Redirecting Human T Cells to Tumors via Transfer of T Cell Receptor Genes:

a study of tumor-specific T cell responses and peptide fine-specificity

Niels Schaft

Redirecting Human T Cells to Tumors via Transfer of T Cell Receptor Genes:

a study of tumor-specific T cell responses and peptide fine-specificity

Het genereren van tumor-specifieke humane T cellen via T cel receptor gen overdracht:

een studie naar tumor-specifieke T cel responsen en peptide fijn-specificiteit

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 5 juni 2003 om 11.00 uur

door

Niels Schaft
geboren te Hoorn

Promotiecommissie

Promotor:	Prof.dr. G. Stoter
Overige leden:	Prof.dr. A.M.M. Eggermont
	Prof.dr. C. Bangma
	Prof.dr. L.F.M.H. de Leij
Copromotor:	Dr. J.E.M.A. Debets

Printed by JPM TISK spol. s r. o., Prague, Czech Republic

The research described in this thesis was performed at the Department of Medical Oncology, Subdivision Clinical & Tumor Immunology at the University Medical Center Rotterdam, the Netherlands. The work was financially supported by a grant from the Dutch Cancer Society (project DDHK 1996-1253).

Voor hen die sterker zijn dan kanker

CONTENTS

Chapter 1

General introduction

Chapter 1: **General introduction**

1.1 Anti-tumor response: the critical role of T lymphocytes

The primary function of T lymphocytes is to recognize the presence of pathogens and to clear them from the body, either directly or by recruiting other immune cells. The mechanism by which the immune system clears pathogens greatly depends on the cellular localization of the pathogen. T lymphocytes distinguish between responses versus extracellular pathogens, such as eukaryotic parasites and some bacteria which exist either in the blood or in tissues, and those versus intracellular pathogens such as viruses and some bacteria.
Also tumor cells leave traces of "harmful proteins" in both the extracellular and intracellular compartments. These antigens can be recognized by T lymphocytes, which play a critical role in the anti-tumor response. How T lymphocytes recognize and respond to antigen is discussed in more detail below.

1.1.1 T lymphocyte : tumor cell interactions

T lymphocyte : tumor cell interaction is a very complex event involving many molecules. These molecules are divided in three groups, i.e., (i) T cell receptor (TCR)/CD3 complexes on T lymphocytes, (ii) major histocompatibility complex (MHC)/peptide complexes on tumor cells, and (iii) co-receptor/co-stimulatory molecules/adhesion molecules either on T cells or tumor cells, which are highlighted separately.

- *TCR/CD3 complex*

Tumor antigens, presented as peptides by MHC-molecules, are recognized by T lymphocytes through their TCR. Most TCR consist of an α and a β chain, while a minor subpopulation of T lymphocytes expresses a $\gamma\delta$ TCR. The α and β chains are members of the immunoglobulin (Ig) superfamily with the extracellular portion having a structure similar to an antibody Fab (fragment antigen binding), i.e., one variable (V) Ig domain, and one constant (C) Ig domain in each chain [1-5]. In between the V and C domains are diversity (D) and joining (J) domains in case of the TCR β and δ chains, and only J domains in case of the TCR α and γ chain [3,6,7]. The human TCR α gene locus consists of 42 functional TCR Vα

gene segments, up to 61 Jα gene segments and one Cα gene segment [8,9]. The human TCR β gene locus consists of 47 functional TCR Vβ gene segments, two D segments, 13 functional human Jβ segments, and two Cβ gene segments [8,10].

Mature TCR chains are encoded by single V (D) and J gene segments joined together by site-specific recombination [11,12]. The random joining of the various V, D, and J elements provides the primary source of TCR diversity, and random association of different chains (α x β or γ x δ) considerably enlarges the potential variability. A secondary source of TCR diversity is provided by junctional diversity, i.e., heterogeneity at the points of joining caused by variable loss of nucleotides from exposed termini or addition of nucleotides to intact termini [13]. Both sources of TCR diversity provide an essentially unlimited repertoire for antigen recognition.

TCR V gene segments encode two of the three complementarity determining regions (CDR) which comprise the antigen-MHC binding site [4]. The junction of V (D) and J domains of both TCR chains form the CDR3 region of the TCR, which has a higher degree of variability than CDR1 and CDR2. It was proposed that the CDR1 and CDR3 regions (especially the CDR3 region of the TCR α chain) contact the peptide antigen, whereas the CDR1 and CDR2 regions contact the MHC molecule [3,14,15]. TCR chains have a single membrane spanning domain, and a very short cytoplasmic tail [16]. Most TCR αβ heterodimers are covalently linked through disulfide bonds.

On T lymphocytes the TCR α and β chains are responsible for the specific recognition of a large number of different antigens presented by a MHC molecule. However, after recognition, due to the short cytoplasmic tails of the α and β chains, the TCR depends on the CD3 complex for further signaling into the T lymphocyte ultimately leading to T cell activation. The variable αβ TCR are associated with the invariant CD3 γ, δ, ε, and ζ proteins, that control TCR/CD3 assembly and signal transduction. CD3 chains again belong to the Ig supergene family. The transmembrane regions of CD3 γ, δ, and ε contain Asp or Glu, which play a dominant role in interchain contacts with the charged residues in the transmembrane regions of TCR α and β chains [17-22]. All three CD3 chains contain an ITAM (Immunoreceptor Tyrosine-based Activation Motif) sequence in their cytoplasmic tails [23-25]. The tyrosine residues in these motifs can be phosphorylated after recognition of antigen/MHC by the αβ TCR, and play an important role in further signal transduction [26-31]. CD3 ζ is found as homodimer in the TCR/CD3 complex at the cell surface. It has a very short extracellular domain and a long cytoplasmic tail with three ITAMs which can be phosphorylated upon T cell triggering through the antigen receptor [27,31-33]. Co-operation between the different elements is required

for a full response during antigen driven T cell activation [34]. In a resting T cell the minimal complex appears to be composed of a TCR$\alpha\beta$/CD3$\gamma\delta\varepsilon_2\zeta_2$ structure, but at the cell membrane, especially after activation, multimers of this minimal structure may be formed [35,36].

- *Peptide/MHC complex*

The TCR cannot recognize foreign antigens directly, but only when it is presented as a short peptide in the context of a self-protein, i.e., a MHC-molecule on an antigen presenting cell (APC), e.g., a tumor cell [37]. There are two different classes of MHC molecules. Class I MHC molecules are expressed on almost all nucleated cells and present antigens derived from cytoplasmic peptides (reviewed in Townsend *et al.* [38]). In short, proteasomes in the cytosol degrade ubiquinated cytoplasmic proteins to peptides, after which the peptides are transported to the lumen of the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) proteins [39]. There further proteolytic processing of the peptide takes place [40]. MHC class I folding requires peptide and takes several steps involving the help of proteins called molecular chaperones, i.e., calnexin, calreticulin and tapasin. These molecular chaperones help to stabilize the class I molecule until it interacts with β2 microglobulin and peptide. Once the peptide is bound, the class I molecule is stable enough to move to the golgi and then out to the cell surface via cellular vesicles (reviewed in Jensen *et al.* [39]). Tumor antigens that are endogenously processed by the tumor cell are also presented by the MHC class I molecules at the cell surface. Class II MHC molecules are expressed on a subset of cells with specific immunological functions (e.g., dendritic cells, macrophages and B cells) and present antigens derived predominantly from extracellular sources (reviewed in Brodsky *et al.* [41]). Extracellular antigen is taken up by the cell via phagocytosis or receptor-mediated endocytosis and enters a series of compartments (i.e., endosome, endolysosome and lysosome) that serve to break the antigen down into peptide. MHC class II molecules are assembled in the ER and transported to an endosomal compartment. The invariant chain associates with the peptide-binding cleft and prevents peptide binding in the ER. In the endosomal compartment the invariant chain is degraded except for a small piece left in the peptide binding cleft called CLIP (class II associated invariant chain peptide). When the vesicle containing class II/CLIP and the vesicle with peptide fuse, the peptide exchange factor DM causes that CLIP is removed and the peptide is loaded in the class II MHC, after which they are transported to the cell surface (reviewed in Jensen *et al.* [39] and Cresswell *et al.* [42]). Tumor proteins or larger tumor cell parts that are released after disruption of a tumor cell can be phagocytosed by macrophages and are presented as peptides in MHC class II molecules.

- *Co-receptor/co-stimulatory molecules/adhesion molecules*

T cells with distinct effector functions recognize class I and class II MHC molecules. Class I molecules present antigen to cytotoxic T lymphocytes (CTLs), that ultimately will kill the presenting cell by release of granules filled with perforin and granzymes, upregulation of Fas-ligand (FasL) which interacts with Fas on the target cell, and INFγ secretion [43-45]. Class II molecules present antigen to T helper cells, that ultimately will activate the presenting cell via release of cytokines and upregulation of CD40-ligand which interacts with CD40 on the presenting cell [46,47]. In addition to the TCR, T cells express a second molecule that is committed to recognize only one class of MHC molecule and stabilizes the TCR: peptide/MHC interaction. For class II MHC-restricted T cells this is the CD4 molecule, and for class I MHC-restricted T cells this is the CD8 molecule [48]. These so called co-receptors are cell membrane proteins, of which the extracellular domain interacts with the respective MHC molecule and the cytoplasmic domain interacts with the T cell-specific protein tyrosine kinase $p56^{lck}$, which plays an important role in the signal transduction after TCR recognition of antigen peptide (see paragraph 1.1.2). Next to the CD4 and CD8 co-receptors several co-stimulatory and adhesion molecules play a role during T lymphocyte activation: (i) CD2 (lymphocyte-function-associated antigen (LFA-)2) on T cells binds to CD58 (LFA-3) on target cells and is important for cell adhesion and T cell activation (CD2 intracellularly binds Lck) [49]; (ii) CD11a/CD18 (LFA-1) on T cells binds CD54 (intracellular adhesion molecule (ICAM)-1) and is involved in cell adhesion and co-stimulation [50]; (iii) CD27 ligation on T cells causes tyrosine-phosphorylation of ZAP-70, a crucial step in T cell activation (see paragraph 1.1.2) [51,52]; (iv) CD28 activates naive T cells and co-stimulates T cell effector functions after binding its ligands CD80 (B7-1) or CD86 (B7-2) [53]; (v) CD134 (OX40) may act as adhesion molecule and co-stimulator; and (vi) CDw137 (4-1BB) is an important co-stimulator of T cell proliferation [54].

1.1.2 T lymphocyte activation

Initial adhesion of a scanning T cell to an APC is antigen independent and is likely to involve various adhesion molecules (e.g., LFA-1 and CD2). When a TCR recognizes and binds to its specific peptide/MHC complex, migration of the T cell is stopped. Intracellularly, the calcium concentration is elevated and protein tyrosine kinases (see below) are activated. These first signals, including signaling through CD3 ITAMs, cause cytoskeleton rearrangements resulting in the formation of the immunological synapse; the site of T lymphocyte : tumor cell interaction. Microclusters of TCRs/CD8 form over a large con-

tact site, and TCRs coalesce in response to co-stimulatory signaling via LFA-1 and CD28, causing a centralized accumulation of TCRs in the central supramolecular activating complex (c-SMAC). Only strong agonistic peptides give rise to this structure. In the c-SMAC TCRs interact with peptide/MHC complexes on the APC. Concentric rings of adhesion and co-stimulatory molecules surround the TCRs. LFA-1 interacts with ICAM-1 in the periphery of the c-SMAC (the p-SMAC), and CD45, a phosphatase of Lck (see below), and the bulky molecule CD43 are excluded from the SMACs (reviewed in Krummel *et al.* [55] and Delon *et al.* [56]). A cross-section of an immunological synapse is schematically represented in figure 1. Termination of cell-surface receptor signaling and T cell activation is accomplished by internalization of the triggered TCR/CD3 complex, and upregulation of CTLA-4 (cytotoxic T-lymphocyte antigen 4) molecules which replace CD28 molecules and send a negative signal [57]. The immunological synapse is broken down, and the T cell detaches from the APC.

Intracellularly, T cell receptor triggering is characterized by calcium influx and activation of a protein tyrosine kinase (PTK) signaling cascade (reviewed in Clements *et al.* [58]). After recognition of antigen/MHC by the TCR, the Src family of protein tyrosine kinases, i.e., the kinase $p56^{lck}$, which is associated with the CD4 or CD8 molecule, and $p59^{fyn}$, which is associated with the cytoplasmic domains of the CD3 complex, are activated by dephosphorylation of negative regulatory sites of tyrosine phosphorylation by CD45. $p56^{lck}$ and $p59^{fyn}$ induce the rapid phosphorylation of the ITAMs of CD3 molecules (i.e., CD3ζ), followed by recruitment and activation via tyrosine phosphorylation of the kinase ZAP-70 (zeta-associated protein of 70 kD). ZAP-70 propagates the signal by phosphorylating the adaptor molecule named linker of activated T cells (LAT), that couples to several downstream signaling pathways, i.e., activation of PLCγ1, Grb-2/Ras/Raf, SLP-76 and Vav. LAT recruits phospholipase C (PLC-)γ1 to the PTKs, and subsequent tyrosine phosphorylation increase the catalytic activity of PLC-γ1. PLC-γ1 induces phosphatidylinositol (PIP_2) metabolism resulting in the generation of inositol polyphosphates (IP_3), which regulate increases in intracellular calcium, and diacylglycerol (DAG), which stimulates protein kinase C (PKC). Intracellular calcium is important for activation of calcineurin. Furthermore, LAT recruits the Grb-2/Sos (son of sevenless) complex, which localizes Sos to the guanine nucleotide binding protein Ras, resulting in Ras activation (i.e., GTP-binding to Ras). Activation of PKC with phorbol esters or DAG also induces the accumulation of Ras-GTP complexes. Ras activates the mitogen activated protein kinase kinase kinase (MAPKKK) Raf-1, which in turn activates a MAP kinase kinase (MAPKK). This MAPKK is an activator kinase and

induces the phosphorylation and activation of MAP kinases or extracellular signal regulated kinases (ERKs). MAP kinases translocate to the nucleus when activated and their known substrates include transcriptional factors. ZAP-70 also phosphorylates SLP-76 (SH2 domain containing leukocyte protein of 76 kD), which forms a complex with Vav and plays an important role in mediating both ERK activation and the release of intracellular calcium following TCR triggering. These pathways ultimately culminate in the activation of several ubiquitous transcription factors, such as activator protein-1 (AP-1), nuclear factor κB (NFκB) and octamer-binding transcription factor-1 (Oct-1), and a unique T cell-specific transcriptional complex termed Nuclear Factor of Activated T cells (NFAT) [59]. These transcription factors induce gene transcription necessary for T cell activation and the appropriate effector functions (reviewed by Nel [60]). T cell receptor triggering and activation of PTK signaling cascades are schematically represented in figure 1.

TCRs are not limited to recognition of one specific peptide. They have a certain flexibility for ligand recognition provided by local conformational changes in the CDR loops that contact peptide in MHC molecules. This means that TCR can perceive structural changes in its ligand (the quality of the ligand) and can translate these changes into distinct signaling patterns that ultimately result in different effector responses (i.e., cytolysis, cytokine production and proliferation). Research involving natural peptide variants and altered peptide ligands provided much insight in how TCR can distinguish between these variants and its natural epitope, and why this results in different effector responses (reviewed by Madrenas [61]). This research leaded to the division of peptide variants into five groups. First, the agonist: those TCR ligands that induce a full array of effector functions on the responding cell. Second, the partial agonist: those variant ligands that can activate some T cell responses but not others which are normally induced by agonists. Some partial agonists induce cytokine production without proliferation, or produce only some cytokines, but not all. Another partial agonist did induce cytolysis of target cells and apoptosis in CTLs, while no IL-2 was produced [62]. Third, the weak agonist: those variant ligands that can induce all effector functions, but require higher concentrations compared to the agonist. The difference between partial agonist and a weak agonist can be seen in titration of effector responses. Fourth, the antagonist: those variant ligands that have an inhibitory effect on T cell responses induced by agonist peptides. And fifth, the supra-agonist: those variant ligands that have an extra stimulatory effect on T cell responses induced by agonist peptides [63]. The different effector functions induced by the variant ligands described above can be explained by differences in signaling patterns. Early signaling events induced by agonist include full phosphorylation of

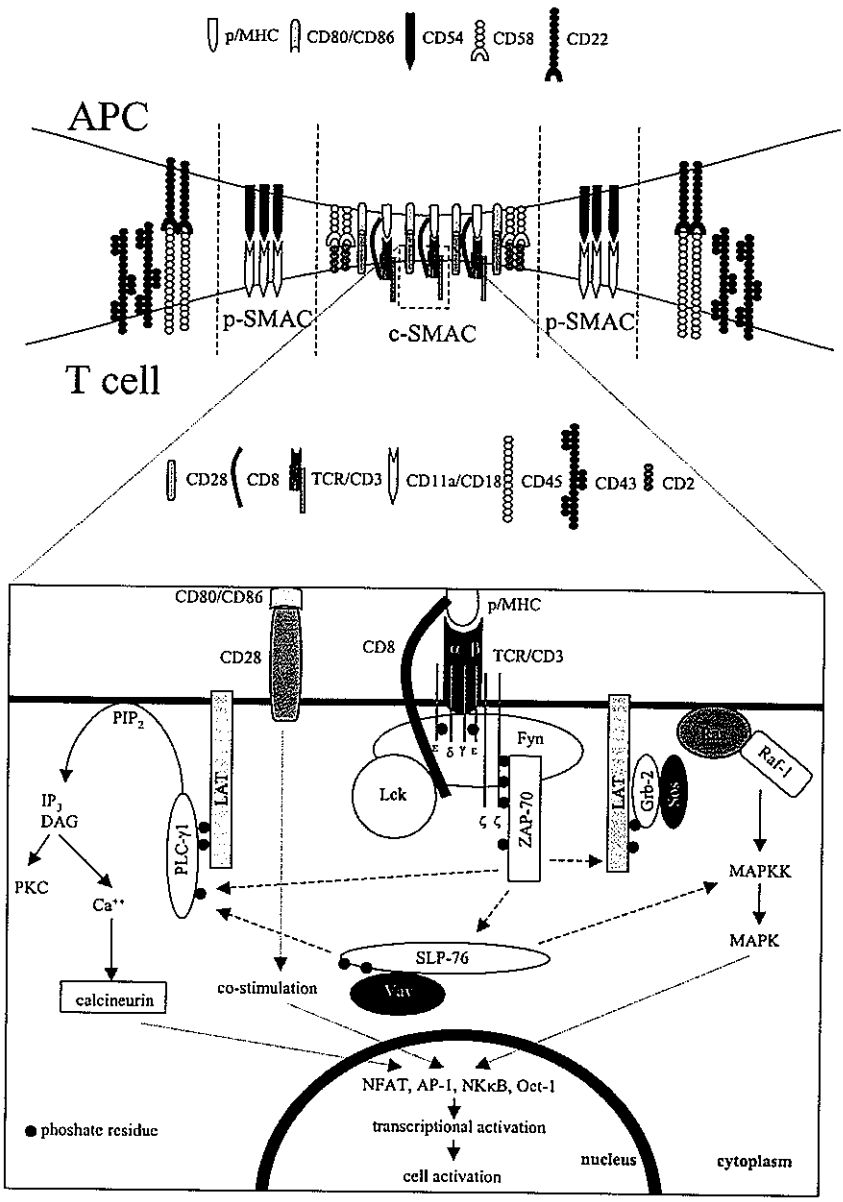

Figure 1. Schematic representation of an immunological synapse formed by a T cell and APC (upper part), and PTK signaling cascades activated by TCR triggering (lower part).

For more details see text of paragraph 1.1.2.

p/MHC, peptide/MHC complex; α, TCRα chain; β, TCRβ chain; γ, CD3γ; δ, CD3δ; ε, CD3ε; ζ, CD3ζ.

all CD3 components, and recruitment, phosphorylation and activation of ZAP-70. Weak agonists reach the threshold for activation when higher concentrations are used. Partial agonists induce differential phosphorylation of CD3ζ, little or no phosphorylation of CD3ε, and recruitment, but no phosphorylation or activation of ZAP-70, whereas antagonists only induce minor phosphorylation of CD3ζ. In contrast, supra-agonists increase ZAP-70 tyrosine kinase activation induced by the natural epitope [63]. In addition, the affinity of TCRs for an agonist/MHC complex is high enough to induce oligomerization of TCRs on the membrane, which is necessary for full activation. TCR affinities for partial agonists and antagonists are lower, resulting in partial oligomerization or no oligomerization of TCRs, respectively. In these cases, recruitment of CD4/CD8 co-receptors and stabilization of the TCR:peptide/MHC interaction can be crucial for quality of TCR-mediated signals (reviewed by Madrenas [61]).

Prior to the first encounter with MHC-presented antigen, T lymphocytes are in a resting state. Among several co-stimulatory pathways, CD28-mediated co-stimulation via ligands of the B7-family expressed on APC is crucial for efficient activation of resting T cells. CD28 co-stimulation results in cellular proliferation, cytokine secretion (i.e., IL-2), CTL-mediated target cell lysis, and prevention of activation-induced T cell death by upregulation of the anti-apoptotic proteins *bcl*-x_L and *bcl*-2 [53,64-66]. CD28 preferentially serves to lower the amount of antigen required to achieve full cellular activation [67] and to amplify and sustain a primary T-cell response [68]. In contrast to resting T cells, the role of CD28/B7 co-stimulation in completely activated T cells is much less clear. In antigen-activated T cells, the triggering of antigen-specific cytolysis via the TCR/CD3 complex appears to be independent of CD28/B7 co-stimulation. Moreover, the proliferation of CD8-positive T cells seems to be uncoupled from their cytolytic activity but is substantially enhanced by B7 co-stimulation [69]. It is of note that most tumor cells do not express B7 molecules on the cell membrane and are not able to provide co-stimulation via CD28 [64]. However, T cell proliferation can be induced by expression of ICAM-1 on the tumor cells, which also provides co-stimulatory activity [70].

1.2 Tumor antigens

Tumor antigens can elicit humoral and cellular reactions in tumor bearing hosts.

The identification of tumor antigens was made possible both by monoclonal antibodies (mAbs) and by the isolation and generation of T lymphocyte lines and clones that recog-

nize MHC-restricted tumor antigens. Therefore, antigens expressed by tumors can be divided in two groups: (i) non-MHC-restricted tumor antigens, and (ii) MHC-restricted tumor antigens. Tumor antigens on which research described in this thesis focused are highlighted.

1.2.1 Non-MHC-restricted tumor antigens

Non-MHC-restricted tumor antigens can be recognized by antibodies as well as lymphokine-activated killer (LAK) cells which lyse cancer cells *in vitro* in a non-MHC-restricted fashion [71]. The SEREX (serologic analysis of recombinant cDNA expression libraries) technique, in which diluted serum from cancer patients is used to detect proteins encoded by cancer cDNA libraries that are expressed in prokaryotes, was very useful to characterize many non-MHC-restricted tumor antigens [72]. Many mAbs specific for tumor antigens have been isolated [73], e.g., mAbs specific for the ganglioside D3 (GD3) melanoma antigen [74], the colon cancer-associated epithelial glycoprotein 40 (EGP40) antigen [75], and the renal cell carcinoma G250-antigen [76].

- *G250-antigen*

The G250-antigen is a renal cell carcinoma tumor associated antigen (RCC-TAA) recognized by the mouse mAb G250. It is a 49,6 kD transmembrane protein identical to the MN gene product, which is identified as a cervix carcinoma associated protein, and it shares 40% homology with carbonic acid anhydrase 6 [77]. To date it is not clear whether the G250-antigen indeed acts as a carbonic acid anhydrase. The G250-antigen is present on more than 90% of the primary tumors and more than 80% of the metastases [76,78,79], and is weakly expressed on the epithelium of larger bile ducts and on gastric mucosal cells [80].

1.2.2 MHC-restricted tumor antigens

MHC-restricted tumor antigens are recognized by T lymphocytes. Therefore, the identification of such tumor antigens is dependent on tumor-specific T lymphocytes. Using such T cells, various types of target cells allowed for the identification of MHC class I-restricted antigens. First, target cells, expressing the appropriate HLA molecule, transfected with complementary DNA libraries from tumor cells [81,82]. Second, APC pulsed with peptides eluted from the surface of human cancer cells [83,84]. And third, intact human cancer cells, in which case tumor-specific T cells were pre-sensitized *in vitro* with candidate tumor proteins [85]. By using these methods, new MHC class I-restricted epitopes coded by, e.g., the MAGE (melanoma antigen) and RAGE (renal tumor antigen) genes were identified [85-87]. Identification

of MHC class II-restricted tumor antigens is more difficult. Transfection of cDNA libraries into target cells is not effective because the encoded proteins are not expressed via the class II pathway. However, identification of MHC class II-restricted tumor antigens, e.g., a mutated form of human CDC27, which gave rise to an HLA-DR4-restricted melanoma antigen [88], or HLA-DR13-restricted epitopes in MAGE-A3 [89], was made possible by fusion of cDNA libraries to genes encoding invariant chain sequences designed to guide the transfected proteins into the class II presentation pathway.

Most human tumor antigens so far identified, have been derived from melanomas, because generation of human T lymphocytes specific for this type of tumor is relatively easy. In addition, many antigens expressed on common epithelial tumors have also been identified. It is of note that many tumor antigens (e.g., MAGE, NY-ESO-1a (New York esophagus 1a)) are now known to be recognized by both T cells and antibodies in the same cancer patients [90], pointing to the expression of different epitopes derived from a single antigen expressed either in a MHC-restricted or non-MHC-restricted fashion, respectively.

Nowadays, MHC-restricted tumor antigens are subdivided into 6 groups (summarized in Renkvist *et al.* [91]):

Group 1: Class I MHC-restricted cancer/testis antigens. These antigens are expressed in histologically different human tumors and in spermatocytes/spermatogonia of testis. Testis do not express class I MHC-molecules, and therefore do not form immunological targets [92]. Cancer/testis antigens result from reactivation of genes normally silent in adult tissues, but that are transcriptionally activated in some tumors [93,94].

- *MAGE-A1 antigen*

MAGE-A1 was the first MHC-restricted tumor antigen to be cloned [86], and is a member of the MAGE family consisting of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10 and MAGE-A12 [91]. Many peptides of MAGE-A1, presented by different HLA molecules, are expressed on melanoma, colon and breast carcinoma, and small cell lung carcinoma [86,93,95].

Group 2: Class I MHC-restricted differentiation antigens. These antigens are shared between tumors and normal tissue from which the tumor arose; most are found in melanomas and normal melanocytes [96].

- *Gp100 antigen*

The glycoprotein 100 kD (gp100) antigen is a class I MHC-restricted melanocyte differentiation antigen and is expressed on normal and neoplastic cells of the same lineage (i.e., melanocytes, skin, retina, peripheral ganglia) [96]. Multiple peptides of gp100 that are recognized by CTL isolated from either peripheral blood or tumor lesions of melanoma patients have been identified [84,97-99].

Group 3: Class I MHC-restricted widely expressed antigens. These antigens are expressed on various normal tissues, probably below the threshold level of T-cell recognition, while their overexpression in tumor cells can trigger an anticancer response.
Group 4: Class I MHC-restricted, tumor-specific antigens. These tumor antigens arise from point mutations of normal genes, whose molecular changes often accompany neoplastic transformation or progression.
Group 5: Class II MHC-restricted antigens. Most of these antigens are cancer/testis antigens or differentiation antigens that, when presented by MHC class II, are capable of provoking an antigen-specific $CD4^+$ T-cell response.
Group 6: Fusion proteins. These antigens result from fusion of distant genes and subsequent expression of fusion proteins, which is seen in several malignancies (e.g., leukaemia). These new epitopes can be recognized by either class I or class II MHC-restricted T lymphocytes.

1.2.3 Virus-related tumor antigens

There is increasing evidence of a relationship between viral infections and the incidence of cancer. Many of the viruses associated with oncogenesis result in the expression of proteins on infected cells that can serve as targets for immune attack and represent a different class of cancer antigens. Examples of virus-related tumor antigens (reviewed by Lowy *et al.* [100]) are: the E6 and E7 epitopes of human papillomavirus on cervical cancers [101], human T-cell lymphotropic virus-1 epitopes on adult T-cell leukaemias [102], epitopes of Epstein-Barr virus (EBV) on lymphomas [103,104].

- *EBV-related tumors*

EBV is associated with a broad range of malignancies of mostly hematopoietic or epithelial origin. EBV is a gamma-1 B-lymphotropic herpesvirus found in all human populations, with a prevalence of over 90% in adults, where it can remain latent and persist for life.

Infection of B cells is mediated through interaction of the viral envelope glycoprotein gp350 with CD21 on B-cells. In addition, regarded as a cofactor for B cell entry, EBV uses the gp42 glycoprotein to bind to MHC class II molecules [105,106]. Once inside the cell, viral DNAs usually take the form of circular episomes, and only a small subset of at least 11 viral genes is expressed in latently infected B-lymphocytes. Based on their protein products, these latent viral genes can be divided into three groups: (i) two genes that encode small, non-polyadenylated RNAs (the EBV-encoded RNAs EBER-1 and EBER-2); (ii) six genes that encode nuclear proteins (the EBV nuclear antigens EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-5); and (iii) three genes that encode integral membrane proteins (the latent membrane proteins LMP-1, LMP-2A and LMP-2B (reviewed by Kieff [107] and Cohen [108]). The expression pattern of these latent genes determines the classification of EBV-associated malignancies. Malignancies with a type I latency express the EBERs and EBNA-1 (i.e., Burkitt's lymphoma and gastric carcinoma) [103,109]. Malignancies with a type II latency express the EBERs, EBNA-1 and all LMPs (i.e., Hodgkin's disease and nasopharyngeal carcinoma) [110-112]. Finally, malignancies with a type III latency express all above mentioned latent genes (i.e., immunosuppression-related lymphomas and X-linked lymphoproliferative disease) [104,108]. In fact, immunosuppressed patients (post-transplant/AIDS) are predisposed to EBV B-cell lymphomas [113], whereas in healthy individuals immune control over EBV exists. Important to the immune control is the recognition of latent antigens by MHC class I-restricted CTL [114,115].

1.3 Immunotherapy of cancer

Despite the fact that tumor-specific antibodies and T lymphocytes can be isolated from patients with many different tumors, the efficiency of tumor-specific antibodies and T lymphocytes to successfully attack tumor cells is too low in most patients. Especially nonmutated tumor-antigens have proven to be poorly immunogenic, most probably because of peripheral tolerance [116-118]. The main goal in the immunotherapy of cancer is to enhance the patient's own immune response against tumors. One strategy to enhance the immune response against tumor-antigens would be to manipulate effector cells of the immune system, such as T cells. A therapeutic anti-tumor T cell response can be established in various ways, as is described below in more detail.

1.3.1 Vaccination therapy

Dendritic cells (DC) are optimal APCs and key regulators in immune responses, capable of priming naive T cells, which, at least in part, is due to their high expression of MHC class I and II molecules as well as co-stimulatory molecules [119-121]. DC can be used as tools to enhance tumor-antigen presentation to both CTL and T helper cells, for which reason DC-based vaccines are being developed for treatment of patients with cancer.

Several methods have been developed to introduce tumor-antigens *ex vivo* in DC. For example DC can be either pulsed with synthetic or eluted peptides derived from known tumor-associated antigens; they can be co-cultivated with full length native or recombinant soluble proteins; they can be transfected with cDNA or RNA encoding known tumor-associated antigens or with whole tumor-derived RNA; they can be transduced with recombinant viruses; they can be loaded with tumor lysate or dying tumor cells (apoptotic bodies); or they can be fused with tumor cells. Depending on the method, tumor-antigens are introduced either into the MHC class I or II processing pathways (reviewed by Brossart *et al.* [122] and Nouri-Shirazi *et al.* [123]).

In vitro studies demonstrated that it is possible to generate CTL specifically directed against tumor-antigens (i.e., melanoma gp100) using peptide-pulsed DC and peripheral blood lymphocytes (PBL) of healthy individuals [98].

In a melanoma mouse model, DC loaded with an MHC class I-restricted peptide derived from a melanocyte differentiation antigen were able to break tolerance and induce protective CTL-mediated immunity against melanoma [124]. Several other animal studies have also demonstrated that DC, when loaded with tumor-antigens or pulsed with peptides and administered to cancer-bearing hosts, can elicit T cell-mediated cancer destruction [125,126].

The observation that peptide-pulsed DC can induce *bona fide* CTL clones capable of lysing tumor cells that endogenously process and present a tumor antigen supports a treatment strategy of tumor patients with such DC. Preliminary results from clinical trials in patients with melanoma, renal cell carcinoma, malignant lymphoma, colorectal carcinoma, lung carcinoma and prostate cancer suggest that immune-therapeutic strategies which take advantage of the unique properties of DC may ultimately prove both efficacious and widely applicable to treat cancer patients (reviewed by Brossart *et al.* [122] and Satthaporn *et al.* [127]).

Other vaccination strategies for the immunotherapy of cancer comprise the use of (genetically modified) tumor cells [128], synthetic peptides [129], antigen-encoding viruses [130], or antigen-encoding naked plasmid DNA [131]. In all these instances an efficient anti-tumor immune-response was generated in mouse-models, consisting of components of cellular and

humoral immunity. An advantage of DNA vaccination is that it allows the incorporation of multiple epitopes derived from the encoded antigen and a broad range of MHC restriction during induction of the immune-response [131]. Presumably all such vaccination protocols induce antigen presentation by DC *in vivo*, after which T lymphocytes and other immune cells become activated.

1.3.2 Adoptive T cell transfer

Apart from aiming immunotherapy at APCs, or DCs in particular, one can also directly use tumor-specific T lymphocytes to treat cancer. Tumor-infiltrating lymphocytes (TIL) can be isolated from some tumor-bearing patients and have the potential to home to tumors [132-135]. Furthermore, tumor-specific T lymphocytes can be generated *in vitro* from PBL of patients by stimulating them with tumor-antigen presenting cells [136-139].

These T lymphocytes can be used for adoptive T cell-mediated immunotherapy in which the (preferably autologous) tumor-specific T lymphocytes are reinfused into the patient in large quantities after culturing. Preservation of the correct homing-signals to the tumor-site seems to be achieved by generation and administration of tumor-specific T cells in ways that mimic physiologic conditions (i.e., stimulation with autologous dendritic cells and very low dose of IL-2, and administration together with low dose of IL-2)[139]. Reinfused TIL can mediate regression of metastatic tumors, such as colon and bladder carcinoma, sarcoma, and melanoma in mouse models [140,141], but administration of autologous TIL to cancer patients resulted only in partial responses in melanoma and renal cell carcinoma [135,142-144]. In addition, adoptive transfer of *in vitro*-generated CTL specific for melanoma antigens (i.e., MART-1, gp100 and tyrosinase) to patients with metastatic melanoma resulted in the objective regression of established tumors, although few patients have shown sustained clinical benefit from such therapy [81,139].

In allogeneic bone marrow transplant (BMT) recipients, receiving immunosuppressive agents, many cases of lymphoproliferative disease, including B-cell lymphomas (BCL), have been reported. The majority of these tumors is EBV-positive and regress when immunosuppressive agents are discontinued. Tumor regression is thought to be secondary to CTL reactive to EBV-infected cells whose function is impaired in patients receiving immunosuppressive agents. EBV-specific CTL, generated *in vitro*, were able to lyse an EBV-expressing B-cell lymphoma in *in vitro* assays, and in a mouse model [145]. Furthermore, in several clinical trials prophylactic or therapeutic adoptive immunotherapy with EBV-specific CTL was also effective in treatment of EBV-positive tumors in allogeneic BMT and solid

organ transplant recipients [146-150]. A note of concern is that this immunotherapy is performed with CTL lines generated from peripheral blood from EBV-seropositive bone marrow donors, and is associated with graft-versus-host disease. Adoptive immuno-therapy with virus-specific CTL also mediates anti-viral effects in BMT recipients with a life-threatening cytomegalovirus (CMV) infection [150-152].

Despite some clinical success, a serious concern of adoptive T-cell mediated immunotherapy is that patients do not always mount a strong and effective *in vivo* cytotoxic T cell response to their tumors. In fact, isolation of tumor-specific T lymphocytes has only been possible in a fraction of patients, most likely due to the fact that, at least in an immunocompetent setting, the peripheral T cell repertoire is devoid of high-avidity tumor-specific CTL due to thymic selection [153]. In addition, these cells and *in vitro* generated tumor-specific T cells only have a limited life-span, and expansion of such T lymphocytes to therapeutic doses is often not feasible [154].

Alternatively, bispecific monoclonal antibodies (bsmAbs), with one binding site directed against a tumor antigen and the other against an activation molecule on T lymphocytes, can be used as tools to bring effector T lymphocytes in close contact with the tumor cells. Large quantities of T lymphocytes pre-coated with the bsmAbs can be delivered to the tumor site. After cross-linking molecules on the cell surface of tumor cells on the one hand and T lymphocytes on the other hand, T lymphocytes become activated and perform various effector functions (i.e., cytokine production and cytolysis of the target cell) [155-157]. Bispecific mAbs very efficiently retargeted T lymphocytes to renal cell carcinoma [158], human colon carcinoma [159], Hodgkin's lymphoma [160], ovarian carcinoma [161], and melanoma [162] both in *in vitro* assays and mouse tumor models. In clinical studies, bsmAbs directed against ovarian carcinoma [163,164], melanoma [165], renal cell carcinoma [166], colon carcinoma [167] and B cell malignancies [168] elicited anti-tumor responses.

Nevertheless, the usage of bsmAbs for the immunotherapy of cancer may have several limitations: (i) bsmAbs are bound to CTL for a limited period of time, and dissociation will diminish anti-tumor effects [157]; (ii) bsmAb-retargeted lymphocytes loose lytic capacity upon their initial encounter with tumor cells, i.e., they cannot "recycle" their lytic capacity [169]; (iii) excess use of bsmAbs could evoke a human anti-mouse Ab (HAMA) response, eventually blocking cytolytic activity [170,171]; and (iv) the use of bsmAbs may be hampered by the inaccessibility of solid tumors [169,172].

1.3.3 T cell retargeting via transfer of tumor-specific receptor genes

Another way to combine tumor-specific mAb or TCR for that matter with effector functions of T lymphocytes would be to introduce such anti-tumor specificity into T lymphocytes by genetic means. Not only T lymphocytes originating from bulk autologous peripheral blood mononuclear cells (PBMC) can be used for genetic retargeting, but also virus- or allospecific T cells (see chapter 6). Various formats of tumor-specific receptors that enable human T cell retargeting to tumors following gene transfer are discussed below.

- *Chimeric antibody-based receptors*

Genetically grafting T lymphocytes with chimeric antibody-based receptors can overcome the above mentioned limitations of bsmAbs [173,174]. Chimeric single chain (sc) Fv receptors generally incorporate the variable domains of both the Ab heavy and light chains, interspersed by a flexible linker [175,176]. The antigen binding domains are coupled to signal transducing elements, in most cases the Fc(ε)RIγ chain of mast cells or the CD3ζ chain. Chimeric antibody-based receptors specific for several tumor-antigens have been functionally expressed in T lymphocytes, and anti-tumor activity of these T lymphocytes was seen in several *in vitro* assays and mouse models [175,177-188]. It is clear from preclinical studies that individual receptor components, such as extracellular spacers and transmembrane domains [189], isolated TCR-CD3 chains or Fc(ε)RIγ signaling domains [176,188,190,191], co-stimulatory molecules or protein tyrosine kinases [192,193], incorporated into chimeric antibody-based receptors greatly affect the expression level of the receptor, its stability, ligand binding affinity and thereby receptor mediated functions. Moreover, the combination of a CD3 component together with a kinase and/or co-stimulatory molecule, i.e., CD28, in a single receptor maximizes chimeric receptor sensitivity and potency [193,194].

A chimeric antibody-based receptor specific for the renal cell carcinoma tumor associated antigen G250 is discussed in more detail in this thesis. Several formats for the chimeric antibody-based receptor were studied [176]. It was decided that the optimal scFv receptor consisted of the variable domains of the heavy and light chain of the mouse mAb G250, connected to each other by a flexible linker and ligated to a stretch of several amino acids of the constant κ chain, the CD4 transmembrane domain and the Fc(ε)RIγ signaling domain (scFv-γ; see figure 2) [176]. The transduction procedure used for primary human T lymphocytes was optimized, allowing for gene transfer into primary human T lymphocytes with high efficiency and at clinical scale meeting the criteria of Good Medical Practice [195]. T cells transduced with the G250-specific chimeric scFv-γ receptor will be used for immuno-genetherapy in a clinical phase I study in metastatic RCC patients.

- *Full length T cell receptors*

Antibody-based receptors recognize MHC-unrestricted tumor-antigens. As described above, there are many tumor-antigens that are presented in the context of an MHC-molecule and are recognized by TCR on T lymphocytes. By genetically grafting T lymphocytes with a TCR, these cells can be redirected to MHC-restricted antigens.

Genes encoding for the full length (i.e., complete) TCR α and β chain (see figure 2), can be cloned from tumor-specific CTL clones, and transferred into primary human T lymphocytes via retroviral transduction.

In several *in vitro* studies, primary human T lymphocytes have been retargeted by transfer of human full length (fl-)TCRα/β genes. The targeted MHC class I-restricted antigens ranged from viral-antigens, i.e., HLA-A3-presented HIV GAG-antigen [196] or HLA-A2-presented EBV LMP2-antigen [197], a melanocyte differentiation antigen, i.e., HLA-A2-presented MART-1 antigen [198] to a tumor-associated oncoprotein, i.e., HLA-A2-presented MDM2-antigen [199]. Also full length mouse TCRα/β genes specific for a MHC class II-restricted antigen were successfully transferred to T lymphocytes [200]. In all mentioned studies, the retargeted primary T lymphocytes were shown to respond specifically towards target cells expressing both the corresponding antigen and the correct HLA-molecule. Recently, it was also shown that mouse T cells that were redirected by TCR gene transfer efficiently promoted the rejection of antigen-expressing tumors *in vivo* [201]. The preservation of both the cytotoxic potency and peptide fine-specificity of CTL following TCR gene transfer, which is crucial to the successful use of TCR genes for immunogene therapy, is a topic dealt with in this thesis (see chapter 3).

- *Chimeric T cell receptors*

In the studies described above, transfer of virus-/tumor-specific full length TCR α and β chain genes was used to retarget T lymphocytes. It is possible that full length TCR chains pair with the endogenous TCR α and β chains of the transduced T lymphocytes (see discussion of chapter 3). This alternative pairing can lead to unpredictable specificities, which may be autoreactive. Although there is limited research with TCR-transduced T cells in a mouse model that suggests no overt autoimmune pathology [201], the formation of self-reactive TCRs is a recognized concern for gene therapy regulatory committees. Furthermore, it was shown that introduction of a full length TCRα transgene in T cells can result in a low and unstable surface expression of TCRα protein [202,203], which, at least in dual-TCR T cells, is probably because of difficulties in the post-translational assembly

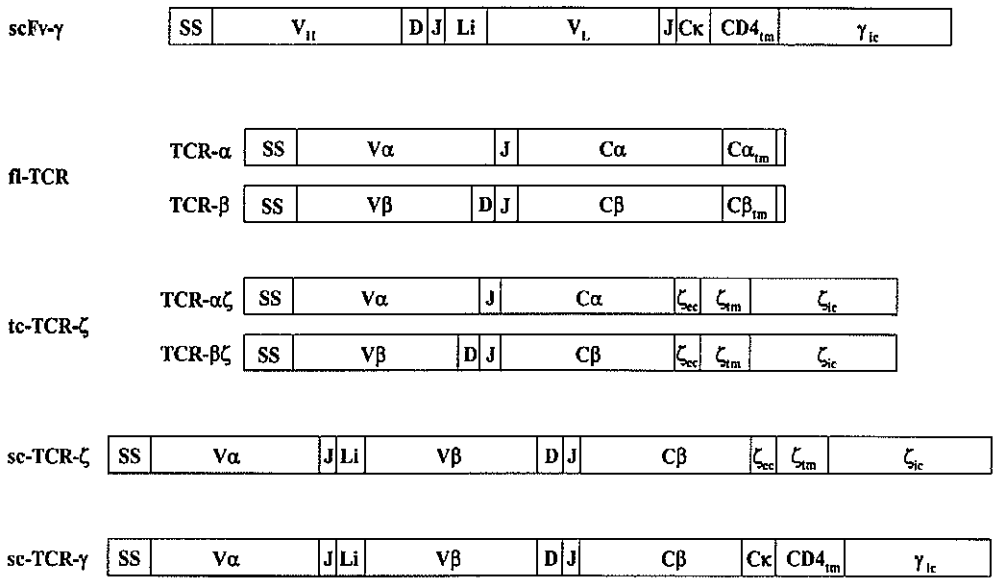

Figure 2. Schematic representation of genes coding for (chimeric) tumor-antigen specific receptors.
The gene encoding the chimeric antibody-based receptor (scFv-γ) specific for the renal cell carcinoma G250 antigen comprises the variable domains of the heavy (V_H) and light (V_L) chains connected by a flexible linker (Li), subsequently ligated to a stretch of amino acids of the constant κ (Cκ) chain, the CD4 transmembrane ($CD4_{tm}$) domain and the Fc(ε)RIγ (γ) signaling domain [176]. The genes coding for the full length TCR consist of the complete DNA coding sequence of the TCR α and β chain. The chimeric tc-TCR-ζ genes consist of the genes coding for the extracellular domains of the TCR α and β chain each coupled to the CD3ζ gene [206]. The chimeric sc-TCR-ζ gene consists of the genes coding for the variable TCR α domain and extracellular domains of the TCR β chain connected by a flexible Li and coupled to the CD3ζ gene [206]. Finally, the chimeric sc-TCR-γ gene consists of the same extracellular domains as described for the sc-TCR-ζ but coupled to a stretch of amino acids of the Cκ chain, the $CD4_{tm}$ and γ signaling domain.
SS, signal sequence; V, variable domain; C, constant domain; D, diversity region; J, joining region; ec, extracellular region; tm, transmembrane region; ic, intracellular region.

process of a second TCR heterodimer [204]. Finally, pairing of introduced TCR α and β chains with endogenous TCR α and β chains, will dilute the expression of the desired TCRαβ heterodimers on T cells (see chapter 3). Therefore, we and others have initiated the development of single and two chain chimeric TCR-based receptors, that are structurally different from full length TCRs, to prevent the problems mentioned above.
Chimeric two-chain TCR-based receptors, comprising CD3ζ signaling molecules

(tc-TCR-ζ), result in exclusive pairing between the introduced TCR α and β chain, and chimeric single chain TCR-based receptors, comprising for example CD3ζ or Fc(ε)RIγ signaling molecules (sc-TCR-ζ or sc-TCR-γ, respectively), do not pair with endogenous TCR chains. In addition, these chimeric TCR-based receptors "rescue" the surface expression of the TCR α chain [203]. Another advantage of the chimeric receptor approach is that receptors chimerized to signaling molecules bypass TCR-mediated proximal signaling events, which are often defective in cancer patients [205]. The receptor formats mentioned above are schematically represented in figure 2.
Using ζ-chimaeras, primary human T lymphocytes transduced for MAGE-1/HLA-A1-specific TCRs showed cytolytic activity and produced cytokines in response towards MAGE-A1^{pos}/HLA-A1^{pos} tumor cells but not MAGE-1^{neg}/HLA-A1^{pos} tumor cells [206].
Careful functional validation of chimeric TCRs after transfer into T lymphocytes (see chapter 2) will result in the development of optimal chimeric receptor designs for immunogene therapy.

1.4 Thesis

1.4.1 Hypothesis

In our studies we aimed to generate human cytotoxic T lymphocytes to treat melanoma and EBV-related tumors. The hypothesis, that directed the research presented in the subsequent chapters, is formulated as follows:

Transfer of full length or chimeric T cell receptor genes into primary human T lymphocytes results in a defined MHC-restricted and tumor- or virus-specific T cell response.

To test this hypothesis, we followed the study design described below.

1.4.2 Study design

To harness primary human T lymphocytes with a melanoma gp100 or EBV BMLF-1, EBNA-3A and EBNA-3B specificity, these cells were retrovirally transduced with genes coding for full length, chimeric two chain or chimeric single chain T cell receptors specific for these antigens. TCR-transduced T lymphocytes were studied by flow-cytometry and several functional assays (as summarized in figure 3).

- *Construction of tumor- and virus-specific receptors*

Several TCR-based receptor formats were used in the research described in this thesis: (i) full length TCR; (ii) tc-TCR-ζ; and (iii) sc-TCR-γ. In addition, a mAb-based scFv-γ receptor was used. Cloning of these receptors is described in more detail in figure 2. Antigen-specificity of the different receptors, CTL-clones from which the TCR-based receptors were cloned, and format of the receptors are summarized in table I.

Table I: Summary of antigen-specificity, parental CTL-clones and format of the different receptors described in this thesis.

