged as a promising approach to monitor T cells in the clinical setting (Ponomarev 2009). PET is currently used in the clinical setting to detect tumors and assess efficacy of cancer therapies by analyzing proliferation of the tumor cells using diverse radioactive traces such as 2-(fluorine-18)-fluoro-2-deoxy-D-glucose (^{18}FDG), 3-deoxy-3-(fluorine-18)-fluorothmidine (^{18}FLT) and methyl-(carbon-11)-L-methionine (^{11}C-MET) (Phelps 2000; Jacobs et al. 2007). ^{18}FDG, a radioactive analog of glucose, is used to assess the growth and localization of the tumor, since the uptake of glucose in tumors is dramatically higher than in most organs. The combination of PET with anatomic imaging computed tomography (CT), PET/CT, allows the 3D anatomic localization of target cells. To render the target T cells radioactive for PET imaging, the target cells can be directly labeled with a radioactive tracer (e.g. ^{18}FDG and ^{64}Cu-Pyruvaldehyde-bis(N^4-methylthiosemicarbzaone)) (Adonai et al. 2002). However, the short life-time of those tracers limits their application in long-term monitoring. Alternatively, the target cells can be equipped with a reporter gene, which can uptake radiotracer and thereafter indirectly enable the radioactive labeling of target cells. The human sodium/iodide symporter (hNIS) gene encodes an endogenous membrane glycoprotein, which cotransports I^- and Na^+ across the thyroid cell membrane, and is an attractive reporter gene for PET imaging by using radioisotopes such as $^{124}I^-$ (Waerzeggers et al. 2009). Non-invasive imaging using the hNIS reporter gene has been achieved in preclinical model (Baril et al. 2010) and clinical trials (Barton et al. 2008). In this study, I sought to exploit PET imaging to monitor TCR-transduced PBMC *in vivo* by using the hNIS reporter-gene.

2.4 TAA

The selection of the right TAA and, in more detail, the most suitable immunogenic peptides (epitopes) contained in the TAA plays an essential role in developing effective T cell-mediated immunotherapy for cancer. Four key criteria have been described by Kessler and Melief to classify TAA as ideal potential targets for immunotherapy: (1). The TAA should be shared between patients and/or cancer types; (2). They should be specifically expressed in tumor and rarely in healthy tissues; (3). They should be of importance for the oncogenesis and/or cancer survival; and (4). Their turnover kinetics should show possible changes as observed in the case of p53 (Kessler and Melief 2007).

2.4.1 Classification of TAA

By now, increasing numbers of TAA have been identified as potential targets and classified into following groups according to tumor specificity (Kessler and Melief 2007):

(1). Individually unique tumor-specific antigens: Unique mutations in a single tumor of one patient result in this group of TAA. One example is the melanoma-associated-mutated antigen-1 (*MUMI*) gene with a unique point mutation.

(2). Lineage-specific differentiation antigens: Antigens from this group are expressed in both the tumor and the original normal tissues. Melanoma/melanocyte antigens such as MART-1, gp100 and tyrosinase and prostate antigens such as PSA belong to this group.

(3). Tumor-specific antigens: This type of antigens is expressed in the tumor but not in normal tissue or only in restricted tissue. The group of the so-called cancer-testis antigens including MAGE, BAGE and GAGE families as well as NY-ESO-1 which are only expressed in testis and/or placental tissue belong to this group. Additionally, viral proteins such as E6 and E7 of human papillomavirus (HPV) also belong to this group.

(4). Overexpressed antigens. This group of antigens are also expressed in healthy tissue, but the expression of the antigens in the tumor is distinctly higher. Among this group are p53, Survivin, PRAME, CEA, HER2/neu and MUC1.

2.4.2 Identification of TAA

TAA and the respective epitopes recognized by T cells can be identified by using different strategies, which are generally classified as ‘direct immunology’ and ‘reverse immunology’ based approaches. The ‘direct immunology’ strategies are based on the natural immunity. One example is the identification of MAGE-1 in the early 1990s (van der Bruggen et al. 1991; Traversari et al. 1992). The cDNA encoding the recognized epitope was identified by a melanoma-specific cytotoxic T lymphocyte (CTL) line, the specificity of which was tested against a melanoma-derived cDNA library. The recognized epitope was further identified by

using cDNA truncation and peptide recognition strategies. Alternatively, CTL clones can also be used to identify peptides by screening the high-performance liquid chromatography (HLPC)-fractions isolated from tumor cell surface. Mass spectrometry can help to identify the epitope sequence, which can be further used to search the entire TAA in a database. In fact, many TAA including Melan-A/MART-1 (Coulie et al. 1994; Kawakami et al. 1994), tyrosinase (Brichard et al. 1993) and gp100 (Bakker et al. 1994) have been found by using this strategy. The serological identification of antigens by the recombination expression cloning (SEREX) strategy is based on the humoral immunity (antibody-mediated) instead of the cellular immunity (T cell-mediated) to define potential TAA. Serum IgG antibody derived from patients can be used to screen proteins encoded by cDNA libraries obtained from the tumor. Cancer-testis antigen NY-ESO-1 is one TAA identified by SEREX strategy (Chen et al. 1997).

With the accumulating knowledge of the interaction between HLA molecules and the specific peptide and the intracellular process of epitope generation and presentation, an additional approach is to predict the putative epitope contained in known TAA by computer algorithms and to exogenously load the peptide on APC to generate specific T cells against the predicted peptide. Subsequently, the obtained specific T cells are tested against tumor cells that express the corresponding TAA and HLA molecule to verify that the predicted peptide is naturally processed and presented and recognized by the peptide-specific T cells. This indirect strategy for epitope identification is known as ‘reverse immunology’ (Kessler and Melief 2007). By now, numerous epitopes have been identified by using this strategy, for example HLA-A2-resctricted epitopes MEGA-$2_{157-166}$, MAGE-$3_{112-120}$, $CEA_{691-699}$, and HER2/$neu_{435-443}$ (Kawashima et al. 1998).

2.4.3 HER2/neu as TAA

HER2/neu (ErbB2), the epidermal growth factor receptor (EGFR) 2, is one well-known TAA. Overexpression of HER2/neu is observed in 25-30% of breast and ovarian cancer (Stern 2000) and is tightly associated with the progression of human ovarian and breast cancer (Slamon et al. 1987). HER2/neu is a transmembrane phosphoglycoprotein receptor tyrosine kinase (RTK). HER2/neu activation triggers the downstream signalling that is involved in cell proliferation, angiogenesis, invasion, and metastasis (Freudenberg et al. 2009; Kruser and Wheeler 2010). Therefore, HER2/neu may play an important role in tumorigensis of human ovarian and breast cancer. With its normal function HER2/neu is involved in the development of the mammary gland (Stern 2003). The underlying mechanisms by which HER2/neu influences tumorigensis in humans remain to be elucidated.

Nevertheless, in the clinic HER2/neu is an attractive clinical therapeutic target. Herceptin^{TM}, a humanized mAb targeting HER2/neu, is approved for clinical application in patients with HER2/neu^{+} advanced breast cancer (Pegram et al. 1998). Unfortunately, occurrence of

resistance to antibody-treatment was observed, which might be mediated by several mechanisms (reviewed by Nahta and Esteva 2006). Clinical trials of vaccination with the HER2/neu-derived E75 peptide (KIFGSLAFL, residue 369-377) in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) revealed that the E75 peptide is safe and can trigger a peptide-specific immune response *in vivo* (Knutson et al. 2002; Murray et al. 2002; Peoples et al. 2005). Moreover, the clinical trial by Peoples et al. showed that the recurrence rate of vaccinated patients with node-positive breast cancer (NPBC) was reduced compared to that of the control group (Peoples et al. 2005). However, further clinical trials are required to determine the clinical efficacy of this strategy. Moreover, adoptive transfer of genetically modified T cells with CAR targeting HER2/neu is currently evaluated in a clinical trial by the Rosenberg group. Unfortunately, a serious adverse event was recently reported: one patient experienced respiratory distress shortly after T cell infusion with marked increases in multiple cytokines in serum and died 5 days after the treatment (Morgan et al. 2010). The observed strong release of many cytokines in this patient, which was consistent with a 'cytokine storm', might result from the recognition of low level of HER2/neu on lung epithelial cells by the transferred T cells after infusion. In addition to immunological strategies, several tyrosine kinase inhibitors have been developed to target HER2/neu. For example, lapatinib inhibits both HER1 and HER2/neu and is able to induce death of human breast cancer cells *in vitro* and *in vivo* (Xia et al. 2002). However, tumor resistance to lapatinib has been observed in *in vitro* studies (Carter et al. 2005). Taken together, currently developed strategies targeting HER2/neu showed a certain level of efficacy for HER2/neu$^+$ cancer treatment, but further optimizations are required to achieve sufficient clinical responses with fewer side effects.

$HER2_{369}$ (E75) as mentioned above is a well-defined epitope derived from HER2/neu. The immunogenicity of $HER2_{369}$ in the context of HLA-A2 molecule has been proven in *in vitro* and *in vivo* experimental models (Fisk et al. 1995; Lustgarten et al. 1997; Anderson et al. 2000) as well as in clinical trials (Zaks and Rosenberg 1998; Knutson et al. 2002 Murray et al. 2002), indicating that $HER2_{369}$ represents an attractive target for T cell-based immunotherapy. To obtain sufficiently effective T cells or TCRs targeting HER2/neu, allorestricted T cell clones with specificity for $HER2_{369}$ have been previously isolated *in vitro* in our laboratory by using the strategy shown in Figure 2.6 (Weigand 2007). Allorestricted cytotoxic T cells against cyclin-D protein as TAA have been previously isolated by using a similar strategy (Sadovnikova et al. 1998). Briefly, $HER2_{369}$ pulsed HLA-A2$^+$ T2 cells, which cannot present endogenous epitopes on MHC I molecules due to TAP-deficiency, were used as APC to stimulate HLA-A2$^-$ T cells derived from a healthy donor (Weigand 2007). Subsequently, the T cell population recognizing $HER2_{369}$ was isolated from the bulk culture by sorting with HLA-A2-$HER2_{369}$ multimers 7 days or 14 days after stimulation. The T cell clones were then

obtained from an enriched $HER2_{369}$-recognizing T cell population by limiting dilution and were tested against peptide-pulsed T2 cells as well as HER2/neu-expressing tumor targets to define peptide recognition and anti-tumor reactivity. As shown in Table 2.1, several clones with $HER2_{369}$-specific peptide recognition and/or anti-tumor reactivity have been successfully isolated (Weigand 2007; Liang et al. 2010). Unfortunately, most T cells could not be cultured for a long period of time. Furthermore, the TCRs harboured in those T cell clones have been previously identified (Liang 2007). These TCRs may represent potential candidates for TCR gene therapy for HER2/neu$^+$ cancer. Further detailed characterization of those TCRs regarding anti-tumor reactivity, potential intrinsic autoreactivity and alloreactivity in both the *in vitro* and *in vivo* settings is required to determine their therapeutic potential for TCR gene therapy and represents a key topic of my PhD thesis.

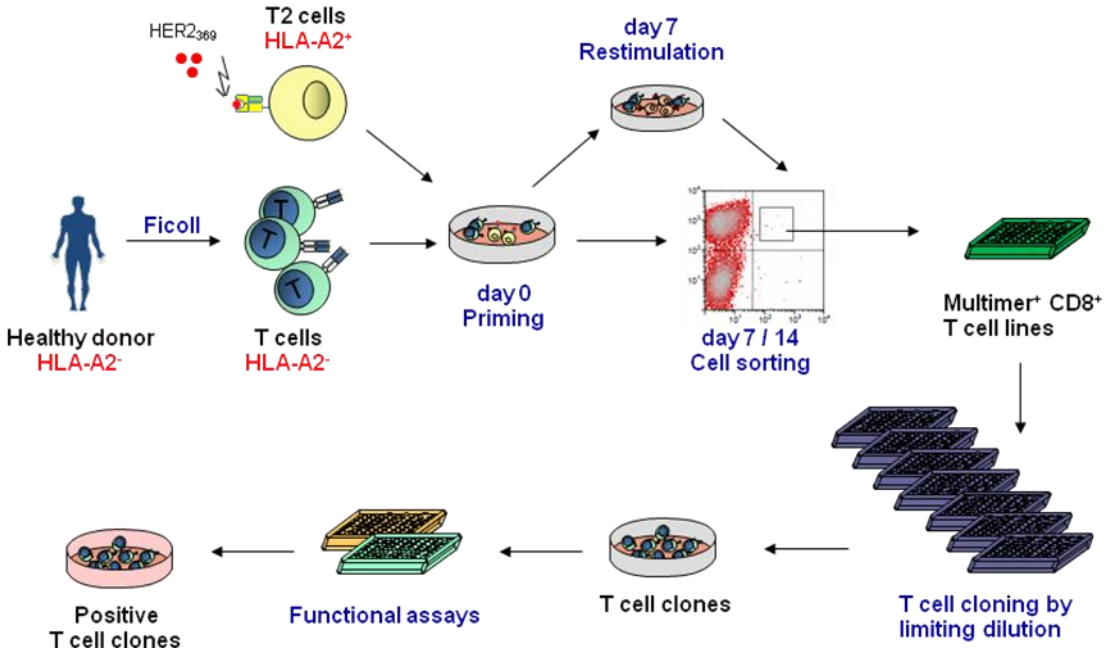

Figure 2.6 The procedure of isolating allorestricted T cell clones with specificity for $HER2_{369}$ by *in vitro* stimulation.

HLA-A2$^-$ T cells obtained from a healthy donor were stimulated *in vitro* with HLA-A2$^+$ TAP-deficient T2 cells, which were pulsed with $HER2_{369}$ peptide and served as APC. HLA-A2-$HER2_{369}$-multimer-specific CD8$^+$ T cells were isolated by cell sorting 7 days after priming or, alternatively, after restimulation with $HER2_{369}$-pulsed T2 cells in the same setting. T cell clones were then obtained from sorted T cells by limiting dilution. The outgrowing T cell clones were tested against peptide-pulsed T2 cells as well as against HER2/neu-expressing tumor cells. Candidate T cell clones with specific peptide-recognition and/or anti-tumor reactivity were chosen for further investigation (Modified from Weigand 2007).

Table 2.1 Peptide recognition and anti-tumor reactivity of selected allorestricted T cell clones with specificity for $HER2_{369}$

Donor	Clone	Cytotoxicity (%)		
		T2 + $HER2_{369}$	T2 + Flu*	SK-Mel 29[&]
1	**HER2-1**	65 ± 6.2	8 ± 7.6	21 ± 8.2
1	**HER2-2**	60 ± 4.7	0 ± 0.4	1 ± 0.9
1	**HER2-3**	64 ± 6.5	6 ± 0.9	29 ± 1.3
2	**HER2-4**	79 ± 4.2	2 ± 2.1	1 ± 0.0

*: peptide Flu served as control peptide

[&]: SK-Mel 29 is a melanoma cell line naturally expressing HER2/neu

2.4.4 FMNL1 as TAA

Formin-like 1 protein (FMNL1) has been previously identified as a novel lymphoma-associated antigen by the SEREX strategy (see section 2.4.2) in chronic lymphocytic leukemia (CLL) (Krackhardt et al. 2002). As a member of the family of formin-related proteins, which are expressed in all eukaryotes and which are involved in the regulation of actin dynamics (Harris et al. 2006), FMNL1 plays an important role in the polarization of the centrosome in T cells (Gomez et al. 2007). Recent work from our group demonstrated that FMNL1 can induce non-apoptotic membrane blebbing, which is regulated by its N-terminal myristoylation site (Han et al. 2009). The expression of FMNL1 at mRNA level in healthy tissues is highly restricted to hematopoietic lineage-derived cells including PBMC and lymphoid organs such as thymus and bone marrow (Figure 2.7 A). Overexpression of FMNL1 has been observed in CLL samples and tumor cells derived from leukemia patients (Figure 2.7 B; Krackhardt et al. 2002; Schuster et al. 2007). Considering the restricted expression in healthy tissues and overexpression in malignant cells, FMNL1 represents an attractive TAA for immunotherapy targeting cancers such as CLL (Schuster et al. 2007).

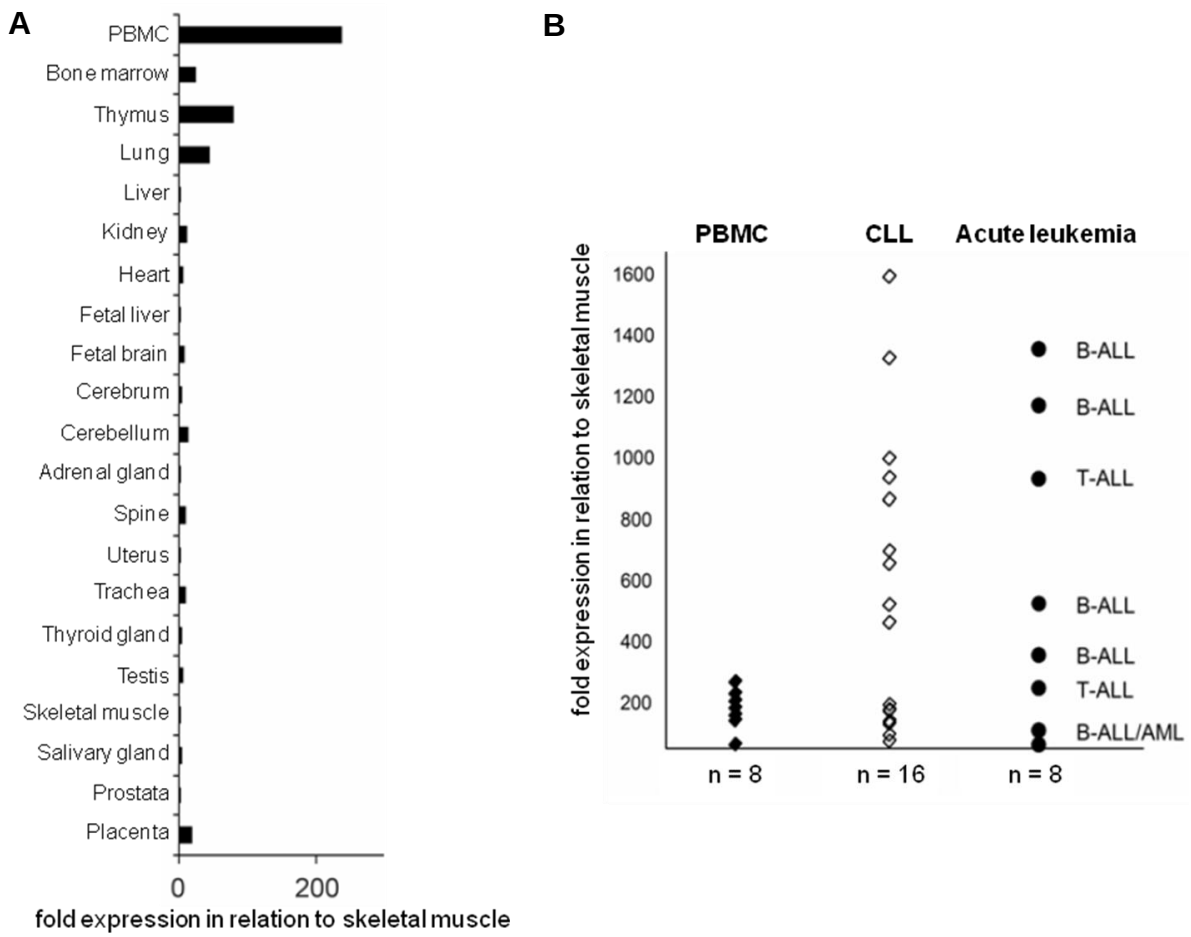

Figure 2.7 Expression profile of FMNL1 in normal and malignant tissues.

(A).The relative mRNA expression of FMNL1 in different tissues obtained from healthy donors was determined by quantitative real-time PCR. The relative quantitative expression compared to skeletal muscle was calculated using the delta-delta Ct method. (B) The relative mRNA expression of FMNL1 in normal peripheral blood mononuclear cells (PBMC), chronic lymphoid leukemia (CLL) samples and tumor cells obtained from patients with B- or T-acute lymphoid leukemia (ALL) as well as acute myeloid leukemia (AML) was measured as described in (A) (Schuster et al. 2007).

In fact, allorestricted T cell clones with specificity for FMNL1-derived peptide PP2 (RLPERMTTL) have been previously isolated by using the same strategy as described above (Figure 2.6). FMNL1-PP2 was predicted as an epitope candidate with potential restriction to HLA-A2 by computer algorithm approaches (Schuster et al. 2007). The isolated T cell clone, designated as SK22, exerted preferential cytotoxicity against FMNL1-expressing HLA-$A2^+$ lymphoma cell lines BJAB and DG75 as well as renal-cell carcinoma cell line RCC26 compared to that against FMNL1-expressing HLA-$A2^-$ lymphoma cell line Raji and renal-cell carcinoma cell line KT187 (Figure 2.8), indicating the potential immunogenicity of FMNL1-PP2 peptide and its HLA-A2 restriction. However, this T cell clone also showed a defined crossreactivity against unknown peptides in the context of HLA-A3303 and HLA-

A6802. The TCR from clone SK22 (TCR SK22) was identified and functionally investigated after retroviral transduction into PBMC. Wildtype TCR SK22 seemed to be a 'week' TCR and was not well expressed in PBMC after transduction (Schuster 2008). The strategies for optimizing TCR pairing and expression improved the surface expression of TCR SK22 on PBMC. Moreover, TCR SK22-transduced PBMC were endowed with specific FMNL1-PP2 recognition and anti-tumor reactivity. Hence, TCR SK22 represents a potential TCR candidate to generate genetically-modified T cells for therapeutic application in lymphoma.

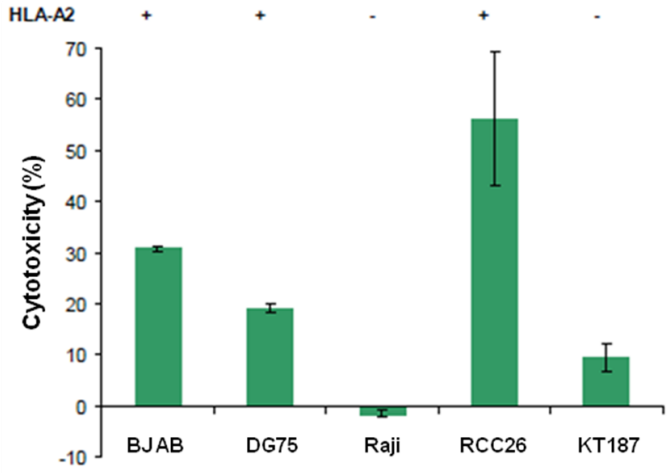

Figure 2.8 Isolated FMNL1-PP2-specific T cell clone SK22 showed killing-capability against nature tumor targets.

The obtained FMNL1-PP2-specific T cell clone SK22 was tested against the lymphoma cell lines BJAB, DG75, and Raji as well as renal-cell carcinoma cell lines RCC26 and KT187 at an effector : target (E:T) ratio of 7.5:1. The HLA-A2 expression profile of the tested tumor cells is shown above the chart. Cytotoxicity of T cell clone SK22 against tumor targets was determined in a ^{51}Cr release assay. The standard deviations of duplicates are shown (Schuster et al. 2007).

2.5 Aim of the project

Previously, allorestricted T cell clones with specificity for $HER2_{369}$ and FMNL1-PP2 have been isolated *in vitro*. The obtained T cells clones showed specific peptide recognition as well as anti-tumor reactivity in *in vitro* experiments (Table 2.1 and Figure 2.8). The aim of my PhD project was to functionally characterize the TCRs derived from these T cell clones *in vitro* (only for $HER2_{369}$-specific TCR) and to assess the immunotherapeutic potential of those allorestricted TCRs with specificity for HER2/neu$^+$ and FMNL1$^+$ cancer *in vivo*. The project procedure is generally outlined in Figure 2.9. Briefly, the TCR encoding genes were cloned either directly or modified into a retroviral vector. Wildtype or modified TCR constructs were then retrovirally transduced into PBMC. PBMC transduced with $HER2_{369}$-specific TCRs were functionally investigated in the aspects of recognition of the specific peptide, anti-tumor

reactivity and autoreactivity against normal cells *in vitro*. In addition, the anti-tumor response of PBMC transduced with TCRs with specificity for $HER2_{369}$ or FMNL1-PP2 was determined in mouse models. Finally, efforts were also made to test the feasibility of the non-invasive monitoring system PET/CT based on the hNIS reporter gene for assessing the migration and localization of TCR-transduced T cells after adoptive transfer.

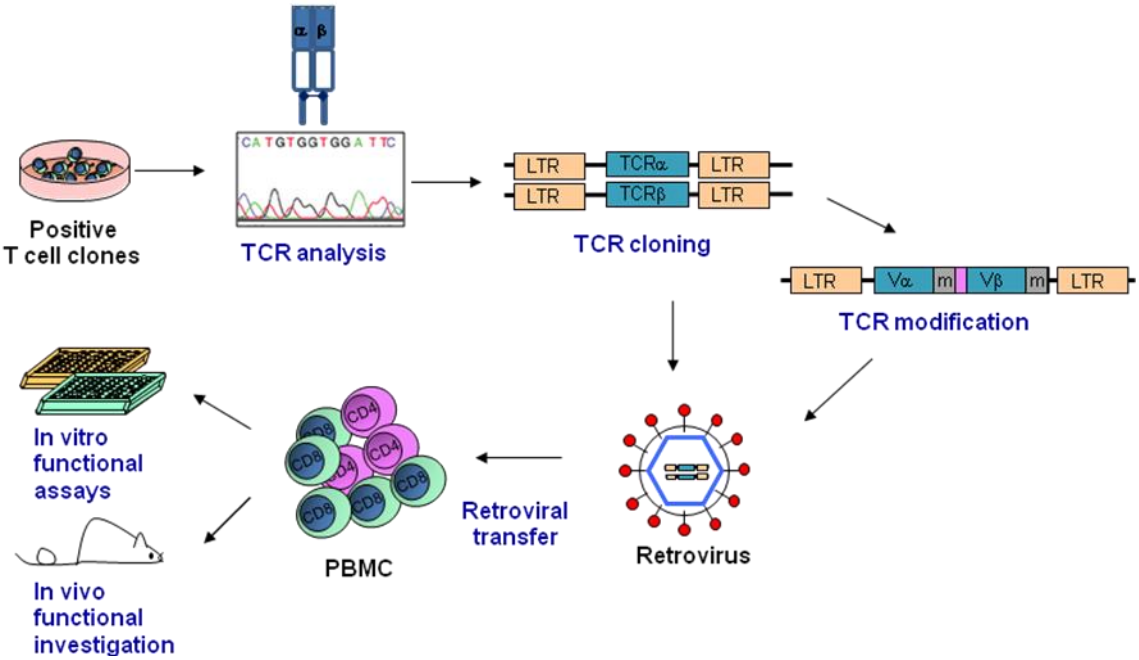

Figure 2.9 Overview about the procedure for further investigation of isolated allorestricted TCRs *in vitro* as well as *in vivo* after retroviral transduction into PBMC.

The TCRs harbored in $HER2_{369}$-specific allorestricted T cell clones were previously identified in my master thesis (Liang 2007). In this study, TCR α- and β-chain genes derived from selected T cell clones were cloned into a retroviral vector and transferred into healthy PBMC. TCR-transduced PBMC were further functionally investigated. Additionally, the TCR α- and β-chain genes-containing retroviral constructs were modified using several strategies to improve the expression of the transduced TCRs in recipient cells. Furthermore, preliminary experiments for investigation of PBMC transduced with modified $HER2_{369}$- or FMNL1-PP2-specific TCRs were initiated in a mouse model.

3 Materials

3.1 Equipments and supplies

Cell culture bottles	Becton Dickinson, Heidelberg
Centrifuge Hettich	Andreas Hettich GmbH, Tuttlingen
Centrifuge 4K15	Sigma Laborzentrifugen GmbH, Osterode am Harz,
Centrifuge 5417R	Eppendorf-Netheler-Hinz-GmbH, Hamburg
Counting chamber	Brand GmbH & Co., Wertheim
Cover film	Neolab, Heidelberg
EIA/RIA Plate	Corning, New York, USA
Embedding molds	Science services GmbH, Munich
FACS Calibur cell sorter	Becton Dickinson, Heidelberg
FACS LSRII cell sorter	Becton Dickinson, Heidelberg
Fluorescence Microscope	Leica Microsystems GmbH, Wetzlar
Gloves latex	Rösner-Mautby Meditrade GmbH, Kiefersfelden
Gloves nitrile	Kimberly-Clark Corporation, Neenah, USA
Gel-documentation system:	
Midi Electrophoresis chamber	Harnischmacher, Kassel
InGenius gel documentation system	Synoptics Ltd, Cambridge, England
Gel-print 2000i	Biophotonics, Ann Arbor, USA
Incubator	Heraeus Holding GmbH, Hanau
Incubator for bacteria	Bachofer, Reutlingen
Incubator shaker	INFORS AG, Bottmingen, Switzerland
Irradiation facility Gammacell 40	Atomic Energy of Canada Limited, Ottawa, Canada
Luma plate	Perkin Elmer, Applied Biosystems, Weiterstadt
Luminex Bio-Plex	System Bio-Rad, Munich
Microplate reader ELISA Reader	SLT Labinstruments, Crailsheim
Microscope	Zeiss AG, Jena
MilliQ System	Millipore GmbH, Schwalbach
MoFlowTM	Dako, Hamburg
Multichannel pipettes	Eppendorf-Netheler-Hinz-GmbH, Hamburg
NanoDrop1000 Spectrophotometer	Thermo Fischer Scientific, Wilmington, USA
PCR thermocycler	Biometra Biomedizinische Analytik GmbH, Göttingen
pH-meter 766	Knick Elektronische Messgeräte GmbH, Berlin
Photometer, ELISA Reader	Tecan, Groot-Bijgaarden, Belgium
Serological pipette 5 ml, 10 ml, 25 ml	Corning, New York, USA
PET/CT imaging system	Siemens AG, Erlangen

Pipette boys	Integra Biosciences, Fernwald
Pipettes	Eppendorf-Netheler-Hinz-GmbH, Hamburg,
Polystyrene round bottom FACS tubes 5ml	Becton Dickinson, Heidelberg
Polystyrene round bottom FACS tubes 1ml	Peske, Heidelberg
Power supply	Pharmacia GmbH, Erlangen
Pursep-A Xpress disinfection agent	Metz Consumer Care GmbH, Frankfurt
Reagent reservoir	Peske, Heidelberg
Sterile bench-1	BDK, Sonnenbühl-Genkingen
Sterile bench-2	Clean Air Techniek B.V. JA Woerden, Netherlands
Sterile polypropylene FACS tubes with cap	Becton Dickinson, Heidelberg
Sterile filters	Techno Plastic Products AG, Trasadingen, Switzerland
Syringes 10ml, 50 ml	Becton Dickinson, Heidelberg
Thermomixer	Eppendorf-Netheler-Hinz-GmbH, Hamburg,
Tips 1–10 µl, 10–200 µl, 100–1000 µl	Greiner Bio-One International AG, Kremsmünster, Austria
Ultraspec1100 UV/vis Spectrophotometer	Biochrom Ltd, Cambridge, England
Vortexer	Bender & Hobei AG, Switzerland
Water bath	Memmert, Schwab Bach
12-, 24- well tissue culture plates	Becton Dickinson, Heidelberg
96 well U-bottom tissue culture plates	Becton Dickinson, Heidelberg
96 well flat-bottom tissue culture plates	Becton Dickinson, Heidelberg
50 ml, 15ml Falcons	Becton Dickinson, Heidelberg
2ml, 1.5 ml, 0.5 ml reagent tubes	Eppendorf-Netheler-Hinz-GmbH, Hamburg,

3.2 Chemicals, cytokines and enzymes

100 bp DNA Ladder	New England Biolabs, Ipswich, England
1-Bromo-3-Chloro-Propane	Sigma Aldrich Chemie GmbH, Taufkirchen
7-Aminoactinomycin D	Sigma Aldrich Chemie GmbH, Taufkirchen
2- Mercaptoethanol	Sigma Aldrich Chemie GmbH, Taufkirchen
Agarose	Biozym, Oldendorf
AIM-V	Gibco, Invitrogen Corporation, Karlsruhe
Blasticidine S hydrochloride	Sigma Aldrich Chemie GmbH, Taufkirchen
Ampicillin	Sigma Aldrich Chemie GmbH, Taufkirchen
Brefeldin A	Becton Dickinson, Heidelberg
Buffer NEB1-4	New England Biolabs, Ipswich, England
$CaCl_2$	Merck, Darmstadt
Chloroform-Isoamylalkohol 24:1	Sigma Aldrich Chemie GmbH, Taufkirchen
Chloroquine	Sigma Aldrich Chemie GmbH, Taufkirchen
Chromium-51	Hartmann analytic GmbH, Braunschweig
DEPC H_2O	Invitrogen Corporation, Karlsruhe
DMEM	Gibco, Invitrogen Corporation, Karlsruhe
DMSO	Sigma Aldrich Chemie GmbH, Taufkirchen
dNTP Mix	Fermentas, St. Leon-Rot
D-PBS	Gibco, Invitrogen Corporation, Karlsruhe
Ethanol	Merck, Darmstadt
Ethidiumbromid	Merck, Darmstadt
F12-Medium	Sigma Aldrich Chemie GmbH, Taufkirchen
Ficoll	Biochrom, Berlin
FCS	PAA Laboratories GmbH, Pasching, Austria
^{18}FDG	GE Healthcare, Munich
Fungizon	Invitrogen Corporation, Karlsruhe
G418	PAA Laboratories GmbH, Pasching, Austria
Gentamycine	PAA Laboratories GmbH, Pasching, Austria
Glucose	Merck, Darmstadt
H_3PO_4	Merck KGaA, Darmstadt
Heparin-Natrium	B. Braun, Melsungen AG
HEPES, 1 M	Gibco, Invitrogen Corporation, Karlsruhe
HEPES, Powder	Biochrom KG, Berlin
Human serum	Helmholtz Zentrum München, Munich
Hygromycin B	Invitrogen Corporation, Karlsruhe
HyperLadder I	BIOLine GmbH, Luckenwalde
Human IFNγ	Peprotech, New Jersey, USA
IL-15	Peprotech, New Jersey, USA
IL-2	Chiron Vaccines International, Marburg
IL-7	Peprotech, New Jersey, USA
IMDM	Gibco, Invitrogen Corporation, Karlsruhe
Ionomycin	Sigma Aldrich Chemie GmbH, Taufkirchen
Isopropanol	Merck, Darmstadt

KCl	Merck, Darmstadt
KOD Hot Start DNA polymerase	Novagen, Darmstadt
Loading buffer	Fermentas, St. Leon-Rot
LB-Agar	Invitrogen Corporation, Karlsruhe
LB-Broth	Invitrogen Corporation, Karlsruhe
L-Glutamine	Gibco, Invitrogen Corporation, Karlsruhe
MB Taq polymerase	Minerva Biolabs GmbH, Berlin
Milk powder	Frema, Luneburg
Mycoplasma-Removal-Agent	MP Biomedicals, Illkirch, French
Murine IL2	Peprotech, New Jersey, USA
Myocyte cell Growth medium	PromoCell, Heidelberg
Na_2CO_3	Merck, Darmstadt
Na_2HCO_3	Merck, Darmstadt
$Na_2HPO_4 \cdot 2H_2O$	Merck, Darmstadt
NaCl	Merck, Darmstadt
NaN_3	Merck KGaA, Darmstadt
Non essential amino acid	Invitrogen Corporation, Karlsruhe
Oligo(dT)-Primer	Promega, Madison, USA
Paraformaldehyde (PFA)	Sigma Aldrich Chemie GmbH, Taufkirchen
PBS Dulbecco's	Biochrom AG, Berlin
pCR®-blunt cloning Kit	Invitrogen Corporation, Karlsruhe
Penicillin Streptomycin	Gibco, Invitrogen Corporation, Karlsruhe
Pfu-Polymerase	Fermentas, St. Leon-Rot
Phenol	Roth, Karlsruhe
PMA	Sigma Aldrich Chemie GmbH, Taufkirchen
Propidium iodide	Sigma Aldrich Chemie GmbH, Taufkirchen
Protaminsulfate	MP Biomedicals, Illkirch, French
RetroNectin	TaKaRa, Japan
RNase Out	Invitrogen Corporation, Karlsruhe
RPMI 1640 (-L-Glutamine)	Gibco, Invitrogen Corporation, Karlsruhe
Sheath fluid	Dako, Glostrup, Denmark
Saponin	Sigma Aldrich Chemie GmbH, Taufkirchen
Sodium chlorid	Sigma Aldrich Chemie GmbH, Taufkirchen
Sodium chlorid solution (0.09%)	Diprom GmbH, Hamburg
Sodium chromat	Hartmann Analytik GmbH, Braunschweig
Sodium pyruvate	Gibco, Invitrogen Corporation, Karlsruhe
Sodium citrate $2H_2O$	Sigma Aldrich Chemie GmbH, Taufkirchen
SuperScript II Reverse Transcriptase	Invitrogen Corporation, Karlsruhe
T4-Ligase	Fermentas, St. Leon-Rot
TAE (Tris Acetat EDTA) Puffer	Invitrogen, Karlsruhe
Taq-Polymerase	Invitrogen Corporation, Karlsruhe
Tissue-Tek® O.C.T Compound	Science services GmbH, Munich
TMB Substrate Reagent Set	Becton Dickinson, Heidelberg
*Trans*IT®-293 reagent	Mirus Bio LLC, Madison, USA
Tri Reagent	Sigma Aldrich Chemie GmbH, Taufkirchen

Trypan blue	Gibco, Invitrogen Corporation, Karlsruhe
Trypsin EDTA 0,5 %	Gibco, Invitrogen Corporation, Karlsruhe
Tween 20	Sigma Aldrich Chemie GmbH, Taufkirchen
β_2 microglobulin	Calbiochem Novabiochem, Darmstadt

Table 3.1 Restriction enzymes

Restriction Enzyme	Buffer	Temperature	Producer
Afl III	NEB 3	37°C	New England Bio Labs, Ipswich, England
BamH I	NEB 2/3/4	37°C	Fermentas, St. Leon-Rot
BstB I	NEB 4	65°C	New England Bio Labs, Ipswich, England
EcoR I	NEB 3	37°C	Fermentas, St. Leon-Rot
Cla I	NEB 4	37°C	Fermentas, St. Leon-Rot
Mfe I	NEB 4	37°C	New England Bio Labs, Ipswich, England
Nhe I	NEB 4	37°C	New England Bio Labs, Ipswich, England
Not I	NEB 3	37°C	Fermentas, St. Leon-Rot
Xba I	NEB 4	37°C	Fermentas, St. Leon-Rot
Xho I	NEB 4	37°C	Fermentas, St. Leon-Rot

3.3 Kits

Bio-Plex Pro Human Cytokine T_H1/T_H2 Assay kit — Bio-Rad, Munich

- Assay buffer
- Wash buffer
- Anti-cytokine conjugated beads (25x)
- Detection antibody dilutent (50x)
- Streptavidin-PE (100x)
- Cytokine Standard

Dynal® Untouched human CD4 T cell Kit — Invitrogen Corporation, Karlsruhe

- Antibody mix
- Depletion Dyna-beads

Dynal® Untouched human CD8 T cell Kit — Invitrogen Corporation, Karlsruhe

- Antibody mix
- Depletion Dyna-beads

Human IFNγ ELISA Set — Becton Dickinson, Heidelberg

- Capture Antibody (Anti-human IFNγ monoclonal antibody)
- Detection Antibody (Biotinylated anti-human IFNγ monoclonal antibody)

- Enzyme Reagent (Streptavidin-horseradish peroxidase conjugate)
- Standards (Recombinant human IFNγ, (Baculovirus-expressed) lyophilized)

JETstar 2.0 Plasmid Purification MAXI KIT — Genomed, Löhne

- E1: cell Resuspending
- E2: cell lysis
- E3: neutralization
- E4: columns equilibration
- E5: column washing
- E6: DNA elution

Mission® lentiviral transfection system — Sigma Aldrich Chemie GmbH, Taufkirchen

- Mission® lentiviral packaging mix
- pLKO.1-puro CMV-TurboGFP™ Control vector

NucleoSpin® Extract II PCR and gel purification kit — Macherey Nagel, Düren

- Buffer NT: binding buffer
- Buffer NT3: wash buffer
- Buffer NE: elution buffer

Venor® Gem-Mycoplasma PCR test kit — Minerva Biolabs GmbH, Berlin

- Primer/Nucleotide mix
- Reaction Buffer (10x)
- Internal control
- Positive control

3.4 Buffers and solutions

Table 3.2 Buffers and solutions

Buffer/solution	Components	Stock-Concentration	Working-Concentration
ACK lysis buffer (10x)	NH4Cl $KHCO_3$ Na-EDTA bidest. H_2O pH=7.4	53.49 g/mol 100.12 g/mol 372.24 g/mol	1.5 M 100 mM 1 mM
DNA agarose gel	Agarose TAE-Buffer Ethidium bromide bidest. H_2O	100 % 10x 10 µg/µl	1.7 % 1x 0.7 µg/ml
DNA elektrophoresis running buffer	TAE-Buffer bidest. H_2O	10x	1x
FACS-buffer	ΔFCS D-PBS (without $CaCl_2$/$MgCl_2$)	100 %	1 %
ΔFCS or ΔHS	Heat-inactivated 20 min at 58 °C		
<u>Calcium-phosphate-precipitation</u>			
2x HBS-Buffer	NaCl KCl Glucose HEPES $Na_2 HPO_4 \cdot 2H_2O$ bidest. H_2O	58.44 g/mol 74.56 g/mol 180.16 g/mol 238.31 g/mol 177.99 g/mol	280 mM 10 mM 12 mM 50 mM 1.5 mM
CaCl2	CaCl2 bidest. H_2O	110.99 g/mol	2 M
<u>ELISA</u>			
Coating buffer	$NaHCO_3$ Na_2CO3 H_2O pH=9.5	84.01 g/mol 105.99 g/mol	0.1 M 0.03 M
Milk buffer	Milk powder PBS	100 %	1 %
Wash buffer	Tween-20 PBS	100 %	0.05% (v/v)
Stop solution	H_3PO_4		
<u>Intracellular staining</u>			
FACS-Azid-buffer	HS EDTA NaN_3 PBS	100% 0.33 M 10%	4% 2 µM 0.1%
0.1% saponin solution	ΔHS Saponin PBS	100% 10%	4% 0.1%
0.35% saponin solution	ΔHS Saponin PBS	100% 10%	4% 0.35%
1% PFA	PFA PBS	10%	1%

3.5 Peptides

Peptides were synthesized by standard fluorenylmethoxycarbonyl (Fmoc) synthesis (Biosyntan GmbH, Berlin). The purity was above 90% as determined by reverse phase high-performance liquid chromatography (RP-HPLC) and verified by mass spectrometry.

Table 3.3 Peptides

Peptide	Sequence	Binding-MHC	Reference
CMV $pp65_{495}$*	NLVPMVATV	HLA-A2	Gavin et al. 1993
Flu (MP58)	GILGFVFTL	HLA-A2	Nijman et al. 1993
FMNL1-PP2	RLPERMTTL	HLA-A2, predicted	Schuster et al. 2007
$GP100_{209}$	ITDQVPFSV	HLA-A2	Parkhurst et al. 1996
HDAC6-$PP1_{862}$	RLAERMTTR	HLA-A2, predicted,	Schuster et al. 2007
$HER1_{364}$	SISGDLHIL	HLA-A2	Conrad et al. 2008
$HER2_{369}$	KIFGSLAFL	HLA-A2	Fisk et al. 1995
$HER3_{356}$	KILGNLDFL	HLA-A2	Conrad et al. 2008
$HER4_{361}$	KINGNLIFL	HLA-A2	Conrad et al. 2008
$Tyrosinase_{369}$	YMDGTMSQV	HLA-A2	Gavin et al. 1993
OVA_{257}	SIINFEKL	H-$2K^b$	Hogquist et al. 1994
$HER2_{369}$ Alanine substitution	AIFGSLAFL KAFGSLAFL KIAGSLAFL KIFASLAFL KIFGALAFL KIFGSAAFL KIFGSLAAL KIFGSLAFA	HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test	tested in this study
$HER2_{369}$ Threonine substitution	TIFGSLAFL KTFGSLAFL KITGSLAFL KIFTSLAFL KIFGTLAFL KIFGSTAFL KIFGSLTFL KIFGSLATL KIFGSLAFT	HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test HLA-A2 for test	tested in this study

*: The peptide was kindly provided by Dr. Andreas Moosmann from Helmholtz Zentrum München

3.6 Antibodies and multimers

3.6.1 Antibodies

Table 3.4 Antibodies

Name	Clone	Isotype	Conj.*	Dilution	Producer
α-human mAb					
IgG1	11711	IgG_1	-	1:25	RnD System, Wiesbaden
IgG1 κ	MOPC-21	IgG_1 κ	FITC	1:25	Becton Dickinson (BD), Heidelberg
IgG1 κ	MOPC-21	IgG_1 κ	PE	1:25	BD, Heidelberg
IgG1 κ	MOPC-21	IgG_1 κ	APC	1:25	BD, Heidelberg
IgG 2b κ	27-35	IgG_{2b} κ	FITC	1:25	BD, Heidelberg
	MPC-11	IgG_{2b} κ	FITC	1:100	BD, Heidelberg
α-hCD3	OKT-3	IgG_{2a}	-	as indicated	Kremmer E., Helmholtz Zentrum München
α-hCD3	UCHT1	IgG_1 κ	FITC	1:25	BD, Heidelberg
α-hCD4	RPA-T4	IgG_1 κ	FITC	1:25	BD, Heidelberg
α-hCD4	RPA-T4	IgG_1 κ	PE	1:25	BD, Heidelberg
α-hCD8	HIT8a	IgG_1 κ	FITC	1:25	BD, Heidelberg
α-hCD8	RPA-T8	IgG_1 κ	PE	1:25	BD, Heidelberg
α-hCD8	RPA-T8	IgG_1 κ	APC	1:25	BD, Heidelberg
α-hCD14	M5E2	IgG_{2a}	PE	1:25	BD, Heidelberg
α-hCD19	HIB19	IgG_1 κ	FITC	1:25	BD, Heidelberg
α-hCD28	CD28.2	IgG_1 κ	-	1:1000	BD, Heidelberg
α-hCD56	B159	IgG_1 κ	PE	1:25	BD, Heidelberg
α-HLA-A2	HB54	IgG_1	-	1:50	Elisabeth Kremmer, Helmholtz Zentrum München
α-HLA-A2	BB7.2	IgG_{2b}	FITC	1:100	BD, Heidelberg
α-MHC I	W6/32	IgG_{2a}	-	1:100	Elisabeth Kremmer, Helmholtz Zentrum München
α-hHLA-ABC	G46-2.6	IgG_1 κ	FITC	1:25	BD, Heidelberg
α-hVβ8	56C5.2	IgG_{2a}	FITC	3:50	Beckman coulter, Krefeld
α-hVβ8	56C5.2	IgG_{2a}	PE	3:50	Beckman coulter, Krefeld
α-hVβ14	CAS1.1.3	IgG_1	FITC	3:50	Beckman coulter, Krefeld
α-hVβ14	CAS1.1.3	IgG_1	PE	3:50	Beckman coulter, Krefeld
α- hTCR αβ	T10B9.1A-31	IgM κ	FITC	1:50	BD, Heidelberg
α- hTCR βγ	11F2	IgG_1	FITC	1:50	BD, Heidelberg
α-hIFNγ	25723.11	IgG_{2b}, κ	FITC	1:10	BD, Heidelberg

Name	Clone	Isotype	Conj.*	Dilution	Producer
α-c-ErbB2/c-Neu (α-HER2/neu)	TA-1	IgG_1	-	1:100	Calbiochem, Darmstadt
α-hNIS	VJ2	-	-	1:10	Dr. Sabine Costagliola, Free University of Brussels (Pohlenz et al. 2000)
α-mouse mAb					
α-mCD16/32 (Fc Blocking)	93	IgG_{2a}, λ	-	1:100	eBioscience, Frankfurt
Rat IgG1, λ	A110-1	IgG_1, λ	PE	1:50	BD, Heidelberg
Rat IgG2a,κ	R35-95	IgG_{2a}, κ	FITC	1:50	BD, Heidelberg
α-mCD3ε	145-2C11	Armenian Hamster IgG_1, κ	-	1:1000	BD, Heidelberg
α-mCD3ε	145-2C11	Armenian Hamster IgG_1, κ	PE	1:50	Biolegend, San Diego, USA
α-mCD4	RM4-5	IgG_{2a}, κ	FITC	1:50	BD, Heidelberg
α-mCD8a	53-6.7	IgG_{2a}, κ	FITC	1:50	BD, Heidelberg
α-mCD11b	M1/70	IgG_{2b}, κ	FITC	1:25	BD, Heidelberg
α-mCD25	PC61	IgG_1, λ	PE	1:50	Biolegend, San Diego, USA
α-mCD28	37.51	Syrian Hamster IgG_2, λ1	-	1:10000	BD, Heidelberg
α-mCD45R/B220	RA3-6B2	IgG_{2a}, κ	PE	1:50	BD, Heidelberg
α-mCD49b	DX5	IgM, κ	FITC	1:25	BD, Heidelberg
α-mVβ5.1/5.2	MR9-4	IgG_1, κ	FITC	3:50	BD, Heidelberg
α-mTCRαβ	H57-597	Armenian Hamster IgG	FITC	1:25	Beckman coulter, Krefeld
Goat α-mouse (H+L)	-	-	PE	1:100	Invitrogen, Karlsruhe

*: conjugation
α: anti
m: mouse antigen
h: human antigen

3.6.2 Multimers

MHC-peptide multimers recognizing T cells with specificities for $HER2_{369}$, FMNL-PP2, $GP100_{209}$, Flu (MP58), $pp65_{495}$, and OVA_{257} were synthesized as elsewhere described and used for detection and sorting of specific TCR (Busch et al. 1998; Knabel et al. 2002).

Table 3.5 Multimers

Multimer	Peptide	Conjugation	Dilution	Producer
HLA-A2-$HER2_{369}$	KIFGSLAFL	PE	1:50	Dirk Busch, TUM
HLA-A2-FMNL1-PP2	RLPERMTTL	PE	1:50	Dirk Busch, TUM
HLA-A2-$GP100_{209}$	ITDYVPFSV	PE	1:50	Dirk Busch, TUM
HLA-A2-Flu	GILGFVFTL	PE	1:50	Dirk Busch, TUM
HLA-A2-$pp65_{495}$	NLVPMVATV	PE	1:50	Beckman Coulter
H-$2K^b$-OVA_{257}	SIINFEKL	PE	1:10	Dirk Busch, TUM

3.7 Cell culture medium

Table 3.6 Cell culture medium

Medium	Components	Stock Concentration	Working Concentration
Freezing medium	ΔFCS	100%	90%
	DMSO	100%	10%
Complete RPMI (cRPMI)	RPMI-1640 – L-Glutamine		
	ΔFCS	100%	10%
	L-Glutamine	200 mM	2 mM
	Non-essential amino acid	100 x	1 x
	Sodium pyruvate	100 mM	1 mM
	Penicillin	10^4 U/ml	100 U/ml
	Streptomycin	10^4 µg/ml	100 µg/ml
Complete DMEM (cDMEM)	DMEM (4.5 g/l Glucose)		
	ΔFCS	100%	10% or 3%
	L-Glutamine	200 mM	2 mM
Hunger medium (3% ΔFCS)	Non-essential amino acid	100 x	1 x
	Sodium pyruvate	100 mM	1 mM
	Penicillin	10^4 U/ml	100 U/ml
	Streptomycin	10^4 µg/ml	100 µg/ml
Complete IMDM (cIMDM)	IMDM		
	ΔFCS	100 %	10 %
	L-Glutamine	200 mM	2 mM
	Penicillin	10^4 U/ml	100 U/ml
	Streptomycin	10^4 µg/ml	100 µg/ml
T cell medium (TCM)	RPMI-1640 – L-Glutamine		
	ΔHS	100%	10%
	L-Glutamine	200 mM	2 mM
	Non-essential amino acid	100 x	1 x
	Sodium pyruvate	100 mM	1 mM
	Penicillin	10^4 U/ml	100 U/ml
	Streptomycin	10^4 µg/ml	100 µg/ml
	HEPES; pH=7.4	1 M	10 mM

Medium	Components	Stock Concentration	Working Concentration
	Gentamycine	10 mg/ml	16.6 µg/ml
Clone medium	RPMI-1640 - L-Glutamine		
	ΔFCS	100%	7.5%
	ΔHS	100%	7.5%
	L-Glutamine	200 mM	2 mM
	Non-essential amino acid	100 x	1 x
	Sodium pyruvate	100 mM	1 mM
	Penicillin	10^4 U/ml	100 U/ml
	Streptomycin	10^4 µg/ml	100 µg/ml
	HEPES; pH=7.4	1 M	10 mM
	Fungizon	250 µg/ml	2.5 µg/ml
Murine T cell medium (mTCM)	RPMI-1640 – L-Glutamine		
	ΔFCS	100 %	10 %
	L-Glutamine	200 mM	2 mM
	Non essential amino acid	100 x	1 x
	Sodium pyruvate	100 mM	1 mM
	Penicillin	10^4 U/ml	100 U/ml
	Streptomycin	10^4 µg/ml	100 µg/ml
	HEPES; pH=7.4	1 M	10 mM
	2-Mercaptoethanol	14.3 M	50 µM
Medium for fetal cardiomyocytes	Myocyte growth medium		
LB-Agar	Dissolve LB-Agar powder in bidest. H_2O and autoclave the medium according to manufacturer's protocol, aliquot in the perish dish after the addition of antibiotic ampicillin (100 ng/ml) or kanamycin (50 ng/ml)		
LB-Broth	Dissolve LB-Broth powder in bidest. H_2O and autoclave the medium according to manufacturer's protocol		
S.O.C medium	2 % Tryptone, 0.5 % Yeast extract, 10 mM NaCl, 2.5 mM KCl,10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose		

3.8 Cell lines, bacteria and mice

3.8.1 Primary cell lines

PBMC from healthy donors were collected with donors` informed consent following the requirements of the local ethical board and the principles expressed in the Helsinki Declaration. PBMC subpopulations from healthy donors were isolated by negative or positive magnetic bead depletion (Invitrogen, Karlsruhe) and the high purity was confirmed by flow cytometric analysis.

3.8.2 Cell lines

Table 3.7 Cell lines and bacteria

Cell line	Description	Culture medium	Reference
BJAB	EBV$^-$ lymphoma cell line, HLA-A2$^+$	cRPMI	Joseph Mautner, Helmholtz Zentrum München
C1R	Human B cell line	cRPMI	Stefan, Stevanovic, University of Tübingen
C1R tA2	C1R, transfected with HLA-A2$^+$	cRPMI + 0.8 mg/ml G418	Stefan, Stevanovic, University of Tübingen
C1R tA2 tHER2	C1R, transfected with HLA-A2 and HER2/neu	cIMDM + 0.3 mg/ml Hygromycin B	Helga Bernhard, TUM
E.G7-OVA	Mouse lymphoma cell line EL-4 transfected with ovalbumin (OVA)	mTCM	ATCC, Wesel (CRL-2113)
EL-4	Mouse lymphoma cell	cRPMI	Ralph Mocikat, Helmholtz Zentrum München
HEK 293T	Human embryonic kidney cell line, HLA-A2$^+$ (ATCC CRL-1573)	cRPMI	Joseph Mautner, Helmholtz Zentrum München
Fetal cardiomyocytes	Human cardiac myocytes	Myocyte growth medium	Jehard Charo, MDC, Berlin
Jurkat76	TCR-deficient CD4$^+$ T cell line	cRPMI	Mirjam Heemskerk, Leiden, Denmark
CD8α^+Jurkat76 (J76CD8)	TCR-deficient CD4$^+$ T cell line, transduced with CD8α	cRPMI	Wolfgang Uckert, MDC, Berlin
K562	Human chronic myeloid leukemia (CML) cell line, MHC class I deficient	cRPMI	Helmholtz Zentrum München (ATCC CCL-243)
Lung fibroblast [a]	134 TK$^-$ fetal lung fibroblasts; HLA-A2$^+$	cRPMI	Ralph Mocikat, Helmholtz Zentrum München
MCF7[a]	Breast carcinoma cell	cRPMI	Irmela Jeremias,

Cell line	Description	Culture medium	Reference
	line; HLA-A2^{+}		Helmholtz Zentrum München
MDA-MB-231[a]	Breast carcinoma cell line; HLA-A2^{+}	cDMEM	Cell lines service, Eppelheim
MRC-5[a]	Lung fibroblasts	cRPMI	Andreas Moosmann Helmholtz Zentrum München
SK-Mel29[a]	Melanoma cell line; HLA-A2^{+}	cRPMI	Helga Bernhard, TUM
SKOV3[a]	Ovarian carcinoma celll line; HLA-A2^{-}	cDMEM	Helga Bernhard, TUM
SKOV3tA2[a]	SKOV3 cell line transfected with HLA-A2	cRPMI	Helga Bernhard, TUM
T2	TAP-deficient hybrid of T- and B-lymphoblastoid cell line, HLA-A2^{+}	cRPMI	Helmholtz Zentrum München (Salter and Cresswell 1986)
628.34 MEL[a]	Melanoma cell line; HLA-A2^{+}	cDMEM	Elfriede Nößner, Helmholtz Zentrum München
TOP10	Chemical competent *E.coli* bacteria strain	LB-Broth-Medium	Invitrogen, Karlsruhe

[a]: adherent cell lines

3.8.3 Mice

Table 3.8 Mice strains

Mouse strain	Nomenclature	Description	Producer
OT-1	C57BL/6-Tg(TcraTcrb) 1100Mjb/J	Transgenic mice with a TCR (Vα2 and Vβ5) specifically recognizing ovalbumin peptide 257-264 in the context of H-2K^{b} in C57BL/6 background	Charles River, Sulzfeld,
NOD/SCID	NOD/MrkBomTac-*Prkdc*^{scid}	A diabetes-susceptible non-obese diabetic (NOD) background with severe combined immune deficiency (SCID) spontaneous mutation, T and B cell deficient.	Taconic, Bomholt, Denmark

3.9 DNA materials

3.9.1 Vectors

Table 3.9 Vectors used for retroviral TCR-Transfer

Vector	Description	Resistance	Reference
pALF10A1 ("env")	Vector with the gene encoding 10A1-virus envelope of murine leukemia virus under the LTR control of friend murine leukemia virus	Ampicillin	Wolfgang Uckert, MDC, Berlin (Stitz et al. 2000)
pcDNA3.1-MLV g/p ("gag-pol")	Vector with genes encoding species-specific antigen and polymerase under CMV promotor	Ampicillin	Wolfgang Uckert, MDC, Berlin, Chrisopher Baum, Hannover (Leisegang et al. 2008)
pMP71-GFP	Retroviral vector with eGFP-gene, mSS and PRE element under MPSV-LTR control	Ampicillin	Wolfgang Uckert, MDC, Berlin (Engels et al. 2003)
pHIV7SF-hNIS	Lentiviral vector with the gene encoding human natrium/Iodide symptor(hNIS) under the control of spleen focus-forming virus (SSFV) promoter and LTR of HIV	Ampicillin	Martina Anton, TUM
TCR-construct:			
pMP71 TCR α	pMP71-PRE with α chain of peptide-specific TCR, no eGFP	Ampicillin	Constructed in this study
pMP71 TCR β	pMP71-PRE with β chain of peptide-specific TCR, no eGFP	Ampicillin	Constructed in this study
pMP71 mod TCR	pMP71-PRE with modified entire fragment of TCR β-P2A-TCRα, no eGFP	Ampicillin	Constructed in this study
pHIV7SF-hNIS-mod TCR	pHIVSF with hNIS gene and modified TCR linked by T2A (hNIS-T2A-TCRβ-P2A-TCRα)	Ampicillin	Constructed in this study

3.9.2 Primers

The primers were synthesized by company Eurofins MWG-Biotech AG (Ebersberg, Germany) or Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and listed in table with 5' → 3' direction.

3.9.2.1 Primers for TCR repertoire

The nomenclature of TCR chains is according to B. Arden (Arden et al. 1995).

TCR α-chain repertoire (Steinle et al. 1995)

Concentration: 2.5 µM, if not indicated.

P-5'αST	CTGTGCTAGACATGAGGTCT	
P-3'αST	CTTGCCTCTGCCGTGAATGT	
3'T-Cα	GGTGAATAGGCAGACAGACTTGTCACTGGA	c = 5 µM
PANVα1	AGAGCCCAGTCTGTGASCCAG	S = C/G
PANVα1.1	AGAGCCCAGTCRGTGACCCAG	R = A/G
Vα2	GTTTGGAGCCAACRGAAGGAG	
Vα3	GGTGAACAGTCAACAGGGAGA	
Vα4	TGATGCTAAGACCACMCAGC	
Vα5	GGCCCTGAACATTCAGGA	
Vα6	GGTCACAGCTTCACTGTGGCTA	
Vα7	ATGTTTCCATGAAGATGGGAG	
Vα8	TGTGGCTGCAGGTGGACT	
Vα9	ATCTCAGTGCTTGTGATAATA	
Vα10	ACCCAGCTGCTGGAGCAGAGCCCT	
Vα11	AGAAAGCAAGGACCAAGTGTT	
Vα12	CAGAAGGTAACTCAAGCGCAGACT	
Vα13	GAGCCAATTCCACGCTGCG	
Vα14.1	CAGTCCCAGCCAGAGATGTC	
Vα14	CAGTCTCAACCAGAGATGTC	
Vα15	GATGTGGAGCAGAGTCTTTTC	
Vα16	TCA GCG GAA GATCAGGTCAAC	
Vα17	GCTTATGAGAACACTGCGT	
Vα18	GCAGCTTCCCTTCCAGCAAT	
Vα19	AGAACCTGACTGCCCAGGAA	
Vα20	CATCTCCATGGACTCATATGA	
Vα21	GTGACTATACTAACAGCATGT	
Vα22	TACACAGCCACAGGATACCCTTCC	
Vα23	TGACACAGATTCCTGCAGCTC	
Vα24	GAACTGCACTCTTCAATGC	
Vα25	ATCAGAGTCCTCAATCTATGTTTA	
Vα26	AGAGGGAAAGAATCTCACCATAA	
Vα27	ACCCTCTGTTCCTGAGCATG	
Vα28	CAAAGCCCTCTATCTCTGGTT	
Vα29	AGGGGAAGATGCTGTCACCA	
Vα30	GAGGGAGAGAGTAGCAGT	
Vα31NEU	TCGGAGGGAGCATCTGTGACTA	
Vα32	CAAATTCCTCAGTACCAGCA	

TCR β-chain repertoire (Steinle et al. 1995)

Concentration: 2.5 µM, if not indicated.

P-5'βST	AAGCAGAGATCTCCCACAC	c = 5 µM
P-3'βST	GAGGTAAAGCCACAGTCTGCT	c = 5 µM
P-3'CβII	GATGGCTCAAACACAGCGACCTC	c = 5 µM
Vβ1	GCACAACAGTTCCCTGACTTGGCAC	c = 5 µM
Vβ2	TCATCAACCATGCAAGCCTGACCT	
Vβ3	GTCTCTACATATGAGAGTGGATTTGTCATT	
Vβ5.1	ATACTTCAGTGAGACACAGAGAAAC	
Vβ5.2	TTCCCTAACTATAGCTCTGAGCTG	Vβ5.2+Vβ5.2T 1:1 MIX, c = 5 µM
Vβ6.1	GCCCAGAGT TTCTGACTTACTTC	
Vβ6.2	ACTCTGASGATCCAGCGCACA	S=C/G
Vβ6.3	ACTCTGAAGATCCAGCGCACA	
Vβ7	CCTGAATGCCCCAACAGCTCTC	
Vβ8	ATTTACTTTAACAACAACGTTCCG	
Vβ8S3	GCTTACTTCCGCAACCGGGCTCCT	c = 5 µM
Vβ9	CCTAAATCTCCAGACAAAGCT	
Vβ10	CTCCAAAAACTCATCCTGTACCTT	
Vβ11	TCAACAGTCTCCAGAATAAGGACG	c = 5 µM
Vβ12	AAAGGAGAAGTCTCAGAT	c = 5 µM
Vβ12S3	GCAGCTGCTGATATTACAGAT	
Vβ13	TCGACAAGACCCAGGCATGG	
Vβ13.1	CAAGGAGAAGTCCCCAAT	
Vβ13.2	GGTGAGGGTACAACTGCC	c = 5 µM
Vβ13S5	ATACTGCAGGTACCACTGGCA	
Vβ14	GTCTCTCGAAAAGAGAAGAGGAAT	
Vβ15	AGTGTCTCTCGACAGGCACAGGCT	c = 5 µM
Vβ16	AAAGAGTCTAAACAGGATGAGTCC	
Vβ17	CAGATAGTAAATGACTTTCAG	
Vβ18	GATGAGTCAGGAATGCCAAAGGAA	
Vβ19	CAATGCCCCAAGAACGCACCCTGC	
Vβ20	AGCTCTGAGGTGCCCCAGAATCTC	
Vβß21	AAAGGAGTAGACTCCACTCTC	
Vβ22.1	CATCTCTAATCACTTATACT	c = 5 µM
Vβ22.2	AAGTGATCTTGCGCTGTGTCCCCA	c = 5 µM
Vβ22.3	CTCAGAGAAGTCTGAAATATTCG	
Vβ23	GCAGGGTCCAGGTCAGGACCCCCA	
Vβ24	ATCCAGGAGGCCGAACACTTCT	c = 5 µM
Vβ25	TGAAAATGTCTTTGATGAAACAG	
Vβ26	CCTAACGGAACGTCTTCCAC	c = 5 µM
Vβ27	ATACTGGAATTACCCAGACAC	c = 5 µM
Vβ28	TACACAATTCCCAAGACACAG	c = 5 µM

TCR β-chain degenerate primer (Zhou et al. 2006)

Concentration: 5µM

VP1	GCIITKTIYTGGTAYMGACA
VP2	CTITKTWTTGGTAYCIKCAG
CP1	GCACCTCCTTCCCATTCAC

3.9.2.2 Primers for cloning of allorestricted TCRs into the retroviral vector pMP71

Concentration: 10 µM

Table 3.10 Primers for cloning of allorestricted TCRs into the retroviral vector pMP71

TCR		5’ forward primer	3’ reverse primer
HER2-1	**α**	TAGCGGCCGCCACCATGCTGACTGCCAGCCTG	TGGAATTCTCAGCTGGACCACAGCCGCAGC
	β	TAGCGGCCGCCACCATGGACTCCTGGACCTTC	TGGAATTCCTAGCCTCTGGAATCCTTTCTC
HER2-2	**α**	TAGCGGCCGCCACCATGGTCCTGAAATTCTCC	TGGAATTCTCAGCTGGACCACAGCCGCAGC
	β	TAGCGGCCGCCACCATGGACTCCTGGACCTTC	TGGAATTCCTAGCCTCTGGAATCCTTTCTC
HER2-3	**α**	TAGCGGCCGCCACCATGACACGAGTTAGCTTGCTG	TGGAATTCTCAGCTGGACCACAGCCGCAGC
	β	TAGCGGCCGCCACCATGGGCACCAGGCTCCTC	TGGAATTCCTAGCCTCTGGAATCCTTTCTC
HER2-4	**α**	TAGCGGCCGCCACCATGGAGACCCTCTTGGGC	TGGAATTCTCAGCTGGACCACAGCCGCAGC
	β	TAGCGGCCGCCACCATGGACTCCTGGACCTTC	TGGAATTCCTAGCCTCTGGAATCCTTTCTC
SK22	**α**	TAGCGGCCGCCACCATGGCATGCCCTGGCTTCCTG	TGGAATTCTCAGCTGGACCACAGCCGCAGC
	β	CAGGCGGCCGCCACCATGGGCCCCCAGC	TGGAATTCCTAGCCTCTGGAATCCTTTCTC
GP100	**α**	TAGCGGCCGCCACCATGGTGAAGATCCGGCAA	TGGAATTCTCAGCTGGACCACAGCCGCAGC
	β	TAGCGGCCGCCACCATGGACTCCTGGACCTTC	TGGAATTCCTAGCCTCTGGAATCCTTTCTC

3.9.2.3 Primers for cloning of modified TCR HER2-1 into the lentiviral vector pHIV7SF

Concentration: 10 µM

Table 3.11 Primers for cloning of modified TCR HER2-1 into the lentiviral vector pHIV7SF

Primer	Sequence
BstB I -NIS -fwd	GACATCTTCGAAGATCTGCCTGGAGTCCCC
NIS-Mfe I-T2A-rev	GGGATTCTCCTCCACGTCACCGCATGTTAGAAGACTTCCTCTGCCC TCCAATTGGAGGTTTGTCTCCTGCTGGTCTCGACCACC
T2A-HER2-1-fwd	GAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAAT CCCGGCCCTATGGACAGCTGGACATTCTGCTGCGTGAGC
HER2-1-Nhe I-rev	TAGCTAGCTCAGCTGCTCCACAGCCGCAGGGT

3.10 Software and websites

Restriction enzyme cut sites and digestion reaction conditions
- http://www.neb.com/nebecomm/default.asp

Analysis of flow cytometric data
- BD CellQuest™ Pro, FlowJo

Analysis of DNA sequences and cloning:
- Sequencher 4.5, Clone Manager 7

Analysis of TCR sequences:
- IMGT (http://imgt.cines.fr)
- PubMed (http://www.ncbi.nlm.nih.gov/BLAST/)

Calculation of standard curves for ELISA data:
- SigmaPlot 10.0

Analysis of Bioplex assay data:
- BioPlex-Manager Software (Version 4.0)

Statistic analysis (mice survival curves):
- GraphPad Prism (Version 5.0)

Analysis of PET/CT imaging data
- Siemens Inveon Research Workplace

4 Methods

4.1 Molecular biology methods

4.1.1 Identification of TCRs in allorestricted T cell clones

The TCR repertoire in isolated allorestricted T cell clones with specificity for $HER2_{369}$ or FMNL1-PP2 was analyzed by using PCR analysis (Liang 2007; Schuster 2008). Briefly, total RNA was extracted from T cell clones and lines using Trizol reagent according to the manufacturer's recommendation. cDNA was next synthesized from isolated total RNA with Superscript II reverse transcriptase and oligo (dT) primers. PCR analysis was then performed by using 34 Vα and 37 Vβ subfamily-specific TCR primers as listed in section 3.9.2.1 followed by gel purification and sequencing of the amplified DNA fragments (Figure 4.1 A). However, only a partial sequence of TCR α- or β-chain was identified by PCR analysis, and the entire TCR α- or β-chain sequence was determined by blasting in PubMed or IMTG database. The TCR nomenclature was according to IMGT (Lefranc et al. 2009).

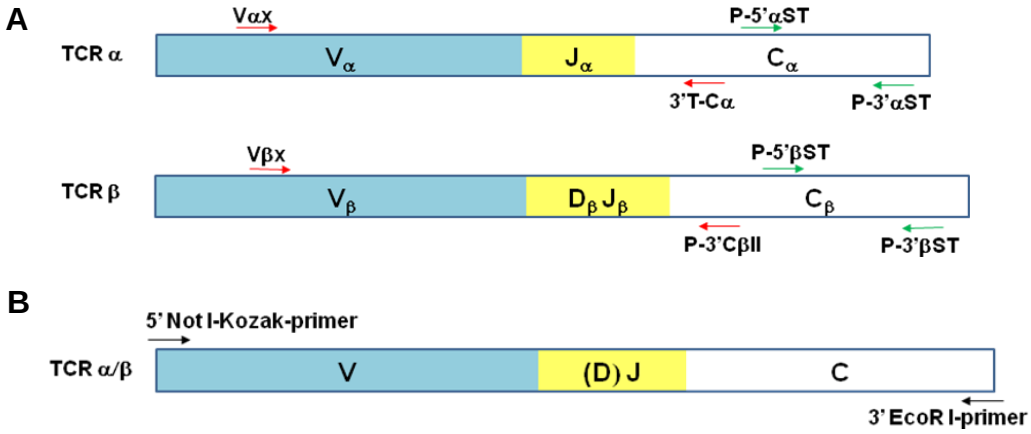

Figure 4.1 **A**. Schematic representation of PCR analysis of the TCR repertoire with primers specific for human TCR α- or β-chain variable-gene segment families. TCR α- or β-chains consist of variable (V), joining (J) and constant (C) regions, whereas the diversity (D) regions exist only in β-chains. $Vα_x/β_x$: forward primers specific for human TCR α- or β-chain variable-gene segment family. 3'T-Cα/ P-3'CβII: reverse primers targeting constant region of TCR α- or β-chain. Two pairs of primers p-5'αST/p-3'αST and p-5'βST/p-3'βST served as positive controls, which bind the constant region of TCR α- and β-chain, respectively. All mentioned primers were listed in section 3.9.2.1. **B.** Schematic representation of PCR amplification of cDNA encoding the entire TCR α- or β-chain with Not I-Kozak- or EcoR I-flanked primers (Table 3.10). The Kozak consensus sequence (CACC), which plays an important role in the initiation of gene translation, was inserted before the start codon to enhance the expression of genes in eukaryotic cells (Kozak 1987).

4.1.2 Cloning of allorestricted TCRs into a retroviral vector

4.1.2.1 Amplification of cDNA encoding allorestricted TCR α- and β-chains

Following the identification TCR α- and β-chains were isolated and cloned into the retroviral vector pMP71. In brief, cDNA encoding the specific TCR α- and β- chains were amplified from isolated T-cell clones using variable chain-specific primers containing a Not I restriction site and a Kozak consensus sequence (CACC) (Figure 4.1 B). In addition, constant region-specific reverse primers containing an EcoR I restriction site were used (Table 3.10). In this study, the following TCRs were used as control TCRs: the CMV-specific TCR JG-9 with specificity for the CMV pp65-derived HLA-A2-restricted peptide NLVPMVATV (kindly provided by A. Moosmann, Schub et al. 2009) and the $GP100_{209}$ specific TCR derived from T cell clone R6C12 (kindly provided by R. Morgen, Morgan et al. 2003).

Not I-EcoR I flanked TCR α- or β-chain fragments

Synthesized cDNA	2 µl
3' Primer (10 µM)	1.6 µl
5' Primer (10 µM)	1.6 µl
dNTP (2 mM)	4 µl
$MgSO_4$ (25 mM)	1.2 µl
Buffer (10x)	4 µl
Pfu Polymerase (5 U/µl)	0.5 µl
DEPC H_2O	to 40µl

PCR program:

1.	Initialization	95°C	2 min
2.	Denaturation	95°C	30 sec
3.	Annealing	65°C	30 sec
4.	Elongation	70°C	2 min
5.	Final elongation	70°C	10 min
6.	Holding	4°C	(step 2 → 4: 35 cycles)

4.1.2.2 Gel electrophoresis and gel purification

The PCR products were mixed with loading buffer, loaded on a 1.7% agarose gel supplemented with 0.7 g/ml ethidium bromide and run in a gel electrophoresis chamber under a voltage of 110 mV for approximately 50 min. The 100 bp DNA ladder (New England Biolabs) served as a size marker. The amplified DNA products were visualized under a UV transilluminator, and the DNA bands of interest were cut out with a scalpel and purified from the gel using the NucleoSpin® gel extraction Kit according to the manufacturer's protocol. The quantity of DNA was determined by the spectrophotometer.

4.1.2.3 Digestion and phenol-chloroform extraction of DNA fragments

To clone the TCR α or β gene separately into the retroviral vector pMP71, the amplified TCR chain gene and vector plasmid pMP71-GFP were first double-digested with the restriction enzymes Not I and EcoR I (Figure 4.2).

TCR α- or β-chain fragment		Vector pMP71-GFP	
PCR product	2.5 µg	Plasmid	40 µg
Not I (10 U/ml)	4 µl	Not I (10 U/ml)	4 µl
EcoR I (10 U/ml)	4 µl	EcoR I (10 U/ml)	4 µl
NEB buffer 4 (10x)	10 µl	NEB buffer 4 (10x)	10 µl
BSA (10x)	10 µl	BSA (10x)	10 µl
DEPC H_2O	to 100µl	DEPC H_2O	to 100µl

Digestion	37°C	2 h
Holding	4°C	
Storage	-20°C	

The digested PCR products were further purified by phenol-chloroform-extraction to remove the small DNA fragments and enzymes. After digestion, 500 µl phenol was added to the digestion mixture and centrifuged at 14000 rpm for 2 min. The upper liquid phase was harvested and mixed thoroughly with 500 µl chloroform-isoamyl alcohol (24:1). After centrifugation at 14000 rpm for 2 min at RT, the upper layer was transferred into a new tube and mixed well with 10 µl Na-acetate (3 M) as well as 1 ml ethanol (99%) followed by centrifugation at 14000 rpm for 25 min at 4°C. Finally the DNA pellet was dried and eluted in 20 µl DEPC H_2O. The amounts of digested vector and TCR gene fragments were determined spectrophotometerically. The digested vector was run on a 1% agarose gel, and the DNA band of 5848 bp was cut out and purified by NucleoSpin® Gel Extract kit.

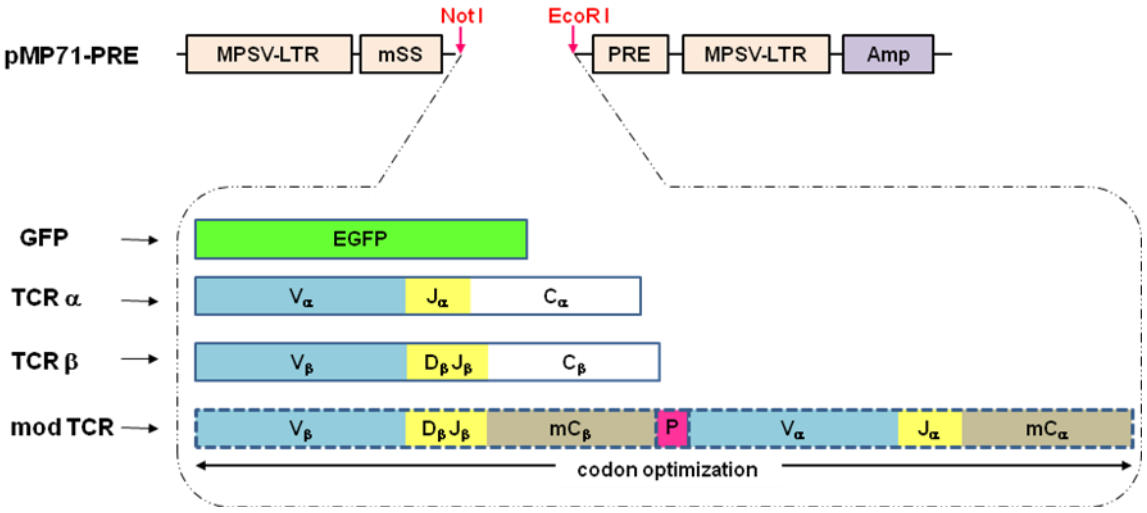

Figure 4.2 Schematic representation of retroviral vector constructs.

The backbone of retroviral vector pMP71-PRE contains these elements: LTR, long terminal repeat from myeloproliferative sarcoma virus (MPSV); mSS, modified mRNA splice site; PRE, posttranscriptional regulatory elements; Amp, ampicillin resistance (Engels et al. 2003). pMP71-GFP contained the enhanced green fluorescent protein gene (EGFP) and was used as control vector. The TCR α- or β-chain fragment was cloned into digested pMP71 with Not I and EcoR I, namely replacing the GFP gene, and the reconstructed vectors were designated as pMP71-TCRα or -TCR β. The allorestricted TCRs were further modified by using several strategies including murinization of TCR α- and β-chain constant regions, linkage of TCR β- and α-chains with a "self-cleaving" 2A peptide from picornavirus (P2A) and codon optimization of the entire fragment to enhance the expression and pairing of transduced TCR chains (see section 4.1.3). mC_α/C_β: murine TCR α- or β-chain constant regions; P: P2A. The constructs harboring modified TCRs were named as pMP71-mod TCR.

4.1.2.4 Ligation and transformation into bacteria

The ligation of TCR α- or β-chain genes into the digested vector pMP71 was done with a 5:1 ratio of insert:vector of molar amount, which was determined as following formula (a). The needed amount of insert was calculated with formula (b)

(a) (b)

$$n = \frac{[Insert]}{[Vector]} = \frac{\frac{m_{insert}}{bp_{insert}}}{\frac{m_{vector}}{bp_{vector}}} \qquad \Longrightarrow \qquad m_{insert} = n \times m_{vector} \times \frac{bp_{insert}}{bp_{vector}}$$

n: the ratio of insert:vector of molar amount
m: the amount of insert or vector

[*Insert/Vector*]: the molar amount of insert or vector
bp: the base-pair length of insert or vector

Ligation mixture:

Insert: TCR α- or β-chain fragment (~900 bp)	279 ng
Vector: pMP71 (5848 bp)	362 ng
T4 ligase (5 U/µl)	1 µl
Ligase Buffer (10x)	1 µl
DEPC-H_20	to 10µl

Ligation	16°C	16 h
Inactivation	65°C	10 min
Holding	4°C	
Storage	-20°C	

The ligation product was transformed into chemically competent *E. coli* TOP10 (Invitrogen, Karlsruhe) according to manufacturer's instruction. Briefly, 2 µl of ligation product was added to thawed TOP10 competent cells and stirred by a tip. After 30 min incubation on ice, the TOP10 were heated for 45 s at 42°C immediately followed by 2 min incubation on ice. After

addition of 200 µl S.O.C medium, the transformation mixture was shake-incubated at 300 rpm for 1 h at 37°C. 50 µl or 100 µl transformation mixture was placed on the pre-warmed LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37°C. The plates could be further preserved at 4°C for maximum one week.

4.1.2.5 Plasmid extraction and control digestion

Theoretically only bacteria transformed with insert- or self-ligated vector containing antibiotic resistance can grow on the selective agar plate. To obtain colonies containing correctly ligated vector, 5-10 colonies were picked and cultured in 5 ml LB medium containing ampicillin (100 µg/ml) overnight at 37°C. Thereafter, the plasmids were extracted from the mini-culture by using JetStar Plasmid Purification kit (Genomed) following the manufacturer's protocol (Mini-scale) and digested with enzyme Not I and Xho I to check primarily the positivity of colonies.

Plasmid		~1 µg
Not I (10 U/ml)		1 µl
Xho I (10 U/ml)		1 µl
NEB buffer 4 (10x)		2 µl
BSA (10x)		2 µl
DEPC H_2O		to 20µl
Digestion	37°C	2 h
Holding	4°C	
Storage	-20°C	

The digestion product was run on a 1% agarose-gel to check the size of digested fragments. One plasmid sample with the expected digestion pattern was further sequenced by Sequiserve (Vaterstetten) or Eurofin MWG (Munich).

pMP71 with	**Enzymes**	**NEB buffer***	**expected band sizes**
TCR HER2-1 α	Xho I, Not I	3	2546 bp, 3781 bp
TCR HER2-1 β	Xho I	4	2247 bp, 4185 bp
TCR HER2-2 α	Xho I, Not I	3	2528 bp, 3781 bp
TCR HER2-2 β	Xho I, Not I	3	2660 bp, 3781 bp
TCR HER2-3 α	Xho I, Not I	3	2549 bp, 3781 bp
TCR HER2-3 β	Xho I, Not I	3	2648 bp, 3781 bp
TCR HER2-4 α	Xho I, Not I	3	2534 bp, 3781 bp
TCR HER2-4 β	Xho I, Not I	3	2654 bp, 3781 bp
TCR GP100 α	Xho I, Not I	3	2541 bp, 3781 bp
TCR GP100 β	Xho I, Not I	3	2652 bp, 3781 bp

TCR SK22 α	Xho I, Not I	3	2540 bp, 3781 bp
TCR SK22 β	Xho I	4	2247 bp, 4179 bp
GFP	Xho I, Not I	3	2437 bp, 3781 bp

*: the selection of appropriate reaction buffer (1, 2, 3, 4) to ensure optimal activity (100%) of the corresponding enzyme is according to the manufacture's recommendation (http://www.neb.com/nebecomm/default.asp).

4.1.2.6 Large-scale (Maxi) preparation of plasmid

To obtain a large amount of plasmid, 150 µl mini-culture of positive colonies verified by sequence analysis were further cultivated in 300 ml LB medium with 100 µg/ml ampicillin. The plasmids were then extracted by using JETstar Plasmid Purification kit according to the manufacturer's instruction (Maxi-scale). The concentration of the plasmid DNA was measured by using a spectrophotometer.

The bacteria transformed with the vector pAIF10A1-GALV ("env") and pcDNA3.1-MLV ("gag-pol") were preserved as glycerol stock at -80°C (kindly provided by W. Uckert, Berlin). To recover plasmids from the glycerol stock, a 200 µl tip was used to stab slightly the glycerol stock and was then transferred into 5 ml LB-medium containing the appropriate antibiotic (see Table 3.9) followed by shake-incubation overnight at 37°C (mini-culture). The following procedure for large-scale plasmid preparation was performed by using the JETstar Plasmid Purification Kit as mentioned above. The obtained plasmids were verified by control digestion using corresponding restriction enzymes.

Vector	**Enzymes**	**NEB buffer**	**Expected band sizes**
pAIF10A1-GALV ("env")	Xba I, Cla I	4	1700 bp, 5000 bp
pcDNA3.1-MLV ("gag-pol")	Xho I	4	4309 bp, 6857 bp

4.1.3 TCR optimization

To enhance the expression and correct pairing of transduced allorestriced TCR α- and β-chains, the TCR constructs were modified in three aspects as showed in Figure 4.2: (1). Murinization of constant regions of TCR α- and β-chains to reduce the mispairing of the transduced TCR α- (β-) chain with endogenous TCR β- (α-) chains (Cohen et al. 2006). (2). Linkage of TCR α- and β-chains with a "self-cleaving" 2A peptide-linker derived from picornavirus (P2A) to transfer the same molar amount of TCR α- and β-chains into recipient cells (Szymczak et al. 2004; Leisegang et al. 2008). (3). Codon optimization of the entire TCRβ-P2A-TCRα genes resulting in an identical amino acid sequence in order to improve the express of genes in recipient cells according to the specific codon usage bias in different organisms (Scholten et al. 2006). Codon usage bias refers to a phenomenon, that synonymous codons (encoding the same amino acid) are used with different frequencies in different organisms. The compatibility of codon usage of heterologous genes and that of

expression host appears to facilitate the expression of heterologous genes (Gustafsson et al. 2004). The modified TCR genes were generated by Geneart (Regensburg) and cloned primarily into a vector provided by the company.

4.1.4 Generation of retroviral constructs with modified TCRs

To clone the modified TCR genes into the vector pMP71, the plasmids containing modified TCR genes derived from Geneart were first amplified by transformation into TOP10 followed by maxi preparation. Thereafter, the plasmids were digested with Not I and EcoR I as shown below.

Plasmid	40 µg
Not I (10 U/ml)	8 µl
EcoR I (10 U/ml)	8 µl
NEB buffer 4 (10x)	20 µl
BSA (10x)	20 µl
DEPC H_2O	to 200 µl

Digestion	37°C	2h
Holding	4°C	
Storage	-20°C	

The bands of interest (approximately 1900 bp) were purified from the agarose gel and ligated into the vector pMP71, which was also digested with EcoR I and Not I, at a 5:1 ratio of insert:vector of molar amount.

Ligation mixture:

Insert: modified TCR fragment (~1900 bp)	588 ng
Vector: pMP71 (5848 bp)	362 ng
T4 ligase (5 U/µl)	1 µl
Ligase Buffer (10x)	1 µl
DEPC H_20	to 10µl

Ligation	16°C	16 h
Inactivation	65°C	10 min
Holding	4°C	
Storage	-20°C	

The ligation product was transformed into competent *E. coli* TOP10, and positive colonies were verified through control digestion with appropriate enzymes. Finally, the plasmids from a verified positive colony were amplified and maxi-prepared as mentioned above.

pMP71 with modified TCR	Enzymes	NEB buffer	Expected band size
HER2-1	Xba I	4	1456 bp, 2591 bp, 3249 bp
HER2-2	Xba I	4	1465 bp, 2573 bp, 3249 bp
HER2-3	Xba I	4	3249 bp, 2594 bp, 1453 bp
GP100	Xba I	4	3249 bp, 2582 bp, 1453 bp
SK22	Xba I	4	3249 bp, 2585 bp, 1450 bp

4.1.5 Generation of a lentiviral construct with hNIS and modified TCR HER2-1

In order to monitor TCR-transduced T lymphocytes *in vivo* for a longer period, the gene encoding hNIS, which can import radioactive substrates such as ^{124}I and render the hNIS-transduced cells traceable, was transferred simultaneously with the allorestricted TCR HER2-1 into T cells. In detail, the modified TCR gene fragment (TCRβ-P2A-TCRα) was fused to the hNIS gene fragment with a 2A linker derived from insect virus *Thosea asigna* (T2A) in the lentiviral vector pHIV7SF-hNIS (Figure 4.3) to generate the construct with a hNIS-T2A-TCRβ-P2A-TCRα fragment by a two-step PCR (Figure 4.4).

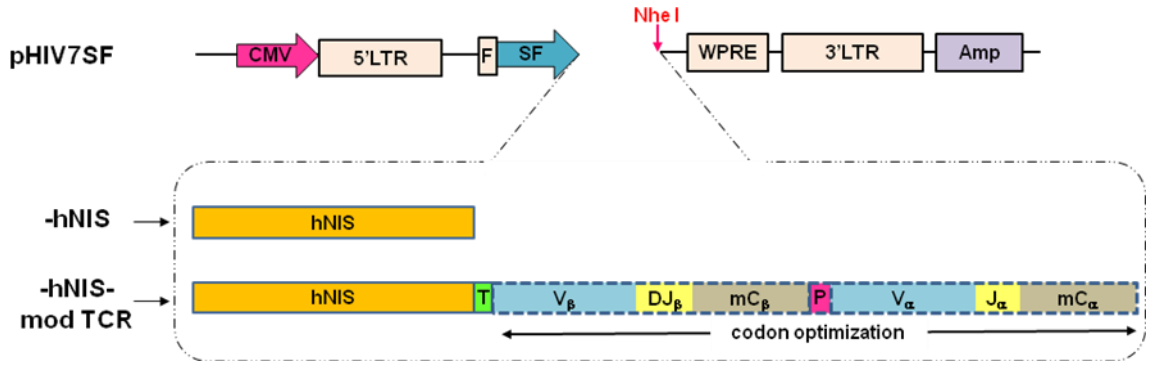

Figure 4.3 Schematic representation of lentiviral vector constructs.

The backbone of the lentiviral vector pHIV7SF consists of the promoters from cytomegalovirus (CMV) and spleen focus-forming virus (SF), 5' and 3' LTR of human immunodeficiency virus (HIV), ampicillin resistance (Amp) and two cis-regulatory elements, namely, a posttranscriptional regulatory element from Woodchuck hepatitis Virus (WPRE) and center DNA flap (F) composed of a polypurine tract sequence and a central termination sequence (Yam et al. 2002). The pHIV7SF-hNIS vector harbors additionally the gene encoding the human sodium-iodide sympoter (hNIS), whereas the vector, which contains the genes encoding hNIS and modified TCR with a "self-cleaving" 2A peptide from *Thosea asigna* (T) in between, was designated as pHIV7SF-hNIS-modTCR. A Nhe I restriction site is located between the hNIS and WPRE element.

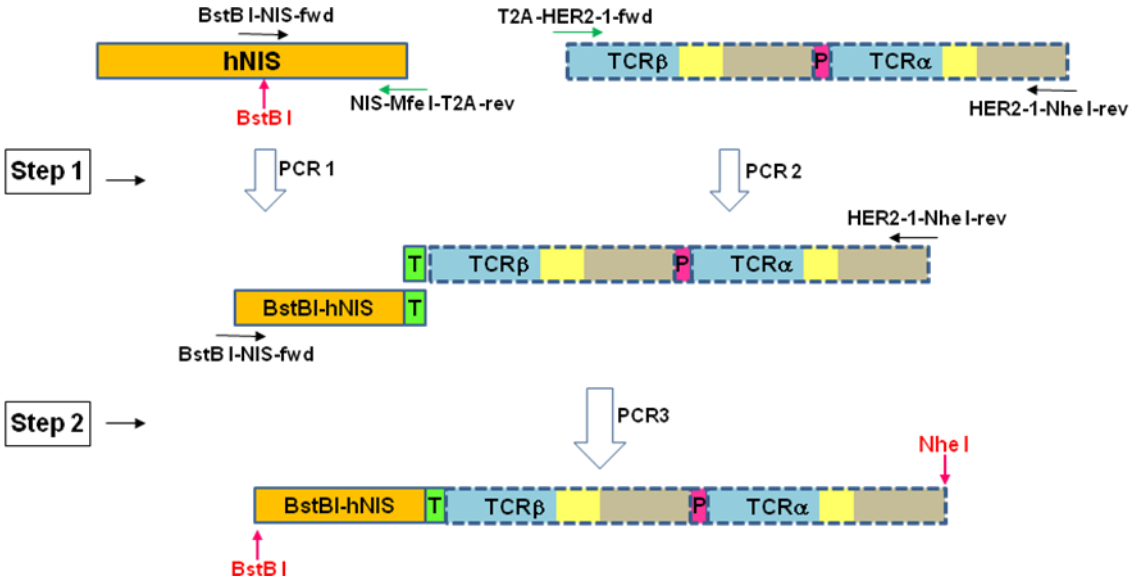

Figure 4.4 Cloning of hNIS-T2A-TCRβ-P2A-TCRα fusion gene by using a two-step PCR.