Antigen-specificity	CTL	Receptor format	Chapter[a]
gp100 (YLEPGPVTA) presented by HLA-A2	CTL-296	• fl-TCR • tc-TCR-ζ	2, 3 and 4
gp100 (YLEPGPVTA) presented by HLA-A2	CTL-MPD	• fl-TCR • tc-TCR-ζ	3
BMLF1 (GLCTLVAML) presented by HLA-A2	A4.5	• tc-TCR-ζ	5
EBNA-3B (FLRGRAYGL) presented by HLA-A11	BK289	• tc-TCR-ζ	5
EBNA-3A (IVTDFSVIK) presented by HLA-B8	CF3	• sc-TCR-γ	5
G250-antigen		• scFv-γ	2

[a] Chapter in which the receptor is described.

- *Retroviral gene-transfer of tumor- or virus-specific receptors*

Genes coding for the TCR-based receptors were cloned in the retroviral vector pBullet [206], which was derived from the pStitch vector [176]. The retroviral vector harboring the TCR-gene was transfected into the packaging cell line phoenix, which thereafter produced retroviral particles containing the TCR RNA. T lymphocytes or Jurkat T cells were transduced with the retroviral supernatant. TCR-based receptor proteins were synthesized inside the T cells, and expressed on the cell surface (Fig. 3).

- *Functional testing of retargeted T lymphocytes*

Receptor-expression on the cell surface of transduced T cells was measured by flow-cyto-

metry. TCR-transduced T lymphocytes were tested for their antigen reactivity, potency (i.e., dose-response studies) and peptide fine-specificities using several functional assays (see also figure 3). First, a *^{51}Cr-release assay*, measuring cytolytic activity. Second, a *TNF α production assay*. And third, *an NFAT-reporter gene assay*, measuring NFAT activation in TCR-transduced Jurkat T cells (see chapter 2). Antigen reactivity was determined using target cells that were peptide-loaded or endogenously expressing tumor-antigen (see chapter 2-5). Potency of TCR-transduced T lymphocytes was determined in peptide titration studies (see chapter 3). Peptide fine-specificities were determined using target cells loaded with a large panel of peptide mutants (see chapter 3 and 4).

1.4.3 Outline of the thesis

In **chapter 2**, we describe the development of a method to functionally validate tumor-specific (chimeric) receptors using an NFAT-reporter gene assay in receptor-transduced Jurkat T cells. The method was carefully validated using a chimeric antibody-based single chain receptor specific for the renal cell carcinoma antigen G250, and full length TCR, as well as chimeric tc-TCR-ζ receptors specific for the HLA-A2-presented gp100 melanoma antigen. Antigen-specific NFAT-activation in receptor-transduced Jurkat T cells was studied using antigen-positive target cells and blocking antibodies. The NFAT-responses in receptor-transduced Jurkat T cells were compared to anti-tumor responses of primary human T cells transduced with identical (chimeric) receptor gene(s). This allowed us to study whether genetically modified Jurkat T cells can be used to functionally analyze chimeric receptors, and whether NFAT-activation can be used as a prognostic value for (chimeric) receptors following transduction into primary human T lymphocytes.

In **chapter 3**, we molecularly characterized two distinct TCRs of the CTL-296 and CTL-MPD clones specific for the same HLA-A2-restricted melanoma gp100 peptide, yet exhibiting different stringencies in peptide requirements. The existence of these two distinct gp100-specific TCRs allowed us to study the preservation of cytotoxic potency and peptide fine-specificity of native TCRα/β, i.e., full length TCR, when engineered for TCR gene transfer into primary human T lymphocytes.

A more detailed study into peptide fine-specificity patterns is described in **chapter 4**. Target cells loaded with a broad panel of single amino acid peptide mutants and a double amino acid peptide mutant were used to test primary human T lymphocytes transduced with fl-296 TCR genes for cytotoxicity, TNFα-production and NFAT-activation in parallel.

TCR gene-transfer was also tested for T cell retargeting to tumor cells bearing viral

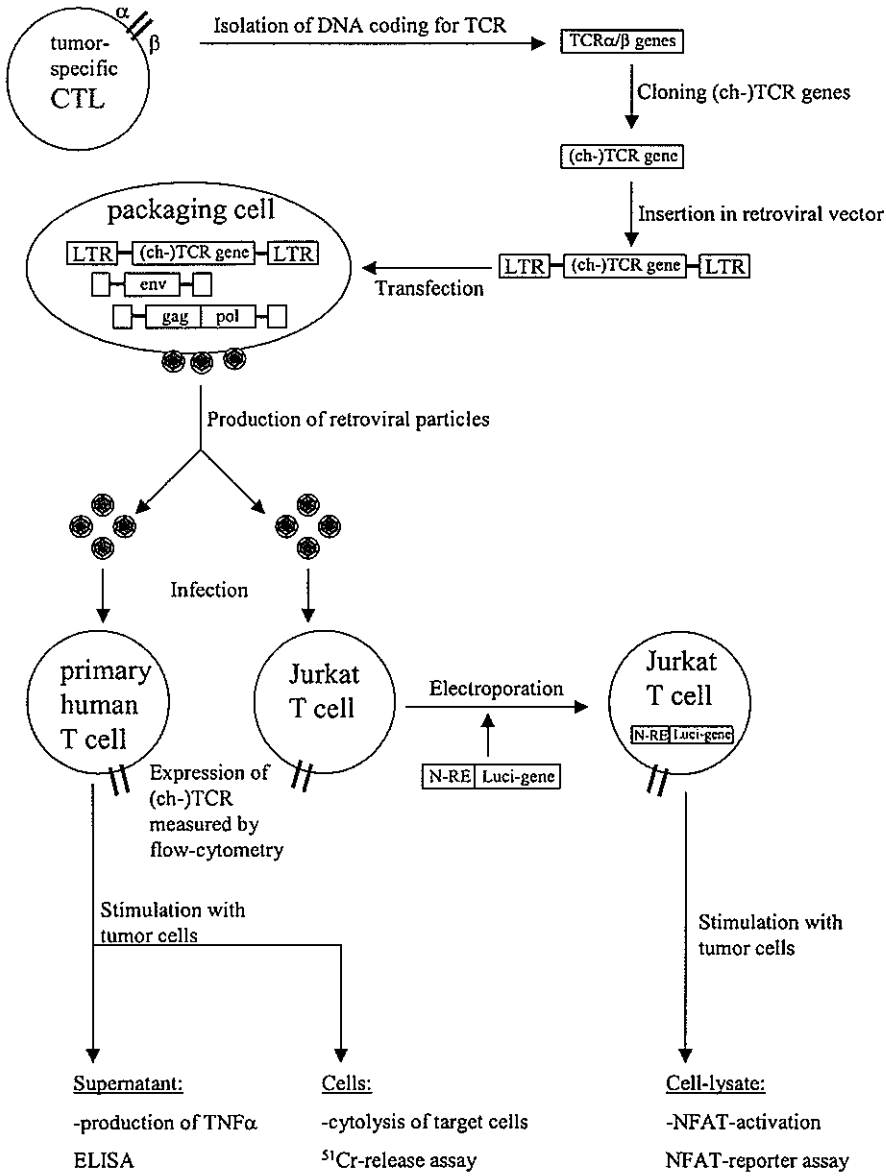

Figure 3. Schematic representation of the study design.

For more details see text of paragraph 1.4.2.

α, TCRα chain; β, TCRβ chain; ch, chimeric; env/gag/pol, retroviral helper constructs; N-RE, NFAT response element; Luci-gene, luciferase gene.

epitopes. **Chapter 5** describes the functional assessment of T lymphocytes transduced with TCR-based receptors specific for various EBV epitopes, i.e., EBNA-3A, BMLF1, and EBNA-3B.

Finally, in **chapter 6** findings reported in this thesis and recent data reported in the literature are discussed, with special emphasis on the feasibility, clinical applicability and future directions of using full length TCR and chimeric TCR-based receptors for the genetic retargeting of T lymphocytes to tumor and viral infected cells.

References

1. Patten,P.; Yokota,T.; Rothbard,J.; Chien,Y.; Arai,K.; and Davis,M.M. "Structure, expression and divergence of T-cell receptor beta-chain variable regions." (1984), *Nature*, **312**:40-46
2. Novotny,J.; Tonegawa,S.; Saito,H.; Kranz,D.M.; and Eisen,H.N. "Secondary, tertiary, and quaternary structure of T-cell-specific immunoglobulin-like polypeptide chains." (1986), *Proc. Natl. Acad. Sci. U. S. A*, **83**:742-746
3. Davis,M.M. and Bjorkman,P.J. "T-cell antigen receptor genes and T-cell recognition." (1988), *Nature*, **334**:395-402
4. Chothia,C.; Boswell,D.R.; and Lesk,A.M. "The outline structure of the T-cell alpha beta receptor." (1988), *EMBO J.*, **7**:3745-3755
5. Claverie,J.M.; Prochnicka-Chalufour,A.; and Bougueleret,L. "Implications of a Fab-like structure for the T-cell receptor." (1989), *Immunol. Today*, **10**:10-14
6. Toyonaga,B. and Mak,T.W. "Genes of the T-cell antigen receptor in normal and malignant T cells." (1987), *Annu. Rev. Immunol.*, **5**:585-620
7. Wilson,R.K.; Lai,E.; Concannon,P.; Barth,R.K.; and Hood,L.E. "Structure, organization and polymorphism of murine and human T-cell receptor alpha and beta chain gene families." (1988), *Immunol. Rev.*, **101**:149-172
8. Arden,B.; Clark,S.P.; Kabelitz,D.; and Mak,T.W. "Human T-cell receptor variable gene segment families." (1995), *Immunogenetics*, **42**:455-500
9. Koop,B.F.; Rowen,L.; Wang,K.; Kuo,C.L.; Seto,D.; Lenstra,J.A.; Howard,S.; Shan,W.; Deshpande,P.; and Hood,L. "The human T-cell receptor TCRAC/TCRDC (C alpha/C delta) region: organization, sequence, and evolution of 97.6 kb of DNA." (1994), *Genomics*, **19**:478-493
10. Toyonaga,B.; Yoshikai,Y.; Vadasz,V.; Chin,B.; and Mak,T.W. "Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor beta chain." (1985), *Proc. Natl. Acad. Sci. U. S. A*, **82**:8624-8628
11. Alt,F.W.; Oltz,E.M.; Young,F.; Gorman,J.; Taccioli,G.; and Chen,J. "VDJ recombination." (1992), *Immunol. Today* , **13**:306-314
12. Schatz,D.G.; Oettinger,M.A.; and Schlissel,M.S. "V(D)J recombination: molecular biology and regulation." (1992), *Annu. Rev. Immunol.*, **10**:359-383
13. Lieber,M.R. "The mechanism of V(D)J recombination: a balance of diversity, specificity, and stability." (1992), *Cell*, **70**:873-876

14. Garcia,K.C.; Degano,M.; Pease,L.R.; Huang,M.; Peterson,P.A.; Teyton,L.; and Wilson,I.A. "Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen." (1998), *Science*, **279**:1166-1172
15. Jorgensen,J.L.; Reay,P.A.; Ehrich,E.W.; and Davis,M.M. "Molecular components of T-cell recognition." (1992), *Annu. Rev. Immunol.*, **10**:835-873
16. Clevers,H.; Alarcon,B.; Wileman,T.; and Terhorst,C. "The T cell receptor/CD3 complex: a dynamic protein ensemble." (1988), *Annu. Rev. Immunol.*, **6**:629-662
17. Morley,B.J.; Chin,K.N.; Newton,M.E.; and Weiss,A. "The lysine residue in the membrane-spanning domain of the beta chain is necessary for cell surface expression of the T cell antigen receptor." (1988), *J. Exp. Med.*, **168**:1971-1978
18. Blumberg,R.S.; Alarcon,B.; Sancho,J.; McDermott,F.V.; Lopez,P.; Breitmeyer,J.; and Terhorst,C. "Assembly and function of the T cell antigen receptor. Requirement of either the lysine or arginine residues in the transmembrane region of the alpha chain." (1990), *J. Biol. Chem.*, **265**:14036-14043
19. Bonifacino,J.S.; Cosson,P.; and Klausner,R.D. "Colocalized transmembrane determinants for ER degradation and subunit assembly explain the intracellular fate of TCR chains." (1990), *Cell*, **63**:503-513
20. Cosson,P.; Lankford,S.P.; Bonifacino,J.S.; and Klausner,R.D. "Membrane protein association by potential intramembrane charge pairs." (1991), *Nature*, **351**:414-416
21. Manolios,N.; Kemp,O.; and Li,Z.G. "The T cell antigen receptor alpha and beta chains interact via distinct regions with CD3 chains." (1994), *Eur. J. Immunol.*, **24**:84-92
22. Shelton,J.G.; Gulland,S.; Nicolson,K.; Kearse,K.P.; and Backstrom,B.T. "Importance of the T cell receptor alpha-chain transmembrane distal region for assembly with cognate subunits." (2001), *Mol. Immunol.*, **38**:259-265
23. Reth,M. "Antigen receptor tail clue." (1989), *Nature*, **338**:383-384
24. Romeo,C.; Amiot,M.; and Seed,B. "Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor zeta chain." (1992), *Cell*, **68**:889-897
25. Cambier,J.C. "New nomenclature for the Reth motif (or ARH1/TAM/ARAM/YXXL)." (1995), *Immunol. Today*, **16**:110
26. Johnson,S.A.; Pleiman,C.M.; Pao,L.; Schneringer,J.; Hippen,K.; and Cambier,J.C. "Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases." (1995), *J. Immunol.*, **155**:4596-4603
27. Perlmutter,R.M.; Levin,S.D.; Appleby,M.W.; Anderson,S.J.; and Alberola-Ila,J. "Regulation of lymphocyte function by protein phosphorylation." (1993), *Annu. Rev. Immunol.*, **11**:451-499
28. Qian,D.; Sperling,A.I.; Lancki,D.W.; Tatsumi,Y.; Barrett,T.A.; Bluestone,J.A.; and Fitch,F.W. "The gamma chain of the high-affinity receptor for IgE is a major functional subunit of the T-cell antigen receptor complex in gamma delta T lymphocytes." (1993), *Proc. Natl. Acad. Sci. U. S. A*, **90**:11875-11879
29. Qian,D.; Griswold-Prenner,I.; Rosner,M.R.; and Fitch,F.W. "Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation." (1993), *J. Biol. Chem.*, **268**:4488-4493
30. Sancho,J.; Franco,R.; Chatila,T.; Hall,C.; and Terhorst,C. "The T cell receptor associated CD3-epsilon protein is phosphorylated upon T cell activation in the two tyrosine residues of a conserved signal transduction motif." (1993), *Eur. J. Immunol.*, **23**:1636-1642

31. Weiss,A. "T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases." (1993), *Cell*, **73**:209-212
32. Weissman,A.M.; Hou,D.; Orloff,D.G.; Modi,W.S.; Seuanez,H.; O'Brien,S.J.; and Klausner,R.D. "Molecular cloning and chromosomal localization of the human T-cell receptor zeta chain: distinction from the molecular CD3 complex." (1988), *Proc. Natl. Acad. Sci. U. S. A*, **85**:9709-9713
33. Sancho,J.; Ledbetter,J.A.; Choi,M.S.; Kanner,S.B.; Deans,J.P.; and Terhorst,C. "CD3-zeta surface expression is required for CD4-p56lck-mediated upregulation of T cell antigen receptor-CD3 signaling in T cells." (1992), *J. Biol. Chem.*, **267**:7871-7879
34. Osman,N.; Turner,H.; Lucas,S.; Reif,K.; and Cantrell,D.A. "The protein interactions of the immunoglobulin receptor family tyrosine- based activation motifs present in the T cell receptor zeta subunits and the CD3 gamma, delta and epsilon chains." (1996), *Eur. J. Immunol.*, **26**:1063-1068
35. Rubin,B.; Alibaud,L.; Huchenq-Champagne,A.; Arnaud,J.; Toribio,M.L.; and Constans,J. "Some hints concerning the shape of T-cell receptor structures." (2002), *Scand. J. Immunol.*, **55**:111-118
36. Fernandez-Miguel,G.; Alarcon,B.; Iglesias,A.; Bluethmann,H.; Alvarez-Mon,M.; Sanz,E.; and de la Hera,A. "Multivalent structure of an alphabetaT cell receptor." (1999), *Proc. Natl. Acad. Sci. U. S. A*, **96**:1547-1552
37. Zinkernagel,R.M. and Doherty,P.C. "Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis." (1974), *Nature*, **251**:547-548
38. Townsend,A. and Bodmer,H. "Antigen recognition by class I-restricted T lymphocytes." (1989), *Annu. Rev. Immunol.*, **7**:601-624
39. Jensen,P.E. "Mechanisms of antigen presentation." (1999), *Clin. Chem. Lab Med.*, **37**:179-186
40. Roelse,J.; Gromme,M.; Momburg,F.; Hammerling,G.; and Neefjes,J. "Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling." (1994), *J. Exp. Med.*, **180**:1591-1597
41. Brodsky,F.M. and Guagliardi,L.E. "The cell biology of antigen processing and presentation." (1991), *Annu. Rev. Immunol.*, **9**:707-744
42. Cresswell,P. "Assembly, transport, and function of MHC class II molecules." (1994), *Annu. Rev. Immunol.*, **12**:259-293
43. Xiao,S.; Matsui,K.; Fine,A.; Zhu,B.; Marshak-Rothstein,A.; Widom,R.L.; and Ju,S.T. "FasL promoter activation by IL-2 through SP1 and NFAT but not Egr-2 and Egr-3." (1999), *Eur. J. Immunol.*, **29**:3456-3465
44. Podack,E.R.; Hengartner,H.; and Lichtenheld,M.G. "A central role of perforin in cytolysis?" (1991), *Annu. Rev. Immunol.*, **9**:129-157
45. Kagi,D.; Ledermann,B.; Burki,K.; Zinkernagel,R.M.; and Hengartner,H. "Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo." (1996), *Annu. Rev. Immunol.*, **14**:207-232
46. Schoenberger,S.P.; Toes,R.E.; van der Voort,E.I.; Offringa,R.; and Melief,C.J. "T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions." (1998), *Nature*, **393**:480-483
47. Swain,S.L.; Bradley,L.M.; Croft,M.; Tonkonogy,S.; Atkins,G.; Weinberg,A.D.; Duncan,D.D.; Hedrick,S.M.; Dutton,R.W.; and Huston,G. "Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development." (1991), *Immunol. Rev.*, **123**:115-144
48. Swain,S.L. "T cell subsets and the recognition of MHC class." (1983), *Immunol. Rev.*, **74**:129-142

49. Bierer,B.E.; Sleckman,B.P.; Ratnofsky,S.E.; and Burakoff,S.J. "The biologic roles of CD2, CD4, and CD8 in T-cell activation." (1989), *Annu. Rev. Immunol.*, **7**:579-599
50. Lub,M.; van Kooyk,Y.; and Figdor,C.G. "Ins and outs of LFA-1." (1995), *Immunol. Today*, **16**:479-483
51. Kobata,T.; Agematsu,K.; Kameoka,J.; Schlossman,S.F.; and Morimoto,C. "CD27 is a signal-transducing molecule involved in CD45RA+ naive T cell costimulation." (1994), *J. Immunol.*, **153**:5422-5432
52. Hintzen,R.Q.; de Jong,R.; Lens,S.M.; and van Lier,R.A. "CD27: marker and mediator of T-cell activation?" (1994), *Immunol. Today*, **15**:307-311
53. June,C.H.; Bluestone,J.A.; Nadler,L.M.; and Thompson,C.B. "The B7 and CD28 receptor families." (1994), *Immunol. Today*, **15**:321-331
54. Schwarz,H.; Blanco,F.J.; von Kempis,J.; Valbracht,J.; and Lotz,M. "ILA, a member of the human nerve growth factor/tumor necrosis factor receptor family, regulates T-lymphocyte proliferation and survival." (1996), *Blood*, **87**:2839-2845
55. Krummel,M.F. and Davis,M.M. "Dynamics of the immunological synapse: finding, establishing and solidifying a connection." (2002), *Curr. Opin. Immunol.*, **14**:66-74
56. Delon,J. and Germain,R.N. "Information transfer at the immunological synapse." (2000), *Curr. Biol.*, **10**:R923-R933
57. Chambers,C.A. and Allison,J.P. "Costimulatory regulation of T cell function." (1999), *Curr. Opin. Cell Biol.*, **11**:203-210
58. Clements,J.L.; Boerth,N.J.; Lee,J.R.; and Koretzky,G.A. "Integration of T cell receptor-dependent signaling pathways by adapter proteins." (1999), *Annu. Rev. Immunol.*, **17**:89-108
59. Rothenberg,E.V. and Ward,S.B. "A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation." (1996), *Proc. Natl. Acad. Sci. U. S. A*, **93**:9358-9365
60. Nel,A.E. "T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse." (2002), *J. Allergy Clin. Immunol.*, **109**:758-770
61. Madrenas,J. "Differential signalling by variant ligands of the T cell receptor and the kinetic model of T cell activation." (1999), *Life Sci.*, **64**:717-731
62. Wei,C.H.; Beeson,C.; Masucci,M.G.; and Levitsky,V. "A partially agonistic peptide acts as a selective inducer of apoptosis in CD8+ CTLs." (1999), *J. Immunol.*, **163**:2601-2609
63. Micheletti,F.; Canella,A.; Vertuani,S.; Marastoni,M.; Tosi,L.; Volinia,S.; Traniello,S.; and Gavioli,R. "Supra-agonist peptides enhance the reactivation of memory CTL responses." (2000), *J. Immunol.*, **165**:4264-4271
64. Greenfield,E.A.; Nguyen,K.A.; and Kuchroo,V.K. "CD28/B7 costimulation: a review." (1998), *Crit Rev. Immunol.*, **18**:389-418
65. Mueller,D.L.; Seiffert,S.; Fang,W.; and Behrens,T.W. "Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells. A model for the long-term survival of memory cells." (1996), *J. Immunol.*, **156**:1764-1771
66. Radvanyi,L.G.; Shi,Y.; Vaziri,H.; Sharma,A.; Dhala,R.; Mills,G.B.; and Miller,R.G. "CD28 costimulation inhibits TCR-induced apoptosis during a primary T cell response." (1996), *J. Immunol.*, **156**:1788-1798
67. Cai,Z. and Sprent,J. "Influence of antigen dose and costimulation on the primary response of CD8+ T cells in vitro." (1996), *J. Exp. Med.*, **183**:2247-2257

68. Kundig,T.M.; Shahinian,A.; Kawai,K.; Mittrucker,H.W.; Sebzda,E.; Bachmann,M.F.; Mak,T.W.; and Ohashi,P.S. "Duration of TCR stimulation determines costimulatory requirement of T cells." (1996), *Immunity.*, **5**:41-52
69. Krummel,M.F.; Heath,W.R.; and Allison,J. "Differential coupling of second signals for cytotoxicity and proliferation in CD8+ T cell effectors: amplification of the lytic potential by B7." (1999), *J. Immunol.*, **163**:2999-3006
70. Deeths,M.J. and Mescher,M.F. "ICAM-1 and B7-1 provide similar but distinct costimulation for CD8+ T cells, while CD4+ T cells are poorly costimulated by ICAM-1." (1999), *Eur. J. Immunol.*, **29**:45-53
71. Rosenberg,S.A.; Lotze,M.T.; Muul,L.M.; Chang,A.E.; Avis,F.P.; Leitman,S.; Linehan,W.M.; Robertson,C.N.; Lee,R.E.; Rubin,J.T.; et al. "A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone." (1987), *N. Engl. J. Med.*, **316**:889-897
72. Chen,Y.T.; Scanlan,M.J.; Sahin,U.; Tureci,O.; Gure,A.O.; Tsang,S.; Williamson,B.; Stockert,E.; Pfreundschuh,M.; and Old,L.J. "A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening." (1997), *Proc. Natl. Acad. Sci. U. S. A*, **94**:1914-1918
73. Scott,A.M. and Welt,S. "Antibody-based immunological therapies." (1997), *Curr. Opin. Immunol.*, 9:717-722
74. Yun,C.O.; Nolan,K.F.; Beecham,E.J.; Reisfeld,R.A.; and Junghans,R.P. "Targeting of T lymphocytes to melanoma cells through chimeric anti-GD3 immunoglobulin T-cell receptors." (2000), *Neoplasia.*, **2**:449-459
75. Daly,T.; Royal,R.E.; Kershaw,M.H.; Treisman,J.; Wang,G.; Li,W.; Herlyn,D.; Eshhar,Z.; and Hwu,P. "Recognition of human colon cancer by T cells transduced with a chimeric receptor gene." (2000), *Cancer Gene Ther.*, 7:284-291
76. Oosterwijk,E.; Ruiter,D.J.; Hoedemaeker,P.J.; Pauwels,E.K.; Jonas,U.; Zwartendijk,J.; and Warnaar,S.O. "Monoclonal antibody G 250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney." (1986), *Int. J. Cancer*, **38**:489-494
77. Grabmaier,K.; Vissers,J.L.; De Weijert,M.C.; Oosterwijk-Wakka,J.C.; Van Bokhoven,A.; Brakenhoff, R.H.; Noessner,E.; Mulders,P.A.; Merkx,G.; Figdor,C.G.; Adema,G.J.; and Oosterwijk,E. "Molecular cloning and immunogenicity of renal cell carcinoma-associated antigen G250." (2000), *Int. J. Cancer*, **85**:865-870
78. Oosterwijk,E.; Debruyne,F.M.; and Schalken,J.A. "The use of monoclonal antibody G250 in the therapy of renal-cell carcinoma." (1995), *Semin. Oncol.*, **22**:34-41
79. van Dijk,J.; Oosterwijk,E.; van Kroonenburgh,M.J.; Jonas,U.; Fleuren,G.J.; Pauwels,E.K.; and Warnaar,S.O. "Perfusion of tumor-bearing kidneys as a model for scintigraphic screening of monoclonal antibodies." (1988), *J. Nucl. Med.*, **29**:1078-1082
80. Oosterwijk,E.; Bander,N.H.; Divgi,C.R.; Welt,S.; Wakka,J.C.; Finn,R.D.; Carswell,E.A.; Larson,S.M.; Warnaar,S.O.; Fleuren,G.J.; et al. "Antibody localization in human renal cell carcinoma: a phase I study of monoclonal antibody G250." (1993), *J. Clin. Oncol.*, **11**:738-750
81. Rosenberg,S.A. "A new era for cancer immunotherapy based on the genes that encode cancer antigens." (1999), *Immunity.*, **10**:281-287
82. Boon,T.; Coulie,P.G.; and Van den Eynde,B. "Tumor antigens recognized by T cells." (1997), *Immunol. Today*, **18**:267-268

83. Hunt,D.F.; Henderson,R.A.; Shabanowitz,J.; Sakaguchi,K.; Michel,H.; Sevilir,N.; Cox,A.L.; Appella,E.; and Engelhard,V.H. "Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry." (1992), *Science*, **255**:1261-1263
84. Cox,A.L.; Skipper,J.; Chen,Y.; Henderson,R.A.; Darrow,T.L.; Shabanowitz,J.; Engelhard,V.H.; Hunt,D.F.; and Slingluff,C.L., Jr. "Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines." (1994), *Science*, **264** :716-719
85. Kawashima,I.; Hudson,S.J.; Tsai,V.; Southwood,S.; Takesako,K.; Appella,E.; Sette,A.; and Celis,E. "The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors." (1998), *Hum. Immunol.*, **59**:1-14
86. van der Bruggen,P.; Traversari,C.; Chomez,P.; Lurquin,C.; De Plaen,E.; Van den Eynde,B.; Knuth,A.; and Boon,T. "A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma." (1991), *Science*, **254**:1643-1647
87. Flad,T.; Spengler,B.; Kalbacher,H.; Brossart,P.; Baier,D.; Kaufmann,R.; Bold,P.; Metzger,S.; Bluggel,M.; Meyer,H.E.; Kurz,B.; and Muller,C.A. "Direct identification of major histocompatibility complex class I-bound tumor-associated peptide antigens of a renal carcinoma cell line by a novel mass spectrometric method." (1998), *Cancer Res.*, **58**:5803-5811
88. Wang,R.F.; Wang,X.; Atwood,A.C.; Topalian,S.L.; and Rosenberg,S.A. "Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen." (1999), *Science*, **284**:1351-1354
89. Chaux,P.; Vantomme,V.; Stroobant,V.; Thielemans,K.; Corthals,J.; Luiten,R.; Eggermont,A.M.; Boon,T.; and van der Bruggen,P. "Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes." (1999), *J. Exp. Med.*, **189**:767-778
90. Jager,E.; Chen,Y.T.; Drijfhout,J.W.; Karbach,J.; Ringhoffer,M.; Jager,D.; Arand,M.; Wada,H.; Noguchi,Y.; Stockert,E.; Old,L.J.; and Knuth,A. "Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes." (1998), *J. Exp. Med.*, **187**:265-270
91. Renkvist,N.; Castelli,C.; Robbins,P.F.; and Parmiani,G. "A listing of human tumor antigens recognized by T cells." (2001), *Cancer Immunol. Immunother.*, **50**:3-15
92. Jassim,A.; Ollier,W.; Payne,A.; Biro,A.; Oliver,R.T.; and Festenstein,H. "Analysis of HLA antigens on germ cells in human semen." (1989), *Eur. J. Immunol.*, **19**:1215-1220
93. De Plaen,E.; Arden,K.; Traversari,C.; Gaforio,J.J.; Szikora,J.P.; De Smet,C.; Brasseur,F.; van der Bruggen,P.; Lethe,B.; Lurquin,C.; et al. "Structure, chromosomal localization, and expression of 12 genes of the MAGE family." (1994), *Immunogenetics*, **40**:360-369
94. De Smet,C.; Lurquin,C.; Lethe,B.; Martelange,V.; and Boon,T. "DNA methylation is the primary silencing mechanism for a set of germ." (1999), *Mol. Cell Biol.*, **19**:7327-7335
95. De Smet,C.; Lurquin,C.; van der Bruggen,P.; De Plaen,E.; Brasseur,F.; and Boon,T. "Sequence and expression pattern of the human MAGE2 gene." (1994), *Immunogenetics*, **39**:121-129
96. Anichini,A.; Maccalli,C.; Mortarini,R.; Salvi,S.; Mazzocchi,A.; Squarcina,P.; Herlyn,M.; and Parmiani,G. "Melanoma cells and normal melanocytes share antigens recognized by HLA- A2-restricted cytotoxic T cell clones from melanoma patients." (1993), *J. Exp. Med.*, **177**:989-998
97. Kawakami,Y.; Eliyahu,S.; Delgado,C.H.; Robbins,P.F.; Sakaguchi,K.; Appella,E.; Yannelli,J.R.; Adema,G.J.; Miki,T.; and Rosenberg,S.A. "Identification of a human melanoma antigen recognized by tu-

mor-infiltrating lymphocytes associated with in vivo tumor rejection." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:6458-6462

98. Bakker,A.B.; Marland,G.; de Boer,A.J.; Huijbens,R.J.; Danen,E.H.; Adema,G.J.; and Figdor,C.G. "Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro." (1995), *Cancer Res.*, **55**:5330-5334
99. Tsai,V.; Southwood,S.; Sidney,J.; Sakaguchi,K.; Kawakami,Y.; Appella,E.; Sette,A.; and Celis,E. "Identification of subdominant CTL epitopes of the GP100 melanoma- associated tumor antigen by primary in vitro immunization with peptide-pulsed dendritic cells." (1997), *J. Immunol.*, **158**:1796-1802
100. Lowy,D.R. and Schiller,J.T. in "Cancer Principles & Practice of Oncology." (2001), DeVita,V.T.; Hellman,S.; Rosenberg,S.A., eds., **6th edition**:3189-3195
101. Ressing,M.E.; Sette,A.; Brandt,R.M.; Ruppert,J.; Wentworth,P.A.; Hartman,M.; Oseroff,C.; Grey,H.M.; Melief,C.J.; and Kast,W.M. "Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA- A*0201-binding peptides." (1995), *J. Immunol.*, **154**:5934-5943
102. Lal,R.B.; Rudolph,D.L.; Griffis,K.P.; Kitamura,K.; Honda,M.; Coligan,J.E.; and Folks,T.M. "Characterization of immunodominant epitopes of gag and pol gene-encoded proteins of human T-cell lymphotropic virus type I." (1991), *J. Virol.*, **65**:1870-1876
103. Rowe,M.; Rowe,D.T.; Gregory,C.D.; Young,L.S.; Farrell,P.J.; Rupani,H.; and Rickinson,A.B. "Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells." (1987), *EMBO J.*, **6**:2743-2751
104. Young,L.; Alfieri,C.; Hennessy,K.; Evans,H.; O'Hara,C.; Anderson,K.C.; Ritz,J.; Shapiro,R.S.; Rickinson,A.; Kieff,E.; et al. "Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease." (1989), *N. Engl. J. Med.*, **321**:1080-1085
105. Fingeroth,J.D.; Weis,J.J.; Tedder,T.F.; Strominger,J.L.; Biro,P.A.; and Fearon,D.T. "Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2." (1984), *Proc. Natl. Acad. Sci. U. S. A*, **81**:4510-4514
106. Li,Q.; Spriggs,M.K.; Kovats,S.; Turk,S.M.; Comeau,M.R.; Nepom,B.; and Hutt-Fletcher,L.M. "Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes." (1997), *J. Virol.*, **71**:4657-4662
107. Kieff,E. "Epstein-Barr virus and its replication." in Fields Virology (1996), Fields,B.N.; Knipe,D.M.; Howley,P.M.; Chanock,R.M.; Melnick,J.L.; Monath,T.P.; Roizman,B.; Straus,S.E., eds., **3rd edition**: 2343-2396
108. Cohen,J.I. "Epstein-Barr virus infection." (2000), *N. Engl. J. Med.*, **343**: 481-492
109. Imai,S.; Koizumi,S.; Sugiura,M.; Tokunaga,M.; Uemura,Y.; Yamamoto,N.; Tanaka,S.; Sato,E.; and Osato,T. "Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**: 9131-9135
110. Deacon,E.M.; Pallesen,G.; Niedobitek,G.; Crocker,J.; Brooks,L.; Rickinson,A.B.; and Young,L.S. "Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells." (1993), *J. Exp. Med.*, **177**:339-349
111. Hitt,M.M.; Allday,M.J.; Hara,T.; Karran,L.; Jones,M.D.; Busson,P.; Tursz,T.; Ernberg,I.; and Griffin,B.E. "EBV gene expression in an NPC-related tumour." (1989), *EMBO J.*, **8**:2639-2651
112. Brooks,L.; Yao,Q.Y.; Rickinson,A.B.; and Young,L.S. "Epstein-Barr virus latent gene transcription in na-

sopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts." (1992), *J. Virol.*, **66**:2689-2697

113. Thomas,J.A.; Allday,M.J.; and Crawford,D.H. "Epstein-Barr virus-associated lymphoproliferative disorders in immunocompromised individuals." (1991), *Adv. Cancer Res.*, **57**:329-380
114. Rickinson,A.B.; Moss,D.J.; Wallace,L.E.; Rowe,M.; Misko,I.S.; Epstein,M.A.; and Pope,J.H. "Long-term T-cell-mediated immunity to Epstein-Barr virus." (1981), *Cancer Res.*, **41**:4216-4221
115. Rickinson,A.B. and Moss,D.J. "Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection." (1997), *Annu. Rev. Immunol.*, **15**:405-431
116. Dyall,R.; Bowne,W.B.; Weber,L.W.; LeMaoult,J.; Szabo,P.; Moroi,Y.; Piskun,G.; Lewis,J.J.; Houghton,A.N.; and Nikolic-Zugic,J. "Heteroclitic immunization induces tumor immunity." (1998), *J. Exp. Med.*, **188**:1553-1561
117. Overwijk,W.W.; Tsung,A.; Irvine,K.R.; Parkhurst,M.R.; Goletz,T.J.; Tsung,K.; Carroll,M.W.; Liu,C.; Moss,B.; Rosenberg,S.A.; and Restifo,N.P. "gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"- reactive, tumoricidal T cells using high-affinity, altered peptide ligand." (1998), *J. Exp. Med.*, **188**:277-286
118. Bowne,W.B.; Srinivasan,R.; Wolchok,J.D.; Hawkins,W.G.; Blachere,N.E.; Dyall,R.; Lewis,J.J.; and Houghton,A.N. "Coupling and uncoupling of tumor immunity and autoimmunity." (1999), *J. Exp. Med.*, **190**:1717-1722
119. Freudenthal,P.S. and Steinman,R.M. "The distinct surface of human blood dendritic cells, as observed after an improved isolation method." (1990), *Proc. Natl. Acad. Sci. U. S. A*, **87**:7698-7702
120. Larsen,C.P.; Ritchie,S.C.; Hendrix,R.; Linsley,P.S.; Hathcock,K.S.; Hodes,R.J.; Lowry,R.P.; and Pearson,T.C. "Regulation of immunostimulatory function and costimulatory molecule (B7- 1 and B7-2) expression on murine dendritic cells." (1994), *J. Immunol.*, **152**:5208-5219
121. Flechner,E.R.; Freudenthal,P.S.; Kaplan,G.; and Steinman,R.M. "Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed-leukocyte reaction." (1988), *Cell Immunol.*, **111**:183-195
122. Brossart,P.; Wirths,S.; Brugger,W.; and Kanz,L. "Dendritic cells in cancer vaccines." (2001), *Exp. Hematol.*, **29**:1247-1255
123. Nouri-Shirazi,M.; Banchereau,J.; Fay,J.; and Palucka,K. "Dendritic cell based tumor vaccines." (2000), *Immunol. Lett.*, **74**:5-10
124. Schreurs,M.W.; Eggert,A.A.; de Boer,A.J.; Vissers,J.L.; van Hall,T.; Offringa,R.; Figdor,C.G.; and Adema,G.J. "Dendritic cells break tolerance and induce protective immunity against a melanocyte differentiation antigen in an autologous melanoma model." (2000), *Cancer Res.*, **60**:6995-7001
125. Hart,D.N. "Dendritic cells: unique leukocyte populations which control the primary immune response." (1997), *Blood*, **90**:3245-3287
126. Mayordomo,J.I.; Zorina,T.; Storkus,W.J.; Zitvogel,L.; Celluzzi,C.; Falo,L.D.; Melief,C.J.; Ildstad,S.T.; Kast,W.M.; Deleo,A.B.; et al. "Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity." (1995), *Nat. Med.*, **1**:1297-1302
127. Satthaporn,S. and Eremin,O. "Dendritic cells (II): Role and therapeutic implications in cancer." (2001), *J. R. Coll. Surg. Edinb.*, **46**:159-167
128. Chen,L.; Ashe,S.; Brady,W.A.; Hellstrom,I.; Hellstrom,K.E.; Ledbetter,J.A.; McGowan,P.; and Linsley,P.S. "Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4." (1992), *Cell*, **71**:1093-1102

129. Mandelboim,O.; Vadai,E.; Fridkin,M.; Katz-Hillel,A.; Feldman,M.; Berke,G.; and Eisenbach,L. "Regression of established murine carcinoma metastases following vaccination with tumour-associated antigen peptides." (1995), *Nat. Med.*, **1**:1179-1183
130. Zhai,Y.; Yang,J.C.; Kawakami,Y.; Spiess,P.; Wadsworth,S.C.; Cardoza,L.M.; Couture,L.A.; Smith,A.E.; and Rosenberg,S.A. "Antigen-specific tumor vaccines. Development and characterization of recombinant adenoviruses encoding MART1 or gp100 for cancer therapy." (1996), *J. Immunol.*, **156**:700-710
131. Schreurs,M.W.; de Boer,A.J.; Figdor,C.G.; and Adema,G.J. "Genetic vaccination against the melanocyte lineage-specific antigen gp100 induces cytotoxic T lymphocyte-mediated tumor protection." (1998), *Cancer Res.*, **58**:2509-2514
132. Belldegrun,A.; Muul,L.M.; and Rosenberg,S.A. "Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity." (1988), *Cancer Res.*, **48**:206-214
133. Adams,D.H.; Yannelli,J.R.; Newman,W.; Lawley,T.; Ades,E.; Rosenberg,S.A.; and Shaw,S. "Adhesion of tumour-infiltrating lymphocytes to endothelium: a phenotypic and functional analysis." (1997), *Br. J. Cancer*, **75**:1421-1431
134. Salmi,M.; Grenman,R.; Grenman,S.; Nordman,E.; and Jalkanen,S. "Tumor endothelium selectively supports binding of IL-2-propagated tumor-infiltrating lymphocytes." (1995), *J. Immunol.*, **154**:6002-6012
135. Dudley,M.E.; Wunderlich,J.R.; Robbins,P.F.; Yang,J.C.; Hwu,P.; Schwartzentruber,D.J.; Topalian,S.L.; Sherry,R.; Restifo,N.P.; Hubicki,A.M.; Robinson,M.R.; Raffeld,M.; Duray,P.; Seipp,C.A.; Rogers-Freezer,L.; Morton,K.E.; Mavroukakis,S.A.; White,D.E.; and Rosenberg,S.A. "Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes." (2002), *Science*, **298**:850-854
136. Rivoltini,L.; Kawakami,Y.; Sakaguchi,K.; Southwood,S.; Sette,A.; Robbins,P.F.; Marincola,F.M.; Salgaller,M.L.; Yannelli,J.R.; Appella,E.; et al. "Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1." (1995), *J. Immunol.*, **154**:2257-2265
137. Slingluff,C.L., Jr.; Darrow,T.L.; and Seigler,H.F. "Melanoma-specific cytotoxic T cells generated from peripheral blood lymphocytes. Implications of a renewable source of precursors for adoptive cellular immunotherapy." (1989), *Ann. Surg.*, **210** :194-202
138. Visseren,M.J.; van der Burg,S.H.; Hawes,G.E.; van der Voort,E.I.; van den Elsen,P.J.; and Melief,C.J. "Affinity, specificity and T-cell-receptor diversity of melanoma-specific CTL generated in vitro against a single tyrosinase epitope." (1997), *Int. J. Cancer*, **72**:1122-1128
139. Yee,C.; Thompson,J.A.; Byrd,D.; Riddell,S.R.; Roche,P.; Celis,E.; and Greenberg,P.D. "Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells." (2002), *Proc. Natl. Acad. Sci. U. S. A*, **99**:16168-16173
140. Rosenberg,S.A.; Spiess,P.; and Lafreniere,R. "A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes." (1986), *Science*, **233**:1318-1321
141. Spiess,P.J.; Yang,J.C.; and Rosenberg,S.A. "In vivo antitumor activity of tumor-infiltrating lymphocytes expanded in recombinant interleukin-2." (1987), *J. Natl. Cancer Inst.*, **79**:1067-1075
142. Arienti,F.; Belli,F.; Rivoltini,L.; Gambacorti-Passerini,C.; Furlan,L.; Mascheroni,L.; Prada,A.; Rizzi,M.; Marchesi,E.; Vaglini,M.; et al. "Adoptive immunotherapy of advanced melanoma patients with inter-

leukin-2 (IL-2) and tumor-infiltrating lymphocytes selected in vitro with low doses of IL-2." (1993), *Cancer Immunol. Immunother.*, **36**:315-322

143. Kradin,R.L.; Kurnick,J.T.; Lazarus,D.S.; Preffer,F.I.; Dubinett,S.M.; Pinto,C.E.; Gifford,J.; Davidson,E.; Grove,B.; Callahan,R.J.; et al. "Tumour-infiltrating lymphocytes and interleukin-2 in treatment of advanced cancer." (1989), *Lancet*, **1**:577-580
144. Rosenberg,S.A.; Yannelli,J.R.; Yang,J.C.; Topalian,S.L.; Schwartzentruber,D.J.; Weber,J.S.; Parkinson,D.R.; Seipp,C.A.; Einhorn,J.H.; and White,D.E. "Treatment of patients with metastatic melanoma with autologous tumor- infiltrating lymphocytes and interleukin 2." (1994), *J. Natl. Cancer Inst.*, **86**:1159-1166
145. DiMaio,J.M.; Van Trigt,P.; Gaynor,J.W.; Davis,R.D.; Coveney,E.; Clary,B.M.; and Lyerly,H.K. "Generation of tumor-specific T lymphocytes for the treatment of posttransplant lymphoma." (1995), *Circulation*, **92**:II202-II205
146. Hoffmann,T.; Russell,C.; and Vindelov,L. "Generation of EBV-specific CTLs suitable for adoptive immunotherapy of EBV-associated lymphoproliferative disease following allogeneic transplantation." (2002), *APMIS*, **110**:148-157
147. Savoldo,B.; Heslop,H.E.; and Rooney,C.M. "The use of cytotoxic t cells for the prevention and treatment of epstein-barr virus induced lymphoma in transplant recipients." (2000), *Leuk. Lymphoma*, **39**:455-464
148. Heslop,H.E.; Ng,C.Y.; Li,C.; Smith,C.A.; Loftin,S.K.; Krance,R.A.; Brenner,M.K.; and Rooney, C.M. "Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes." (1996), *Nat. Med.*, **2**:551-555
149. Heslop,H.E. and Rooney,C.M. "Adoptive cellular immunotherapy for EBV lymphoproliferative disease." (1997), *Immunol. Rev.*, **157**:217-222
150. Gahn,B.; Hunt,G.; Rooney,C.M.; and Heslop,H.E. "Immunotherapy to reconstitute immunity to DNA viruses." (2002), *Semin. Hematol.*, **39**:41-47
151. Riddell,S.R.; Walter,B.A.; Gilbert,M.J.; and Greenberg,P.D. "Selective reconstitution of CD8+ cytotoxic T lymphocyte responses in immunodeficient bone marrow transplant recipients by the adoptive transfer of T cell clones." (1994), *Bone Marrow Transplant.*, 14 Suppl **4**:S78-S84
152. Walter,E.A.; Greenberg,P.D.; Gilbert,M.J.; Finch,R.J.; Watanabe,K.S.; Thomas,E.D.; and Riddell,S.R. "Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor." (1995), *N. Engl. J. Med.*, **333**:1038-1044
153. Theobald,M.; Biggs,J.; Hernandez,J.; Lustgarten,J.; Labadie,C.; and Sherman,L.A. "Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes." (1997), *J. Exp. Med.*, **185**:833-841
154. Bolhuis,R.L. and Gratama,J.W. "Genetic re-targeting of T lymphocyte specificity." (1998), *Gene Ther.*, **5**:1153-1155
155. Staerz,U.D.; Kanagawa,O.; and Bevan,M.J. "Hybrid antibodies can target sites for attack by T cells." (1985), *Nature*, **314**:628-631
156. Perez,P.; Hoffman,R.W.; Shaw,S.; Bluestone,J.A.; and Segal,D.M. "Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody." (1985), *Nature*, **316**:354-356
157. Lanzavecchia,A. and Scheidegger,D. "The use of hybrid hybridomas to target human cytotoxic T lymphocytes." (1987), *Eur. J. Immunol.*, **17**:105-111
158. van Dijk,J.; Warnaar,S.O.; van Eendenburg,J.D.; Thienpont,M.; Braakman,E.; Boot,J.H.; Fleuren,G.J.; and

Bolhuis,R.L. "Induction of tumor-cell lysis by bi-specific monoclonal antibodies recognizing renal-cell carcinoma and CD3 antigen." (1989), *Int. J. Cancer*, **43**:344-349