A BstB I restriction site is located within the hNIS fragment gene. An Mfe I site was incorporated into construct in order to facilitate subsequent cloning of other TCRs into the lentiviral vector. T: a "self-cleaving" 2A sequence derived from the insect virus *Thosea asigna* (T2A). The first-step PCR amplified two fragments, (1) the modified TCR and (2) a part of hNIS gene. Both fragments were flanked with T2A sequence at the 5' or 3' end, respectively. The second-step TCR, an annealing PCR, amplified the fused fragments of hNIS-T2A-mod TCR by using the PCR products from the first step as template. The obtained fragment contained BstB I and Nhe I restriction sites at the end and were temporally cloned into a pCR-blunt vector (Invitrogen) followed by double digestion (see section 4.1.5.3) and ligation into the lentiviral vector pHIV7SF-hNIS (see section 4.1.5.4).

4.1.5.1 PCR amplification of T2A-flanked TCR HER2-1 or hNIS fragments

The hNIS fragment and mod TCR HER2-1 gene flanked with the T2A sequence at the 3' or 5' end were amplified from the template plasmids pHIV7SF-hNIS or pMP71-mod HER2-1 using the primer pair BstB I-NIS-fwd/ NIS-MfeI-T2A-rev and T2A-HER2-1-fwd/HER2-1-NheI-rev (Table 3.11), respectively. A BstB I restriction site is located at the position 998 of the hNIS gene, thus, only part of the hNIS gene was amplified. An Mfe I cleavage site was introduced between hNIS and the T2A sequence in order to facilitate subsequent cloning of other TCRs into the pHIV7SF-hNIS vector by using the MfeI-NheI cleavage sites.

PCR1: BstBI- hNIS-T2A fragment		**PCR2**: T2A-HER2-1 fragment	
pHIV7SF-NIS	3µg	pMP71- mod HER2-1	3 µl
5' P: BstBI-NIS-fwd (10 µM)	3µl	5' P: T2A-HER2-1-fwd (10 µM)	3 µl
3' P: NIS-Mfe I-T2A-rev (10 µM)	3µl	3' P: HER2-1-Nhe I-rev (10 µM)	3 µl
dNTP (2 mM)	10µl	dNTP (2 mM)	10 µl
$MgSO_4$ (25 mM)	6µl	$MgSO_4$ (25 mM)	6 µl
Buffer (10x)	10µl	Buffer (10x)	10 µl

KOD Polymerase (1 U/µl)	2µl	KOD Polymerase (1 U/µl)	2 µl
DEPC H_2O	to 100µl	DEPC H_2O	to 100 µl

PCR1 and 2 program:

1.	Initialization	95°C	2 min
2.	Denaturation	95°C	30 s
3.	Annealing	65°C	30 s
4.	Elongation	70°C	2 min
5.	Final elongation	70°C	10 min
6.	Holding	4°C	(step 2 → 4: 35 cycles)

The PCR reaction without template plasmid served as control. The PCR products were run on a 1% agarose gel, and the amplified 993 bp fragment from PCR1 and 1860 bp fragment from PCR2 were purified from the gel and the quantity of DNA was determined on a spectrophotometer.

4.1.5.2 Annealing PCR for amplification of the hNIS-T2A-HER2-1 construct

The purified PCR fragments were further used to amplify a hNIS-T2A-TCR construct by annealing PCR with the primer pair BstBI-NIS-fwd/ HER2-1-Nhe I-rev.

PCR3: BstB I-hNIS-T2A-HER2-1 fragment

PCR1 product: BstB I-NIS-T2A fragment	100 ng
PCR2 product: T2A-HER2-1 fragment	100 ng
dNTP (2 mM)	10 µl
$MgSO_4$ (25 mM)	6 µl
Buffer (10x)	10 µl
KOD Polymerase (1 U/µl)	2 µl
DEPC H_2O	to 100µl

Gradient PCR program:

1.	Initialization	95°C	2 min	
2.	Denaturation	95°C	10 s	
3.	Annealing	40°C	10 s	(step 2→3: cooling down with10°C /min)
4.	Elongation	72°C	2 min	(step 2→4: 5 cycles)
5.	Holding	4°C		

5’ Primer: NIS-BstB I-fwd (10µM)	3 µl
3’ Primer: HER2-1-Nhe I-rev (10µM)	3 µl
KOD Polymerase (1U/µl)	2 µl

Long PCR program:

1.	Initialization	95°C	2 min
2.	Denaturation	95°C	30 s
3.	Annealing	55°C	30 s
4.	Elongation	70°C	4 min
5.	Final elongation	70°C	10 min
6.	Holding	4°C	(step 2 →4: 35 cycles)

The amplified fragment (size: 2805 bp) was purified from a gel and the quantity of DNA was spectrophotometrically determined.

4.1.5.3 Cloning of hNIS-T2A-HER2-1 construct into the pCR-Blunt vector

The obtained hNIS-T2A-HER2-1 construct was first cloned into the pCR-Blunt vector (Sigma, Taufkirchen) with a 5:1 ratio of insert:vector of molar amout in order to verify the sequence of annealed PCR product.

Insert (BstBI-NIS-T2A-HER2-1, 2805bp):	102 ng
Vector (pCR-Blunt vector, 3512bp):	25 ng
T4 ligase (1U/µl)	1 µl
Ligase Buffer (10x)	1 µl
DEPC H_2O	to 10µl

Ligation	16°C	16h
Inactivation	65°C	10min
Holding	4°C	
Storage	-20°C	

The ligation product was transformed into Top10 competent cells as described above. On the following day, 10 colonies grown on the agar plates containing kanamycin as selection antibiotic were picked and checked for their positivity by control digestion following mini-preparation of the plasmid. The insert fragment may ligate into the pCR-Blunt-vector in both directions, resulting in two possible size patterns after digestion as shown below. One plasmid sample with the expected digestion pattern was further sequenced by Eurofin MWG (Munich).

pCR-Blunt vector with	**Enzyme**	**NEB buffer**	**Expected band sizes**
BstB I-hNIS-T2A-mod HER2-1	Xba I	4	1997 bp, 4320 bp
		or	1572 bp, 4745 bp

4.1.5.4 Cloning of hNIS-T2A-HER2-1 fragment into pHIV7SF-hNIS vector

One plasmid sample with verified sequence by sequencing was digested with BstB I and Nhe I and ligated into the pHIV7SF-hNIS vector, which was cut using the same enzymes.

Plasmid (pHIV7SF-hNIS or pCR-Blunt-NIS-T2A-mod HER2-1)	40 µg
Nhe I	8 µl
NEB buffer 4 (10x)	20 µl
BSA (10x)	20 µl
DEPC H_2O	to 200 µl

	Digestion	37°C 2h
BstB I		8 µl
	Digestion	65°C 2h
	Holding	4°C
	Storage	-20°C

The expected two fragments, NIS-T2A-HER2-1 (2792 bp) and pHIV7SF-hNIS (8324 bp), were eluted from the gel and further used for ligation with a 4:1 ratio of insert:vector of molar amount.

Insert (NIS-T2A-HER2-1, 2792 bp):	231 ng
Vector (pHIV7SF-hNIS, 8324 bp):	165 ng
T4 ligase (1 U/µl)	1.5 µl
Ligase Buffer (10x)	1.5 µl
DEPC H_2O	to 15µl

Ligation	16°C	16 h
Inactivation	65°C	10 min
Holding	4°C	
Storage	-20°C	

The ligation product was transformed into competent *E. coli* TOP10, and the positivity of colonies was determined by control digestion with appropriate enzymes. Finally, the plasmids from a verified positive colony were maxi-prepared as mentioned above.

pHIV7SF vector with	**Enzymes**	**NEB buffer**	**Expected band sizes**
hNIS-T2A-mod HER2-1	Xba I,XhoI	4	2184 bp, 2892 bp, 6040 bp,

4.1.6 Mycoplasma contamination testing PCR

The infection of cultured cells with mycoplasma bacteria was determined using Venor®GeM-Mycoplasma Detektions Kit (Minerva Biolabs). Samples were derived from cell cultures, which were at 90-100% confluence. Cell culture supernatants or cell pellets following DNA extraction were tested for mycoplasma C infection according to the manufacturer's recommendation. The contaminated samples show the positive PCR amplification product (265-278 bp) with specific primers targeting mycoplasma DNA. The kit provided an internal

control resulting in a PCR product of 191 bp, which should be present in each sample. Mycoplasma-contaminated cells were cultivated with culture medium supplemented with 10 µg/ml ciprofloxacin (HEXAL).

Approximately 100 µl of cell culture supernatant was harvested in a 1.5 ml tube and boiled for 5 min at 95°C. After short centrifugation, 2 µl supernatant was used for the PCR-reaction as shown below.

Sample supernatant:	2 µl
Primer/nucleotide mix:	2.5 µl
Buffer (10x):	2.5 µl
Internal control:	2.5 µl
Mq-polymerase:	0.2 µl
H_2O:	to 20 µl

PCR program:

1.	Initialization	94°C 2 min	
2.	Denaturation	94°C 30 s	
3.	Annealing	55°C 30 s	
4.	Elongation	72°C 2 min	
5.	Holding	4°C	(Step 2→ 4: 39 cycles)

4.2 Cell biology methods

4.2.1 General cell culture conditions

All cell lines and primary cells used in this study were kept in tissue-culture treated multi-well plates or flasks if not otherwise indicated and cultivated in the incubator at 37°C with 5 to 6% CO_2 and 95% humidity. Cell culture was performed under sterile conditions. The experiments with retrovirus, lentivirus or non-tested blood sample (testing for HIV, Hepatitis B and C virus) were carried out according to the security level S2 (gene technology law) and L2 (biosafety law).

4.2.1.1 Cultivation of cells (cell passenger)

The suspension cells were cultured by replacing old medium with fresh medium in appropriate dilution every 3-4 days. The adherent cells were harvested and placed in new flasks with fresh medium in appropriate dilution every 3-4 days. To do this, cells were washed once with PBS, incubated with trypsin for several minutes, and harvested in complete culture medium (see Table 3.6 and 3.7). After centrifugation at 1800 rpm for 5 min, the cells were resuspended in fresh culture medium and transferred to new culture flasks in a 1:3 to 1:40 split according to a defined cell type.

4.2.1.2 Freezing and thawing cells

The cells were cryo-preserved in freezing medium containing 90% ΔFCS and 10% DMSO (1.8 ml/vial) first at -80°C for at least 24 h and then stored in a liquid nitrogen tank for long-term storage.

To thaw the frozen cells, the cryo-preserved vials were first briefly incubated at 37°C in a water bath until the thawing of the frozen medium and immediately transferred into fresh RPMI medium. Thereafter, cells were washed and cultivated in corresponding medium.

4.2.1.3 Determination of the cell number

To determine cell numbers, the samples were diluted in trypan blue for analysis of the proportion of dead cells. Trypan blue can only penetrate dead cells resulting in blue staining of dead cells. Unstained living cells present in two to four large squares were counted under the microscope using a Neubauer-counting chamber. The cell number was calculated by the following formula:

$$c[cells/ml] = \frac{\text{number of alive cell}}{\text{number of large squares}} \times \text{dilution factor} \times 10^4$$

4.2.2 Isolation of PBMC from the whole blood

PBMC were obtained from healthy donors with informed consent according to the regulations of the local ethical board and the principles in Helsinki Declaration. PBMC were isolated by Ficoll density gradient centrifugation. Briefly, heparinized blood was diluted using an equal volume of RPMI. Thereafter, 35 ml of diluted blood was carefully transferred on the top of 15 ml Ficoll solution. After centrifugation at 2000 rpm for 30 min with slow acceleration and without brake, the Buffy coat layer was carefully harvested and washed twice with fresh RPMI medium. Finally, the PBMC pellet was resuspended in 20 ml TCM, counted and used for further experiments.

4.2.3 Enrichment of $CD4^+$ and $CD8^+$ T lymphocytes with magnetic beads

$CD4^+$ or $CD8^+$ T lymphocytes were enriched from PBMC using the human untouched CD4 or CD8 negative isolation kit (Dynal, Invitrogen). The principle of such isolation is: the primary mouse anti-human antibody-mixture containing anti-CD4 or anti-CD8, but always anti-CD14, anti-CD16, anti-CD19, anti-CD36, anti-CD56, anti-CDw123, and anti-CD235a, labels all PBMC-derived cells with the exception of the targeted $CD4^+$ T or $CD8^+$ T cell population. Antibody-labeled cells were then recognized by magnetic bead-conjugated secondary antibody targeting the Fc fragment of the primary antibody. At the end, the cell population of interest was separated from magnetic bead-bound residual cells using a strong magnet.

The isolation procedure was performed according to the manufacturer's instruction. Briefly, freshly isolated PBMC were resuspended in ΔFCS and incubated with antibody-mixture for 30 min at 4°C under continuous rotation. After washing once (PBS with 1%ΔFCS), the cells were mixed with pre-washed depletion beads under continuous rotation for 30 min at RT. The cell-bead mixture was then placed in a magnet field twice for 2 min. The supernatant comprising the untouched cell population of interest was harvested, washed and resuspended in TCM. The purity of the cell population was about 97% as determined by flow cytometry.

4.2.4 Nonspecific stimulation of PBMC, $CD4^+$ and $CD8^+$ T cell subsets

The isolated PBMC ($1x10^6$/ml/well) from blood samples were nonspecifically stimulated with anti-CD3 (OKT3, 50 ng/ml) and IL-2 (50 U/ml) in TCM in 24-well plates for 48 to 72 h prior to retroviral transduction. The enriched $CD4^+$ or $CD8^+$ T lymphocyte populations ($1x10^6$/ml/well) were nonspecifically stimulated in TCM with IL-2 (50 U/ml) in anti-CD3 (5 µg/ml) and anti-CD28 (1 µg/ml) antibody-coated non-treated 24-well plates for 48 h prior to retroviral transduction.

For antibody-coating of non-treated 24-well plates, 0.5 ml PBS supplemented with 5 µg/ml anti-CD3 and 1 µg/ml anti-CD28 antibody was added into each well and incubated for 2 h at 37°C. Thereafter, the wells were blocked with 2% BSA solution in H_2O for 30 min at 37°C. After washing once with PBS, the coated plates were used for the experiments.

4.2.5 Generation and expansion of human T cell clones

T cell clones transduced with allorestricted TCR were generated from TCR-transduced PBMC by limiting dilution: TCR-transduced PBMC were diluted and cultivated at a concentration of 1 cell/well in 96-well plates 12 days after retroviral TCR transduction. TCR-transduced cells were stimulated with feeder cells ($5x10^4$ allogeneic PBMC/well), which were pooled from 3 donors and irradiated with 60Gy, and culture medium was supplemented with OKT-3 (30 ng/ml), IL-2 (50 U/ml), IL-7 (5 ng/ml), and IL-15 (5 ng/ml). Every 3 or 4 days 50 U/ml IL-2 was added and every two weeks the T cell clones were nonspecifically restimulated with different PBMC pools and supplementation of OKT-3 (30 ng/ml), IL-2 (50 U/ml), IL-7 (5 ng/ml), and IL-15 (5 ng/ml).

4.3 Fluorescence activated cell sorting (FACS)

4.3.1 Surface staining

The cell samples of interest were washed once with FACS buffer and stained with fluorescence-conjugated mAbs or multimers specific for certain surface markers under

suitable dilution in FACS buffer (see Table 3.4 and 3.5). After 30 min incubation, the cells were washed once or twice and resuspended in a suitable volume of FACS buffer followed by measurement by the flow cytometer (Calibur or LSRII, BD). In case of multimer-staining in combination with mAb-staining, the cells were first stained with multimers for 20 min on ice in the dark followed by conventional mAb-staining as described above. After staining, cells were eventually fixed with 1% Paraformaldehyde A (PFA) solution and analyzed on the same or the following day.

4.3.2 IFNγ-intracellular staining

The IFNγ secretion of TCR-transduced $CD8^+$ T cells in response to diverse targets was determined by intracellular staining with specific antibodies against human IFNγ to investigate IFNγ-production of defined cell populations. Namely, T cells and diverse targets were cocultivated for 6 h with an ratio of effector (E) : target (T) as indicated. During the incubation, Brefeldin A (BFA, 10 µg/ml), inhibiting the transport of proteins from the ER to the Golgi apparatus, was added to retain the produced cytokines within cytoplasma instead of secretion into the supernatant. Subsequently, the stimulated cells were stained with surface antibodies or multimers for 20 min on ice in combination with 7-AAD (10 µg/ml), which stained the nucleus enabling to differentiate dead/live cell populations. After washing twice with PBS, the cells were fixed with 1% PFA in PBS for 20 min on ice. Thereafter, the cells were again washed twice with PBS and permeabilized with Saponin- and serum-supplemented PBS in two steps subsequently: (1) 0.1% Saponin and (2) 0.35% Saponin (Sigma). The permeabilized cells were incubated with FITC-conjugated anti-human IFNγ antibody for 30 min on ice. Following washing with 0.1% Saponin and FACS-Azide-buffer, the stained samples were fixed with 1% PFA and analyzed at the LSRII flow cytometer.

4.3.3 Flow cytometry

The stained cell samples were measured at the Calibur or LSRII flow cytometer following the instruction of the manufacturers. Generally approximately 10 000 vital cells were acquired during the measurement. The acquired data were further analyzed by using the Flowjo program. The gating strategies used for analysis are shown in Figure 4.5. For the standard surface staining, vital cells were first gated by using SSC-A and FSC-A parameters. The gated population was then dot plotted on FSC-H versus FSC-A to exclude the duplex or multiplex cells. Therefore, only vital and single cells were analyzed for the staining. For intracellular staining of stimulated T cells, the T cell population was generally gated with the parameters SSC-A and FSC-A. Thereafter, 7-AAD negative cells, namely the vital cell population, were selected. Finally, $CD8^+$ T cells were gated and assessed for the fluorescence staining.

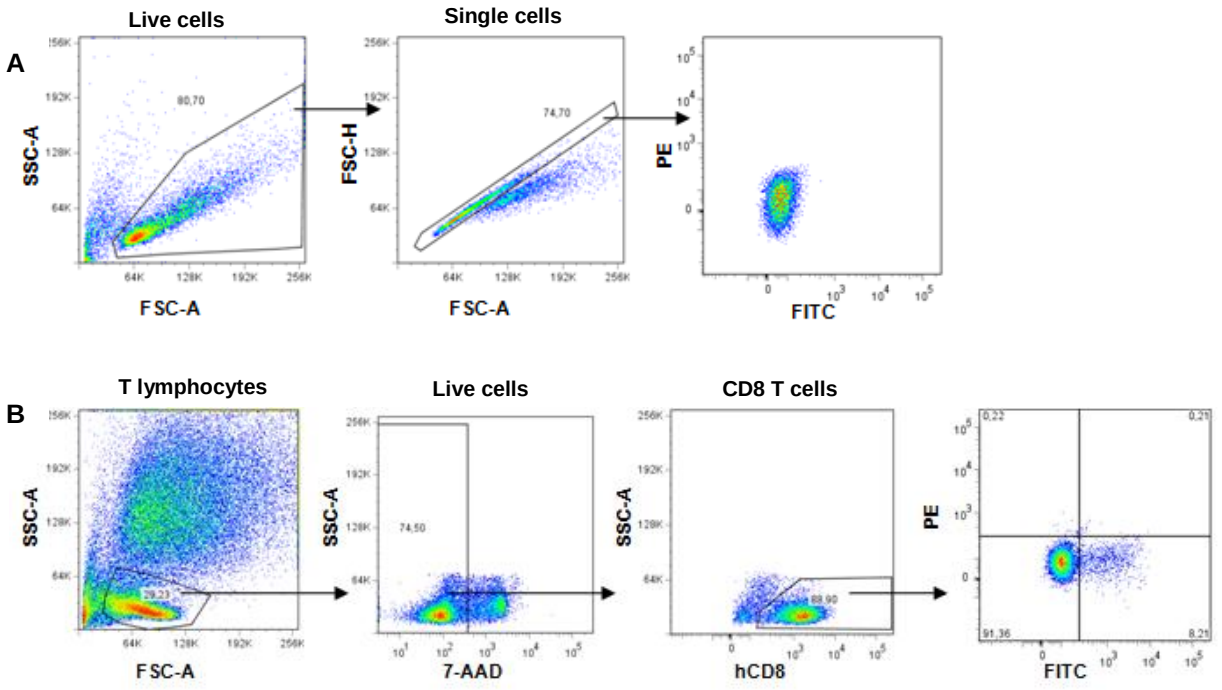

Figure 4.5 Gating strategies for analysis of FACS data of surface (A) and intracellular staining (B) using the Flowjo software.

FSC: Forward Scatter correlating with the cell volume. SSC: Side Scatter depending on the inner complexity of particles. A: area; H: height; Plots of FSC-H versus FSC-A are used for the discrimination of cell aggregates. Single cells have linearly correlated FSC-A and FSC-H signals resulting in a relatively straight line in the display of FSC-H versus FSC-A, whereas cell aggregates have larger FSC-A signals compared to their FSC-H signals and therefore are located off the diagonal formed by single cells; 7-AAD, a fluorescent chemical compound binding DNA in dead cells through permeabilized membrane but not in viable cells. PE and FITC were fluorescent molecules with different emission wavelength.

4.3.4 Sorting of interested cell populations via FACS sorter

To enrich the cell population of interest, the cell samples were stained with mAbs or multimers specifically recognizing the cell populations of interest followed by FACS sorting (MoFlow™). The staining procedure is similar to the conventional FACS surface staining. However, all procedures were performed under sterile conditions and the stained cells were filtrated through a cell filter to reduce the formation of cell-clot before sorting. Sorted cells (10000 cells/well) were restimulated in TCM in the presence of OKT3, IL-2, IL-7, and IL-15 as well as an allogeneic PBMC pool as mentioned above (see section 4.2.5).

4.4 Retroviral transfer of TCRs

4.4.1 Retrovirus production via calcium-phosphate-precipitation

To generate amphotropic retroviruses including TCR genes, the package cell line human embryonic kidney 293T (HEK 293T) was cotransfected with the retroviral vector pMP7-TCR as well as with 2 vectors harboring the retroviral genes *env* derived from gibbon ape leukemia virus (pAIF10A1-GALV) and *gag/pol* derived from murine leukemia virus (pcDNA3.1-MLV) by calcium phosphate precipitation. The genes *env*, *gag*, and *pol* encode envelope, gag polyprotein and reverse transcriptase, respectively, which are essential proteins for the assembly of virus particles.

HEK 293T cells were seeded in 6-well plates (0.5-0.6 Mio cells in 3 ml cDMEM per well) one day prior to transfection. The culture medium was exchanged by hunger medium about 1 h before transfection. 18 µg of total plasmid DNA was mixed with $CaCl_2$ and Chloroquin in H_20 as shown in Table 4.1. Thereafter, the plasmid-mixture was added drop by drop into the same volume 2x HBS solution, which was freshly adjusted to pH 7.6 and mixed by pipette boy under generation of bubbles. After 30 min incubation at RT, the plasmid-HBS mixture was transferred drop by drop into HEK 293T cells (300 µl per well). After 6 h incubation at 37°C, the hunger medium was replaced with fresh cDMEM. 48 h later, the supernatant containing virus particles (virus supernatant) was harvested, centrifuged for 5 min at 1800 rpm (in order to get rid of cell detritus), and filtrated through a 0.45 µm filter. The filtrated virus supernatant was used for immediate retroviral transduction or was preserved at -20°C.

Table 4.1 Solution containing plasmids, $CaCl_2$, Chloroquin, and HBS used for calcium-phosphate transfection of HEK 293T cells

	C_{stock}	C_{end}	V_{end}	V_{stock}	
				3 vectors	4 vectors
$CaCl_2$	2 M	100 mM	300 µl	15 µl	15 µl
Chloroquin	100 mM	126.7 µM	300 µl	0.38 µl	0.38 µl
Plasmid-mixture		18 µg	300 µl		
gag-pol				6 µg	4.5 µg
env				6 µg	4.5 µg
mod TCR				6 µg	
TCRα					4.5 µg
TCRβ					4.5 µg
Sterile H_2O	-	-	-	to 150 µl	to 150 µl
2x HBS	2x	1x	300 µl	150 µl	150 µl

4.4.2 Retroviral transduction of CD8α^+ Jurkat 76

The expression of transduced TCR could be easily analyzed in CD8α^+ Jurkat76 (J76CD8) cells as J76CD8 is a TCR-deficient T cell line. For retroviral transduction of J76CD8 cells, $1x10^5$ J76CD8 cells kept in 1 ml cRPMI were transferred into non-treated 24-well cell culture plates coated with RetroNectin® and spinoculated with 1 ml fresh or thawed virus supernatant supplemented with protaminsulfate (4 µg/ml) and HEPEs (10 mM) at 2000 rpm for 90 min at 32°C. Following 48 h incubation at 37°C, the culture medium was replaced with fresh cRPMI and the cells were analyzed by flow cytometry 3 or 4 days after transduction. J76CD8 cells transduced with pMP71-GFP were treated as mock control.

To prepare RetroNectin®-coated non-treated 24-well cell culture plates, 0.4 ml RetroNectin® (12.5 µg/ml) were transferred into each well and incubated for 2 h at RT. Thereafter, the RetroNectin® solution was replaced with 0.5 ml 2%BSA followed by 30 min incubation at 37°C. Finally, coated wells were washed once with PBS solution containing 25 mM HEPES and were used for transduction experiments.

4.4.3 Retroviral transduction of PBMC

The following procedure was performed for retroviral transduction of a defined TCR. Stimulated PBMC ($1x10^6$ cells) were plated in 1 ml TCM per well on RetroNectin®-coated non-treated 24-well plates (see section 4.4.2). Cells were mixed with 1 ml fresh or thawed virus supernatant and protaminsulfate (4 µg/ml), HEPEs (10 mM) as well as IL-2 (100 U/ml) was added. Thereafter, plates were spinoculated at 2000 rpm for 90 min at 32°C. After 24 h incubation at 37°C, the transduced PBMC were harvested, washed, and repeatedly spinoculated with virus supernatant with $3x10^5$ cells per well. After 24 h cultivation, the culture medium was replaced by fresh TCM supplemented with IL-2 (100 U/ml). Transduced PBMC were cultured with low dose IL-2 (30 U/ml) every 3 or 4 days and analyzed by flow cytometric or functional assays at indicated time points after transduction. GFP-transduced PBMC were used as mock control.

4.5 Lentiviral transfer of TCRs and hNIS

4.5.1 Lentivirus generation via TransIT®-293-mediated transfection

The TransIT®-293 reagent was used for lentiviral vector transfection. TransIT®-293-mediated transfection is less toxic, less vector-consuming and does not require hunger medium prior to transfection when compared to calcium-phosphate transfection. 6 µl TransIT®-293 reagent was mixed thoroughly with 200 µl free DMEM and incubated for 20 min at RT. Thereafter, 3 µl packaging vector mixture (Sigma, Taufkirchen) as well as 3 µg lentiviral vector pHIV7SF-hNIS-modTCR were added to the TransIT-medium mixture and

incubated for 30 min at RT. Subsequently, the TransIT-plasmid mixture was transferred into pre-seeded HEK 293T cells drop by drop as mentioned in section 4.4.1. After 6 h incubation, the medium was exchanged with fresh cDMEM. Finally, the viral supernatant was harvested in the same way as that described in section 4.4.1.

4.5.2 Lentiviral transfer of a defined TCR into the recipient cells J76CD8

The lentiviruses containing a TCR as well as the hNIS gene were produced in HEK 293T packaging cells through TransIT®-293-mediated transfection. For lentiviral transfer of a defined TCR into J76CD8 cells, the same following procedure of virus transduction was used as previously described for retroviral transduction (see section 4.4.2). The expression of the transduced TCR and hNIS genes was determined by antibody- and multimer-staining followed by flow cytometric analysis 4 days after transduction.

4.6 Cell function assays

4.6.1 Peptide pulsing

Peptide pulsing is the process of loading a peptide of interest onto APC, which will serve later on as a target for peptide-specific T cells. HLA-$A2^+$ TAP-deficient T2 cells were used as APC, since only a limited set of endogenous peptides was presented by T2 cells due to the deficiency of TAP. Prior to peptide-pulsing, T2 cells were washed twice with RPMI medium in order to remove peptides present in the cell culture. Cells were then resuspended in AIM-V medium at a concentration of $2x10^6$/ml. Different concentrations of defined peptides as well as β_2-microglobulin (10 µmol/l) were added followed by incubation for 2 h at 37°C. The reaction tubes were shortly and gently shaken every 20 min to improve the contact of T2 cells with peptides during the incubation. Finally, peptide-pulsed T2 cells were washed once with RPMI medium and resuspended in a specific medium defined by the following assays.

4.6.2 Stimulation assay

To investigate the specific reactivity of TCR-transduced T cells against diverse targets by cytokine release, TCR-transduced T cells were co-cultured with peptide-pulsed T2 cells or tumor target cells at 37°C for 24 h in a 5:1 ratio of E : T using 50.000:10.000 cells if not otherwise indicated. TCR-transduced PBMC were harvested 10-11 days after transduction or restimulation, washed once with RPMI medium, and resuspended in fresh TCM. After determination of the cell number, 50 000 T cells diluted in 100 µl TCM were transferred to each well in a 96-well round-bottom plate. Meanwhile, the targets were also resuspended in TCM, counted, and added to the corresponding wells in the same volume (100 µl with 10 000

cells per well). GFP-transduced or non-transduced T cells were used as controls. The stimulation assays were performed in duplicates or triplicates as indicated.

4.6.3 Enzyme-Linked Immunosorbent Assay (ELISA)

After 24 h stimulation, the supernatant of co-culture was transferred to new 96-well round-bottom plates and analyzed for IFNγ release by using human IFNγ ELISA Kits according to the recommendations of the manufacturer. Shortly, the 96-well ELISA plate was at first coated with 50 µl capture antibody per well and incubated with a cover-film overnight at 4°C. After washing three times with wash buffer (0.05% Tween-20 in PBS), wells were then blocked with 300 µl milk buffer per well for 1 h at RT. After washing three times with wash buffer, 50 µl of supernatant samples or control probes containing a defined concentration of the specific cytokine, which will be used for evaluation of the standard curve, was transferred to the plate. The control probes were used in duplicates. The samples were tested in duplicates or triplicates as indicated. After 1 h incubation at RT, the wells were washed five times with wash buffer. 50 µl of biotinylated anti-IFNγ detection antibody and avidin-conjugated horse-radish peroxidase (HRP) enzyme in milk buffer was then added to bind the captured IFNγ on the bottom. After 1 h incubation at RT and subsequent washing for seven times with wash buffer, a mixture of hydrogen peroxide and tetramethylbenzidine solution (substrate A and B) in a volume of 100 µl was added serving as substrate for HRP and plates were subsequently incubated for about 15 min in the dark. Finally, the color enzyme reaction (blue) was stopped by the addition of 50 µl phosphate acid (1 M), and the color intensity correlating with the amount of captured IFNγ in the plate was measured on an ELISA Reader at 450nm. IFNγ concentrations present in different samples were determined by using the standard curve, which was calculated by Sigma Plot 10.0 following a formula for a sigmoid curve with 5 parameters.

4.6.4 Multiplex-Bioplex

Multiple cytokines present in the supernatant after specific stimulation with defined target cells were simultaneously detected using the Multiplex-analysis Kit. Cytokine-specific antibodies are conjugated to polystyrene-beads, which have maximally 100 different fluorescent colour codes, thus allowing theoretically the detection of 100 different cytokines at the same time. Another set of cytokine-specific antibodies, which were conjugated with biotin, was simultaneously used. The binding of the conjugated biotin to PE-coupled streptavidin allows the quantification of each cytokine. A BioPlex kit for detection of 9 T_H1/T_H2 human cytokines was used to analyze the following distinct cytokines: IL-2, IL-4, IL-5, IL-10, IL-13, IL-12, GM-CSF, IFNγ, TNFα, according to the manufacturer's recommendation (Bio-Rad, Munich). Briefly, 50 µl of bead-coupled capture antibodies were first added to the plate.

Subsequently, the plate was washed twice with wash buffer (100 µl/well), and 50 µl of probe supernatant or standard dilution was transferred to the capture antibody-coated plate. After binding of the cytokines to the capture antibodies (shake-incubation for 30 min at RT in the dark) followed by washing three times, 25µl of biotinlyated-detection antibodies were added to recognize a second epitope of defined cytokines (shake-incubation for 30 min at RT in the dark). After washing three times, 25µl of PE-conjugated streptavidin was added to bind biotinylated-detection antibodies (shake-incubation for 10 min at RT in the dark). After washing three times, the probes were resuspended in assay-buffer (125 µl/well). Finally, the fluorescence of probes was measured by Luminex BioPlex systems (Bio-Rad, Munich), and the amount of each secreted cytokine was determined by the bead-fluorescence in combination with the intensity of PE. Using standard curves of different cytokines, the concentrations of various cytokines in the same sample were automatically quantified by using BioPlex-Manager software. The standard dilutions were assessed in duplicates.

4.6.5 Chromium (^{51}Cr)-release Assay

The cytotoxicity of the T cells was determined by a 4 h standard ^{51}Cr release assay. T cells transduced with peptide-specific TCRs were generally tested 10 days after transduction. Effector cells were not treated with cytokines 2-3 days before the co-incubation with target cells. Target cells such as tumor cells or peptide-pulsed T2 cells were harvested, resuspended in 100 µl ΔFCS, and incubated with approximately 50 µCi (= 1.85 MBq) ^{51}Cr for 1-1.5 h at 37°C. After washing three times with cRPMI, the ^{51}Cr-labelled target cells were counted, diluted to 2000 cells/50 µl in cRPMI medium, and co-incubated with T cells (50 µl/well) in 96-well plates for 4 h at 37°C at diverse E:T ratios as indicated (the total volume of medium per well was 100 µl). After incubation, 50 µl of supernatant from each well was transferred to a luma plate (Applied Biosystems, Weiterstadt) and air-dried overnight. Finally, the release of radioactivity (cpm, counts per minute) in supernatants was measured by the Top count NXT (PerkinElmer, Waltham, USA). The relative specific cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity}(\%) = \frac{\text{cpm(sample)} - \text{cpm(spontaneous)}}{\text{cpm(maximum)} - \text{cpm(spontaneous)}} \times 100$$

The maximum release was calculated using the target cells, which were directly transferred to the Luma plate after labelling (50 µl), while the spontaneous release was calculated using the 50 µl supernatant from the wells, where target cells was co-cultivated with 50 µl cRPMI instead of effector cells. Since only half (50 µl) of the total supernatant (100 µl) from sample and spontaneous wells was measured, the value of maximum release used for the formula was the half of the measured value.

4.7 Mouse experiments

4.7.1 Cell preparation and injection

The tumor cells or T cells, which were used for injection into mice, were harvested from culture and resuspended in PBS. After counting, the cells were diluted in PBS at a certain concentration as indicated, transferred into 2 ml tubes and incubated on ice. Prior to injection, cells were gently mixed by reverting the tube, slowly aspirated into the 1ml syringe, and injected into mice subcutaneously, intraperitoneally or intravenously as indicated. (1). Subcutaneous application (s.c.): Cells were injected in the left or right flank; (2). Intraperitoneal application (i.p.): Cells was injected on the right side of the murine abdomen; (3). Intravenous application (i.v.): Cells were injected into the tail vein. To render the vein more visible, the tails of mice were placed under a red-light lamp.

4.7.2 Isolation and stimulation of mouse splenocytes

Mice were sacrificed and the spleens were harvested into centrifugation tubes filled with mTCM. The old medium was replaced by 12 ml fresh mTCM, and the spleens were squashed through a 40 µm cell strainer using the piston of a syringe. The cells were resuspended in 12 ml fresh mTCM and transferred into a new 12 ml tube followed by centrifugation for 5 min at 1500 rpm. The supernatant was discarded and the pellet was loosened before all red blood cells were lysed with 2 ml ACK lysis buffer (see Table 3.2) for 1.5 min. The lysis was stopped with 12 ml fresh medium and the cells were washed three times by repeated centrifugation and resuspension in 12 ml fresh mTCM. Prior to the following stimulation, the cells were filtered through a 40 µm cell strainer to get rid of any dead and fat cells and counted afterwards. For the specific activation of OT-1 splenocytes, the cells were specifically stimulated with anti-mCD3 (1 µg/ml), anti-mCD28 (0.1 µg/ml) antibodies, and the peptide SIINFEKL (1 µg/ml) in 24-well cell culture plates.

4.7.3 Adoptive T cell transfer

To determine the anti-tumor efficacy of TCR-transduced human T cells *in vivo*, 8-9 week-old NOD/SCID were inoculated with 5×10^6 human tumor cells (lymphoma BJAB cells i.v., breast carcinoma cells MCF7 i.p.). One day later, mice were randomly grouped (n=5-6) and received 5×10^6 TCR-transduced human PBMC in the tail vein. As a control, mice were treated with 5×10^6 GFP-transduced human PBMC. Mice were then monitored for signs of disease. Diseased mice were sacrificed and the organs of interest were dissected for further analysis (e.g. flow cytometry by anti-HLA-A2 antibody staining).

The OT-1 mice model was also used for PET/CT imaging. Therefore, 8 week-old NOD/SCID mice were treated with $5x10^6$ OVA^+ E.G7 and OVA^- EL-4 tumor cells s.c. at the left and right

flank, respectively. The tumor-bearing mice were treated with $5x10^6$ stimulated OT-1 splenocytes i.p. 6 days after the inoculation of tumor cells and monitored for tumor growth by using PET/CT imaging at indicated time points (see section 4.7.5).

4.7.4 Dissection

The mice were euthanized with CO_2 or isoflurane in a closed cabinet and analyzed for tumor-manifestations or organ abnormalities. The organs of interest were further extracted. For immunohistochemistry analysis, the selected organs were embedded in Tissue-Tek O.C.T. medium and frozen at -80°C for long-term storage. For flow cytometric analysis, single cell suspensions were generated by screwing the organs through 0.45 m cell strainers using syringe pistols followed by lysis of red blood cells using ACK lysis buffer as mentioned above (see section 4.7.2). Prior to FACS staining, the cells were blocked with mouse Fc-Blocking antibody for 10 min on the ice, and cells were stained with antibodies and multimers as previously described (see section 4.3.1).

4.7.5 Non-invasive molecular Imaging

A micro PET/CT (microPET/CT) was used to investigate non-invasively the localization and growth of tumors in mice. PET signal was generated principally from the annihilation of positrons emitted from tracers with electrons in the circumstance. The 2 annihilation gamma rays are emitted in an opposite direction and detected in coincidence as a signal. The combination of functional imaging by PET scans and anatomic imaging by CT scans in PET/CT imaging system allows the exact 3D anatomic localization of tracers *in vivo*.

For PET/CT imaging, mice were starved for 12 h before imaging and approximately 12 MBq of ^{18}FDG were administrated i.v. at indicated time points. ^{18}FDG has a half-time of 110 min and is an analog of glucose. It is quickly metabolized in the metabolically active tumor-tissue. 45 min after administration of ^{18}FDG, the mice were anesthetized by using 2.5% isoflurane and monitored for 15 min in the microPET/CT scanner (Siemens AG, Erlangen). The acquired data were reconstructed by using a filtered back projection and normalized and corrected for randoms, dead time and decay. The obtained PET/CT images were analyzed by using Siemens Inveon Research Workplace software (Siemens AG, Erlangen).

5 Results

5.1 Functional investigation of allorestricted TCRs *in vitro*

5.1.1 TCR repertoire in generated peptide-specific allorestricted T cells

Allorestricted T cell lines or clones with specificity for $HER2_{369}$ or FMNL-PP2 were previously isolated by stimulation of HLA-$A2^-$ CD8 T cells from healthy donors with HLA-$A2^+$ $HER2_{369}$ or FMNL1-PP2 pulsed T2 cells followed by sorting with HLA-A2-peptide-specific multimers (Schuster et al. 2007; Weigand 2007). However, most of the isolated T cell lines or clones could not be cultivated *in vitro* for a long period of time. In my Master's thesis the $HER2_{369}$-specific TCRs were further identified by PCR-analysis using cDNA templates, which were generated from total RNA extracted from the T cell lines or clones, and 35 Vα and 37 Vβ variable segment-specific primers (Liang 2007). The identified variable and joining region segments as well as CDR3 amino acid sequences of the TCRs from the $HER2_{369}$-specific T cell clones HER2-1 to HER2-4 are listed in Table 5.1a and 5.1b and are designated according to the name of clone they are derived from. This study focuses on the characterization of the $HER2_{369}$-specific TCRs. TCR R6C12 and JG-9 were isolated from T cell clones with specificity for $GP100_{209}$ (Morgan et al. 2003) and CMV $pp65_{495}$ peptide (Schub et al. 2009), respectively, with HLA-A2 restriction and were used as control TCRs in this study. TCR SK22 was isolated from allorestricted T cell clone SK22 with specificity for FMNL-1-PP2 in the context of HLA-A2 (Schuster et al. 2007), and was functionally investigated *in vitro* by Ingrid Schuster in her PhD project (Schuster 2008). In this study TCR SK22 was further analyzed in *in vivo* experiments. As shown in Table 5.1a and 5.1b TCR α-chains derived from diverse Vα families. In contrast, the TRBV12 family was predominantly used within $HER2_{369}$-specific TCR β-chains. Three of four $HER2_{369}$-specific TCRs used the same variable β chain segment but different CDR3 regions.

Table 5.1a Variable and joining region family affiliation and CDR3 sequences of α-chains of $HER2_{369}$-specific and control TCRs.

Clone	Specificity	TRAV	TRAJ	CDR3	Reference
HER2-1	HER2 (369)	19*01	24*02	CALYTTDSWGKLQF	FJ795357[#]
HER2-2	HER2 (369)	27*01	20*01	CAGVPSNDYKLSF	FJ795359
HER2-3	HER2 (369)	38-1*01	28*01	CAFIDSGAGSYQLTF	FJ795361
HER2-4	HER2 (369)	21*01	20*01	CAVRPQNDYKLSF	FJ795363
R6C12	GP100(209)	41*01	54*01	CAASLIQGAQKLVF	Morgan et al. 2003
JG-9	pp65(495)	35*02	50*01	CAGPMKTSYDKVIF	FJ795368
SK22	FMNL1-PP2	38-2/DV8*01	41*01	CAYENSGYALNF	FJ795365

Table 5.1b Variable and joining region family affiliation and CDR3 sequences of β-chains of $HER2_{369}$-specific and control TCRs.

Clone	Specificity	TRBV	TRBJ	TRBD	CDR3	Reference
HER2-1	HER2 (369)	12-3*01	2-3*01	-	CASSFVLGDTQYF	FJ795358[#]
HER2-2	HER2 (369)	12-3*01	2-7*01	2*02	CASSPPLGSGIYEQYF	FJ795360
HER2-3	HER2 (369)	7-8*01	2-7*01	2*01	CASSLAADEQYF	FJ795362
HER2-4	HER2 (369)	12-3*01	2-1*01	2*01	CASSSWTSGDEQFF	FJ795364
R6C12	GP100(209)	12-3*01	2-1*01	2*01	CASSPGGNEQFF	Morgan et al. 2003
JG-9	pp65(495)	12-4*01	1-2*01	1*01	CASSSANYGYTF	FJ795367
SK22	FMNL1-PP2	27*01	2-5*01	2*01	CASSFLGETQYF	FJ795366

[#] indicated the corresponding reference sequence number in NCBI database;

5.1.2 Expression of retrovirally transferred TCRs in recipient cells

The isolated $HER2_{369}$-specific allorestricted TCRs were cloned into the retroviral vector pMP71 as described in section 4.1.2. The pMP71 retroviral constructs containing control TCR R6C12 and TCR JG-9 were directly obtained from the Morgan and the Moosmann group, respectively (Morgan et al. 2003; Schub et al. 2009). The TCR retroviral constructs were transferred into J76CD8 cells and PBMC for functional characterization. J76CD8 is a TCR-deficient T cell hybridoma. Since there is no competition with endogenous TCR chains in J76CD8 cells, the expression of the transduced TCRs could be well investigated in these cells. All four $HER2_{369}$-specific TCRs as well as control TCR R6C12 were highly expressed in J76CD8 4 days after transduction and recognized specific-multimers but not control multimers (Figure 1A). However, the functionality of the TCRs cannot be investigated in this

cell line because J76CD8 cells do not secrete cytokines and are not able to kill target cells. Therefore, the TCRs were further retrovirally transferred into PBMC and functionally investigated. TCR-transduced PBMC were also used for adoptive transfer in a mouse model later on. As shown in Figure 5.1B, all TCR-transduced PBMC showed a detectable population with specific multimer staining, although the proportions of multimer$^+$ cells were obviously lower than those in J76CD8. TCR HER2-1 was the only $HER2_{369}$-specific TCR demonstrating a significant proportion of multimer$^+$ cells in the CD8$^-$ population, which indicates CD8-independency of $HER2_{369}$-multimer recognition by TCR HER2-1. In addition, the expression of TRBV12 β-chain in both CD8$^-$ and CD8$^+$ populations was significantly enhanced in TCR-transduced PBMC compared to the non-transduced counterpart, indicating high transduction efficiency. However, the percentage of specific TRBV12$^+$ cells was significantly higher than that of specific multimer+ cells, revealing that the transduced TCR α- and β-chains might be not well assembled to be recognized by specific multimer or that mispairing of the transduced TCR β(α)-chain with endogenous TCR α(β)-chain might occur.