159. Titus,J.A.; Garrido,M.A.; Hecht,T.T.; Winkler,D.F.; Wunderlich,J.R.; and Segal,D.M. "Human T cells targeted with anti-T3 cross-linked to antitumor antibody prevent tumor growth in nude mice." (1987), *J. Immunol.*, **138**:4018-4022
160. Renner,C.; Jung,W.; Sahin,U.; Denfeld,R.; Pohl,C.; Trumper,L.; Hartmann,F.; Diehl,V.; van Lier,R.; and Pfreundschuh,M. "Cure of xenografted human tumors by bispecific monoclonal antibodies and human T cells." (1994), *Science*, **264**:833-835
161. Mezzanzanica,D.; Garrido,M.A.; Neblock,D.S.; Daddona,P.E.; Andrew,S.M.; Zurawski,V.R., Jr.; Segal,D.M.; and Wunderlich,J.R. "Human T-lymphocytes targeted against an established human ovarian carcinoma with a bispecific F(ab')2 antibody prolong host survival in a murine xenograft model." (1991), *Cancer Res.*, **51**:5716-5721
162. Cochlovius,B.; Perschl,A.; Adema,G.J.; and Zoller,M. "Human melanoma therapy in the SCID mouse: in vivo targeting and reactivation of melanoma-specific cytotoxic T cells by bi-specific antibody fragments." (1999), *Int. J. Cancer*, **81**:486-493
163. Canevari,S.; Stoter,G.; Arienti,F.; Bolis,G.; Colnaghi,M.I.; Di Re,E.M.; Eggermont,A.M.; Goey,S.H.; Gratama,J.W.; Lamers,C.H.; et al. "Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody." (1995), *J. Natl. Cancer Inst.*, **87**:1463-1469
164. Bolhuis,R.L.; Lamers,C.H.; Goey,S.H.; Eggermont,A.M.; Trimbos,J.B.; Stoter,G.; Lanzavecchia,A.; di Re,E.; Miotti,S.; Raspagliesi,F.; et al. "Adoptive immunotherapy of ovarian carcinoma with bs-MAb-targeted lymphocytes: a multicenter study." (1992), *Int. J. Cancer Suppl*, 7:78-81
165. Nitta,T.; Sato,K.; Yagita,H.; Okumura,K.; and Ishii,S. "Preliminary trial of specific targeting therapy against malignant glioma." (1990), *Lancet*, **335**:368-371
166. Kroesen,B.J.; Buter,J.; Sleijfer,D.T.; Janssen,R.A.; van der Graaf,W.T.; The,T.H.; de Leij,L.; and Mulder,N.H. "Phase I study of intravenously applied bispecific antibody in renal cell cancer patients receiving subcutaneous interleukin 2." (1994), *Br. J. Cancer*, **70**:652-661
167. Weiner,L.M.; Clark,J.I.; Davey,M.; Li,W.S.; Garcia,d.P., I; Ring,D.B.; and Alpaugh,R.K. "Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB- 2 and Fc gamma RIII." (1995), *Cancer Res.*, **55**:4586-4593
168. de Gast,G.C.; Van Houten,A.A.; Haagen,I.A.; Klein,S.; De Weger,R.A.; Van Dijk,A.; Phillips,J.; Clark,M.; and Bast,B.J. "Clinical experience with CD3 x CD19 bispecific antibodies in patients with B cell malignancies." (1995), *J. Hematother.*, **4**:433-437
169. Blank-Voorthuis,C.J.; Braakman,E.; Ronteltap,C.P.; Tilly,B.C.; Sturm,E.; Warnaar,S.O.; and Bolhuis, R.L. "Clustered CD3/TCR complexes do not transduce activation signals after bispecific monoclonal antibody-triggered lysis by cytotoxic T lymphocytes via CD3." (1993), *J. Immunol.*, **151**:2904-2914
170. Weiner,L.M.; Clark,J.I.; Ring,D.B.; and Alpaugh,R.K. "Clinical development of 2B1, a bispecific murine monoclonal antibody targeting c-erbB-2 and Fc gamma RIII." (1995), *J. Hematother.*, **4**:453-456
171. Lamers,C.H.; Gratama,J.W.; Warnaar,S.O.; Stoter,G.; and Bolhuis,R.L. "Inhibition of bispecific monoclonal antibody (bsAb)-targeted cytolysis by human anti-mouse antibodies in ovarian carcinoma patients treated with bsAb-targeted activated T-lymphocytes." (1995), *Int. J. Cancer*, **60**:450-457

172. Jain,R.K. "Delivery of novel therapeutic agents in tumors: physiological barriers and strategies." (1989), *J. Natl. Cancer Inst.*, **81**:570-576
173. Gross,G.; Waks,T.; and Eshhar,Z. "Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity." (1989), *Proc. Natl. Acad. Sci. U. S. A*, **86**:10024-10028
174. Eshhar,Z. and Gross,G. "Chimeric T cell receptor which incorporates the anti-tumour specificity of a monoclonal antibody with the cytolytic activity of T cells: a model system for immunotherapeutical approach." (1990), *Br. J. Cancer Suppl*, **10**:27-29
175. Eshhar,Z.; Waks,T.; Gross,G.; and Schindler,D.G. "Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors." (1993), *Proc. Natl. Acad. Sci. U. S. A*, **90**:720-724
176. Weijtens,M.E.; Willemsen,R.A.; Hart,E.H.; and Bolhuis,R.L. "A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes." (1998), *Gene Ther.*, **5**:1195-1203
177. Stancovski,I.; Schindler,D.G.; Waks,T.; Yarden,Y.; Sela,M.; and Eshhar,Z. "Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors." (1993), *J. Immunol.*, **151**:6577-6582
178. Brocker,T.; Peter,A.; Traunecker,A.; and Karjalainen,K. "New simplified molecular design for functional T cell receptor." (1993), *Eur. J. Immunol.*, **23**:1435-1439
179. Moritz,D.; Wels,W.; Mattern,J.; and Groner,B. "Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:4318-4322
180. Hwu,P.; Shafer,G.E.; Treisman,J.; Schindler,D.G.; Gross,G.; Cowherd,R.; Rosenberg,S.A.; and Eshhar,Z. "Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain." (1993), *J. Exp. Med.*, **178**:361-366
181. Roberts,M.R.; Qin,L.; Zhang,D.; Smith,D.H.; Tran,A.C.; Dull,T.J.; Groopman,J.E.; Capon,D.J.; Byrn,R.A.; and Finer,M.H. "Targeting of human immunodeficiency virus-infected cells by CD8+ T lymphocytes armed with universal T-cell receptors." (1994), *Blood*, **84**:2878-2889
182. Weijtens,M.E.; Willemsen,R.A.; Valerio,D.; Stam,K.; and Bolhuis,R.L. "Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity." (1996), *J. Immunol.*, **157**:836-843
183. Hombach,A.; Muche,J.M.; Gerken,M.; Gellrich,S.; Heuser,C.; Pohl,C.; Sterry,W.; and Abken,H. "T cells engrafted with a recombinant anti-CD30 receptor target autologous CD30(+) cutaneous lymphoma cells." (2001), *Gene Ther.*, **8**:891-895
184. Hwu,P.; Yang,J.C.; Cowherd,R.; Treisman,J.; Shafer,G.E.; Eshhar,Z.; and Rosenberg,S.A. "In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes." (1995), *Cancer Res.*, **55**:3369-3373
185. Hekele,A.; Dall,P.; Moritz,D.; Wels,W.; Groner,B.; Herrlich,P.; and Ponta,H. "Growth retardation of tumors by adoptive transfer of cytotoxic T lymphocytes reprogrammed by CD44v6-specific scFv:zeta-chimera." (1996), *Int. J. Cancer*, **68**:232-238
186. Altenschmidt,U.; Klundt,E.; and Groner,B. "Adoptive transfer of in vitro-targeted, activated T lymphocytes results in total tumor regression." (1997), *J. Immunol.*, **159**:5509-5515
187. McGuinness,R.P.; Ge,Y.; Patel,S.D.; Kashmiri,S.V.; Lee,H.S.; Hand,P.H.; Schlom,J.; Finer,M.H.; and

McArthur,J.G. "Anti-tumor activity of human T cells expressing the CC49-zeta chimeric immune receptor." (1999), *Hum. Gene Ther.*, **10**:165-173

188. Haynes,N.M.; Snook,M.B.; Trapani,J.A.; Cerruti,L.; Jane,S.M.; Smyth,M.J.; and Darcy,P.K. "Redirecting mouse CTL against colon carcinoma: superior signaling efficacy of single-chain variable domain chimeras containing TCR-zeta vs Fc epsilon RI-gamma." (2001), *J. Immunol.*, **166**:182-187
189. Patel,S.D.; Moskalenko,M.; Smith,D.; Maske,B.; Finer,M.H.; and McArthur,J.G. "Impact of chimeric immune receptor extracellular protein domains on T cell function." (1999), *Gene Ther.*, **6**:412-419
190. Roberts,M.R.; Cooke,K.S.; Tran,A.C.; Smith,K.A.; Lin,W.Y.; Wang,M.; Dull,T.J.; Farson,D.; Zsebo,K.M.; and Finer,M.H. "Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains." (1998), *J. Immunol.*, **161**:375-384
191. Nolan,K.F.; Yun,C.O.; Akamatsu,Y.; Murphy,J.C.; Leung,S.O.; Beecham,E.J.; and Junghans, R.P. "Bypassing immunization: optimized design of "designer T cells" against carcinoembryonic antigen (CEA)-expressing tumors, and lack of suppression by soluble CEA." (1999), *Clin. Cancer Res.*, **5**:3928-3941
192. Fitzer-Attas,C.J.; Schindler,D.G.; Waks,T.; and Eshhar,Z. "Harnessing Syk family tyrosine kinases as signaling domains for chimeric single chain of the variable domain receptors: optimal design for T cell activation." (1998), *J. Immunol.*, **160**:145-154
193. Geiger,T.L.; Nguyen,P.; Leitenberg,D.; and Flavell,R.A. "Integrated src kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes." (2001), *Blood*, **98**:2364-2371
194. Hombach,A.; Sent,D.; Schneider,C.; Heuser,C.; Koch,D.; Pohl,C.; Seliger,B.; and Abken,H. "T-cell activation by recombinant receptors: CD28 costimulation is required for interleukin 2 secretion and receptor-mediated T-cell proliferation but does not affect receptor-mediated target cell lysis." (2001), *Cancer Res.*, **61**:1976-1982
195. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
196. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
197. Orentas,R.J.; Roskopf,S.J.; Nolan,G.P.; and Nishimura,M.I. "Retroviral transduction of a T cell receptor specific for an Epstein- Barr virus-encoded peptide." (2001), *Clin. Immunol.*, **98**:220-228
198. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
199. Stanislawski,T.; Voss,R.H.; Lotz,C.; Sadovnikova,E.; Willemsen,R.A.; Kuball,J.; Ruppert,T.; Bolhuis, R.L.; Melief,C.J.; Huber,C.; Stauss,H.J.; and Theobald,M. "Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer." (2001), *Nat. Immunol.*, **2**:962-970
200. Fujio,K.; Misaki,Y.; Setoguchi,K.; Morita,S.; Kawahata,K.; Kato,I.; Nosaka,T.; Yamamoto,K.; and Kitamura,T. "Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex." (2000), *J. Immunol.*, **165**:528-532
201. Kessels,H.W.; Wolkers,M.C.; van den Boom,M.D.; van der Valk,M.A.; and Schumacher,T.N. "Immunotherapy through TCR gene transfer." (2001), *Nat. Immunol.*, **2**:957-961

202. Chung,S.; Wucherpfennig,K.W.; Friedman,S.M.; Hafler,D.A.; and Strominger,J.L. "Functional three-domain single-chain T-cell receptors." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:12654-12658

203. Debets,R.; Willemsen,R.; and Bolhuis,R. "Adoptive transfer of T-cell immunity: gene transfer with MHC-restricted receptors." (2002), *Trends Immunol.*, **23**:435-436

204. Sant'Angelo,D.B.; Cresswell,P.; Janeway,C.A., Jr.; and Denzin,L.K. "Maintenance of TCR clonality in T cells expressing genes for two TCR heterodimers." (2001), *Proc. Natl. Acad. Sci. U. S. A*, **98**:6824-6829

205. Nakagomi,H.; Petersson,M.; Magnusson,I.; Juhlin,C.; Matsuda,M.; Mellstedt,H.; Taupin,J.L.; Vivier,E.; Anderson,P.; and Kiessling,R. "Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma." (1993), *Cancer Res.*, **53**:5610-5612

206. Willemsen,R.A.; Weijtens,M.E.; Ronteltap,C.; Eshhar,Z.; Gratama,J.W.; Chames,P.; and Bolhuis,R.L. "Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR." (2000), *Gene Ther.*, **7**:1369-1377

Chapter 2

Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT

Niels Schaft, Birgit Lankiewicz, Jan Willem Gratama, Reinder L.H. Bolhuis, Reno Debets

Journal of Immunological Methods *in press*

Abstract

Tumor-specific receptors may provide effective tools for anti-tumor immunogene therapy. However, the functional analysis of primary human T cells engrafted with tumor-specific receptors is laborious and emphasizes the need for a fast and sensitive method to validate such receptors. To this end, we have set up a Jurkat T cell-based reporter gene assay and tested receptors with various formats, i.e., receptors based on either a monoclonal antibody (mAb), a full length T cell receptor (fl-TCR)αβ or a chimeric (ch-)TCRαβ, and various antigen specificities for their ability to mediate tumor-specific activation of Nuclear Factor of Activated T cells (NFAT). The mAb-based receptor specifically mediates NFAT activation after stimulation with tumor antigen-positive target cells. The observed receptor-mediated NFAT responses were validated by the use of ligand- and receptor-specific mAbs, as well as cyclosporin A (CsA) and a dominant negative mutant of NFAT. Furthermore, anti-TCR mAbs, peptide-loaded tumor cells, and antigen-positive tumor cells *all* resulted in specific NFAT activation in TCR/CD8 co-transduced Jurkat T cells, irrespective of the TCR format used. Importantly, receptor-mediated NFAT responses parallel tumor-specific cytolysis and TNFα production of receptor-transduced primary human T lymphocytes. In fact, inhibition of NFAT activation compromises the immune responses of primary human T lymphocytes, pointing to a central involvement of NFAT in anti-tumor T cell responses. Taken together, receptor-mediated activation of NFAT constitutes a representative measure of anti-tumor T cell responses and the genetically modified Jurkat T cells provide a flexible and sensitive tool with which to select rapidly tumor-specific (chimeric) receptors for immunogene therapy.

Introduction

Transfer of genes encoding tumor-specific receptors into human T lymphocytes is a promising strategy to induce antigen-specific immunity. Tumor-specific receptors used to functionally redirect human T cells exist in various single or two-chain receptor formats, including mAb, full length TCRαβ, as well as chimeric TCRαβ-based receptors [1-6]. T lymphocytes equipped with tumor-specific receptors, with or without an MHC-restriction element, are not only able to recognize the relevant antigen, but also produce cytokines and kill tumor target cells upon antigenic stimulation both *in vitro* and *in vivo* [1-8]. Individual receptor components, such as the transmembrane and intracellular domains, greatly affect expression and/or function of these receptors in human T lymphocytes [1,9]. The design and validation of mAb- or TCR-based receptors that permit optimal antigen-specific T cell activation are therefore of critical importance to the clinical implementation of receptor genes in the treatment of cancers. To date, the validation of tumor-specific receptors is labor intensive and time consuming since primary human T lymphocytes have to be transduced, enriched for chimeric receptor expression and subsequently tested for antigen-specific function *in vitro*, with anti-tumor cell cytotoxicity and cytokine production being the accepted read-outs.

In this paper, we studied whether receptor-mediated activation of NFAT would constitute an easy-to-use and reliable alternative to validate such receptor genes. Reporter genes under the control of NFAT are considered valid tools to measure T cell activation [10-12] with the human Jurkat T cell line often used as a model cell line to study antigen-specific responses mediated by introduced full length TCRαβ chains [13-15]. Nevertheless, TCR-mediated activation of NFAT in CD4-positive Jurkat T cells equipped with exogenous TCRαβ chains and, in some cases, the coreceptor CD8α could only be detected in response to peptide-loaded target cells but not tumor-antigen positive target cells [13-15]. Here, we introduce a flexible and sensitive method that detects receptor-mediated activation of NFAT, irrespective of whether a mAb-based receptor, full length TCRαβ chains or TCRαβ chains fused to CD3ζ are used in response to native tumor target cells. The NFAT reporter gene assay described relies on Jurkat E6.1 T cells retrovirally transduced with tumor-specific receptors and, in the case of TCR-based receptors, CD8α coreceptor, and allows for costimulation which is especially relevant for TCR-mediated NFAT activation. The specificity of the receptor-mediated responses was confirmed by blocking the activation of NFAT via ligand- or receptor-specific antibodies or specific

inhibitors of NFAT activation, such as CsA and a dominant-negative NFAT mutant [16]. It is important to note that NFAT responses in Jurkat T cells clearly reflect the tumor-specific cytolysis and TNFα production by primary human T cells transduced with the identical receptor genes. In fact, blocking experiments with CsA show that anti-tumor responses of primary human T cells depend on NFAT activation.
Thus, receptor-mediated activation of NFAT constitutes a representative measure of anti-tumor T cell responses, with the Jurkat T cell-based NFAT reporter gene assay allowing for rapid testing and selection of tumor-specific (chimeric) receptors for immunogene therapy of cancers and viral infections.

Materials and methods

Cells and reagents

The Jurkat T cell clone E6.1, the G250^{neg} renal cell carcinoma (RCC)-derived SKRC-17 clone 1 and the G250 cDNA-transfected SKRC-17 clone 4 (both kindly provided by E. Oosterwijk, Nijmegen, The Netherlands) were cultured with RPMI 1640 medium supplemented with 200 nM L-glutamine, 10% Bovine Calf Serum (BCS, Hyclone, Logan, UT), and the antibiotics streptomycin (100 μg/ml) and penicillin (100 U/ml). The human amphotropic packaging cell line Phoenix, the endogenously G250^{pos} RCC-derived A75 cell line (generated in our laboratory by [17]), the gp100^{pos}/HLA-A2^{pos} melanoma cell line FM3 (kind gift from G. Adema, Nijmegen, the Netherlands) and the TAP-deficient HLA-A2^{pos} TxB hybrid T2 cells were grown in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% BCS and antibiotics. The gp100^{neg}/HLA-A2^{pos} melanoma cell line BLM and BLM cells transfected with human gp100-encoding cDNA (BLMgp100) were cultured as described previously [18]. Expression of the G250 or gp100 tumor-antigens on these cell lines was verified by flow cytometry. The mAbs used in this study were: G250 mAb; anti-idiotypic mAb NUH-82 (both from E. Oosterwijk, Nijmegen, The Netherlands); PE-conjugated goat-anti-mouse (GaM^{PE}) Ig mAb (ITK, Uithoorn, The Netherlands); anti-TCRVβ8 mAb; anti-TCRVβ14 mAb; PE-conjugated anti-TCRVβ14 mAb (TCR mAbs all from Beckman Coulter, Marseille, France); FITC-conjugated anti-CD8α mAb (Becton Dickinson Biosciences, San Jose, CA); mouse gamma globulin (mIg; Jackson Immuno Research Laboratories, West Grove, PA); and anti-CD28 mAb (clone 15E8; CLB, Amsterdam, The Netherlands). Other reagents used in this study were: RetroNectin (human fibronectin fragments CH-296; Takara Shuzo Co. Ltd., Otsu, Japan); PMA (Phorbol

12-myristate 13-acetate; Sigma, Zwijndrecht, The Netherlands); ionomycin (Calbiochem, La Jolla, CA); Cyclosporin A (CsA; Sandoz Pharmaceuticals, NJ); INFγ; IL-1β (both from PeproTech, NJ); gp100 wild type (wt) peptide (YLEPGPVTA); irrelevant HLA-A2-binding EBV-peptide (GLCTLVAML); and a dominant negative mutant of NFAT (dnNFATmut; kindly provided by R.J. Davis, Worcester, MA [16]).

Construction of chimeric receptor genes

The chimeric antibody based single chain (sc)FvG250:γ receptor was constructed as described elsewhere [2]. Briefly, the scFvG250:γ receptor comprises the variable domains of the human Ig heavy chain and κ light chain of the G250 mAb connected by a flexible linker. The antigen-binding part is coupled to a few amino acids of the constant domain of the κ light chain, the transmembrane domain of the human CD4 molecule and the signaling domain of Fc(ε)RIγ (i.e., scFvG250:γ). The chimeric antibody based scFvG250:γ receptor was subsequently cloned into the retroviral vector pSTITCH [2]. The full length (fl-)296 TCRαβ consisted of the complete TCR α and β chain, whereas the chimeric two chain (tc-)296 TCRαβ:ζ consisted of the extracellular domains of the TCR α and β chain, each linked to the human CD3ζ molecule [3]. TCR α and β DNAs were obtained by PCR using cDNA of the CTL 296 clone (described by [19]) as template DNA. Specific primer sequences to amplify the TCR domains will be provided upon request. The TCR genes were subsequently cloned into the retroviral vector pBullet containing a heterologous signal peptide [3].

Retroviral gene transduction of (chimeric) receptors into Jurkat T cells

Jurkat T cells were retrovirally transduced according to a protocol optimized for primary human T lymphocytes as described by Lamers *et al.* [20]. In short, 24 well culture plates were coated with RetroNectin and pre-treated with receptor-positive retroviral particles, derived from receptor-positive Phoenix cells. Next, 10^6 Jurkat T cells per well were centrifuged in fresh retrovirus containing supernatant, and cultured for 4-5 h at 37°C/5% CO_2. Cells were allowed to recover in culture medium overnight prior to a second transduction cycle, after which cells were harvested and transferred to T25 culture flasks. After sufficient numbers were obtained, cells were analyzed for receptor expression by flow cytometry and used in the NFAT reporter gene assay. The fl-296 TCRαβ and tc-296 TCRαβ:ζ genes were introduced into CD8-transduced Jurkat T cells.

Cytofluorometric analysis of retrovirally transduced Jurkat T cells

Transduced Jurkat T cells were analyzed for transgene expression by flow cytometry using either the anti-idiotypic NUH-82 mAb for the scFvG250:γ receptor or PE-conjugated anti-TCRVβ14 mAb for the fl-296 TCRαβ and tc-296 TCRαβ:ζ. FITC-conjugated anti-CD8α mAb was used to analyze expression of the CD8α molecule. For immunostaining, 0.25-0.5x10^6 transduced Jurkat T cells were incubated with the primary mAbs on ice for 30 min (in the case of the NUH-82 mAb this was followed by a wash step and a second incubation with GaM^{PE}), washed, fixed with 1% PFA and analyzed on a flowcytometer (Becton-Dickinson, San Jose, CA). Jurkat T cells were subsequently enriched for receptor-positive cells via the primary mAbs mentioned and GaM-coated magnetic beads (Dynal, Oslo, Norway) or anti-PE MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufaturers' instructions.

NFAT reporter gene assay

For reporter gene assays, conditions for transfection, lysis and measurement have been optimized, resulting in the following procedure (in part described by [21]): exponentially growing receptor-transduced Jurkat T cells (4-5 x10^6) were transfected by electroporation (300 V, 1920 μF, 129 Ω) with 5 μg of both the NFAT-luciferase construct (containing four NFAT binding sites; Stratagene, La Jolla, CA) and β-galactosidase construct. Twenty hours post-transfection, Jurkat T cells were transferred to round-bottom 96-well plates (Costar, Corning, NY) at 2x10^5 cells/well and were stimulated for 6 h with mAbs or target cells (at 10^5 cells/well) in RPMI 1640 medium supplemented with 1% BCS at 37°C/5% CO_2. The scFvG250:γ^{pos} Jurkat T cells were stimulated with the following RCC-derived tumor target cells: SKRC-17 clone 1, SKRC-17 clone 4, and the A75 cell line. The RCC specificity of NFAT activation in scFvG250:γ^{pos} Jurkat T cells was tested via blocking experiments with the G250 mAb (at 25μg/ml final) or the NUH82 mAb (at 40μg/ml final) added at the start of cocultivation. The fl-296 TCR$\alpha\beta^{pos}$ and tc-296 TCRαβ:ζ^{pos} Jurkat T cells were stimulated with anti-TCR mAbs, peptide-loaded T2 target cells, and the melanoma cell lines FM3, BLM and BLMgp100. For mAb stimulations, TCR-positive Jurkat T cells were added to wells precoated with anti-TCRVβ8 mAb, anti-TCRVβ14 mAb or control mIg (at 0.1 μg/well final). T2 cells were peptide-loaded for 30 min at 37°C/5% CO_2 with 1 μM of the gp100 wt peptide or irrelevant HLA-A2-binding EBV-peptide prior to their use in NFAT reporter gene assays. FM3, BLM and BLMgp100 cells were pre-incubated O/N with INFγ (10 ng/ml) and

IL-1β (30 ng/ml), and cocultivation of these melanoma cells with TCR-transduced Jurkat T cells was performed in the presence of anti-CD28 mAb (2μg/ml). Stimulations with PMA (10 ng/ml) and ionomycin (1 μM) served as positive controls. For NFAT inhibition experiments, CsA (at 100 nM final) was added to the Jurkat T cells 30 min prior to the 6 h co-culture experiments. In some experiments, a dominant negative mutant of NFAT (10 μg) was co-electroporated with the NFAT reporter construct and the β-galactosidase construct into Jurkat T cells prior to stimulation experiments. Amounts of DNA were kept constant by adding pcDNA3.1 (backbone vector of dnNFATmut). After stimulation, cells were collected, lysed with Cell Lysis Buffer (Promega, Madison, WI), and luciferase and β-galactosidase activities were assessed using chemiluminescent substrates according to the manufacturer's instructions (Mediators, Vienna, Austria). Samples were analyzed in a 96 well plate luminometer (Mediators), and luciferase activities were normalized on the basis of β-galactosidase activities and expressed (in RLU) relative to non-stimulated conditions (medium only: set to 1.0).

Cytotoxicity assay

Primary human T cells expressing the same receptors as tested in the NFAT reporter gene assay were assayed in a standard 6 h ^{51}Cr-release assay [22] using the target cells described above. To this end, primary human T cells were retrovirally transduced with the scFvG250:γ, fl-296 TCRαβ, or tc-296 TCRαβ:ζ receptors resulting in receptor expression levels of over 45%. The significance of NFAT activation to receptor-mediated cytotoxicity was also directly assayed by adding CsA (100 nM final concentration, or as indicated) to the T lymphocyte effector cells 30 min prior to cytotoxicity assays.

TNFα production

Receptor-positive primary human T lymphocytes were also tested for antigen-specific TNFα production as described [3]. To inhibit NFAT activation CsA was added to the T lymphocyte effector cells as described above. Supernatants were harvested and levels of TNFα weremeasured by standard ELISA (CLB, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Results

Expression of (chimeric) mAb- and TCR-based receptors on transduced Jurkat T cells

In order to employ the Jurkat T cell line E6.1 as a tool to functionally analyze tumor-specific receptors, we retrovirally transduced these cells with a chimeric mAb-based single chain receptor specific for the renal cell carcinoma antigen G250 (scFvG250:γ) [2], a full length (fl-) 296 TCRαβ and a two chain 296 TCR chimerized to human CD3ζ (tc-296 TCRαβ:ζ), both specific for the HLA-A2-presented gp100 melanoma antigen. Cell surface expression of the mAb- and TCR-based receptors was determined by flow cytometry using anti-G250 idiotypic and anti-TCRVβ14 mAbs, respectively. Transduction of Jurkat T cells, followed by selection for receptor-positive cells, resulted in expression levels of 92% (mean fluorescence intensity (MFI)=432), 93% (MFI=204) and 82% (MFI=223) for the scFvG250:γ receptor, the fl-296 TCRαβ and the tc-296 TCRαβ:ζ, respectively (Fig. 1). Jurkat T cells used for the transduction of TCR-based receptors were co-transduced with the human CD8α gene (expression level 100%; MFI=590), and

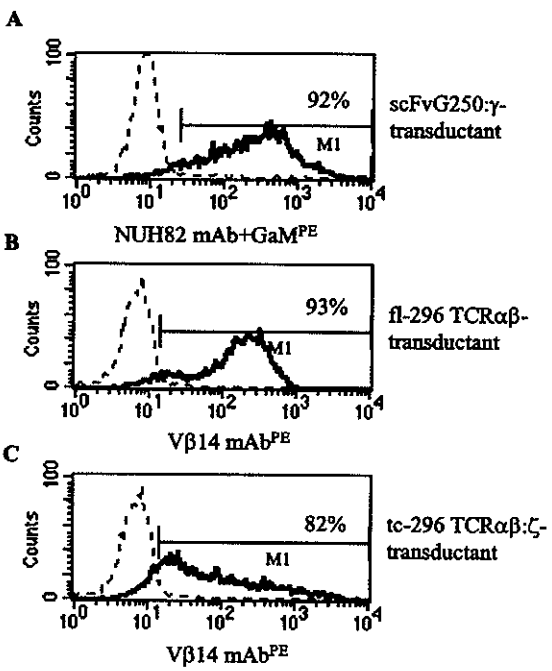

Figure 1. Cell surface expression of mAb- and TCR-based receptors on Jurkat T cells.

Jurkat T cells were transduced with the scFvG250:γ receptor, fl-296 TCRαβ or tc-296 TCRαβ:ζ and subsequently enriched for receptor-positive cells. Receptor expression was analyzed by flow cytometry using the anti-idiotypic NUH82 mAb followed by GaM^{PE} mAb (for scFvG250:γ transductants: solid line in A), or with the PE-labeled anti-TCRVβ14 mAb (for fl-296 TCRαβ and tc-296 TCRαβ:ζ transductants: solid lines in B and C, respectively). Non-transduced Jurkat T cells were used as a negative control (A, B, C; dotted line). Marker M1 (< 5% positive staining) was set in the histogram of non-transduced Jurkat T cells, and the percentage of positively stained receptor-transductants relative to M1 is indicated in the figure.

TCR/CD8α-grafted Jurkat T cells specifically bound gp100/HLA-A2 tetramer complexes, whereas the binding of an irrelevant EBV/HLA-A2 complex was negligible (data not shown).

The scFvG250:γ receptor induces antigen-specific activation of NFAT in Jurkat T cells

Single chain FvG250:γ-mediated activation of NFAT was analyzed by electroporation of the scFvG250:γ^{pos} Jurkat T cells with an NFAT-luciferase reporter construct, after which these cells were cocultivated for 6 h with $G250^{neg}$ and $G250^{pos}$ renal cell carcinoma cell lines, derived from carcinoma biopsies. Antigen-induced activation of NFAT was seen when scFvG250:γ^{pos} Jurkat T cells were stimulated with the $G250^{pos}$ cell line SKRC-17 cl.4 but not when stimulated with the $G250^{neg}$ cell line SKRC-17 cl.1 (Fig. 2). Antigen-induced NFAT activation was also seen after stimulation with the $G250^{pos}$ cell line A75 (data not shown). The antigen-specific activation of NFAT by scFvG250:γ^{pos} Jurkat T cells in response to the $G250^{pos}$ cell line SKRC-17 cl.4 was completely inhibited by adding either G250 mAb or NUH-82 mAb, but not control mIg (Fig. 2). Non-transduced Jurkat T cells did not show any NFAT activation upon stimulation with target cells.

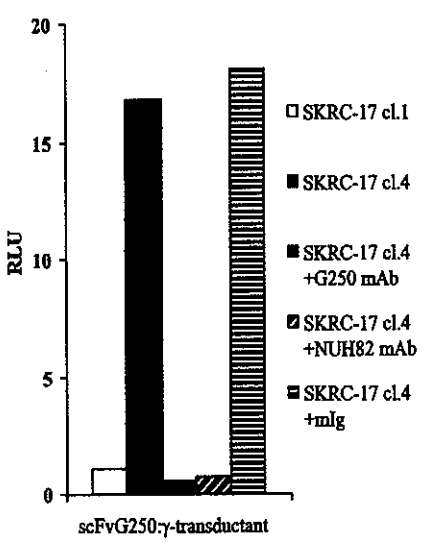

Figure 2. The scFvG250:γ receptor specifically mediates activation of NFAT in response to $G250^{pos}$ target cells.
Jurkat T cells expressing the scFvG250:γ receptor were transfected with 5 μg of both the NFAT-reporter and β-galactosidase constructs and stimulated for 6 h with the following target cells: the $G250^{neg}$ SKRC-17 clone 1 (white bar) and the $G250^{pos}$ SKRC-17 clone 4, the latter target in the absence (black bar) or presence of G250 mAb (grey bar; 25μg/ml), NUH82 mAb (diagonally striped bar; 40μg/ml), or control mouse gamma globulin (mIg; horizontally striped bar; 40μg/ml). Luciferase activities were determined in cell lysates, normalized for β-galactosidase activities, and expressed relative to a non-stimulated condition (i.e., medium only: RLU=0.04 which is set to 1.0). Stimulation with PMA (10 ng/ml) and ionomycin (1μM) was used as a positive control. Non-transduced Jurkat T cells did not respond to stimulation with any target cell (data not shown). Results of one (out of two) representative experiment are shown.

The fl-296 TCRαβ and tc-296 TCRαβ:ζ mediate activation of NFAT in response to native gp100 antigen

Receptor-mediated activation of NFAT was analyzed in the fl-296 TCRαβ^{pos}/CD8^{pos} and tc-296 TCRαβ:ζ^{pos}/CD8^{pos} Jurkat T cells after stimulation with anti-TCRVβ14 mAb as well as gp100^{pos}/HLA-A2^{pos} melanoma target cells. When stimulated with anti-TCRVβ14 mAb, activation of NFAT was seen in TCR-transduced Jurkat T cells, whereas control mIg was not able to induce NFAT activation in these cells (Fig. 3A). As a positive control, Jurkat T cells

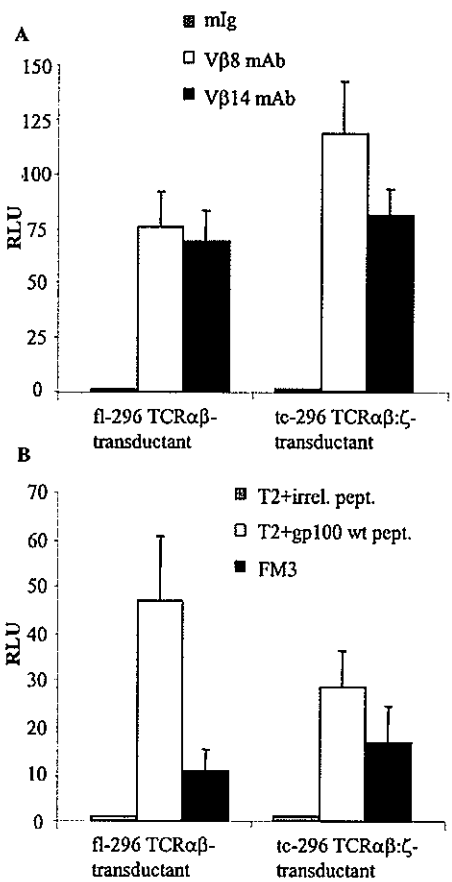

Figure 3. The fl-296 TCRαβ and tc-296 TCRαβ:ζ mediate NFAT activation in response to antigen-positive melanoma cells.

A, Anti-TCR mAb induces receptor-mediated activation of NFAT in TCR-transduced Jurkat T cells. Jurkat T cells transduced with the fl-296 TCRαβ and the tc-296 TCRαβ:ζ were transfected with 5 μg of both NFAT-reporter and β-galactosidase constructs and subsequently stimulated for 6 h with anti-TCRVβ14 mAb (black bar), anti-TCRVβ8 mAb (open bar) or control mIg (grey bar) (0,1 μg/well final). Luciferase activities were determined, normalized for β-galactosidase activities and expressed relative to the control mIg response (fl-296 TCRαβ: RLU=0.08; and tc-296 TCRαβ:ζ: RLU=0.07, which are both set to 1.0). Non-transduced Jurkat T cells only responded to stimulation with anti-TCRVβ8 mAb (data not shown). Averages of 3 to 5 experiments are shown, with error bars indicating standard errors of the mean. B, Peptide-loaded and native antigen-positive melanoma cells induce antigen-specific activation of NFAT in TCR-positive Jurkat T cells. TCR-transduced Jurkat T cells were transfected with the NFAT-reporter construct and subsequently co-cultured for 6 h with T2 cells loaded with either the gp100 wt peptide (open bars) or an irrelevant HLA-A2-binding peptide (grey bars), or with the gp100^{pos} FM3 melanoma cell line (black bars). Luciferase activities were determined and expressed relative to either T2 cells loaded with irrelevant peptide (fl-296 TCRαβ: RLU=0.06; and tc-296 TCRαβ:ζ: RLU=0.36, both set to 1.0) or medium only (fl-296 TCRαβ and tc-296 TCRαβ:ζ: RLU of both=0.04 and set to 1.0). Note: the high endogenous β-galactosidase activities of melanoma cells did not permit the normalization of luciferase activities for introduced β-galactosidase activities. Non-transduced Jurkat T cells did not respond to stimulation with any target cell (data not shown).

were stimulated with anti-TCRVβ8 mAb, which is specific for the endogenous TCRβ expressed on Jurkat T cells (Fig. 3A). As expected, non-transduced Jurkat T cells only showed NFAT activation upon stimulation with anti-TCRVβ8 mAb, confirming their ability to mediate activation of NFAT upon TCR triggering, but not with anti-TCRVβ14 mAb. When the TCR-transduced Jurkat T cells were co-cultured with T2 cells that were loaded with gp100 wild type (wt) peptide, we observed an activation of NFAT, whereas T2 cells loaded with an irrelevant HLA-A2-binding peptide did not induce NFAT activation in these cells (Fig. 3B). More importantly, a clear activation of NFAT was observed when TCR-transduced Jurkat T cells were cocultivated with the endogenously gp100^{pos}/HLA-A2^{pos} FM3 melanoma cell line (Fig. 3B). Non-transduced Jurkat T cells did not show any NFAT activation upon stimulation with peptide-pulsed T2 cells or FM3.

Validation of the NFAT reporter assay with Jurkat T cells

We further validated the NFAT reporter assay using specific inhibitors of NFAT activation. First, blocking experiments were performed with CsA, a specific inhibitor of calcineurin and consequently of nuclear translocation and activation of NFAT. CsA (at 100 nM final) was added to the Jurkat T cells 30 min prior to the 6 h co-culture experiments. CsA completely inhibited the activation of NFAT triggered by the G250^{pos} cell line SKRC-17 cl.4 (Fig. 4A). Next, a dominant negative mutant of NFAT was used [16], which competes with endogenous NFAT for binding to calcineurin and thereby selectively inhibits NFAT-mediated gene expression. The dnNFATmut (10μg) was co-electroporated with the NFAT-reporter and the β-galactosidase constructs into scFvG250:γ^{pos} Jurkat T cells prior to cocultivation experiments. The dnNFATmutant also completely inhibited the G250 antigen-induced activation of NFAT (Fig. 4B).

Antigen-specific activation of NFAT in Jurkat T cells parallel antigen-specific immune responses of primary human T lymphocytes

To investigate whether receptor-mediated NFAT activation is a representative measure of anti-tumor responses of human T cells, we compared NFAT activation in Jurkat T cells to immune responses of primary human T cells, such as cytotoxicity and cytokine production, following antigen-specific stimulation. Data obtained with scFvG250:γ^{pos} primary human T lymphocytes is summarized in Table IA. Single chain FvG250:γ^{pos} T lymphocytes were cytolytic and produced TNFα when co-cultured with G250^{pos} but not G250^{neg} target cells. In addition to NFAT activation, the G250 mAb also inhibited the antigen-induced cytolysis and

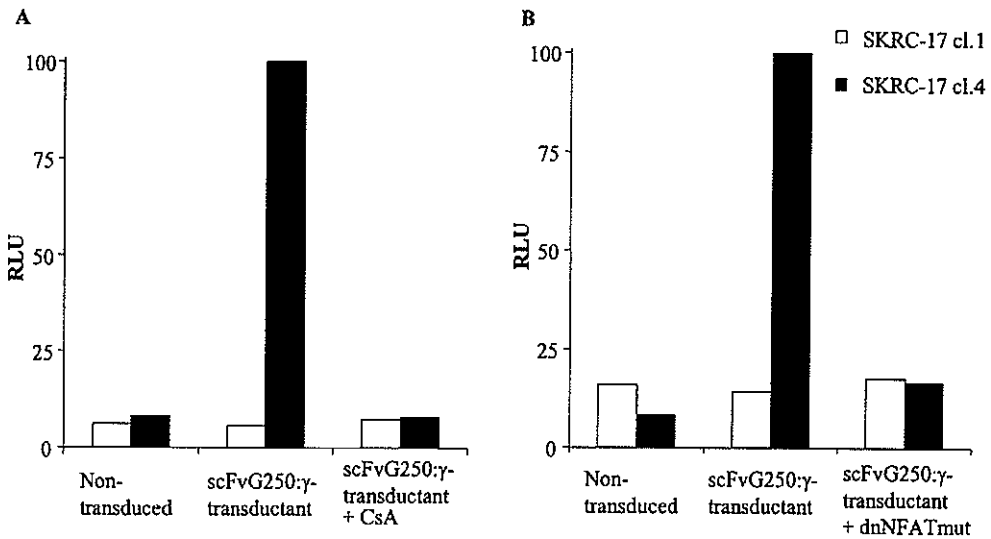

Figure 4. Antigen-specific responses in Jurkat T cells are inhibited by specific inhibitors of NFAT.
A, Inhibition of antigen-specific activation of NFAT in Jurkat T cells by CsA. scFvG250:γ-transduced Jurkat T cells were transfected with 5 μg of both NFAT-reporter and β-galactosidase constructs and subsequently co-cultured for 6 h with the G250^{neg} SKRC-17 clone 1 (open bars) or the G250^{pos} SKRC-17 clone 4 (black bars), both in the absence or presence of CsA (100nM). Luciferase activities were determined, normalized for β-galactosidase activities and expressed relative to the SKRC-17 cl.4 signal obtained without CsA (RLU relative to medium only=7.0; set to 100%). Stimulation with PMA and ionomycin was used as a positive control (data not shown). Non-transduced Jurkat T cells did not respond to stimulation with any target cell. Results of one (out of four) representative experiment are shown. B, Inhibition of antigen-specific activation of NFAT in Jurkat T cells by a dominant negative mutant. Jurkat T cells transduced with the chimeric scFvG250:γ receptor were transfected with 5 μg of both NFAT-reporter and β-galactosidase constructs and 10 μg of dnNFATmut construct, with amounts of DNA kept constant by adding pcDNA3.1 (backbone vector of dnNFATmut). Subsequently, cells were co-cultured for 6 h with the same target cells as described in the legend to Fig. 4A, after which luciferase activities were determined, normalized and expressed relative to the SKRC-17 cl.4 signal obtained without dnNFATmut.

TNFα production by scFvG250:γ-transduced primary human T lymphocytes. Data obtained with fl-296 TCRαβ^{pos} and tc-296 TCRαβ:ζ^{pos} primary human T lymphocytes is summarized in Table IB and C, respectively. Again, the fl-296 TCRαβ^{pos} and tc-296 TCRαβ:ζ^{pos} T lymphocytes showed cytolytic activity and produced TNFα when co-cultured with gp100 peptide-loaded T2 cells and gp100^{pos}/HLA-A2^{pos} melanoma cells but not gp100^{neg} target cells. To assay directly the significance of NFAT activation to antigen-specific responses of primary human T lymphocytes, we added CsA to the T lymphocyte effector cells 30 min prior to

Table I: Antigen-specific activation of NFAT parallels antigen-specific immune responses of primary human T lymphocytes

A. scFvG250:γ

Target cells	**CTX[a] (% ^{51}Cr-release)**	**TNFα production[b] (pg/ml)**	**NFAT activity[c] (RLU)**	
			Exp.1	Exp.2
SKRC-17 cl.1	1	0	1.1	0.4
SKRC-17 cl.1+CsA	0	2		0.5
SKRC-17 cl.4	45	361	16.8	7.0
SKRC-17 cl.4+G250 mAb	2	0	0.6	
SKRC-17 cl.4+CsA	27	8		0.6

B. fl-296 TCRαβ

Target cells	**CTX[a] (% ^{51}Cr-release)**	**TNFα production[b] (pg/ml)**	**NFAT activity[c] (RLU)**
T2+irrel.pept.	0	0	1.7
T2+gp100 wt pept.	93	665	22.0
T2+gp100 wt pept.+CsA	81	3	0.7
BLM	1	0	1.1
BLMgp100	35	945	2.2
BLMgp100+CsA	18	0	0.6
FM3	30	243	16.0
FM3+CsA	19	0	0.5

C. tc-296 TCRαβ:ζ

Target cells	**CTX[a] (% ^{51}Cr-release)**	**TNFα production[b] (pg/ml)**	**NFAT activity[c] (RLU)**
T2+irrel.pept.	16	0	5.3
T2+gp100 wt pept.	85	283	140.0
T2+gp100 wt pept.+CsA	71	0	1.4
BLM	15	0	1.9
BLMgp100	25	145	9.4
BLMgp100+CsA	12	0	1.1
FM3	36	25	32.0
FM3+CsA	24	0	1.3

[a] Percentages of ^{51}Cr release from various target cells when cocultivated with primary human T lymphocytes expressing the scFvG250:γ receptor (Table IA), fl-296 TCRαβ (Table IB), and tc-296 TCRαβ:ζ (Table IC). Single chain FvG250:γ-positive T lymphocytes were used at an effector to target cell (E:T) ratio of 5:1. The fl-296 TCRαβ^{low} and tc-296 TCRαβζ^{low} T lymphocytes were co-cultured with peptide-loaded T2 target cells at an E:T ratio of 7.5:1, whereas TCR-transduced T lymphocytes were co-cultured with the melanoma cell lines BLM, BLMgp100 and FM3 at an E:T ratio of 30:1. T2 cells were peptide-loaded for 30 min at 37°C/5% CO_2 prior to their use in cytotoxicity assays. Final concentrations of CsA, G250 mAb and peptides are 100nM, 25μg/ml and 1 μg/ml, respectively. Abbreviations used: irrel. pept.=irrelevant peptide; gp100 wt pept.=gp100 wild type peptide.
[b] TNFα production (determined by ELISA; expressed in pg/ml) by receptor-positive primary human T lymphocytes that were co-cultured with the same target cells as described in (a) in the absence or presence of G250 mAb or CsA. E:T ratio used was 3:1.
[c] NFAT activity (in RLU) of receptor-positive Jurkat T cells that were co-cultured with the target cells as described in (a) in the absence or presence of the G250 mAb or CsA. BLM, BLMgp100 and FM3 cells were pre-treated with INFγ and IL-1β, and used in combination with anti-CD28 mAb to stimulate TCR-transduced Jurkat T cells. Luciferase activities were determined in cell lysates, normalized for β-galactosidase activities, and expressed relative to a non-stimulated condition (i.e., medium only). See the Materials and Methods section for details.

cytotoxicity and TNFα production assays. CsA inhibited 35-50% of the antigen-specific cytotoxicity of primary human T lymphocytes expressing either mAb- or TCR-based tumor-specific receptors, whereas CsA completely inhibited the antigen-specific TNFα production in these T cell transductants (Table I).

Discussion

We set up an NFAT reporter gene assay based on Jurkat T cells to test functionally tumor-specific receptors in a fast and sensitive manner, as opposed to the standard but more labor-intensive testing of tumor cell cytotoxicity and cytokine production of receptor-transduced primary human T lymphocytes. The NFAT reporter gene assay in Jurkat T cells is validated along the following lines: (i) receptor-positive Jurkat T cells are easy to obtain via retroviral transduction and, when necessary, to enrich via MACS; (ii) mAb-based as well as TCR-based anti-tumor receptors, having different (chimeric) receptor formats, mediate antigen-specific NFAT activation; (iii) various stimuli such as anti-receptor mAbs and tumor target cells that are either loaded with peptides or natively express the antigen or peptide/MHC complex of interest trigger an NFAT response; (iv) ligand- or receptor-specific antibodies as well as the immunosuppressor CsA and a dominant negative NFAT mutant block antigen-specific NFAT activation; and importantly (v) receptor-mediated NFAT

responses parallel tumor-specific cytolysis and TNFα production of receptor-transduced primary human T lymphocytes, and the immune responses of receptor-engrafted primary human T lymphocytes are, in fact, compromised by inhibition of NFAT activation.

Transduction of Jurkat T cells with retroviral vectors harboring mAb- or TCR-based receptor gene(s) using phoenix-derived viruses and retronectin as a substrate on average resulted in levels of expression of 30%, being slightly less but in good agreement with results obtained with primary human T lymphocytes [20]. Receptor-positive Jurkat T cells can easily be enriched resulting in levels of expression of over 80% (Fig. 1) and avoiding the need for cloning and antibiotic selection. Moreover, TCR/CD8 co-transduction resulted in specific binding of gp100/HLA-A2 tetramers, showing preservation of ligand binding affinity of TCR chains following gene transfer in immortalized T cells. Antigen-specific function of the introduced receptors was evident from their ability to mediate NFAT activation. A clear NFAT activation was observed in Jurkat T cells transduced with either the G250-specific mAb-based chimeric scFvG250:γ receptor or the gp100/HLA-A2-specific TCR-based 296 receptors (i.e., fl-296 TCRαβ or tc-296 TCRαβ:ζ) when stimulated with the $G250^{pos}$ target cell (Fig. 2), and anti-TCR mAb or gp100 peptide-loaded target cells (Figs. 3A and B), respectively. More importantly, TCR-transduced Jurkat T cells show NFAT activation in response to melanoma cells presenting endogenously processed gp100 peptide (Fig. 3B).

The observed native response of TCR-reconstituted Jurkat T cells is in contrast to previous reports showing that TCR-reconstituted Jurkat T cells only respond to peptide-loaded target cells but not to tumor cells [13-15]. We ascribe the detection of native antigen responses mediated by TCR-based receptors to the following. First, the retroviral transduction protocol we employed results in high expression levels of receptor and CD8α on Jurkat T cells that can then be easily enriched. High expression levels of the introduced receptor as well as CD8α facilitate antigen-specific T cell responses [23]. The lack of CD8α expression on Jurkat T cells is in fact suggested to explain the inability of Jurkat T cells transfected with MART-1-specific TCR to produce IL-2 in response to MART-1 positive tumor cells [13]. Noteworthy in this respect is our finding that TCR-expressing Jurkat T cells with a low expression level of CD8α do not respond to gp100 positive melanoma cells (data not shown), in line with the observation by Aarnoudse and colleagues that additional introduction of CD8α in Jurkat T cells increased the weak TCR-mediated response to melanoma cells [15]. Second, in-house flow cytometry experiments show that the Jurkat E6.1 T cell clone, in sharp contrast to other Jurkat T cell clones often used for TCR-reconstitution

experiments such as the TCRβ_{neg} Jurkat RT3-T3.5 T cell clone [14,15,24], expresses high levels of endogenously expressed CD2, CD5, CD11α, CD18 and CD28 molecules (data not shown). The expression of such costimulatory and adhesion molecules is particularly important when the expression level of tumor antigens on target cells is low as is the case for endogenously expressed peptide/MHC molecules on melanoma cells. In fact, the use of Jurkat E6.1 T cells allowed us to provide these cells a costimulatory signal via an anti-CD28 mAb when testing TCR-mediated NFAT activation in response to tumor cells, by analogy to the stimulation conditions used by Liu and colleagues to study TCR-mediated IL-2 production in Jurkat E6.1 T cells in response to native p53-positive tumor cells [24]. Third, cytokine-stimulation of tumor target cells prior to our NFAT activation assay, to increase the expression of MHC class I and adhesion molecules on tumor cells, further enhances antigen-specific responses. Finally, the NFAT reporter gene assay has been optimized for various parameters with the number of response elements present in the NFAT reporter construct being important to the assay's sensitivity and facilitating the detection of antigen-specific NFAT activation.

The NFAT responses in receptor-transduced Jurkat T cells are in parallel with the antigen-specific immune responses (i.e., cytotoxicity and cytokine production) of primary human T lymphocytes transduced with identical receptor gene(s) (Table I). In addition, the sensitivity of antigen-specific cytotoxicity and TNFα of primary human T lymphocytes to CsA provides direct evidence of the contribution of NFAT activation to these immune responses. CsA inhibited 30-50% and 100% of the antigen-specific cytolytic and TNFα responses of primary human T cells transductants, respectively, irrespective of the receptor format used (Table I). Inhibition of cytotoxicity of TCR-transduced T lymphocytes in response to peptide-loaded target cells was less efficient (Table IB and C), most likely due to the fact that a peptide stimulus is non-physiological for TCR-mediated cytotoxicity. Our observation that at least 50% of the cytotoxic response of primary human T cells are not affected by CsA is most likely due to the existence of two major pathways to induce cytotoxicity: 1) the FasL pathway, with an NFAT dependence of the transcriptional activation of FasL in cytotoxic T cells [25], and 2) the granzyme/perforin release pathway, which is NFAT independent [26].

The observation that tumor-specific receptors coupled to the intracellular signaling domains of Fc(ε)RIγ (scFvG250:γ) or CD3ζ (tc-296 TCR$\alpha\beta$:ζ) mediate NFAT activation points to the usefulness of the described NFAT reporter gene assay to test the function of immune receptors containing various building blocks. By analogy to the Fc(ε)RIγ-crosslinking on mast cells, receptors containing a γ domain probably mediate antigen-specific NFAT activa-

tion as a result of activation of the protein kinases Lyn and Syk followed by calcium mobilization and activation of MAP kinases [27,28], whereas CD3ζ-containing receptors are able to recruit and activate ZAP-70, which propagates intracellular signaling resulting in the activation of NFAT and other transcription factors [29].

Taken together, receptor-mediated activation of NFAT is a representative measure of anti-tumor T cell responses and the genetically modified Jurkat T cells described in this paper provide a flexible and sensitive tool for the analysis and selection of chimeric receptors for immunogene therapy. In addition, the genetically engineered Jurkat T cells enable studies on whether and how (chimeric) immune-receptors (and other receptors, such as homing receptors or adhesion molecules, for that matter) interact with other T cell molecules, and provide an approach to the study of intracellular signaling pathways.

Acknowledgements

This work was supported by the Dutch Cancer Society. The 96 well plate luminometer used in this study was financed by the Nijbakker-Morra Foundation, Leiden, the Netherlands. We thank Dr. E. Oosterwijk for providing the SKRC-17 clones 1 and 4, the G250 and NUH-82 mAbs, Dr. G. Adema for the melanoma cell lines FM3, BLM and BLMgp100 as well as the fl-296 TCRαβ sequence, Dr. J.W. Drijfhout for peptide synthesis, Dr. Davis for providing the dominant negative NFAT mutant, T. van de Wetering for optimization of the NFAT reporter assay, C. M. Groot-van Ruijven for performing cytotoxicity assays, and Drs. R.A. Willemsen, C.A. Aarnoudse and P.I. Schrier for fruitful discussions.

References

1. Eshhar,Z.; Waks,T.; Gross,G.; and Schindler,D.G. "Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors." (1993), *Proc. Natl. Acad. Sci. U. S. A*, **90**:720-724
2. Weijtens,M.E.; Willemsen,R.A.; Hart,E.H.; and Bolhuis,R.L. "A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes." (1998), *Gene Ther.*, **5**:1195-1203
3. Willemsen,R.A.; Weijtens,M.E.; Ronteltap,C.; Eshhar,Z.; Gratama,J.W.; Chames,P.; and Bolhuis,R.L. "Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR." (2000), *Gene Ther.*, **7**:1369-1377

4. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
5. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
6. Kessels,H.W.; Wolkers,M.C.; van den Boom,M.D.; van der Valk,M.A.; and Schumacher,T.N. "Immunotherapy through TCR gene transfer." (2001), *Nat. Immunol.*, **2**:957-961
7. Hwu,P.; Yang,J.C.; Cowherd,R.; Treisman,J.; Shafer,G.E.; Eshhar,Z.; and Rosenberg,S.A. "In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes." (1995), *Cancer Res.*, **55**:3369-3373
8. McGuinness,R.P.; Ge,Y.; Patel,S.D.; Kashmiri,S.V.; Lee,H.S.; Hand,P.H.; Schlom,J.; Finer,M.H.; and McArthur,J.G. "Anti-tumor activity of human T cells expressing the CC49-zeta chimeric immune receptor." (1999), *Hum. Gene Ther.*, **10**:165-173
9. Patel,S.D.; Moskalenko,M.; Smith,D.; Maske,B.; Finer,M.H.; and McArthur,J.G. "Impact of chimeric immune receptor extracellular protein domains on T cell function." (1999), *Gene Ther.*, **6**:412-419
10. Fiering,S.; Northrop,J.P.; Nolan,G.P.; Mattila,P.S.; Crabtree,G.R.; and Herzenberg,L.A. "Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor." (1990), *Genes Dev.*, **4**:1823-1834
11. Karttunen,J. and Shastri,N. "Measurement of ligand-induced activation in single viable T cells using the lacZ reporter gene." (1991), *Proc. Natl. Acad. Sci. U. S. A*, **88**:3972-3976
12. Sanderson,S. and Shastri,N. "LacZ inducible, antigen/MHC-specific T cell hybrids." (1994), *Int. Immunol.*, **6**:369-376
13. Cole,D.J.; Weil,D.P.; Shilyansky,J.; Custer,M.; Kawakami,Y.; Rosenberg,S.A.; and Nishimura,M.I. "Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen." (1995), *Cancer Res.*, **55**:748-752
14. Calogero,A.; Hospers,G.A.; Kruse,K.M.; Schrier,P.I.; Mulder,N.H.; Hooijberg,E.; and de Leij,L.F. "Retargeting of a T cell line by anti MAGE-3/HLA-A2 alpha beta TCR gene transfer." (2000), *Anticancer Res.*, **20**:1793-1799
15. Aarnoudse,C.A.; Kruse,M.; Konopitzky,R.; Brouwenstijn,N.; and Schrier,P.I. "TCR reconstitution in Jurkat reporter cells facilitates the identification of novel tumor antigens by cDNA expression cloning." (2002), *Int. J. Cancer*, **99**:7-13
16. Chow,C.W.; Rincon,M.; and Davis,R.J. "Requirement for transcription factor NFAT in interleukin-2 expression." (1999), *Mol. Cell Biol.*, **19**:2300-2307
17. Weijtens,M.E.; Willemsen,R.A.; Valerio,D.; Stam,K.; and Bolhuis,R.L. "Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity." (1996), *J. Immunol.*, **157**:836-843
18. Bakker,A.B.; Schreurs,M.W.; de Boer,A.J.; Kawakami,Y.; Rosenberg,S.A.; Adema,G.J.; and Figdor,C.G. "Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes." (1994), *J. Exp. Med.*, **179**:1005-1009
19. Zarour,H.; De Smet,C.; Lehmann,F.; Marchand,M.; Lethe,B.; Romero,P.; Boon,T.; and Renauld,J.C. "The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes

of a melanoma patient recognize an antigenic peptide derived from gene Pmel17/gp100." (1996), *J. Invest Dermatol.*, **107**:63-67

20. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
21. Debets,R.; Timans,J.C.; Churakowa,T.; Zurawski,S.; de Waal,M.R.; Moore,K.W.; Abrams,J.S.; O'Garra,A.; Bazan,J.F.; and Kastelein,R.A. "IL-18 receptors, their role in ligand binding and function: anti-IL- 1RAcPL antibody, a potent antagonist of IL-18." (2000), *J. Immunol.*, **165**:4950-4956
22. Lamers,H.J.; Gratama,J.W.; van Putten,W.L.; Stoter,G.; and Bolhuis,R.L. "Exogenous interleukin 2 recruits in vitro lymphokine-activated killer activity by in vivo activated lymphocytes." (1991), *Cancer Res.*, **51**:2324-2328
23. Nel,A.E. "T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse." (2002), *J. Allergy Clin. Immunol.*, **109**:758-770
24. Liu,X.; Peralta,E.A.; Ellenhorn,J.D.; and Diamond,D.J. "Targeting of human p53-overexpressing tumor cells by an HLA A*0201- restricted murine T-cell receptor expressed in Jurkat T cells." (2000), *Cancer Res.*, **60**:693-701
25. Xiao,S.; Matsui,K.; Fine,A.; Zhu,B.; Marshak-Rothstein,A.; Widom,R.L.; and Ju,S.T. "FasL promoter activation by IL-2 through SP1 and NFAT but not Egr-2 and Egr-3." (1999), *Eur. J. Immunol.*, **29**:3456-3465
26. Kagi,D.; Ledermann,B.; Burki,K.; Zinkernagel,R.M.; and Hengartner,H. "Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo." (1996), *Annu. Rev. Immunol.*, **14**:207-232
27. Jouvin,M.H.; Numerof,R.P.; and Kinet,J.P. "Signal transduction through the conserved motifs of the high affinity IgE receptor Fc epsilon RI." (1995), *Semin. Immunol.*, **7**:29-35
28. Hutchinson,L.E. and McCloskey,M.A. "Fc epsilon RI-mediated induction of nuclear factor of activated T-cells." (1995), *J. Biol. Chem.*, **270**:16333-16338
29. Clements,J.L.; Boerth,N.J.; Lee,J.R.; and Koretzky,G.A. "Integration of T cell receptor-dependent signaling pathways by adapter proteins." (1999), *Annu. Rev. Immunol.*, **17**:89-108

Chapter 3

Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR$\alpha\beta$ genes into primary human T lymphocytes

Niels Schaft, Ralph A. Willemsen, Jolanda de Vries, Birgit Lankiewicz, Bram W. L. Essers, Jan-Willem Gratama, Carl G. Figdor, Reinier L. H. Bolhuis, Reno Debets, Gosse J. Adema

Journal of Immunology **170**:2186-2194 (2003)

Abstract

TCR with known anti-tumor reactivity can be genetically introduced into primary human T lymphocytes, and provide promising tools for immunogene therapy of tumors. We molecularly characterized two distinct TCRs specific for the same HLA-A2-restricted peptide derived from the melanocyte differentiation antigen gp100, yet exhibiting different stringencies in peptide requirements. The existence of these two distinct gp100-specific TCRs allowed us to study the preservation of peptide fine-specificity of native TCRα/β when engineered for TCR gene transfer into human T lymphocytes. Retroviral transduction of primary human T lymphocytes with either one of the two sets of TCRα/β constructs enabled T lymphocytes to specifically kill and produce TNFα when triggered by native gp100^{pos}/HLA-A2^{pos} tumor target cells as well as gp100 peptide-loaded HLA-A2^{pos} tumor cells. Peptide titration studies revealed that the cytolytic efficiencies of the T lymphocyte transductants were in the same range as those of the parental CTL clones. Moreover, primary human T lymphocytes expressing either one of the two engineered gp100-specific TCRs show cytolytic activities in response to a large panel of peptide mutants that are identical to those of the parental CTL. The finding that two gp100-specific TCR, derived from two different CTL, can be functionally introduced into primary human T lymphocytes without loss of the antigen reactivity and peptide fine-specificity, holds great promise for the application of TCR gene transfer in cancer immunotherapy.

Introduction

Cytotoxic T lymphocytes specific for tumor-associated antigens have the capacity to mediate efficient anti-tumor immune responses *in vivo*. The availability of tumor-specific CTLs enabled the molecular identification and characterization of a large panel of class I MHC-binding peptides derived from tumor and viral antigens (for reviews, see refs [1] and [2]). Of the melanoma differentiation antigen gp100, multiple peptides have been identified that are recognized by CTL isolated either from peripheral blood or tumor lesions of melanoma patients [3-6]. Recently, we have demonstrated that it is possible to generate specific CTL directed against gp100 using peptide-pulsed APC and PBL of healthy individuals [5]. In these studies, we made use of dendritic cells (DC) as the APC. DC are key regulators in immune responses, capable of priming naive T cells, which, at least in part, is due to their high expression of MHC class I and II molecules as well as co-stimulatory molecules [7-9]. Currently, DC-based vaccines are used to induce anti-tumor immunity in man.

Alternatively, adoptive transfer of antigen-specific, MHC-restricted CTL may be successful in the eradication of tumors in patients with metastatic melanoma and in e.g. allogeneic bone marrow transplant recipients with EBV positive tumors [10-13]. Unfortunately, patients do not always mount a strong and effective *in vivo* cytotoxic T cell response to their tumors. In fact, isolation of tumor-specific T lymphocytes has only been possible in a fraction of patients, most likely due to the fact that, at least in an immunocompetent setting, the peripheral T cell repertoire is devoid of high-avidity tumor-specific CTL due to thymic selection [14]. In addition, these cells only have a limited life-span, and expansion of such T lymphocytes to therapeutic doses is often not feasible [15,16]. To overcome these problems, T lymphocytes can be harnessed with anti-tumor/virus specificities via genetic means. Genes encoding for TCRα and β chain can be cloned from the tumor-specific CTL clones, and transfer of these TCRα/β genes into primary human T lymphocytes reprograms T lymphocytes with a specificity against a tumor antigen. In several *in vitro* studies, primary human T lymphocytes have been retargeted with a tumor- or virus-specificity by transfer of TCRα/β genes, and were shown to respond specifically towards target cells expressing both the corresponding antigen and the correct HLA-molecule [17-22]. Recently, it was also shown that mouse T cells that were redirected by TCR gene transfer efficiently promoted the rejection of antigen-expressing tumors *in vivo* [23].

To date very little is known about the preservation of both the cytotoxic potency and peptide fine-specificity of CTL following TCR gene transfer, which is crucial to the successful use

of TCR genes for immunogene therapy. Here we describe the characterization of the TCRs of two anti-gp100/HLA-A2 CTL clones: one obtained from PBL of a healthy donor via stimulation with peptide-pulsed DC [5], and the other obtained from PBL of a melanoma patient via stimulation with autologous melanoma cells [24]. These CTL clones showed identical cytotoxic responses against gp100^{pos}/HLA-A2^{pos} target cells, but clearly displayed different fine-specificities for gp100 amino acid substitution mutants. The existence of two gene sets each encoding for TCRα/β molecules specific for gp100 peptide but with distinct gp100 peptide fine-specificties enabled us to analyze the preservation of both the cytotoxic potency and peptide fine-specificity of the physiological TCRs when engineered for TCR gene transfer into human T lymphocytes. Peptide-loaded HLA-A2^{pos} and native gp100^{pos}/HLA-A2^{pos} tumor target cells triggered lysis and TNFα production by TCR-transduced T lymphocytes. The cytolytic efficiencies of the TCR-transduced T lymphocytes were in the same range as those of the parental CTL clones. Most importantly, the gp100 peptide fine-specificities of the transduced TCRs were identical to those of the parental CTLs. Taken together, this report shows for the first time that human TCR genes can be functionally transferred to human T lymphocytes without loss of cytolytic efficiency and peptide fine-specificity.

Materials and Methods

Cells and cell lines

PBL from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density=1.077 g/cm^3; Pharmacia Biotech, Uppsala, Sweden). Transduced primary human T lymphocytes were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 200 mM L-glutamine, 10% human serum and 360 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands), and stimulated every 2 weeks with a mixture of irradiated allogeneic feeder-cells, as described elsewhere [25]. The melanoma cell lines BLM (HLA-A2^{pos}), BLM transfected with human gp100-encoding cDNA (BLMgp100) and MEL624 (gp100^{pos}/HLA-A2^{pos}) were cultured as described previously [26,27]. The human amphotropic packaging cell line Phoenix, the melanoma cell lines FM3 (gp100^{pos}/HLA-A2^{pos}; a kind gift from J. Zeuthen, Copenhagen, Denmark) and LB265-MEL, as well as the TAP-deficient TxB cell hybrid T2 cells (HLA-A2^{pos}) were grown in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% Bovine Calf Serum (BCS; Hyclone, Logan, UT). The B-LCL BSM (HLA-A2^{pos}) and K562, a chronic

myelogenic leukemic cell line, were cultured in RPMI 1640 medium supplemented with 200 mM L-glutamine and 10% BCS. The B-cell lines LG2-EBV and JY-EBV were cultured in IMDM as described previously [28]. Contamination of cell lines and cells with mycoplasm species was excluded by frequent PCR testing of cellular DNA with mycoplasm specific primers (Gen-Probe Inc., San Diego, CA). HLA-A2 expression of melanoma cell lines was checked by flow cytometry and expression of gp100 was checked by staining cytospin preparations with anti-gp100 polyclonal antibody (HMB45).

Peptides and peptide binding assays

Peptides used in this study were: an irrelevant HLA-A2-binding EBV-peptide (GLCTLVAML), $gp100_{280\text{-}288}$ wild type (wt) peptide YLEPGPVTA, and the gp100 peptide mutants (A1 to A8, and G9, with the A or G referring to an alanine or glycine residue, respectively and the number corresponding to the position of the aa in the gp100 wt peptide). Peptides were synthesized with a free carboxy-terminus either by f-moc peptide chemistry using an ABIMED Multiple Synthesizer or by t-boc chemistry on a Biosearch SAM2 peptide synthesizer. All peptide preparations were >90% pure as analyzed by analytical HPLC. Peptides were dissolved in 100% DMSO and stored at –20°C. The HLA-A2 stabilization assay on T2 cells has been described previously [29]. Briefly, peptides at various dilutions were incubated with 10^5 T2 cells for 14 hours at 37°C/5% CO_2 in serum-free medium in the presence of 3 µg/ml human β2-microglobulin (Sigma, St. Louis, MO) in a total volume of 100 µl. Peptide-mediated stabilization of HLA-A2 molecules at the cell surface of T2 cells was analyzed by flow cytometry using the anti-HLA-A2 mAb BB7.2, and expressed as an Fluorescence Index: (experimental mean fluorescence/background mean fluorescence)-1. The background mean fluorescence values were obtained by incubating T2 cells with an HLA-A2 non-binding peptide (WMAFKERKV) at a similar concentration as the experimental peptide. A second, competition-based HLA-A2 peptide binding assay using JY-EBV cells was performed as described previously [30]. In short, HLA molecules on JY-EBV cells were stripped via a mild acid treatment. Stripped cells were subsequently incubated with various concentrations of peptides for 24 hours at 4°C in the presence of 150 nM of a Fluorescein (FL)-labeled reference peptide (FLPSDC(-FL)FPSV) and 1.5 µg/ml human β2-microglobulin, and analyzed by flow cytometry. The binding capacity of a peptide is expressed as the concentration required to inhibit 50% of the binding of the FL-labeled reference peptide (IC_{50}).

Generation of CTL

CTL-MPD, specific for the HLA-A2-presented $gp100_{280-288}$ peptide, was cloned via limiting dilution from a bulk anti-gp100 CTL culture that was induced *in vitro* using gp100 wt peptide-pulsed DC as described previously [5]. After several weeks of restimulation, stable clones were obtained. CTL-MPD was expanded weekly: CTL were mixed with LB265-MEL, LG2-EBV and JY-EBV cells, the latter two pulsed with the $gp100_{280-288}$ wt peptide, in IMDM supplemented with 3% human serum, 120 IU/ml human rIL-2 and 5 ng/ml human rIL-7 (Genzyme, Cambridge, MA). CTL-296, also specific for the HLA-A2-presented $gp100_{280-288}$ peptide, was generated from PBL of a melanoma patient [24]. The CTL-296 was maintained via co-culture of this CTL with gp100 wt peptide-pulsed T2 cells, LG2-EBV and JY-EBV cells in IMDM containing 10% human serum, 200 mM L-glutamine and 50 IU human rIL2. The CTL cultures were restimulated weekly.

Analysis of TCR Vα/β gene usage of CTL-296 and CTL-MPD

RNA was isolated from 10^6 CTL and used for reverse transcriptase reactions performed with Superscript (Gibco BRL) according to the manufacturer's instructions. The TCRα chain variable regions were amplified using a set of sense primers, specific for the TCRAV1 to the TCRAV29 segment (kindly provided by Dr. T. Logtenberg, Crucell BV., Leiden, the Netherlands; and Dr. P. van der Elsen, LUMC, Leiden), in combination with a TCRAC antisense consensus primer. Nested PCR was performed on TCRVA products before gel-electrophoresis. The TCRβ chain variable regions were amplified using a combination of degenerate TCRBV sense primers (kindly provided by Dr. H. Dolstra, University Hospital Nijmegen, the Netherlands) and a TCRBC2 antisense primer [31]. The Vα and Vβ PCR products were run on agarose gels, blotted onto Hybond filters and hybridized with either a labeled TCRAC or a TCRBC2 probe. Positive PCR products were cloned, and plasmid DNAs from at least 5 independent colonies were sequenced using both the CTAB sequencing protocol and the T7 sequencing kit (Pharmacia Biotech).

Cloning of the gp100-specific MPD and 296 TCR genes and transduction of human T lymphocytes

Sequence analysis of the TCR Vα and Vβ genes used by CTL-MPD and CTL-296 allowed for the design of specific primers to amplify the full length (fl) TCRα and β DNAs using CTL-derived cDNA as template DNA. Primers used to amplify the TCRα and TCRβ DNAs without their signal peptide sequences are as follows:

fl-MPD TCRα chain:
TCRAMPD: 5'-CTC TCC ATG GAG ACT CTC CTG AAA GTG C-3'
HCA: 5'-CTC TCT CGA GGG ATC CTC AGC TGG ACC ACA GCC GCA GC-3'
fl-MPD TCRβ chain:
TCRBMPD: 5'-CTC TCC ATG GAC TCC TGG ACC TTC TGC TG-3'
HCB: 5'-CTC TCT CGA GGG ATC GCT AGC CTC TGG AAT CCT TTC TC-3'
fl-296 TCRα chain:
TCRA296: 5'-CTC TCC ATG GCA TCC ATT CGA GCT GTA TTT-3'
HCA: 5'-CTC TCT CGA GGG ATC CTC AGC TGG ACC ACA GCC GCA GC-3'
fl-296 TCRβ chain:
TCRB296: 5'-CTC TCC ATG GGC CCC CAG CTC CTT GGC TAT G-3'
HCB: 5'-CTC TCT CGA GGG ATC GCT AGC CTC TGG AAT CCT TTC TC-3'

The PCR products (i.e., the TCR cDNAs) were cloned into the retroviral vector pBullet containing an heterologous signal peptide, as described elsewhere [21] and checked by sequence analysis. Human T lymphocytes of healthy donors were activated with anti-CD3 mAbs and transduced with retroviruses harboring the TCRα and TCRβ transgenes. The TCR-encoding retroviruses were produced by the packaging cell line Phoenix. The transduction procedure used for primary human T lymphocytes was optimized and described by Lamers *et al.* [32]. In short, 24 well culture plates were coated with human fibronectin fragments CH-296 (Takara Shuzo co. Ltd., Otsu, Japan) and pre-treated with retroviral particles by centrifugation at 2400 rpm for 1h with 2 ml retrovirus-containing supernatant per well. Next, 10^6 human T cells were centrifuged in 2 ml of fresh virus-supernatant, and cultured for 4-5 h at 37°C/5% CO_2. T cells were allowed to recover in normal T cell medium O/N prior to a second transduction cycle, after which cells were harvested and transferred to T25 culture flasks.

Flowcytometry of TCR-transduced T lymphocytes
TCR-transduced T cells were analyzed for TCR and CD8α expression by flow cytometry using PE-conjugated anti-TCRVβ8 mAb (i.e., recognizing fl-MPD TCR), anti-TCRVβ14 mAb (i.e., recognizing fl-296 TCR) (both from Beckman Coulter, Marseille, France) or APC-labeled gp100/HLA-A2 tetramers, and FITC-conjugated anti-CD8 mAb (Becton Dickinson Biosciences, San Jose, CA), respectively. For immuno-staining, 0.1-0.5x10^6 transduced cells were washed with ice-cold PBS containing 0.5% BSA and 0.02% EDTA,

and incubated with mAbs on ice for 30 min or tetramers at room temperature for 1 h. For immuno double stainings, cells were first incubated with tetramers, washed and subsequently incubated with anti-TCRVβ mAb. Upon completion of the immuno-stainings, cells were washed again, fixed (with 1% paraformaldehyde) and analyzed on a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA) using CellQuest software.

Cytotoxicity assay

Cytotoxic activity of both gp100/HLA-A2-specific CTL clones and T lymphocytes that were retrovirally transduced with the fl-MPD and fl-296 TCR genes was routinely measured in a standard 5-6h ^{51}Cr-release assay [33]. Target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4/10^6$ cells for at least 1h at 37°C/5% CO_2. T2, BLM and BSM cells were pulsed with gp100 peptides for 45 min at 37°C/5% CO_2 prior to co-cultivation with effector T cells. To correct for NK cell-activity, cold target cell inhibition assays were performed by adding non-labeled K562 cells at the indicated cold target : hot target ratios. In experiments aimed at specific blocking of the cytolytic activities of fl-MPD and fl-296 TCR-transductants, anti-TCRVβ8 mAb (at 1 μg/ml final), anti-TCRVβ14 mAb (1 μg/ml), anti-MHC class I mAb (clone W6/32; Sera-Lab, Crawley Down, UK) (10 μg/ml), or mouse gamma globulin (mIg; Jackson Immuno Research Laboratories, West Grove, PA) (10 μg/ml) was added at the onset of the cytotoxicity assay. Cytolytic efficiencies of parental CTL and TCR-transductants were assayed by pulsing T2 cells with various amounts of the gp100 wt peptide, whereas peptide fine-specificities were determined by loading T2 cells with a series of gp100 peptide mutants (all at 1 μM final) prior to cytotoxicity assays. Percentage specific cytolysis, i.e., specific ^{51}Cr-release, was calculated as described previously [34].

TNFα production

To quantify TNFα production by TCR-transduced T lymphocytes after antigen-specific stimulation, $6x10^4$ T cells were cultured in the presence of $2x10^4$ tumor cells or peptide-pulsed T2 cells for 18 h. As a positive control, TCR-transduced T lymphocytes were stimulated with PHA and PMA. Supernatants were harvested and levels of TNFα were measured by standard ELISA (CLB, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Results

Two distinct anti-gp100 CTL use a highly similar TCRβ region

We generated an HLA-A2-restricted $gp100_{280-288}$-specific CTL by stimulating PBL from a healthy donor with autologous peptide-pulsed DC [5]. Limiting dilution subsequently yielded the CTL clone-MPD which showed a cytotoxic reactivity against peptide-pulsed and native $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cells (see Fig. 1) that was identical to the reactivity pattern of the CTL bulk culture. No lysis of the $gp100^{neg}$/HLA-$A2^{pos}$ melanoma cell line BLM was observed, whereas BLM cells pulsed with the gp100 wild type (wt) peptide or transfected with human gp100-encoding cDNA (BLMgp100) were efficiently lysed by CTL-MPD (Fig. 1). In addition, the $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cell lines MEL624 and FM3 were also lysed by this CTL clone (Fig. 1). PCR-based typing of the TCRα/β genes of CTL-MPD and sequence analysis of cDNA clones revealed that the TCRα/β chains of CTL-MPD comprised the segments AV29s1/JA49 and BV8s2/D1s1/JB2s1 (see Fig. 2). In fact, sequence analysis of 17 different CTL clones showed that they all express the same TCRβ chain and are therefore derived from one parental CTL (data not shown).

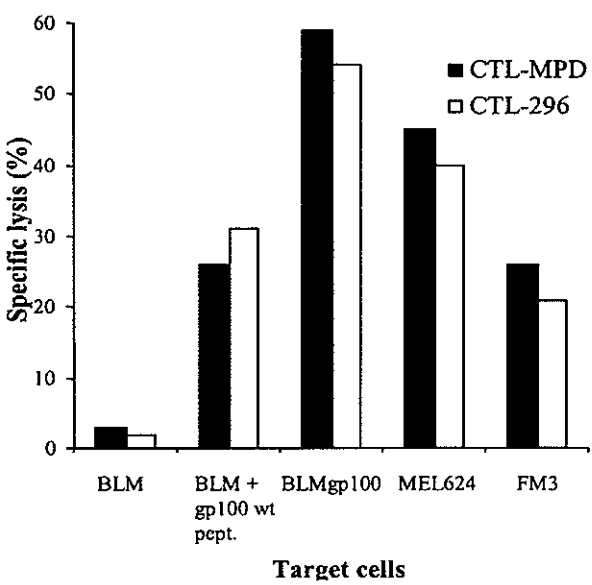

Figure 1. CTL-MPD and CTL-296 lyse melanoma target cells positive for the human $gp100_{280-288}$ epitope presented in the context of HLA-A2.

CTL-MPD (black bar) and CTL-296 (white bar) were tested in a standard 5h ^{51}Cr-release assay as described in the Materials and Methods section. Target cells were: the $gp100^{neg}$/HLA-$A2^{pos}$ melanoma cell line BLM, with or without exogenous gp100 wt peptide (pre-incubation with peptide for 45 min at 37°C at a final concentration of 1 μM), BLM cells transfected with the $hgp100_{280-288}$ cDNA (BLMgp100), and the $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cell lines MEL 642 and FM3. The effector to target cell ratio was 10:1.

Interestingly, homology searches revealed the existence of an almost identical TCRβ chain of another HLA-A2 restricted anti-gp100$_{280-288}$ CTL clone, i.e., CTL-296/147, which was generated by stimulating PBL from an HLA-A2^{pos} melanoma patient with autologous gp100^{pos} tumor cells [24]. The overall cytotoxic response of this CTL appeared to be identical to that of CTL-MPD (Fig. 1). Sequence analysis performed on cDNA clones from this melanoma patient-derived CTL-296 showed that its TCRα/β consisted of the segments AV8s1/JAJ and BV14s1/D1s1/JB2s7 (Fig. 2). Although both CTL-MPD and CTL-296 clearly use different Vβ and Jβ gene segments, the TCRβ complementarity determining (CDR)3 regions of both clones are strikingly homologous at the amino acid level, and in fact identical in size (Fig. 2). The resemblance between the TCRα CDR3 regions of both CTL clones is less pronounced.

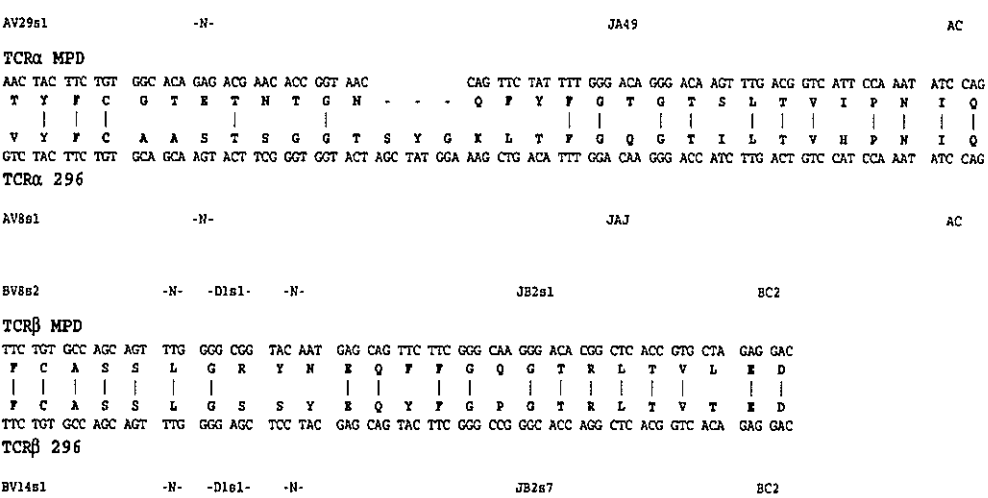

Figure 2. Alignment of CDR3 regions of the TCRα as well as β chain of CTL-MPD and CTL-296.
Nucleotide and amino acid sequence alignment of the CDR3 regions of the TCRα as well as β chains of both CTL-MPD and CTL-296. Identical amino acids are indicated by a vertical line. CDR3 regions were defined according to Chothia *et al.* EMBO J. 7:3745 (1988), and TCR variable segments were designated according to Arden *et al.* Immunogenetics 42:455 (1995).

CTL-MPD and CTL-296 differ in their gp100 peptide fine-specificity

To determine the cytolytic efficiencies of CTL-MPD and CTL-296, T2 target cells were pulsed with various amounts of the gp100 wt peptide and specific lysis was measured in a ^{51}Cr-release assay. From figure 3A it is clear that the amount of gp100 wt peptide

required to obtain 50% of the maximum lysis of peptide-pulsed T2 cells (ED_{50}) was similar for both CTL-MPD and CTL-296 (ED_{50}=100 and 50 pM peptide, respectively). A control HLA-A2-binding peptide was not recognized by both CTL, even at higher concentrations (data not shown). These findings show that both CTL display similar cytolytic efficiencies towards gp100 wt peptide-loaded target cells.

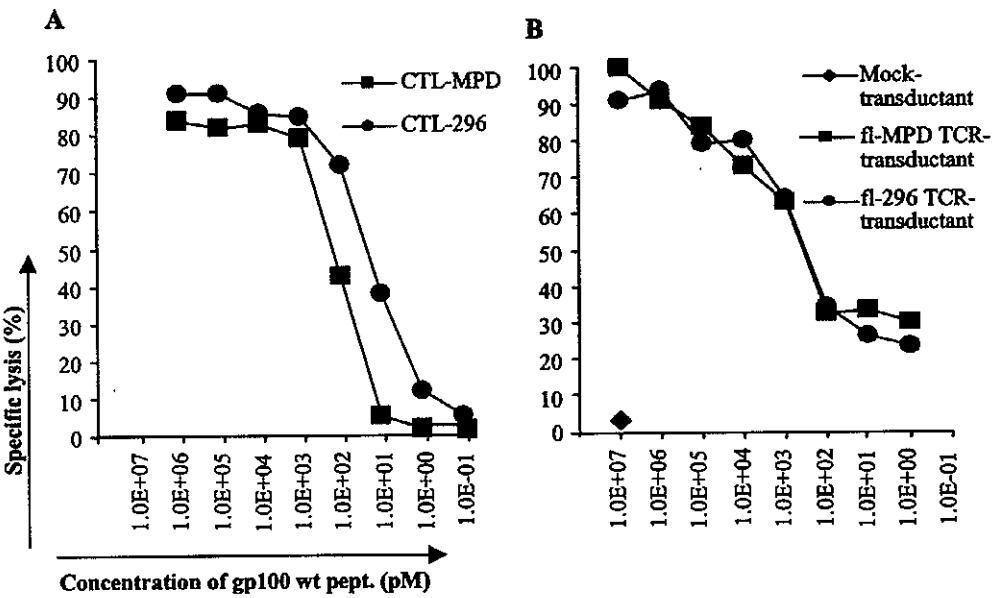

Figure 3. Cytolytic efficiencies of CTL-MPD and CTL-296 are preserved following transfer of engineered TCRα/β genes into primary human T lymphocytes.

A, CTL-MPD and CTL-296 show a similar efficiency in gp100 wt peptide-induced cytotoxicity. CTL-MPD (squares) and CTL-296 (circles) were tested in a 5 h ^{51}Cr-release assay using T2 cells, pre-incubated with various amounts of gp100 wt peptide for 45 min at 37°C, as target cells. The effector to target cell ratio was 10:1. The peptide concentration corresponding to 50% of the maximum lysis (ED_{50}), used as a measure of cytolytic efficiency, was 100 and 50 pM for the CTL-MPD and CTL-296, respectively. B, Cytolytic efficiencies of fl-MPD and fl-296 TCR^{tm} human T lymphocytes are in the same range as the cytolytic efficiencies of the parental CTL clones. Human T cells transduced with the fl-MPD and fl-296 TCR genes (squares and circles, respectively) and mock-transduced human T lymphocytes (diamond) were tested in a 6 h ^{51}Cr-release assay. T2 cells, pre-incubated with various amounts of gp100 wt peptide, were used as target cells. The effector to target cell ratio was 15:1. Cold K562 target cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. The peptide concentration corresponding to the ED_{50} of both fl-MPD TCR and fl-296 TCR-transductants was 300 pM. The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. A second donor provided similar data.

Next, we studied the importance of each individual amino acid residue in the $gp100_{280-288}$ peptide with respect to TCR:peptide/MHC interactions. A series of peptide analogues was synthesized, in which the native aa of the gp100 wt peptide, i.e., YLEPGPVTA, were substituted for an alanine or glycine. The peptides were first assayed for their binding to HLA-A2 via both an indirect binding assay, using the antigen processing defective cell line T2 [29], and a peptide binding competition assay using JY cells [30]. Both assays showed that only amino acid substitutions at positions 2 and 9 have a drastic effect on the peptide binding to HLA-A2 molecules (Table I), consistent with the notion that the aa at positions 2 and 9 are the known anchor residues involved in HLA-A2 binding [35]. Subsequently, the

Table I: HLA-A2 binding capacity of gp100 peptide mutants.

Peptide	**Stabilization assay w/ T2 cells[a] 50μM**		**Competition assay w/ JY cells[b] 25μM**
WMAFKERKV[c]	0.00	0.00	
FLPSDFFPSV[d]	1.76	1.34	0.6
YLEPGPVTA[e]	1.25	1.30	9.6
A1	0.87	0.73	53.3
A2	0.00	0.00	41000.0
A3	1.29	1.54	2.3
A4	1.36	0.82	3.9
A5	1.56	1.35	9.3
A6	0.98	1.11	14.6
A7	1.30	1.51	11.5
A8	1.61	1.70	6.5
G9	0.13	0.09	3367.3

[a] HLA-A2 stabilization by peptides was analyzed by incubating the processing defective cell line T2 with the indicated peptide concentrations. Values indicate Fluorescence Index: (experimental mean fluorescence/background mean fluorescence)-1. See Materials and Methods section for details.

[b] Binding capacity of peptides was determined using the competition-based assay with JY cells. The binding capacity of a peptide is expressed as the concentration required to inhibit 50% of binding of a FL-labeled reference peptide (IC_{50} in μM). See Materials and Methods section for details.

[c] WMAFKERKV is a non-HLA-A2 binding peptide.

[d] FLPSDFFPSV is used as an HLA-A2-binding reference peptide in the competition assay.

[e] YLEPGPVTA is the $gp100_{280-288}$ wt peptide. Gp100 peptide mutants are encoded as described in the legend to figure 4A.

cytotoxic CTL responses towards T2 cells loaded with the various gp100 peptide mutants were assayed to determine the functional peptide requirements of the TCRα/β of both CTL-MPD and CTL-296. It is apparent from Figure 4A that gp100 peptide mutants with substitutions at the anchor positions still initiated a cytotoxic response by both CTL clones,

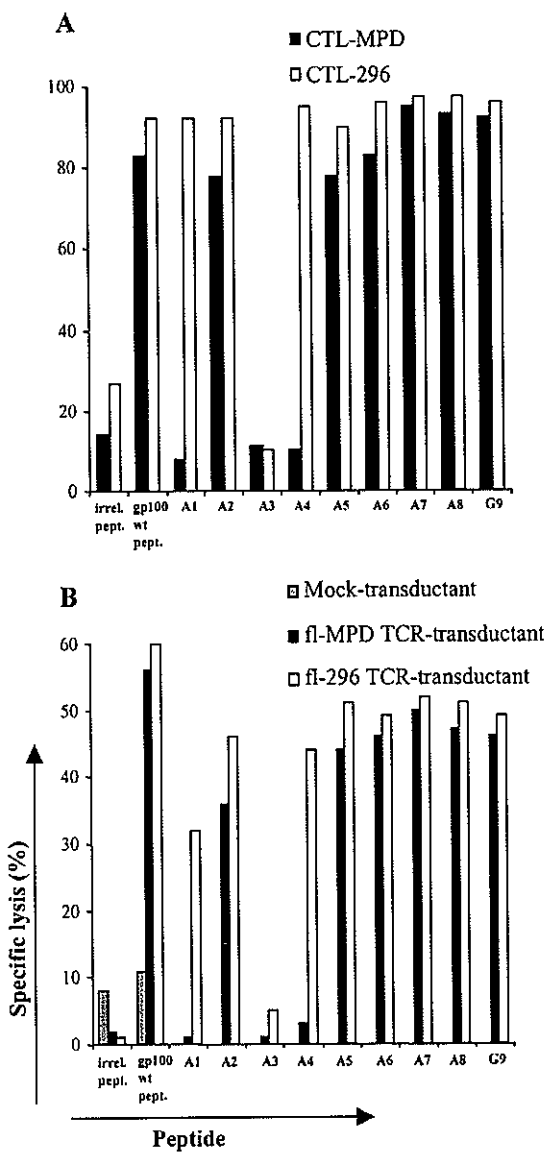

Figure 4. Differences in peptide fine-specificities between CTL-MPD and CTL-296 are preserved following transfer of engineered TCRα/β genes into primary human T lymphocytes.

A, CTL-MPD and CTL-296 differ in their gp100 peptide fine-specificity. CTL-MPD (black bar) and CTL-296 (white bar) were tested in a 5 h ^{51}Cr-release assay. T2 cells were pre-incubated with gp100 peptide mutants (at 1 μM final, see Materials and Methods section for details), and used as target cells. As a negative control an irrelevant HLA-A2-binding $gp100_{154-162}$-peptide (irrel. pept.), and as a positive control the gp100 wt peptide ($gp100_{280-288}$ pept.) were used. Gp100 peptide mutants are encoded as follows: the letter indicates an alanine (A) or glycine (G) substitution, and the number corresponds to the aa position of the substituted amino acid relative to wt peptide. The effector to target cell ratio was 10:1. B, fl-MPD and fl-296 TCR-transduced human T lymphocytes show gp100 peptide fine-specificities identical to those of the parental CTL clones. Human T cells transduced with the fl-MPD and fl-296 TCR genes (black and white bars, respectively) and mock-transduced human T lymphocytes (grey bars) were tested in a 6 h ^{51}Cr-release assay using T2 cells, pulsed with gp100 peptide mutants, as target cells. The effector to target cell ratio was 15:1. Cold K562 cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. A second donor provided similar data.

despite their poor binding to HLA-A2. This is in line with the observation that only a few peptide/MHC complexes on target cells are sufficient to trigger CTL responses [36]. However, the gp100 peptide with an alanine substitution at position 3 did not trigger lysis by either CTL clone (see Fig. 4A), indicating that the glutamic acid at position 3 of the gp100 peptide is crucial for TCR recognition. Furthermore, no substitutions were allowed at positions 1 and 4 of the gp100 peptide for peptide-mediated lysis by CTL-MPD. In contrast, peptide analogues with substitutions at positions 1 and 4 were still capable of sensitizing T2 cells for lysis by CTL-296 (Fig. 4A). Peptide titration experiments confirmed that the alanine 1 and 4 mutants were as efficient as the gp100 wt peptide with respect to peptide-induced cytolysis by CTL-296 but not CTL-MPD (data not shown). Taken together, the CTL-MPD displays a peptide requirement that is more stringent than that of CTL-296.

TCR gene transfer to primary human T lymphocytes

The existence of two gp100-specific CTL clones with distinct gp100 peptide requirements allowed us to investigate the preservation of peptide fine-specificity of native TCRs following TCR gene transfer to human T lymphocytes. To this end, we isolated full length (fl) TCRα/β cDNAs from both CTL, engineered them for retroviral expression using the vector pBullet [21] and the packaging cell line Phoenix, and transferred them into primary human T lymphocytes. It is important to note that the employed retroviral transduction protocol (described in detail in [32]) allows for high transduction efficiencies of primary human T lymphocytes that can then be directly assayed in functional tests and does not necessitate subsequent enrichment or cloning of TCR-transduced T lymphocytes.

Approximately 50% of the human T lymphocytes stably expressed the fl-MPD and fl-296 TCR transgenes, as determined by flow cytometry using anti-TCRVβ8 and anti-TCRVβ14 mAbs, respectively (Fig. 5A). Primary human T cell transductants were checked weekly for expression of the introduced TCR chains by flow cytometry, and TCR expression remained stable for a period of at least 3 months in continuous culture. Expression of CD8 was always above 50% (data not shown). In addition, the TCR-transduced human T lymphocytes specifically bound $gp100_{280\text{-}288}$/HLA-A2 tetramers (Fig. 5B), whereas the binding of an irrelevant EBV/HLA-A2 tetramers was negligible. Interestingly, when analyzing the TCRβ and gp100/HLA-A2 tetramer stainings at the single cell level, we observed that of T cells expressing the introduced TCRβ only 50-60% is able to bind the gp100/HLA-A2 tetramer. Results were confirmed when looking within the CD8-positive fraction of the T cell transductants.

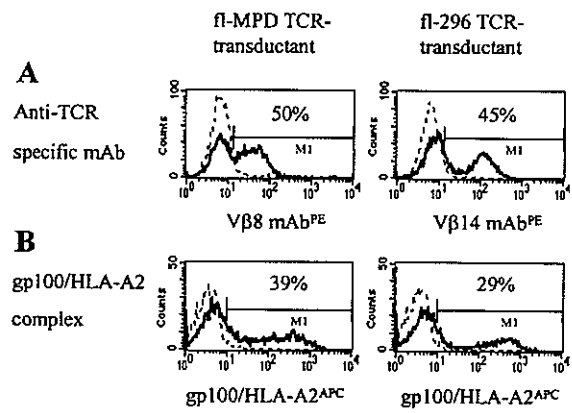

Figure 5. Cell surface expression of gp100-specific fl-MPD and fl-296 TCR on transduced primary human T lymphocytes.
A, Human T lymphocytes retrovirally transduced with genes encoding gp100-specific TCRα/β show cell surface expression of the TCR transgenes. T lymphocytes transduced with the fl-MPD and fl-296 TCRα/β genes specific for gp100 (solid lines) were labeled with the PE-conjugated TCR family-type specific anti-TCRVβ8 and anti-TCRVβ14 mAbs, respectively, and analyzed by flow cytometry. B, T lymphocytes transduced with gp100-specific TCR genes specifically bind gp100 peptide/MHC complexes. The gp100 TCR-transductants described in the legend to Figure A were labeled with APC-conjugated $gp100_{209\text{-}288}$/HLA-A2 tetramers prior to flow cytometric analysis. Mock-transduced human T lymphocytes (dotted lines) served as negative controls in figures A and B. Marker M1 was set in the correspondinghistogram of mock-transduced T cells at a 5% expression level, and percentages given reflect the fraction of stained TCR-transductants relative to M1. The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. Three additional donors provided similar data. The average transduction efficiencies were 49% (range: 27-60%) and 46% (range: 39-54%) for the MPD and 296 receptors, respectively.

Table II: fl-MPD and fl-296 TCR-transduced T lymphocytes produce TNFα after stimulation with $gp100^{pos}$/$HLA\text{-}A2^{pos}$ target cells.

	fl-MPD TCR-transductants	fl-296 TCR-transductants
Target cells		
None	0[a]	2
BLM	8	4
BLMgp100	378	716
FM3	141	329
MEL624	120	223
T2+irrel. pept.	40	14
T2+gp100 wt pept.	365	810

[a] fl-MPD and fl-296 TCR^{pos} human T lymphocytes were co-cultivated for 18 h with BLM, BLMgp100, FM3, MEL624, and T2 cells, pulsed with irrelevant HLA-A2-binding EBV-peptide (irrel. pept.) or gp100 wt peptide (both at 1 μM final), at an effector to target cell ratio of 3:1. Cell-free supernatants were harvested and analyzed in a TNFα ELISA. Levels of TNFα are expressed in pg/ml. Mock-tranduced T lymphocytes did not produce TNFα after stimulation with any target cell. PHA/PMA-stimulated T lymphocytes produced more than 1500 pg/ml TNFα.

As shown in figure 6A, the fl-MPD and fl-296 TCR-transduced human T lymphocytes lysed the gp100 wt peptide-loaded HLA-A2^{pos} B-LCL BSM and the gp100^{pos}/HLA-A2^{pos} melanoma cell lines BLMgp100, FM3 and MEL624 very efficiently, whereas the HLA-A2^{pos} BLM or BSM cells loaded with an irrelevant EBV-peptide were not lysed. Mock-transduced human T lymphocytes did not lyse any of the tumor cell lines. The cytolytic responses of the fl-MPD and fl-296 TCR^{pos} T lymphocytes against the MEL624 melanoma cell line were almost completely inhibited by anti-TCRVβ mAb (CTL-MPD: anti-TCRVβ8 mAb, and CTL-296: anti-TCRVβ14 mAb) and anti-MHC class I mAb (Fig. 6B). Mouse gamma globulin (mIg) as a control Ab did not block the cytolytic responses of either TCR-transductants (Fig. 6B). In addition, the fl-MPD and fl-296 TCR-transduced human T lymphocytes were also specifically triggered to produce TNFα in response to peptide-loaded and native gp100^{pos} tumor target cells (Table II).