5.1.3 Functional reactivity of $HER2_{369}$-specific TCR-transduced PBMC

The specific peptide recognition and anti-tumor reactivity of $HER2_{369}$-specific TCR-transduced PBMC *in vitro* was determined by measurement of IFNγ cytokine-release after stimulation with peptide-pulsed T2 cells and diverse tumor cell lines. $HER2_{369}$-specific and control TCR R6C12-transduced PBMC showed cytokine release in response to T2 cells pulsed with the specific peptide in a dose-dependent manner and exerted an intermediate functional avidity, as measured by the half-maximal cytokine-release concentration (EC_{50}), in a range from 10^{-6} M and 10^{-8} M (Figure 5.2 A and B). TCR-transduced PBMC did not recognize T2 cells pulsed with a set of irrelevant peptides (Figure 5.2 C). In respect to anti-tumor reactivity, the highest reactivity was observed for TCR HER2-3-transduced PBMC which significantly recognized the HER2/neu-expressing tumor cell lines MCF7, MDA-MB-231 and 624.38MEL. TCR HER2-1 showed marginal reactivity towards the HER2/neu$^+$ cell line SK-Mel 29 (Figure 5.2 D). In contrast, PBMC transduced with TCR HER2-2 and HER2-4 exerted no significant reactivity against the tested tumor cells when compared to GFP-transduced PBMC as a control. The PBMC transduced with the melanoma antigen-specific control TCR R6C12 showed high reactivity against 624.38MEL melanoma cells expressing GP100.

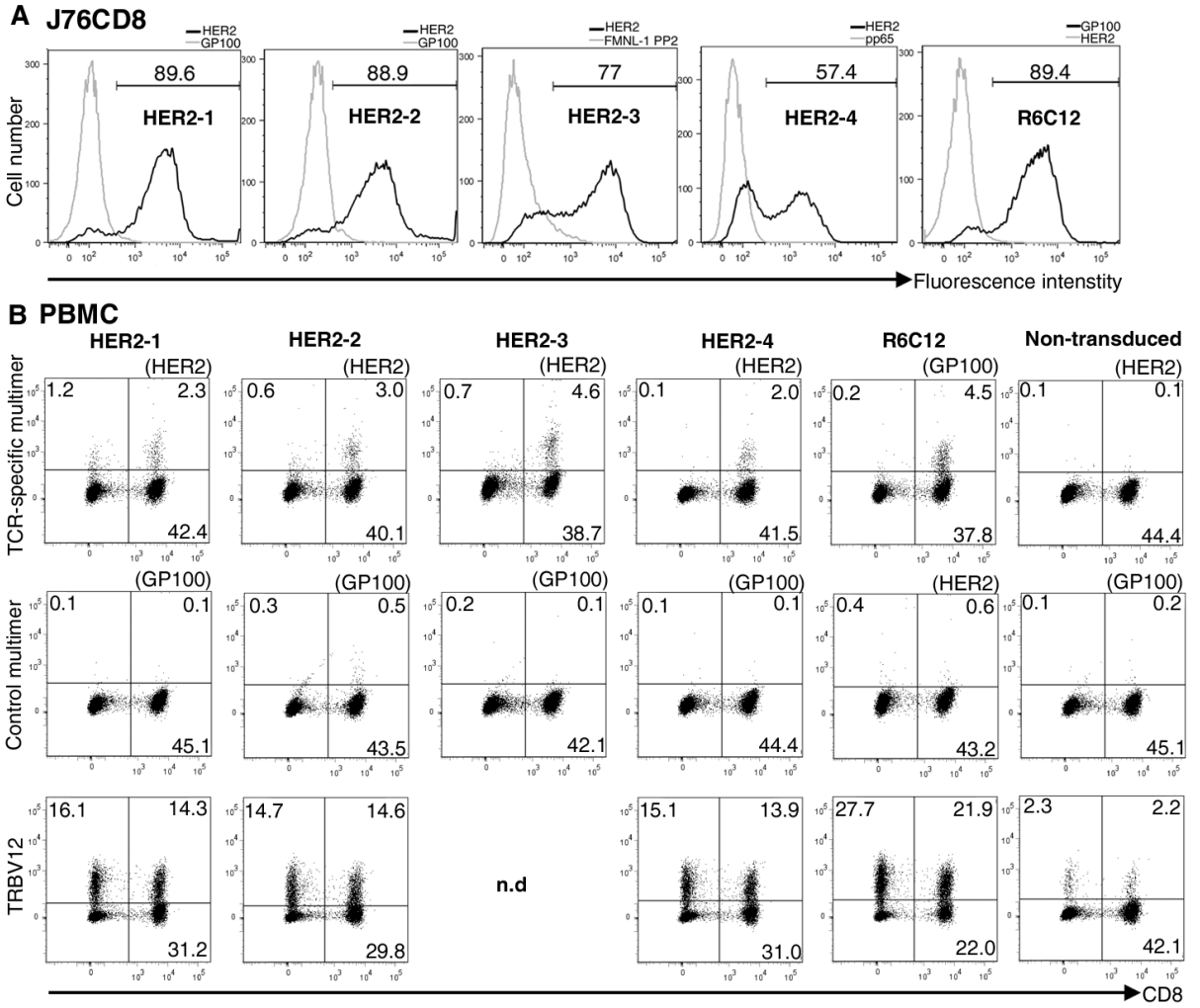

Figure 5.1 Transduced wildtype TCR chains were expressed in J76CD8 cells (A) and PBMC (B) determined by flow cytometry.

A. J76CD8 cells were stained with TCR-specific (black line) or control multimers 4 days after retroviral TCR transduction (grey line). The percentage of specific-multimer$^+$ cells is indicated. B. TCR-transduced PBMC were stained with TCR-specific (upper row) or control multimers (middle row) or β-chain TRBV12-specific antibody (lower row) in combination with anti-CD8 antibody 4 days after transduction. The proportion of the cell population in corresponding quadrant is shown in the respective corner of the dot plots.

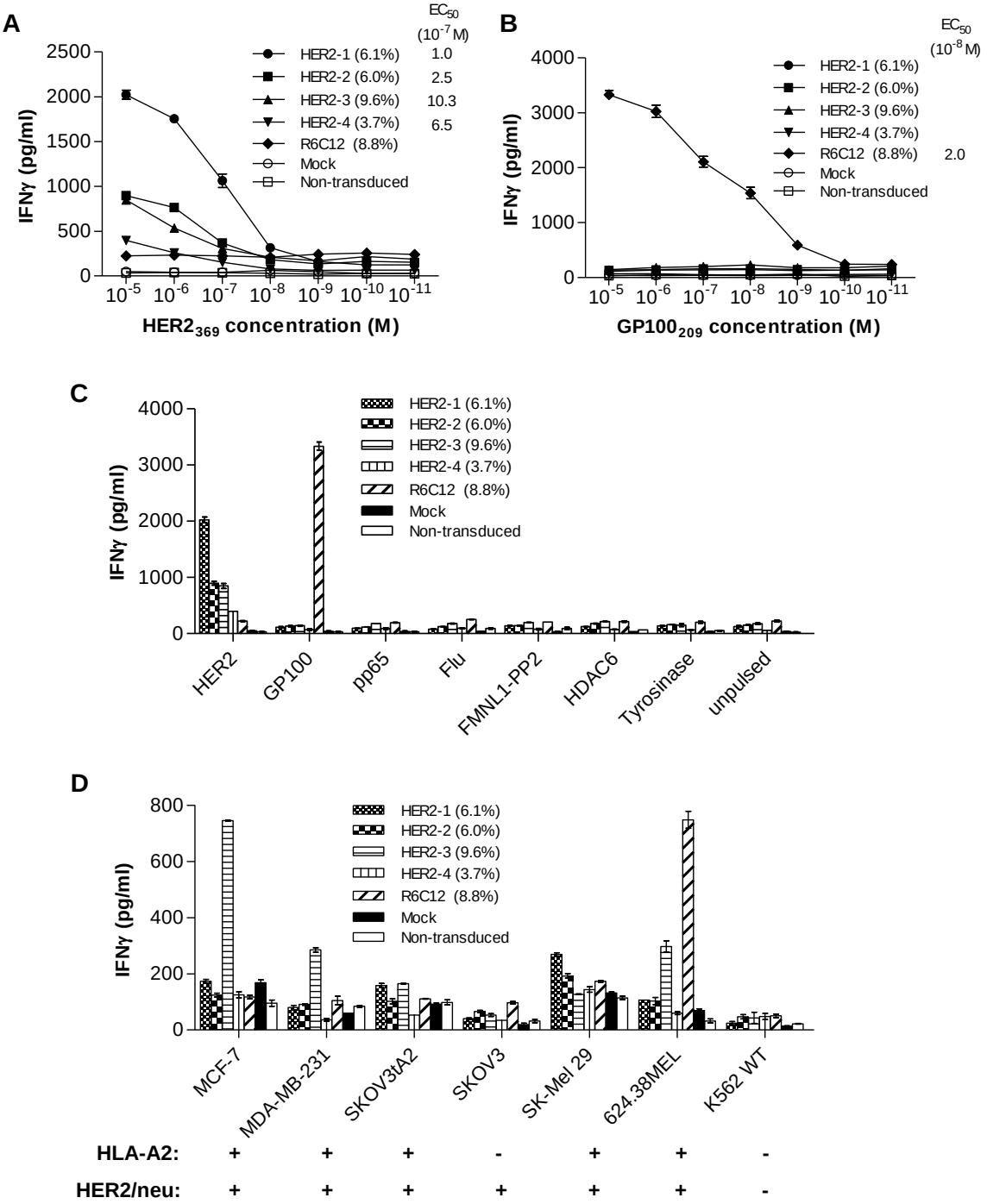

Figure 5.2 PBMC transduced with wildtype HER2-specific TCRs showed specific peptide recognition and anti-tumor reactivity.

(A) and (B). PBMC transduced with $HER2_{369}$-specific TCRs as well as control TCR R6C12 were stimulated 11 days post-transduction with T2 cells pulsed with peptide $HER2_{369}$ (A) or GP100 (B) at defined concentrations at an effector (E) : target (T) ratio of 5:1. Cell culture supernatants were analyzed for IFNγ release determined by IFNγ ELISA after 24 hours of co-cultivation. The half-maximum effective concentration (EC_{50}) is indicated. (C) Similarly, TCR-transduced PBMC were tested for their reactivity against T2 cells pulsed with either the specific peptide or a panel of irrelevant

peptides (see Table 3.3 in detail) in a concentration of 10^{-5} M at an E:T ratio of 5:1. (D) The reactivity of TCR-transduced PBMC against selected tumor cells was assessed in a similar way at an E:T ratio of 5:1. The tumor cell lines were treated with IFNγ (100 U/ml) 48 h prior to the stimulation assay in order to enhance the endogenous peptide presentation (Leggatt et al. 2002). The surface-expression profile of the tumor cells in respect of HLA-A2 and HER2/neu was determined by flow cytometry and is shown below the graph indicating presence (+) or lack (-) of expression. 624.38MEL melanoma cells naturally express GP100 and were used in this assay as a positive target for TCR R6C12-transduced PBMC. K562, a MHC I deficient leukemia cell line, is a target for nature killer cells (NK) and was used as control to evaluate non-TCR-dependent NK cell reactivity. The numbers in parenthesis indicate the proportion of specific-multimer$^+$ cells in the TCR-transduced bulk population. GFP-transduced (Mock) and non-transduced PBMC served as control. Standard deviations from triplicates are presented.

5.2 Functional investigation of cells transduced with modified allorestricted TCRs *in vitro*

Considering the relative low expression and anti-tumor reactivity of $HER2_{369}$-specific TCR-transduced PBMC, retroviral constructs containing the genes, which encode the $HER2_{369}$-specific TCRs with highest peptide-reactivity (TCR HER2-1, HER2-2 and HER2-3) as well as control TCR R6C12, were modified by murinization of the TCR constant regions (Cohen et al. 2006), linkage of β- and α-chain genes with a “self-cleaving” 2A sequence derived from picornavirus (P2A) (Leisegang et al. 2008) and codon optimization of the entire β-P2A-α genes (Scholten et al. 2006) to improve the correct pairing and expression of transduced α- and β-chains (details see section 4.1.3). PBMC transduced with modified TCRs were further investigated regarding TCR expression, peptide recognition and anti-tumor reactivity.

5.2.1 Expression of the modified TCRs in PBMC after retroviral transfer

As shown in Table 5.2, PBMC transduced with the modified TCRs showed significantly higher proportions of specific-multimer$^+$ cells 4 and 11 days after transduction compared to PBMC transduced with wildtype TCRs. Furthermore, the proportion of specific-multimer$^+$ cells correlated better with that of TRBV12$^+$ cells, which indirectly indicates improved pairing of the transduced TCR α-and β-chains.

Table 5.2 Proportions of specific-multimer$^+$ and TRBV12$^+$ PBMC 4 or 11 days after retroviral transduction with vector constructs containing either wildtype or modified TCR chain genes.

TCR		D4			D11
		Multimer$^+$ (%)	TRBV12$^+$ (%)	GFP$^+$ (%)	Multimer$^+$ (%)
HER2-1	WT	4.8	30.4		6.1
	Mod	14.1	19.9		15.6
HER2-2	WT	5.6	29.3		6.0
	Mod	8.8	17.3		16.2
HER2-3	WT	5.3	n.d.		9.6
	Mod	7.3	n.d.		20.3
HER2-4	WT	2.7	27.9		3.7
	Mod	n.d.	n.d.		n.d.
R6C12	WT	6.7	49.5		8.8
	Mod	9.1	21.5		13.8
Non transduced		0.2	4.5		0.3
Mock		n.d.	n.d.	31.3	n.d.

WT: wildtype, usage of vector constructs containing unmodified TCR α- or β-chain genes
Mod: modified, usage of bicistronic vector constructs containing TCR α- and β-chain genes with murinized constant regions and codon optimization
n.d.: not determined

5.2.2 Functional reactivity of modified TCR-transduced PBMC *in vitro*

PBMC transduced with vector constructs containing modified TCR genes were functionally investigated regarding functional avidity, peptide recognition and anti-tumor reactivity in a similar way as described for PBMC transduced with wildtype TCRs. As shown in Figure 5.3, PBMC transduced with the modified TCR HER2-1, HER2-2 as well as the control TCR R6C12 showed enhanced responsiveness to T2 cells pulsed with the specific peptide in respect to IFNγ secretion when compared to PBMC transduced with the respective wildtype TCR, although the functional avidity was not affected (Figure 5.3 A). PBMC transduced with the modified TCRs specifically recognized their cognate peptide and did not show significant reactivity towards diverse irrelevant peptides, similar to the PBMC transduced with the respective wildtype TCR (Figure 5.3 B). Furthermore, PBMC transduced with the modified TCR HER2-1 and control TCR R6C12 exhibited improved reactivity against tumor cell lines naturally expressing the tumor antigen (Figure 5.3 C). However, for TCR HER2-3-transduced

PBMC both specific peptide recognition and anti-tumor reactivity were not enhanced by TCR modification. PBMC transduced with this TCR showed reduced proliferation (data not shown) potentially suggesting self-reactivity of this TCR. PBMC transduced with modified TCR HER2-2 did not show significant reactivity against tumor targets, similar to the wildtpye TCR HER2-2-transduced PBMC. Taken together, the modification strategies improved the functional reactivity of TCR HER2-1, HER2-2 and control TCR R6C12 but not of TCR HER2-3.

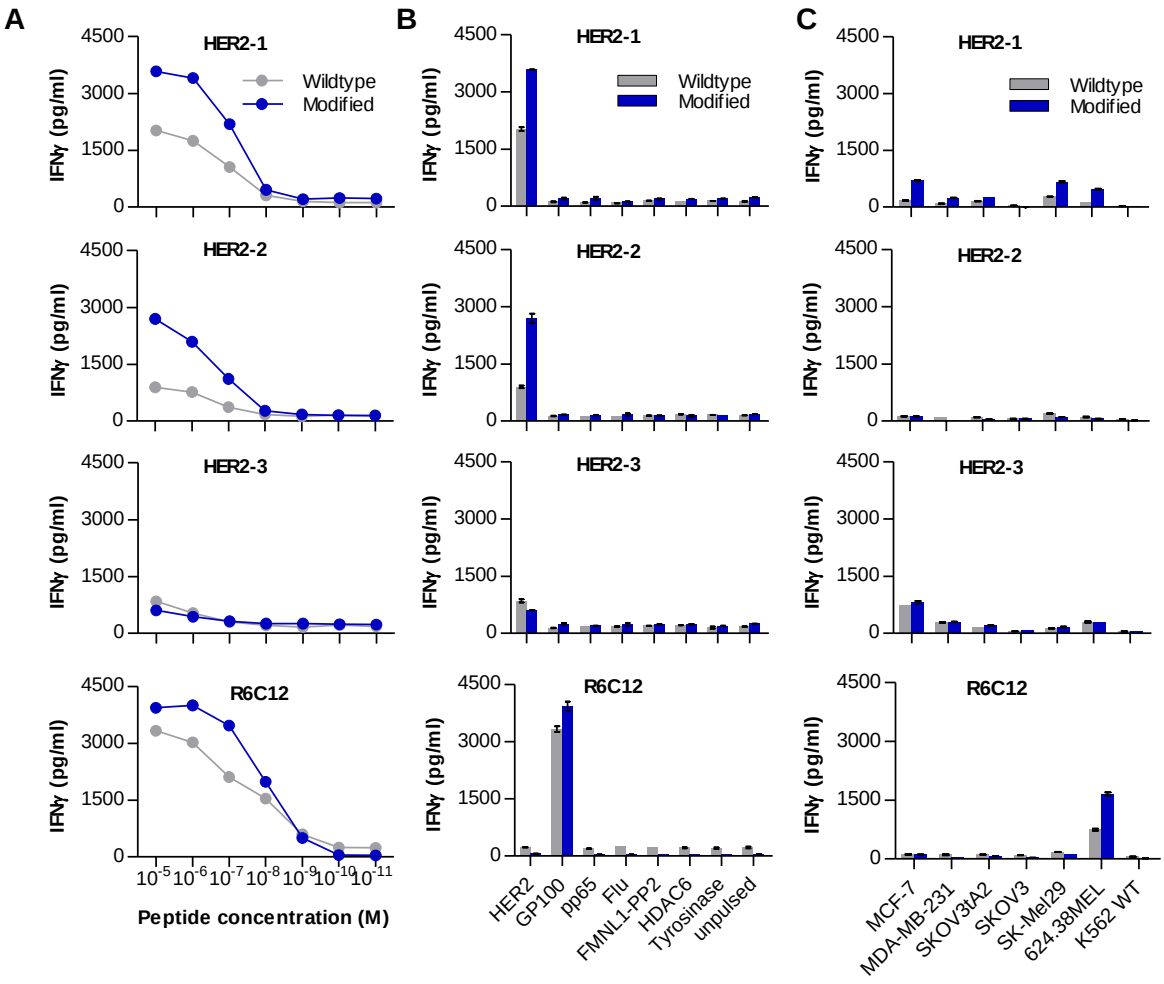

Figure 5.3 PBMC transduced with modified TCRs showed enhanced functionality with preserved peptide specificity.

PBMC transduced with either wildtype or modified TCR α- and β-chain genes were stimulated 11 days after transduction with diverse target cells for 24 h at an E:T ratio of 5:1. IFNγ secretion in the cell culture supernatants was then analyzed by using IFNγ ELISA. The proportions of multimer$^+$ cells in the bulk transduced population are listed in Table 5.2. Error bars represent standard deviations of triplicates. (A) TCR-transduced PBMC were stimulated with T2 cells pulsed with different concentrations of the specific peptide. $HER2_{369}$-peptide was used for stimulation of PBMC transduced

with TCR HER2-1, HER2-2 and HER2-3, while $GP100_{209}$-peptide was used for TCR R6C12-transduced PBMC. (B) TCR-transduced PBMC were tested against T2 cells pulsed with either their specific peptide or a panel of alternative peptides at a concentration of 10^{-5} M. (C) TCR-transduced PBMC were co-cultivated with selected tumor cell lines, which were treated with IFNγ (100 U/ml) 48 h prior to the stimulation assay. After 24 h co-culture, the amount of secreted IFNγ in the cell culture supernatants was measured by using IFNγ ELISA.

5.2.3 Functional reactivity of modified TCR-transduced $CD4^+$ and $CD8^+$ T cells

Although PBMC transduced with retroviral constructs containing the wildtpye or modified TCR chain genes of TCR HER2-2, HER2-3, and HER2-4, respectively, did not show substantial anti-tumor reactivity even after modification, PBMC transduced with the modified TCR HER2-1 showed specific peptide recognition and anti-tumor response suggesting this TCR to be a potential candidate for further investigations. Hence, TCR HER2-1 was functionally analyzed in more detail after transduction in $CD4^+$ and $CD8^+$ T cell subsets, and the modified TCR HER2-1 construct was used for further *in vitro* as well as *in vivo* analysis.

5.2.3.1 Specific peptide recognition by TCR-transduced $CD4^+$ and $CD8^+$ T cells

To assess peptide specificity of TCR HER2-1 in more detail, TCR HER2-1- transduced $CD4^+$ and $CD8^+$ T cells were tested against T2 cells pulsed with homologous peptides derived from the EGF receptor family proteins including HER1, HER3, and HER4. TCR HER2-1-transduced $CD4^+$ and $CD8^+$ T cells recognized $HER2_{369}$, but not $HER1_{364}$, $HER3_{356}$, or $HER4_{361}$, indicating high peptide specificity (Figure 5.4). In addition, I used $HER2_{369}$ analogous peptides containing alanine or threonine substitutions in each amino acid position. $CD4^+$ and $CD8^+$ T cells transduced with TCR HER2-1 showed a similar response pattern against the panel of point-mutated peptides, although $CD8^+$ T cells generally secreted high amounts of IFNγ compared to $CD4^+$ T cells (Figure 5.4). The exchange of most important amino acids with threonine resulted in dramatically reduced recognition by TCR HER2-1, whereas several exchanges with alanine were better tolerated. Exchange of the amino acid at positions 3 (phenylalanine), 6 (leucine) and 8 (phenylalanine) by either alanine or threonine nearly abolished peptide recognition by TCR HER2-1-transduced $CD4^+$ and $CD8^+$ T cells, indicating that these amino acids are most essential for TCR-peptide-MHC interaction.

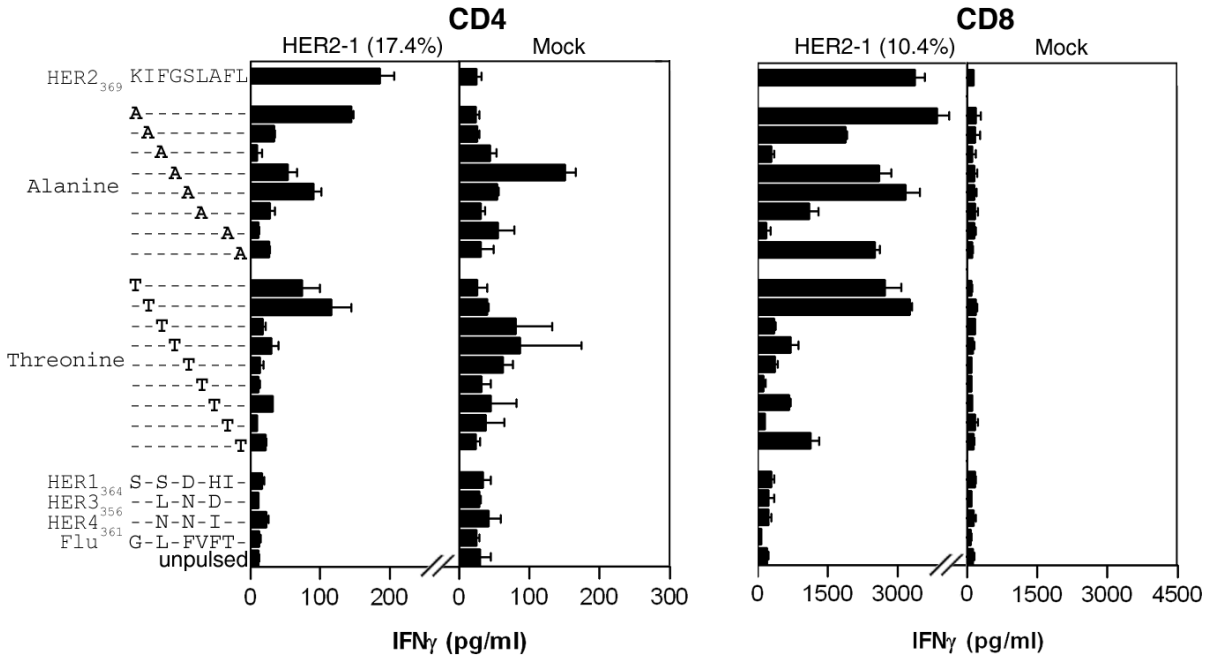

Figure 5.4 Recognition of $HER2_{369}$ analogous peptides containing substitutions of alanine or threonine by $CD4^+$ and $CD8^+$ T cell subsets transduced with TCR HER2-1.

$CD4^+$ and $CD8^+$ T cell subsets were enriched from PBMC by depleting other cell populations by using a CD4 or CD8 negative isolation kit (Invitrogen). Cells were then stimulated (see section 4.2.4) and retrovirally transduced with TCR HER2-1 as well as GFP according to the same protocol as used for PBMC. Eleven days after transduction, TCR HER2-1 transduced $CD4^+$ and $CD8^+$ T cells were tested for their reactivity against T2 cells pulsed with a panel of single amino acid analogues of $HER2_{369}$ peptide with alanine and threonine substitution as well as the homologous peptides derived from EGF receptor family proteins, such as HER1, HER3, and HER4, at a concentration of 10^{-5} M and an E:T ratio of 5:1. Unpulsed T2 cells and T2 cells pulsed with Flu-peptide serve as control targets. After 24 h stimulation, IFNγ release was analyzed by IFNγ ELISA. GFP-transduced $CD4^+$ and $CD8^+$ T cells (Mock) were used as controls. The proportion of HLA-A2-$HER2_{369}$-multimer positive cells at the time point of the IFNγ release assay is indicated in parentheses. Standard deviations of triplicates are shown.

5.2.3.2 Tumor recognition by TCR-transduced $CD4^+$ and $CD8^+$ T cell subsets

In order to analyze the recognition of naturally presented epitopes by TCR HER2-1, TCR HER2-1-transduced $CD4^+$ and $CD8^+$ T cells were stimulated with tumor cell lines expressing HER2/neu. TCR HER2-1-transduced $CD8^+$ T cells recognized C1R cells transfected with HLA-A2 and HER2/neu, but not wildtype C1R cells or C1R cells only transfected with HLA-A2, while the $CD4^+$ T cells transduced with TCR HER2-1 did not show significant reactivity against all tested C1R cells (Figure 5.5 A). Moreover, I sought to more specifically define the effector cell population recognizing the tumor targets. Flow cytometric analysis of the TCR HER2-1-transduced $CD8^+$ T cell subsets directly after stimulation with the HER2/neu- and

HLA-A2-expressing C1R cells revealed that the T cell subpopulation, which expressed the transduced TCR ($HER2_{369}$-multimer$^+$/TRBV12$^+$), was also positive for intracellular IFNγ (Figure 5.5 B). This suggests that T cells harboring the transduced TCR HER-1 represent the effector cell population. In detail, TCR HER2-1-transduced $CD8^+$ T cells preferentially recognized C1R transfected with HLA-A2 and HER2/neu, although they also showed reactivity against C1R transfected only with HLA-A2 to some extent. Moreover, HER2/neu$^+$ MCF-7 cells were specifically recognized by T cells expressing TCR HER2-1.

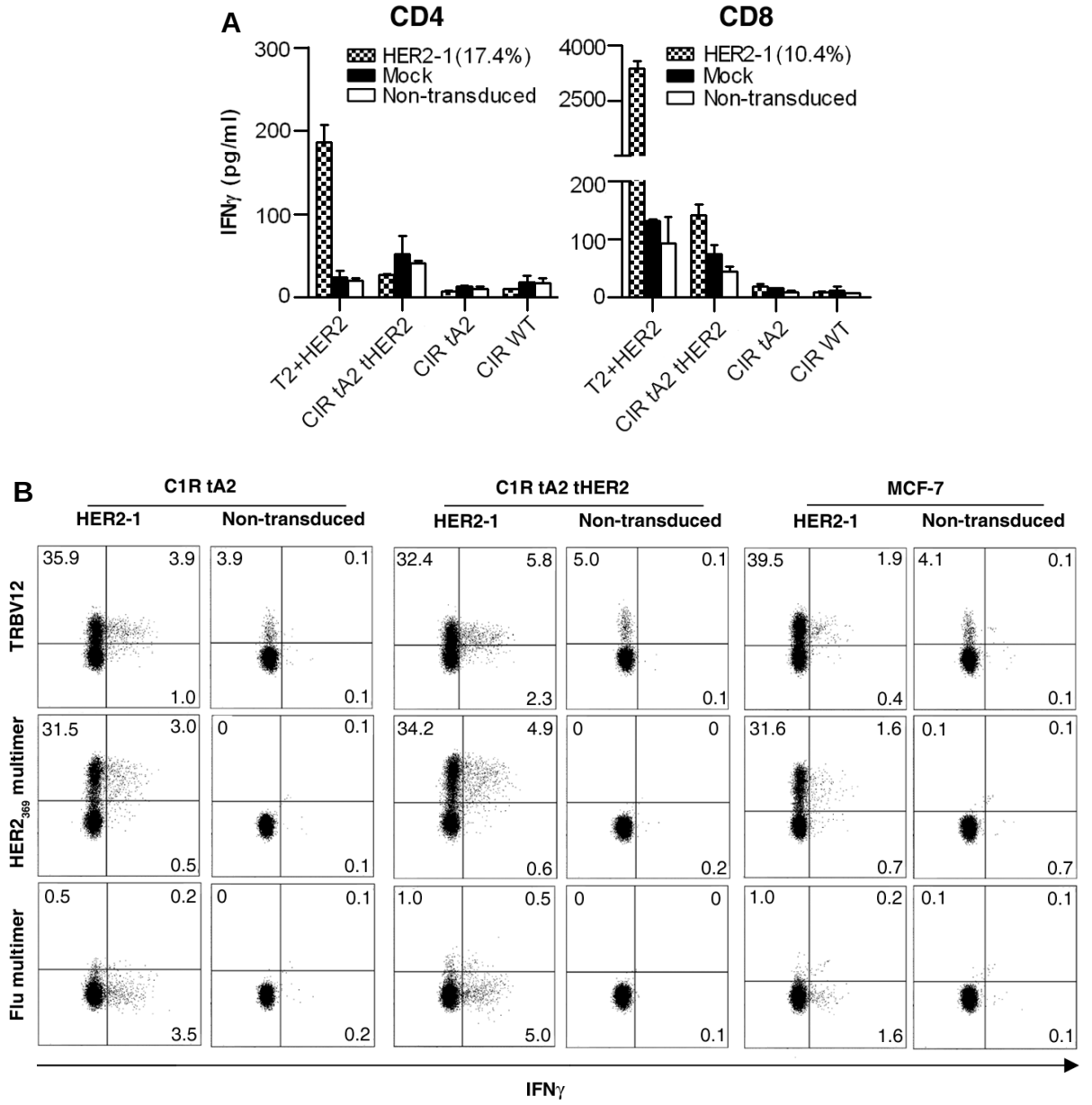

Figure 5.5 T cells transduced with the TCR HER2-1 recognized specifically tumor cells expressing HER2/neu.

(A). Eleven days after transduction, $CD4^+$ and $CD8^+$ T cells transduced with TCR HER2-1 were tested for their reactivity against tumor cell lines, such as wildtype C1R (C1R WT), C1R transfected either with HLA-A2 alone (C1R tA2) or in combination with HER2/neu (C1R tA2 tHER2), and HER2/neu$^+$

MCF-7 cells at an E:T ratio of 5:1. After 24 h stimulation, the IFNγ-response was determined by IFNγ ELISA. $HER2_{369}$-pulsed T2 cells were used as a positive control. GFP-transduced (Mock) and non-transduced $CD4^+$ and $CD8^+$ T cells served as controls for non-TCR-specific reactivity. The proportion of $HER2_{369}$-multimer$^+$ cells is indicated in parentheses. The standard deviations of triplicates are shown. (B) To define the effector cell population in response to tumor cell lines in the bulk culture by IFNγ-intracellular flow cytometry, $CD8^+$ T cells transduced with TCR HER2-1 were co-cultivated with target cells 12 days after transduction at an E:T ratio of 1:1 by using 200 000:200 000 cells in the presence of Brefeldin A (BFA), which is a Golgi inhibitor that inhibits the secretion of proteins (like IFNγ). After 6 h incubation, the cells were harvested and stained with anti-TRBV12 antibody (upper row), $HER2_{369}$-multimer (middle row) or Flu-multimer (lower row) in combination with anti-CD8 antibody followed by fixation, permeabilization, staining with anti-IFNγ antibody and flow cytometric analysis. Only $CD8^+$ cells were included into analysis in order to exclude target cells from analysis. The numbers represent the proportion of the respective cell population in the corresponding quadrant. Non-transduced T cells were used as control.

In addition to cytokine release, cytotoxicity of HER2-1 transduced $CD4^+$ and $CD8^+$ T cells against tumor cells was assessed by using a ^{51}Cr release assay. $CD8^+$ but not $CD4^+$ T cells transduced with the TCR HER2-1 showed significant cytolytic response to the HER2/neu$^+$ tumor cell line MCF-7 and C1R cells transferred with HLA-A2 and HER2/neu (Figure 5.6). Moreover, $CD8^+$ T cell exhibited preferential cytotoxicity toward C1R transferred with HLA-A2 and HER2/neu compared to C1R cells transferred with HLA-A2 alone. However, wildtype C1R cells were also slightly lysed.

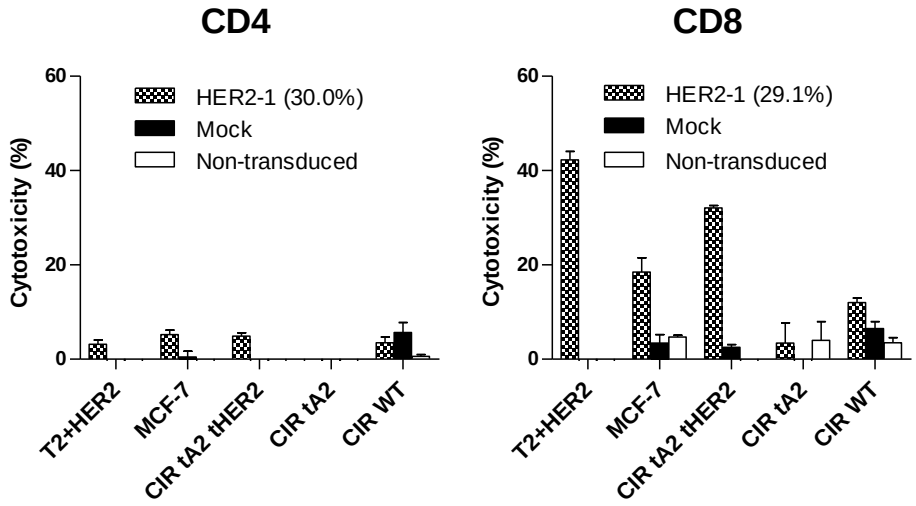

Figure 5.6 $CD8^+$ T cell subsets transduced with TCR HER2-1 showed specific cytolytic reactivity in response to T2 cells pulsed with $HER2_{369}$ peptide as well as HER2/neu-expressing HLA-$A2^+$ tumor targets.

Cytotoxicity of T cells transduced with TCR HER2-1 was determined by a standard ^{51}Cr-release assay. Twelve days after transduction, $CD4^+$ and $CD8^+$ T cells transduced with TCR HER2-1 were co-cultivated with ^{51}Cr-labeled target cells at an E:T ratio of 15:1 by using 30 000:2 000 cells. After 4 h incubation, cell-culture supernatants were harvested and the amount of released ^{51}Cr was measured by using a γ-counter. The relative cytotoxicity (%) of effector cells against target cells was calculated as described in the method section 4.6.5. The proportion of $HER2_{369}$-multimer$^+$ cells is indicated in brackets. GFP-transduced (Mock) and non-transduced $CD4^+$ and $CD8^+$ T cells were used as control. Standard deviations of triplicates are shown.

5.2.3.3 Alloreactivity of TCR-transduced $CD4^+$ and $CD8^+$ T cell subsets

As $CD8^+$ T cells transduced with TCR HER2-1 secreted low amounts of IFNγ in response to C1R cells transfected with HLA-A2 alone (Figure 5.5 B) and showed slightly cytotoxic reactivity against wildtype C1R (Figure 5.6), I investigated the alloreactivity of TCR HER2-1-transduced $CD4^+$ and $CD8^+$ T cells more deeply. A panel of HLA-$A2^+$ non-transformed cells including lung fibroblast, fetal cardiomyocytes, $CD4^+$ and $CD8^+$ T cells, and activated PBMC were used as target cells. The release of IFNγ by the TCR-transduced cells was analyzed by IFNγ ELISA. As shown in Figure 5.7, there was no enhanced IFNγ secretion of TCR HER2-1-transduced $CD4^+$ as well as $CD8^+$ T cells in response to all tested target cells. Activated PBMC was recognized by transduced as well as non-transduced PBMC, suggesting that a bystander cell population may be responsible for the observed background reactivity.

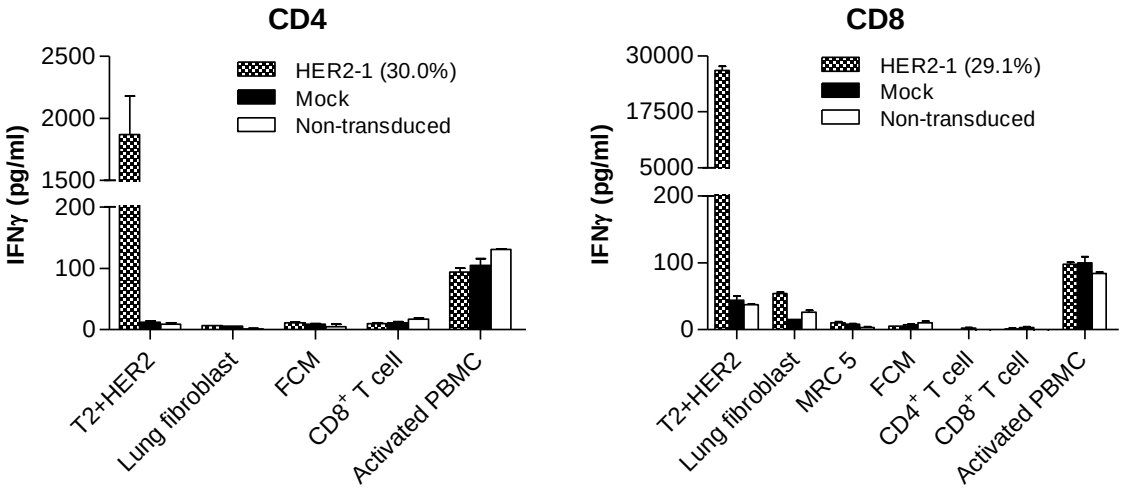

Figure 5.7 $CD4^+$ and $CD8^+$ T cell subsets transduced with TCR HER2-1 did not recognize lung fibroblast, fetal cardionmyocyte and PBMC derived cells with low expression of HER2/neu.
Eleven days after transduction, $CD4^+$ and $CD8^+$ T cells transduced with modified TCR HER2-1 were incubated with $HER2_{369}$-pulsed T2 cells (10^{-5} M) as a positive control as well as with a number of HLA-$A2^+$ cells with only low HER2/neu expression, such as lung fibroblasts, fetal cardiomyocytes (FCM),

negative-isolated $CD4^+$ and $CD8^+$ T cell subsets, and PBMC, which had been previously activated with OKT3 (50 ng/ml) and IL-2 (50 U/ml). Supernatants were investigated for IFNγ release by IFNγ ELISA after 24 h co-culture. The error bars represent deviations of duplicates. The propotion of $HER2_{369}$-$multimer^+$ cells is indicated in parentheses. GFP-transduced (Mock) as well as non-tranduced PBMC were used as negative controls.

5.2.4 Functional reactivity of modified TCR-transduced T cell clones

In order to further exclude background and bystander reactivity of non-transduced effector cells, $CD8^+$ T cell clones expressing the transduced TCR HER2-1 were isolated by limiting dilution. Growing T cell clones were analyzed for expression of TCR HER2-1 by multimer staining. Two TCR HER2-1-positive clones ($HER2_{369}$-$multimer^+$ T cell Clone 1 and Clone 2) as well as one TCR HER2-1-negative T cell clone ($HER2_{369}$-$multimer^-$ T cell Clone 3) were functionally investigated regarding peptide recognition and anti-tumor reactivity. Both positive T cell clones demonstrated IFNγ secretion in response to $HER2_{369}$-pulsed T2 cells and C1R cells transfected with HLA-A2 and HER2/neu but no reactivity was detected in response to T2 cells pulsed with the control peptide Flu, wildtype C1R cells as well as C1R cells transfected with HLA-A2 alone, indicating $HER2_{369}$-specific recognition by TCR HER2-1 (Figure 5.8 A). Moreover, Multi-plex assays were performed in order to analyze the spectrum of cytokines released by the TCR HER2-1-transduced T cell clones in response to C1R cells transfected with HLA-A2 and HER2/neu or HLA-A2 alone. Both positive T cell clones secreted preferentially multiple cytokines including IL-2, IL-4, IL-5, IL-13, GM-CSF, and IFNγ in response to $HLA\text{-}A2^+$ C1R cells expressing HER2/neu (Figure 5.8 B). IL-10, an anti-inflammatory cytokine, was secreted to a similar extent by the two positive clones as well as the negative control clone. Interestingly, T cell Clone 1 exerted relative low amounts of IFNγ and IL-2 but high amounts of cytokines such as IL-4, IL-5, and IL-13 in response to HER2/neu-expressing $HLA\text{-}A2^+$ C1R cells when compared to T cell Clone 2, which demonstrated a different cytokine secretion pattern.

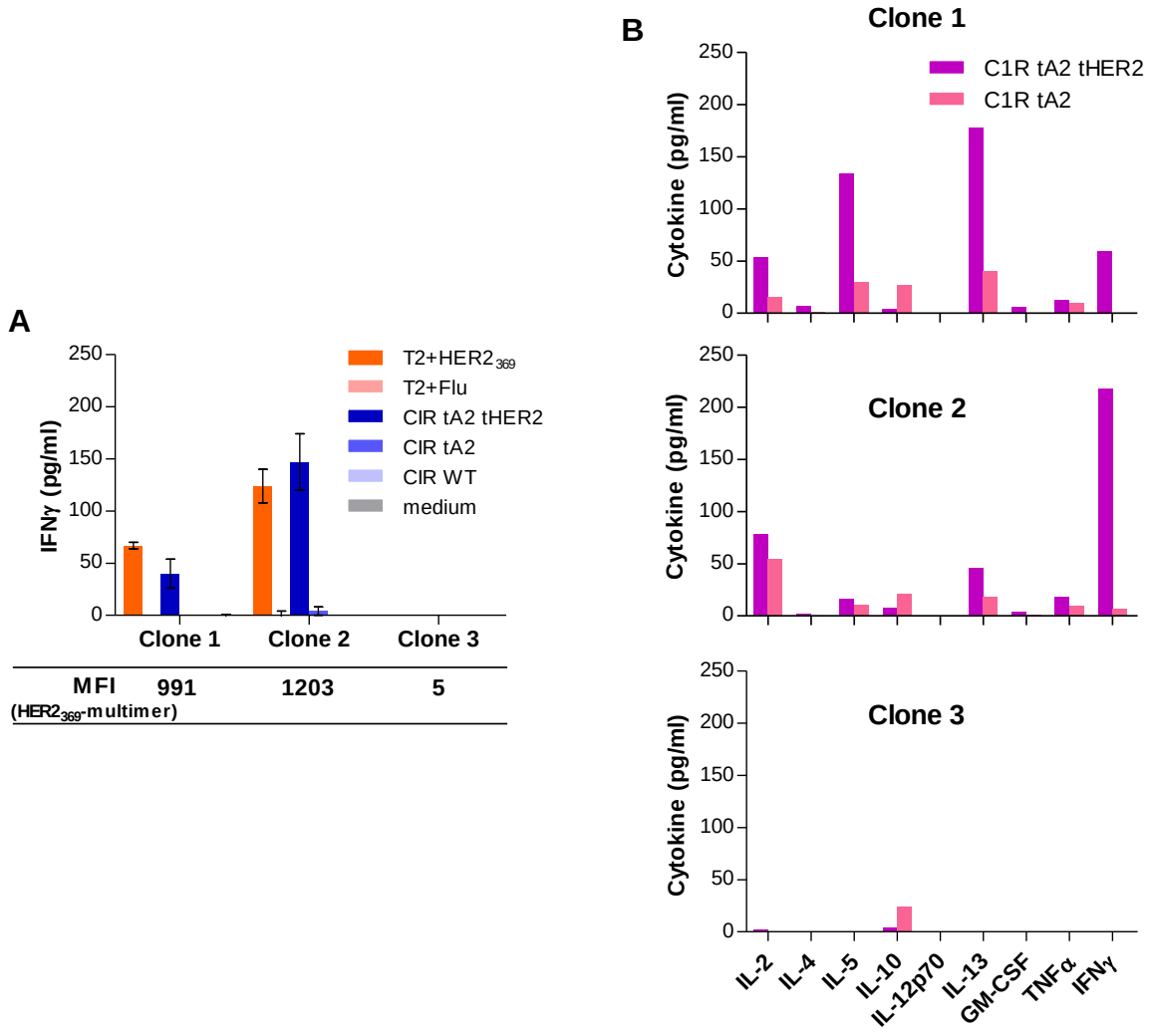

Figure 5.8 T cell clones transduced with TCR HER2-1 showed specific peptide recognition, anti-tumor reactivity, and distinct patterns of cytokine release.