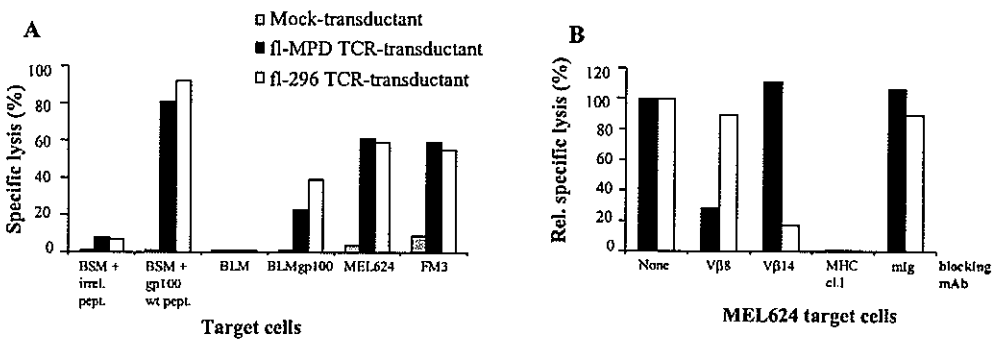

Figure 6. Human T lymphocytes transduced with fl-MPD and fl-296 TCR genes show antigen-specific cytolysis of gp100^{pos}/HLA-A2^{pos} tumor cells.

A, fl-MPD and fl-296 TCR^{pos} human T lymphocytes lyse melanoma cells positive for the human gp100$_{280\text{-}288}$ peptide presented in the context of HLA-A2. Human T lymphocytes transduced with the fl-MPD and fl-296 TCR genes (black and white bars, respectively) and mock-transduced human T lymphocytes (grey bars) were tested in a 6 h ^{51}Cr-release assay. The following target cells were used: the HLA-A2^{pos} B-LCL BSM, pulsed with either gp100 wt peptide or an irrelevant HLA-A2-binding EBV-peptide (both at 10 μM final), BLM, BLMgp100, FM3 and MEL624 (as described in the legend to Fig. 1). The effector to target cell ratio was 15:1. Cold K562 target cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. The results reflect fl-TCR transductions of primary human T lymphocytes of one representative donor out of two. B, The cytotoxic response of fl TCR-transduced human T lymphocytes towards gp100^{pos} melanoma cells is specifically blocked by anti-TCRVβ and MHC class I mAbs. Cytotoxicity assays were performed with fl-MPD and fl-296 TCR^{pos} T lymphocytes as effector cells (black and white bars, respectively) and the gp100^{pos}/HLA-A2^{pos} melanoma cell line MEL624 as target cells under the same conditions as described above but in the presence of anti-TCRVβ8 mAb (at 1 μg/ml final), anti-TCRVβ14 mAb (1 μg/ml), anti-MHC class I mAb (10 μg/ml) or mouse gamma globulin (mIg, at 10 μg/ml). Immune specific lysis was expressed relative to the lysis of MEL624 in the absence of mAbs (set to 100%: corresponding to 41% specific lysis for both the fl-MPD and fl-296 TCR-transductants). The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. A second donor provided similar data.

Next to the antigen-reactivity, we studied the cytolytic efficiencies of the fl-MPD and fl-296 TCR^{pos} human T lymphocytes by measuring specific lysis towards T2 target cells loaded with several dilutions of the gp100 wt peptide. The ED_{50} of gp100 wt peptide is approximately 300 pM for both fl-MPD and fl-296 TCR^{pos} human T lymphocytes (Fig. 3B), which is in the same range as the cytolytic efficiencies of the parental CTL-MPD and CTL-296 (ED_{50} =100 pM and 50 pM, respectively; Fig. 3A). T2 cells loaded with a high concentration of an irrelevant HLA-A2-binding EBV-peptide (10 μM final) were not lysed by the TCR-transduced human T lymphocytes (data not shown), and mock-transduced human T lymphocytes did not lyse T2 cells loaded with gp100 wt peptide (at 10 μM final) (Fig. 3B).
In total, the TCR-transduced human T lymphocytes show specific immune responses to $gp100^{pos}$/$HLA\text{-}A2^{pos}$ target cells, and exhibit similar cytolytic efficiencies as their corresponding parental CTL clones.

Peptide requirement of gp100-specific TCR-transduced primary human T lymphocytes is identical to that of parental CTL

The preservation of not only the cytotoxic potency but also the peptide fine-specificity of CTL by TCR gene transfer is crucial to the potential use of TCR genes for immunotherapy. To address this issue, the peptide fine-specificities of the fl-MPD and fl-296 TCR-transductants were determined in a ^{51}Cr-release assay with T2 cells pulsed with a series of gp100 peptide analogues (as described in legend to Fig. 4A). The gp100 peptide with an alanine substitution at position 3 did not trigger lysis by either TCR-transductant (Fig. 4B). In addition to position 3, no substitutions were allowed at positions 1 and 4 for peptide-mediated lysis by the fl-MPD TCR^{pos} T lymphocytes, whereas these latter peptide analogues were still capable of sensitizing T2 cells for lysis by fl-296 TCR^{pos} T lymphocytes (Fig. 4B). T2 cells loaded with irrelevant HLA-A2-binding EBV-peptide were not lysed by the TCR-transduced human T lymphocytes, and mock-transduced human T lymphocytes did not lyse target cells loaded with gp100 wt peptide (Fig. 4B). We conclude from these experiments that the TCR-transductants show gp100 peptide fine-specificities that are identical to those of the parental CTL (compare Fig. 4A with Fig. 4B).

Discussion

This paper reports on the molecular and functional characterization of two distinct CTL clones each specific for the native melanoma differentiation antigen gp100 presented by HLA-A2, and the preservation of specific antigen reactivity and peptide fine-specificity following transfer of engineered TCR genes into primary human T lymphocytes. The CTL clones described in this paper have different origins, one CTL being derived from a healthy individual using peptide-pulsed DC (i.e., CTL-MPD) and the other derived from a melanoma patient using autologous melanoma cells as APC (i.e., CTL-296), and express TCRα/β chains using different TCRVα and Vβ gene segments. The two CTL clones are both specific for the same $gp100_{280\text{-}288}$ epitope but clearly differ in their response to amino acid substitutions in this gp100 epitope. Full length TCRα/β genes of either CTL clone, when engineered for gene transfer into primary human T lymphocytes, completely preserve the observed gp100-specific antigen reactivity and cytolytic efficiency. Moreover, the characterized peptide fine-specificities of the two native anti-gp100 TCRs were also functionally exhibited by T lymphocytes transduced with the full length TCRs.

The TCR chains of the gp100-specific CTL-MPD and CTL-296 have strictly homologous CDR3β but not CDR3α regions (see Fig. 2), implying the selection of a specific amino acid sequence in the CDR3β region. The same pattern of sequence and size conservation of the CDR3 regions of the TCRα and β chains was observed in a panel of CTL with the same antigen-specificity by Moss and colleagues [37]. These findings fit the idea that the TCRα and TCRβ chains contribute differently to the recognition of the peptide/MHC complex. This notion is further supported by the crystal structure of a TCR bound to a viral peptide/HLA-A2 complex [38]. Although several studies show that conservation of sequence and size of CDR3β of CTL is not an absolute determinant of antigen recognition, TCR CDR3 regions do appear to govern peptide fine-specificity of CTL [39,40].

Analysis of the cytotoxic responses of CTL-MPD and CTL-296 showed that both CTL clones have an identical antigen reactivity towards $gp100^{pos}$/HLA-$A2^{pos}$ melanoma target cells (see Fig. 1). Furthermore, both CTL clones lyse gp100 wt peptide-pulsed target cells with efficiencies in the pM range (see Fig. 3A) suggesting that the TCR of both CTLs bind the gp100 peptide/HLA-A2 complex on target cells with similar affinities. Nevertheless, the specificities of CTL-MPD and CTL-296 for gp100 peptides with amino acid substitu-

tions are strikingly different (see Fig. 4A). The CTL responses towards gp100 peptide mutants indicate that the aa in the gp100 epitope important for TCR:peptide/MHC interaction of either CTL clone overlap but are not identical. Both parental CTL clones are equally sensitive to a substitution of the glutamic acid at amino acid position 3 of the gp100 peptide for alanine, but only CTL-MPD is sensitive to alanine substitutions at amino acid positions 1 and 4. Since the tested amino acid changes do not affect binding of the gp100 peptide to HLA-A2 (see Table I), a peptide mutant may be incorrectly positioned in the MHC molecule and result in inadequate TCR recognition and CTL response.

Our observation that peptide-pulsed DC can induce *bona fide* CTL clones capable of lysing melanoma tumor cells endogenously processing and presenting the tumor antigen *in vitro*, supports a treatment strategy of melanoma patients with DC pulsed with peptides derived from melanoma-associated antigens, which we and others currently employ. The molecular analysis of the TCRα/β chains expressed by gp100 reactive CTLs allows for TCR gene transfer into primary human T lymphocytes, a possible alternative to immunotherapy protocols. The feasibility of transferring full length as well as chimeric two chain and single chain TCR genes to impose an anti-tumor or anti-virus specificity onto various T cells has been reported by us and others [17,18,20-23,41-43]. The present study demonstrates for the first time that TCR gene transfer not only preserves peptide specificity, but even peptide fine-specificity, which is crucial to the use of TCR genes for immunogene therapy. Important to TCR gene transfer studies, but often unaccounted for, is the efficiency which one can obtain to introduce TCR genes into primary human T lymphocytes. We used a retroviral transduction protocol that results in high levels of surface expression of TCR chains on human T lymphocytes (approximately 50% of bulk cultures of transduced T lymphocytes expressed the appropriate TCRβ chain, and 35% of these bulk cultures bound gp100 $^{280\text{-}288}$/HLA-A2 tetramers, see Fig. 5). This is in contrast to previous reports that show only poor retroviral transduction efficiencies of TCR genes, necessitating antibiotic selection, enrichment for CD8 positive T cells or even cloning to obtain a tumor or virus-specific response of retargeted T cells [44-47]. Parameters that greatly affect retroviral transduction efficiencies, such as transduction-mediating agents and type of retroviral vector, have been carefully optimized by our group and others, allowing for gene transfer into primary human T lymphocytes with high efficiency [21,34,48] and at clinical scale meeting the criteria of Good Medical Practice [49].

Retargeted T cells were analyzed functionally for their lytic activity and ability to produce TNFα in response to the same panel of target cells as used to analyze the CTL clones (see Fig. 6A and Table II). The antigen reactivities of the TCR-transductants are identical to those of the parental CTL clones (compare Figs. 1 and 6A), and were confirmed by blocking studies with family-type specific anti-TCR Vβ as well as anti-MHC class I antibodies (see Fig. 6B). Subsequent peptide titration experiments revealed that the cytolytic efficiencies of the T lymphocyte transductants are in the same pM range as those of the parental CTL clones (compare Figs. 3A and 3B). Importantly, primary human T lymphocytes positive for either gp100/HLA-A2 specific TCR show cytolytic activities in response to a large panel of peptide mutants that are identical to those of the parental CTL clones (compare Figs. 4A and 4B). Moreover, production levels of TNFα of the TCR-transductants in response to the gp100 peptide mutants reflect the peptide fine-specificity observed in the cytotoxicity assays (not shown). The observation that TCRs engineered for gene transfer and introduced into primary human T lymphocytes completely preserve the peptide fine-specificity of the native TCRs implies that the exogenous TCR genes, in particular their CDR3 regions, do properly fold when expressed on T cell membranes.

The preservation of the peptide fine-specificity of parental CTL by transfer of full length TCR genes makes TCR-transductants promising tools for immunogene therapy. However, an important issue that needs to be addressed regarding the clinical use of such TCR gene-transduced T lymphocytes is the possible formation of new, potentially autoimmune TCRα/β heterodimers comprising exogenous and endogenous TCR chains. Flow cytometry at the single cell level shows that following transfer of both TCRα and β genes only a fraction (i.e., 50-60%) of CD8-positive T cells expressing the introduced TCRβ chain are able to bind the gp100/HLA-A2 tetramer. These findings agree with the observation by Stanislawski and colleagues that upon transfer of MDM-2-specific TCRα/β genes into primary human T cells only 30-50% of T cells positive for the introduced TCRβ was able to bind the MDM-2-specific tetramer [50], and suggest that the introduced and surface-expressed TCRβ chains do pair with endogenous TCRβ chains. The transfer of chimeric TCR genes linked to genes encoding signal transduction molecules, such as CD3ζ, has proven advantageous in this respect since it facilitates pairing between two CD3ζ-containing proteins [21,51], thereby maximizing the expression of the introduced TCR and reducing the risk that alternative TCR complexes are formed. Another advantage of tumor-specific receptors chimerized to CD3ζ or other signaling

molecules is that they bypass TCR-mediated proximal signaling events [52], which are often defective in cancer patients [53,54]. Recently we have made chimeric genes comprising the Vα and Vβ domains of the fl-MPD and fl-296 TCR genes coupled to various signaling molecules, such as CD3ζ and Fc(ε)RIγ, which are currently tested for their efficiency in T cell retargeting.

Taken together, in this report gp100-specific TCR, derived from two different CTL, have been molecularly analyzed and functionally introduced into primary human T lymphocytes without loss of the antigen reactivity and peptide fine-specificity. These results confirm the notion that TCR gene transfer holds promise for immunogene therapy.

Acknowledgements

We thank Drs. H. Dolstra, P. van der Elsen and T. Logtenberg for providing PCR primers, J.W. Drijfhout for peptide synthesis, H. Spits for providing $gp100_{280-288}$/HLA-A2 tetramers, H. Zarour and P. van der Bruggen for the CTL-296 clone, and M.W.J. Schreurs, J.L.M. Vissers and A.B.H. Bakker for fruitful discussions.

References

1. Rosenberg,S.A. "Progress in human tumour immunology and immunotherapy." (2001), *Nature*, **411**:380-384
2. Renkvist,N.; Castelli,C.; Robbins,P.F.; and Parmiani,G. "A listing of human tumor antigens recognized by T cells." (2001), *Cancer Immunol. Immunother.*, **50**:3-15
3. Kawakami,Y.; Eliyahu,S.; Delgado,C.H.; Robbins,P.F.; Sakaguchi,K.; Appella,E.; Yannelli,J.R.; Adema,G.J.; Miki,T.; and Rosenberg,S.A. "Identification of a human melanoma antigen recognized by tumor- infiltrating lymphocytes associated with in vivo tumor rejection." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:6458-6462
4. Cox,A.L.; Skipper,J.; Chen,Y.; Henderson,R.A.; Darrow,T.L.; Shabanowitz,J.; Engelhard,V.H.; Hunt,D.F.; and Slingluff,C.L., Jr. "Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines." (1994), *Science*, **264**:716-719
5. Bakker,A.B.; Marland,G.; de Boer,A.J.; Huijbens,R.J.; Danen,E.H.; Adema,G.J.; and Figdor,C.G. "Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro." (1995), *Cancer Res.*, **55**:5330-5334
6. Tsai,V.; Southwood,S.; Sidney,J.; Sakaguchi,K.; Kawakami,Y.; Appella,E.; Sette,A.; and Celis,E. "Identification of subdominant CTL epitopes of the GP100 melanoma- associated tumor antigen by primary in vitro immunization with peptide- pulsed dendritic cells." (1997), *J. Immunol.*, **158**:1796-1802

7. Freudenthal,P.S. and Steinman,R.M. "The distinct surface of human blood dendritic cells, as observed after an improved isolation method." (1990), *Proc. Natl. Acad. Sci. U. S. A*, **87**:7698-7702
8. Larsen,C.P.; Ritchie,S.C.; Hendrix,R.; Linsley,P.S.; Hathcock,K.S.; Hodes,R.J.; Lowry,R.P.; and Pearson,T.C. "Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells." (1994), *J. Immunol.*, **152**:5208-5219
9. Flechner,E.R.; Freudenthal,P.S.; Kaplan,G.; and Steinman,R.M. "Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed-leukocyte reaction." (1988), *Cell Immunol.*, **111**:183-195
10. Collins,R.H., Jr.; Shpilberg,O.; Drobyski,W.R.; Porter,D.L.; Giralt,S.; Champlin,R.; Goodman,S.A.; Wolff,S.N.; Hu,W.; Verfaillie,C.; List,A.; Dalton,W.; Ognoskie,N.; Chetrit,A.; Antin,J.H.; and Nemunaitis,J. "Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation." (1997), *J. Clin. Oncol.*, **15**:433-444
11. Heslop,H.E. and Rooney,C.M. "Adoptive cellular immunotherapy for EBV lymphoproliferative disease." (1997), *Immunol. Rev.*, **157**:217-222
12. Rosenberg,S.A. "A new era of cancer immunotherapy: converting theory to performance." (1999), *CA Cancer J. Clin.*, **49**:70-3, 65
13. Hanson,H.L.; Donermeyer,D.L.; Ikeda,H.; White,J.M.; Shankaran,V.; Old,L.J.; Shiku,H.; Schreiber,R.D.; and Allen,P.M. "Eradication of established tumors by CD8+ T cell adoptive immunotherapy." (2000), *Immunity.*, **13**:265-276
14. Theobald,M.; Biggs,J.; Hernandez,J.; Lustgarten,J.; Labadie,C.; and Sherman,L.A. "Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes." (1997), *J. Exp. Med.*, **185**:833-841
15. Bolhuis,R.L. and Gratama,J.W. "Genetic re-targeting of T lymphocyte specificity." (1998), *Gene Ther.*, **5**:1153-1155
16. Bolhuis,R.L.; Willemsen,R.A.; and Gratama,J.W. "Clinical applications of redirected cytotoxicity." in Cytotoxic Cells (2000), Sitkovsky,M.V.; Henkart,P.A., eds., **30**: 423-440
17. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
18. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
19. Fujio,K.; Misaki,Y.; Setoguchi,K.; Morita,S.; Kawahata,K.; Kato,I.; Nosaka,T.; Yamamoto,K.; and Kitamura,T. "Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex." (2000), *J. Immunol.*, **165**:528-532
20. Orentas,R.J.; Roskopf,S.J.; Nolan,G.P.; and Nishimura,M.I. "Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide." (2001), *Clin. Immunol.*, **98**:220-228
21. Willemsen,R.A.; Weijtens,M.E.; Ronteltap,C.; Eshhar,Z.; Gratama,J.W.; Chames,P.; and Bolhuis,R.L. "Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR." (2000), *Gene Ther.*, **7**:1369-1377
22. Stanislawski,T.; Voss,R.H.; Lotz,C.; Sadovnikova,E.; Willemsen,R.A.; Kuball,J.; Ruppert,T.; Bolhuis,R.L.; Melief,C.J.; Huber,C.; Stauss,H.J.; and Theobald,M. "Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer." (2001), *Nat. Immunol.*, **2**:962-970

23. Kessels,H.W.; Wolkers,M.C.; van den Boom,M.D.; van der Valk,M.A.; and Schumacher,T.N. "Immunotherapy through TCR gene transfer." (2001), *Nat. Immunol.*, **2**:957-961
24. Zarour,H.; De Smet,C.; Lehmann,F.; Marchand,M.; Lethe,B.; Romero,P.; Boon,T.; and Renauld,J.C. "The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene Pmel17/gp100." (1996), *J. Invest Dermatol.*, **107**:63-67
25. van de Griend,R.J.; Van Krimpen,B.A.; Bol,S.J.; Thompson,A.; and Bolhuis,R.L. "Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells." (1984), *J. Immunol. Methods*, **66**:285-298
26. Bakker,A.B.; Schreurs,M.W.; de Boer,A.J.; Kawakami,Y.; Rosenberg,S.A.; Adema,G.J.; and Figdor,C.G. "Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes." (1994), *J. Exp. Med.*, **179**:1005-1009
27. Kawakami,Y.; Zakut,R.; Topalian,S.L.; Stotter,H.; and Rosenberg,S.A. "Shared human melanoma antigens. Recognition by tumor-infiltrating lymphocytes in HLA-A2.1-transfected melanomas." (1992), *J. Immunol.*, **148**:638-643
28. Bakker,A.B.; van der Burg,S.H.; Huijbens,R.J.; Drijfhout,J.W.; Melief,C.J.; Adema,G.J.; and Figdor,C.G. "Analogues of CTL epitopes with improved MHC class-I binding capacity elicit anti-melanoma CTL recognizing the wild-type epitope." (1997), *Int. J. Cancer*, **70**:302-309
29. Nijman,H.W.; Houbiers,J.G.; Vierboom,M.P.; van der Burg,S.H.; Drijfhout,J.W.; D'Amaro,J.; Kenemans,P.; Melief,C.J.; and Kast,W.M. "Identification of peptide sequences that potentially trigger HLA-A2.1- restricted cytotoxic T lymphocytes." (1993), *Eur. J. Immunol.*, **23**:1215-1219
30. van der Burg,S.H.; Ras,E.; Drijfhout,J.W.; Benckhuijsen,W.E.; Bremers,A.J.; Melief,C.J.; and Kast,W.M. "An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301." (1995), *Hum. Immunol.*, **44**:189-198
31. Lessin,S.R.; Rook,A.H.; and Rovera,G. "Molecular diagnosis of cutaneous T-cell lymphoma: polymerase chain reaction amplification of T-cell antigen receptor beta-chain gene rearrangements." (1991), *J. Invest Dermatol.*, **96**:299-302
32. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
33. Gratama,J.W.; Bruin,R.J.; Lamers,C.H.; Oosterom,R.; Braakman,E.; Stoter,G.; and Bolhuis,R.L. "Activation of the immune system of cancer patients by continuous i.v. recombinant IL-2 (rIL-2) therapy is dependent on dose and schedule of rIL-2." (1993), *Clin. Exp. Immunol.*, **92**:185-193
34. Weijtens,M.E.; Willemsen,R.A.; Hart,E.H.; and Bolhuis,R.L. "A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes." (1998), *Gene Ther.*, **5**:1195-1203
35. Ruppert,J.; Sidney,J.; Celis,E.; Kubo,R.T.; Grey,H.M.; and Sette,A. "Prominent role of secondary anchor residues in peptide binding to HLA- A2.1 molecules." (1993), *Cell*, **74**:929-937
36. Sykulev,Y.; Joo,M.; Vturina,I.; Tsomides,T.J.; and Eisen,H.N. "Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response." (1996), *Immunity.*, **4**:565-571

37. Moss,P.A. and Bell,J.I. "Comparative sequence analysis of the human T cell receptor TCRA and TCRB CDR3 regions." (1996), *Hum. Immunol.*, **48**:32-38
38. Garboczi,D.N.; Ghosh,P.; Utz,U.; Fan,Q.R.; Biddison,W.E.; and Wiley,D.C. "Structure of the complex between human T-cell receptor, viral peptide and HLA-A2." (1996), *Nature*, **384**:134-141
39. Visseren,M.J.; van der Burg,S.H.; Hawes,G.E.; van der Voort,E.I.; van den Elsen,P.J.; and Melief,C.J. "Affinity, specificity and T-cell-receptor diversity of melanoma-specific CTL generated in vitro against a single tyrosinase epitope." (1997), *Int. J. Cancer*, **72**:1122-1128
40. Romero,P.; Pannetier,C.; Herman,J.; Jongeneel,C.V.; Cerottini,J.C.; and Coulie,P.G. "Multiple specificities in the repertoire of a melanoma patient's cytolytic T lymphocytes directed against tumor antigen MAGE-1.A1." (1995), *J. Exp. Med.*, **182**:1019-1028
41. Dembic,Z.; Haas,W.; Weiss,S.; McCubrey,J.; Kiefer,H.; von Boehmer,H.; and Steinmetz,M. "Transfer of specificity by murine alpha and beta T-cell receptor genes." (1986), *Nature*, **320**:232-238
42. Cole,D.J.; Weil,D.P.; Shilyansky,J.; Custer,M.; Kawakami,Y.; Rosenberg,S.A.; and Nishimura,M.I. "Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen." (1995), *Cancer Res.*, **55**:748-752
43. Liu,X.; Peralta,E.A.; Ellenhorn,J.D.; and Diamond,D.J. "Targeting of human p53-overexpressing tumor cells by an HLA A*0201- restricted murine T-cell receptor expressed in Jurkat T cells." (2000), *Cancer Res.*, **60**:693-701
44. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
45. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
46. Fujio,K.; Misaki,Y.; Setoguchi,K.; Morita,S.; Kawahata,K.; Kato,I.; Nosaka,T.; Yamamoto,K.; and Kitamura,T. "Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex." (2000), *J. Immunol.*, **165**:528-532
47. Liu,X.; Peralta,E.A.; Ellenhorn,J.D.; and Diamond,D.J. "Targeting of human p53-overexpressing tumor cells by an HLA A*0201- restricted murine T-cell receptor expressed in Jurkat T cells." (2000), *Cancer Res.*, **60**:693-701
48. Pollok,K.E.; Hanenberg,H.; Noblitt,T.W.; Schroeder,W.L.; Kato,I.; Emanuel,D.; and Williams,D.A. "High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments." (1998), *J. Virol.*, **72**:4882-4892
49. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Clinical protocol for gene transduction and expansion of human T lymphocytes for immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
50. Stanislawski,T.; Voss,R.H.; Lotz,C.; Sadovnikova,E.; Willemsen,R.A.; Kuball,J.; Ruppert,T.; Bolhuis,R.L.; Melief,C.J.; Huber,C.; Stauss,H.J.; and Theobald,M. "Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer." (2001), *Nat. Immunol.*, **2**:962-970
51. Chang,H.C.; Bao,Z.; Yao,Y.; Tse,A.G.; Goyarts,E.C.; Madsen,M.; Kawasaki,E.; Brauer,P.P.; Sacchettini,J.C.; Nathenson,S.G.; et al. "A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:11408-11412

52. Fitzer-Attas,C.J.; Schindler,D.G.; Waks,T.; and Eshhar,Z. "Harnessing Syk family tyrosine kinases as signaling domains for chimeric single chain of the variable domain receptors: optimal design for T cell activation." (1998), *J. Immunol.*, **160**:145-154
53. Nakagomi,H.; Petersson,M.; Magnusson,I.; Juhlin,C.; Matsuda,M.; Mellstedt,H.; Taupin,J.L.; Vivier,E.; Anderson,P.; and Kiessling,R. "Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma." (1993), *Cancer Res.*, **53**:5610-5612
54. Finke,J.H.; Zea,A.H.; Stanley,J.; Longo,D.L.; Mizoguchi,H.; Tubbs,R.R.; Wiltrout,R.H.; O'Shea,J.J.; Kudoh,S.; Klein,E.; et al. "Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma." (1993), *Cancer Res.*, **53**:5613-5616

Chapter 4

An altered gp100 TCR peptide ligand shows a decreased binding to TCR-transduced human T lymphocytes and dissects cytotoxicity from TNFα production and NFAT activation

Niels Schaft, Birgit Lankiewicz, Christiaan Knoop, Ralph Willemsen, Jolanda M. de Vries, Gosse J. Adema, Reno Debets

Submitted

Abstract

Altered peptide ligands provide useful tools to study T cell activation, and may proof beneficial for vaccination purposes. Partial agonist peptides, for instance, selectively stimulate one type of T cell response without affecting another type of response, most likely due to their inability to initiate certain signal cascades. To study anti-gp100 T cell functions in more detail, we analyzed cytotoxicity, TNFα production and NFAT activation mediated by a defined TCRαβ in response to a broad panel of $gp100_{280-288}$ peptide mutants. To this end, we cloned the TCRαβ genes from the gp100/HLA-A2-specific CTL clone 296, and transferred these genes into primary human T cells and Jurkat T cells. We demonstrate that neither gp100-specific cytotoxicity, TNFα production nor NFAT activation are affected by single amino acid (aa) substitutions of the gp100 wt peptide, except for an aa substitution at position 3 (E to A) which was unable to elicit any T cell response. A gp100 double aa mutant (P to S at positions 4 and 6), however, was able to elicit a cytotoxic response but only low TNFα production and no NFAT activation. T cells transduced with the gp100-specific TCR did not bind the E to A mutant complexed to HLA-A2, but were able to bind the P to S double aa mutant complexed to HLA-A2, although to a lesser extent than the parental gp100 peptide/HLA-A2 complexes. Taken together, all single aa mutants of the $gp100_{280-288}$ peptide studied function as gp100 peptide agonists, except for the E to A peptide mutant, which is not bound by the TCR and functions as a non-responsive ligand. The P to S double aa mutant of the gp100 peptide, acting as a partial agonist, showed that a decrease in TCR binding dissects receptor-mediated cytotoxicity from TNFα production and NFAT activation.

Introduction

Cytotoxic T lymphocytes (CTL) mediate efficient anti-tumor and anti-virus immune responses *in vivo*. T cell receptor (TCR) genes derived from such tumor or virus-specific CTL can be transferred to T lymphocytes of patients to improve their immune response against tumor cells or virus-infected cells. In several *in vitro* studies TCRαβ genes were cloned from CTL to retarget primary human T lymphocytes with an antigen specificity either against tumors (i.e., MDM-2 [1], MART-1 [2], MAGE-1 [3], and gp100 [4]) or viruses (i.e., HIV-1 [5] and EBV [6], (and Schaft *et al.*, Epstein-Barr virus-specific retargeting of primary human T lymphocytes following transfer of chimeric TCR genes, submitted). Retargeted T lymphocytes respond specifically towards peptide-loaded target cells or target cells that endogenously express the antigen in the context of the correct HLA-molecule. Potency and antigen-specificity of cytotoxic responses of CTL-clones are preserved following TCR gene transfer into primary human T lymphocytes. More importantly, cytotoxic responses of TCR-transductants towards a panel of gp100 alanine peptide mutants are identical to those of the parental CTL clones [4].

In this paper, we studied the peptide requirements of various TCR-mediated functions such as cytotoxicity, TNFα production and NFAT activation in human T cells in more detail. To this end, we transferred a defined TCR, i.e., a gp100/HLA-A2-specific TCRαβ derived from CTL-clone 296 [4], into human T cells, and designed variants of the $gp100_{280\text{-}288}$ wildtype (wt) peptide to study the effect of individual amino acid (aa) substitutions and a double aa substitution on TCR-mediated responses. Receptor-mediated cytotoxicity and TNFα production of full length 296 TCRαβ-transduced primary human T lymphocytes were qualitatively identical in response to single aa gp100 substitution variants. In addition, the peptide fine-specificity of fl-296 TCRαβ-mediated NFAT activation, as measured via a reporter gene assay in Jurkat T cells stimulated with single aa gp100 variants, paralleled those of fl-296 TCRαβ-mediated immune responses. All single aa gp100 variants including those with mutated anchor residues (i.e., substitutions at position 2 or 9) functioned as gp100 agonists, except the glutamic acid to alanine substitution mutant (i.e., the E to A substitution at position 3: gp100 A3 mutant) which functioned as a null ligand for the 296 TCRαβ. These findings agree with the observed peptide requirement for the cytotoxic responses of the anti-gp100 296 parental CTL-clone [4], and demonstrate that the agonist and null ligand properties of the single aa gp100 peptide variants extend to T cell TNFα production and NFAT activation. The serine substitution peptide variant, containing two serines instead of prolines (i.e., P to S substitutions at positions 4 and 6:

gp100 S4S6 mutant), however, did not induce activation of NFAT and only a low production of TNFα, whereas it was able to induce a cytotoxic response in TCR-transduced primary human T lymphocytes. Dose-response studies confirmed the partial agonistic nature of this gp100 peptide variant. The difference in responses elicited by the gp100 A3 and S4S6 mutants were not due to differences in their binding to HLA-A2 but rather to differences in the binding of peptide/HLA-A2 complexes to anti-gp100 TCR. The gp100 A3 and S4S6 mutants complexed to HLA-A2 showed no or lowered binding to 296 fl-TCRαβ^{pos} human T cells, most likely explaining the null and partial responses of these altered gp100 ligands, respectively. Taken together, we demonstrate that a defined TCRαβ allows the functional analysis of altered peptide ligands in primary human T lymphocytes. Our analyses of altered gp100 peptide ligands revealed that most single aa mutants retain their agonistic properties, whereas other peptide variants clearly affected binding to TCR and functioned as a null ligand or a partial agonist.

Materials and Methods

Cells and cell lines

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm^3; Pharmacia Biotech, Uppsala, Sweden). TCR-transduced primary human T lymphocytes were cultured as described elsewhere [7]. The TAP-deficient TxB cell hybrid T2, and the melanoma cell line FM3 (HLA-A2^{pos}/gp100^{pos}) were maintained in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% Bovine Calf Serum (BCS: Hyclone, Logan, UT), and the antibiotics streptomycin (100 μg/ml) and penicillin (100 U/ml). The HLA-A2^{pos} melanoma cell lines BLM and BLMgp100 were cultured as described previously [8,9]. The Jurkat T cell clone E6.1 was expanded in RPMI 1640 medium supplemented with L-glutamine, 10% BCS, and antibiotics.

Peptides

Peptides used in this study were: the gp100$_{280\text{-}288}$ wild type (wt) peptide YLEPGPVTA, the gp100 peptide mutants (A1 to A8, G9, V9 and S4S6, indicating an alanine, glycine, valine or serine substitution at the indicated amino acid position of gp100$_{280\text{-}288}$ wt peptide), and an irrelevant HLA-A2-binding EBV-peptide (GLCTLVAML). Peptide preparations were synthesized as described [4] and found to be >90% pure as analyzed by analytical HPLC. MHC class I binding of peptides was analyzed via stabilization of HLA-A2 on T2 cells, as well as competition of HLA-A2 peptide-binding using JY-EBV cells, as described previously [10,11].

Transfer of gp100-specific TCRαβ genes to human T cells

CTL clone 296 (CTL-296), specific for the HLA-A2-presented $gp100_{280-288}$ peptide, was generated from PBL of a melanoma patient [12]. TCR genes expressed by this CTL clone were PCR-amplified and cloned into the retroviral vector pBullet, as described previously [4]. Primary human T lymphocytes of healthy donors, pre-activated with anti-CD3 mAbs were transduced with TCR-positive retroviruses produced by the packaging cell line Phoenix. The transduction procedure used was optimized for human T lymphocytes and described by Lamers *et al.* [13]. A similar procedure was used for human Jurkat T cells except that these cells were cotransduced for both TCR and human CD8α.

Cytofluorometric analysis of TCR-transduced T cells

TCR-transduced T cells were analyzed for transgene expression by flow cytometry using either PE-conjugated anti-TCRVβ14 mAb (Beckman-Coulter, Marseille, France) or PE-conjugated gp100/HLA-A2 tetramer complexes (ProImmune Ltd., Oxford, UK). For immuno-staining, $0.1\text{-}0.5x10^6$ transduced T cells were incubated with the mAb on ice for 30 min or tetramer complexes at room temperature for 1 h, washed, fixed (1% PFA) and analyzed on a FACSCalibur (Becton-Dickinson, San Jose, CA). TCR^{pos} T cells were subsequently MACS-enriched according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Gp100/HLA-A2 tetramers comprise the gp100 A3 mutant, the gp100 S4S6 mutant, the gp100 wt peptide or the EBV-derived BMFL-1 peptide, and were freshly prepared by incubating $streptavidin^{PE}$ and the corresponding soluble gp100 peptide/HLA-A2 monomers at a 1:4 molar ratio for 1 h at 4°C prior to immuno-staining.

Cytotoxicity assay

Cytotoxic activity of TCR^{pos} primary human T lymphocytes was assayed in a standard 6 h ^{51}Cr-release assay [14] using the following target cells: $HLA\text{-}A2^{pos}$ T2 cells pulsed with 1 mM of either the gp100 wt peptide, the gp100 peptide mutants or an irrelevant EBV-peptide, or the melanoma cell lines BLM, BLMgp100 or FM3. For peptide-loading, peptides were pre-incubated for 30 min at 37°C/5% CO_2 with target cells. Antigen-specificity was confirmed by the addition of anti-TCRVβ14 mAb or mouse immunoglobulin (mIg; Jackson Immuno Research Laboratories, West Grove, PA) (both at 1 μg/ml final) to the effector cells prior to their cocultivation with gp100-positive melanoma cells. To inhibit NK-activity during the cytotoxicity assay, non-labeled K562 cells (cold targets) were added at the indicated cold : hot target cell ratio.

TNFα production

Receptor-positive primary human T lymphocytes were tested for antigen-specific TNFα production as described [3] using the same peptide-loaded and melanoma target cells as in cytotoxicity assays. Stimulation with PHA (1 μg/ml) and PMA (5 ng/ml) was used as a positive control. Supernatants were harvested and levels of TNFα were measured by standard ELISA (CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions.

NFAT reporter gene assay

NFAT reporter gene assays were performed as described in detail [15]. In short, exponentially growing TCR/CD8-cotransduced Jurkat T cells ($5x10^6$) were transiently transfected by electroporation with the NFAT-luciferase (Stratagene, La Jolla, CA) and β-galactosidase constructs. Twenty hours post-transfection, Jurkat T cells were transferred to round-bottom 96-well plates (Costar, Corning, NY) at $2x10^5$ cells/well and TCR-stimulated for 6 h in RPMI 1640 medium supplemented with 1% BCS at 37°C/5% CO_2, after which cells were lysed and luciferase and β-galactosidase activities were determined. Luciferase activities were normalized on the basis of β-galactosidase activities and expressed relative to a non-stimulated condition (i.e., medium only; set to 1.0). Antibodies used to stimulate TCR-transduced Jurkat T cells comprise anti-TCRVβ8 mAb (Beckman-Coulter), anti-TCRVβ14 mAb and mIg, and were immobilized to round-bottom 96 well plates at 0,1 μg/well. Target cells used to stimulate TCR-transduced Jurkat T cells were as described for the cytotoxicity and TNFα production assays, and were seeded at 10^5 cells per well. FM3 cells were pre-incubated O/N with INFγ (10 ng/ml) and IL-1β (30 ng/ml), and cocultivation of these melanoma cells with TCR-transduced Jurkat T cells was performed in the presence of anti-CD28 mAb (2 μg/ml). As a positive control for NFAT activation, cells were stimulated with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA: Sigma, Zwijndrecht, The Netherlands) and 1 μM iomycin (Calbiochem, La Jolla, CA).

Results

Expression of gp100-specific TCRαβ on transduced T cells

In order to study the gp100 peptide requirements of various TCR-mediated responses, both primary human T lymphocytes and the human Jurkat T cell line E6.1 were retrovirally transduced with full length 296 TCR α and β genes, originating from the gp100/HLA-A2-specific CTL clone 296. Transduction was followed by enrichment of receptor-positive cells to obtain equal expression levels of the introduced TCR in both cell types. Expression levels were 90% (mean

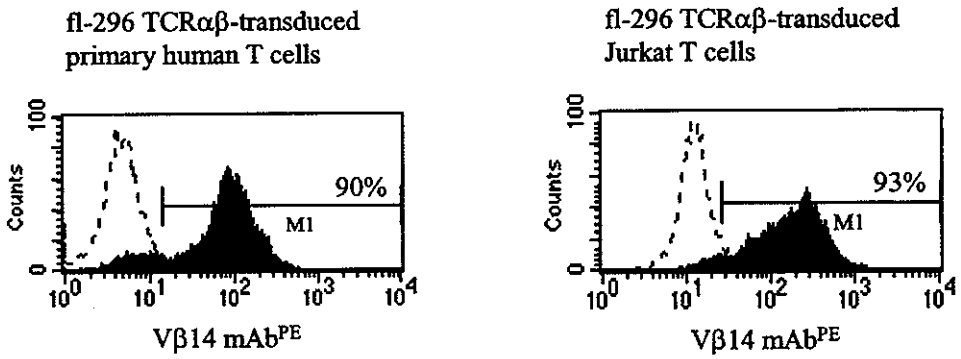

Figure 1. Cell surface expression of gp100-specific fl-296 TCRαβ on transduced T cells.
Primary human T lymphocytes (left panel) and Jurkat T cells (right panel) transduced with the fl-296 TCRαβ genes specific for gp100 (filled histograms) were labeled with the PE-conjugated anti-TCRVβ14 mAbs and analyzed via flow cytometry. Mock-transduced human T lymphocytes served as negative controls (dotted lines). Marker M1 was set in the histogram of mock-transduced T cells at a 5% expression level. The percentage of positively stained TCR-transductants relative to M1 is indicated in the figure. Results of one (out of six) experiment are shown.

fluorescence intensity (MFI)=103) and 93% (MFI=214) on primary human T lymphocytes and Jurkat T cells, respectively, as determined by flow cytometry using anti-TCRVβ14 mAb (see Fig. 1). Jurkat T cells were cotransduced with the human CD8α gene (expression level 100%; MFI=590). CD8 expression on primary human T cells was higher than 50% (data not shown).

Gp100-reactivity of TCR-transduced T cells

Gp100-specific responses of fl-296 TCRαβ^{pos} T cells were assayed at the level of cytotoxicity, cytokine production and NFAT activation. TCR-transduced primary human T lymphocytes were tested for their cytotoxic reactivity and TNFα production in response to native gp100^{pos} melanoma cells. As shown in figure 2A, the fl-296 TCRαβ-transduced human T lymphocytes were able to lyse the gp100^{pos}/HLA-A2^{pos} melanoma cell lines BLMgp100 and FM3, whereas the gp100^{neg}/HLA-A2^{pos} melanoma cell line BLM was not lysed. The gp100 specificity of this response was further confirmed by the use of anti-TCRVβ14 mAb which blocked the cytotoxicity of fl-296 TCRαβ^{pos} T cells (Fig. 2A). In addition, the fl-296 TCRαβ-transduced human T lymphocytes produced TNFα in response to the melanoma cell lines BLMgp100 and FM3 but not in response to the melanoma cell line BLM (Fig. 2B). NFAT activation in response to native gp100^{pos} melanoma cells was tested in TCR-retargeted Jurkat T cells. As shown in figure 2C, NFAT was activated in fl-296 TCRαβ-transduced Jurkat T cells again in response to the mela-

noma cell lines BLMgp100 and FM3, but not in response to the melanoma cell line BLM. Mock-transduced human T lymphocytes showed neither cytotoxic reactivity, TNFα production nor NFAT activation in response to any of the tumor cell lines tested (Fig.2).

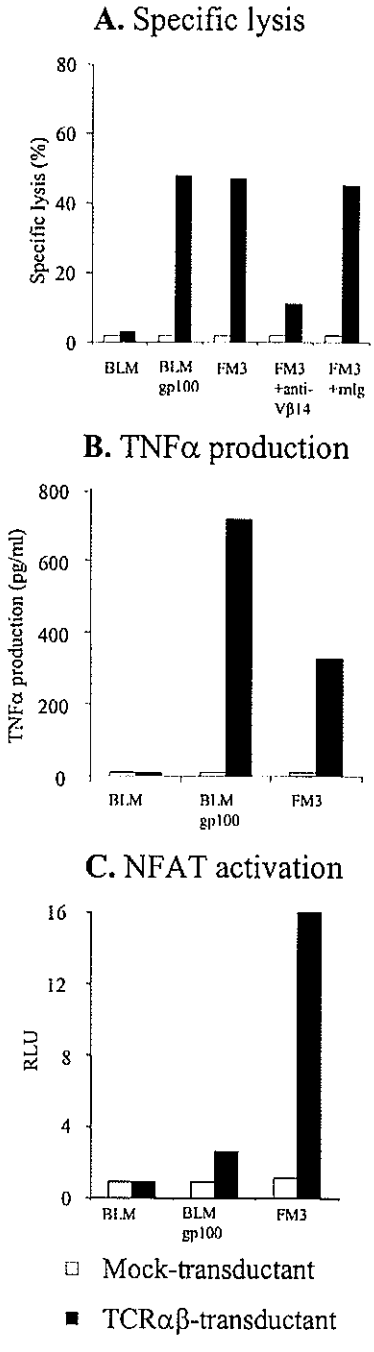

Figure 2. Fl-296 TCR^{pos} primary human T lymphocytes show specific reactivity against gp100^{pos}/HLA-A2^{pos} melanoma cells.
A, Fl-296 TCRαβ-transduced human T cells specifically lyse gp100^{pos}/HLA-A2^{pos} melanoma cells. Primary human T lymphocytes transduced with the fl-296 TCRαβ genes (black bars) and mock-transduced T cells (white bars) were tested in a 6 h ^{51}Cr-release assay with the following target cells: the gp100^{neg}/HLA-A2^{pos} melanoma cell line BLM, BLM cells transfected with the hgp100$_{280-288}$ cDNA (BLMgp100), and the gp100^{pos}/HLA-A2^{pos} melanoma cell line FM3. Inhibition studies were performed with fl-296 TCRαβ^{pos} T lymphocyte effector cells and FM3 target cells using anti-TCRVβ14 mAb or mIg (both at 1 μg/ml final). The effector to target cell ratio was 30:1. Cold K562 target cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. Specific lysis was calculated, and results of one (out of two) representative experiment are shown. B, Fl-296 TCRαβ-transduced human T cells produce TNFα in response to gp100^{pos}/HLA-A2^{pos} melanoma cells. Primary human T cells transduced with the fl-296 TCRαβ genes (black bars) and mock-transduced T cells (white bars) were cocultivated for 18 h with the same target cells as described in the legend to figure 2A. Effector to target cell ratio was 3:1. TNFα production was determined by ELISA, and is expressed in pg/ml. Results of one (out of three) representative experiment are shown. C, Fl-296 TCRαβ-transduced human T cells activate NFAT in response to gp100^{pos}/HLA-A2^{pos} melanoma cells. Jurkat T cells transduced with the fl-296 TCRαβ genes (black bars) and non-transduced T cells (white bars) were transfected with 5 μg of NFAT-reporter construct and subsequently cocultivated for 6 h with the same target cells as described in the legend to figure 2A. Luciferase activities were determined in cell lysates and expressed relative to medium only (RLU (relative light units) of non- and TCR-transduced Jurkat T cells are 0.018 and 0.024, respectively, and are both set to 1.0). FM3 cells were pre-treated with cytokines, and used in combination with anti-CD28 mAb to stimulate TCR-transduced Jurkat T cells (see Materials & Methods section for details). Note: the high endogenous β-galactosidase activities of melanoma cells did not allow for normalization of luciferase activities for introduced β-galactosidase activities. Results of one (out of two) representative experiment are shown.

Single aa substitutions of the gp100 wt peptide do not affect TCR-mediated functions except for an E to A substitution which results in a non responsive peptide ligand

Next to the antigenic-responsiveness of the fl-296 TCRαβ^{pos} T cells, we studied the peptide fine-specificities of immune responses mediated by this receptor in human T cells using single aa substitution variants. First, the peptide requirement of the fl-296 TCRαβ was determined in a cytotoxicity assay with T2 target cells pulsed with gp100 peptide

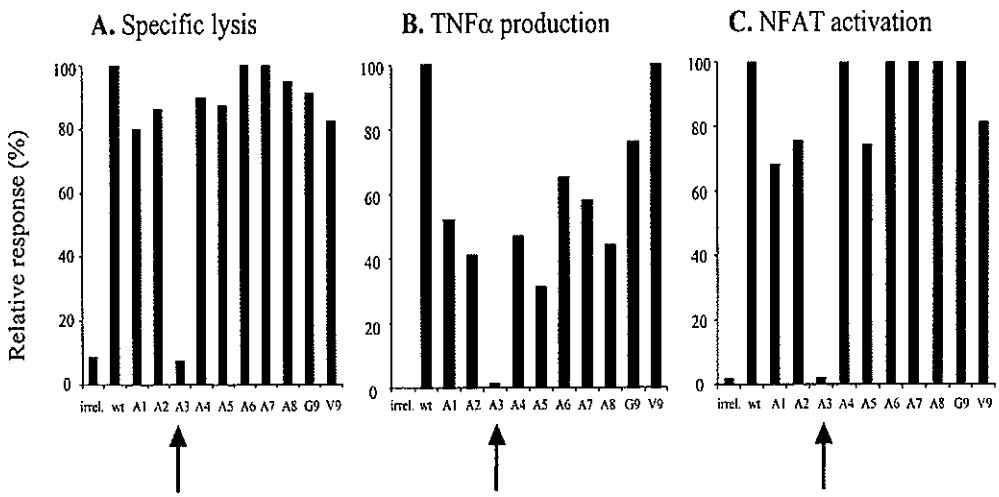

Figure 3. Single aa substitutions of the gp100 wt peptide function as agonists except for the gp100 A3 mutant which functions as a null ligand.