TCR HER2-1-transduced $CD8^+$ T cells were cloned by limiting dilution 12 days after transduction as described in the method section 4.2.5. Growing T cell clones were analyzed for specific TCR expression by multimer-staining and flow cytometric analysis. (A). Two positive T cell clones with 99% purity, Clone 1 and Clone 2, were tested against T2 cells pulsed with peptides at a concentration of 10^{-5} M as well as selected tumor cells at an E:T ratio of 1:5 by using 2 000 : 10 000 cells. Clone 3 did not exhibite $HER2_{369}$-multimer binding and was used as a negative control. The mean fluorescence intensity (MFI) of $HER2_{369}$-multimer staining is indicated. After 24 h stimulation, cell culture supernatants were analyzed for IFNγ release by IFNγ ELISA. Deviations of duplicates are shown. (B). The supernatants were additionally investigated for the presence of multiple cytokines by using Multi-cytokine Bio-plex kit (BioRad, Munich).

5.3 Functional investigation of chimeric TCRs *in vitro*

A predominant usage of the TRBV12 chain in $HER2_{369}$-allorestricted TCR repertoire was observed (Table 5.1b). It has been previously reported, that restricted usage of variable chains is involved in detection of a defined antigen (Sensi et al. 1993; Dietrich et al. 2003) and mixed TCR α- and β-chain combinations derived from diverse TCRs with a defined antigen-specificity showed again the defined antigen-specificity (Heemskerk et al. 2003). I therefore sought to investigate the capability of chimeric combinations of TCR α- and β-chains derived from different TCRs to form TCRs with specificity for $HER2_{369}$. The diverse chimeric TCR combinations were retrovirally transferred into recipient cells such as J76CD8 cells and PBMC and further investigated in aspects of specific-multimer binding, functional avidity and anti-tumor response.

5.3.1 Expression of chimeric TCRs in recipient cells

Diverse combinations of retroviral vectors containing single α- and β-chain genes derived from TCRs with different specificities (Table 5.1a and 5.1b) were co-transfected into the packaging cell line to produce retrovirus harboring chimeric TCR genes. Thereafter, J76CD8 cells, which lack the expression of endogenous TCRs, and PBMC were retrovirally transduced. Firstly, the transduced J76CD8 cells were stained with $HER2_{369}$-multimer as well as control multimer and analyzed by flow cytometry for specific binding. The chimeric TCRs were expressed on the cell surface as shown by positive staining for anti-CD3 and anti-TCRαβ antibody (data not shown), suggesting that mispairing of TCR α- and β-chains from different TCRs occurs easily. Interestingly, mixed TCRs containing the α-chain from TCR HER2-2 (HER2-2α) in combination with different β-chains derived from the TRBV 12 family recognized $HER2_{369}$-multimer (Table 5.3). Two of those β-chains were derived from TCRs with other specificities than $HER2_{369}$, namely from $GP100_{209}$-specific (R6C12) and CMV-$pp65_{495}$-specific (JG-9) TCR. However, chimeric TCRs did not bind control Flu-multimers or the specific-multimers of the original TCRs, $GP100_{209}$-multimers or CMV-$pp65_{495}$-multimers, respectively (data not shown), indicating $HER2_{369}$–specific recognition by chimeric TCRs. In contrast, $HER2_{369}$-specific recognition was not observed using HER2-2α in combination with β-chains from other families such as TRBV7 (HER2-3β) and TRBV27 (SK22β).

Table 5.3 Specific $HER2_{369}$-multimer staining after transduction of chimeric TCR chain combinations into J76CD8 cells.

		BV						
	$HER2_{369}$ multimer^{+}	**HER2-1** 12-3*01	**HER2-2** 12-3*01	**HER2-3** 7-8*01	**HER2-4** 12-3*01	**R6C12** 12-3*01	**JG-9** 12-4*01	**SK22** 27*01
AV	**HER2-1** 19*01	+	-	-	n.d.	-	n.d.	n.d.
	HER2-2 27*01	+	+	-	+	+	+	-
	R6C12 41*01	-	-	n.d.	n.d	-	n.d.	n.d.

\+ specific $HER2_{369}$-multimer recognition
\- no $HER2_{369}$-multimer recognition
n.d. not determined

The chimeric TCRs containing HER2-2α in combination with diverse β-chains derived from the TRBV12 family resulting in $HER2_{369}$-multimer recognition were further retrovirally transferred to PBMC. PBMC transduced with HER2-2α in combination with TRBV12 β-chains derived from TCRs with diverse specificities showed the identical multimer-recognition pattern as in J76CD8 cells: PBMC transduced with those chimeric TCRs recognized $HER2_{369}$-multimers but not control $GP100_{209}$-multimers or specific-multimers of original TCRs, $GP100_{209}$-multimers or CMV-$pp65_{495}$-multimers, respectively (Figure 5.9 A). Moreover, transduction of PBMC with single TCR α-chain HER2-2α alone resulted in a significant population specifically binding $HER2_{369}$-multimers. This $HER2_{369}$-multimer positive population expressed mainly TRBV12 chain as indicated by staining with anti-TRBV12 antibody 4 and 12 days after transduction, respectively (Figure 5.9 B). Taken together, these data revealed that HER2-2α is a dominant TCR α-chain specially recognizing $HER2_{369}$ peptide in combination with diverse β-chains from the TRBV12 family. PBMC transduced with HER2-2α in combination with β-chains from TCR HER2-4 (HER2-4β) or R6C12 (R6C12β) contained a $CD8^{-}$ population with specific $HER2_{369}$-multimer binding (Figure 5.9 A), while this was not observed in PBMC transduced with the original TCR chain combination (Figure 5.1 B), suggesting CD8 independency of multimer-binding of these two chimeric TCRs. Chimeric TCRs composed of HER2-2α and HER2-1β (HER2-2α/HER2-1β) or R6C12β (HER2-2α/R6C12β) were further modified by using the TCR optimization strategies mentioned above (see section 5.2) resulting in efficient $HER2_{369}$-multimer binding after transduction in PBMC (Figure 5.10).

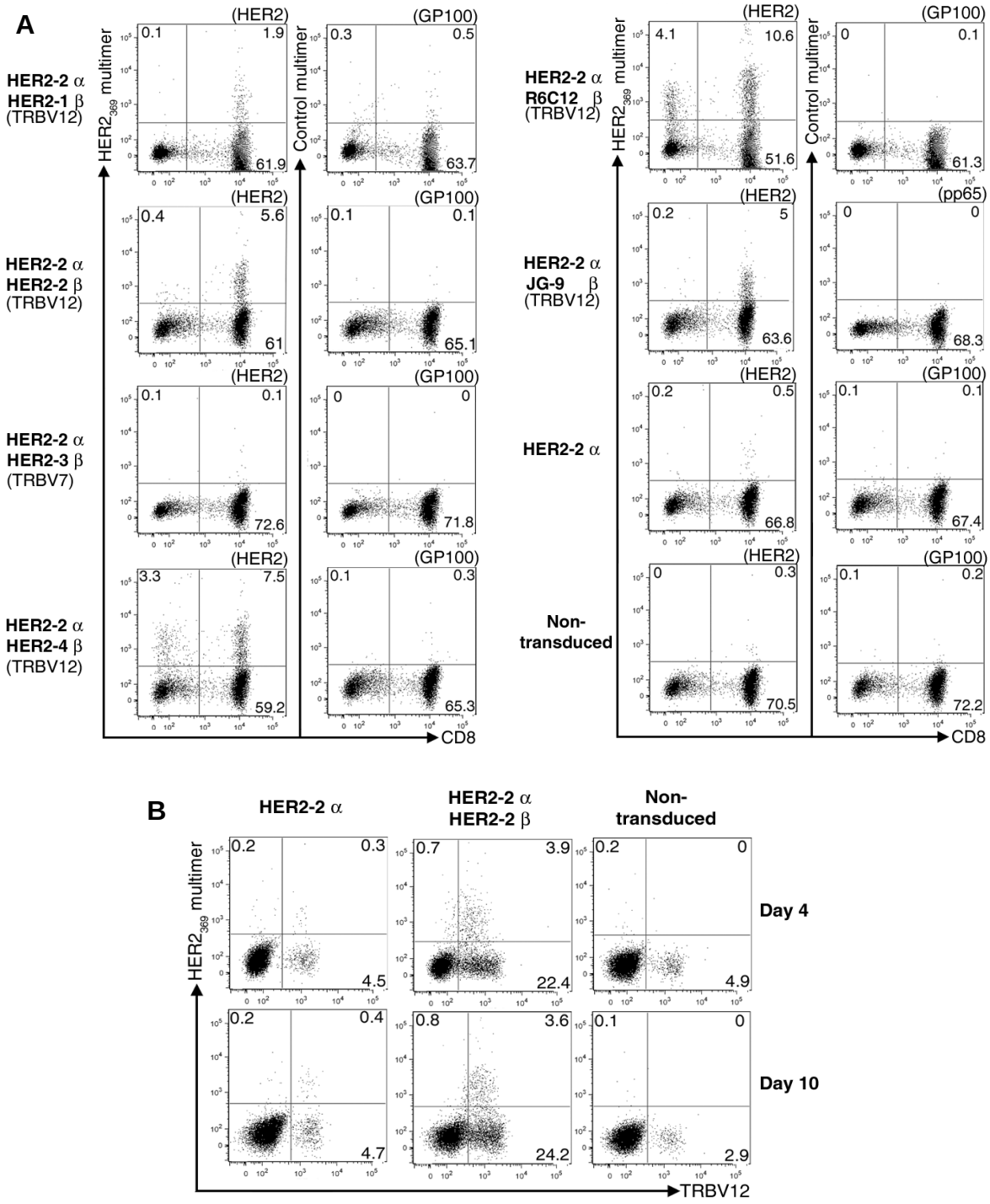

Figure 5.9 PBMC transduced with the $HER2_{369}$-specific TCR α-chain HER2-2α alone or in combination with diverse TRBV12 chains derived from TCRs with different specificities showed specific recognition of $HER2_{369}$-multimer.

(A). PBMC transduced with different TCR α- and β-chain combinations or single HER2-2α chain were stained with $HER2_{369}$-multimers or control multimers in combination with aniti-CD8 antibody 4 days after transduction and analyzed by flow cytometry. The control multimer used is indicated in brackets above the graph. The proportion of cells in the corresponding quadrant is indicated. (B). PBMC

transduced with single HER2-2α chain were stained with $HER2_{369}$-multimer and anti-TRBV12 antibody 4 days (upper row) and 10 days (lower row) after transduction, respectively. HER2-2αβ-transduced and non-transduced PBMC served as positive and negative control, respectively. The numbers represent the proportion of cells in the respective quadrant.

5.3.2 Functional reactivity of chimeric TCR-transduced PBMC

Since chimeric TCRs containing HER2-2α in combination with TCR β-chains derived from the TRBV12 family were expressed on the cell surface and bound specifically to $HER2_{369}$-multimer after transduction in PBMC, I further investigated the functionality of PBMC transduced with the chimeric TCRs focusing on peptide recognition, functional avidity and anti-tumor reactivity by IFNγ-response.

5.3.2.1 Peptide recognition by chimeric TCR-transduced PBMC

PBMC transduced with diverse combinations of chimeric TCR were investigated for their reactivity against T2 cells pulsed with $HER2_{369}$ and a panel of irrelevant peptides, respectively. The TCR combination HER2-2α/HER2-4β and HER2-2α/R6C12β showed enhanced IFNγ secretion in response to $HER2_{369}$-pulsed T2 cells compared to the original TCR HER2-2 and HER2-4 (Figure 5.10 A and 5.2 A). Moreover, the functional avidity of HER2-2α/HER2-4β was one log higher compared to the original TCR combinations HER2-2 and HER2-4 (Figure 5.10 B). However, this chimeric combination resulted not only in high $HER2_{369}$-peptide specificity but also in an increased IFNγ-secretion in response to T2 cells pulsed with alternative peptides or not pulsed at all (Figure 5.10 C). Two other TCR combinations, HER2-2α/HER2-1β and HER2-2α/JG-9β, recognized $HER2_{369}$ peptide only at a high peptide concentration (Figure 5.10 A and C), although both chimeric TCRs showed positive $HER2_{369}$-multimer binding (Figure 5.9 A). The TCR combination containing HER2-2α/HER2-3β, which was not $HER2_{369}$-multimer$^+$, did not exert any IFNγ secretion in response to all peptides tested.

5.3.2.2 Anti-tumor reactivity of PBMC transduced with chimeric TCR combinations

Although PBMC transduced with the original TCR HER2-2 and HER2-4 showed no tumor recognition (Figure 5.2 D), PBMC transduced with mixed TCR combination HER2-2α/HER2-4β showed strong anti-tumor reactivity against HLA-$A2^+$ tumor cell lines as MCF7, MDA-MB-231, SKOV3tA2, SK-MEL-29, and 624.38MEL (Figure. 5.10 D). Whereas PBMC transduced with chimeric TCR combinations HER2-2α/HER2-1β, HER2-2α/R6C12β, or HER2-2α/JG-9β showed positive $HER2_{369}$-multimer staining, they did not demonstrate significant anti-tumor reactivity when compared to mock- or non-transduced PBMC.

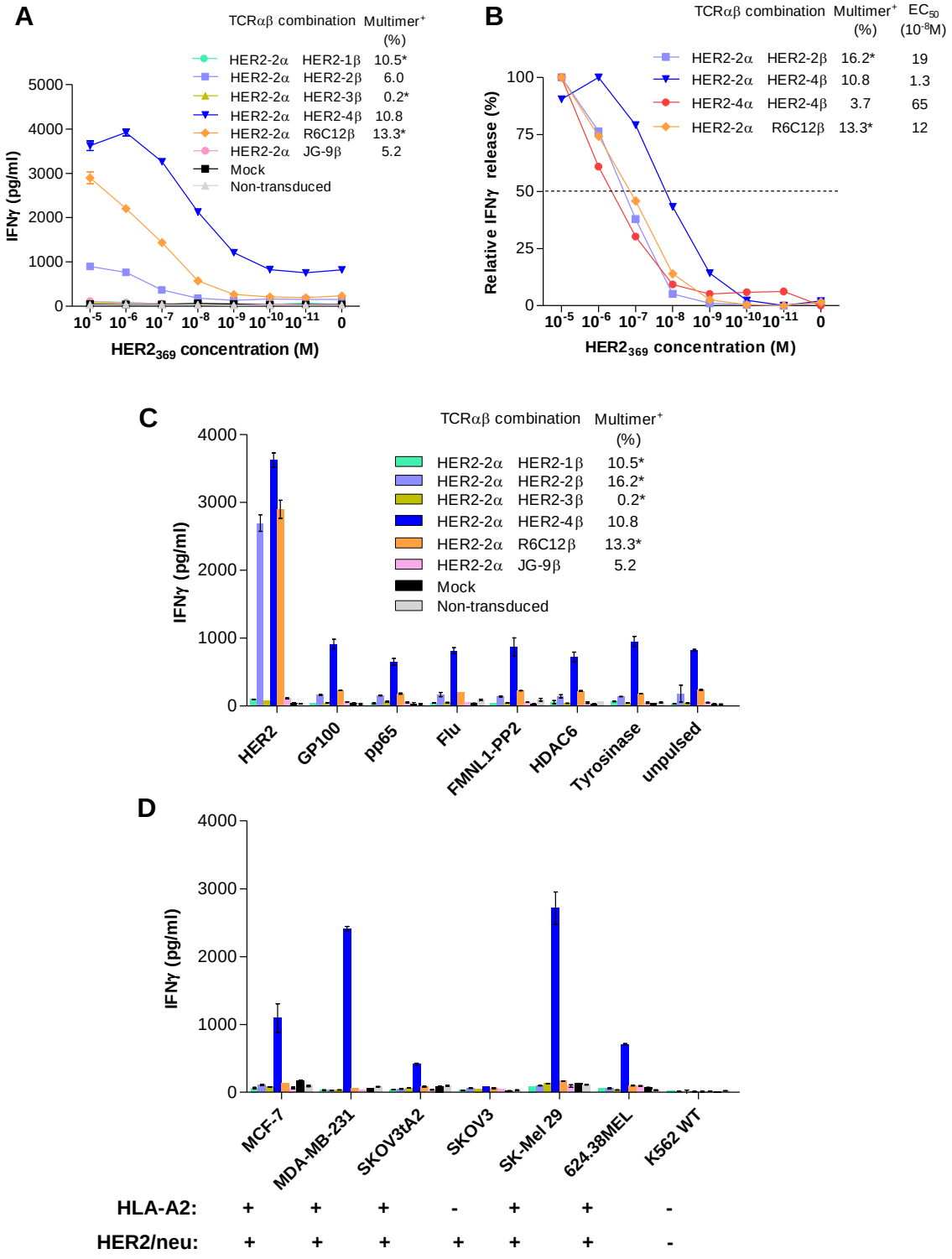

Figure 5.10 Transduction of PBMC with chimeric TCR combinations resulted in specific peptide recognition, enhanced functional avidity and anti-tumor reactivity.

PBMC transduced with diverse chimeric TCR combinations were tested 11 days after transduction against T2 cells pulsed with $HER2_{369}$ peptide at different peptide concentrations (A and B), T2 cells pulsed with a set of alternative peptides at a concentration of 10^{-5} M (C) and tumor cell lines (D). Tumor cell lines were treated with IFNγ (100 U/ml) 48 h prior to the stimulation assay. An E:T ratio of

5:1 was used. After 24 h stimulation, supernatants were analyzed for IFNγ secretion by using IFNγ ELISA. The proportions of $HER2_{369}$-multimer$^+$ cells within the chimeric TCR-transduced bulk populations are indicated. Asterisks indicate the usage of modified TCR constructs. Standard deviations of triplicates are shown. GFP-transduced (Mock) and non-transduced PBMC were used as negative controls. The expression pattern of tumor cells for HLA-A2 and HER2/neu are shown below the graph. The relative IFNγ release in Figure 5.10 B was calculated by using the following formula and the half-maximum effective concentration (EC_{50}) is indicated.

$$\text{Relative IFN}\gamma\text{ release (\%)} = \frac{\text{sample release - minimum release}}{\text{maximum release - minimum release}} \times 100$$

5.3.2.3 Functional reactivity of CD8-depleted chimeric TCR-transduced PBMC

As PBMC transduced with the TCR chain combinations of HER2-2α/HER2-4β and HER2-2α/R6C12β showed CD8-independent binding of $HER2_{369}$-multimer (Figure 5.9 A), I sorted the CD8$^-$ population after TCR transduction and investigated the functional peptide recognition and anti-tumor reactivity of this subpopulation. CD8-depleted PBMC transduced with HER2-2α/HER2-4β and HER2-2α/R6C12β showed the same response pattern to target cells as the transduced counterpart without sorting (Figure 5.11 and 5.10). CD8-depleted effector cells transduced with the TCR chain combination HER2-2α/HER2-4β demonstrated high functional avidity, peptide-specific recognition of $HER2_{369}$ and anti-tumor reactivity. However, again enhanced background reactivity in response to T2 cells pulsed with alternative peptides or unpulsed was observed. CD8-depleted PBMC transduced with the TCR chain combination HER2-2α/R6C12β showed specific recognition of $HER2_{369}$ but no anti-tumor reactivity.

5.3.2.4 Proliferation of chimeric TCR-transduced CD4$^+$ or CD8$^+$ T cell subsets

Autoreactivity against healthy tissues including PBMC might be a major risk using T cells transduced with TCRs specifically recognizing overexpressed self antigens in cancer patients. Moreover, the chimeric TCR chain combination HER2-2α/HER2-4β showed enhanced background reactivity against T2 cells pulsed with alternative peptides (Figure 5.11 C). I therefore investigated the proliferation capacity of HLA-A2$^-$ and HLA-A2$^+$ PBMC subpopulations transduced with HER2-2α/HER4-β as well as TCR HER2-1. HLA-A2$^-$ CD4$^+$ and CD8$^+$ T cells transduced with HER2-2α/HER2-4β or TCR HER2-1 proliferated similarly compared to the proliferation tendency of mock and non-transduced controls, although the multimer$^+$ HLA-A2$^-$ CD8$^+$ population transduced with HER2-2α/HER2-4β proliferated slower compared to the HLA-A2$^-$ CD8$^+$ population transduced with TCR HER2-1. Regarding the HLA-A2$^+$ PBMC population, slight inhibition of proliferation in cells transduced with TCR

HER2-1 was observed, whereas proliferation of cells transduced with HER2-2α/HER2-4β was extensively inhibited.

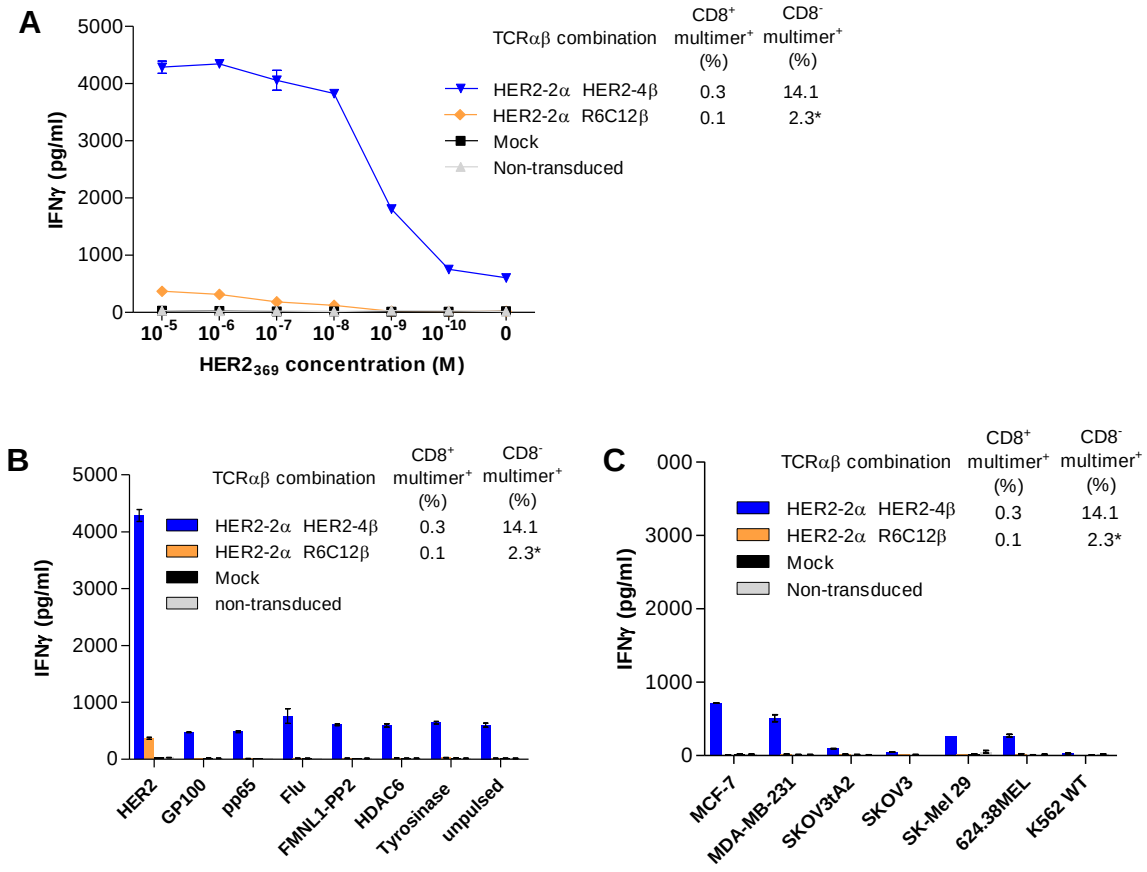

Figure 5.11 CD8-depleted PBMC transduced with chimeric TCR chain combinations containing HER2-2α in combination with HER2-4β and R6C12β preserved functional avidity, peptide specificity and anti-tumor reactivity.

CD8-depleted PBMC transduced with the chimeric TCR chain combination HER2-2α/HER2-4β or HER2-2α/R6C12β were tested 14 days after sorting (25 days after transduction) for their reactivity against T2 cells pulsed with different concentrations of $HER2_{369}$ (A), T2 cells pulsed with a set of alternative peptides at a concentration of 10^{-5} M (B) and tumor cells (C) (E:T=5:1). After 24 h stimulation, supernatants were investigated for IFNγ secretion by IFNγ ELISA. The proportions of $HER2_{369}$-multimer$^+$ cells within the $CD8^-$ or $CD8^+$ cell populations are shown. Asterisks indicate the usage of modified TCR constructs. Standard deviations of triplicates are indicated. Similarly, sorted GFP-transduced (Mock) and non-transduced PBMC were used as negative controls.

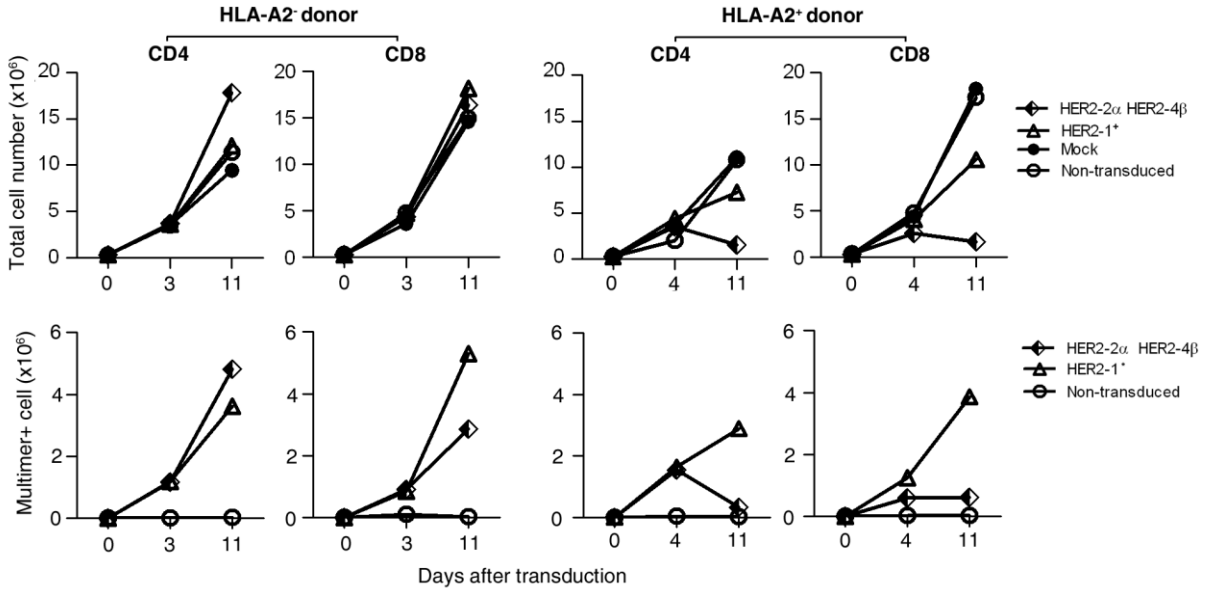

Figure 5.12 Proliferation of HLA-A2$^-$ and HLA-A2$^+$ PBMC after transduction with TCR HER2-1 and the chimeric TCR chain combination HER2-2α/HER2-4β.

T cells from HLA-A2$^-$ and HLA-A2$^+$ donors were counted at the indicated time point after TCR-transduction. The number of multimer$^+$ cells was calculated depending from the proportion of multimer$^+$ cells measured by flow cytometry at the corresponding time point. Asterisks indicate the usage of modified TCR constructs.

5.4 Anti-tumor reactivity of allorestricted TCR-transduced PBMC *in vivo*

I have functionally investigated the isolated HER2$_{369}$-specific TCRs as well as the novel chimeric TCRs containing HER2-2α in combination with diverse TRBV12 chains after transduction in T cells *in vitro*. All four original TCRs (TCR HER2-1 to HER 2-4) and two chimeric TCRs (HER2-2α/HER2-4β and HER2-2α /R6C12β) showed significant HER2$_{369}$ recognition. Remarkably, TCR HER2-1 and the chimeric TCR chain combination HER2-2α/HER2-4β showed also reactivity against tumor cells *in vitro* (Figure 5.3 C, 5.6 and 5.10 D). Another TCR (TCR SK22) with specificity for FMNL1, an antigen overexpressed in leukemia and lymphoma and aberrantly expressed in diverse tumor cell lines (Krackhardt et al. 2002; Schuster et al. 2007), was extensively investigated *in vitro* by Ingrid Schuster in her PhD project (Schuster 2008), and specific peptide-recognition and anti-tumor reactivity were observed after TCR transfer to PBMC. Within my PhD project, first analyses of the three mentioned HER2$_{369}$- or FNML1-specific TCRs (TCR HER2-1, HER2-2α/HER2-4β, and TCR SK22) in regard to anti-tumor reactivity *in vivo* were performed.

5.4.1 Establishment of human tumor xenografts in immunodeficient NOD/SCID mice

As MCF-7 tumor cells were well recognized by PBMC transduced with TCR HER2-1 or HER2-2α/HER2-4β *in vitro* (Figure 5.3 C and 5.10 D), and BJAB tumor cells were recognized by original T cell clone SK22 (Schuster et al. 2007), these tumor cell lines were used to establish human tumor xenografts expressing antigens of interest in mice. MCF-7 is a HLA-$A2^+$ human breast carcinoma cell line MCF-7 expressing HER2/neu and BJAB is a HLA-$A2^+$ B lymphoma cell line expressing FMNL1. The MCF-7 and BJAB tumor xenografts were then used to analyze anti-tumor reactivity of $HER2_{369}$-specific TCRs (TCR HER2-1 and HER2-2α/HER2-4β) and FMNL-1-specific TCR SK22, respectively, *in vivo*.

As shown in Figure 5.13 A and C, immunodeficient NOD/SCID mice receiving MCF-7 cells at a dose of at least 5 x 10^6 cells intraperitoneally (i.p.) suffered from severe hemorrhagic ascites in the peritoneal cavity 68 days (one of three mice is ill, 1/3) or 90 days (2/3) after tumor cell injection, indicating peritoneal carcinosis as demonstrated by immunohistochemistry analysis (data not shown). In contrast, this tumor manifestation was only observed in one mouse (1/3) within the group inoculated with MCF-7 at a dose of 5 x 10^5 cells 124 days after tumor injection, and another mouse within this group developed thymic lymphoma (thymoma), which has been previously reported to occur spontaneously in NOD/SCID mice (Kato et al. 2009). Moreover, NOD/SCID mice did not show visible tumor manifestation after intravenous (i.v.) or subcutaneous (s.c.) application of MCF-7 cells (data not shown). These data revealed that i.p. inoculation of MCF-7 cells at a dose of 5 x 10^6 cells is able to induce tumor metastasis and this dose might be required for successful tumor establishment. Therefore, this approach was chosen for further experiments to investigate anti-tumor reactivity of $HER2_{369}$-specific TCR *in vivo*.

On the other hand, female NOD/SCID mice were injected i.v. with 5 x 10^6 BJAB cells. These animals all developed ovarian tumor manifestations at day 33 (2/3) or 34 (1/3) (Figure 5.13 B and D). Application of 5 x 10^5 cells resulted in ovarian tumor manifestations in one mouse at day 50 (1/3). Animals receiving lower tumor cell dosages remained healthy at least until day 95. In addition, a cell suspension was prepared from the dissected ovarian tumors. The tumor cells showed HLA-A2 expression as determined by flow cytometeric analysis (data not shown), indicating that the ovarian tumors are derived from human BJAB tumor cells. Male NOD/SCID mice did not show tumor manifestation after i.v. injection of 5 x 10^6 BJAB cells (data not shown), suggesting that BJAB-derived tumor development in the recipient mice after i.v. application may be gender-dependent. Hence, I used female mice with i.v. inoculation of 5 x 10^6 BJAB cells as tumor model for following *in vivo* investigation. As it is

difficult to measure tumor size during tumor development in these two models, the kinetics of tumor growth have not been analyzed.

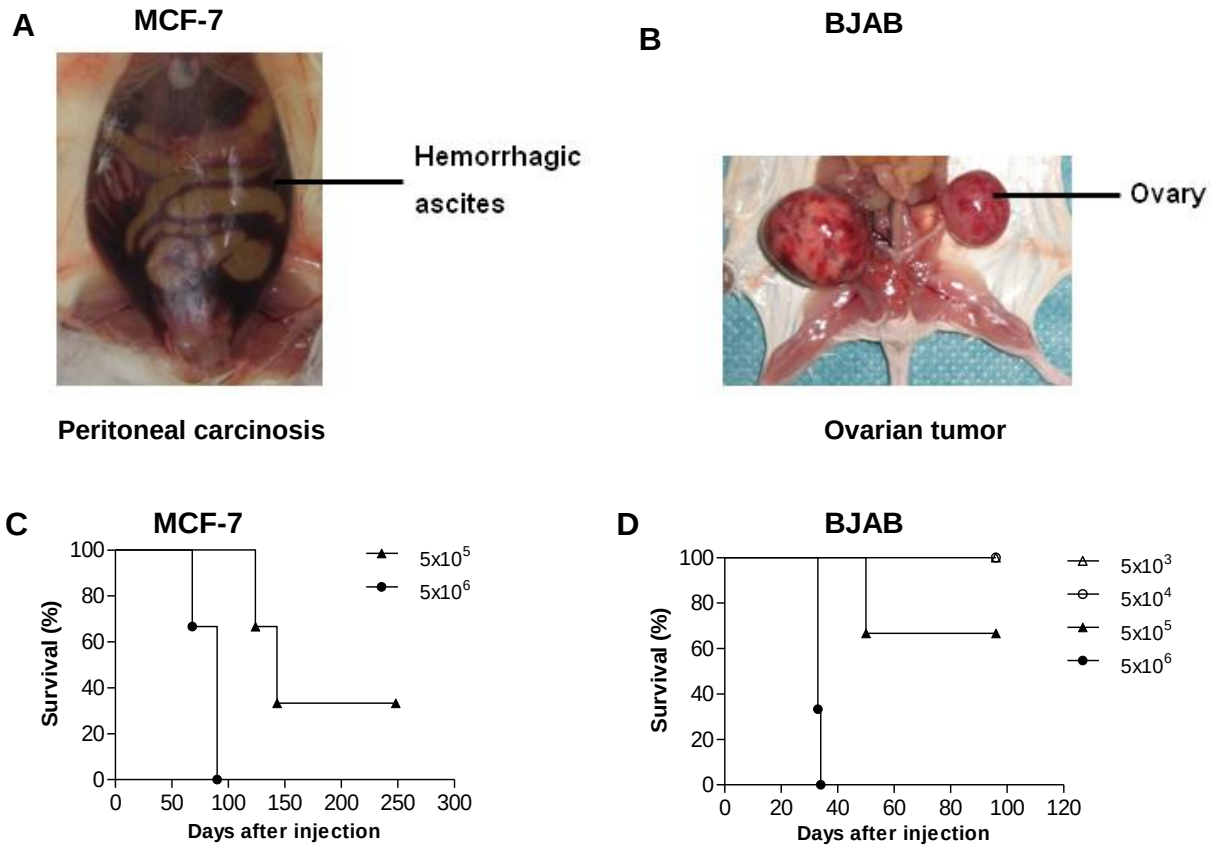

Figure 5.13 Tumor manifestations in immunodeficient NOD/SCID mice and overall survival after application of human tumor cells.

8-9 week-old NOD-SCID mice were intraperitoneally (i.p.) and intravenously (i.v.) infused with human MCF-7 and BJAB cells in different doses as indicated, and monitored for evidences of tumor establishment (n=3). Mice with signs of severe illness were sacrificed and dissected. (A). Mice with inoculation of 5 x 10^6 MCF-7 cells showed severe hemorrhagic ascites in the peritoneal cavity. (B). Mice receiving 5 x 10^6 BJAB cells developed ovarian tumor manifestations. (C) and (D). Kaplan-Meier survival curves of mice with MCF-7 and BJAB tumor xenografts are shown. The survival percentage of mice inoculated with MCF-7 or BJAB cells was determined from the day of tumor injection until the day of death or sacrifice. Data are representative for two experiments.

5.4.2 Anti-tumor reactivity of PBMC transduced with allorestricted TCRs *in vivo*

5.4.2.1 Anti-tumor reactivity of $HER2_{369}$-specific allorestricted TCR-transduced PBMC *in vivo*

To investigate the anti-tumor reactivity of PBMC transduced with $HER2_{369}$-specific allorestricted TCR HER2-1 and HER2-2α/HER2-4β, TCR-transduced PBMC were adoptively transferred into NOD/SCID mice one day after inoculation of MCF-7 cells (Figure 5.14 A). All mice suffered from severe hemorrhagic ascites and had to be sacrificed. As shown in Figure 5.14 B, mice receiving PBMC transduced with either TCR HER2-1 (average survival of 56 days) or HER2-2α/HER2-4β (58 days) demonstrated increased survival compared to the control group treated with GFP-transduced PBMC (50 days). The observed difference was statistically significant ($P<0.05$). In addition, no significant survival difference was observed between mice treated with TCR HER2-1- and HER2-2α/HER2-4β-transduced PBMC ($P>0.05$). However, considering the relatively low expression of introduced TCRs in the transduced T cells and generally lower survival compared to that observed during the establishment of the tumor model (Figure 5.13 C), these data need to be considered with caution and additional experiments are necessary. Moreover, survival and functionality of adoptively transferred T cells have not been investigated in this experiment whereas comparable studies were not able to provide evidence for human T cells survival in mice (data not shown). Therefore, it would also be essential to assess the survival of the transferred T cells and to track the T cells in the tumor sites in tumor-bearing mice by flow cytometry or immunohistrochemistry.

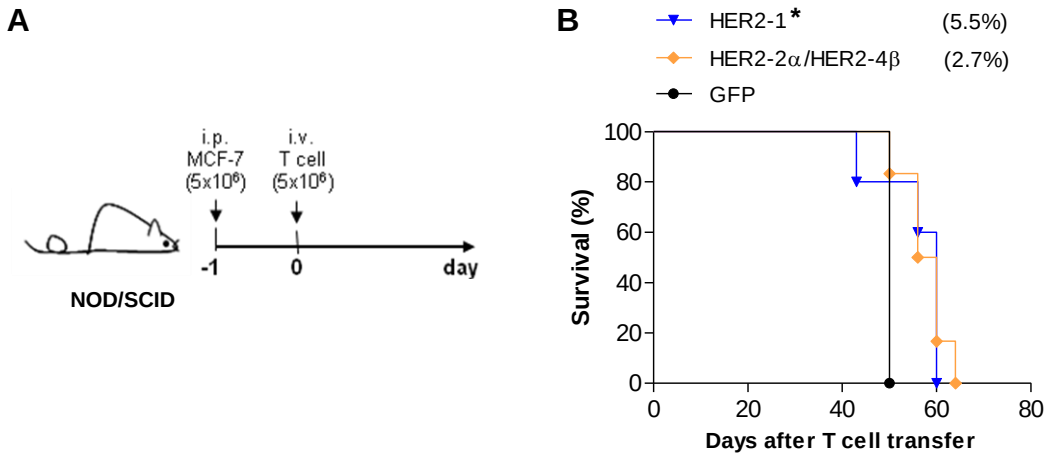

Figure 5.14 Application of PBMC transduced with $HER2_{369}$-specific TCR HER2-1 and HER2-2α/HER2-4β results in enhanced survival of treated NOD/SCID mice in one experiment.

The *in vivo* anti-tumor reactivity of PBMC transduced with $HER2_{369}$-specific TCR HER2-1 or HER2-2α/HER2-4β was investigated in immunodeficient NOD/SCID mice. (A). Overview of the experimental procedure. 5 x 10^6 TCR HER2-1- or HER2-2α/HER2-4β-transduced PBMC were adoptively transferred (i.v.) into NOD/SCID mice one day after inoculation of 5 x 10^6 MCF-7 cells (i.p.). (B). Mice with signs of severe illness were sacrificed. Kaplan-Meier survival curves of each group are shown. Mice treated with GFP-transduced PBMC were used as a negative control. Asterisk indicates the usage of modified TCR construct. The proportions of $HER2_{369}$-multimer$^+$ cells within the TCR-transduced PBMC population used for transfer are shown in the parentheses. P values of Figure 5.14 B: HER2-1 group (n=6) versus GFP group (n=5): 0.049; HER2-2α/HER2-4β group (n=6) versus GFP group: 0.005; HER2-1 group versus HER2-2α/HER2-4β group: 0.783 (Mantel-Cox test).

5.4.2.2 Anti-tumor reactivity of FMNL1-specific allorestricted TCR-transduced PBMC *in vivo*

Similarly, anti-tumor reactivity of PBMC transduced with the FMNL1-PP2-specific TCR SK22 *in vivo* was analyzed in NOD/SCID mice. TCR SK22-transduced PBMC were adoptively transferred into mice one day after inoculation with BJAB tumor cells (Figure 5.15 A). Mice receiving TCR SK22-transduced PBMC demonstrated marginally improved survival compared to the control group treated with GFP-transduced PBMC, which was not statistically significant (Figure 5.15 B). All sacrificed mice developed ovarian tumors. One mouse in the control group did not develop a tumor, which might be due to unsuccessful tumor injection by i.v. application. Similar as in the MCF-7 model, low expression of TCR SK22 in the TCR-transduced PBMC (1.1%) used for adoptive transfer may have had a major impact on the result of this experiment. Therefore, it is difficult to precisely evaluate the anti-tumor reactivity of TCR SK22 after transduction into T cells *in vivo* from these data. To better analyze anti-tumor reactivity of TCR SK22-transduced PBMC, further experiments using PBMC which contain a higher frequency of TCR SK22-expressing T cells are required. Furthermore, as mentioned above for $HER2_{369}$-specific TCRs it is also important to assess the survival of the transferred T cells and the migration of transferred T cells into the tumor sites in tumor-bearing mice.

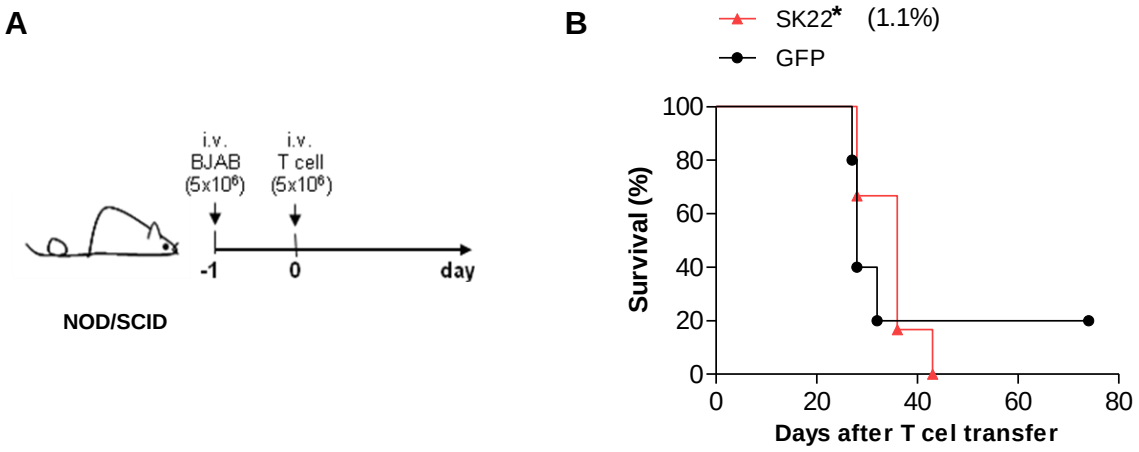

Figure 5.15 Application of PBMC transduced with the FMNL1-specific TCR SK22 results in marginally non-statistically significant survival in treated mice *in vivo* in one experiment.
(A).Overview of the experimental procedure. 8-9 week-old NOD/SCID mice received 5 x 10^6 TCR SK22-transduced PBMC (i.v.) one day after inoculation of 5 x 10^6 BJAB tumor cells (i.v.). (B).Kaplan-Meier survival curves of each group are shown. Mice treated with GFP-transduced PBMC served as control. Asterisk indicates the usage of modified TCR construct. The number in parenthesis indicates the proportion of FMNL1-PP2-multimer$^+$ cells within the TCR-transduced PBMC population used for transfer. P values of Figure 5.15 B: SK22 group (n=6) versus GFP group (n=5): 0.686 (Mantel-Cox test).

5.5 *In vivo* monitoring of T cells after adoptive transfer

Importantly, the persistence and migration capability of tumor-specific T cells into tumor sites after adoptive transfer have been shown to correlate with their clinical efficacy (Pockaj et al. 1994; Dudley et al. 2002; Robbins et al. 2004). Therefore, in this study efforts were also made to trace the T cells transduced with allorestricted TCRs long-term *in vivo*. To test the feasibility of non-invasive molecule imaging system PET/CT using the hNIS reporter gene for monitoring of survival and spatial distribution of TCR-modified T cells *in vivo*, TCR-transduced PBMC were firstly equipped with the hNIS reporter gene by lentiviral transfer.

5.5.1 Lentiviral transfer of hNIS and allorestricted TCR genes into recipient cells

To allow the expression of hNIS as well as the specific TCRs in the same T cell after viral transduction, a bicistronic lentiviral vector containing both genes with a “self-cleaving” T2A in between was constructed as described in section 4.1.5. The expression of hNIS and the allorestricted TCR HER2-1 was at first investigated in TCR-deficient J76CD8 cells by flow cytometry. As shown in Figure 5.16, J76CD8 cells showed a significant population positive for $HER2_{369}$-multimer and anti-hNIS antibody 4 days after lentiviral transduction, although the

proportions of positive cells were low. PBMC transduced with the same lentiviral vector expressed also TCR HER2-1 and hNIS, whereas the expression was even lower when compared to J76CD8 cells (data not shown). The construction of the bicistronic lentiviral vector seemed to be successful, since the expression of both hNIS and TCR HER2-1 was observed. However, the low gene expression in recipient cells suggests that the protocol used for lentiviral transduction needs to be further optimized in order to improve the transduction efficiency.

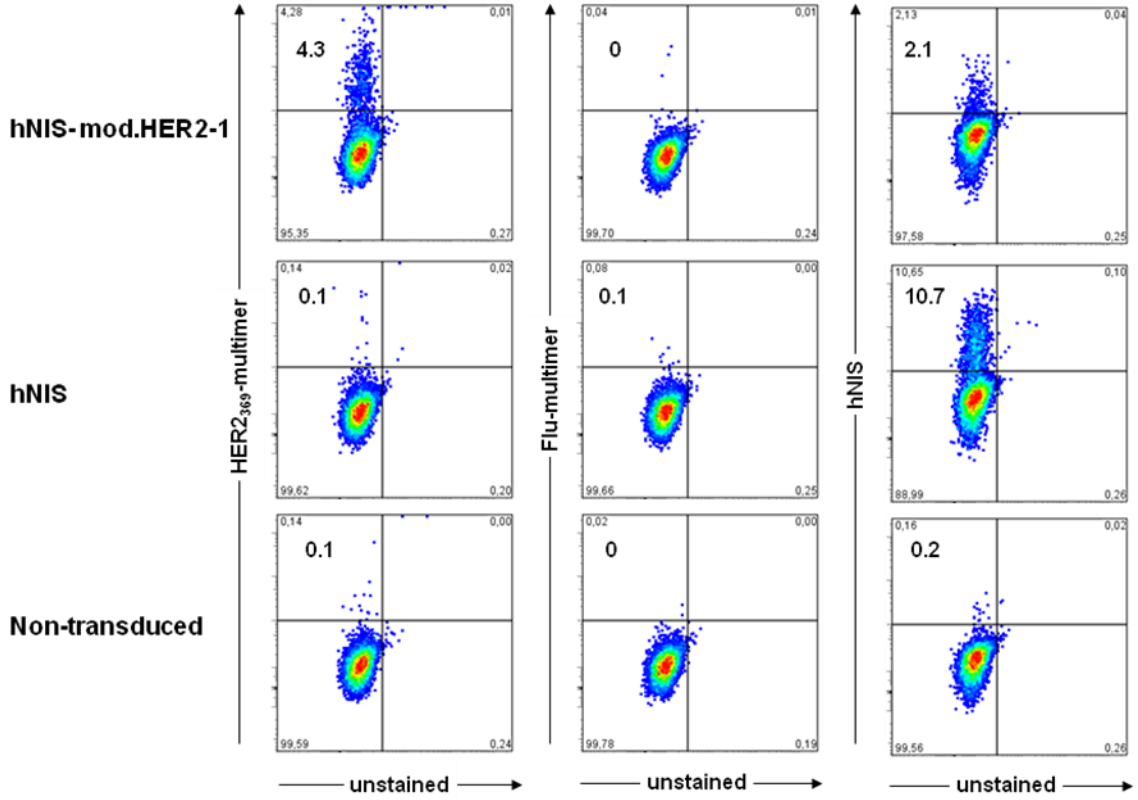

Figure 5.16 J76CD8 cells transduced with hNIS and the modified TCR HER2-1 by using a bicistronic lentiviral vector showed expression of TCR HER2-1 and hNIS.

J76CD8 cells were stained with $HER2_{369}$-multimer, Flu-multimer or anti-hNIS antibody 4 days after lentiviral transduction of hNIS and TCR HER2-1 and analyzed by flow cytometry. Flu-multimer was used as control multimer. The proportions of multimer$^+$ or h-NIS$^+$ cells are indicated.

5.5.2 Assessment of the anti-tumor reactivity of OT-1 transgenic T cells by PET/CT

To principally establish the *micro*PET/CT imaging system (PET/CT for small animal), I firstly used a previously established mouse model to assess the anti-tumor reactivity of the well characterized OT-1 transgenic murine T cells. OT-1 transgenic T cells harbor a TCR specifically recognizing the peptide OVA_{257} (SIINFEKL, residues 257-266) derived from ovalbumin (OVA) in the context of murine MHC molecule H-$2K^b$ (Figure 5.17 B) (Hogquist et

al. 1994) and eradicate successfully established OVA-expressing tumors in a mouse model (Helmich and Dutton 2001). In this study, NOD/SCID mice were s.c. inoculated with wildtype EL-4 and OVA-transfected EL-4 (E.G7-OVA) lymphoma cells on the right and left flank, respectively, and received activated OT-1 transgenic T cells i.p. 6 days after tumor cell application (Figure 5.17 A). Mice were monitored by *micro*PET/CT system after i.v. infusion of ^{18}FDG. It was observed that two subcutaneous regions with high accumulation of ^{18}FDG 7 days after inoculation of tumor cells (one day after T cell transfer), demonstrating the growth of E.G7-OVA and EL-4 tumors on the left and right flank (Figure 5.17 A and C). However, the E.G7-OVA tumors expressing OVA were not visible in both mice 7 days after adoptive transfer of activated OT-1 transgenic T cells, whereas the control EL-4 tumors continued to grow, indicating that the transgenic OT-1 T cells exert specific tumor-killing *in vivo* (Figure 5.17C). Of note, some background ^{18}FDG signals were detected at heart and bladder (Figure 5.17C). The successful monitoring of the anti-tumor reactivity of OT-1 transgenic T cells with PET/CT imaging as previously published (Su et al. 2006) will be the basis for future *in vivo* tracking of the described tumor-specific TCR-transduced T cells *in vivo*.

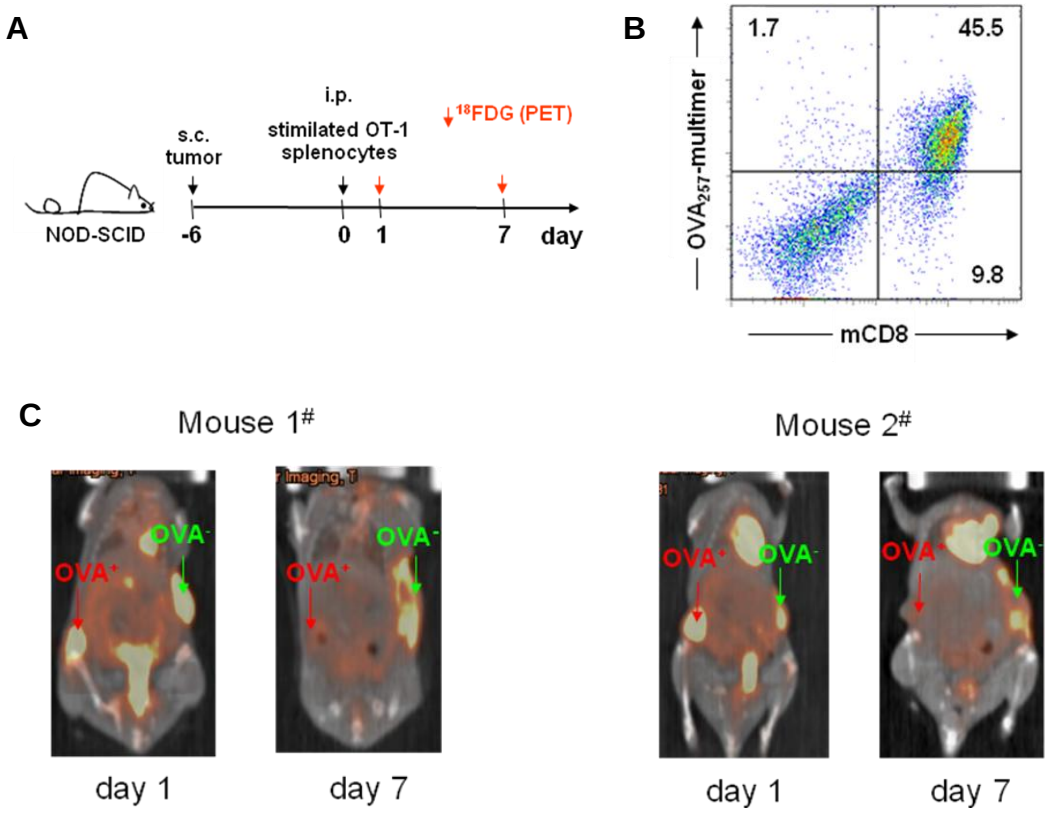

Figure 5.17 Assessment of the anti-tumor reactivity of OT-1 transgenic T cells *in vivo* by *micro*PET/CT imaging.

(A). Overview of the experimental procedure. 7 week-old NOD/SCID mice were inoculated with 5x10^6 wildtype murine lymphoma EL-4 cells as well as EL-4 cells transfected with ovalbumin (E.G7-OVA)

s.c. on the right and left flank, respectively (day -6). Subsequently, $5x10^6$ stimulated OT-1 splenocytes were injected i.p. 6 days after tumor injection (day 0). The tumor growth was measured by microPET/CT at indicated time points after T cell transfer following i.v. infusion of radioactive ^{18}FDG i.v.. (B). The splenocytes isolated from OT-1 transgenic mice were specifically stimulated with OVA_{257} peptide and anti-mCD3 and anti-mCD28 antibodies for 48 h. The stimulated splenocytes were stained with OVA_{257}-multimer and anti-mCD8 antibody and analyzed by flow cytometry before adoptive transfer. (C). The PET imaging data from two treated mice are shown at indicated time points after T cell transfer. Red arrows indicate the E.G7-OVA tumor expressing ovalbumin, while green arrows indicate the EL-4 tumor cells without expression of ovalbumin.

6 Discussion

Adoptive transfer of tumor-specific T cells has emerged as an attractive therapeutic approach to treat malignant diseases. The pivotal clinical studies by the Rosenberg group have demonstrated that infusion of autologous TIL derived from the tumors represents an effective strategy to treat patients with melanoma (Rosenberg et al. 1988; Dudley et al. 2002; Rosenberg and Dudley 2004). However, several shortcomings limit the broad application of this strategy for the treatment of diverse cancer types: (1) Isolation and cultivation of TIL is difficult and time-consuming; (2) Effective TIL cannot be easily obtained from most tumors other than melanoma; (3) Isolated TIL may not show high avidity toward tumor antigens, since T cells with high avidity toward self-antigens in the context of self MHC molecules have been mostly eliminated from the repertoire during thymic selection. Alternatively, alloreactive T cells specifically recognizing self-antigens presented by foreign MHC molecules are not controlled by central tolerance and therefore, represent a source of tumor-specific effector cells for adoptive T cell therapy (Sadovnikova and Stauss 1996). In fact, infusion of lymphocytes derived from HLA-mismatched donors into leukemia patients after allogeneic stem cell transplantation induces GVL effects in the clinical setting (Kolb et al. 1995). Unfortunately, besides the desired GVL effects, harmful side effects such as GVHD often occur. The TCR mediates the antigen recognition by a T cell and transfer of genes encoding TCR α- and β-chains redirects the specificity of recipient T cells (Dembic et al. 1986; Clay et al. 1999; Calogero et al. 2000). Moreover, TCR-modified T cells can be expanded to sufficient numbers for transfer in a relatively short period of time, allowing the rapid generation of tumor-specific T cells. Hence, genetic modification of T cells by transfer a defined TCR with specificity for TAA might overcome some drawbacks of the strategies mentioned above and be a therapeutic option for the treatment of cancer. In fact, the feasibility and efficacy of this approach (namely TCR gene therapy) have been demonstrated in clinical trials (Morgan et al. 2006; Johnson et al. 2009). However, a broad range of TCRs with specificity for diverse classes of TAA need to be characterized for broader application and further assessment of potential benefits and side effects of TCR gene therapy.

This study focuses on two TAA, HER2/neu and FMNL1. HER2/neu is a TAA overexpressed in diverse malignancies and has been demonstrated to be a preferable target for anti-cancer therapies using antibodies (Slamon et al. 2001). In our group, allorestricted T cell clones with specificity for HER2/neu epitope $HER2_{369}$ have been previously isolated (Weigand 2007) by using the allorestricted approach (Sadovnikova et al. 1998) and TCRs from those T cell clones were also indentified (Liang 2007). In this study the functionality of those isolated

$HER2_{369}$-specific TCRs after transfer into recipient cells was investigated *in vitro* as well as *in vivo*.

Another TAA, FMNL1, was previously identified by SEREX and showed restricted natural expression in hematopoietic lineage-derived cells in healthy tissues and overexpression in CLL as well as in other malignant cells (Krackhardt et al. 2002; Schuster et al. 2007), demonstrating an attractive target antigen for immunotherapy of CLL. In addition, the allorestricted T cell clone SK22 with specificity for FMNL1-derived peptide PP2 has been previously isolated and demonstrated specific-peptide recognition and anti-tumor reactivity. Moreover, transfer of the isolated TCR (TCR SK22) into PBMC endowed the recipient cells with recognition of both peptide FMNL1-PP2 and FMNL1-expressing tumor cells *in vitro* (Schuster et al. 2007). In this study the anti-tumor reactivity of this TCR after transfer into PBMC was further assessed *in vivo*.

6.1 Retroviral transfer of $HER2_{369}$-specific allorestricted TCRs into recipient cells

Retroviral transfer of $HER2_{369}$-specific allorestricted TCRs into TCR-deficient J76CD8 cells as well as into PBMC resulted in a significant population specifically binding $HER2_{369}$-multimer, although the proportions of $HER2_{369}$-multimer$^+$ cells in TCR-transduced PBMC were low when compared to their J76CD8 counterpart likely due to the lack of competition of endogenous TCR chains in J76CD8 cells. Additionally, PBMC transduced with $HER2_{369}$-specific allorestricted TCRs exerted high peptide specificity, whereas anti-tumor reactivity was primarily low. Modification of TCR constructs including usage of a bicistronic vector construct using virus-derived “self-cleaving” linker P2A, murinization of TCR constant chain genes and codon optimization as previously described (Cohen et al. 2006; Scholten et al. 2006; Leisegang et al. 2008) resulted in enhanced surface expression of transduced TCRs as determined by $HER2_{369}$-multimer staining. Especially, TCR HER2-1 demonstrated significantly improved anti-tumor reactivity after modification. The observed enhancement of expression and functionality of the modified TCRs is most likely due to reduced mispairing between transduced α- (β-) chains and endogenous β- (α-) chains and improved stable association with CD3 molecules (Cohen et al. 2006). In fact, reduced mispairing of modified TCR chains with endogenous TCR chains was also observed in this study. Actually, reducing the mispairing of exogenous and endogenous TCRs is of great importance for adoptive transfer of TCR-modified effector cells (also called TCR gene therapy), because such mispairing not only reduces the expression of correctly paired transduced TCRs but also may result in new TCR chain combinations with unknown specificities and thereby may induce unexpected autoimmunity as discussed in detail in section 6.3 (Fernandez-Miguel et al.

1999). Additional modification strategies reducing the risk for mispairing might be applied for TCR HER2-1: (1) introducing a second disulfide bridge between transduced TCR α- and β-chains through sited-cysteine mutation (Cohen et al. 2007); (2) modifying the interface of the constant regions of transduced TCR α- and β-chains by mutation of paired amino acid residues to form a 'knob-in-hole' interaction and thereby enhance the interaction between TCR chains (Voss et al. 2008); (3) incorporating entire of ξ chain derived from the CD3 complex into TCR α- and β-chains (Sebestyen et al. 2008); (4) generating a single chain TCR containing variable region of α-chain and the entire β-chain, which is fused to TCR ξ-chain (Voss et al. 2010).

A potential problem using murinized TCRs and virus-derived P2A linker in TCR gene therapy is that parts of these foreign sequences may be presented by MHC molecules and thereby may induce immune responses to reject transferred T cells by the host. However, recent clinical data showed effectivity and *in vivo* persistence of effector cells transduced with a murine TCR (Johnson et al. 2009) suggesting that TCRs containing murine sequences should not be prematurely deleted from further investigation. With respect to P2A linker sequence, no epitopes could be predicted currently to bind on most commonly expressed human MHC I molecule HLA-A2, although its potential immunogenicity could not be excluded (Leisegang et al. 2008).

6.2 TCR HER2-1 represents a potential TCR candidate targeting HER2/neu for clinical application

6.2.1 Peptide specificity of TCR HER2-1-transduced T cells

As PBMC transduced with modified TCR HER2-1 showed specific peptide recognition and anti-tumor reactivity representing a potential candidate for clinical application, this TCR was functionally analyzed in more detail after transduction in $CD4^+$ and $CD8^+$ T cell subsets. In contrast to a previously reported allorestricted T cell clone with specificity for $HER2_{369}$ (Conrad et al. 2008), TCR HER2-1 exclusively recognized T2 cells pulsed with $HER2_{369}$ but not pulsed with the analogous peptides $HER1_{364}$, $HER3_{356}$, or $HER4_{361}$ derived from HER1, HER3, and HER4, respectively. Additionally, I tested the recognition of single amino acid peptide analogues of $HER2_{369}$ in the context of HLA-A2 by the TCR HER2-1 using threonine and alanine for substitution. Whereas peptides with several single substitutions with the amino acid alanine were still recognized, exchange by threonine was less tolerated by this TCR potentially due to the polarity as well as longer side chain of threonine. Limited crossreactivity for analogous peptides has been previously reported for other TCRs with specificity for viral antigens (Hausmann et al. 1999) and this might reflect the general

crossreactive potential reported for TCRs (Felix et al. 2007). Of note, exchange of the HLA-A2 anchor residues in position 2 and 9 by alanine or threonine was tolerated at high peptide concentrations of 10^{-5} M. However, melanoma epitopes have been previously described containing alanine or threonine on position 2 and alanine on position 9 (Kawakami et al. 1995). In addition, it has been previously published that position 3 may represent a secondary anchor and that aromatic residues are frequent in this position (Ruppert et al. 1993; Nicholls et al. 2009). Substitution of phenylalanine at position 3 with either alanine or threonine completely abolished recognition by TCR HER2-1 potentially pointing to a role of the aromatic residue at this position in peptide binding of $HER2_{369}$ to HLA-A2 as well as recognition by TCR HER2-1.

6.2.2 Anti-tumor reactivity of TCR HER2-1-transduced T cells

In parallel to peptide specificity, $CD8^+$ T cells transduced with TCR HER2-1 exerted both IFNγ-secretion and cytotoxicity in response to HLA-$A2^+$ tumor cells naturally expressing HER2/neu. Moreover, $CD8^+$ T cells expressing transduced TCR HER2-1 were shown to be the effector cells for the tumor recognition as determined by intracellular IFNγ-staining in combination with $HER2_{369}$-multimer binding. These results demonstrate that transfer of TCR HER2-1 endows the recipient cells with anti-tumor reactivity. Additionally, two TCR HER2-1-expressing $CD8^+$ T cell clones (Clone 1 and Clone 2), which were isolated from TCR HER2-1-transduced $CD8^+$ T cell populations, also showed anti-tumor response, whereas distinct release patterns of multiple cytokines were displayed. T cell Clone 1 preferentially produced IL-4, IL-5, and IL-13 in response to HER2/neu expressing HLA-$A2^+$ C1R tumor cells, while Clone 2 secreted more IFNγ and IL-2, suggesting the heterogeneity of $CD8^+$ T cell populations transduced with $HER2_{369}$-specific TCRs and that different functions might be mediated by each population *in vivo*. The observed heterogeneity might result from differentiation of naïve $CD8^+$ T cells after TCR transduction or transfer of TCRs into different $CD8^+$ T cell subpopulations. In fact, $CD8^+$ T cells can be also classified into two subtypes, similar to $CD4^+$ T cells, based on their cytokine secretion pattern: Tc1 cells (secreting type 1 cytokines IFNγ and IL-2) and Tc2 cells (secreting type 2 cytokines as IL-4, IL-5 and IL-13), whereas the functions of different $CD8^+$ T cell subpopulations *in vivo* remain to be further clarified (Mosmann et al. 1997).

By contrast, $CD4^+$ T cell subsets showed in general marginal IFNγ secretion and cytotoxicity in response to HER2/neu-expressing tumor cells after transduction with TCR HER2-1 compared to their $CD8^+$ counterpart, which might be due to the reduced functional avidity of TCR-HER2-1-transduced $CD4^+$ T cells lacking CD8, or might correlate to the main role of $CD4^+$ T cells as helper cells in the immune system by secreting diverse cytokines. To better clarify this point, it might be necessary to determine the functional avidity of $CD4^+$ T cells

transduced with TCR HER2-1 and the secretion pattern of multiple cytokines in response to various targets. In fact, tumor-specific $CD4^+$ T cells play also an essential role in inducing systemic anti-tumor immunity by activation of $CD8^+$ effector T cells and maintenance of immune memory or induction of eosinophils and macrophages to produce superoxide and nitric oxide (Hung et al. 1998) or potentially direct cytotoxic activity (Williams and Engelhard 1996; Porakishvili et al. 2004; Zaunders et al. 2004). Therefore, increasing effort is given to exploit tumor-specific $CD4^+$ T cells for immunotherapy of cancer (Perez-Diez et al. 2007; Hunder et al. 2008; Muranski et al. 2008).

6.2.3 Crossreactivity of TCR HER2-1-transduced T cells

Besides efficient anti-tumor reactivity, lack of crossreactivity is an essential prerequisite of TCR-transduced T cells used for TCR gene therapy of cancer. Importantly, PBMC transduced with TCR HER2-1 did not show reactivity against non-malignant HLA-$A2^+$ targets as fibroblast cell lines, cardiomyocytes and non-stimulated or stimulated PBMC suggesting that peptide-independent HLA-A2 recognition or crossreactivity against a peptide broadly presented by HLA-A2 may not play a role in target recognition by TCR HER2-1. However, C1R cells transfected with HLA-A2 induced limited intracellular IFNγ production in TCR HER2-1-transduced $CD8^+$ T cells and transduction of this TCR in HLA-$A2^+$ PBMC resulted in slightly decelerated proliferation after longer *in vitro* culture suggesting that proliferating cells of hematopoietic origin may represent a target of TCR HER2-1-transduced PBMC. This is in accordance with the published data demonstrating that mature hematopoietic cells express low levels of HER2/neu and increase expression of HER2/neu mRNA as well as HER2/neu protein after mitogenic stimulation (Leone et al. 2003). Transient leukopenia may be tolerated during therapy. However, in order to further assess the value of HER2/neu-specific TCRs in general and, especially, of TCR HER2-1 for clinical application, it will be necessary to investigate the crossreactivity against a broad panel of HLA-$A2^+$ healthy tissues *in vitro* as well as *in vivo* (Jorritsma et al. 2007).

6.2.4 Intermediate functional avidity of TCR HER2-1

Allorestricted T cells represent a source of high avidity TCRs for tumor antigens, since they are not negatively deleted in the thymus during T cell development and mostly elicit a vigorous response (Whitelegg and Barber 2004). High avidity T cells have been isolated previously by other groups by using the allogeneic approach (Sadovnikova and Stauss 1996; Stanislawski et al. 2001). It is interesting in this regard that the most promising TCR candidate (TCR HER2-1) showed only an intermediate functional avidity (EC_{50} is 10^{-7} M), although this TCR has been selected in an allogeneic environment. This may rely on several factors (Gonzalez et al. 2005). Firstly, T cells with high avidity for $HER2_{369}$ might undergo

apoptosis during the *in vitro* selection procedure using $HER2_{369}$-multimer, since persistent binding of multimers as nature ligands on TCRs may result in overstimulation of T cells and lead to cell death (Knabel et al. 2002). Secondly, binding of $HER2_{369}$ to HLA-A2 has been reported to have a relative short half life (Kuhns et al. 1999), which may have an impact on the observed intermediate functional avidities. It has been reported that peptides displaying high dissociation rates bind MHC molecules less stably and thereby might be less immunogenic (van der Burg et al. 1996). Thirdly, the discriminative $HER2_{369}$ epitope density between tumor cells and non-transformed cells might be marginal. In fact, $HER2_{369}$ peptide has been previously shown to be presented only at low levels on tumor cells (Weidanz et al. 2006). Moreover, overexpression of HER2/neu seems to result in proteasomal dysfunction and therefore reduced peptide presentation on tumor cells overexpressing this protein (Vertuani et al. 2009). Hence, it might be possible that TCRs with higher avidity have not been selected on the T cell clone level due to enhanced crossreactivity. TCRs with high avidity might be problematic when targeting overexpressed self antigens and an individual optimal functional TCR avidity potentially needs to be determined for any specific MHC-peptide complex.

6.3 Novel chimeric TCR chain combinations with recognition of $HER2_{369}$

6.3.1 Specific $HER2_{369}$-recognition by novel chimeric TCR chain combinations

As the other selected TCRs seemed at first less interesting as they demonstrated either limited peptide specificity (TCR HER2-3) or lack of tumor reactivity (TCR HER2-2 and HER2-4), they were not chosen as potential candidates for further investigation. However, as a preferential usage of β-chains of the TRBV12 family was observed in the isolated $HER2_{369}$-specific TCR repertoire, random combinations of isolated single TCR α- und β-chains of these TCR were further investigated. Thereby one TCR α-chain (HER2-2α) with specific $HER2_{369}$-peptide recognition in combination with diverse β-chains all belonging to the TRBV12 family was identified. Combinations with β-chains of two other TRBV families did not result in $HER2_{369}$-multimer positive cells or peptide specific target recognition. Importantly, all different TRBV12-derived β-chains contributing to $HER2_{369}$-specific multimer staining in combination with HER2-2α possessed different CDR3 regions and were not only derived from allorestricted $HER2_{369}$-specific TCRs from diverse HLA-$A2^-$ donors but also from TCRs with alternative specificities as $GP100_{209}$ and $CMVpp65_{495}$. These latter TCRs were selected in HLA-$A2^+$ individuals, which means in an autologous environment. Moreover, $HER2_{369}$-$multimer^+$ PBMC transduced with HER2-2α alone showed an enhanced frequency of $TRBV12^+$ cells, further emphasizing that HER2-2α is dominantly responsible for HLA-A2-

restricted $HER2_{369}$-specific recognition in combination with diverse TCR β-chains of the TRBV12 family.

6.3.2 Dominant TCR α-chain HER2-2α with $HER2_{369}$ specificity

A prevalent role of the TCR α-chain in the selection of the preimmune TCR repertoire specific for Melan-A has been previously reported (Sensi et al. 1993; Dietrich et al. 2003). Heemskerk et al. described novel chimeric TCR chain combinations containing single TCR chains derived from diverse HA-2-specific TCR resulting again in HA-2-specificity (Heemskerk et al. 2003). Moreover, Yokosuka et al. reported a murine HIV-specific TCR α-chain in TCR-transgenic mice responsible for recognition of HIVgp160 peptide/H-2Dd complexes in combination with a variety of TCR β-chains (Yokosuka et al. 2002). In these experiments one third of randomly picked β-chains of the same TRBV family could reconstitute for specific peptide recognition in combination with the HIVgp160 peptide-specific TCR α-chain. Here in this study it is shown that such TCR single chains with dominant peptide recognition in combination with TCR β-chains derived from TCRs with diverse specificities are present in the circulating T cell pool of humans.

MHC-peptide recognition by random combinations of TCR α- and β-chains has been previously suggested to be related to an intrinsic affinity of the TCRs towards MHC (Blackman et al. 1986) and a genetic bias towards MHC recognition has been proposed (Huseby et al. 2005; Maynard et al. 2005). More recently, germline interaction codons have been structurally defined for TCR α- and β-chains contacting specific MHC molecules (Feng et al. 2007; Dai et al. 2008). The observations in this study can be well explained by the existence of such germline restrictions of TCR-MHC-peptide complexes and favour a preferential germline restriction of β-chains of the TRBV12 family towards HLA-A2 when paired to HER2-2α. However, the CDR3 regions of the matching β-chains still play an important role in $HER2_{369}$ peptide recognition of HER2-2α and determined the dimensions of CD8 dependency, functional avidity and anti-tumor reactivity. Two β-chains of the TRBV12 family (HER2-1β and JG-9β) resulted in only low percentages of $HER2_{369}$ multimer$^+$ cells lacking specific functions. This might potentially result from low inter-chain affinity of the novel chimeric TCR chain combination. In addition, the β-chain of JG-9 represented another subtype (TRBV12-4). In contrast, two other TCR β-chains (HER2-4β and R6C12β) potentiated peptide-specific antigen recognition. Moreover, transduction of HER2-2α in combination with either HER2-4β or R6C12β into PBMC results in $HER2_{369}$-multimer binding of a $CD8^-$ cell population, which was not observed in PBMC transduced with original TCR chain combinations (TCR HER2-2, HER2-4, and R6C12) indicating enhanced CD8 independency of multimer binding of these two chimeric TCR chain combinations. It has

been reported by Choi et al. and Johnson et al. that CD8 dependency of multimer-binding is associated with low T cell avidity (Choi et al. 2003; Johnson et al. 2006). In their studies multimers with mutations in the CD8 binding site were utilized to inhibit CD8 binding. T cells with low avidity showed multimer staining only in the presence of CD8 binding, whereas T cells with high avidity bound multimers in the absence of CD8. The observations in this study seem to be consistent with their studies, as PBMC demonstrating CD8 independent multimer staining after transduction of chimeric TCR chain combinations (HER2-2α/HER2-4β and HER2-2α/R6C12β) showed also increased functional avidity compared to other mismatched combinations. The combination of HER2-2α and HER2-4β additionally resulted in potent anti-tumor reactivity. Both TCR single chains derived from different $HER2_{369}$-specific TCRs and, when dimerized with their native partners, primarily exhibited low functional avidities for peptide-pulsed T2 cells and lack of anti-tumor reactivity. However, combination of HER2-2α and R6C12β demonstrating significant $HER2_{369}$-multimer staining did not result in significant anti-tumor recognition, which might be due to its relatively low functional avidity for $HER2_{369}$ compared to HER2-2α/HER2-4β.

Currently, the frequency of such TCR single chains with dominant peptide recognition in combination with diverse β-chains is unknown and it is not clear if TCR β-chains may have similar properties. It will be interesting to further evaluate the docking pattern on MHC-peptide complexes of these TCRs. Apart from that, the existence of these TCR single chains with dominant peptide recognition may have an important impact on the further development of TCR transfer strategies. Chimeric TCR combinations involving such TCR single chains with dominant peptide recognition may be a source for TCRs with improved properties as enhanced functional avidity for TAA and preferential recognition of natural tumor targets. Otherwise, effector T cells transduced with the chimeric TCR HER2-2α/HER2-4β also showed increased background reactivity against T2 cells pulsed with alternative peptides as well as strong reactivity against HLA-$A2^+$ PBMC. This reactivity might result from enhanced germline MHC-interactions but also from recognition of low level $HER2_{369}$ on these target cells. TCRs with enhanced affinity due to mutations within the CDR3 regions have been previously demonstrated to be crossreactive for self peptides and might therefore harbour risks when used for clinical approaches with TCR-transgenic T cells (Holler et al. 2003; Chervin et al. 2009). Thus, the presence of such single TCR chains in the circulating T cell pool displaying dominant peptide recognition with different affinities in combination with diverse partner chains derived from introduced TCRs may represent a risk for TCR transfer in PBMC.

6.3.3 Potential side effects resulting from mispairing between exogenous and endogenous TCR chains

It was observed in this study that transduction of random TCR chain combinations derived from different TCRs, which harbour either the same specificity as $HER2_{369}$ or not, results in surface expression of transduced TCRs in J76CD8 cells which lack endogenous TCR expression. This observation indicated that TCR mispairing can occur easily. Actually the capability of TCR mispairing has been previously published by several groups (Heemskerk et al. 2003; Kuball et al. 2007; Thomas et al. 2007). By now, the efficiency of TCR pairing remains unknown. As there is only one germline gene segment encoding for the constant region of the TCR α-chain and only two gene segments encoding for two slightly different constant regions of the TCR β-chain, it is likely that the variable regions of TCR chains mainly contribute to inter-chain affinity and efficient TCR α- and β- chain pairing. Mispairing of exogenous (transduced) and endogenous TCR chains is one of the key concerns in exploiting TCR-transduced T cells for adoptive T cell therapy, as formation of such 'mixed' TCRs not only reduces the expression of the correctly paired transduced TCRs and thereby impairs the efficacy of modified T cells, but also may result in novel TCRs with unknown specificities which might result in self-recognition and thereby induce autoimmunity (Fernandez-Miguel et al. 1999). The results in this study show that mispairing can indeed lead to new TCR specificities: transduction of the β-chain derived from TCR R6C12 with specificity for $GP100_{209}$ together with the dominant α-chain HER2-2α showed specific $HER2_{369}$ recognition lacking recognition of $GP100_{209}$. Therefore, if recipient T cells harbour such dominant TCR α-chains, mismatched TCRs composed of dominant α-chains and transduced TCR β-chains might possess specificities for self-antigens and lead to autoreactivity. Although the frequency of such TCR single chains with dominant peptide recognition in combination with diverse β-chains is unknown, it provides a possibility by which mispairing of transduced and endogenous TCR chain can induce unexpected side effects.

Similarly, recently published studies by von Loenen et al. and Bendle et al. demonstrated that mispaired TCRs composed of transduced and enogenous TCR chains can possess new reactivity and induce severe side effects *in vitro* as well as in mice (van Loenen et al. 2010 Bendle et al. 2010). Von Loenen and colleagues demonstrated that introduction of different TCRs targeting hematopoiesis-restricted mHAgs into several virus-specific T cell lines elicits neoreactivity against different lymphoblastoid cell lines (LCLs). The observed neoreactivity of transduced T cells is mediated by mixed TCR dimers, as exclusive introduction of a single TCR α- or β-chains derived from those mHAg-specific TCRs resulted in neoreactivity against LCLs. Bendle et al. observed severe GVHD pathology in recipient mice after adoptive transfer of T cells modified with ovalbumin-specific OT-1 TCR and IL-2 administration,

closely mimicking the clinical TCR gene therapy setting. In contrast, mice receiving either OT-1 transgenic T cells or GFP-transduced T cells under the same condition did not develop lethal autoimmune pathology, and the whole group of treated mice survived, demonstrating that the observed fatal pathology was due to the transduction of TCR genes into the infused T cells. In addition, either recipients of T cells transduced with only OT-1 TCR α-chain or recipients of only TCR β-chain transduced T cells suffered from similar GVHD pathology, and antibody-mediated specific depletion of single TCR chain transduced T cells after infusion significantly reduced the incidence of GVHD pathology. These data demonstrate that the formation of mixed TCRs may induce severe side effects in TCR gene therapy.

Taken together, this study as well as recently published studies show the potential side effects resulting from mismatched TCRs consisting of introduced and endogenous TCR chains. Hence, strategies addressing TCR mispairing are of great importance for TCR gene therapy. Currently, several strategies have been exploited to facilitate matched pairing of transduced TCR chains mainly through improving the preferred interaction of introduced TCR α- and β- chains as described above (section 6.1). Additionally, Okamoto et al. developed a strategy to 'knock down' the expression of endogenous TCR chains by specific siRNA silencing and thereby reduce the mispairing indirectly (Okamoto et al. 2009,). Although all mentioned strategies addressing TCR mispairing show significant efficiency, formation of mixed TCR dimers cannot be absolutely excluded using those approaches. Therefore, if potential $HER2_{369}$ specific TCR candidates such as TCR HER2-1 and the novel TCR chain combination HER2-2α/HER2-4β are further evaluated in the preclinical or clinical settings, possibilities for selective deletion of TCR-transduced PBMC might be considered in order to avoid undesired side effects after adoptive transfer (Kieback et al. 2008): (1) equipment of transduced T cells with suicide genes such as HSV thymidine kinase (HSV-TK) or apoptosis-inducing fusion constructs in combination with activating prodrugs; (2) co-introduction of a defined surface tag such as CD20 surface molecule or a 10-amino acid tag of human c-myc protein (directly fused to the transduced TCR sequence) that can be targeted by specific antibodies.

As a matter of fact, in addition to the mispairing of introduced and endogenous TCR chains, there are other potential mechanisms, by which TCR gene therapy might be associated with side effects. Firstly, activation of ignorant self-reactive T cells either through ex vivo stimulation during transduction or by *in vivo* triggering via the introduced TCRs could lead to the expansion and differentiation of autoreactive cells, inducing 'off-target' autoimmunity (Bendle et al. 2009). Secondly, recognition of allogeneic MHC molecules, which are absent in the original donors from which the TCR were derived, could result in 'off-target' autoimmune damage. To date, neither mice models nor clinical trials have provided evidence that these two mechanisms represent a substantial reason for safety concerns. Thirdly, if an

overexpressed TAA was chosen as target, normal tissues expressing such TAA in low level could be recognized by high-avidity TCR-transduced T cells, resulting in 'on-target' autoimmune toxicity (Offringa 2009). Unexpected destruction of normal melanocytes in the eye and ear, which was observed in a clinical trial targeting overexpressed MART1 and GP100 in melanoma patients (Johnson et al. 2009), as well as inhibited growth of HLA-A2$^+$ PBMC in culture after retroviral transfer of TCR HER2-2α/HER2-4β might be explained by this mechanism, whereas direct evidence is still lacking. Hence, the choice of the TAA for targeting and a suitable avidity of the TCR-transduced T cells used for transfer are of great importance for TCR gene therapy. Finally, cellular transformation caused by retroviral insertional mutagenesis after TCR gene transfer could lead to leukemia and thereby represents a more general safety concern of TCR gene therapy. Such side effects were observed in preclinical (Li et al. 2002) and clinical studies (Hacein-Bey-Abina et al. 2008) using hematopoietic stem cells genetically modified with retroviral vectors. However, no case of leukemia resulting from insertional mutagenesis after TCR gene transfer into mature T cells has been reported by now. In addition, Newrzela et al. have shown in a mouse model that the genotoxic risk of retroviral gene transfer is substantially lower for mature T cells in compared to hematopoietic stem cells (Newrzela et al. 2008). Considering these potential risk factors in TCR gene transfer, safety strategies which enable the selective depletion of the transferred T cells are essential for TCR gene therapy.

6.4 Functionality of T cells transduced with HER2$_{369}$- or FMNL1-PP2-specific allorestricted TCRs *in vivo*

As the HER2$_{369}$-specific allorestricted TCR HER2-1 as well as the chimeric TCR chain combination HER2-2α/HER2-4β exerts specific recognition of HER2$_{369}$ peptide and tumor cells naturally expressing HER2/neu *in vitro*, I have also assessed the anti-tumor reactivity of these two TCRs *in vivo*. Immunodeficient NOD/SCID mice receiving either TCR HER2-1 or HER2-2α/HER2-4β transduced PBMC one day after inoculation of MCF7 tumor cells showed significantly prolonged survival compared to control mice treated with GFP-transduced PBMC in one experiment performed. However, considering that only few T cells within the TCR-transduced bulk population expressed the HER2$_{369}$-specific TCRs and that generally shorter survival was observed in these mice compared to that observed during the establishment of the *in vivo* tumor model, it is not possible to evaluate the contribution of the HER2$_{369}$-specific TCR-transduced T cells to the improved survival in this experiment. Therefore, TCR-transduced T cells with sufficient expression of the specific TCR are required for future experiments *in vivo*. Moreover, survival and functionality of adoptively transferred T cells *in vivo* have not been investigated in this experiment whereas comparable studies were

not able to provide evidence for human T cells survival *in vivo* (data not shown). Thus, to prove any contribution of TCR-transduced T cells to the increased survival in this setting, it would be essential to analyze the survival of the transferred T cells and the migration of the transferred T cells into the tumor sites in tumor-bearing mice by using flow cytometry or immunohistrochemistry.

On the other hand, all NOD/SCID mice receiving MCF-7 tumor cells i.p. developed severe hemorrhagic ascites and peritoneal carcinosis and were used as *in vivo* tumor model. However, one drawback of this tumor model is that it is inconvenient to determine the location and size of the tumor, which renders the assessment of anti-tumor efficacy of TCR-transduced T cells regarding inhibition of tumor growth and migration of T cells into the tumor site difficult. Although no visible tumors grew in NOD/SCID mice after inoculation s.c. of MCF-7 tumor cells, it has been reported that co-inoculation s.c. of matrigel, an extract of basement membrane proteins, facilitates the tumorigenicity of MCF-7 tumor cells in nude mice (Noel et al. 1992). Therefore, it might be meaningful to test the feasibility of the establishment of MCF-7 tumors s.c in NOD/SCID mice with matrigel co-inoculation and to assess the anti-tumor reactivity of $HER2_{369}$-specific TCR-transduced T cells in this model.

Similarly, the FMNL1-specifc allorestricted TCR SK22 demonstrating peptide recognition and anti-tumor reactivity *in vitro* (Schuster 2008) was functionally investigated *in vivo*. NOD/SCID mice treated with TCR SK22-transduced PBMC one day after inoculation of $FMNL1^+$ BJAB tumor cells survived marginally longer compared to control mice treated with GFP-transduced PBMC, which was not statistically significant. In fact, only few T cells expressing TCR SK22 were present within the TCR-transduced PBMC population (2.5%) used for transfer. This might have a major impact on the result of this experiment, because it has been reported that the precursor frequency of transduced TCR-expressing T cells within the cell population used for transfer is of importance for effective anti-tumor response of T cells *in vivo* (de Witte et al. 2008b). Hence, further experiments using higher frequencies of TCR SK22-transduced T cells are required to accurately assess the functionality of this TCR *in vivo*. Importantly, similar to the $HER2_{369}$-specific TCRs as mentioned above, determination of the survival of transferred T cells *in vivo* as well as of the migration of TCR SK22-transduced T cells into tumor sites is also of great importance for assessment of the anti-tumor response of TCR SK22 after transduction in PBMC *in vivo*. In addition, the growth of ovarian tumors derived from BJAB cells is not measurable in this model by extern observation and depends on the gender of the recipient mice. Considering that, it might be meaningful to establish BJAB tumors using i.p. inoculation, which was shown to result in palpable peritoneal tumors in nude/SCID mice (Secchiero et al. 2010), to practically optimize the experimental setting.

Besides the precursor frequency of transduced-TCR expressing T cells within the graft, there are other factors that have been reported to contribute greatly to the anti-tumor efficacy of

TCR-transduced T cells *in vivo*: (1) the number of TCR-modified T cells that are transferred and (2) the expansion and persistence of infused T cells (de Witte et al. 2008b). Hence, the amount of T cells used for infusion should be assessed in further experiments to achieve effective anti-tumor response *in vivo*. In respect to expansion and persistence of infused T cells, in clinical trials administration of IL-2 has been shown to effectively enhance the proliferation and survival of transferred T cells in the patients (Yee et al. 2002). Furthermore, vaccination with antigens recognized by the TCR-transduced T cells (de Witte et al. 2008a) and host pre-conditioning in the form of non-myeloablative sublethal total body irradiation (Morris et al. 2005) or myeloablative lymphodepleting chemotherapy (Morgan et al. 2006) have also been utilized to promote proliferation of infused T cells *in vivo*. The underlying mechanisms for the beneficence from lymphodepletion might rely on the increased availability of cytokines such as IL-7 and IL-15 and the decreased amount of T_{reg} cells (Muranski et al. 2006). As NOD/SCID mice utilized in our experiment are immunodeficient, lymphodepletion conditioning might be less beneficial in this case. However, additional strategies including IL-2 administration and vaccination could be beneficial to facilitate the expansion and long-term persistence of T cells transduced with $HER2_{369}$- or FMNL1-PP2-specific TCRs and enhance anti-tumor efficacy *in vivo*.

Moreover, conventional T cells are classified into several subtypes including naive T cells (T_N) and antigen experienced central memory T cells (T_{CM}) and effector memory T cells (T_{EM}), which demonstrate distinct phenotypic and functional characteristics. These intrinsic properties of the recipient T cells used for TCR transduction have also an impact on the fate of the TCR-transduced T cells *in vivo* (Sallusto et al. 2004). In line with this, Riddell and colleagues investigated the persistence and function of CMV-specific $CD8^+$ T cells derived from either T_{CM} or T_{EM} compartments in a nonhuman primate model and observed that T_{CM} derived $CD8^+$ T cells demonstrated significantly longer persistence (more than 11 months after the infusion) with high frequencies in lymphoid organs when compared to the T_{EM} derived counterpart (less than 14 days) and induced an effective response to antigen challenge *in vivo* (Berger et al. 2008). Therefore, utilization of T_{CM} as target cells for TCR transduction might be an attractive strategy to improve anti-tumor effectiveness of the potential TCR candidates *in vivo*.

6.5 Monitoring of TCR-transduced T cells after transfer

To non-invasively monitor TCR-transduced T cells after infusion for a longer period of time using PET/CT imaging, a bicistronic lentiviral vector (pHIV7SF-hNIS-T2A-mod HER2-1), containing genes encoding for the $HER2_{369}$-specific TCR HER2-1 chains and the reporter gene encoding for the transmembrane protein hNIS, was constructed. hNIS can incorporate

radioactive tracers such as $^{124}I^-$ and allow the detection of T cells by PET imaging. TCR HER2-1 as well as hNIS protein was successfully expressed on the cell surface of J76CD8 cells and PBMC after lentiviral transduction. However, the proportion of cells expressing the TCR HER2-1 and hNIS was low in both recipient cell types. By contrast, lentiviral transduction using the control vector pLKO.1-puro CMV-TurboGFP™ (Sigma, Taufkirchen) resulted in a high proportion of GFP^+ CD8J76 cells (91.8%, data not shown). Since the identical packaging plasmid mixture containing the minimal set of lentiviral genes, which encode the indispensible proteins for the viral structure and packaging function, were used during lentiviral transfer of hNIS/TCR HER2-1 or GFP genes in the same procedure, the low expression of hNIS or TCR HER2-1 likely lies in the lentiviral vector pHIV7SF-hNIS-T2A-mod HER2-1.

In detail, following factors might be responsible for the limited gene expression: (1) The relatively big size of the lentiviral vector containing hNIS and TCR HER2-1 chain genes (approximately 11 kb) compared to GFP-containing control vector (approximately 8 kb) might render transfection of the bicistronic lentiviral vector into the packaging cell line HEK 293T inefficient, which results in a low virus titer in the supernatant used for transduction; (2) the expression of the multiple introduced genes at the transcriptional and (or) translational level might be low, which might be associated to construct elements such as promoters and (or) self-cleaving processing post-translation. The determination of the respective virus titer using HIV p24 antigen ELISA Kit (Zeptometrix, New York, USA) might give a hint if the potential factors contributing to low expression lie in the procedure of transfection or transduction. Additionally, an increase of the total amount of vectors used for transfection and the adjustment of the ratio of the lentiviral vector to the packaging plasmid might improve the efficiency of transfection and thereby the virus titer. On the other hand, Yang and co-workers have demonstrated that lentivirus-mediated TCR gene expression can be significantly optimized by addition of amino-acid spacer sequences (GSG or SGSG) before the 2A sequence and by addition of a furin cleavage site followed by a V5 peptide tag (Yang et al. 2008). Hence, introduction of such modifications into our lentiviral vectors might be also beneficial to enhance the expression of hNIS and TCRs in recipient cells. Alternatively, hNIS- and TCR-expressing T cells after transduction even with low efficiency might be enriched by flow cytometric sorting using anti-hNIS antibody and thereafter be used for adoptive transfer into mice for further experiments.

Successful monitoring of anti-tumor efficacy of transgenic OT-1 T cells *in vivo* using PET/CT imaging as previously published (Su et al. 2006) provides the possibility to non-invasively assess anti-tumor reactivity and migration of T cells transduced with $HER2_{369}$- or FMNL1-PP2-specific allorestricted TCRs *in vivo*. The optimization strategies for predictable and measurable tumor engraftment (discussed in section 6.4) as well as for enhancement of

hNIS and TCR expression as mentioned above may render the assessment of TCR-transduced T cells using PET/CT easier and more effective. However, several parameters such as the uptake of the radioactive tracer by hNIS-expressing T cells *in vivo* and the sensitivity of PET/CT imaging with the hNIS-reporter gene need be determined before the functional assessment of TCR-transduced T cells *in vivo*.