Human T lymphocytes transduced with the fl-296 TCRαβ genes were tested in a ^{51}Cr-release assay (A), a TNFα production assay (B) and a NFAT reporter gene assay (C). Target cells used were T2 cells pre-incubated with 1 μM of the gp100 wt peptide (wt), gp100 peptide mutants or irrelevant EBV-peptide (irrel.). Gp100 peptide mutants are encoded as follows: letter indicates an alanine (A), glycine (G), valine (V), or serine (S) residue and number indicates the amino acid position of the substitution relative to gp100 wt peptide. In a 6 h cytotoxicity assay, the effector to target cell ratio was 15:1, and cold K562 target cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. In a TNFα production assay, the effector to target cell ratio was 3:1. TNFα production was determined after 18 h of cocultivation by ELISA. In a NFAT reporter gene assay, TCR-transduced Jurkat T cells were transfected with 5 μg of both the NFAT reporter and β-galactosidase constructs and subsequently cocultured for 6 h with target cells. Luciferase activities were determined and normalized for β-galactosidase activity. Peptide-induced responses in all three assays are expressed relative to T2 target cells pulsed with gp100 wt peptide (specific lysis of 80%, 1015 pg/ml TNFα and an RLU of 34.73, all set to 100%). Mock-transduced human T cells did not show activity in response to the single aa gp100 peptide mutants. Results of one (out of three) representative experiment are shown. Note the null responses of the gp100 A3 mutant (arrows).

analogues. All single aa gp100 peptide variants tested (n=11) were able to sensitize T2 target cells for TCR-mediated lysis, except for the gp100 peptide mutant with an E to A substitution at position 3 (i.e., gp100 A3 mutant), which does not trigger lysis (Fig. 3A). Second, we analyzed TNFα production by the fl-296 TCRαβ^{pos} T lymphocytes after cocultivation with gp100 peptide-pulsed T2 cells (Fig. 3B). Although the quantity of TNFα produced varied somewhat depending on the peptide used, the TCR-transductants produced TNFα in response to all peptide mutants, except for the gp100 A3 mutant. Third, we analyzed the effect of single aa substitutions on the gp100 peptide induced activation of NFAT in fl-296 TCRαβ^{pos} Jurkat T cells. Again, we observed a clear activation of NFAT irrespective of the gp100 peptide used, except for the gp100 A3 mutant (Fig. 3C). Mock-transduced T cells did not respond in any of the readouts upon stimulation with any of the gp100 peptides tested. T2 cells loaded with irrelevant HLA-A2 binding EBV-peptide induced negligible activity in TCR-transduced T lymphocytes. These observations are in extension to previous data showing that the cytotoxic responses of the parental anti-gp100 CTL versus single aa gp100 peptide variants are preserved following genetic transfer of the fl-TCRαβ chains into primary human T lymphocytes. In this study, we show that cytotoxicity, TNFα production and NFAT activation all show an identical response to single aa substitutions of the gp100 wt peptide, with the E to A mutant being a non-responsive ligand in all three read-outs.

A double P to S substitution variant of the gp100 wt peptide functions as a partial agonist and allows for cytotoxicity, low TNFα production but no NFAT activation

To study the effects of two prolines at positions 4 and 6 in the gp100 wt peptide on TCR-mediated functions, we designed a double P to S substitution variant of the gp100 wt peptide (i.e., gp100 S4S6 mutant). This double aa peptide variant was exposed to the same set of assays as described above. T2 target cells pulsed with 1 μM of the gp100 S4S6 peptide induced a cytotoxic response in fl-296 TCRαβ^{pos} primary human T cells (see Fig. 4A). However, this peptide variant resulted only in low TNFα production and was unable to induce activation of NFAT in human T cells (see Figs. 4B and C). To follow up on these observations, we performed dose-response studies of the gp100 wt and S4S6 peptides using fl-296 TCR-retargeted primary human T cells. In both cytotoxicity and TNFα production assays, the S4S6 peptide variant used at concentrations lower than 1 μM showed a tenfold decrease in its activity relative to the gp100 wt peptide. Dose-response experiments using the NFAT reporter gene assay show that S4S6 is unable to

induce activation of this transcription factor, confirming the partial agonistic nature of this peptide variant. An overview of the T cell functions mediated by the fl-296 TCRαβ in response to all gp100 peptide variants is given in Table I.

TCR-mediated responses against gp100 peptide variants correlate with binding of peptide/MHC ligands

The observed responsiveness of fl-296 TCR^{pos} T cells against gp100 peptide variants may depend on the efficiency of peptide-presentation by HLA-A2 molecules and/or recognition of the peptide/MHC ligands by the fl-296 TCR. To study the efficiency with which gp100 peptide variants are presented by HLA-A2, we performed an HLA-A2 stabilization assay using T2 cells as well as a competition-based binding assay using JY-EBV cells. These assays showed that the binding of all single aa gp100 peptide variants, including the gp100 A3 mutant, are as efficient as the binding of the gp100 wt peptide to HLA-A2 (for instance, stabilization factors on

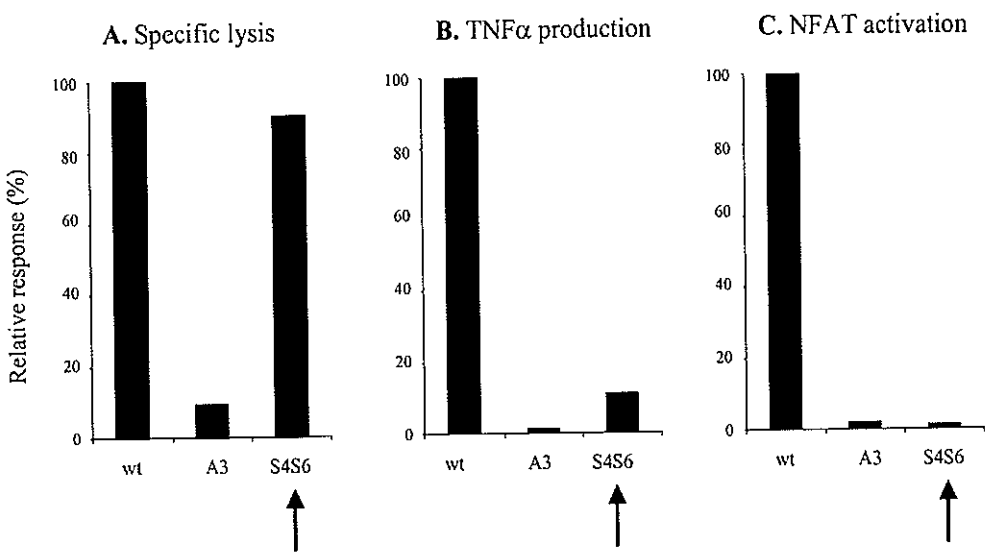

Figure 4. A double P to S substitution variant of the gp100 wt peptide functions as a partial agonist.
Human T lymphocytes transduced with the fl-296 TCRαβ genes were tested in a ^{51}Cr-release assay (A), a TNFα production assay (B) and a NFAT reporter gene assay (C). Target cells used were T2 cells pre-incubated with 1 μM of the gp100 wt peptide (wt), the gp100 A3 mutant (A3) (see Figure 3) or the gp100 S4S6 mutant (S4S6). See legend to figure 3 and the Materials and Methods section for details on performed assays. Peptide-induced responses in all three assays are expressed relative to the gp100 wt peptide response (see legend to figure 3 for absolute responses). Mock-transduced human T cells did not show activity in response to the gp100 peptides. Results of one (out of three) representative experiment are shown. Note the partial response of the gp100 S4S6 mutant (arrows).

Table I. Peptide requirements of fl-296 TCRαβ-meditated cytotoxicity, TNFα production and NFAT activation in human T cells[a]

	Cytotoxicity	TNFα production	NFAT activation
Peptide[b]:			
irrelevant	N[c]	N	N
wt gp100	Y[d]	Y	Y
A1	Y	Y	Y
A2	Y	Y	Y
A3	N	N	N
A4	Y	Y	Y
A5	Y	Y	Y
A6	Y	Y	Y
A7	Y	Y	Y
A8	Y	Y	Y
V9	Y	Y	Y
G9	Y	Y	Y
S4S6	Y	L[e]	N

[a] Cytolytic reactivity and TNFα production of fl-296 TCRαβ^{low} primary human T lymphocytes and NFAT activation in fl-296 TCRαβ^{low} Jurkat T cells in response to peptide-loaded T2 target cells.

[b] T2 target cells were pulsed with the gp100 wt peptide, the panel of gp100 peptide mutants or an irrelevant HLA-A2-binding peptide (see the legend to Fig. 3).

[c] N=No response

[d] Y=Positive response

[e] L=Low response, at least 10x less relative to gp100 wt peptide

T2 cells at 50 μM are 1.29 and 1.25 for gp100 A3 and wt peptide, respectively). The gp100 S4S6 mutant was also able to stabilize HLA-A2 (stabilization factor at 50 μM being 1.14). The only exceptions to our observations that HLA-A2 molecules are able to present gp100 peptide variants, are peptides with a non-conservative mutation in one of the anchoring residues (i.e., positions 2 and 9: gp100 A2 and G9 (but not V9) mutants) which are not bound by HLA-A2 (stabilization factors being 0). The A2 and G9 gp100 peptide mutants clearly induce cytotoxicity, TNFα production and activation of NFAT (see Fig. 3), most likely indicating that only a few peptide-loaded MHC class I molecules are needed to induce T cell activation, and arguing against a dominant role for the efficiency of peptide/MHC presentation in T cell responses.

In a further effort to explain the non- and partial responses of TCR-transduced T cells to the gp100 A3 and S4S6 mutants, respectively, we analyzed the binding of the corresponding peptide/MHC tetramers by TCR-transduced primary human T cells. Flow cytometric analysis using biotinylated monomers of HLA-A2 and gp100 peptide variants complexed with streptavidin^{PE}, revealed that TCR-transduced T cells were able to bind gp100 S4S6 mutant/HLA-A2 complexes, although to a lesser extent than gp100 wt peptide/HLA-A2 complexes (Fig. 5). TCR-transduced T cells did neither bind HLA-A2 complexed to the gp100 A3 mutant nor an irrelevant EBV-derived peptide (Fig. 5). Mock-transduced T cells did not bind any of the mentioned peptide/HLA-A2 multimers. In fact, phenotypical data of the TCR-transduced human T cells show a perfect correlation with the functional data described above.

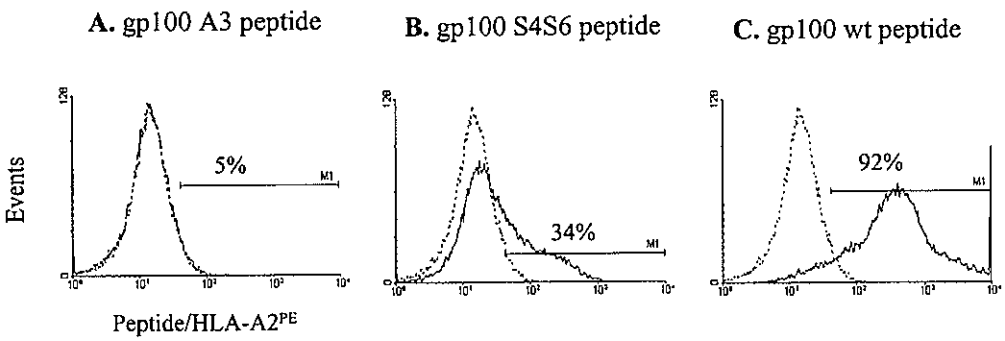

Figure 5. T cell responsiveness towards gp100 peptide variants is in agreement with TCR binding of gp100/HLA-A2 complexes.

Primary human T lymphocytes were transduced with the fl-296 TCRαβ genes, labeled with PE-conjugated complexes of HLA-A2 and gp100 peptide variants, and analyzed via flow cytometry. The gp100/HLA-A2 tetramers used (solid lines) comprise either the gp100 A3 mutant (A), the gp100 S4S6 mutant (B) or the gp100 wt peptide (C), and were made by incubating streptavidin^{PE} and the corresponding soluble gp100 peptide/HLA-A2 monomers at a 1:4 molar ratio for 1 h at 4° C. Tetrameric complexes based on the EBV-derived BMFL-1 peptide served as a negative control (dotted lines). Marker M1 was set at a 5% expression level of T lymphocytes labeled with BMFL-1/HLA-A2 tetramers. The percentages of positively stained TCR-transduced T lymphocytes relative to M1 are indicated in the figure. Results of one (out of two) experiment are shown.

Discussion

In this study, we characterized the gp100 peptide requirements of a defined TCR for various T cell responses in parallel: cytotoxicity, TNFα production and NFAT activation. To this end, the TCRαβ originating from the $gp100_{280-288}$-specific 296 CTL clone (i.e., fl-296 TCR)

was transferred in human T cells and receptor-mediated responses were tested versus a broad panel of gp100 peptide mutants. The single amino acid (aa) mutants of the gp100 peptide studied function as gp100 peptide agonists. The E to A peptide mutant (i.e., gp100 A3) is an exception to this, as it is not bound by the fl-296 TCR and functions as a null ligand. The P to S double aa mutant of the gp100 peptide (i.e., gp100 S4S6) showed a lowered binding to TCR-transduced human T cells and dissected cytotoxicity from TNFα production and NFAT activation.

Expression of the introduced fl-296 TCRαβ and antigen-specific function of this receptor was evident from its ability to mediate lysis, TNFα production and NFAT activation in response to $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cells in human T cells (Figs. 1 and 2) and extended previous findings with fl-296 TCRαβ-transduced T cells [4].

Using the fl-296 TCR, we showed that neither gp100-specific cytotoxicity, TNFα production nor NFAT activation were affected by single aa substitutions of the gp100 wt peptide, except for the gp100 A3 mutant which was unable to elicit any T cell response. Our observations that TCR-mediated cytotoxicity, TNFα production and NFAT activation in human T lymphocytes have identical single aa peptide requirements (Fig. 3) were confirmed for a second anti-gp100 TCR. Primary human T cells retargeted with the fl-TCRαβ derived from the $gp100_{280-288}$-specific MPD CTL clone (i.e., fl-MPD TCR) [16] showed gp100 peptide-specific cytolytic responses which were in complete accordance with TNFα production: all gp100 peptide mutants elicited a response except the variants A1, A3 and A4 ([4]; and data not shown). The single aa gp100 peptide mutant analysis demonstrated that, relative to gp100 wt peptide, all but one peptide mutant act as agonists. The gp100 A3 mutant behaved as a non-responder, a true null ligand in cytotoxicity, TNFα production and NFAT activation assays (Fig. 3 and Table I). The non-responsiveness is not caused by less efficient presentation of the gp100 A3 mutant since the binding capacity of this mutant to HLA-A2 molecules is in the same range as that of the gp100 wt peptide. It is unlikely that the gp100 A3 mutant is a weak or partial agonist, because a ten-fold increase in peptide concentration (i.e., 10 μM final) had no effect on the amount of TNFα produced. In addition, we were unable to show an inhibitory effect of excess gp100 A3 mutant on wt peptide-induced responses in fl-296 TCR-transduced human T cells, suggesting that this peptide variant is not acting as an antagonist (data not shown). The possibility that the gp100 A3 mutant acts as a supra-agonist, i.e. a null ligand that enhances the reactivation of memory CTL responses [17], is currently under investigation.

Studies with the gp100 S4S6 mutant, classified this peptide variant as a partial agonist as it was able to elicit a cytotoxic response but only low production of TNFα and no activation of NFAT

(Fig. 4). HLA-A2 stabilization experiments show that the peptide binding capacity of the gp100 S4S6 mutant is in the same range as the gp100 wt peptide. Moreover, the A2 and G9 peptide mutants, which hardly bind to HLA-A2, clearly induce NFAT activation (Fig. 4).
The induction of cytotoxic activity requires a small amount of antigenic peptide and hardly any TCR downregulation because cytolytic proteins, e.g., perforin and granzymes, are already synthesized, and cytolysis is therefore usually triggered by partial agonists [18-20]. However, induction of *de novo* synthesis of TNFα, which is dependent on NFAT activation, requires a higher peptide concentration and a much stronger TCR signal, i.e., high TCR occupancy and downregulation [19], and may not be triggered by a partial agonist. To confirm that the S4S6 peptide mutant constituted a partial agonist, dose-response studies with fl-296 TCR-retargeted human T cells were performed, showing that the gp100 S4S6 peptide mutant was able to induce cytotoxicity, although to a lesser extent than the gp100 wt peptide, produced low amounts of TNFα and did not activate NFAT. Partially agonistic peptides can selectively stimulate some T cell effector functions, i.e., cytotoxicity or apoptosis, without inducing other functions, i.e., proliferation or cytokine production, by inducing a pattern of signal transduction that is qualitatively different from the pattern induced by any concentration of the native peptide [21-24]. Partial agonistic signaling patterns are characterized by differential phosphorylation of TCR subunits, recruitment but no activation of ZAP-70, activation of MAP kinases (although shortened) and/or phenotypically distinct Ca^{2+} fluxes [25]. A shortened MAP kinase activation and/or weakened Ca^{2+} flux could explain the observed lack of NFAT activation in TCR-transduced T cells following stimulation with the gp100 S4S6 mutant. Translocation of NFAT is reported to take place during 'the TCR and coreceptor microclustering stage' in the formation of an immunological synapse [26]. It would be interesting to find out whether the gp100 S4S6 mutant would allow for TCR engagement but not the subsequent proceeding to TCR microclustering and/or its coalescence into a central synapse, and whether this peptide mutant would allow for co-engagement of TCR and the CD8 coreceptor.
Finally, we showed that TCR-mediated responses against gp100 peptide variants correlate with TCR binding of the corresponding peptide/MHC ligands (see Fig 5). The non-TCR binding of the gp100 A3 mutant and the lowered binding affinity of the gp100 S4S6 mutant relative to the gp100 wt peptide for the fl-296 TCR favors a kinetic model of TCR engagement and/or activation. Taken together, using a defined anti-gp100 TCRαβ this report identified $gp100_{280\text{-}288}$ peptide mutants that act either as a full agonist, a partial agonist or a null ligand, and our analyses with a partial agonist showed that cytotoxicity can be dissected from TNFα-production and NFAT activation.

Acknowledgements

This work was supported by the Dutch Cancer Society. We thank Dr. J.W. Drijfhout for peptide synthesis and Mrs. C. Groot-van Ruijven for her technical assistance.

References

1. Stanislawski,T.; Voss,R.H.; Lotz,C.; Sadovnikova,E.; Willemsen,R.A.; Kuball,J.; Ruppert,T.; Bolhuis,R.L.; Melief,C.J.; Huber,C.; Stauss,H.J.; and Theobald,M. "Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer." (2001), *Nat. Immunol.*, **2**:962-970
2. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
3. Willemsen,R.A.; Weijtens,M.E.; Ronteltap,C.; Eshhar,Z.; Gratama,J.W.; Chames,P.; and Bolhuis,R.L. "Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR." (2000), *Gene Ther.*, **7**:1369-1377
4. Schaft,N.; Willemsen,R.A.; De Vries,J.; Lankiewicz,B.; Essers,B.W.; Gratama,J.W.; Figdor,C.G.; Bolhuis,R.L.; Debets,R.; and Adema,G.J. "Peptide Fine Specificity of Anti-Glycoprotein 100 CTL Is Preserved Following Transfer of Engineered TCRalphabeta Genes Into Primary Human T Lymphocytes." (2003), *J. Immunol.*, **170**:2186-2194
5. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
6. Orentas,R.J.; Roskopf,S.J.; Nolan,G.P.; and Nishimura,M.I. "Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide." (2001), *Clin. Immunol.*, **98**:220-228
7. van de Griend,R.J.; Van Krimpen,B.A.; Bol,S.J.; Thompson,A.; and Bolhuis,R.L. "Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells." (1984), *J. Immunol. Methods*, **66**:285-298
8. Bakker,A.B.; Schreurs,M.W.; de Boer,A.J.; Kawakami,Y.; Rosenberg,S.A.; Adema,G.J.; and Figdor,C.G. "Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes." (1994), *J. Exp. Med.*, **179**:1005-1009
9. Kawakami,Y.; Zakut,R.; Topalian,S.L.; Stotter,H.; and Rosenberg,S.A. "Shared human melanoma antigens. Recognition by tumor-infiltrating lymphocytes in HLA-A2.1-transfected melanomas." (1992), *J. Immunol.*, **148**:638-643
10. Nijman,H.W.; Houbiers,J.G.; Vierboom,M.P.; van der Burg,S.H.; Drijfhout,J.W.; D'Amaro,J.; Kenemans,P.; Melief,C.J.; and Kast,W.M. "Identification of peptide sequences that potentially trigger HLA-A2.1- restricted cytotoxic T lymphocytes." (1993), *Eur. J. Immunol.*, **23**:1215-1219
11. van der Burg,S.H.; Ras,E.; Drijfhout,J.W.; Benckhuijsen,W.E.; Bremers,A.J.; Melief,C.J.; and Kast,W.M. "An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301." (1995), *Hum. Immunol.*, **44**:189-198

12. Zarour,H.; De Smet,C.; Lehmann,F.; Marchand,M.; Lethe,B.; Romero,P.; Boon,T.; and Renauld,J.C. "The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene Pmel17/gp100." (1996), *J. Invest Dermatol.*, **107**:63-67
13. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
14. Lamers,H.J.; Gratama,J.W.; van Putten,W.L.; Stoter,G.; and Bolhuis,R.L. "Exogenous interleukin 2 recruits in vitro lymphokine-activated killer activity by in vivo activated lymphocytes." (1991), *Cancer Res.*, **51**:2324-2328
15. Schaft,N.; Lankiewicz,B.; Gratama,J.W.; Bolhuis,R.L.; and Debets,R. "Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT." (2003), *J. Immunol. Methods, in press*
16. Bakker,A.B.; Marland,G.; de Boer,A.J.; Huijbens,R.J.; Danen,E.H.; Adema,G.J.; and Figdor,C.G. "Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro." (1995), *Cancer Res.*, **55**:5330-5334
17. Micheletti,F.; Canella,A.; Vertuani,S.; Marastoni,M.; Tosi,L.; Volinia,S.; Traniello,S.; and Gavioli,R. "Supra-agonist peptides enhance the reactivation of memory CTL responses." (2000), *J. Immunol.*, **165**:4264-4271
18. Hollsberg,P.; Weber,W.E.; Dangond,F.; Batra,V.; Sette,A.; and Hafler,D.A. "Differential activation of proliferation and cytotoxicity in human T-cell lymphotropic virus type I Tax-specific CD8 T cells by an altered peptide ligand." (1995), *Proc. Natl. Acad. Sci. U. S. A* , **92**:4036-4040
19. Valitutti,S.; Muller,S.; Dessing,M.; and Lanzavecchia,A. "Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy." (1996), *J. Exp. Med.*, **183**:1917-1921
20. Griffiths,G.M. and Mueller,C. "Expression of perforin and granzymes in vivo: potential diagnostic markers for activated cytotoxic cells." (1991), *Immunol. Today*, **12** :415-419
21. Sloan-Lancaster,J. and Allen,P.M. "Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology." (1996), *Annu. Rev. Immunol.*, **14**:1-27
22. Chau,L.A.; Bluestone,J.A.; and Madrenas,J. "Dissociation of intracellular signaling pathways in response to partial agonist ligands of the T cell receptor." (1998), *J. Exp. Med.*, **187**:1699-1709
23. Hemmer,B.; Stefanova,I.; Vergelli,M.; Germain,R.N.; and Martin,R. "Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells." (1998), *J. Immunol.*, **160**:5807-5814
24. Wei,C.H.; Beeson,C.; Masucci,M.G.; and Levitsky,V. "A partially agonistic peptide acts as a selective inducer of apoptosis in CD8+ CTLs." (1999), *J. Immunol.*, **163**:2601-2609
25. Madrenas,J. "Differential signalling by variant ligands of the T cell receptor and the kinetic model of T cell activation." (1999), *Life Sci.*, **64**:717-731
26. Krummel,M.F. and Davis,M.M. "Dynamics of the immunological synapse: finding, establishing and solidifying a connection." (2002), *Curr. Opin. Immunol.*, **14**:66-74

Chapter 5

Epstein-Barr virus-specific retargeting of primary human T lymphocytes following transfer of chimeric TCR genes

Niels Schaft, Birgit Lankiewicz, Denis J. Moss, Victor Levitsky,
Marc Bonneville, Stephen P. Lee, Andrew J. McMichael, Ralph Willemsen,
Jan-Willem Gratama, Reinier L.H. Bolhuis, Reno Debets

Submitted

Abstract

Epstein-Barr virus (EBV) is associated with a broad range of malignancies. Adoptive immunotherapy of these tumors with EBV-specific cytotoxic T lymphocytes (CTL) proved useful. In this study, we generated a panel of human T lymphocytes specific to various EBV tumor rejection antigens via transfer of chimeric T cell receptor (ch-TCR) genes. TCR genes were derived from CTL clones with specifities ranging from EBNA-3A and B to BMLF-1 and were engineered into two or single chain formats coupled to either CD3ζ or Fc(ε)RIγ. This panel of EBV-specific ch-TCR was retrovirally transduced into primary human T cells from 25 donors and resulted in high expression levels of the introduced TCR as determined by binding to peptide/MHC tetramers and anti-TCRVβ mAbs. Twenty to 60% of the EBV-specific ch-TCR-transduced T cell populations specifically lysed EBV-peptide-loaded target cells. This MHC-restricted EBV-peptide specific killing of target cells showed a clear dependence on the ch-TCR format, and hence antigen specificity: with the two chain TCR directed against EBNA-3B being the most effective TCR. Surprisingly, no response towards tumor cells that express native EBV-tumor antigens was seen, in contrast to melanoma-specific ch-TCR^{pos} T cells, which did kill native melanoma cells. The observed non-responsiveness was not due to functional anergy of the transduced T cells. In fact, EBV-specific ch-TCR^{pos} T cells stimulated with anti-TCR mAbs or peptide/MHC complexes but not EBV-positive target cells specifically induced NFAT activation. Taken together, ch-TCR allow for EBV-specific retargeting of T lymphocytes. However, antigen-binding affinity/triggerability of EBV-specific ch-TCR needs to be improved prior to their use for immunogene therapy of EBV-induced disease.

Introduction

EBV is a gamma-1 B-lymphotropic herpesvirus found in all human populations, with a prevalence of over 90% in adults, and it can remain latent and persist for life in its natural hosts. Important to the immune control of EBV are EBV-specific CTL. In healthy individuals EBV-specific CTL recognize MHC class I-restricted latent antigens and prevent viral spreading [1,2]. Only a small subset of at least 11 viral genes is expressed in latently infected B-lymphocytes and recognized by EBV-specific CTL. These latent viral gene products can be divided into three groups: (i) the EBV-encoded RNAs EBER-1 and 2; (ii) the EBV nuclear antigens EBNA-1, 2, 3A, 3B, 3C, and 5; and (iii) the latent membrane proteins LMP-1, 2A and 2B (reviewed in [3,4]). EBV is associated with a broad range of malignancies of mostly hematopoietic or epithelial origin, which can be divided into the following types of latencies based on EBV latent gene expression patterns: type I latency expresses the EBER and EBNA-1 genes; type II latency expresses the EBER, EBNA-1, and the LMP genes; and type III latency expresses the EBER, all EBNA and LMP genes. These types of expression patterns are unique to various forms of (immunosuppression-related) lymphomas, carcinomas, Hodgkin's disease and AIDS [5-11].

Adoptive immunotherapy of EBV^{pos} tumors in allogeneic bone marrow transplant recipients with HLA-restricted, EBV-specific T lymphocytes has proven useful. This immunotherapy, performed with lymphocytes from EBV-seropositive bone marrow donors, is associated with graft-versus-host disease [12,13]. In addition, EBV-specific CTL generated in vitro by stimulation with autologous EBV-transformed B-lymphoblast cell lines were useful in the treatment of relapsed EBV^{pos} Hodgkin's disease [14] and post-transplant lymphoproliferative disease in solid organ transplant patients [15,16]. Although results were promising, they did not include cures. Identification, isolation and expansion of tumor-specific T lymphocytes are laborious and time consuming, and have only been successful in a fraction of patients [17,18].

Alternatively, autologous T cells can be genetically programmed to express MHC-restricted antigen-specific receptors. Primary human T lymphocytes can be retargeted to antigen-expressing cells by transfer of human T cell receptor α/β genes. Successfully targeted HLA class I-restricted antigens ranged from melanoma cancer/testis antigens (i.e., MAGE-1/HLA-A1 [19]) and melanocyte differentiation antigens (i.e., MART-1/HLA-A2 [20] or gp100/HLA-A2 [21] to viral-antigens (i.e., HIV gag/HLA-A3 [22]

or EBV LMP2/HLA-A2 [23]). The retargeted primary human T lymphocytes were shown not only to bind the relevant peptide/MHC ligand but also to produce cytokines and kill target cells upon antigen-specific stimulation.

The therapeutic use of full length TCR genes, however, may face problems. The induction of unknown and possibly dangerous specificities may constitute a theoretical side effect of TCR gene transfer [19,22]. In addition, the introduction of a TCRα transgene into T cells results in a low and unstable surface expression of TCRα protein [24]. Moreover, the formation of new TCRα/β heterodimers comprising both exogenous and endogenous TCR chains dilutes the expression of the desired TCRα/β heterodimers. Indeed, we recently observed that only a fraction of T cells expressing the introduced TCRβ chain, after transfer of TCRα and β genes, is able to bind the corresponding peptide/MHC tetramer [21], suggesting that the introduced and surface expressed TCRβ chain pairs with the endogenous TCRα chain. We have pioneered the use of chimeric two-chain (tc-) and single-chain (sc-) TCR molecules which offers a solution to problems associated with full length TCR gene transfer [19]. The chimeric two-chain TCR-based receptors, that for instance comprise CD3 signaling molecules, e.g., CD3ζ, result in exclusive pairing between the introduced TCRα and β chains, and 'rescue' the surface expression of the introduced TCRα chain.

In this paper we studied whether the chimeric (ch-)TCR approach is applicable to treat EBV-induced tumors. EBV-specific CTL clones which recognize EBNA-3A, EBNA-3B or BMLF-1 epitopes were used to generate single or two chain TCRαβ gene constructs, which were chimerized to either CD3ζ or Fc(ε)RIγ. This panel of EBV-specific ch-TCR was retrovirally transduced into primary human T cells from 25 donors and resulted in high expression levels of the introduced TCR. Twenty to 60% of the transduced T cell populations specifically lysed EBV-peptide-loaded target cells, with the tc-TCR directed against EBNA-3B being the most effective TCR. Surprisingly, EBV-specific ch-TCR^{pos} T lymphocytes showed no response towards tumor cells that express native EBV-tumor antigens, in contrast to melanoma-specific ch-TCR^{pos} T cells, which were able to kill native melanoma target cells. The observed non-responsiveness was not due to donor variation or functional anergy of the transduced T cells. To study receptor functions in more detail, Jurkat T cells were transduced with the EBV-specific ch-TCR constructs and subjected to NFAT reporter gene assays. Specific NFAT activation was seen in EBV-specific ch-TCR^{pos} Jurkat T cells in response to anti-TCR mAbs or peptide/MHC complexes, which induce strong TCR signals. Activation of

NFAT was not observed in response to peptide-loaded or native target cells, again in contrast to melanoma-specific ch-TCR^{pos} Jurkat T cells which did show NFAT activation following stimulation with melanoma target cells.
Taken together, this report shows that several chimeric TCR genes with different formats and EBV antigen specificities have been functionally transferred to primary human T cells. The functional characterization of the ch-TCR suggests that antigen-binding affinity/triggerability of these ch-TCR needs to be increased prior to their use as effective tools in the immunogene therapy of EBV-related tumors.

Materials and Methods

Cells and reagents

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density=1.077 g/cm^3; Pharmacia Biotech, Uppsala, Sweden). Transduced primary human T lymphocytes, the EBV-specific CTL clones BK289 (specific for the HLA-A11-presented EBNA-3B peptide IVTDFSVIK (IVT/A11) [25,26]), A4.5 (specific for the HLA-A2-presented BMLF-1 peptide GLCTLVAML (GLC/A2) [27]) and CF3 (specific for the HLA-B8-presented EBNA-3A peptide FLRGRAYGL (FLR/B8) [28]), and the melanoma-specific CTL clone 82/30 (specific for the HLA-A1-presented melanoma peptide EADPTGHSY (EAD/A1)) were all cultured with RPMI 1640 medium supplemented with 25 mM Hepes, 200 nM L-glutamine, 10% human serum, 360 IU/ml rIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands) and the antibiotics streptomycin (100 µg/ml) and penicillin (100 U/ml), and stimulated every 2 weeks with a mixture of irradiated allogeneic feeder-cells, as described elsewhere [29]. The EBV-transformed B lymphoblast cell lines (B-LCL) BSM (GLC^{neg}/HLA-A2^{pos}), CJO (IVT^{pos}/HLA-A11^{pos}), HAR (FLR^{neg}/HLA-B8^{pos}), APD (EAD^{neg}/HLA-A1^{pos}) (all four target cells kindly provided by Dr. M. Giphart, Leiden, the Netherlands), RL, CFpuy (both FLR^{pos}/HLA-B8^{pos}), BK (IVT^{pos}/HLA-A11^{pos}) and the Jurkat T cell clone E6.1 were cultured with RPMI 1640 medium supplemented with 200 nM L-glutamine, 10% BCS (BCS; Hyclone, Logan, UT) and antibiotics. The human amphotropic packaging cell line Phoenix, the TAP-deficient TxB cell hybrid T2 cells, HLA-A11 transfected T2 cells (T2-A11), and the melanoma cell lines G43 (EAD^{pos}/HLA-A1^{pos}) and FM3 (YLE^{pos}/HLA-A2^{pos}) were grown in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% Bovine Calf Serum and antibiotics. The mAbs used in this study

comprised anti-TCRVβ8 mAb; anti-TCRVβ22 mAb; PE-conjugated anti-TCRVβ22 mAb; PE-conjugated anti-TCRVβ4 mAb (TCR mAbs all from Beckman-Coulter, Marseille, France); mouse immunoglobulin (mIg; Jackson Immuno Research Laboratories, West Grove, PA); and anti-MHC class I mAb (clone W6/32, Sera-Lab, Crawley Down, UK). Other reagents used in this study were: RetroNectin (human fibronectin fragments CH-296; Takara Shuzo Co. Ltd., Otsu, Japan); FLR/HLA-B8 monomers; streptavidin-PE (Becton Dickinson Biosciences, San Jose, CA); GLC-peptide (GLCTLVAML); IVT-peptide (IVTDFSVIK); FLR-peptide (FLRGRAYGL), the gp100 peptide YLEPGPVTA, the MAGE-1 peptide EADPTGHSY (all peptides from Dr. Drijfhout, Leiden, the Netherlands); PMA (Sigma, Zwijndrecht, The Netherlands); and ionomycin (Calbiochem, La Jolla, CA).

Construction of EBV-specific chimeric T-cell receptor genes

EBV-specific chimeric TCR were constructed as follows. TCR α and β DNA was obtained by PCR using template cDNA of the CTL clones BK289 (which expresses the TCR genes Vα1s1b/Jα45; Vβ22s1/Jβ2s1), A4.5 (Vα15s1/Jα23; Vβ4s1/Jβ1s4) and CF3 (Vα2s1/Jα9s14; Vβ8s6/Jβ1s2). Chimeric tc-TCRαβ:ζ specific for EBNA-3B or BMFL-1 were constructed by linking the extracellular domains of the TCR α and β chain to the CD3ζ molecule (i.e., VαCαζ and VβCβζ) (as described in [19]). A chimeric sc-TCRαβ:γ specific for EBNA-3A was constructed by coupling the TCR Vα and VβCβ domains, interspersed by a flexible linker, to a few amino acids of the constant domain of the κ light chain, the transmembrane domain of the human CD4 molecule and the signaling domain of Fc(ε)RIγ (i.e., VαVβCβCκCD4γ) (as described in [30]). As control receptors tc-TCRαβ:ζ specific for the melanoma antigens MAGE-1 [19] or gp100 [31] were used. Specific primer sequences to amplify the TCR and other domains will be given upon request. The chimeric TCR genes were subsequently cloned into the retroviral vector pBullet containing a heterologous signal peptide, as described elsewhere [19].

Retroviral gene transduction of chimeric TCR into human T cells

Primary human T lymphocytes of healthy donors were depleted for cells endogenously expressing TCRVβ22 and TCRVβ4 by using anti-TCRVβ22 and anti-TCRVβ4 mAbs and MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) or magnetic beads (Dynal, Oslo, Norway), and pre-activated with anti-CD3 mAbs prior to retroviral transductions. These primary human T cells (number of donors for ch-TCR specific for EBNA-3B,

BMFL-1 and EBNA-3A were 9, 6 and 10, respectively) as well as Jurkat T cells were transduced with retrovirus, containing the ch-TCR genes, produced by the packaging cell line Phoenix. The transduction procedure used was optimized for human T lymphocytes and described by Lamers *et al.* [32]. In short, 24 well culture plates were coated with RetroNectin and pre-treated with retroviral particles by centrifugation. Next, 10^6 human T cells were centrifuged in fresh retroviral supernatant, and cultured for 4-5 h at 37°C/5% CO_2. T cells were allowed to recover in normal T cell medium O/N prior to a second transduction cycle, after which cells were harvested and transferred to T25 culture flasks. After sufficient numbers were obtained, ch-TCR-transduced T cells were analyzed for receptor expression by flow cytometry.

Flowcytometric analysis of retrovirally transduced T cells

TCR-transduced T cells were analyzed for transgene expression by flow cytometry using PE-conjugated anti-TCR mAb (Vβ22 or Vβ4) or FLR/HLA-B8 tetramer complexes. Soluble FLR/HLA-B8 tetramers were made by incubating streptavidin-PE and biotinylated FLR/HLA-B8 monomers at a 1:4 molar ratio for 1 h at 4° C. For immuno-staining, 0.25-0.5x10^6 transduced T cells were incubated with the mAbs or tetramers on ice for 30 min, washed, fixed (1% paraformaldehyde) and analyzed on a flowcytometer (Becton-Dickinson, San Jose, CA). TCR-transduced T cells were subsequently enriched for ch-TCR expression via magnetic beads or MicroBeads according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxic activity of CTL clones and ch-TCR^{pos} primary human T lymphocytes versus indicated target cells was assayed in a standard 6 h ^{51}Cr-release assay [33]. CTL clones and ch-TCR^{pos} T lymphocytes were cocultivated with either peptide-pulsed target cells or target cells expressing the native antigen of interest. Peptide loading was performed by addition of the peptide (10 μM final) to the target cells prior to incubation with effector T lymphocytes. Specific blocking of cytolytic activity was studied by adding anti-MHC class I mAb or mIg (both at 10 μg/ml final) to the target cells 15 min prior to cocultivation with effector T cells.

NFAT reporter gene assay

NFAT reporter gene assays were performed as described before [31]. In short, exponentially growing ch-TCR-transduced Jurkat T cells ($5x10^6$) were transiently transfected by electroporation with 5 μg of both the NFAT-luciferase (Stratagene, La Jolla, CA) and β-galactosidase constructs. Twenty hours post-transfection, Jurkat T cells were transferred to round-bottom 96-well plates (Costar, Corning, NY) at $2x10^5$ cells/well and were TCR-stimulated for 6 h with anti-TCR mAbs, peptide/MHC complexes or target cells (at 10^5 cells/well) in RPMI 1640 medium supplemented with 1% BCS at 37°C/5% CO_2, after which cells were lysed and luciferase and β-galactosidase activities were determined. Luciferase activities were normalized on the basis of β-galactosidase activities and expressed relative to a non-stimulated condition (i.e., medium only; set to 1.0). Antibodies used to stimulate ch-TCR-transduced Jurkat T cells comprise anti-TCRVβ22 or Vβ8 mAb or mIg. The mAbs were pre-coated in round-bottom 96 well tissue culture treated plates (Costar, Corning, NY) at 0,1 μg/well. For stimulations with FLR/HLA-B8 complexes, biotinylated BSA (at 1 μg/ml final) was pre-coated in 96 well plates (O/N at 4°C), after which wells were PBS-washed, and streptavidin (10 μg/ml) was bound to the biotin for 45 min at room temperature. Plates were washed again and biotinylated FLR/HLA-B8 complexes (at indicated concentrations) were added to wells and incubated for 30 min at room temperature. Finally, for stimulations with target cells, CJO, T2-A11 cells and CFpuy cells were peptide-loaded for 30 min at 37°C/5% CO_2 with 10 μM of the relevant peptide prior to their use in NFAT reporter gene assays. As a positive control for NFAT activation, cells were stimulated with 10 ng/ml PMA and 1 μM ionomycin for 6 h at 37°C/5% CO_2.

Results

Expression of EBV-specific ch-TCR on transduced T cells

Primary human T lymphocytes and the human Jurkat T cell line E6.1 were retrovirally transduced with ch-TCRs originating from various EBV-specific CTL clones. Transduced T cells were enriched for receptor-positive cells to obtain an equal expression level of the introduced ch-TCR on both cell types. Expression levels on primary human T lymphocytes were 88% (mean fluorescence intensity (MFI)=440), 65% (MFI=312), and 67% (MFI=315) for the chimeric tc-TCRαβ:ζ specific for EBNA-3B and BMFL-1, and the sc-TCRαβ:γ specific for EBNA-3A, respectively,

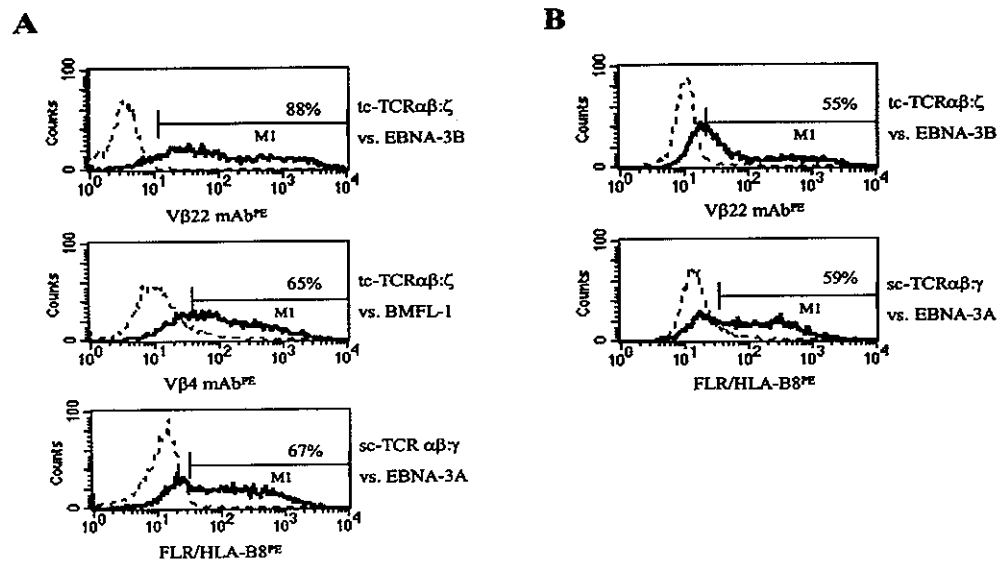

Figure 1. Cell surface expression of EBV-specific ch-TCR on transduced human T lymphocytes.
Primary human T lymphocytes (A) or Jurkat T cells (B) transduced with chimeric TCRαβ genes and enriched for receptor-positive cells, were labeled with PE-conjugated anti-TCRVβ mAbs or tetramers, and analyzed by flow cytometry. Mock-transduced human T lymphocytes (dotted lines) served as negative controls. Marker M1 was set in the corresponding histogram of mock-transduced T cells at a 5% expression level, and percentages given reflect the fraction of stained ch-TCR-transductants relative to M1. For each ch-TCR-construct results of one representative donor are shown.

as determined by flow cytometry (Fig. 1A). Expression levels on Jurkat T cells were 55% (MFI=450) and 59% (MFI=290) for the tc-TCRαβ:ζ specific for EBNA-3B and the sc-TCRαβ:γ specific for EBNA-3A, respectively (Fig. 1B). Jurkat T cells were co-transduced with the human CD8α gene (expression level 100%; MFI=590). CD8 expression on primary human T cells was higher than 50% (data not shown). Mock-transduced T lymphocytes did not bind anti-TCRVβ mAbs or tetramers specific for the introduced TCR (Fig.1).

Peptide-loaded target cells are specifically lysed by primary human T lymphocytes transduced with EBV-specific chimeric TCR

The ch-TCR^{pos} human T lymphocytes were first tested for their cytolytic reactivity versus target cells loaded with EBV-peptides. For peptide-loading experiments we chose target cells that do not endogenously express the corresponding EBV-epitope, i.e., T2-A11 and

HAR (HLA-B8 positive). All EBV-transformed B-LCL express latent viral proteins which are not recognized by TCRs that specifically interact with the lytic cycle protein-derived BMLF-1 epitope. Primary human T cells of 5 out of 9 (56%), 2 out 6 (33%), and 2 out of 10 (20%) different donors transduced with the chimeric tc-TCRαβ:ζ specific for EBNA-3B or BMFL-1, and the sc-TCRαβ:γ specific for EBNA-3A, respectively, specifically lysed target cells that were loaded with 10 μM of the corresponding peptide. Target cells without peptide were not lysed (Table I, and data not shown). A representative example of a cytotoxic response of ch-TCR-transduced primary human T cells is shown in figure 2A. Primary human T cells of the same donor transduced with the MAGE-1/HLA-A1-specific tc-TCRαβ:ζ [19], which served as a positive control, were also able to lyse MAGE-1 peptide-pulsed target cells (Fig. 2B). Peptide pulsed target cells were

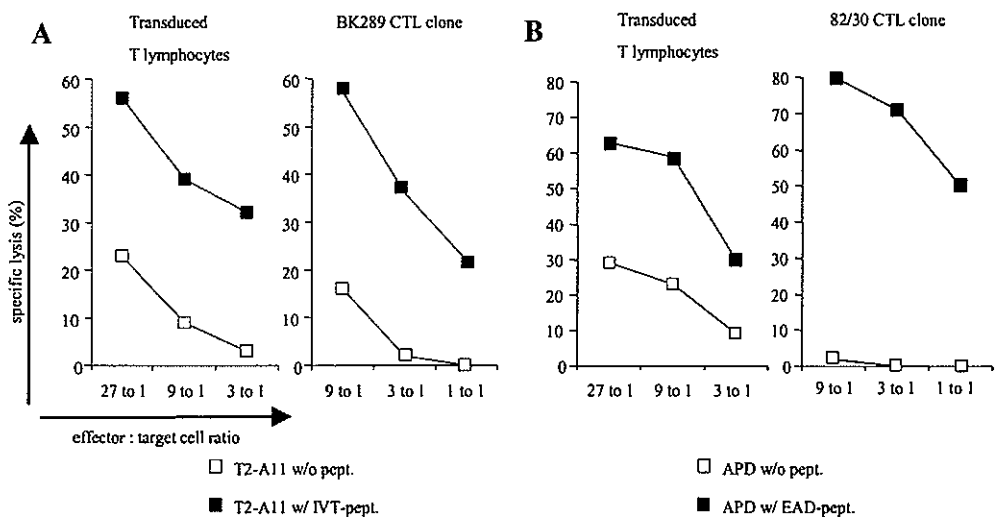

Figure 2. Primary human T cells transduced with EBV-specific chimeric TCR specifically lyse peptide-loaded target cells.

Human T lymphocytes transduced with the tc-TCRαβ:ζ specific for EBNA-3B (A) or MAGE-1 (B) were tested in a 6 h ^{51}Cr-release assay. The following target cells were used: the IVT$^{EBNA-3B}$/A11^{pos} T2-A11 cell line (A) or the EAD$^{EBNA-3B}$/A1^{pos} B-LCL APD (B) in the absence (open symbols) or presence (closed symbols) of the relevant peptide. Target cells were pre-incubated with peptide for 30 min at 37°C at a final concentration of 10 μM. The effector cell to target cell ratio is indicated in the figure. Data of one representative donor is shown. Antigen-specificity was confirmed by blocking experiments with anti-MHC class I mAb or control mIg (10 μg/ml final) which was added to the target cells 30 min prior to cocultivation with T cells (data not shown). The EBNA-3B- and MAGE-1-specific parental CTL clones were used as positive controls. Mock-transduced primary human T lymphocytes were not cytolytic towards the mentioned targets.

also cocultivated with the various parental CTL clones (EBV as well as MAGE-1-specific CTLs) and in all cases target cells pulsed with the corresponding peptide were specifically lysed, whereas target cells without peptide were not lysed (see Fig. 2A/B for an example). Mock-transduced T lymphocytes were not able to lyse any target cells.

Table I: Retargeting of primary human T lymphocytes with EBV-specific chimeric TCR

			Level of surface expression[c]		Cytotoxic response versus target cells	
Format[a]	**Specificity[b]**	**#donors**	**TCR**	**CD8**	**Native[d]**	**Pept. load.[e]**
tc-TCRαβ:ζ	IVT/A11	9	73± 5.4%	86± 0.9%	0 of 9	5 of 9
tc-TCRαβ:ζ	GLC/A2	6	55± 5.8%	91± 3.6%	0 of 6	2 of 6
sc-TCRαβ:γ	FLR/B8	10	45± 3.9%	82± 6.8%	0 of 8	2 of 10

[a] Format of the chimeric TCR. Abbreviations used: tc-TCRαβ:ζ, chimeric two chain TCRαβ coupled to CD3ζ; sc-TCRαβ:γ, chimeric single chain TCRαβ coupled to Fc(ε)RIγ (see Materials and Methods section for details).

[b] Antigen specificity of the parental CTL clone (EBV-peptide/HLA-subtype).

[c] Average cell surface expression (± standard error of the mean) of chimeric TCR and CD8 on transduced primary human T lymphocytes.

[d] Target cells that express endogenously processed EBV-peptide.

[e] Peptide-loaded target cells.

Primary human T lymphocytes transduced with EBV-specific chimeric TCR do not lyse target cells that express endogenously processed antigen

Next, EBV-specific ch-TCR^{pos} T lymphocytes were tested for their cytolytic reactivity versus target cells that express natively processed antigen. Since there is no cell line that natively expresses the epitope recognized by the BMFL-1-specific TCR, the tc-TCRαβ:ζ^{pos} T lymphocytes specific for this EBV-epitope were only tested versus peptide-loaded target cells. tc-TCRαβ:ζ^{pos} primary human T cells specific for EBNA-3B of 9 different donors were cocultivated with the native IVT^{pos}/A11^{pos} EBV-transformed BK B-LCL (Table I). Surprisingly, only exogenously peptide-loaded BK cells were lysed by the chimeric tc-TCRαβ:ζ^{pos} T cells, whereas BK cells without exogenously added peptide were not lysed (Fig. 3A). Also the IVT^{pos}/A11^{pos} EBV-transformed B-LCL CJO was only lysed by tc-TCRαβ:ζ^{pos} T cells after addition of IVT-peptide (data not shown). T lymphocytes positive for sc-TCRαβ:γ specific for EBNA-3A of 10 different donors were tested for cytolytic reactivity versus the FLR^{pos}/B8^{pos} EBV-transformed B-LCL target cells RL or CFpuy (Table I). Again, only peptide-loaded target cells were lysed by chimeric sc-TCRαβ:γ^{pos} T lymphocytes, whereas target cells without

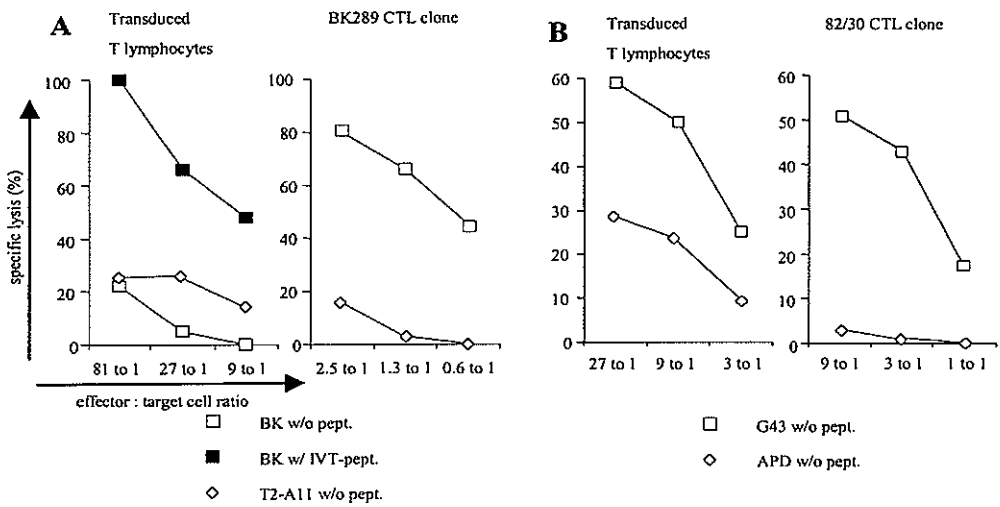

Figure 3. Native target cells are not lysed by primary human T lymphocytes transduced with EBV-specific chimeric TCR.

Ch-TCR-transduced human T lymphocytes with specificities for EBNA-3B (A) or MAGE-1 (B) were tested in a 6 h ^{51}Cr-release assay, with the following target cells: the IVT^{pos}/A11^{pos} B-LCL BK in the absence (open squares) or presence (closed squares) of the IVT-peptide, the IVT^{neg}/A11^{pos} T2-A11 cell line (open diamonds), the EAD^{neg}/A1^{pos} melanoma cell line G43 (open squares) or the EAD^{neg}/A1^{pos} B-LCL APD (open diamonds). Target cells were pre-incubated with peptide for 30 min at 37°C at a final concentration of 10 µM. The effector cell to target cell ratio is indicated in the figure. Data of one representative donor is shown. Antigen-specificity was confirmed by blocking experiments as described in the legend to figure 2 (data not shown). The parental CTL clones were used as positive controls. Mock-transduced primary human T lymphocytes were not cytolytic towards the above mentioned target cells.

exogenous peptide were not lysed. Even at high effector to target cell ratios (81:1), ch-TCR^{pos} T lymphocytes were not able to lyse native targets (see Fig. 3A). In contrast, primary human T cells transduced with the MAGE-1/A1-specific control ch-TCR did lyse target cells that express the natively processed MAGE-1 epitope without addition of exogenous peptide (Fig. 3B). The parental CTL clones, including the EBV-specific ones, were also able to lyse target cells that express natively processed antigen (Fig. 3 A/B). Again mock-transduced T lymphocytes were not able to lyse any of the target cells.

Receptor-specific activation of NFAT via EBV-specific chimeric TCR in Jurkat T cells

The observed non-responsiveness of EBV-specific ch-TCR^{pos} T lymphocytes, i.e., cytolytic responses versus peptide-loaded target cells by only a fraction of the donors and no cytolytic responses versus native targets of any of the donors, was followed up by NFAT

reporter gene assays in Jurkat T cells. Receptor-mediated activation of NFAT in ch-TCR^{pos} Jurkat T cells was analyzed by stimulation with anti-TCRVβ mAbs, peptide/MHC complexes or tumor target cells. Receptor-specific activation of NFAT was seen in tc-TCRαβ:ζ^{pos} and sc-TCRαβ:γ^{pos} Jurkat T cells specific for EBNA-3B and EBNA-3A, respectively, following stimulation with anti-TCRVβ mAb or peptide/MHC complexes (Fig. 4A). Cocultivation of tc-TCRαβ:ζ^{pos} Jurkat T cells specific for EBNA-3B with CJO tumor cells and T2-A11 target cells loaded with or without IVT-peptide, or cocultivation of sc-TCRαβ:γ^{pos} Jurkat T cells specific for EBNA-3A with CFpuy tumor cells loaded with or without FLR-peptide, did not result in NFAT activation (Fig. 4A and data not shown). In con-

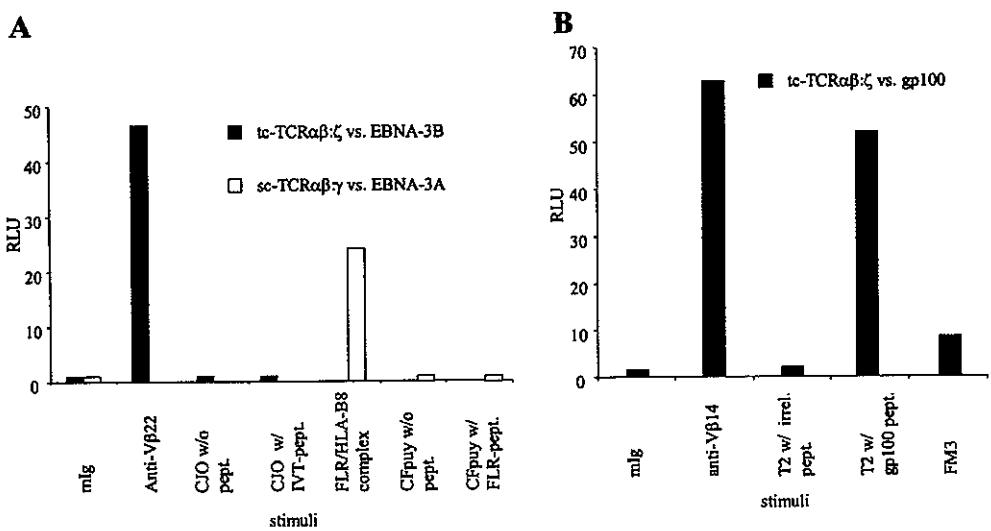

Figure 4. EBV-specific ch-TCR mediate NFAT activation following strong TCR stimuli but not following cocultivation with antigen-positive target cells.

Jurkat T cells transduced with the tc-TCRαβ:ζ specific for EBNA-3B, sc-TCRαβ:ζ specific for EBNA-3A (A: black and white bars, respectively) or tc-TCRαβ:ζ specific for gp100 (B: black bars) were transfected with 5 μg of both the NFAT-reporter and β-galactosidase constructs. TCR-transduced Jurkat T cells were stimulated for 6 h with anti-TCRVβ mAb, control mIg (both at 0,1 μg/well final), peptide/MHC complexes (20 μg/ml final) or target cells. T2 target cells, the EBNA-3B^{pos} CJO and the EBNA-3A^{pos} CFpuy B-LCLs were pre-incubated with peptide for 30 min at 37°C at a final concentration of 1 μM. FM3 cells (gp100^{pos}/HLA-A2^{pos}) were pre-incubated O/N with cytokines, and cocultivation of these melanoma cells with TCR-transduced Jurkat T cells was performed in the presence of anti-CD28 mAb (Schaft *et al.* J. Imunnol. Methods, *in press*). Luciferase activities were determined in cell lysates, normalized for β-galactosidase activities, and expressed relative to medium only (tc-TCRαβ:ζ specific for EBNA-3B: RLU=0.09; sc-TCRαβ:ζ specific for EBNA-3A: RLU=0.05; and tc-TCRαβ:ζ specific for gp100: RLU=0.02, which are all set to 1.0). Results of one (out of three) representative experiment are shown.

trast, we did demonstrate receptor-specific activation of NFAT in Jurkat T cells transduced with a melanoma gp100/A2-specific tc-TCRαβ:ζ in response to not only anti-TCRVβ mAb, but also T2 cells pulsed with the gp100 peptide as well as native gp100^{pos}/A2^{pos} FM3 melanoma cells (Fig. 4B). Non-transduced Jurkat T cells only showed NFAT activation upon stimulation with anti-TCRVβ8 mAb (specific for the endogenous TCRβ of Jurkat T cells), but not with anti-TCRVβ22 mAb, anti-TCRVβ14 mAb, FLR/HLA-B8 peptide/MHC complexes or target cells.

Discussion

This paper reports on gene transfer into human T lymphocytes and functional characterization of EBV-specific chimeric T cell receptors (ch-TCR). We generated a panel of ch-TCR with specificities to various EBV-tumor antigens such as EBNA-3A, 3B and BMFL-1 as potential tools for immunogene therapy of EBV-induced diseases. Our analysis of the antigen reactivity and specificity of ch-TCR-transduced human T cells demonstrated that EBV-specific ch-TCR mediate a cytolytic response versus peptide-loaded target cells but not native EBV^{pos} tumor cells. In addition, EBV-specific ch-TCR are able to mediate NFAT activation only after a strong TCR-specific stimulus but not in response to antigen-positive target cells.

Table I shows that 20 to about 60% of the TCR-transduced T cell populations specifically lysed EBV-peptide-loaded target cells (see also Fig. 2). This MHC-restricted killing of target cells showed a clear dependence on the ch-TCR used, and hence antigen specificity, with the tc-TCRαβ:ζ directed against EBNA-3B (i.e., IVT/A11) being the most effective TCR. Antigen specificity was confirmed in blocking experiments with anti-TCRVβ mAb or anti-MHC cl. I mAb (data not shown). EBV-specific ch-TCR^{pos} T lymphocytes showed no response towards tumor cells that express endogenously processed antigen (Table I and Fig. 3). This was expected for T cells transduced with ch-TCR specific for BMFL-1 since B-LCLs express latent viral proteins that are not recognized by TCRs specific for this lytic cycle protein-derived epitope. However, the HLA-A11-presented EBNA-3B antigen is expressed by CJO and BK cells, whereas the HLA-B8-presented EBNA-3A antigen is expressed by RL and CFpuy cells. Yet, ch-TCR^{pos} T lymphocytes specific for either EBNA-3B or 3A were not able to kill the corresponding antigen-positive target cells (Fig. 3A). Technical or perhaps donor-related aspects as a possible explanation for the absence of cytolysis of native targets by EBV-specific ch-TCR^{pos} T cells are highly unlikely since a melanoma ch-TCR, when introduced into T lymphocytes from the same donor, is

able to mediate cytotoxicity versus native target cells (Fig. 3B). Moreover, primary human T cells transduced with EBV-specific ch-TCR genes were not in an anergic state, since these T cells were able to lyse anti-CD3 mAb-coated target cells in a reverse ADCC assay (data not shown). Also, transduction of primary human T lymphocytes with retrovirus produced by other packaging cells (i.e., 293T or PG13) had no effect on responsiveness.
The Jurkat T cell-based NFAT reporter gene assay provides a fast en sensitive method to functionally validate ch-TCRs [31], and was used to follow up on the inability of EBV-specific ch-TCRs to mediate a cytolytic response versus antigen-positive B-LCLs. Jurkat T cells expressing ch-TCRs specific either for EBNA-3B or 3A activated NFAT after stimulation with anti-TCRVβ22 mAb and FLR/HLA-B8 peptide/MHC complexes, respectively (Fig. 4A). However, no NFAT response was seen when these EBV-specific ch-TCR^{pos} Jurkat T cells were cocultivated with either peptide-pulsed target cells or native tumor cells. In contrast, a melanoma-specific chimeric tc-TCRαβ:ζ expressed on Jurkat T cells was able to induce NFAT-activation after stimulation with peptide-pulsed target cells as well as native antigen-positive melanoma cells (Fig. 4B). These data indicate that EBV-specific ch-TCRs are functional and are, at least following a strong TCR stimulus, able to initiate a signal transduction cascade ultimately leading to activation of NFAT. The ability of these ch-TCR to initiate a signal was confirmed in primary human T cells which show CD3ζ phosphorylation in response to strong TCR stimuli (data not shown).
In line with our data, Orentas and co-workers showed that primary human T lymphocytes transduced with full length, i.e., non-chimeric TCR α and β chains specific for the EBV-LMP2 antigen only responded to peptide-loaded target cells but not to native EBV^{pos} cells [23]. We believe the non-responsive property of EBV-specific TCR-transduced T cells may be inherent to a suboptimal balance between receptor density and tumor-antigen density and/or a low ligand-binding affinity/triggerability of the receptors themselves. We have shown earlier that a functional balance exists between chimeric receptor density on the one hand and tumor-antigen density on the other [34]. A low density of EBV-specific ch-TCR on the cell surface of transduced T lymphocytes does not appear to explain the observed non T cell response, because our retroviral transduction protocol and subsequent enrichment of receptor-positive T cells allowed for high ch-TCR densities (Fig. 1 and Table 1). Nevertheless, the expression level of ch-TCR was about 15% lower in T lymphocytes from donors lacking a cytotoxic T cell response against peptide-loaded target cells. This suggests that high chimeric receptor density on transduced T cells compensates for low antigen density on target cells. Our findings are in extension to data of Cooper and co-workers who

transferred full length TCR α and β chains specific for HIV-gag to primary human T lymphocytes and demonstrated that T cells expressing diminished levels of the introduced TCR chains exhibit a reduced capacity to lyse target cells [22]. The reduced capacity to lyse target cells became increasingly prominent when the target antigen density was also reduced. In fact, a high level of TCR density appears to be required for the TCR to function in response to physiological antigen levels [22]. We speculate that the density of EBV-antigen on *in vitro* cultured B-LCL target cells is too low to functionally interact with a bulk culture of human T lymphocytes transduced with EBV-specific ch-TCR, whereas it is sufficiently high to trigger lysis by the highly differentiated EBV-specific CTL clones. Introduction of recombinant vaccinia viruses expressing EBV antigens into B-LCL may provide a mean to increase antigen densities and study the responses of EBV-specific ch-TCR towards native antigen. EBV-specific T cell activation may require special properties of the effector cell (e.g., higher expression of co-stimulatory/adhesion molecules and signaling molecules). Choosing alternative recipient cells for TCR transduction, such as virus- or allo-specific T cells [35,36] which may express the necessary co-stimulatory/adhesion and signaling molecules, could benefit EBV-specific T cell retargeting.

Another approach to optimize the EBV-specific responses of receptor-transduced primary human T cells is to improve the receptor's ligand-binding affinity or triggerability. Our observation that EBV-specific ch-TCR in contrast to melanoma-specific ch-TCR are unable to mediate a T cell response following cocultivation with antigen-positive target cells may imply a lower antigen binding affinity of these receptors relative to for instance melanoma-specific receptors. Recently, a chimeric TCR-like Fab-based receptor specific for MAGE-1/A1 was developed by using phage display technology [37,38], and the affinity of this TCR-like Fab could be enhanced 18-fold. Chimeric receptors comprising this affinity-matured Fab mediated improved immune responses (i.e., cytolysis and cytokine production) in response to native MAGE-1^{pos}/$A1^{pos}$ tumor cells relative to the non-affinity-matured receptor [39]. The development of a high affinity and MHC-restricted EBV-specific Fab molecule and/or further optimization of the chimeric receptor format may overcome the EBV-related problems mentioned with receptor-transduced T cells.

Taken together, several EBV-specific ch-TCR constructs with different formats and antigen specificities have been introduced into primary human T lymphocytes and allow for EBV-specific T cell responses. Nevertheless, in order to treat EBV-induced diseases with EBV-specific retargeted T cells, the receptor's antigen-binding affinity/triggerability needs to be further increased.

Acknowledgements

This work was supported by the Dutch Cancer Society (DDHK 1996-1253). We thank Drs. T. Boon and P. Coulie for providing the 82/30 CTL clone and G43 melanoma cell line, Dr. M. Giphart for the BSM, CJO, HAR, and APD B-LCLs and Dr. J.W. Drijfhout for peptide synthesis.

References

1. Rickinson,A.B.; Moss,D.J.; Wallace,L.E.; Rowe,M.; Misko,I.S.; Epstein,M.A.; and Pope,J.H. "Long-term T-cell-mediated immunity to Epstein-Barr virus." (1981), *Cancer Res.*, **41**:4216-4221
2. Rickinson,A. and Kieff,E. "Epstein-Barr Virus." in Fields Virology (1996), Fields,B.N.; Knipe,D.M.; Howley,P.M.; Chanock,R.M.; Melnick,J.L.; Monath,T.P.; Roizman,B.; Straus,S.E., eds., **3rd edition**: 2397-2446
3. Kieff,E. "Epstein-Barr virus and its replication." in Fields Virology (1996), Fields,B.N.; Knipe,D.M.; Howley,P.M.; Chanock,R.M.; Melnick,J.L.; Monath,T.P.; Roizman,B.; Straus,S.E., eds., **3rd edition**: 2343-2396
4. Cohen,J.I. "Epstein-Barr virus infection." (2000), *N. Engl. J. Med.*, **343**:481-492
5. Rowe,M.; Rowe,D.T.; Gregory,C.D.; Young,L.S.; Farrell,P.J.; Rupani,H.; and Rickinson,A.B. "Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells." (1987), *EMBO J.*, **6**:2743-2751
6. Imai,S.; Koizumi,S.; Sugiura,M.; Tokunaga,M.; Uemura,Y.; Yamamoto,N.; Tanaka,S.; Sato,E.; and Osato,T. "Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:9131-9135
7. Deacon,E.M.; Pallesen,G.; Niedobitek,G.; Crocker,J.; Brooks,L.; Rickinson,A.B.; and Young,L.S. "Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells." (1993), *J. Exp. Med.*, **177**:339-349
8. Hitt,M.M.; Allday,M.J.; Hara,T.; Karran,L.; Jones,M.D.; Busson,P.; Tursz,T.; Ernberg,I.; and Griffin,B.E. "EBV gene expression in an NPC-related tumour." (1989), *EMBO J.*, **8**:2639-2651
9. Brooks,L.; Yao,Q.Y.; Rickinson,A.B.; and Young,L.S. "Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts." (1992), *J. Virol.*, **66**:2689-2697
10. Young,L.; Alfieri,C.; Hennessy,K.; Evans,H.; O'Hara,C.; Anderson,K.C.; Ritz,J.; Shapiro,R.S.; Rickinson,A.; Kieff,E.; et al. "Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease." (1989), *N. Engl. J. Med.*, **321**:1080-1085
11. Thomas,J.A.; Allday,M.J.; and Crawford,D.H. "Epstein-Barr virus-associated lymphoproliferative disorders in immunocompromised individuals." (1991), *Adv. Cancer Res.*, **57**:329-380
12. Papadopoulos,E.B.; Ladanyi,M.; Emanuel,D.; Mackinnon,S.; Boulad,F.; Carabasi,M.H.; Castro-Malaspina,H.; Childs,B.H.; Gillio,A.P.; Small,T.N.; et al. "Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation." (1994), *N. Engl. J. Med.*, **330** :1185-1191

13. Heslop,H.E.; Brenner,M.K.; and Rooney,C.M. "Donor T cells to treat EBV-associated lymphoma." (1994), *N. Engl. J. Med.*, **331**:679-680
14. Rooney,C.M.; Roskrow,M.A.; Suzuki,N.; Ng,C.Y.; Brenner,M.K.; and Heslop,H. "Treatment of relapsed Hodgkin's disease using EBV-specific cytotoxic T cells." (1998), *Ann. Oncol.*, **9 Suppl 5**:S129-S132
15. Khanna,R.; Bell,S.; Sherritt,M.; Galbraith,A.; Burrows,S.R.; Rafter,L.; Clarke,B.; Slaughter,R.; Falk,M.C.; Douglass,J.; Williams,T.; Elliott,S.L.; and Moss,D.J. "Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease." (1999), *Proc. Natl. Acad. Sci. U. S. A*, **96**:10391-10396
16. Comoli,P.; Labirio,M.; Basso,S.; Baldanti,F.; Grossi,P.; Furione,M.; Vigano,M.; Fiocchi,R.; Rossi,G.; Ginevri,F.; Gridelli,B.; Moretta,A.; Montagna,D.; Locatelli,F.; Gerna,G.; and Maccario,R. "Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication." (2002), *Blood*, **99**:2592-2598
17. Bolhuis,R.L. and Gratama,J.W. "Genetic re-targeting of T lymphocyte specificity." (1998), *Gene Ther.*, **5**:1153-1155
18. Bolhuis,R.L.; Willemsen,R.A.; and Gratama,J.W. "Clinical applications of redirected cytotoxicity." in Cytotoxic Cells (2000), Sitkovsky,M.V.; Henkart,P.A., eds., **30**: 423-440
19. Willemsen,R.A.; Weijtens,M.E.; Ronteltap,C.; Eshhar,Z.; Gratama,J.W.; Chames,P.; and Bolhuis,R.L. "Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR." (2000), *Gene Ther.*, **7**:1369-1377
20. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
21. Schaft,N.; Willemsen,R.A.; de Vries,J.; Lankiewicz,B.; Essers,B.W.; Gratama,J.W.; Figdor,C.G.; Bolhuis,R.L.; Debets,R.; and Adema,G.J. "Peptide Fine Specificity of Anti-Glycoprotein 100 CTL Is Preserved Following Transfer of Engineered TCRalphabeta Genes Into Primary Human T Lymphocytes." (2003), *J. Immunol.*, **170**:2186-2194
22. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
23. Orentas,R.J.; Roskopf,S.J.; Nolan,G.P.; and Nishimura,M.I. "Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide." (2001), *Clin. Immunol.*, **98**:220-228
24. Chung,S.; Wucherpfennig,K.W.; Friedman,S.M.; Hafler,D.A.; and Strominger,J.L. "Functional three-domain single-chain T-cell receptors." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:12654-12658
25. Campos-Lima,P.O.; Levitsky,V.; Imreh,M.P.; Gavioli,R.; and Masucci,M.G. "Epitope-dependent selection of highly restricted or diverse T cell receptor repertoires in response to persistent infection by Epstein- Barr virus." (1997), *J. Exp. Med.*, **186**:83-89
26. Levitsky,V.; Campos-Lima,P.O.; Frisan,T.; and Masucci,M.G. "The clonal composition of a peptide-specific oligoclonal CTL repertoire selected in response to persistent EBV infection is stable over time." (1998), *J. Immunol.*, **161**:594-601
27. Lim,A.; Trautmann,L.; Peyrat,M.A.; Couedel,C.; Davodeau,F.; Romagne,F.; Kourilsky,P.; and Bonneville,M. "Frequent contribution of T cell clonotypes with public TCR features to the chronic res-

ponse against a dominant EBV-derived epitope: application to direct detection of their molecular imprint on the human peripheral T cell repertoire." (2000), *J. Immunol.*, **165**:2001-2011

28. Burrows,S.R.; Silins,S.L.; Moss,D.J.; Khanna,R.; Misko,I.S.; and Argaet,V.P. "T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen." (1995), *J. Exp. Med.*, **182**:1703-1715
29. Van de Griend,R.J.; Van Krimpen,B.A.; Bol,S.J.; Thompson,A.; and Bolhuis,R.L. "Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells." (1984), *J. Immunol. Methods*, **66**:285-298
30. Weijtens,M.E.; Willemsen,R.A.; Hart,E.H.; and Bolhuis,R.L. "A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes." (1998), *Gene Ther.*, **5**:1195-1203
31. Schaft,N.; Lankiewicz,B.; Gratama,J.W.; Bolhuis,R.L.; and Debets,R. "Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT." (2003), *J. Immunol. Methods, in press*
32. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
33. Lamers,H.J.; Gratama,J.W.; van Putten,W.L.; Stoter,G.; and Bolhuis,R.L. "Exogenous interleukin 2 recruits in vitro lymphokine-activated killer activity by in vivo activated lymphocytes." (1991), *Cancer Res.*, **51**:2324-2328
34. Weijtens,M.E.; Hart,E.H.; and Bolhuis,R.L. "Functional balance between T cell chimeric receptor density and tumor associated antigen density: CTL mediated cytolysis and lymphokine production." (2000), *Gene Ther.*, **7**:35-42
35. Kershaw,M.H.; Westwood,J.A.; and Hwu,P. "Dual-specific T cells combine proliferation and antitumor activity." (2002), *Nat. Biotechnol.*, **20**:1221-1227
36. Rossig,C.; Bollard,C.M.; Nuchtern,J.G.; Rooney,C.M.; and Brenner,M.K. "Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy." (2002), *Blood*, **99**:2009-2016
37. Chames,P.; Hufton,S.E.; Coulie,P.G.; Uchanska-Ziegler,B.; and Hoogenboom,H.R. "Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library." (2000), *Proc. Natl. Acad. Sci. U. S. A*, **97**:7969-7974
38. Willemsen,R.A.; Debets,R.; Hart,E.H.; Hoogenboom,H.R.; Bolhuis,R.L.H.; and Chames,P. "A phage display selected Fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes." (2001), *Gene Ther.*, **8**:1601-1608
39. Chames,P.; Willemsen,R.A.; Rojas,G.; Dieckmann,D.; Rem,L.; Schuler,G.; Bolhuis,R.L.; and Hoogenboom,H.R. "TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity." (2002), *J. Immunol.*, **169**:1110-1118

Chapter 6

General discussion

Chapter 6: **General discussion**

The human immune-system is powerful in sensing and protecting the body from "danger" [1], including microbial attacks, viral infections and even tumors. The patient's own immune-system is therefore considered a primary tool in the treatment of cancer, and has been used as such in various ways. In most approaches cytotoxic T lymphocytes play a prominent role. Tumor-specific CTL can either be isolated from a patient and used for adoptive T cell-mediated immunotherapy [2-8], or be generated in vivo by dendritic cell-induced T cell immunity [9-11]. An exciting alternative is to permanently retarget CTL to tumor cells by genetic means.
In this thesis, we have described the development and *in vitro* functional testing of TCR-based (chimeric) receptors specific for melanoma and Epstein-Barr virus antigens.

6.1 Activation of NFAT as a measure to functionally validate tumor-specific receptors

In chapter 2 we describe the development of a fast and easy-to-perform biological assay to functionally analyze tumor-specific (chimeric) receptors that relies on Jurkat T cells transfected with an NFAT-controlled luciferase reporter gene. This assay is a good tool for the validation of receptor function, because NFAT is a crucial transcription factor for T cell functions [12] and plays an important role in FasL-mediated cytotoxicity [13]. In addition, Jurkat T cells are human T cells that were used before to show functional retargeting after full length TCR$\alpha\beta$ transfer [14-16].
Jurkat T cells are easy to transduce with retroviral vectors encoding a mAb-based receptor as well as TCR-based receptors, after which receptor-positive Jurkat T cells can be enriched avoiding the need for cloning and antibiotic selection. All receptors tested were able to mediate NFAT activation in response to various stimuli such as anti-receptor mAbs and tumor target cells that were either loaded with peptides or natively express the antigen or peptide/MHC complex of interest. CD8/TCR co-transduced Jurkat T cells even bound gp100/HLA-A2 tetramers, showing preservation of ligand binding in immortalized T cells. Antigen specificity of the activation of NFAT was confirmed by blocking with

ligand- or receptor-specific mAbs and specific inhibitors of NFAT activation such as cyclosporin A (CsA) and a dominant negative NFAT mutant (chapter 2).
Importantly, data presented in chapter 2 demonstrate that TCR-transduced Jurkat T cells also activated NFAT in response to melanoma cells presenting endogenously processed gp100 peptide, in contrast to previous reports which show that TCR-reconstituted Jurkat T cells respond to peptide-loaded target cells but not or only very weakly to tumor cells [14-16]. We believe the following characteristics of the NFAT reporter gene assay we have set up contribute to its sensitivity to TCR-mediated responses. First, the expression of high levels of the endogenous adhesion and co-stimulatory molecules CD2, CD5, CD11a, CD18 and CD28 on the Jurkat E6.1 T cell clone, in sharp contrast to the TCRβ^{neg} Jurkat RT3-T3.5 T cell clone often used for TCR-reconstitution experiments [15,16]. Second, high expression levels of receptor and CD8α on Jurkat T cells [14,16,17]. Third, an optimized stimulation protocol to further enhance antigen-specific responses which uses cytokine stimulated target cells to increase the expression of MHC class I and adhesion molecules on tumor cells on the one hand, and anti-CD28 mAb as a co-stimulatory signal for Jurkat T cells on the other hand. Finally, an NFAT reporter gene assay which has been optimized for various parameters, such as the number of response elements present in the NFAT reporter construct (see chapter 2).
To investigate whether receptor-mediated NFAT activation is a functional measure of anti-tumor responses of human T cells, we compared NFAT responses in receptor-transduced Jurkat T cells with antigen-specific responses, such as cytotoxicity and TNFα-production, of primary human T lymphocytes transduced with identical receptor gene(s), and found them to be in complete accordance (chapter 2). In fact, both cytotoxic responses as well as cytokine production of primary human T lymphocytes were blocked by the NFAT inhibitor CsA. The antigen-specific cytotoxic responses of primary human T lymphocytes expressing either G250-specific mAb-based or gp100/HLA-A2-specific TCR-based receptors were partially inhibited by CsA. The NFAT dependence of the transcriptional activation of FasL and the initiation of the FasL cytotoxicity pathway in T cells [13], which coexists next to the NFAT independent granzyme/perforin release pathway [18], may explain the partial inhibition of receptor-mediated cytotoxicity responses by CsA. Antigen-specific cytokine responses of primary human T lymphocytes were completely inhibited by CsA, being in line with previous reports [19,20]. The contribution of NFAT activation to immune responses of primary human T cells is not different for the various receptor formats tested in the reporter gene assay, i.e., a mAb-based scFv-γ, a fl-TCR and a tc-TCR-ζ receptor. The different signaling

molecules used in the chimeric receptors (i.e., the γ chain of the Fc(ε)RIγ from mast cells in the scFv-γ receptor and CD3ζ from T cells in the tc-TCR-ζ receptor) may induce different protein-tyrosine kinase signaling cascades, i.e., the Lyn/Syk pathway [21] and Lck/ZAP-70 pathway [22,23], respectively, but ultimately activate NFAT via induction of calcium mobilization and MAP kinase pathways [12,23,24]. We therefore expect that chimeric receptors containing a γ or CD3ζ signaling molecule will also use these pathways to enable antigen-specific activation of NFAT.

EBV-specific TCRs were also tested for their capacity to mediate antigen-specific NFAT activation. The IVT/A11-specific tc-TCR-ζ^{pos} and the FLR/B8-specific sc-TCR-γ^{pos} Jurkat T cells were able to activate NFAT following stimulation with anti-TCRVβ mAb and MHC/peptide complexes demonstrating that these receptors initiate a signal transduction cascade, which ultimately leads to activation of NFAT (see chapter 5). However, no NFAT response was seen when these EBV-specific chimeric TCR^{pos} Jurkat T cells were cocultivated with target cells pulsed with the corresponding peptide. The EBV-specific chimeric TCRs appear to need a strong stimulus, such as mAbs or MHC/peptide complexes, to reach a signaling threshold. Remarkably, only 20 to 56% of the primary human T cell populations transduced with the EBV-specific receptors described above specifically lysed EBV-peptide-loaded target cells. No response towards tumor cells that express native EBV-tumor antigens was seen (chapter 5). EBV-specific chimeric TCRs in primary human T cells seem to need a strong stimulus to reach a signaling threshold as well (discussed in more detail in paragraph 6.2.3), which is in line with the NFAT data.

From the data described above we conclude that NFAT activation in receptor-transduced Jurkat T cells reflects the immune responses of primary human T cells transduced with identical receptor gene(s), and that the NFAT-reporter assay can be used to functionally analyze tumor-specific (chimeric) receptors.

The NFAT-reporter assay also enabled us to perform a detailed $gp100_{280-288}$ peptide mutant analysis. Primary human T lymphocytes and Jurkat T cells were transduced with the gp100/HLA-A2-specific fl-296 TCR, and cytotoxicity, TNFα production and NFAT activation in response to a panel of gp100 peptide mutants were studied (see chapter 4). Neither gp100-specific cytotoxicity, TNFα production nor NFAT-activation were affected by single amino acid (aa) substitutions of the gp100 wt peptide, except for an aa substitution at position 3 (E to A) which was unable to elicit any T cell response. The S4S6 (P to S at positions 4 and 6) double aa gp100 peptide mutant, however, was able to induce cytotoxicity and low TNFα production by TCR-transduced primary human T lymphocytes, but was not able to

activate NFAT in TCR-transduced Jurkat T cells. The wt, A3 and S4S6 peptide mutants bound with approximately the same capacity to HLA-A2 molecules. Moreover, the A2 (L to A at position 2) and G9 (A to G at position 9) peptide mutants, which hardly bind to HLA-A2, clearly induced cytotoxicity, TNFα production and NFAT activation. Therefore, inefficient presentation by HLA-A2 could not explain the observed results. Antagonistic peptides have an inhibitory effect on T cell responses induced by agonist peptides, whereas partially agonistic peptides can selectively stimulate some T cell effector functions, i.e., cytotoxicity or apoptosis, without inducing other functions, i.e., proliferation or cytokine production, by inducing a pattern of signal transduction that is qualitatively different from the pattern induced by any concentration of the native peptide [25-28]. Inhibition of immune responses induced by wt peptide was not seen in competition assays with the A3 peptide mutant. In addition, fl-296 TCR-transduced T cells did not bind tetramer complexes consisting of HLA-A2 molecules loaded with the A3 peptide mutant, which explained the absence of T cell activation. From these data we concluded that the A3 peptide mutant is a null ligand and not an antagonist. The possibility that the gp100 A3 mutant acts as a supra-agonist, i.e., a null ligand that enhances the reactivation of anti-gp100 memory CTL responses [29], is currently under investigation. Furthermore, dose-response curves with the S4S6 peptide in the various assays, which confirmed our initial observations, together with the finding that fl-296 TCR-transduced T cells weakly bound to S4S6/HLA-A2 tetramer complexes, which may result in a qualitatively different pattern of signal transduction, made us conclude that this double aa gp100 peptide mutant is a partial agonist. Translocation of NFAT is reported to take place during the TCR and co-receptor micro-clustering stage in the formation of an immunological synapse [30]. It would be interesting to find out whether the S4S6 mutant would allow for TCR engagement but not the proceeding to TCR micro-clustering and/or its coalescence into a central synapse, and whether this peptide mutant allows for co-engagement of TCR and the CD8 co-receptor.

Taken together, the gp100 peptide mutant analysis showed that all studied single aa gp100 peptide mutants function as agonists, except for the A3 peptide mutant, which is a non-responsive ligand. The S4S6 mutant, acting as a partial agonist, showed that a decrease in TCR binding dissects receptor-mediated cytotoxicity from TNFα production and NFAT activation.

In addition to using the genetically engineered Jurkat T cells as a fast, flexible and sensitive tool to screen new improved receptors, which may facilitate rapid selection of optimal receptors for the immunogene therapy of cancer and viral infections, these cells enable

studies on whether and how (chimeric) immune-receptors (and other receptors, such as homing receptors or adhesion molecules, for that matter) interact with other T cell molecules. Furthermore, these Jurkat T cells can help to unravel intracellular signaling pathways by using reporter genes under control of other transcription factors, and/or specific inhibitors and dominant negative mutants of molecules involved in signal transduction.

6.2 Receptor formats and building blocks

6.2.1 T cell retargeting via mAb-based receptors

From preclinical studies with mAb-based tumor-specific chimeric receptors it is clear that individual receptor components incorporated into these chimeric receptors greatly affect the expression level of the receptor, its stability, ligand binding affinity and thereby receptor mediated functions. Therefore, changing of these receptor components can lead to an optimized mAb-based tumor-specific chimeric receptor. Extracellular spacer and hinge regions extend the distance between the antigen-binding moiety and the T-cell membrane, and allow for flexibility and enhanced expression of the chimeric receptor on T cells [31,32]. The choice of transmembrane domain can influence expression and function of mAb-based chimeric receptors. For instance, CD3ζ transmembrane domains, in contrast to CD4 or CD8 transmembrane domains, may associate with endogenous TCR/CD3 components, adversely affecting surface expression and responsiveness of the chimeric receptors [33,34]. Furthermore, incorporation of different CD3 or Fc(ε)RIγ signaling domains will probably result in distinct induction of subsequent signaling pathways. The CD3ζ, CD3ε and Fc(ε)RIγ signaling domains have been incorporated into mAb-based chimeric receptors and resulted in successful retargeting of T cells [35-37]. However, it is apparent from normal TCR/CD3 triggering that distinct CD3 components (e.g., CD3ζ and CD3ε) have varying binding affinities for, or show selective binding to, intracellular signal transduction molecules [38,39], which will result in differential patterns of protein phosphorylation and calcium mobilization [40]. Moreover, the combination of a CD3 component together with a kinase and/or co-stimulatory molecule, i.e., CD28 (described in more detail below), in a single receptor enhanced chimeric receptor sensitivity and potency [41-43].

A co-stimulatory signal in addition to a TCR/CD3 signal is required for full activation of resting T cells resulting in cellular proliferation, cytokine secretion (i.e., IL-2), CTL-mediated target cell lysis, and prevention of activation induced anergy [44,45]. Among several co-stimulatory pathways, CD28-mediated co-stimulation via ligands of the B7-family

is crucial for efficient T cell activation and prevents activation-induced T cell death by upregulation of the anti-apoptotic proteins *bcl*-x_L and *bcl*-2 [46,47].

Several *in vitro* and *in vivo* studies with mAb-based chimeric receptors showed that chimeric receptor-transduced T lymphocytes were not activated, rapidly lost function, and failed to expand *in vivo*, presumably due to lack of co-stimulatory molecules on tumor cells and the inherent limitations of signaling exclusively through the chimeric receptor [33,48,49]. Indeed, most tumor cells function poorly as antigen-presenting cells in part because they do not express co-stimulatory molecules [45]. Efficient and sustained expansion and activation of chimeric receptor-transduced T lymphocytes *in vivo* will obviously benefit from the presence of co-stimulatory signals. Furthermore, it was shown that CD28 co-stimulation is required for IL-2 secretion and receptor-mediated T-cell proliferation, but not for receptor-mediated target cell lysis, by T lymphocytes transduced with a recombinant receptor [43,48]. These results leaded to the development of chimeric receptors that integrated CD3ζ signaling and CD28 co-stimulation into one combined CD28/CD3ζ signaling domain (reviewed in Abken *et al.* [50]). T cells transduced with these mAb-based receptors were able to secrete IL-2 without exogenous B7/CD28 co-stimulation in response to antigen-positive target cells [51]. In addition, a mouse model showed that T lymphocytes transduced with an erbB2-specific mAb-based receptor with a CD28/CD3ζ signaling domain had a greater capacity to secrete cytokines (i.e., INFγ), induce T-cell proliferation, and inhibit established tumor growth and metastases, than a receptor with a CD3ζ signaling domain [52]. T lymphocytes transduced with chimeric anti-tumor receptors that can mediate cytokine production following antigen-recognition will not only favor their own proliferation, but will also attract additional effector cells at the tumor site, e.g., NK cells [53].

Target antigens recognized by mAb-based chimeric receptors are highly heterogenous, and structure and composition of the target antigen are crucial to efficiently cluster of receptor molecules. It is therefore unlikely that there exists one optimal mAb-based receptor format for all tumor-antigens. An optimal design of a mAb-based receptor may depend on the type of antigen, and each receptor may have to be optimized individually [54]. In contrast, T cell receptors recognize specific peptide antigen exclusively in the context of an MHC molecule. Slight differences in the overall structure of the peptide-MHC class I - TCR-complex allow for a more generalized mode of antigen-recognition [55]. As a consequence, one could argue that optimization of chimeric TCR-based receptors would yield a chimeric receptor format that is universally applicable to retarget T cells to MHC-restricted antigens.

Analogous to various mAb-based receptors, we have currently developed chimeric TCR-based receptors in which CD3ε/CD3ζ and CD28 signaling are integrated into one molecule. We expect these receptors to provide an efficient antigen-specific co-stimulation to receptor-transduced T lymphocytes and induce proliferation and cytokine production *in vivo* (Willemsen *et al.* unpublished data).

6.2.2 T cell retargeting via full length T cell receptors

Up to now, researchers have successfully performed full length TCR gene transfer in T lymphocytes using TCR specificities for viral antigens such as HIV-1 [56], EBV [57], and influenza [58] as well as tumor antigens such as MDM-2 [59] and MART-1 [60]. In *in vitro* and *in vivo* experiments these cells proved to be reactive against target cells expressing the antigen in the context of the correct HLA-molecule. In addition, we show in chapter 3 that full length TCRα/β genes of two gp100/A2-specific CTL clones, when engineered for gene transfer into primary human T lymphocytes, completely preserve the observed gp100-specific antigen reactivity and cytolytic efficiency. These fl-TCR gene transfer studies were made possible by using an optimized transduction protocol that results in high levels of surface expression of TCR chains on human T lymphocytes [61]. Tumor-specific responses of retargeted T cells were obtained without the necessity of antibiotic selection, enrichment for CD8 positive T cells, or cloning of transduced T cells, which is in contrast to other studies [14,56,60,62]. In extension to previous reports, we also showed that the peptide fine-specificities of the two native anti-gp100 TCRs were preserved following TCR gene transfer into primary human T lymphocytes. Furthermore, we compared induction of NFAT activation, cytotoxicity and TNFα production by the fl-296 TCR, and found that these three read-outs paralleled each other when induced by single amino acid (aa) gp100 peptide mutants (chapter 4).

Important when using tumor-specific receptor-transduced T lymphocytes for the immunogene therapy of cancer is the persistence and function *in vivo*. A long lasting anti-tumor response of T cells grafted with the tumor-specific receptor is anticipated to be crucial for the therapeutic efficacy. In addition to highly efficient target cell lysis, one expects retargeted T cells to proliferate produce cytokine *in situ* in an antigen-driven way. Recent work provided indications that this can be reached with adoptively transferred T cells. In these papers adoptively transferred, genetically unmodified melanoma-specific T cells were shown to persist *in vivo*, preferentially localize to tumor sites, proliferate *in vivo*, and mediate an antigen-specific immune response characterized by the elimination of antigen-positive tumor cells in patients with metastatic melanoma [2,3]. Important for persistence of

adoptively transferred T lymphocytes is also the formation of T cell memory. Promising results in that direction came from Kessels *et al.* who showed in an *in vivo* model with T lymphocytes transduced with an influenza A-specific full length TCR, that these cells proliferated following influenza A infection, and after challenge with tumor cells expressing the influenza peptide. The frequency of tumor-specific T cells in peripheral blood of these mice decreased after the initial challenge, but rapidly increased after a rechallenge with tumor cells 81 days after initial tumor inoculation, and no expansion of tumor growth was observed, indicating a long lasting antigen-reactivity of TCR-transduced T cells and the possible formation of T cell memory [58].

Concerns associated to the use of full length TCR in the immunogene therapy of cancer remain. For instance, the introduction of transgenic full length TCR α and β chains into mature T cells can lead to the heterologous pairing of either TCR chain with endogenous TCR α and β chains. These TCR-pairs will have unpredictable antigenic specificity and may potentially lead to auto-reactivity. Alternative TCR pairing is suggested by our flow-cytometry experiments with primary human T lymphocytes transduced with the two different gp100/A2-specific fl-TCRs. Only 50-60% of CD8-positive T cells expressing the introduced TCR β chain were able to bind the gp100/HLA-A2 tetramer (chapter 3). The remaining TCR β chain may have paired with the endogenous TCR α chain resulting in a TCR complex that cannot bind the gp100/HLA-A2 tetramers. Also Stanislawski *et al.* observed that upon retroviral transfer of a full length MDM2-specific TCR only 30-50% of the TCRVβ^{pos} T lymphocytes were able to bind the correct tetramer [59], confirming our results with the gp100-specific fl-TCRs. Other concerns are that introduction of a full length TCR α transgene in T cells can result in a low and unstable surface expression of TCRα protein [63,64], and that the formation of new TCRαβ heterodimers comprising both exogenous and endogenous TCR chains will dilute the expression of correct TCR chain pairs. This will result in low expression of the desired TCR αβ heterodimers on T cells.

Unpredictable TCR specificities may be prevented from being generated by redesigning the interface of transduced TCR α and β chains. An alternative would be the transfer of chimeric TCR genes linked to genes encoding signal transduction molecules, such as CD3ζ, which excludes heterologous TCR pairing [65,66]. Chimeric TCR thereby have the potential to maximize the expression of the introduced TCR and reducing the risk that alternative TCR complexes are formed. An additional advantage of the chimeric receptor approach is that receptors chimerized to signaling molecules bypass TCR-mediated proximal signaling events, which are often defective in cancer patients [67]. Development of chimeric TCR-based receptors is discussed below.

6.2.3 T cell retargeting via chimeric T cell receptors

In this thesis, we have generated and functionally assessed several TCR-based receptors specific for melanoma gp100 and EBV antigens having various formats, i.e., tc and sc receptors coupled either to CD3ζ or CD4-γ signaling modules (see Fig. 2 of chapter 1). Primary human T lymphocytes transduced with fl-TCR and chimeric TCR specific for gp100/A2 (chapters 2, 3 and 4) were able to recognize not only peptide-loaded target cells, but also tumor cells that present endogenously processed antigen. This in contrast to data obtained with primary human T lymphocytes transduced with the EBV-specific chimeric TCR (chapter 5). Although high expression levels of chimeric TCR were obtained (i.e., 45%-73%), only 20 to 56% of the transduced T cell populations (25 donors in total) specifically lysed EBV-peptide-loaded target cells, with the tc-TCR-ζ specific for EBNA-3B/A11 being the most efficient TCR. However, no response towards tumor cells that express native EBV-tumor antigens was seen. This is in line with data of Orentas *et al.* who showed that primary human T lymphocytes transduced with a full length TCR specific for the EBV LMP-2 antigen only responded to peptide-loaded target cells but not to native EBV-positive cells [57]. The lack of kill of native EBV-positive cells by our TCR-transduced T lymphocytes was not due to functional anergy, because these cells showed phosphorylation of CD3ζ and NFAT activation when stimulated with anti-TCR mAbs and peptide/MHC ligands. These data suggest that EBV-specific chimeric TCRs in primary human T cells need a strong stimulus to reach a signaling threshold. Several explanations for the absence of lysis of native EBV-positive cells are possible. First, the chimeric receptor density on the transduced T cells may be too low to functionally interact with the target cells. It is important to note that the expression level of chimeric TCR was on average 15% lower in T lymphocytes from donors lacking a cytotoxicity response against peptide-loaded target cells compared to reactive T cells. This is in line with data of Cooper *et al.* who transferred full length TCR α and β chains specific for HIV-gag to primary human T lymphocytes and showed that T cells expressing diminished levels of the introduced TCR chains exhibited a reduced capacity to lyse targets. The reduced capacity to lyse targets became increasingly prominent when the target antigen density was also reduced (see second point). In fact, a high level of TCR density is required for TCR functioning in response to physiological antigen levels [56]. Second, the EBV-antigen density on *in vitro* cultured target cells may be too low to functionally interact with TCR-transduced T cells. Testing the EBV-specific TCR-transduced T cells on a panel of distinct EBV-positive tumor cells, as well as target cells infected with vaccinia virus with EBV peptide-encoding genes, will give more insight in the importance of antigen density. Third, EBV-specific T cell activation may require special properties of the effector cell (e.g., higher

expression of co-stimulatory/adhesion molecules and signaling molecules), which can be fulfilled by highly differentiated EBV-specific CTL clones, but not by bulk-cultured, TCR-transduced primary human T cells. By choosing different recipient cells for TCR transduction, such as virus- or allo-specific T cells [68,69], which may express the necessary co-stimulatory/adhesion and signaling molecules, the functional interaction of TCR-transduced T cells with tumor cells could be enhanced. Furthermore, the 'triggerability' of the EBV-specific chimeric receptors could be optimized by increasing the ligand-binding affinity or incorporating co-stimulatory and/or signaling domains into the chimeric receptor as described for mAb-based receptors in paragraph 6.2.1.