6.6 Potential of $HER2_{369}$- or FMNL-PP2-specific allorestricted TCRs in clinical application

In vitro, $HER2_{369}$-specific TCR HER2-1 showed specific peptide-recognition and anti-tumor reactivity. In addition, no significant crossreactivity to non-transformed cells was observed. *In vivo*, tumor-bearing SCID mice treated with TCR HER2-1-transduced PBMC showed an increased survival when compared to control mice treated with GFP-transduced PBMC. However, these experiments are very preliminary and several factors challenge these results: (1) Survival and functionality of T cells in vivo has not been demonstrated yet whereas data from comparable experiments were not able to show survival of human T cells in mice; (2) Low frequency of $HER2_{369}$-specific TCR expressing T cells within the TCR-transduced bulk population and (3) relatively shorter survival of tumor-bearing mice compared to that observed during the establishment of tumor model. Thus, the mouse model first need to be further established in order to evaluate the anti-tumor efficacy of these TCRs *in vivo*. The *in vitro* data, however, imply that TCR HER2-1 might be a candidate for TCR gene therapy of HER2/neu$^+$ cancer. As TCR HER2-1 recognizes the epitope $HER2_{369}$ in the context of HLA-A2, this TCR is only suitable for HLA-A2$^+$ patients. Moreover, overexpression of HER2/neu is reported to result in proteasomal dysfunction and therefore reduced peptide presentation on tumor cells (Vertuani et al. 2009). Hence, TCR gene therapy using TCR HER2-1 with intermediate avidity might be more effective in patients with tumors expressing HER2/neu at intermediate and low levels. Furthermore, since slight inhibition of proliferation of HLA-A2$^+$ PBMC after transduction with TCR HER2-1 was observed in culture, the anti-tumor efficacy and persistence of HLA-A2$^+$ PBMC transduced with TCR HER2-1 *in vivo* are required to be determined. Although autologous T cells (i.e. HLA-A2$^+$ T cells derived from the patient) transduced with tumor-specific TCRs principally are used in clinical application (Figure 6.1) and have advantages over their allogeneic counterpart (i.e. HLA-A2$^-$ T cells derived from the donor) for adoptive transfer concerning potential side effects due to mismatched MHC, transfer of allogeneic T cells modified with TCRs might be less problematic, if the crossreactivity and alloreactivity of these T cells is well characterized *in vitro*.

The novel chimeric TCR chain combination HER2-2α/HER2-4β demonstrated also anti-tumor reactivity *in vitro* and potentially also in the mouse model (preliminary data), for which further

experiments are required. As the proliferation of HLA-A2$^+$ PBMC transduced with HER2-2α/HER2-4β was extensively inhibited after transfer of this TCR, HLA-A2$^-$ T cells are more suitable for transduction of this TCR. Moreover, significant background recognition of HLA-A2$^+$ T2 cells unpulsed or pulsed with irrelevant peptides by HER2-2α/HER2-4β-transduced PBMC was observed, suggesting that 'off-target' toxicity resulting from $HER2_{369}$-independent recognition or 'on-target' toxicity resulting from recognition of $HER2_{369}$ presented on normal tissue in low level might occur *in vivo*. Therefore, the application of HER2-2α/HER2-4β in the clinical setting might need more caution.

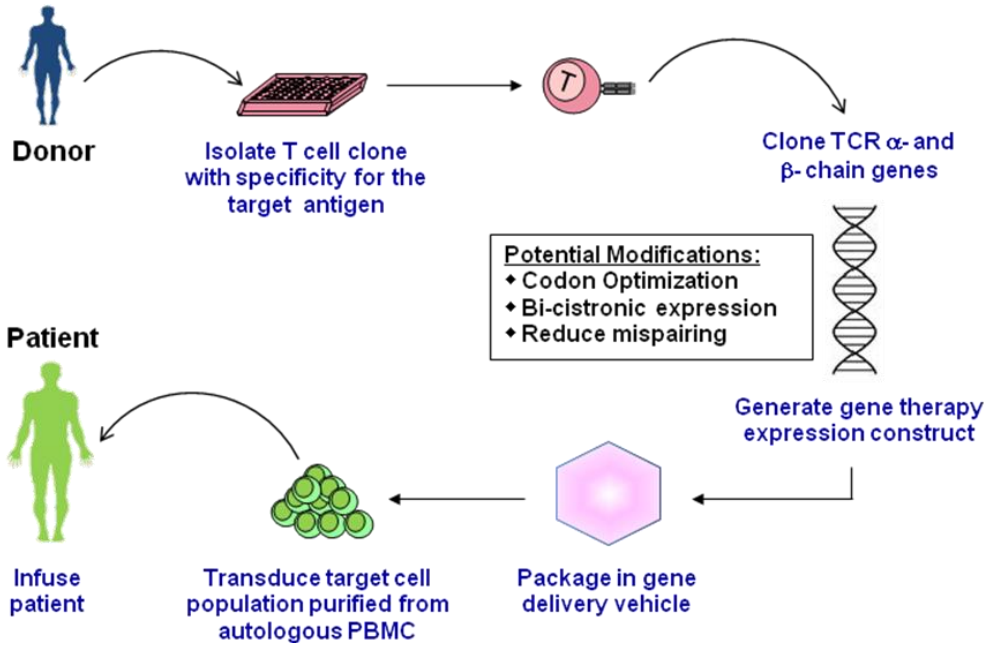

Figure 6.1 Overview of TCR gene therapy in the clinical setting.

A T cell clone harboring a TCR with specificity for the target antigen is firstly isolated. This is often done by culturing TILs or PBMC together with APC pulsed with an epitope in the context of a defined HLA molecule. Thereafter, the TCR α- and β-chain genes of the isolated T cell clone are identified and isolated by molecular cloning. Subsequently, an expression construct containing the TCR α- and β-chain genes of interests is generated. Potential modifications (see section 4.1.3) are introduced into the expression construct to allow stable and high-level expression of both TCR chains in recipient human T cells. The delivery vehicle for TCR genes (generally a γ-retrovirus or lentivirus) is then generated and used to transduce the target T cell population (generally purified from patient PBMC). Finally, transduced T cells are expanded and then transferred into the patient (Modified from Schmitt et al. 2009).

The FMNL1-PP2-specific TCR SK22 exerted specific peptide-recognition as well as anti-tumor reactivity *in vitro* (Schuster 2008). One preliminary mouse experiment demonstrated that tumor-bearing SCID mice treated with TCR SK22-transduced PBMC showed a slightly increased survival when compared to control mice treated with GFP-transduced PBMC,

which was not statistically significant. Again, these experiments are very preliminary and as mentioned above several factors highly challenge these results. As FMNL1 is naturally expressed in hematopoietic-linage cells such as T cells, HLA-$A2^+$ T cells might be potential targets for this TCR and be not suitable to be used for TCR transduction. In addition, TCR SK22 showed crossreactivity against unknown peptides in the context of HLA-A3303 and HLA-A6802 (Schuster et al. 2007). Therefore, TCR SK22 might be therapeutically effective in clinical application by using well characterized donor T cells and recruiting patients with a well-defined MHC pattern.

In conclusion, diverse TCRs with specificity for HER2/neu-derived peptide 369 as potential candidates for TCR transfer were isolated in an allogeneic environment *in vitro*. One TCR (TCR HER2-1) showed preferential characteristics for further investigation regarding clinical application. I additionally identified a single TCR α-chain with dominant $HER2_{369}$ peptide recognition in combination with β-chains of the TRBV12 family derived from TCRs with diverse specificities, demonstrating that pairing with novel partner chains can result in an increase of peptide-specific avidity, CD8 independency and anti-tumor reactivity. Although the frequency of such TCR is currently unknown, they may represent interesting tools for TCR optimization resulting in enhanced functionality when paired to novel partner chains. However, mispairing with novel partner chains may also result in enhanced crossreactivity or self reactivity, and these data further indicate that enhanced efforts for improved chain pairing (Cohen et al. 2007; Sebestyen et al. 2008; Voss et al. 2008) as well as selective deletion of TCR-transduced PBMC (Kieback et al. 2008) may be important. Although preliminary studies in a xenogenic mouse model have been performed using T cells transduced with these TCRs, these experiments do not allow any conclusion at this time point. Future studies using a well established model will need to be performed in order to investigate the efficacy of these TCRs *in vivo*. Finally, the reporter gene hNIS for PET/CT imaging as well as the TCR HER2-1 chain genes were successfully cloned into one lentiviral vector and expressed in recipient cells after lentiviral transfer. However, the expression of both hNIS and TCR HER2-1 was low. Again, further optimization strategies are required to obtain T cells with high expression of these genes and allow the tracing of TCR-transduced T cells after adoptive transfer *in vivo* using PET/CT imaging.

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8 Abbreviations

7-AAD	7- aminoactinomycin D
ACT	Adoptive T-cell therapy
ALL	Acute lymphatic leukemia
AML	Acute myeloid leukemia
APC (Immune system)	Antigen-presenting cells
APC (FACS)	Allophycocyanin
bidest. H_2O	Double-distilled water
BCR	B cell receptor
BFA	Brefeldin A
BLI	Bioluminescence imaging
bp	Base pair
BSA	Bovine serum albumin
C	Constant region in T cell receptor
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	Complementary-DNA
CDR	Complementarity-determining region
C_{end}	End concentration
Ci	Curie
CLL	Chronic lymphocytic leukaemia
^{11}C-MET	Methyl-(carbon-11)-L-methionine
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CPM	Counts per minute
C_{stock}	Stock concentration
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
^{51}Cr	Chromium 51
D	Diversity region in T cell receptor
DC	Dendritic cells
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
D-PBS	Dulbecco’s Phosphate buffered saline
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum

^{18}FDG	2-(fluorine-18)-fluoro-2-deoxy-D-glucose
^{18}FLT	3-deoxy-3-(fluorine-18)-fluorothmidine
FITC	Fluorescein isothiocyanate
FMNL1	Formin-like 1
FSC	Forward Scatter (FACS)
GFP	Green fluorescent protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factory
GvHD	Graft-versus-Host-Disease
GvL	Graft-versus-leukemia
Gy	Gray
HBS	HEPEs buffered saline
HEK 293T	Human embryonic kidney cell line 293T
HIV	Human immunodeficiency virus
HLA	Human Leukocyte antigen
hNIS	Human sodium-iodide symporter
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HS	Human serum
IFN	Interferon-γ
IMDM	Iscove's Modified Dulbecco's Medium
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
J	Joint Region in T cell receptor
LCL	Lymphoblastic cell line
LPS	Lipopolysaccharide
mAb	monoclonal antibody
MBq	Megabecquerel (1Ci=37000MBq)
mHAg	Minor histocompatibility antigen
MHC	Major histocompatibility complex
min	Minute
Mio	Million
MPSV	Myeloproliferative sarcoma virus
MRA	Mycoplasma-Removal Agent
NHL	Non-Hodgkin lymphoma
NK	Nature killer cell
NOD/SCID	Non-obese diabetes/ Severe combined immunodeficiency
NPBC	Node-positive breast cancer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PET	Positron emission tomography
PFA	Paraformaldehyde
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
RCC	Renal-cell carcinoma
RNA	Ribonucleic acid
rpm	Rounds per minute

RPMI 1640	Roswell Park Memorial Institute Medium 1640
RT	Room temperature
RTK	Receptor tyrosine kinase
s	Second
scTCR	Single chain TCR
SEREX	Serological identification of antigens by the recombination expression cloning
SSC	Side scatter (FACS)
svFv	Single-chain variable fragment
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
T_H	Helper T cells
T_N	Naive T cells
TAA	Tumor-associated antigens
TAE	Tris-acetate-EDTA buffer
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TIL	Tumor-infiltrating lymphocyte
TNFα	Tumor-necrosis factor α
T_{reg}	Regulatory T cells
U	Unit
UV	Ultraviolet
V	Variable region in T cell receptor
β_2m	β_2-microglobulin
Δ	Heat-inactivated

9 Acknowledgement

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10 Curriculum Vitae

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EDUCATION

01/2010 – present	**PhD student** in III. Medicine department, Technical University Munich (the same project) Supervisor: Prof. Dr. med. Angela Krackhardt External promotion in faculty of WZW, Technical University Munich Supervisor: Prof. Dr. Iris Antes Title of PhD thesis: Functional characterization of allorestricted T cell receptors (TCRs) with specificity for Her2/neu and FMNL1 *in vitro* and *in vivo* for potential clinical application.
12/2007 – 12/2009	PhD student at Institute of Molecular Immunology, Helmholtz Zentrum München – German Research Center for Environmental Health Supervisor: Prof. Dr. med. Angela Krackhardt
10/2005 – 10/2007	**Postgraduate student** at department of Biology, Faculty of WZW, Technical University Munich Majors: Biochemistry, Microbiology, and Immunology Title of Master thesis: Structural and functional characterization of the TCR repertoire of $Her2_{369}$-specific allorestricted T cells
09/2001 – 07/2005	**Bachelor student** at department of Biology science, College of life science, Zhejiang University in Hangzhou, China Title of Bachelor thesis: The function of *Caenorhabditis elegans* anti-apoptosis gene *ced-9* in tobacco and *Arabidopsis.*
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PUBLICATIONS

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A Single TCR α-Chain with Dominant Peptide Recognition in the Allorestricted HER2/neu-Specific T Cell Repertoire
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Formin-like 1 (FMNL1) is regulated by N-terminal myristoylation and induces polarized membrane blebbing.
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11 Erklärung

Ich erkläre an Eides statt, dass ich die der Fakultät für

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Functional characterization of allorestricted T cell receptors (TCRs) with specificity for HER2/neu and FMNL1 *in vitro* and *in vivo* for potential clinical application

in **Fachgebiet für Protein Modelling**

unter der Anleitung und Betreuung durch

Univ.-Prof. Dr. Iris Antes

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Unterschrift

A Single TCRα-Chain with Dominant Peptide Recognition in the Allorestricted HER2/neu-Specific T Cell Repertoire

Xiaoling Liang,*,[1] Luise U. Weigand,*,[1] Ingrid G. Schuster,* Elfriede Eppinger,* Judith C. van der Griendt,* Andrea Schub,†,‡ Matthias Leisegang,§ Daniel Sommermeyer,§ Florian Anderl,¶ Yanyan Han,* Joachim Ellwart,* Andreas Moosmann,†,‡ Dirk H. Busch,¶,‖,# Wolfgang Uckert,§, Christian Peschel,†† and Angela M. Krackhardt*,††**

T cells can recognize tumor cells specifically by their TCR and the transfer of TCR-engineered T cells is a promising novel tool in anticancer therapies. We isolated and characterized four allorestricted TCRs with specificity for the HER2/neu-derived peptide 369 ($HER2_{369}$) demonstrating high peptide specificity. PBMCs transduced with especially one TCR, HER2-1, mediated specific tumor reactivity after TCR optimization suggesting that this TCR represents a potential candidate for targeting HER2 by TCR-transduced effector cells. Another TCR showed high-peptide specificity without tumor reactivity. However, the TCRα-chain of this TCR specifically recognized $HER2_{369}$ not only in combination with the original β-chain but also with four other β-chains of the same variable family deriving from TCRs with diverse specificities. Pairing with one β-chain derived from another $HER2_{369}$-specific TCR potentiated the chimeric TCRs in regard to functional avidity, CD8 independency, and tumor reactivity. Although the frequency of such TCR single chains with dominant peptide recognition is currently unknown, they may represent interesting tools for TCR optimization resulting in enhanced functionality when paired to novel partner chains. However, undirected mispairing with novel partner chains may also result in enhanced cross-reactivity and self-reactivity. These results may have an important impact on the further design of strategies for adoptive transfer using TCR-transduced T cells. ***The Journal of Immunology*****, 2010, 184: 1617–1629.**

Adoptive transfer of TCR-transduced effector cells represents an interesting novel strategy to treat a variety of viral and malignant diseases by specific targeting. This therapy has already been applied in the clinic, demonstrating feasibility of this approach (1, 2). However, the outcome of the first trial (1) showed limited antimelanoma reactivity when compared with the application of tumor-infiltrating lymphocytes potentially because of the intermediate avidity of the TCRs used for transfer. TCRs with higher avidity for a tumor-associated self-Ag might be rarely isolated from autologous T cells, but rather be selected from T cells that recognize their target peptide in the context of a foreign MHC molecule or recognize allogeneic or xenogeneic peptides (3–6). In fact, in the second clinical trial (2) two TCRs with specificity for MART1 and GP100 exhibiting higher avidity were transduced into PBMCs. This trial demonstrated improved response rates compared with the first trial. However, unexpected oculotoxicity and ototoxicity occurred, likely originating from specific targeting of MART1 and GP100 in these tissues. Recognition of low amounts of peptide on normal tissue therefore represents a major concern and these results emphasize the delicate choice of target Ag to be selected for this approach. Moreover, additional mechanisms may be involved in unexpected self-reactivity using TCR-transduced T cells. Introduced TCRα- and TCRβ-chains can pair with endogenous TCR chains potentially resulting in self-reactive T cells (3, 7). In addition, potential intrinsic cross-reactivity, alloreactivity, and xenoreactivity of each TCR candidate must be carefully evaluated, as these TCRs have not been negatively selected for the MHC environment of the novel host. Thus, TCRs with multiple specificities for diverse classes of tumor-associated Ags need to be investigated to further explore mechanisms involved in target specificity and potential self-reactivity to better evaluate the potential of this therapeutic approach for a broader application.

We previously identified an allorestricted T cell clone with specificity for the FMNL1-derived peptide PP2 (8). This clone recognizes the FMNL1-PP2 peptide with high specificity in the context of HLA-A2, but also demonstrates a defined cross-reactivity against an unknown peptide presented in the context of HLA-A*3303. We similarly targeted the well-described tumor-associated Ag, HER2/neu (HER2), which is overexpressed in diverse malignancies (9, 10). We isolated and characterized several allo-HLA-A2–restricted T cell clones and TCRs with specificity for the HER2-derived peptide 369 ($HER2_{369}$). PBMCs transduced with these TCRs demonstrated high peptide-specificity and some tumor reactivity. One TCR specifically recognized endogenously processed HER2 representing a potential candidate for further evaluation for clinical studies. Moreover, investigation of mixed chain chimeras revealed one α-chain derived from a $HER2_{369}$-

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The online version of the article contains supplemental material.

Abbreviations used in this paper: HER2, HER2/neu; J76, Jurkat 76; J76CD8, J76 transduced with CD8α; Mod, modified; NCBI, National Center for Biotechnology Information; TRBV, TCR gene variable β-chain; WT, wild type.

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specific TCR, recognizing the specific peptide in combination with β-chains derived from TCRs with diverse specificities. Importantly, one novel combination of TCRαβ-chains derived from two $HER2_{369}$-specific TCRs primarily lacking tumor reactivity resulted in enhanced functional avidity, CD8 independency, and tumor-target recognition. These data may have a significant impact on the further development of adoptive T cell therapies using TCR-transgenic T cells.

Materials and Methods

Cells and cell lines

PBMCs from healthy donors were collected with donors' informed consent according to the requirements of the local ethical board and the principles expressed in the Helsinki Declaration. PBMC subpopulations from healthy donors were isolated by negative or positive magnetic bead depletion (Invitrogen, Karlsruhe, Germany), and high purity was confirmed by flow cytometric analysis. The T2 cell line that is a somatic cell hybrid of human B- and T-lymphoblastoid cell lines (ATCC CRL-1992, Manassas, VA) has been reported to be defective in TAP molecules and to be deficient in peptide presentation (11). However, T2 cells have been demonstrated to express HLA-A2 at some level and to present a limited set of endogenous peptides (12). Peptide-pulsed T2 cells were used for priming and restimulation of HLA-A2–negative T cells (8). The TCR-deficient T cell line Jurkat76 (J76) (13) and J76 transduced with CD8α (J76CD8) cells were used for TCR-transfer experiments. The following malignant cell lines were used as targets to test tumor reactivity and cross-reactivity: HLA-A2–positive breast carcinoma cell lines MCF-7 (ATCC HTB-22) and MDA-MB 231 (CLS, Eppelheim, Germany), the HLA-A2–negative ovarian cancer cell lines SKOV3 and SKOV3 transfected with HLA-A2 (SKOV3tA2) (kindly provided by H. Bernhard, Munich, Germany) (14), the HLA-A2–positive melanoma cell lines SK-Mel 29, 624.38MEL (kindly provided by E. Noessner, Munich, Germany), wild-type (WT) K562 (ATCC CCL-243), HLA-A2–positive 143 TK^- lung fibroblasts (kindly provided by R. Mocikat, Munich, Germany), MRC-5 lung fibroblasts (CCL-171), and the human B cell lines C1R untransfected and transfected with HLA-A*0201 (kindly provided by S. Stevanovic, Tübingen, Germany) (15). CIR cells transfected with HLA-A*0201 and HER2, as well as fetal cardiomyocytes, were kindly provided by J. Charo (Berlin, Germany) (16).

Peptides

We used the following peptides for pulsing of APCs: the HLA-A2–restricted HER2 369–377 (KIFGSLAFL) (17), single amino acid analog peptides of HER2 369–377 substituting all amino acids at all positions by either alanine or threonine, the HER1-derived peptide 364–372 (SISGDLHIL) (14), the HER3-derived peptide 356–364 (KILGNLDFL) (14), the HER4-derived peptide 361–369 (KINGNLIFL) (14), the HLA-A2–restricted influenza matrix peptide MP58 (GILGFVFTL) (18), the HLA-A2–restricted tyrosinase-derived peptide 369–377 (YMNGTMSQV) (19), the Formin-related protein in leukocytes (FMNL1)-derived HLA-A2–binding peptide PP2 (RLPERMTTL) (8), the HDAC6-derived peptide (RLAERMTTR) (8), the HLA-A2–restricted GP100-derived peptide 209–217 (ITDQVPFSV) (20), and the CMV-phosphoprotein (pp) 65-derived HLA-A2–restricted peptide 495–503 (NLVPMVATV) (21). Peptides were synthesized by standard fluorenylmethoxycarbonyl (Fmoc) synthesis (Biosyntan, Berlin, Germany). Purity was above 90% as determined by reverse phase HPLC and verified by mass spectrometry.

MHC-peptide multimers and Abs

MHC-peptide tetramers and streptamers (multimers) detecting T cells with specificities for HLA-A2–$HER2_{369}$, HLA-A2–Flu (MP58), and HLA-A2–$GP100_{209}$ were synthesized as previously reported and used for detection and sorting of specific TCRs (22, 23). Streptamers were only used once for sorting of $HER2_{369}$-specific T cells before T cell cloning, resulting in isolation of HER2-3. For selecting $HER2_{369}$-specific T cells, multimer staining assays were performed essentially as previously described (24). The following Abs were used to characterize PBMC-derived cells, primary tumor cells, and malignant cell lines: anti-CD3–FITC (UCHT1, BD Diagnostic Systems, Heidelberg, Germany), anti-CD4–FITC (RPA-T4, BD, Heidelberg, Germany), anti-CD8–FITC (V5T-HIT8a, BD Diagnostic Systems), anti-CD8–PE and –APC (RPA-T8, BD), anti-IFN-γ–FITC (25723.11, BD Diagnostic Systems), anti-CD19–FITC and –PE (HIB19, BD Diagnostic Systems), anti-CD14–PE (M5E2, BD Diagnostic Systems), anti-CD56–PE (B159, BD Diagnostic Systems), anti-HLA-A2–FITC (BB7.2, ATCC), anti-αβ-TCR–FITC (T10B9.1A-31, BD Diagnostic Systems), anti-TCR gene variable β-chain (TRBV)12 Ab (IM1233, Beckman Coulter, Krefeld, Germany), and anti-TRBV27 Ab (CAS1.1.3, Beckman Coulter).

Table I. *Peptide recognition and tumor reactivity of allorestricted $HER2_{369}$-specific T-cell clones*

Donor	Clone	Cytotoxicity (%)		
		T2 + $HER2_{369}$	T2 + Flu	SK-Mel29
1	HER2-1	65 ± 6.2	8 ± 7.6	21 ± 8.2
1	HER2-2	60 ± 4.7	0 ± 0.4	1 ± 0.9
2	HER2-3	64 ± 6.5	6 ± 0.9	29 ± 1.3
1	HER2-4	79 ± 4.2	2 ± 2.1	1 ± 0.0

CTLs

CTLs were generated from PBMCs using peptide-pulsed T2 cells for specific stimulation. T2 cells were pulsed with specific peptides (10^{-5} M and 10^{-7} M) and used for CTL priming at stimulator/effector cell ratios of 1:10 and for restimulation at stimulator/effector cell ratios of 1:100. Cytokines were added as follows: IL-2 (50 U/ml) (Chiron Vaccines International, Marburg, Germany), IL-7 (10 ng/ml) (Peprotech, London, U.K.), and IL-15 (10 ng/ml) (Peprotech). Peptide-specific T cells were detected by flow cytometry using PE-conjugated peptide-presenting HLA-A2 multimers and sorted by a high-performance cell sorter (MoFlo, Dako, Hamburg, Germany). Sorted cells were cloned by limiting dilution and nonspecifically restimulated every 2 wk using pooled allogeneic irradiated PBMCs, together with anti-CD3 Ab (OKT3), IL-2, IL-7, and IL-15.

Functional assays

Primary clones were tested for their specificity by standard ^{51}Cr-release assay as previously described (8). TCR-transduced PBMCs were analyzed for their specificity by cytokine release. For stimulation assays, effector and target cells were incubated for 24 h at E:T ratio = 5:1 using 50,000:10,000 cells if not otherwise indicated. Supernatants were collected and the presence of IFN-γ was analyzed by ELISA (BD Diagnostics Systems), according to the recommendations of the manufacturer. In addition, the specificity of IFN-γ–producing TCR-transduced $CD8^+$ T cells was determined by surface multimer and β-chain staining in combination with intracellular staining of IFN-γ. Effector and target cells were cocultivated at E:T ratio = 1:1 for 6 h in the presence of brefeldin A (BD Diagnostics Systems). Subsequently, TCR-transduced $CD8^+$ T cells were at first stained with surface Ab or multimers as indicated, followed by fixation with 1% paraformaldehyde A (Sigma-Aldrich, Munich, Germany) and permeabilization with serum-supplemented PBS containing Saponin (Sigma-Aldrich). Intracellular staining of IFN-γ was performed using the anti-human IFN-γ–FITC (25723.11, BD Diagnostics Systems) Ab. Stained samples were analyzed using the LSRII flow cytometer (BD Diagnostics Systems).

Table II. *Variable and joining region family affiliation and CDR3 sequences of $HER2_{369}$-specific and control TCR*

Clone	Specificity	TRAV	TRAJ	CDR3	NCBI	TRBV	TRBJ	TRBD	CDR3	NCBI
HER2-1	HER2 (369)	19*01	24*02	CALYTTDSWGKLQF	FJ795357	12-3*01	2-3*01		CASSFVLGDTQYF	FJ795358
HER2-2	HER2 (369)	27*01	20*01	CAGVPSNDYKLSF	FJ795359	12-3*01	2-7*01	2*02	CASSPPLGSGIYEQYF	FJ795360
HER2-3	HER2 (369)	38-1*01	28*01	CAFIDSGAGSYQLTF	FJ795361	7-8*01	2-7*01	2*01	CASSLAADEQYF	FJ795362
HER2-4	HER2 (369)	21*01	20*01	CAVRPQNDYKLSF	FJ795363	12-3*01	2-1*01	2*01	CASSSWTSGDEQFF	FJ795364
R6C12 (33)[a]	GP100(209)	41*01	54*01	CAASLIQGAQKLVF	—	12-3*01	2-1*01	2*01	CASSPGGNEQFF	—
JG-9 (32)[a]	pp65(495)	35*02	50*01	CAGPMKTSYDKVIF	FJ795368	12-4*01	1-2*01	1*01	CASSSANYGYTF	FJ795367
SK22 (8)[a]	FMNL1-PP2	38-2/DV8*01	41*01	CAYENSGYALNF	FJ795365	27*01	2-5*01	2*01	CASSFLGETQYF	FJ795366

[a]These TCRs have been previously described as indicated.
NCBI, National Center for Biotechnology Information.

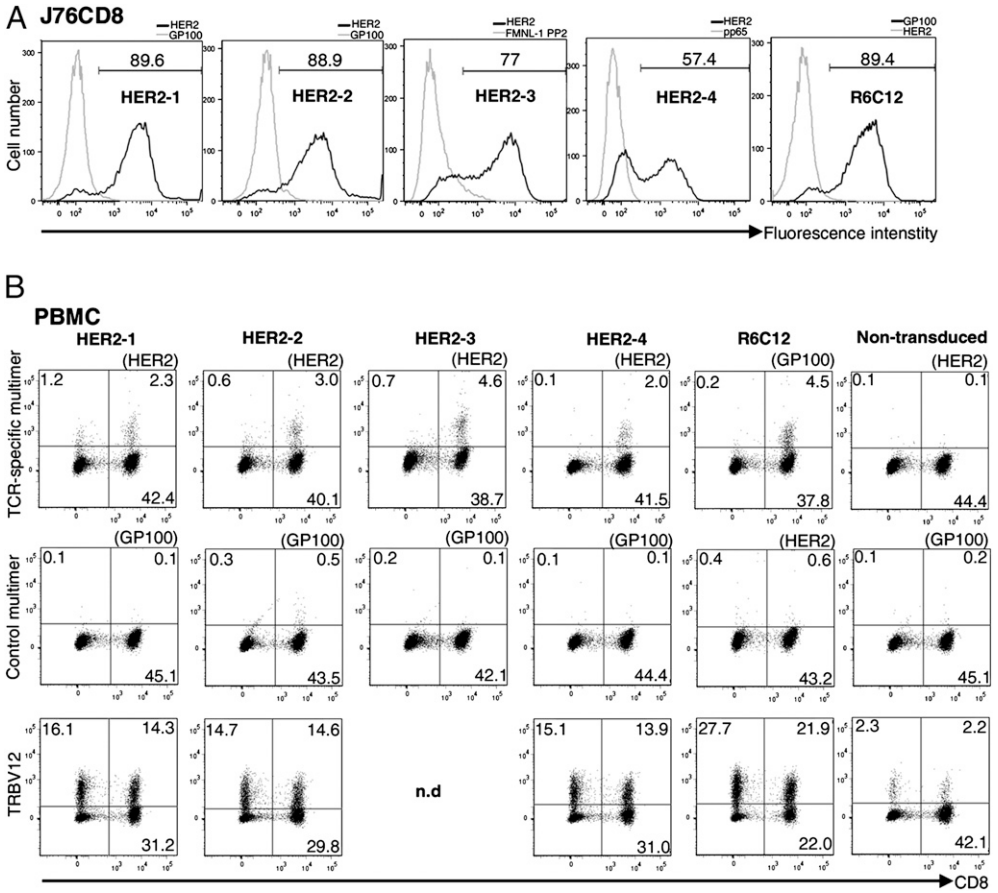


FIGURE 1. $HER2_{369}$-specific TCRs are expressed after retroviral gene transfer. *A*, TCRα- and β-chain genes of the $HER2_{369}$-specific TCR HER2-1, HER2-2, HER2-3, and HER2-4 as well as the control TCR R6C12 with specificity for $GP100_{209}$ were retrovirally transduced into J76CD8 and analyzed by flow cytometry 4 d after transduction. Transduced cells were analyzed for specific TCR expression by staining with the specific multimer (thick line) as well as control multimers (thin line). *B*, Similarly, single TCR chains of $HER2_{369}$-specific TCR and control TCR were retrovirally transduced into PBMCs and stained with the specific multimer (*first panel*), the control multimer (*middle panel*), as well as the specific TRBV Ab (as available) 4 d after transduction. Nontransduced PBMC were used as control.

TCR analysis

TCR-PCR analysis of $HER2_{369}$-specific T cell clones was performed as previously described (25). Total RNA from T cell clones and lines was extracted according to the manufacturer's recommendation (Trizol reagent, Invitrogen). cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primers. Subfamily-specific TCR-PCR was performed using 34 Vα and 37 Vβ primers, followed by gel isolation (NucleoSpin, Macherey-Nagel, Düren, Germany) and direct DNA sequencing of the amplified products. The TCR nomenclature was used according to International Immuno-Genetics Database (26). Sequences of isolated $HER2_{369}$-specific and selected control TCR (Table II) have been deposited in Genbank, National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/Genbank/).

Cloning of the $HER2_{369}$-specific TCRs

TCR cloning was performed as previously described (27). Shortly, the specific TCRα- and β-chain coding cDNA of the allo-HLA-A2–restricted T cell clones were amplified from isolated T cell clones using variable chain-specific oligonucleotides containing a NotI restriction site (Supplemental Table I). In addition, constant chain-specific primers containing an EcoRI restriction site were selected (Supplemental Table I). The TCR genes were first cloned separately as single TCR genes into the retroviral vector pMP71-PRE. Murinization of the constant chains (28) and codon optimization of the whole murinized TCRs (Geneart, Regensburg, Germany) (29, 30) were performed as indicated. In addition, bicistronic constructs with TCR single-chain genes separated by the picorna virus-derived peptide element P2A were cloned as previously described (31). A GFP-encoding MP71 vector was used as a mock control. All TCR cassettes used in this study were verified by sequence analysis (Eurofins, Ebersberg, Germany). The following TCRs were used as control TCRs: The FMNL1-specific TCR SK22 (8), the CMV-specific TCR JG-9 with specificity for the CMVpp65-derived HLA-A2–restricted peptide NLVPMVATV (32) and the previously described $GP100_{209}$-specific TCR derived from clone R6C12 (33).

Retroviral transfer into PBMCs

The TCR-containing retroviral vector plasmids pMP71-TCR-PRE were cotransfected with plasmids harboring retroviral genes for gag/pol derived from Moloney murine leukemia virus (pcDNA3.1-Mo-MLV) and env (pALF-10A1) into 293 T cells by calcium phosphate precipitation to generate amphotropic vector particles (31). PBMCs were activated for 2–3 d with IL-2 (50 U/ml) and OKT3 (50 ng/ml), whereas sorted $CD8^+$ and $CD4^+$ cells were stimulated with IL-2 (50 U/ml), plate-coated OKT3 (5 μg/ml), and CD28 mAb (clone CD28.2, BD, 1 μg/ml). Activated cells were transduced twice with retrovirus-containing supernatant in 24-well nontissue culture plates coated with RetroNectin (Takara, Apen, Germany) containing protamine sulfate (4 μg/ml) and IL-2 (100 U/ml). After addition of retroviral supernatant, the plates were spinoculated with 800 × *g* for 1.5 h at 32°C. Medium was replaced by fresh medium 24 h after second transduction. Transduced PBMCs were cultured with low-dose IL-2 (50 U/ml) every 3 d and analyzed for multimer staining and surface markers, as well as functional assays at different time points after transduction as indicated. PBMCs transduced with a GFP-containing MP71 vector were used as mock control. For analysis of CD8-depleted cell populations after TCR-transfer, cells were stained with anti-CD8 and then sorted by the high-performance cell sorter (MoFlo; Dako) by negative selection. TCR-transduced PBMCs were depleted from $CD8^+$ cells by flow cytometry sorting where indicated and re-stimulated with OKT3 (30 ng/ml), IL-2 (50 U/ml), IL-7 (5 ng/ml), and IL-15 (5 ng/ml) as well as allogeneic PBMCs pooled from three different donors. Cells were used for further analysis 2 wk after sorting

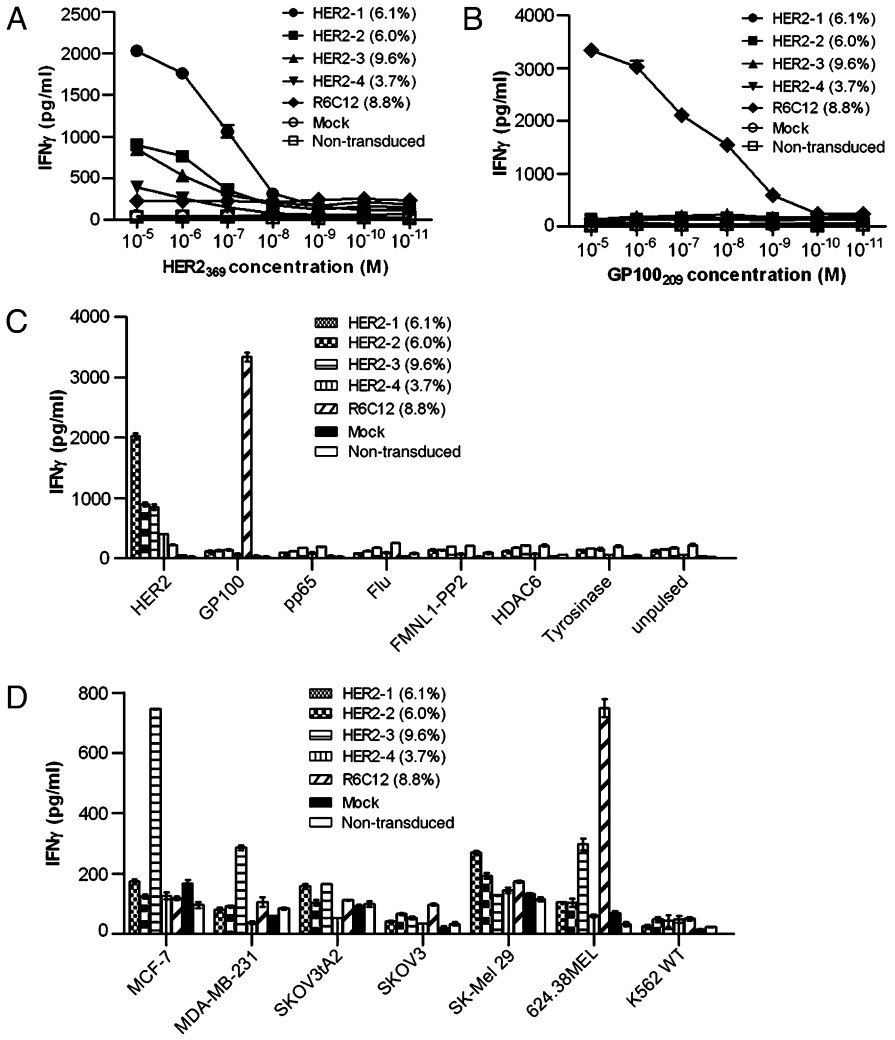


FIGURE 2. $HER2_{369}$-specific TCRs show peptide-specific function after retroviral gene transfer. PBMCs transduced with the $HER2_{369}$-specific TCR as well as the control TCR R6C12 were incubated 11 d after transduction for 24 h with T2 cells pulsed with a range of titrated concentrations of $HER2_{369}$ (*A*), $GP100_{209}$ (*B*), or a panel of control peptides at 10 μM (*C*). Selected tumor cell lines were used as target cells for TCR-transduced PBMCs (*D*). Tumor target cells were treated with IFN-γ (100 U/ml) 48 h prior to the stimulation assay. Supernatants were analyzed by IFN-γ ELISA. The numbers in brackets indicate the percentage of cells stained positive with the specific multimer. SDs of triplicates are shown.

(4 wk after transduction). In addition, TCR-transduced T cells were cloned by limiting dilution 12 d after transduction and phenotypically, as well as functionally, analyzed.

Results

Isolation of $HER2_{369}$-specific HLA-A2–allorestricted T cell clones after stimulation of HLA-$A2^-$–$CD8^+$ cells with $HER2_{369}$-pulsed T2 cells

Allo–HLA-A2–restricted T cells specific for $HER2_{369}$ were isolated by MHC-peptide multimers after stimulation of HLA-$A2^-$–$CD8^+$ T cells with peptide-pulsed T2 cells (22). A total of 0.05–0.8% of cells stained positive for the specific $HER2_{369}$ multimer after stimulation and before sorting (data not shown). $HER2_{369}$ multimer$^+$ cells were then enriched by flow cytometric sorting and cloned by limiting dilution. A total of 33 different clones with peptide specificity for $HER2_{369}$ were generated in three independent stimulation experiments using T cells derived from two different healthy donors. Most clones could not be cultured over a longer period preventing further extensive functional testing. However, RNA from four clones with high $HER2_{369}$ specificity but low background reactivity against irrelevant peptides (Table I) was harvested and the TCR repertoire of these selected clones was determined (Table II). Interestingly, three of these four clones (HER2-1, HER2-2, and HER2-4) used a TCRβ-chain of the TRBV12-3 family (Table II).

Retroviral TCR transfer of $HER2_{369}$-specific HLA-A2–allorestricted TCR into recipient cells results in positive $HER2_{369}$-multimer staining as well as peptide-specific function and tumor reactivity

The TCRα- and β-chain genes derived from these four different clones demonstrating peptide-specificity were used for cloning of TCR chain genes for transfer studies (Table II). In addition, we used the $GP100_{209}$-specific TCR derived from clone R6C12 (33), a CMV-$pp65_{495}$–specific TCR (JG-9) (32), and the FMNL1-PP2–specific TCR SK22 (8) as control TCR with defined specificities for other Ags than HER2 (Table II). Retroviral TCR gene transfer with unmodified TCRα- and β-chain genes using single TCR chain vectors into TCR knockout J76CD8 cells or PBMCs resulted in cells positive for the specific MHC-peptide multimer but negative for control multimer (Fig. 1*A*, 1*B*). TCR HER2-1 was the only $HER2_{369}$-specific TCR demonstrating a significant percentage of multimer$^+$ cells in the $CD8^-$ population (Fig. 1*B*) corresponding to multimer positivity of HER2-1–transduced J76 lacking CD8 (data not shown). Although the percentage of multimer$^+$ cells was overall low, we observed high expression of the specific TRBV12 chain in $CD4^+$ and $CD8^+$ populations indicating efficient transduction and suggesting that either transduced TCRs are not well enough assembled to be detected by the specific multimer or that mispairing with endogeneous α-chains occurs. PBMCs transduced with unmodified TCR chain pairs from $HER2_{369}$-specific T cell clones, as

Table III. *Percentage of specific multimer$^+$ and TRBV12$^+$PBMCs after retroviral transduction with vector constructs containing either WT or modified TCR chain genes*

TCR		Day 4 Multimer$^+$ (%)	Day 4 TRBV12$^+$ (%)	Day 4 GFP$^+$ (%)	Day 11 Multimer$^+$ (%)
HER2-1	WT	4.8	30.4		6.1
	Mod	14.1	19.9		15.6
HER2-2	WT	5.6	29.3		6.0
	Mod	8.8	17.3		16.2
HER2-3	WT	5.3	ND		9.6
	Mod	7.3	ND		20.3
HER2-4	WT	2.7	27.9		3.7
	Mod	ND	ND		ND
R6C12	WT	6.7	49.5		8.8
	Mod	9.1	21.5		13.8
Nontransduced		0.2	4.5		0.3
Mock		ND	ND	31.3	ND

WT, Wild type usage of vector constructs containing unmodified TCR chain genes; Mod, Modified usage of bicistronic vector constructs containing TCRα- and β-chain genes with murinized constant chains and codon optimization.

well as the control TCR R6C12, exerted reactivity toward the specific peptide in a dose-dependent manner (Fig. 2*A*, 2*B*). All transduced TCR demonstrated high-peptide specificity and did not respond to a panel of irrelevant peptides (Fig. 2*C*). Analyzing the tumor reactivity of PBMCs transduced with diverse HER2$_{369}$-specific TCRs, we observed mainly tumor reactivity of HER2-3 against diverse tumor cell lines as MCF-7 and MDA-MB 231, as well as marginal tumor reactivity of HER2-1 against SK-Mel 29 (Fig. 2*D*).

TCR modifications improve transgenic TCR expression and specific functions of TCR-transduced PBMCs

To improve expression and reactivity of the three TCRs with the highest HER2$_{369}$ reactivity, we introduced modifications into TCR constructs of HER2-1, HER2-2, and HER2-3. We first murinized constant chains as previously described (28). We additionally performed codon optimization and cloned both TCRβ- and α-chain genes in one vector separated by the picorna virus-derived peptide element P2A as previously described (29, 31). These modifications improved multimer staining and reduced expression of the specific Vβ chain indicating that these modifications resulted in a reduction of mispairing (Table III). Moreover, these modifications improved peptide-specific function (Fig. 3*A*, 3*B*) of TCR HER2-1 and HER2-2. Functional avidity of PBMCs transduced with HER2-1 was increased by these modifications when compared with transduction of unmodified chain genes (Fig. 3*A*). In addition, these modifications improved tumor reactivity of HER2-1, but not HER2-2 and HER2-3 (Fig. 3*C*). Modification of TCR HER2-3 revealed only improvement of multimer staining, but not HER2$_{369}$-specific function and tumor reactivity (Fig. 3*A*–*C*). HLA-A2$^-$ and HLA-A2$^+$ PBMCs transduced with this TCR showed reduced proliferation (data not shown) indicating a more general alloreactive potential of this TCR.

As the optimized TCR HER2-1 demonstrated the most specific reactivity pattern, including evidence of multimer$^+$ cells in the CD4$^+$ and CD8$^+$ T cell population as well as potent antitumor reactivity, we analyzed T cell populations transduced with the optimized TCR HER2-1 to further test specificity and tumor reactivity of this TCR (Figs. 4, 5). In contrast to a previous report about a HER2$_{369}$-specific allorestricted T cell clone (14), TCR HER2-1 specifically recognized

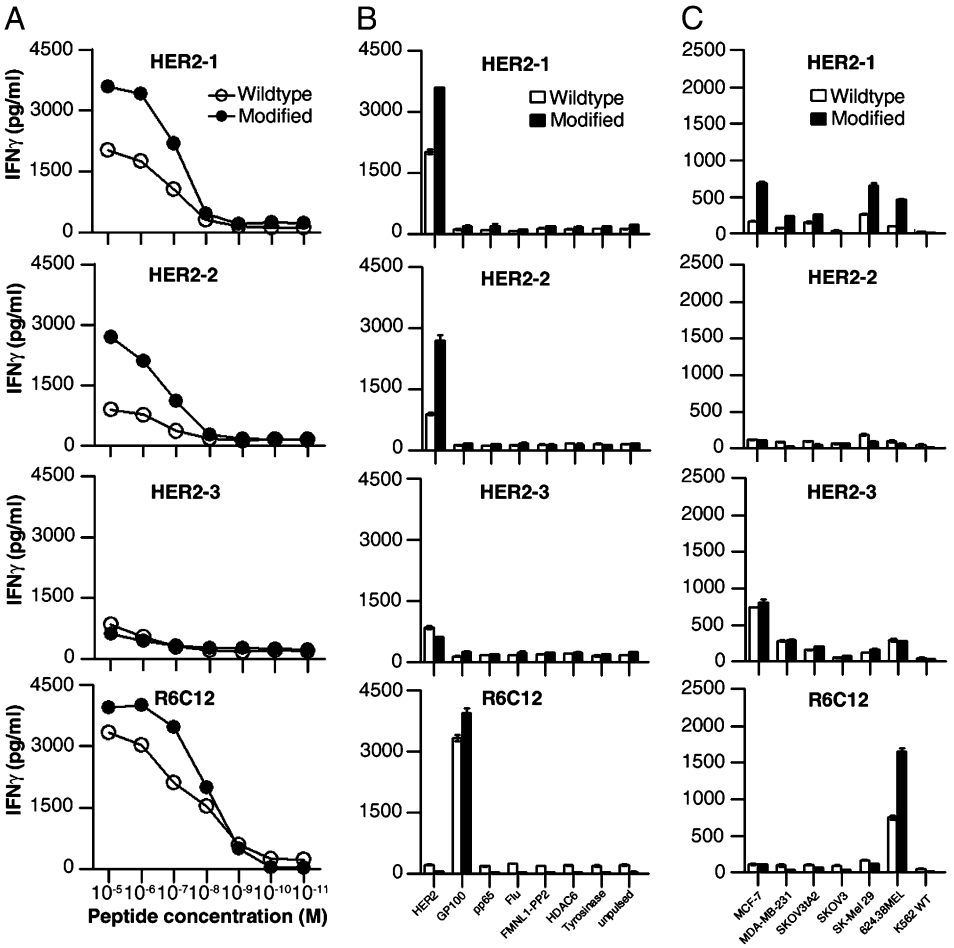


FIGURE 3. PBMCs transduced with modified TCR constructs show enhanced functions with preserved peptide specificity. PBMCs transduced with either single TCR chains (WT) or modified constructs (modified) were stimulated 11 d after transduction for 24 h with target cells at E:T ratios of 5:1. Supernatants were then harvested and analyzed by IFN-γ ELISA. The percentage of multimer$^+$ cells in the effector cell population is shown in Table III. Nontransduced PBMCs as well as mock-transduced PBMCs were used as controls. SDs of triplicates are shown. *A*, TCR-transduced PBMCs were tested against T2 cells pulsed with a range of titrated concentrations of specific peptide. HER2$_{369}$ was used for TCR HER2-1, HER2-2, and HER2-3; GP100$_{209}$ was used for TCR R6C12. *B*, TCR-transduced PBMCs were tested against T2 cells pulsed with a set of alternative peptides at a concentration of 10^{-5} M. *C*, TCR-transduced PBMCs were tested against selected tumor cell lines. Tumor target cells were treated with IFN-γ (100 U/ml) 48 h prior to the stimulation assay.

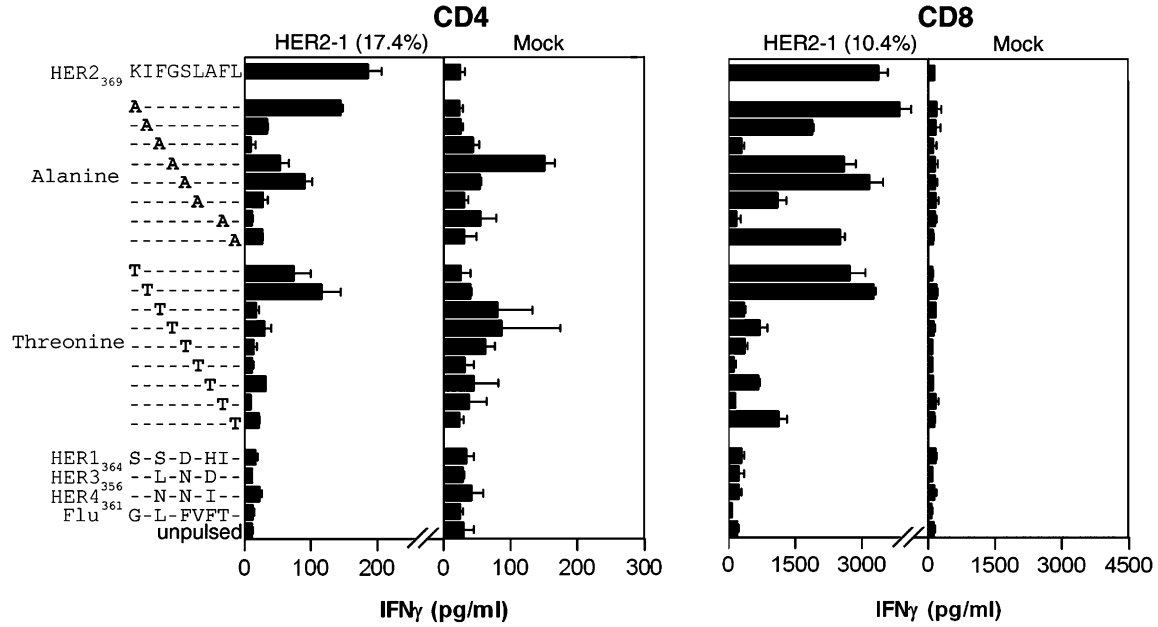


FIGURE 4. Detailed peptide-specificity of PBMC populations transduced with the modified construct of TCR HER2-1. Sorted CD4$^+$ and CD8$^+$ cell populations transduced with TCR HER2-1 were tested for their reactivity against T2 cells pulsed with the following peptides at concentrations of 10^{-5} M: $HER2_{369}$ as well as single amino acid analogs of $HER2_{369}$ using alanine and threonine for substitution, $HER1_{364}$, $HER3_{356}$, $HER4_{361}$, and Flu (E:T = 5:1). Supernatants were harvested after 24 h and analyzed by IFN-γ ELISA. SDs of triplicates are shown. Nontransduced PBMCs (data not shown) as well as mock-transduced PBMCs were used as controls. The number in brackets indicate the percentage of multimer$^+$ cells in the given cell population. Transduction efficiencies were determined 4 d after transduction by multimer staining, TRBV12 staining, and GFP (mock control) as shown in Supplemental Table II.

T2 cells pulsed with $HER2_{369}$ but not $HER1_{364}$, $HER3_{356}$, or $HER4_{361}$ (Fig. 4). We additionally used single-peptide analoga substituting all amino acids of $HER2_{369}$ by either alanine or threonine. Although exchange of most central amino acids by threonine mainly abolished recognition by this TCR, several exchanges by alanine were tolerated. However, several peptide amino acid positions as 3 aa (phenylalanine), 6 aa (leucine), and 8 aa (phenylalanine) were essential for target recognition (Fig. 4). To analyze the specificity for endogenously presented HER2, we analyzed the reactivity against C1R cells transfected with HLA-A2 and HER2. CD8$^+$ T cells transduced with HER2-1 showed specific IFN-γ–secretion in response to HLA-A2$^+$ C1R cells transfected with HER2 (Fig. 5*A*). Moreover, HER2-multimer$^+$ T cell clones generated from HER2-1–transduced T cell lines preferentially recognized C1R cells transfected with HLA-A2 and HER2 (Fig. 5*B*). Specificity of IFN-γ expression in bulk cultures was further analyzed by investigation of the presence of intracellular IFN-γ in combination with the expression of the specific TCR or β-chain indicating preferential recognition of C1R cells transfected with HLA-A2 and HER2 (Fig. 5*C*). Moreover, MCF-7 cells were specifically recognized by multimer$^+$ cells. Limited recognition of HLA-A2$^+$ C1R cells by TCR-transduced CD8$^+$ cells as investigated by intracellular IFN-γ staining may indicate alloreactive recognition of HLA-A2$^+$. We therefore investigated the recognition of a panel of nontransformed HLA-A2$^+$ targets as lung fibroblasts, fetal cardiomyocytes, PBMC subpopulations, and activated PBMCs demonstrating no major recognition by CD4$^+$ or CD8$^+$ populations transduced with TCR HER2-1 as analyzed by IFN-γ ELISA (Fig. 5*D*).

Mispairing of the TCRα-chain derived from HER2-2 with different β-chains of the TRBV12 family derived from TCR with diverse specificities results in $HER2_{369}$-specific multimer binding

By investigating the TCR repertoire of selected T cell clones with specificity for $HER2_{369}$, we detected a preferential usage of β-chains of the TRBV12 family in these clones (Table II and data not shown). Restricted usage of variable chains involved in detection of a defined Ag has been previously described (34, 35). Moreover, it has been previously reported that the usage of mixed TCR chain combinations derived from diverse TCR with a defined Ag-specificity resulted again in the defined Ag-specificity (13). We therefore analyzed the capability of chimeric chain combinations to form TCRs with specificity for $HER2_{369}$. We transduced J76CD8 and PBMCs with random combinations of unmodified TCR single chains derived from diverse TCR with specificity for HER2 but also alternative specificities and investigated these cells for TCR expression and specific multimer binding. All combinations showed surface expression of TCR or CD3 in transduced J76CD8 indicating that the TCR single chains easily mispair with novel partner TCR chains (data not shown). Interestingly, combinations of HER2-2α with diverse β-chains belonging to the TRBV12 family resulted in $HER2_{369}$-multimer$^+$ cells, whereas this was not observed using combinations with β-chains of other TRBV families (Table IV and Fig. 6*A*). Two of the β-chains belonging to the TRBV12 family were derived from receptors with specificities other than $HER2_{369}$, namely, $GP100_{209}$ (R6C12) and CMV-$pp65_{495}$ (JG-9). PBMCs transduced with HER2-2α in combination with these TCRβ-chains did not stain positive with multimers specific for the original TCRs, HLA-A2–$GP100_{209}$ or HLA-A2–CMV-$pp65_{495}$, respectively (Fig. 6*A*). Moreover, transduction of single TCR HER2-2α-chain in PBMCs resulted in a slight increase of $HER2_{369}$-multimer$^+$ cells (Fig. 6*A*, 6*B*). These $HER2_{369}$-multimer$^+$ cells stained mainly concomitantly positive for anti-TRBV12 (Fig. 6*B*). Interestingly, combinations of HER2-2α with HER2-4β as well as R6C12β resulted in $HER2_{369}$-multimer binding of TCR-chain–transduced CD8$^-$ PBMCs suggesting that specific multimer staining of these chimeric TCRs was less dependent on CD8 (Fig. 6*A*). Murinization of TCR constant chains, cloning of HER2-2α and HER2-1β or R6C12β in a bicistronic vector and codon optimization of chimeric TCR chain genes was performed on selected chain genes of chimeric TCRs and resulted in efficient $HER2_{369}$-multimer binding of surface expressed chimeric TCRs after transduction in PBMCs.

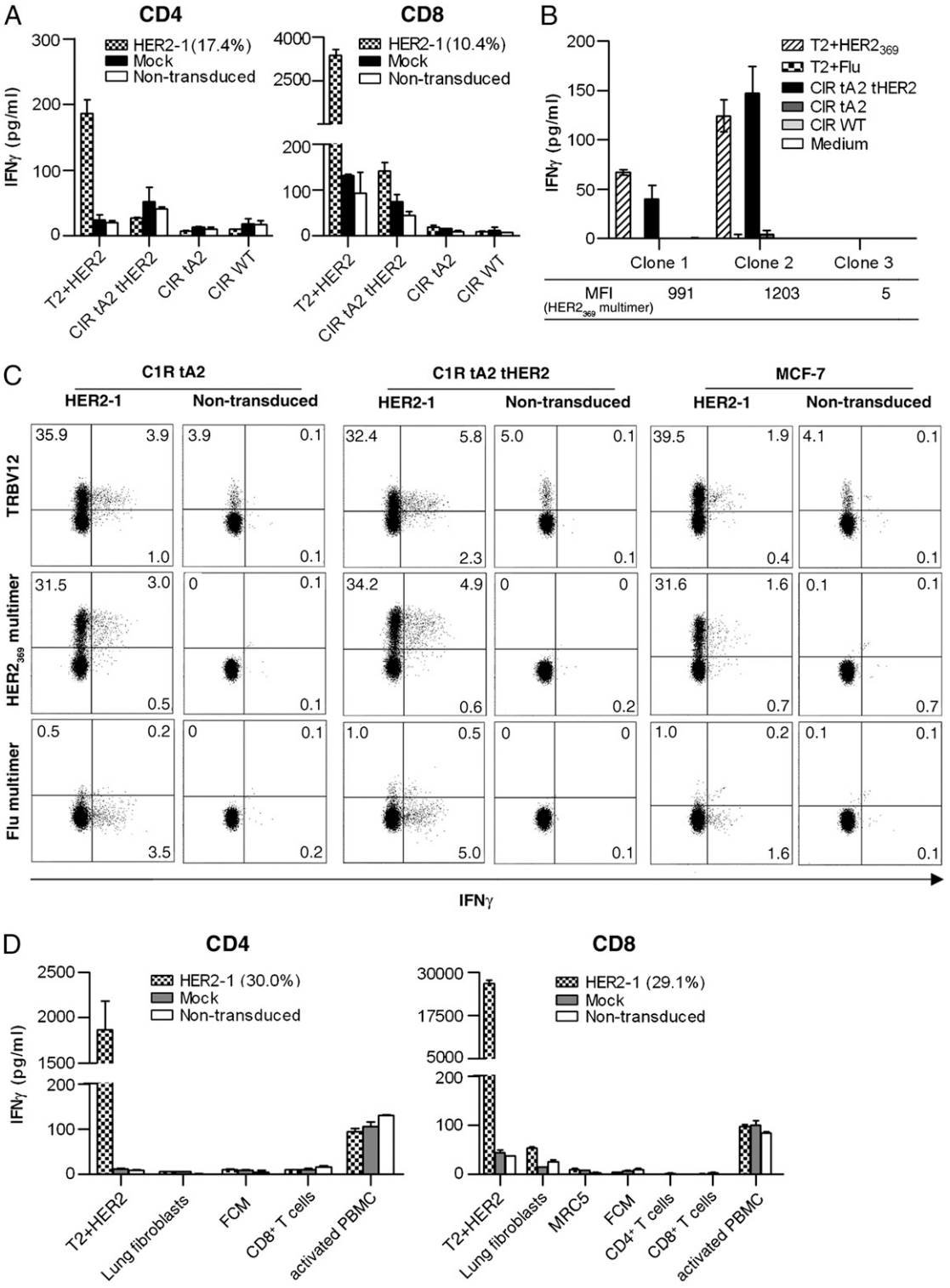


FIGURE 5. Specified target recognition of PBMC populations transduced with the modified construct of TCR HER2-1. *A*, HER2-1–transduced $CD4^+$ and $CD8^+$ cells were stimulated 11 d after transduction for 24 h with WT C1R cells, C1R cells transfected with HLA-A2 as well as HLA-A2 and HER2. Standard deviations of triplicates are shown (E:T = 5:1). *B*, $CD8^+$ cells transduced with HER2-1 were cloned by limited dilution and growing T cell clones were analyzed with flow cytometry for specific multimer binding. The mean fluorescence intensity is shown for selected clones. Selected clones were analyzed for their $HER2_{369}$-specificity with specified target cells at E:T ratios of 1:5. SDs of duplicates are shown. *C*, HER2-1–transduced $CD8^+$ cells were cocultivated 12 d after transduction with indicated target cells (E:T = 1:1). Cells were harvested after 6 h and stained with CD8, TCRBV12-specific Abs as well as specific and control multimers. Cells were then fixed and permeabilized, followed by treatment with anti-human IFN-γ Ab and analysis by flow cytometry. IFN-γ secretion of CD8-gated cells is shown. *D*, HER2-1–transduced $CD4^+$ and $CD8^+$ cells were stimulated 11 d after transduction with $HER2_{369}$-pulsed T2 cells as positive control, lung fibroblasts, fetal cardiomyocytes, $CD4^+$ and $CD8^+$ sorted PBMCs as well as PBMCs stimulated with OKT3 and IL-2 (E:T = 5:1). SDs of duplicates are shown. For IFN-γ ELISA analysis, supernatants were harvested after 24 h and then analyzed. Nontransduced PBMCs as well as mock-transduced PBMCs were used as controls. The number in brackets indicate the percentage of $multimer^+$ cells in the given cell population. Transduction efficiencies were determined at day 4 after transduction by multimer staining, TRBV12 staining, and GFP (mock control) and are shown in Supplemental Table II.

Table IV. *Specific $HER2_{369}$-multimer staining after transduction of chimeric TCR combinations into J76CD8 cells*

$HER2_{369}$ Multimer$^+$	HER2-1 TRBV 12-3*01	HER2-2 TRBV 12-3*01	HER2-3 TRBV 7-8*01	HER2-4 TRBV 12-3*01	R6C12 TRBV 12-3*01	JG-9 TRBV 12-4*01	SK22 TRBV 27*01
HER2-1 TRAV19*01	+	−	−	ND	−	ND	ND
HER2-2 TRAV27*01	+	+	−	+	+	+	−
R6C12 TRAV41*01	−	−	ND	ND	−	ND	ND

(+) indicates specific $HER2_{369}$-multimer recognition.
(−) indicates no $HER2_{369}$-multimer recognition.

Mispairing of the TCRα-chain derived from HER2-2 with diverse β-chains of the TRBV12 family results in enhanced $HER2_{369}$-specific functional avidity, CD8-independency, and tumor reactivity

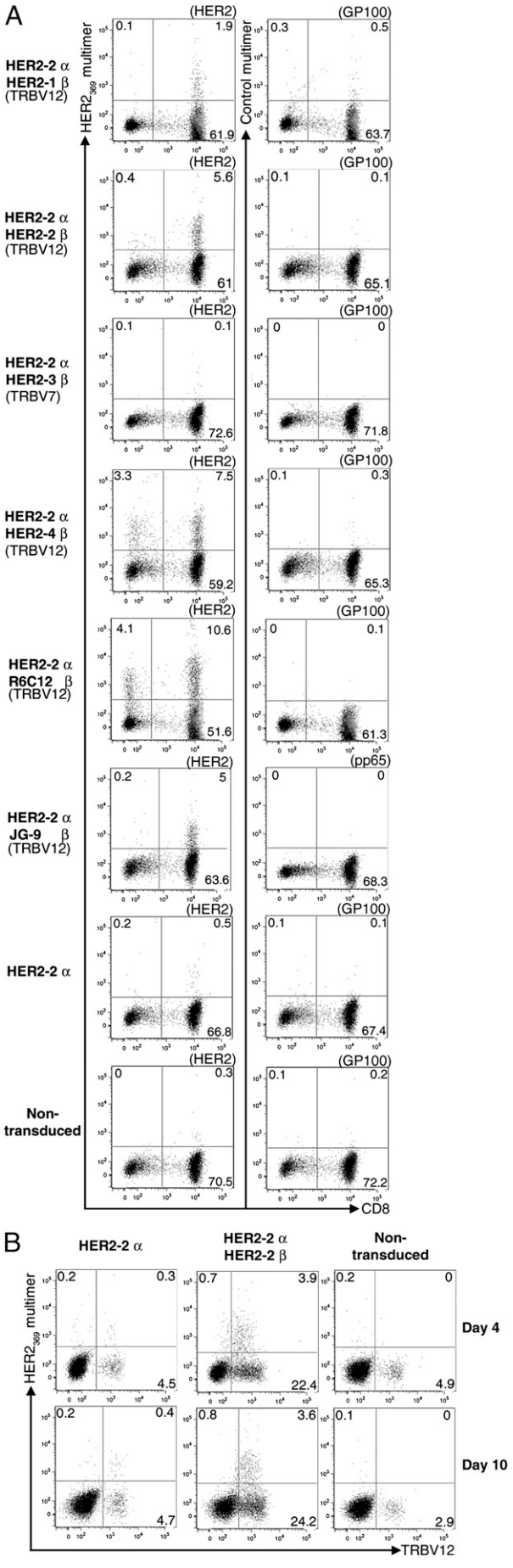
In parallel to the specific multimer binding, combinations of HER2-2α with different β-chains of the TRBV12 family displayed specific reactivity for T2 cells pulsed with $HER2_{369}$ in a dose-dependent manner (Fig. 7*A*, Table V). Whereas chimeric TCR combinations of HER2-2α with HER2-1β or JG-9β resulted in only marginal recognition of T2 cells pulsed with high concentrations of $HER2_{369}$ (Table V), combinations of HER2-2α with R6C12β but especially HER2-4β resulted in an increased functional avidity in peptide titration experiments when compared with the original chain combination (Fig. 7*A*). Moreover, the combination of HER2-2α and HER2-4β resulted in reactivity against diverse tumor cell lines (Fig. 7*B*, Table V), although the original TCR chain combinations HER2-2αβ and HER2-4αβ showed no tumor reactivity. The combination of HER2-2α and HER2-4β demonstrated high-peptide specificity for $HER2_{369}$ but also revealed increased reactivity in response to T2 cells pulsed with alternative peptides or unloaded T2 cells (Table V).

In parallel to the CD8-independent multimer staining of chimeric TCR combinations, mixed combinations of HER2-2α with HER2-4β or R6C12β expressed in $CD8^-$ cells sorted after TCR-transfer by flow cytometry resulted in CD8-independent peptide recognition in a dose-dependent manner demonstrating again a high-functional avidity of these chimeric chain combinations (Table VI). Sorted $CD8^-$ cells transduced with HER2-2α in combination with HER2-4β also demonstrated high-peptide specificity, although background reactivity against T2 cells pulsed with alternative peptides was again noticed (Table VI). In addition, reactivity against selected tumor cell lines was observed (Fig. 7*C*, Table VI). In contrast to HER2-1, T cells transduced with HER2-2α/HER2-4β showed similar recognition of HLA-$A2^+$ C1R cells either transfected with HER2 or not (data not shown). As autoreactivity against PBMCs might be a major risk of transgenic T cells targeting overexpressed self-Ags, we analyzed proliferation of HLA-$A2^-$ and HLA-$A2^+$ cells after transduction of TCR HER2-1 as well as HER2-2α/HER2-4β (Fig. 7*D*). HLA-$A2^-$ PBMCs transduced with either HER2-1 or HER2-2α/HER2-4β showed similar proliferation as mock-transduced or nontransduced PBMCs although proliferation of multimer$^+$ cells was reduced in the $CD8^+$ but not $CD4^+$ cell population transduced with HER2-2α/HER2-4β. Using HLA-$A2^+$ PBMC populations, we observed a slightly decelerated proliferation of cells transduced with HER2-1. In contrast, proliferation of CD4 and CD8 PBMC populations was completely abrogated when transduced with HER2-2α/HER2-4β (Fig. 7*D*).

Discussion

Transfer of Ag-specific TCR chains might be a therapeutic option for the treatment of cancer and has been previously shown to be a feasible and effective approach (1, 2). However, for broader application and further assessment of potential benefits and side effects, a broad range of TCRs with specificity for diverse classes of tumor-associated Ags need to be characterized. HER2 is a tumor-associated Ag overexpressed in diverse malignancies and has been previously demonstrated to be a preferable target for anticancer therapies using Abs (36). In this study, we investigated the function of $HER2_{369}$-specific T cell clones that were generated using the allorestricted approach (4), and their specific TCR after transfer into diverse recipient cells.

Transfer of $HER2_{369}$-specific allorestricted TCRs into PBMCs resulted in high-peptide specificity, whereas tumor reactivity was primarily low. Peptide specificity and tumor reactivity of especially one TCR, HER2-1, could be significantly enhanced after codon optimization, usage of a bicistronic vector construct, and murinization of constant chain genes as previously described (28, 29, 31). Moreover, these modifications also reduced mispairing with endogenous TCR chains. Murine sequences may induce immune responses. However, recent clinical data show effectivity and in vivo persistence of effector cells transduced with a murine TCR (2) suggesting that TCRs containing murine sequences should not be prematurely deleted from further investigation. However, other modifications reducing the risk for mispairing might be applied for HER2-1 (37–39). High specificity of HER2-1 was proven by recognition of endogenously presented $HER2_{369}$ as well as lack of cross-reactivity against $HER1_{364}$, $HER3_{356}$, or $HER4_{361}$. We additionally tested recognition of single amino acid peptide analoga of $HER2_{369}$ presented in the context of HLA-A2 using threonine and alanine for substitution. Although several single substitutions with the inert amino acid alanine were still recognized, exchange by threonine was less accepted by this TCR potentially because of the polarity as well as a longer side chain of this amino acid. Limited cross-reactivity for analog peptides has been previously reported for other TCRs with specificity for viral Ags (40) and this might reflect the general cross-reactive potential reported for TCRs (41). Of note, exchange of the HLA-A2 anchor residues in positions 2 and 9 by alanine or threonine was tolerated at high-peptide concentrations of 10 μM. However, melanoma epitopes have been previously described containing alanine or threonine on position 2 and alanine on position 9 (42). In addition, it has been previously published that position 3 may represent a secondary anchor and that aromatic residues are frequent in this position (43, 44). Substitution of phenylalanine at position 3 with either alanine or threonine completely abolished recognition by HER2-1 potentially pointing to a role of the aromatic residue at this position in peptide binding of $HER2_{369}$ to HLA-A2 as well as recognition by TCR HER2-1. Importantly, PBMCs transduced with HER2-1 did not show reactivity against nonmalignant HLA-$A2^+$ targets as fibroblast cell lines, cardiomyocytes, and unstimulated or stimulated PBMCs suggesting that peptide-independent HLA-A2 recognition or cross-reactivity against a peptide broadly presented by HLA-A2 may not play a role in target recognition. However, C1R cells transfected with HLA-A2 induced limited intracellular IFN-γ production in HER2-1–transduced $CD8^+$ cells and transduction of this TCR in HLA-$A2^+$ PBMCs resulted in slightly decelerated proliferation after longer in vitro culture suggesting that proliferating cells of hematopoietic origin may represent a target of HER2-1–transduced PBMCs. This is in common with previously published data demonstrating that mature hematopoietic cells express low levels of HER2 and increase HER2 mRNA and protein expression after mitogenic stimulation (45). Transient



leukopenia may be tolerated during therapy. However, to further assess the value of HER2-specific TCRs and especially TCR HER2-1 for clinical application, it will be necessary to investigate the reactivity against a broad panel of HLA-A2$^+$ healthy tissues in vitro and in vivo. Moreover, possibilities for selective deletion of TCR-transduced PBMCs (46) may be considered to avoid prolonged undesired side effects when targeting this Ag. It is interesting in this regard that the most promising TCR candidate (HER2-1) showed only an intermediate functional avidity, although this TCR has been selected in an allogeneic environment. This may rely on several factors (47). First, binding of $HER2_{369}$ to HLA-A2 has been reported to have a relative short $t_{1/2}$ (48) that may have an impact on the intermediate functional avidities observed. Second, the discriminative $HER2_{369}$ epitope density between tumor cells and nontransformed cells might be marginal for $HER2_{369}$. In fact, $HER2_{369}$ peptide has been previously shown to be presented only at low levels on tumor cells (49). Moreover, overexpression of HER2 seems to result in proteasomal dysfunction and therefore reduced peptide presentation on tumor cells overexpressing this protein (50). It might be therefore possible that TCRs with higher avidity have not been selected on the T cell clone level because of enhanced cross-reactivity. TCRs with high avidity might be therefore problematic when targeting overexpressed self-Ags and an individual optimal functional TCR avidity potentially needs to be determined for any specific MHC-peptide complex.

The other selected TCRs seemed at first less interesting as they demonstrated either limited peptide specificity (HER2-3) or lack of tumor reactivity (HER2-2 and HER2-4). However, as we observed a preferential usage of β-chains of the TRBV12 family in the isolated HER2-specific TCR repertoire, we investigated random combinations of isolated single TCRα- and β-chains of these TCRs. We thereby identified a TCRα-chain (HER2-2α) with specific $HER2_{369}$ peptide recognition in combination with diverse β-chains, all belonging to the TRBV12 family. Combinations with β-chains of two other TRBV families did not result in $HER2_{369}$-multimer$^+$ cells or peptide-specific target recognition. Importantly, all different TRBV12-derived β-chains contributing to $HER2_{369}$-specific multimer staining in combination with HER2-2α possessed different CDR3 regions and were not only derived from allorestricted $HER2_{369}$-specific TCRs from diverse HLA-A2$^-$ donors, but also from TCRs with alternative specificities as $GP100_{209}$ and $CMVpp65_{495}$. These latter TCRs were selected in HLA-A2–expressing individuals. Moreover, $HER2_{369}$-multimer$^+$ PBMCs transduced with HER2-2α alone stained mainly positive for anti-TRBV12 further emphasizing that HER2-2α is dominantly responsible for HLA-A2–restricted $HER2_{369}$-specific recognition in combination with diverse TCRβ-chains of the TRBV12 family. A prevalent role of the TCRα-chain in the selection of the preimmune TCR repertoire specific for melan-A has been previously reported (34, 35). Heemskerk et al. described novel chimeric TCR chain combinations containing single TCR chains derived from diverse HA-2–specific TCR resulting again in HA-2 specificity (13). Moreover,

FIGURE 6. Transduction of PBMCs with HER2-2α alone or in combination with β-chains derived from the TRBV12 family with diverse specificities results in significant $HER2_{369}$-specific multimer binding. *A*, PBMCs transduced with TCR chain genes of HER2-2α alone or in combination with β-chains derived from six different receptors with diverse specificities were stained 10 d after transduction with $HER2_{369}$-specific multimer and control multimer (*left* and *right data sets*). The specific multimers used are indicated in brackets above the graphs. *B*, PBMCs transduced with HER2-2α alone, HER2-2α/HER2-2β, and nontransduced PBMCs were stained with the $HER2_{369}$-multimer and anti-TRBV12 Ab at day 4 and day 10 after transduction and analyzed by flow cytometry.

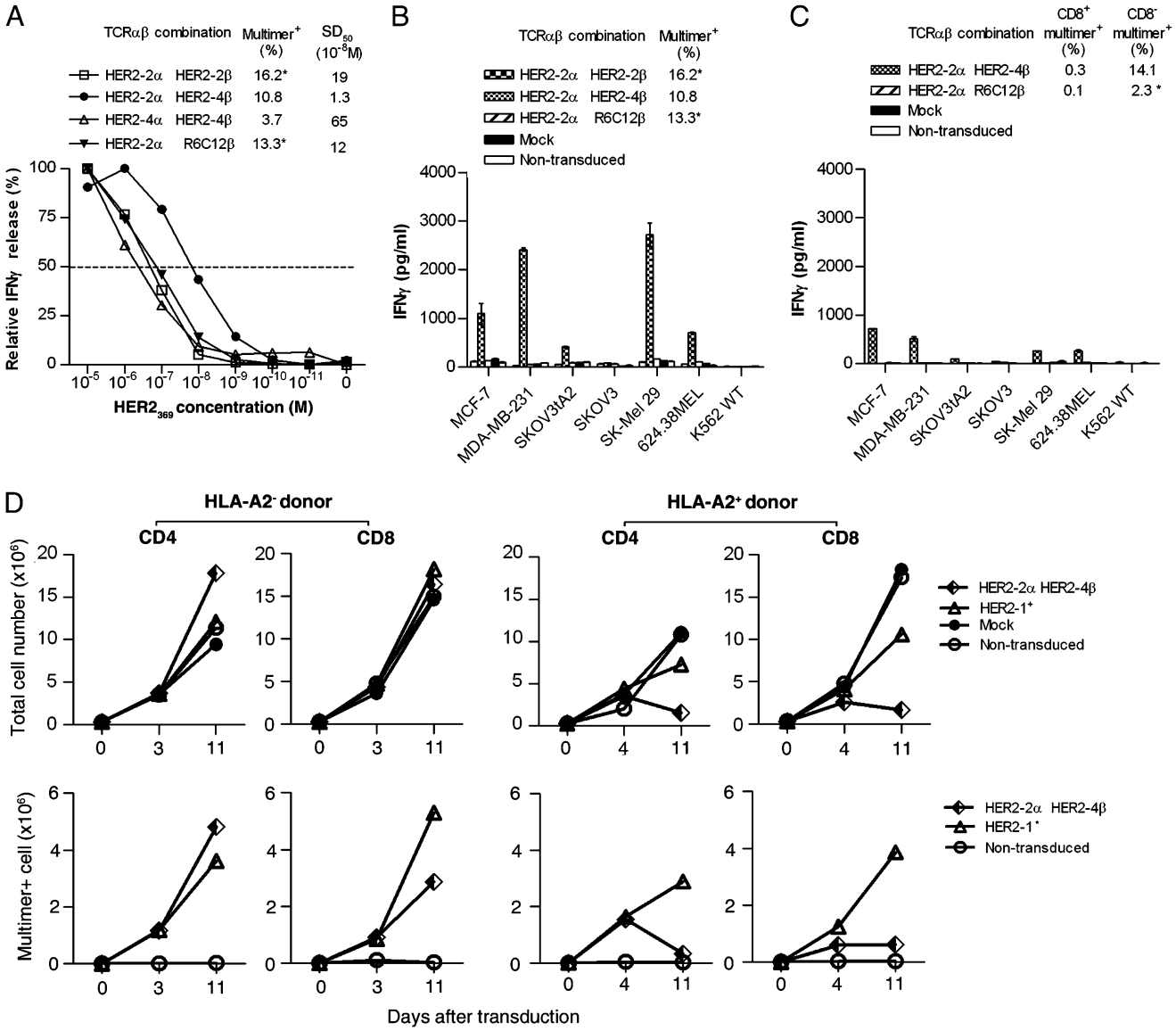


FIGURE 7. Enhanced peptide-specific functional avidity as well as recognition of natural targets by PBMC populations transduced with novel chimeric TCR chain combinations of HER2-2α with HER2-4β and R6C12β. *A*, PBMCs transduced with TCR chain genes of HER2-2α in combination with β-chains derived from HER2-4β and R6C12β, as well as the native TCR combinations of HER2-2αβ and HER2-4αβ, were stimulated with T2 cells pulsed with a range of titrated concentrations of $HER2_{369}$ at E:T ratios of 5:1. Supernatants were harvested after 24 h and analyzed by IFN-γ ELISA. The percentage of maximum IFN-γ release and the SD50 are shown. *B* and *C*, PBMCs (*B*) and CD8-depleted populations (*C*) transduced with TCR chain genes of HER2-2α in combination with β-chains derived from HER2-4β and R6C12β were stimulated 11 d (*B*) and 25 d (*C*) after TCR-transduction with diverse target cells at E:T ratios of 5:1. The percentage of multimer$^+$ cells in the effector cell population is indicated. Nontransduced PBMCs as well as mock-transduced PBMCs were used as controls. SDs of triplicates are shown. *Usage of optimized constructs. *D*, Proliferation of total cell counts (*upper panel*) and multimer$^+$ cells (*lower panel*) of CD4$^+$ and CD8$^+$ cells of HLA-A2$^-$ (*left four panels*) and HLA-A2$^+$ (*right four panels*) donors are shown after transduction with the chimeric TCR combination HER2-2α/HER2-4β as well as HER2-1. Mock- and nontransduced cells were used as controls. Transduction efficiencies were primarily determined at day 4 after transduction by multimer staining, TRBV12 staining, and GFP (mock control) and are shown in Supplemental Table III.

Yokosuka et al. reported a murine HIV-specific TCRα-chain in TCR-transgenic mice responsible for recognition of HIVgp160 peptide/H-2Dd complexes in combination with a variety of TCRβ-chains (51). In these experiments, one third of randomly picked β-chains of the same TRBV family could reconstitute for specific peptide recognition in combination with the HIVgp160 peptide-specific TCRα-chain. In this study, we report that such TCR single chains with dominant peptide recognition in combination with TCRβ-chains derived from TCRs with diverse specificities are present in the circulating T cell pool of humans. MHC-peptide recognition by random combinations of TCRα- and β-chains has been previously suggested to be related to an intrinsic affinity of the TCRs toward MHC (52) and a genetic bias toward MHC recognition has been proposed (53, 54). More recently, germline interaction codons have been structurally defined for TCRα- and β-chains contacting specific MHC molecules (55, 56). Our data can be well explained by the existence of such germline restrictions of TCR–MHC-peptide complexes and favor for a preferential germline restriction of β-chains of the TRBV12 family toward HLA-A2 when paired to HER2-2α. However, the CDR3 regions of the matching β-chains still play an important role in $HER2_{369}$ peptide recognition of HER2-2α and determined the dimensions of functional avidity, CD8 dependency, and tumor reactivity. Two β-chains of the TRBV12 family (HER2-1β and JG-9β) resulted in only low percentages of $HER2_{369}$-multimer$^+$ cells lacking specific functions. This might potentially result from low interchain affinity of the novel chimeric TCR chain combination. In addition, the β-chain of JG-9

Table V. *Peptide and tumor recognition of PBMCs transduced with HER2-2α in combination with different TCR β-chains*

α-Chain	HER2-2 AV27							
β-Chain ($HER2_{369}$-Multimer$^+$%)	HER2-1 BV12 (10.5)[a]	HER2-2 BV12 (16.2)[a]	HER2-3 BV7 (0.2)[a]	HER2-4 BV12 (10.8)	R6C12 BV12 (13.3)[a]	JG-9 BV12 (5.2)	Mock	Nontransduced
T2 + 10^{-5} M $HER2_{369}$	99 (2)[b]	2697 (123)	74 (0)	3624 (108)	2899 (132)	111 (8)	48 (3)	33 (5)
T2 + 10^{-6} M $HER2_{369}$	86 (4)	2094 (93)	57 (3)	3924 (81)	2202 (63)	68 (3)	42 (9)	39 (8)
T2 + 10^{-7} M $HER2_{369}$	53 (8)	1114 (40)	52 (3)	3261 (51)	1436 (4)	62 (17)	41 (4)	37 (3)
T2 + 10^{-8} M $HER2_{369}$	60 (10)	276 (2)	45 (2)	2129 (21)	573 (16)	43 (1)	61 (5)	30 (12)
T2 + 10^{-9} M $HER2_{369}$	35 (5)	175 (0)	53 (1)	1208 (42)	265 (19)	52 (18)	47 (8)	20 (3)
T2 + 10^{-10} M $HER2_{369}$	36 (10)	158 (19)	38 (1)	826 (7)	208 (4)	49 (8)	28 (1)	28 (3)
T2 + 10^{-11} M $HER2_{369}$	70 (7)	147 (7)	42 (6)	754 (5)	194 (11)	42 (4)	31 (3)	24 (7)
T2 unpulsed	32 (2)	183 (123)	44 (4)	820 (13)	236 (6)	51 (3)	35 (5)	25 (5)
T2 + $GP100_{209}$	48 (1)	163 (4)	49 (2)	911 (70)	228 (3)	61 (2)	41 (7)	31 (8)
T2 + $pp65_{495}$	38 (9)	155 (6)	62 (8)	650 (50)	182 (7)	53 (4)	35 (15)	31 (8)
T2 + Flu_{58}	45 (2)	168 (32)	50 (3)	813 (44)	207 (0)	61 (0)	40 (2)	89 (12)
T2 + FMNL1-PP2	42 (1)	140 (6)	46 (4)	870 (131)	225 (1)	57 (2)	33 (7)	92 (20)
T2 + $HDAC6_{862}$	56 (21)	142 (24)	40 (3)	721 (73)	221 (6)	48 (11)	36 (2)	65 (0)
T2 + $Tyrosinase_{369}$	66 (4)	139 (2)	45 (2)	951 (74)	181 (1)	49 (9)	37 (3)	51 (7)
MCF-7 ($A2^+$, $HER2^+$)	66 (9)	112 (9)	82 (4)	1094 (13)	134 (0)	67 (10)	168 (10)	95 (11)
MDA-MB-231 ($A2^+$, $HER2^+$)	34 (4)	29 (2)	35 (2)	2412 (33)	56 (0)	38 (0)	60 (0)	84 (3)
SKOV3 tA2 ($A2^+$, $HER2^+$)	40 (1)	51 (4)	67 (4)	413 (17)	85 (7)	37 (4)	92 (4)	98 (9)
SKOV3 ($A2^-$, $HER2^+$)	31 (4)	64 (3)	54 (0)	82 (0)	63 (5)	47 (0)	19 (5)	32 (6)
SK-Mel 29 ($A2^+$, $HER2^+$)	91 (0)	100 (4)	133 (1)	2716 (238)	169 (2)	94 (18)	132 (3)	114 (6)
624.38 MEL ($A2^+$, $HER2^+$)	61 (0)	61 (4)	37 (1)	709 (10)	102 (4)	95 (4)	69 (6)	32 (7)
K562 WT ($A2^-$, $HER2^-$)	26 (0)	14 (2)	8 (23)	10 (4)	9 (4)	15 (2)	14 (1)	22 (1)
Medium	21 (2)	24 (1)	25 (5)	21 (5)	10 (7)	18 (4)	21 (5)	4 (0)

[a]Indicates usage of TCR constructs modified by codon optimization, usage of murinized constant chains, and bicistronic vectors.
[b]SDs of triplicates are shown in parentheses.

represented another subtype (TRBV12-4). In contrast, two other TCRβ-chains potentiated peptide-specific Ag recognition (HER2-4β and R6C12β). These chimeric TCR chain combinations resulted in increased peptide-specific avidity and CD8 independent function. Combination of HER2-2α and HER2-4β additionally resulted in potent tumor reactivity. Both TCR single chains derived from different $HER2_{369}$-specific TCRs and, when dimerized with their native partners, primarily exhibited low-functional avidities for peptide-pulsed T2 cells and a lack of tumor reactivity. Currently, the frequency of such TCR single chains with dominant peptide recognition in combination with diverse β-chains is unknown and it is not clear whether TCRβ-chains may have similar properties. It will be interesting to further evaluate the docking pattern on MHC-peptide complexes of these TCRs. Apart from that, the existence of these TCR single chains with dominant peptide recognition may have an important impact on the further development of TCR transfer strategies.

Table VI. *Peptide and tumor recognition of PBMCs transduced with HER2-2α in combination with different TCR β-chains followed by depletion of $CD8^+$ cells*

α-Chain	HER2-2 AV27			
β-Chain ($CD8^-$,$HER2_{369}$ multimer$^+$%)	HER2-4 BV12 (14.1)	R6C12 BV12 (2.3)[a]	Mock	Nontransduced
T2 + 10^{-5} M $HER2_{369}$	4287 (105)[b]	369 (20)	27 (1)	30 (1)
T2 + 10^{-6} M $HER2_{369}$	4347 (55)	315 (20)	24 (4)	19 (2)
T2 + 10^{-7} M $HER2_{369}$	4059 (174)	185 (8)	16 (0)	22 (5)
T2 + 10^{-8} M $HER2_{369}$	3827 (44)	126 (5)	14 (1)	16 (3)
T2 + 10^{-9} M $HER2_{369}$	1806 (25)	18 (3)	11 (1)	31 (4)
T2 + 10^{-10} M $HER2_{369}$	750 (9)	14 (3)	11 (2)	26 (1)
T2 unpulsed	602 (35)	18 (2)	17 (1)	15 (4)
T2 + $GP100_{209}$	472 (6)	23 (1)	19 (5)	17 (2)
T2 + $pp65_{495}$	484 (17)	14 (2)	14 (1)	14 (1)
T2 + Flu_{58}	762 (129)	16 (2)	16 (4)	21 (0)
T2 + FMNL1-PP2	606 (16)	18 (3)	19 (1)	14 (3)
T2 + $HDAC6_{862}$	593 (28)	21 (2)	15 (2)	16 (2)
T2 + $Tyrosinase_{369}$	644 (24)	28 (5)	20 (2)	18 (2)
MCF-7 ($A2^+$, $HER2^+$)	715 (3)	7 (2)	19 (2)	16 (1)
MDA-MB-231 ($A2^+$, $HER2^+$)	511 (47)	1 (1)	12 (2)	11 (4)
SKOV3 tA2 ($A2^+$, $HER2^+$)	95 (1)	17 (2)	13 (3)	9 (3)
SKOV3 ($A2^-$, $HER2^+$)	47 (2)	22 (1)	12 (2)	10 (0)
SK-Mel 29 ($A2^+$, $HER2^+$)	271 (0)	16 (0)	21 (2)	50 (19)
624.38 MEL ($A2^+$, $HER2^+$)	271 (19)	25 (2)	11 (2)	13 (5)
K562 WT ($A2^-$, $HER2^-$)	29 (2)	8 (1)	7 (3)	16 (7)
Medium	22 (2)	11 (3)	9 (2)	5 (1)

[a]Indicates usage of TCR constructs modified by codon optimization, usage of murinized constant chains and bicistronic vectors.
[b]SDs of triplicates are shown in parentheses.

Chimeric TCR combinations involving such TCR single chains with dominant peptide recognition may be a source for TCRs with improved properties as enhanced functional avidity for tumor-associated Ags and preferential recognition of natural tumor targets. Otherwise, effector T cells transduced with the chimeric TCR HER2-2α/HER2-4β also showed increased background reactivity against T2 cells pulsed with alternative peptides as well as strong reactivity against HLA-A2$^+$ PBMCs. This reactivity might result from enhanced germline MHC interactions but also from recognition of low level $HER2_{369}$ on these target cells. TCRs with enhanced affinity because of mutations within the CDR3 region have been previously demonstrated to be cross-reactive for self-peptides and might therefore harbor risks when used for clinical approaches with TCR-transgenic T cells (57, 58). Thus, the presence of such single TCR chains in the circulating T cell pool displaying dominant peptide recognition with different affinities in combination with diverse partner chains derived from introduced TCR may represent a risk for TCR transfer in PBMCs.

In conclusion, we isolated and characterized diverse TCRs with specificity for the $HER2_{369}$ as potential candidates for TCR transfer. One TCR showed preferential characteristics for further investigation regarding clinical application. We additionally identified a single TCRα-chain with dominant $HER2_{369}$ peptide recognition in combination with β-chains of the TRBV12 family derived from TCRs with diverse specificities demonstrating that pairing with novel partner chains can result in an increase of peptide-specific avidity as well as CD8 independency. Although the frequency of such TCRs is currently unknown, they may represent interesting tools for TCR optimization resulting in enhanced functionality when paired to novel partner chains. However, mispairing with novel partner chains may also result in enhanced cross-reactivity or self-reactivity, and these data further indicate that enhanced efforts for improved chain pairing (37–39), as well as selective deletion of TCR-transduced PBMCs (46), may be important.

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Disclosures

X. L., L. W., and A. K. have a patent application for the described HER2-specific TCR sequences.

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