From studies with the gp100/A2-specific chimeric TCR we know that the chimeric tc-296 TCR-ζ receptor expressed on T cells potently recognized peptide-loaded and native gp100/A2^{pos} tumor cells (chapter 2). This receptor even showed the same peptide fine-specificity as fl-296 TCR in cytotoxicity assays (Schaft *et al.*, unpublished data). The chimeric tc-296 TCR-ζ turned out to be less efficient in TNFα production in response to peptide-loaded and native target cells than the fl-296 TCR (chapter 2). In contrast to the tc-296 TCR-ζ receptor, a chimeric sc-296 TCR-γ receptor expressed on T cells only weakly recognized peptide-loaded target cells (Schaft *et al.*, unpublished data). Furthermore, chimeric TCR-based receptors (i.e., tc-TCR-ζ and sc-TCR-ζ receptors; see Fig. 2 of chapter 1) specific for the HLA-A1 presented melanoma MAGE-1 antigen were functional in transduced primary human T lymphocytes. Peptide-loaded melanoma tumor cells as well as melanoma cells that endogenously express the target antigen were lysed by these T lymphocytes. In addition, these T lymphocytes produced cytokines in response to antigen-positive melanoma cells [65]. Taken together, the data obtained in the described experiments show that chimeric TCR can be potent receptors, and can be used for retargeting of primary human T lymphocytes to viral infected cells or tumors. It is not yet clear how the chimeric tc-TCR-ζ, sc-TCR-ζ and sc-TCR-γ receptors interact with the endogenous signaling machinery in T lymphocytes. The NFAT reporter assay may facilitate the screening of new receptor formats for functionality.

6.2.4 T cell retargeting via other MHC-restricted receptors

Recently, another type of MHC-restricted, tumor-specific receptor was developed. The combined use of soluble peptide/MHC complexes and the phage display technology allowed for the screening of libraries of randomly paired combinations of variable domains of Ab heavy and light chains, and resulted in an Ab with an MHC-restricted melanoma

specificity [70]. There are several advantages of these MHC-restricted Abs. First, Abs generated by the phage display system are completely human, are well-tolerated, and in this way overcome problems with immunogenicity, which has proved to be a major drawback in clinical applications of other Abs generated for example in mice. Second, MHC-restricted specificities can be generated independent of available tumor-specific CTL clones and their TCR genes. And third, the ligand-binding affinity of the MHC-restricted Abs can be manipulated by affinity maturation and further selection.

The phage-Ab G8, specific for MAGE-A1 presented by HLA-A1, was used to generate a chimeric TCR-like Fab-based receptor. When this chimeric TCR-like Fab-based receptor was transduced into primary human T lymphocytes, these cells were functionally retargeted to melanoma cells expressing MAGE-A1 in the context of HLA-A1 [71]. The ligand-binding affinity of the TCR-like Fab could be enhanced 18-fold by affinity maturation. Chimeric receptors created with this affinity-matured Fab still had the same peptide fine-specificity and retargeted T lymphocytes were more efficient in cytolysis and cytokine production than T cells transduced with the non-affinity-matured receptors [72].

Due to affinity maturation, TCR-like Fab-based receptors can have a higher binding affinity for peptide/MHC complexes when compared to normal TCR. It is important to note that it is unclear if this has a negative or positive effect on peptide/MHC recognition and subsequent intracellular signaling and T cell activation.

6.3 A perspective to treat cancer: TCR gene transfer

Recent work of two groups showed that genetically unmodified, adoptively transferred T cells specific for melanoma antigens persisted *in vivo*, preferentially localized to tumor sites, proliferated, and mediated antigen-specific regression of the patient's metastatic melanoma [2,3]. This indicates that antigen-specific T cells can persist and function *in vivo* and possess the correct signals for homing to tumors. This is very important for the use of tumor-specific receptor-transduced T cells in the immunogene therapy of cancer, because a long lasting anti-tumor response of T cells grafted with the tumor-specific receptor is anticipated to be crucial for the therapeutic efficacy.

Several clinical trials with T lymphocytes expressing mAb-based receptors specific for different tumor associated antigens (i.e., folate receptor (ovarian cancer), TAG72 (adenocarcinoma), CEA (adenocarcinoma), and CD19 (lymphoma) have been initiated [34]. We designed a phase I clinical protocol in which T cells transduced with the G250-specific

chimeric scFv-γ receptor will be used for immunogene therapy in metastatic RCC patients. These clinical trials will give us essential knowledge on toxicity and clinical efficacy of T lymphocytes transduced with tumor-specific chimeric receptors. In this respect, promising data have been obtained in a clinical trial with human T cells genetically engineered to express human immunodeficiency virus (HIV)-specific chimeric receptors. Adoptive transfer of these cells was reported to be safe, and, importantly, these cells persisted and specifically homed to target tissues in HIV infected patients [73,74].

Next to tumor-specific mAb-based receptors that recognize non-MHC-restricted tumor-antigens we and others developed a second generation of receptors: the MHC-restricted receptors. Clinical applicability of any of these tumor-specific receptors greatly depends on a high percentage of receptor-positive cells after transduction, which makes selection or cloning of receptor-expressing T cells unnecessary. This is now made possible with an optimized transduction and expansion protocol [61].

Several MHC-restricted receptor formats were investigated, i.e., full length TCR, chimeric two chain TCR, chimeric single chain TCR, and TCR-like Fab-based receptors. At the moment, there still exist concerns about the clinical applicability of full length TCR, since introduction of unwanted TCR specificities, low TCRα chain expression, and low expression levels of desired TCRαβ heterodimers cannot be ruled out (see paragraph 6.2.2).

Using chimeric tc- and sc-TCR prevents these problems. That, together with the advantage that chimeric TCR chimerized to signaling molecules will bypass TCR-mediated proximal signaling events, which are often defective in cancer patients, makes these receptors more suitable for clinical use. According to our data, the tc-TCR-ζ format seems to be more efficient in induction of an immune response, when stimulated with peptide-loaded as well as native antigen-positive tumor cells, than the sc-TCR-γ format (chapter 2, chapter 5 and Schaft *et al.*, unpublished data). However, in a direct comparison of MAGE-1/A1-specific tc-TCR-ζ and sc-TCR-ζ constructs, both receptors proved to be efficient in induction of immune responses [65].

Distinct intracellular signaling domains in chimeric TCRs may lead to different interaction of this chimeric receptors with endogenous molecules expressed on the cell surface of T lymphocytes, and may have consequences for chimeric-receptor expression and function. This interaction was studied by using three sc-TCR constructs, all consisting of MAGE-1/A1-specific extracellular TCR domains, but coupled to either Fc(ε)RIγ, CD3ε or CD3ζ signaling domains (Debets *et al.*, unpublished data). Analysis by flow cytometry of TCR-transduced T cells showed that the expression of sc-TCR-ζ was least stable, whereas

that of sc-TCR-γ was most stable over time. The fluorescence resonance energy transfer (FRET) technique was used to study the physical association of endogenous CD3ε and CD8α with the chimeric TCR in T lymphocytes. Only upon TCR triggering, a clear chimeric receptor-induced association between the sc-TCRs and endogenous CD3ε and CD8α was seen. These data showed that sc-TCR molecules do not constitutively associate with endogenous CD3 or CD8, yet are able to mediate the formation of an immunological synapse containing the sc-TCR, CD3 and CD8 components. Furthermore, in sc-TCR-transduced Jurkat T cells, significant activation of NFAT was seen for all three sc-TCR constructs when the sc-TCR^{pos} Jurkat T cells were stimulated with anti-sc-TCR antibodies. However, when MAGE-1 peptide-loaded melanoma target cells were used, the sc-TCR-ε receptor was far more potent in mediating NFAT activation relative to the other two receptors. In addition, the sc-TCR-ε was also the most potent receptor in mediating antigen-specific production of INFγ. This indicates that distinct intracellular signaling domains had indeed consequences for chimeric receptor expression and function.

Analogous to the optimization of mAb-based receptors (see paragraph 6.2.1), it should be possible to optimize chimeric TCR-based receptor formats, for example by incorporating a CD28 domain next to a CD3ζ domain in the intracellular signaling region. Optimization can lead to a chimeric TCR-based receptor format that guarantees efficient recognition of target cells that express endogenously processed antigen, irrespective of the type of antigen (e.g., viral or tumor antigens), with an unchanged peptide fine-specificity. Analysis by FRET and NFAT reporter assays will provide more insight in the functionality of the newly developed receptors.

TCR-like Fab-based receptors might have some advantages over TCR-based receptors (see paragraph 6.2.4). However, it should be noted that TCR-like Fab-based receptors could have a higher binding affinity for peptide/MHC complexes than normal TCR. Preliminary results of experiments with Fab- and TCR-based receptors containing a CD28 region in the signaling domain show that the increased affinity enhances sensitivity, but reduces receptor specificity (Willemsen *et al.*, unpublished data).

Results described here and elsewhere indicate that T lymphocytes harboring an MHC-restricted tumor-specific receptor are promising tools to treat cancer. However, pre-clinical studies in mouse models, in which T lymphocytes transduced with MHC-restricted (chimeric) receptors are compared for their persistence, anti-tumor activity, and homing capacity, will give more insight in the advantages, disadvantages and applicability of different receptor formats *in vivo*.

It is important to note that tumor cells that have a very heterogenous antigen expression (i.e., MHC-restricted and non-MHC-restricted) and/or lose the expression of certain antigens (i.e., antigen-loss variants) may escape immune rejection when human T lymphocytes transduced with a single receptor specific for one antigen are used in the immunogene therapy of cancer. Therefore, retargeting T lymphocytes with several mAb-based, TCR-based and TCR-like Fab-based chimeric receptors, specific for different tumor-antigens may be advantageous for immunogene therapy. With the described retroviral transduction protocol, this can now be achieved. Nevertheless, retroviral transductions may have one drawback. The provirus can integrate at random in the genome of the transduced cells. Thus, it can also integrate in genes involved in cell cycle control, and subsequently disturb cell growth. This insertional mutagenesis has been thought to be unlikely with retroviral vectors since they cannot reproduce themselves within the cell and cannot repeatedly insert into the cell's chromosomes, which would increase the likelihood of oncogenesis. Recent data of a genetherapeutic clinical trial in severe combined immunodeficiency (SCID) patients suggests differently. In this trial, autologous hematopoietic stem cells were retrovirally transduced with a vector containing a gene encoding the common γ chain, which is defective in SCID patients [75]. Initially, patients receiving this genetherapy showed normal outgrowth of T and NK cells, which is, due to the absence of the common γ chain, not seen in SCID. However, one patient showed an unexpectedly large clonal expansion of γδ T cells. These leukaemia-like symptoms were caused by integration of the provirus in the LMO-2 oncogene, which is crucial for normal haematopoiesis and serves a regulatory function in normal progenitor cell development [76]. Further investigation is necessary to understand whether there are other factors that contributed to the development of the leukaemia-like process in the patient [76,77]. Although the danger of insertional mutagenesis seems to preferentially affect retroviral transduction of stem cells [75,78] and not of primary human T cells, it should be kept in mind when using retroviral transduction for gene-transfer.

It may be beneficial for immunogene therapy of cancer to transfer tumor-specific chimeric receptors to other anti-tumor effector cells such as neutrophils, NK cells, γδ T lymphocytes and monocytes, which was already shown for chimeric mAb-based receptors [79]. The homing pattern, cytokine repertoire and cytolytic mechanism of these cells differ from T lymphocytes and thereby can target different tumor cells and attract particular components of the immune system. Optimal targeting of different recipient cells to tumor cells may require different receptor formats.

In addition to induction of an immune response, T lymphocytes retargeted to tumors by tumor-specific receptors can be applied for the delivery of pro-apoptotic or anti-angiogenic molecules at the tumor site [80,81]. Furthermore, cytotoxic T lymphocytes can be retargeted to tumor vasculature by introducing chimeric molecules that cause homing to the vasculature and its subsequent destruction. For instance, a chimeric molecule consisting of a vascular endothelial growth factor (VEGF) sequence linked to intracellular signaling sequences of CD3ζ was transduced into T cells. These T cells efficiently homed to VEGF-receptor expressing tumor vasculature in mice, and strongly inhibited the growth of tumors [82]. Finally, next to using gene-transduced T cells to treat viral infections and tumors, receptor-transduced T cells can also be used to treat autoimmunity. A chimeric receptor, consisting of an autoantigenic peptide in its restricting MHC and an intracellular CD3ζ domain, was developed. Engagement of this chimeric receptor by the TCR of autoreactive T cells activated the receptor-modified T cells, inducing proliferation and cytolysis *in vitro* and *in vivo* [83].

Taken together, the ability to efficiently transduce primary human T lymphocytes with antigen-specific mAb-based, TCR-based and TCR-like Fab-based (chimeric) receptors with an optimal receptor design will provide us with powerful new tools to treat viral infections and cancer. Preclinical experiments and clinical trials with receptor-transduced T lymphocytes will have to prove the efficacy of this type of immunogene therapy.

References

1. Gallucci,S. and Matzinger,P. "Danger signals: SOS to the immune system." (2001), *Curr. Opin. Immunol.*, **13**:114-119
2. Yee,C.; Thompson,J.A.; Byrd,D.; Riddell,S.R.; Roche,P.; Celis,E.; and Greenberg,P.D. "Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells." (2002), *Proc. Natl. Acad. Sci. U. S. A*, **99**:16168-16173
3. Dudley,M.E.; Wunderlich,J.R.; Robbins,P.F.; Yang,J.C.; Hwu,P.; Schwartzentruber,D.J.; Topalian,S.L.; Sherry,R.; Restifo,N.P.; Hubicki,A.M.; Robinson,M.R.; Raffeld,M.; Duray,P.; Seipp,C.A.; Rogers-Freezer,L.; Morton,K.E.; Mavroukakis,S.A.; White,D.E.; and Rosenberg,S.A. "Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes." (2002), *Science*, **298**:850-854
4. Rosenberg,S.A. "A new era for cancer immunotherapy based on the genes that encode cancer antigens." (1999), *Immunity.*, **10**:281-287
5. Heslop,H.E. and Rooney,C.M. "Adoptive cellular immunotherapy for EBV lymphoproliferative disease." (1997), *Immunol. Rev.*, **157**:217-222
6. Rooney,C.M.; Aguilar,L.K.; Huls,M.H.; Brenner,M.K.; and Heslop,H.E. "Adoptive immunotherapy of

EBV-associated malignancies with EBV-specific cytotoxic T-cell lines." (2001), *Curr. Top. Microbiol. Immunol.*, **258**:221-229

7. Walter,E.A.; Greenberg,P.D.; Gilbert,M.J.; Finch,R.J.; Watanabe,K.S.; Thomas,E.D.; and Riddell,S.R. "Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor." (1995), *N. Engl. J. Med.* , **333**:1038-1044
8. Riddell,S.R. and Greenberg,P.D. "T-cell therapy of cytomegalovirus and human immunodeficiency virus infection." (2000), *J. Antimicrob. Chemother.*, **45 Suppl T3**:35-43
9. Brossart,P.; Wirths,S.; Brugger,W.; and Kanz,L. "Dendritic cells in cancer vaccines." (2001), *Exp. Hematol.*, **29**:1247-1255
10. Nouri-Shirazi,M.; Banchereau,J.; Fay,J.; and Palucka,K. "Dendritic cell based tumor vaccines." (2000), *Immunol. Lett.*, **74**:5-10
11. Banchereau,J.; Schuler-Thurner,B.; Palucka,A.K.; and Schuler,G. "Dendritic cells as vectors for therapy." (2001), *Cell*, **106**:271-274
12. Rao,A.; Luo,C.; and Hogan,P.G. "Transcription factors of the NFAT family: regulation and function." (1997), *Annu. Rev. Immunol.*, **15**:707-747
13. Xiao,S.; Matsui,K.; Fine,A.; Zhu,B.; Marshak-Rothstein,A.; Widom,R.L.; and Ju,S.T. "FasL promoter activation by IL-2 through SP1 and NFAT but not Egr-2 and Egr-3." (1999), *Eur. J. Immunol.*, **29**:3456-3465
14. Cole,D.J.; Weil,D.P.; Shilyansky,J.; Custer,M.; Kawakami,Y.; Rosenberg,S.A.; and Nishimura,M.I. "Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen." (1995), *Cancer Res.*, **55**:748-752
15. Calogero,A.; Hospers,G.A.; Kruse,K.M.; Schrier,P.I.; Mulder,N.H.; Hooijberg,E.; and de Leij,L.F. "Retargeting of a T cell line by anti MAGE-3/HLA-A2 alpha beta TCR gene transfer." (2000), *Anticancer Res.*, **20**:1793-1799
16. Aarnoudse,C.A.; Kruse,M.; Konopitzky,R.; Brouwenstijn,N.; and Schrier,P.I. "TCR reconstitution in Jurkat reporter cells facilitates the identification of novel tumor antigens by cDNA expression cloning." (2002), *Int. J. Cancer*, **99**:7-13
17. Dembic,Z.; Haas,W.; Zamoyska,R.; Parnes,J.; Steinmetz,M.; and von Boehmer,H. "Transfection of the CD8 gene enhances T-cell recognition." (1987), *Nature*, **326**:510-511
18. Kagi,D.; Ledermann,B.; Burki,K.; Zinkernagel,R.M.; and Hengartner,H. "Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo." (1996), *Annu. Rev. Immunol.*, **14**:207-232
19. Goldfeld,A.E.; McCaffrey,P.G.; Strominger,J.L.; and Rao,A. "Identification of a novel cyclosporin-sensitive element in the human tumor necrosis factor alpha gene promoter." (1993), *J. Exp. Med.*, **178**:1365-1379
20. Macian,F.; Garcia-Rodriguez,C.; and Rao,A. "Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun." (2000), *EMBO J.*, **19**:4783-4795
21. Jouvin,M.H.; Numerof,R.P.; and Kinet,J.P. "Signal transduction through the conserved motifs of the high affinity IgE receptor Fc epsilon RI." (1995), *Semin. Immunol.*, **7**:29-35
22. Clements,J.L.; Boerth,N.J.; Lee,J.R.; and Koretzky,G.A. "Integration of T cell receptor-dependent signaling pathways by adapter proteins." (1999), *Annu. Rev. Immunol.*, **17**:89-108
23. Nel,A.E. "T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways,

and signal integration at the T-cell antigen receptor synapse." (2002), *J. Allergy Clin. Immunol.*, **109**:758-770

24. van Leeuwen,J.E. and Samelson,L.E. "T cell antigen-receptor signal transduction." (1999), *Curr. Opin. Immunol.* , **11**:242-248
25. Sloan-Lancaster,J. and Allen,P.M. "Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology." (1996), *Annu. Rev. Immunol.*, **14**:1-27
26. Madrenas,J. "Differential signalling by variant ligands of the T cell receptor and the kinetic model of T cell activation." (1999), *Life Sci.*, **64**:717-731
27. Chau,L.A.; Bluestone,J.A.; and Madrenas,J. "Dissociation of intracellular signaling pathways in response to partial agonist ligands of the T cell receptor." (1998), *J. Exp. Med.*, **187**:1699-1709
28. Hemmer,B.; Stefanova,I.; Vergelli,M.; Germain,R.N.; and Martin,R. "Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells." (1998), *J. Immunol.*, **160**:5807-5814
29. Micheletti,F.; Canella,A.; Vertuani,S.; Marastoni,M.; Tosi,L.; Volinia,S.; Traniello,S.; and Gavioli,R. "Supra-agonist peptides enhance the reactivation of memory CTL responses." (2000), *J. Immunol.*, **165**:4264-4271
30. Krummel,M.F. and Davis,M.M. "Dynamics of the immunological synapse: finding, establishing and solidifying a connection." (2002), *Curr. Opin. Immunol.*, **14**:66-74
31. Weijtens,M.E.; Willemsen,R.A.; Hart,E.H.; and Bolhuis,R.L. "A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes." (1998), *Gene Ther.*, **5**:1195-1203
32. Patel,S.D.; Moskalenko,M.; Smith,D.; Maske,B.; Finer,M.H.; and McArthur,J.G. "Impact of chimeric immune receptor extracellular protein domains on T cell function." (1999), *Gene Ther.*, **6**:412-419
33. Brocker,T. and Karjalainen,K. "Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes." (1995), *J. Exp. Med.*, **181**:1653-1659
34. Willemsen,R.A.; Debets,R.; Chames,P.; and Bolhuis,R.L. "Genetic engineering of T cell specificity for immunotherapy of cancer." (2003), *Hum. Immunol.*, **64**:56-68
35. Eshhar,Z.; Waks,T.; Gross,G.; and Schindler,D.G. "Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors." (1993), *Proc. Natl. Acad. Sci. U. S. A*, **90**:720-724
36. Weijtens,M.E.; Willemsen,R.A.; Valerio,D.; Stam,K.; and Bolhuis,R.L. "Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity." (1996), *J. Immunol.*, **157**:836-843
37. Nolan,K.F.; Yun,C.O.; Akamatsu,Y.; Murphy,J.C.; Leung,S.O.; Beecham,E.J.; and Junghans,R.P. "Bypassing immunization: optimized design of "designer T cells" against carcinoembryonic antigen (CEA)-expressing tumors, and lack of suppression by soluble CEA." (1999), *Clin. Cancer Res.*, **5**:3928-3941
38. Isakov,N.; Wange,R.L.; Burgess,W.H.; Watts,J.D.; Aebersold,R.; and Samelson,L.E. "ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity." (1995), *J. Exp. Med.*, **181**:375-380
39. Yamazaki,T.; Hamano,Y.; Tashiro,H.; Itoh,K.; Nakano,H.; Miyatake,S.; and Saito,T. "CAST, a novel CD3epsilon-binding protein transducing activation signal for interleukin-2 production in T cells." (1999), *J. Biol. Chem.*, **274**:18173-18180

40. Wegener,A.M.; Letourneur,F.; Hoeveler,A.; Brocker,T.; Luton,F.; and Malissen,B. "The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules." (1992), *Cell*, **68**:83-95
41. Fitzer-Attas,C.J.; Schindler,D.G.; Waks,T.; and Eshhar,Z. "Harnessing Syk family tyrosine kinases as signaling domains for chimeric single chain of the variable domain receptors: optimal design for T cell activation." (1998), *J. Immunol.*, **160**:145-154
42. Geiger,T.L.; Nguyen,P.; Leitenberg,D.; and Flavell,R.A. "Integrated src kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes." (2001), *Blood*, **98**:2364-2371
43. Hombach,A.; Sent,D.; Schneider,C.; Heuser,C.; Koch,D.; Pohl,C.; Seliger,B.; and Abken,H. "T-cell activation by recombinant receptors: CD28 costimulation is required for interleukin 2 secretion and receptor-mediated T-cell proliferation but does not affect receptor-mediated target cell lysis." (2001), *Cancer Res.*, **61**:1976-1982
44. June,C.H.; Bluestone,J.A.; Nadler,L.M.; and Thompson,C.B. "The B7 and CD28 receptor families." (1994), *Immunol. Today*, **15**:321-331
45. Greenfield,E.A.; Nguyen,K.A.; and Kuchroo,V.K. "CD28/B7 costimulation: a review." (1998), *Crit Rev. Immunol.*, **18**:389-418
46. Mueller,D.L.; Seiffert,S.; Fang,W.; and Behrens,T.W. "Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells. A model for the long-term survival of memory cells." (1996), *J. Immunol.*, **156**:1764-1771
47. Radvanyi,L.G.; Shi,Y.; Vaziri,H.; Sharma,A.; Dhala,R.; Mills,G.B.; and Miller,R.G. "CD28 costimulation inhibits TCR-induced apoptosis during a primary T cell response." (1996), *J. Immunol.*, **156**:1788-1798
48. Krause,A.; Guo,H.F.; Latouche,J.B.; Tan,C.; Cheung,N.K.; and Sadelain,M. "Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes." (1998), *J. Exp. Med.*, **188** :619-626
49. Brocker,T. "Chimeric Fv-zeta or Fv-epsilon receptors are not sufficient to induce activation or cytokine production in peripheral T cells." (2000), *Blood*, **96**:1999-2001
50. Abken,H.; Hombach,A.; Heuser,C.; Kronfeld,K.; and Seliger,B. "Tuning tumor-specific T-cell activation: a matter of costimulation?" (2002), *Trends Immunol.*, **23**:240-245
51. Hombach,A.; Wieczarkowiecz,A.; Marquardt,T.; Heuser,C.; Usai,L.; Pohl,C.; Seliger,B.; and Abken,H. "Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule." (2001), *J. Immunol.*, **167**:6123-6131
52. Haynes,N.M.; Trapani,J.A.; Teng,M.W.; Jackson,J.T.; Cerruti,L.; Jane,S.M.; Kershaw,M.H.; Smyth,M.J.; and Darcy,P.K. "Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors." (2002), *Blood*, **100**:3155-3163
53. Morel,P.A. and Oriss,T.B. "Crossregulation between Th1 and Th2 cells." (1998), *Crit Rev. Immunol.*, **18**:275-303
54. Hombach,A.; Heuser,C.; and Abken,H. "The recombinant T cell receptor strategy: insights into structure and function of recombinant immunoreceptors on the way towards an optimal receptor design for cellular immunotherapy." (2002), *Current Gene Therapy*, **2**:211-226
55. Bjorkman,P.J.; Saper,M.A.; Samraoui,B.; Bennett,W.S.; Strominger,J.L.; and Wiley,D.C. "The foreign

antigen binding site and T cell recognition regions of class I histocompatibility antigens." (1987), *Nature*, **329**:512-518

56. Cooper,L.J.; Kalos,M.; Lewinsohn,D.A.; Riddell,S.R.; and Greenberg,P.D. "Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes." (2000), *J. Virol.*, **74**:8207-8212
57. Orentas,R.J.; Roskopf,S.J.; Nolan,G.P.; and Nishimura,M.I. "Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide." (2001), *Clin. Immunol.*, **98**:220-228
58. Kessels,H.W.; Wolkers,M.C.; van den Boom,M.D.; van der Valk,M.A.; and Schumacher,T.N. "Immunotherapy through TCR gene transfer." (2001), *Nat. Immunol.*, **2**:957-961
59. Stanislawski,T.; Voss,R.H.; Lotz,C.; Sadovnikova,E.; Willemsen,R.A.; Kuball,J.; Ruppert,T.; Bolhuis,R.L.; Melief,C.J.; Huber,C.; Stauss,H.J.; and Theobald,M. "Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer." (2001), *Nat. Immunol.*, **2**:962-970
60. Clay,T.M.; Custer,M.C.; Sachs,J.; Hwu,P.; Rosenberg,S.A.; and Nishimura,M.I. "Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity." (1999), *J. Immunol.*, **163**:507-513
61. Lamers,C.H.; Willemsen,R.A.; Luider,B.A.; Debets,R.; and Bolhuis,R.L. "Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer." (2002), *Cancer gene therapy*, **9**:613-623
62. Fujio,K.; Misaki,Y.; Setoguchi,K.; Morita,S.; Kawahata,K.; Kato,I.; Nosaka,T.; Yamamoto,K.; and Kitamura,T. "Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex." (2000), *J. Immunol.*, **165**:528-532
63. Chung,S.; Wucherpfennig,K.W.; Friedman,S.M.; Hafler,D.A.; and Strominger,J.L. "Functional three-domain single-chain T-cell receptors." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:12654-12658
64. Debets,R.; Willemsen,R.; and Bolhuis,R. "Adoptive transfer of T-cell immunity: gene transfer with MHC-restricted receptors." (2002), *Trends Immunol.*, **23**:435-436
65. Willemsen,R.A.; Weijtens,M.E.; Ronteltap,C.; Eshhar,Z.; Gratama,J.W.; Chames,P.; and Bolhuis,R.L. "Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR." (2000), *Gene Ther.*, **7**:1369-1377
66. Chang,H.C.; Bao,Z.; Yao,Y.; Tse,A.G.; Goyarts,E.C.; Madsen,M.; Kawasaki,E.; Brauer,P.P.; Sacchettini,J.C.; Nathenson,S.G.; et al. "A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments." (1994), *Proc. Natl. Acad. Sci. U. S. A*, **91**:11408-11412
67. Nakagomi,H.; Petersson,M.; Magnusson,I.; Juhlin,C.; Matsuda,M.; Mellstedt,H.; Taupin,J.L.; Vivier,E.; Anderson,P.; and Kiessling,R. "Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma." (1993), *Cancer Res.*, **53**:5610-5612
68. Rossig,C.; Bollard,C.M.; Nuchtern,J.G.; Rooney,C.M.; and Brenner,M.K. "Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy." (2002), *Blood*, **99**:2009-2016
69. Kershaw,M.H.; Westwood,J.A.; and Hwu,P. "Dual-specific T cells combine proliferation and antitumor activity." (2002), *Nat. Biotechnol.*, **20**:1221-1227
70. Chames,P.; Hufton,S.E.; Coulie,P.G.; Uchanska-Ziegler,B.; and Hoogenboom,H.R. "Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library." (2000), *Proc. Natl. Acad. Sci. U. S. A*, **97**:7969-7974

71. Willemsen,R.A.; Debets,R.; Hart,E.H.; Hoogenboom,H.R.; Bolhuis,R.L.H.; and Chames,P. "A phage display selected Fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes." (2001), *Gene Ther.*, **8**:1601-1608
72. Chames,P.; Willemsen,R.A.; Rojas,G.; Dieckmann,D.; Rem,L.; Schuler,G.; Bolhuis,R.L.; and Hoogenboom,H.R. "TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity." (2002), *J. Immunol.*, **169**:1110-1118
73. Walker,R.E.; Bechtel,C.M.; Natarajan,V.; Baseler,M.; Hege,K.M.; Metcalf,J.A.; Stevens,R.; Hazen,A.; Blaese,R.M.; Chen,C.C.; Leitman,S.F.; Palensky,J.; Wittes,J.; Davey,R.T., Jr.; Falloon,J.; Polis,M.A.; Kovacs,J.A.; Broad,D.F.; Levine,B.L.; Roberts,M.R.; Masur,H.; and Lane,H.C. "Long-term in vivo survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection." (2000), *Blood*, **96**:467-474
74. Mitsuyasu,R.T.; Anton,P.A.; Deeks,S.G.; Scadden,D.T.; Connick,E.; Downs,M.T.; Bakker,A.; Roberts,M.R.; June,C.H.; Jalali,S.; Lin,A.A.; Pennathur-Das,R.; and Hege,K.M. "Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects." (2000), *Blood*, **96**:785-793
75. Hacein-Bey-Abina,S.; Le Deist,F.; Carlier,F.; Bouneaud,C.; Hue,C.; De Villartay,J.P.; Thrasher,A.J.; Wulffraat,N.; Sorensen,R.; Dupuis-Girod,S.; Fischer,A.; Davies,E.G.; Kuis,W.; Leiva,L.; and Cavazzana-Calvo,M. "Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy." (2002), *N. Engl. J. Med.*, **346**:1185-1193
76. Buckley,R.H. "Gene therapy for SCID – a complication after remarkable progress." (2002), *Lancet*, **360**:1185-1186
77. Marshall,E. "Clinical research. Gene therapy a suspect in leukemia-like disease." (2002), *Science*, **298**:34-35
78. Li,Z.; Dullmann,J.; Schiedlmeier,B.; Schmidt,M.; von Kalle,C.; Meyer,J.; Forster,M.; Stocking,C.; Wahlers,A.; Frank,O.; Ostertag,W.; Kuhlcke,K.; Eckert,H.G.; Fehse,B.; and Baum,C. "Murine leukemia induced by retroviral gene marking." (2002), *Science*, **296**:497
79. Roberts,M.R.; Cooke,K.S.; Tran,A.C.; Smith,K.A.; Lin,W.Y.; Wang,M.; Dull,T.J.; Farson,D.; Zsebo,K.M.; and Finer,M.H. "Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains." (1998), *J. Immunol.*, **161**:375-384
80. Jin,N.; Chen,W.; Blazar,B.R.; Ramakrishnan,S.; and Vallera,D.A. "Gene therapy of murine solid tumors with T cells transduced with a retroviral vascular endothelial growth factor – immunotoxin target gene." (2002), *Hum. Gene Ther.*, **13**:497-508
81. Vallera,D.A.; Jin,N.; Baldrica,J.M.; Panoskaltsis-Mortari,A.; Chen,S.Y.; and Blazar,B.R. "Retroviral immunotoxin gene therapy of acute myelogenous leukemia in mice using cytotoxic T cells transduced with an interleukin 4/diphtheria toxin gene." (2000), *Cancer Res.*, **60**:976-984
82. Niederman,T.M.; Ghogawala,Z.; Carter,B.S.; Tompkins,H.S.; Russell,M.M.; and Mulligan,R.C. "Antitumor activity of cytotoxic T lymphocytes engineered to target vascular endothelial growth factor receptors." (2002), *Proc. Natl. Acad. Sci. U. S. A*, **99**:7009-7014
83. Jyothi,M.D.; Flavell,R.A.; and Geiger,T.L. "Targeting autoantigen-specific T cells and suppression of autoimmune encephalomyelitis with receptor-modified T lymphocytes." (2002), *Nat. Biotechnol.*, **20**:1215-1220

Summary

The human immune-system is powerful in protecting the body from microbial attacks, viral infections and even tumors. The patient's own immune-system is therefore considered a primary tool in the treatment of cancer, and has been used as such in various ways. In most approaches the cytotoxic T lymphocyte plays a prominent role. One of these approaches is to retarget T lymphocytes to tumor cells by genetic means. Chimeric mAb- and TCR-based receptors, composed of a tumor recognition part, i.e., variable domains of antibody or T cell receptor chains, linked to a transmembrane and signaling module, can provide T lymphocytes with a permanent anti-tumor-antigen reactivity. Thus, these receptors, if generally applicable, may provide effective tools for immunogene therapy of tumors and viral infections.

As a general introduction to tumor immunology **chapter 1** describes the molecules present on the surfaces of T cells and tumor cells involved in antigen recognition and the subsequent T cell activation. Furthermore, it provides an overview of the different strategies that can be followed for the immunotherapy of cancer.

The primary goal of this thesis was to generate and functionally characterize full length and chimeric T cell receptors, specific for melanoma and Epstein-Barr virus antigens, following gene transfer into primary human T lymphocytes. The hypothesis and study design are given in the last part of **chapter 1**.

Transduction of genes encoding tumor-specific (chimeric) receptors into primary human T cells and *in vitro* analysis of antigen-specific responses are laborious and time consuming, and emphasize the need for a fast and sensitive assay to validate such receptors. In **chapter 2** we describe the development of such a method to functionally validate tumor-specific receptors using an NFAT-reporter gene assay in receptor-transduced Jurkat T cells. The method was set up using a chimeric mAb-based single chain receptor specific for the renal cell carcinoma antigen G250 as well as a full length TCR and a chimeric two chain TCR-ζ receptor specific for the HLA-A2-presented gp100 melanoma antigen. Jurkat T cells transduced with these receptors specifically activated NFAT after stimulation with antigen-positive tumor cells. The assay's sensitivity was evident from the detection of NFAT activation in TCR-transduced Jurkat T cells following stimulation with native gp100/HLA-A2^{pos} melanoma cells, a response that was not observed in previous studies. In our opinion the presented NFAT-reporter assay is sufficiently sensitive to functionally validate tumor-specific receptors. The assay's sensitivity can be ascribed to high CD8α expression, choice of Jurkat T cell line, reporter-gene construct and optimal transduction and

stimulation protocol. In all cases the NFAT-response in receptor-transduced Jurkat T cells reflected the anti-tumor cytolysis and TNFα production by primary human T cells transduced with the same receptor gene(s). Inhibition experiments with CsA revealed a clear contribution of NFAT-activation in these tumor-specific responses. We concluded that the described assay provides a flexible and sensitive tool to functionally analyze tumor-specific chimeric receptors, allowing for a more rapid selection of such receptors for immunogene therapy.

The preservation of cytotoxic potency and peptide fine-specificity of CTL following transfer of native TCRα/β, i.e., full length TCR, into primary human T lymphocytes, which is crucial to the potential use of (chimeric) TCR genes for immuno therapy, was studied in **chapter 3**. To address this issue, we used two distinct TCRs which were derived from the CTL-296 and CTL-MPD clones, respectively, and showed the same HLA-A2-restricted melanoma gp100 specificity but exhibited different stringencies in peptide requirements. Retroviral transduction of human T lymphocytes with either one of the two TCRα/β constructs resulted in high levels of membrane expression of the TCR chains and significant binding of gp100 peptide/HLA-A2 tetramers. Peptide-loaded and native gp100/HLA-A2^{pos} tumor target cells were able to trigger lysis and production of TNFα by the fl-TCR-transduced T lymphocytes. Peptide titration studies revealed that the cytolytic efficiencies of the T lymphocyte transductants were in the same range as those of the parental CTL clones. Moreover, primary human T lymphocytes expressing either one of the two engineered gp100-specific TCRs showed cytolytic activities in response to a large panel of peptide mutants that were identical to those of the parental CTL. Thus, TCR, derived from different CTL, can be functionally introduced into primary human T lymphocytes without loss of the antigen reactivity, cytolytic potency and peptide fine-specificity.

In **chapter 4** we studied anti-gp100 T cell functions in more detail. Primary human T lymphocytes and Jurkat T cells were transduced with a gp100/HLA-A2-specific full length TCR, and cytotoxicity, TNFα production and NFAT activation in response to a panel of gp100 peptide mutants were measured. Neither gp100-specific cytotoxicity, TNFα production nor NFAT-activation were affected by single amino acid (aa) substitutions of the gp100 wt peptide, except for an aa substitution at position 3 (E to A) which was unable to elicit any T cell response. A gp100 double aa mutant (P to S at positions 4 and 6), however, was able to elicit a cytotoxic response but only low TNFα production and no NFAT activation. These observations were confirmed in dose-response curves with this peptide in the various assays. Furthermore, T cells transduced with the gp100-specific TCR did not bind the E to A mutant complexed to HLA-A2, but were able to bind the P to S double aa

mutant complexed to HLA-A2, although to a lesser extent than the parental gp100 peptide/HLA-A2 complexes. From this gp100 peptide mutant analysis we concluded that all single aa mutants of the gp100 peptide studied function as peptide agonists, except for the E to A peptide mutant, which is not bound by the TCR and functions as a non-responsive ligand. The P to S double aa mutant of the gp100 peptide, acting as a partial agonist, showed that a decrease in TCR binding dissects receptor-mediated cytotoxicity from TNFα production and NFAT activation.

To investigate whether chimeric TCR gene-transfer could also retarget human T lymphocytes to viral epitopes, chimeric TCR constructs specific for three different Epstein-Barr viral epitopes which are expressed on EBV-related tumors, i.e., EBNA-3A, BMLF-1, and EBNA-3B, were cloned (described in **chapter 5**). A receptor-specific activation of NFAT was seen when chimeric TCR^{pos} Jurkat T cells were stimulated with strong stimuli, such as anti-TCR mAbs and peptide/MHC complexes, but not when stimulated with antigen-positive target cells. Furthermore, only 20 to 56% of the transduced T cell populations specifically lysed EBV-peptide-loaded target cells, with the two chain TCR-ζ specific for EBNA-3B being the most efficient TCR. However, no response towards tumor cells that express native EBV-tumor antigens was seen. This was not due to functional anergy, because these cells showed phosphorylation of CD3ζ and NFAT activation when stimulated with anti-TCR mAbs and peptide/MHC ligands. These data suggested that the EBV-specific chimeric TCRs needed a strong stimulus to reach a signaling threshold. Absence of lysis of native EBV-positive cells may be explained in several ways. First, chimeric receptor density on TCR-transduced T cells might be too low to interact functionally with the target cells. Second, EBV-antigen density on *in vitro* cultured target cells might be too low to interact functionally with TCR-transduced T cells. And third, EBV-specific T cell activation might require special properties of the effector cell (e.g., higher expression of co-stimulatory/adhesion molecules and signaling molecules), which are not fulfilled by bulk-cultured, TCR-transduced primary human T cells. This study demonstrated that retargeting human T lymphocytes to EBV-infected cells with chimeric TCR is feasible, but receptors lack sufficient triggerability and need further optimization.

The studies described in chapters 2 to 5 are discussed in **chapter 6**. Results described in chapter 2, 4 and 5 confirm that the NFAT-reporter assay can be used to functionally analyze tumor-specific (chimeric) receptors and provides a good measure of immune functions mediated by these receptors in transduced primary human T lymphocytes. This assay may therefore facilitate a more rapid selection of receptors for the immunogene therapy of cancer and viral infections. Receptor-transduced Jurkat T cells may also help to

clarify interactions of receptors with other T cell molecules, and unravel intracellular signaling pathways.

Studies with mAb-based receptors have shown that individual receptor components can have a large impact on receptor expression and function, and that changing of these components can lead to optimized receptors. The initiated clinical trials with T lymphocytes expressing chimeric mAb-based receptors will give us essential knowledge on toxicity and clinical efficacy of these cells. Findings reported in this thesis on full length and chimeric TCR-based receptors specific for tumor- and viral-antigens demonstrate their feasibility for the genetic retargeting of T lymphocytes. Chimeric TCR-based receptors may have advantages over full length TCR, i.e., introduction of unwanted TCR specificities, low TCRα chain expression and low expression levels of desired TCR$\alpha\beta$ heterodimers are prevented by using chimeric TCR. Analogous to mAb-based receptors, chimeric TCR-based receptor formats can be optimized. Next to TCR-based MHC-restricted receptors, MHC-restricted Fab-based receptors were developed. These MHC-restricted specificities can be generated independent of available tumor-specific CTL clones and their TCR genes. In addition, ligand binding affinity of these Fabs can be increased by affinity maturation. It is, however, unclear how enhanced affinity of the Fab-based receptor effects T cell activation.

Taken together, the ability to efficiently transduce primary human T lymphocytes with antigen-specific mAb-based, TCR-based and TCR-like Fab-based (chimeric) receptors with an optimal receptor design will provide us with powerful new tools to treat viral infections and cancer. Preclinical studies in mouse models and clinical trials with receptor-transduced T lymphocytes will have to prove the efficacy of this type of immunogene therapy.

Samenvatting

Het humane afweersysteem kan het lichaam krachtig beschermen tegen aanvallen van micro-organismen, virale infecties en zelfs tumoren. Het eigen afweersysteem van de patiënt wordt daarom beschouwd als een primair instrument in de behandeling van kanker, en wordt als zodanig op verschillende manieren gebruikt. In de meeste benaderingen spelen de cytotoxische T cellen (CTL) een prominente rol. Een van deze benaderingen is het herrichten van T lymfocyten naar tumor cellen met hulp van genetische manipulatie. Chimere antistof (As-) en T cel receptor (TCR-)gebaseerde receptoren, bestaande uit een tumor herkenningsdeel, i.e., variable domeinen van As of TCR receptor ketens, gekoppeld aan een transmembraan en signaleringsmodule, kunnen T lymfocyten van een permanente anti-tumor-antigeen reactiviteit voorzien. Dus deze receptoren, als ze algemeen toepasbaar zijn, vormen mogelijk effectieve instrumenten voor immuno-gen therapie van tumoren en virale infecties.

Als een algemene inleiding in de tumor immunologie beschrijft **hoofdstuk 1** de moleculen die aanwezig zijn op het oppervlak van T cellen en tumor cellen, en tevens betrokken zijn bij antigeen herkenning en de daaropvolgende T cel activatie. Bovendien geeft het een overzicht van de verschillende strategiën die gevolgd kunnen worden in de immuno-therapie van kanker.

Het primaire doel van dit proefschrift was het genereren en functioneel karakteriseren van complete keten (ck) en chimere T cel receptoren, specifiek voor melanoma en Epstein-Barr virus antigenen, na gen-transfer in primaire humane T lymfocyten. De hypothese en studieopzet zijn beschreven in het laatste deel van **hoofdstuk 1**.

Transductie van genen, coderend voor tumor-specifieke (chimere) receptoren, in primaire humane T cellen en *in vitro* analyse van antigeen-specifieke responsen zijn arbeidsintensief en tijdrovend, en beklemtonen de noodzaak van een snelle en gevoelige assay voor de validatie van zulke receptoren. In **hoofdstuk 2** beschrijven wij de ontwikkeling van zo'n assay voor de functionele validatie van tumor-specifieke receptoren, welke gebruik maakt van een NFAT-reporter gen assay in receptor-getransduceerde Jurkat T cellen. De methode is opgezet met een chimere As-gebaseerde enkel keten receptor specifiek voor het niercarcinoom antigeen G250, en daarnaast een ck-TCR en een chimere dubbel keten (dk-)TCR-ζ receptor specifiek voor het HLA-A2-gepresenteerde gp100 melanoma antigeen. Jurkat T cellen, getransduceerd met deze receptoren, induceerden na stimulatie met antigeen-positieve tumor cellen een specifieke NFAT activatie. De gevoeligheid van de assay bleek uit de detectie van NFAT activatie in TCR-getransduceerde Jurkat T cellen na stimulatie met natief gp100/HLA-$A2^{pos}$ melanoma cellen; een respons die niet te zien was in vorige studies. Naar

onze mening is de gepresenteerde NFAT-reporter assay gevoelig genoeg om tumor-specifieke receptoren functioneel te valideren. De gevoeligheid van de assay kan worden toegeschreven aan hoge CD8α expressie, keuze van Jurkat T cel lijn, reporter-gen construct en een optimale transductie en stimulatie protocol. In alle gevallen reflecteerde de NFAT-respons in receptor-getransduceerde Jurkat T cellen de anti-tumor cytolyse en TNFα productie door primaire T cellen getransduceerd met dezelfde receptor genen. Remmings experimenten met cyclosporine A toonden een duidelijke bijdrage van NFAT-activatie in deze tumor-specifieke responsen aan. Wij concludeerden dat de beschreven assay een flexibel en gevoelig instrument vormt voor de functionele analyse van tumor-specifieke chimere receptoren, wat een snellere selectie van zulke receptoren voor immuno-gen therapie toestaat.

Het behoud van cytotoxische potentie en peptide fijn-specificiteit van CTL na overdracht van natieve TCRα/β, i.e., ck-TCR, in primaire humane T lymfocyten, wat cruciaal is voor het eventuele gebruik van (chimere) TCR genen voor immuno-therapie, is bestudeerd in **hoofdstuk 3**. Om deze kwestie te onderzoeken, hebben wij twee verschillende TCR van respectievelijk de CTL-296 en CTL-MPD clones, specifiek voor het zelfde HLA-A2-gerestricteerde melanoma gp100 peptide, maar een verschillende peptide fijn-specificiteit vertonend, gebruikt. Retrovirale transductie van humane T lymfocyten met zowel het een als het andere TCRα/β construct resulteerde in hoge niveaus van membraan expressie van de TCR ketens en significante binding van gp100 peptide/HLA-A2 tetrameren. Peptide-beladen en natief gp100/HLA-A2^{pos} tumor target cellen induceerden lysis en productie van TNFα door de TCR-getransduceerde T lymfocyten. Peptide titratie studies toonden aan dat de cytolytische efficienties van de T lymfocyt transductanten van dezelfde omvang waren als die van de parentale CTL clones. Bovendien, primaire T lymfocyten die de een dan wel de ander gp100-specifieke TCR tot expressie brachten, lieten cytolytische activiteiten zien in respons op een groot panel van peptide mutanten die identiek waren aan die van de parentale CTL. Dus TCR, afkomstig van verschillende CTL, kunnen functioneel geïntroduceerd worden in primaire humane T lymfocyten zonder verlies van antigeen activiteit, cytolitische potentie en peptide fijn-specificiteit.

In **hoofdstuk 4** bestudeerden wij de anti-gp100 T cel functies gedetailleerder. Primaire humane T lymfocyten en Jurkat T cellen werden getransduceerd met een gp100/HLA-A2-specifieke ck-TCR, en cytotoxiciteit, TNFα productie en NFAT activatie in respons op een panel van gp100 peptide mutanten werden gemeten. Noch gp100-specifieke cytotoxiciteit, TNFα productie, noch NFAT-activatie werden beinvloed door enkelvoudige aminozuur (az) substituties van het gp100 wt peptide; uitgezonderd een enkelvoudige az substitutie op positie 3 (E naar A), welke niet in staat was een T cel respons te induceren. Een tweevoudige az gp100 mutant (P naar S op positie 4 en 6), daarentegen, induceerde een cytotoxische re-

spons maar induceerde alleen lage TNFα productie en geen NFAT activatie. Deze observaties werden bevestigd in dosis-respons curves met dit peptide in de verschillende assays. Verder bonden T cellen getransduceerd met de gp100-specifieke TCR niet aan de E naar A mutant gecomplexeerd met HLA-A2. Deze cellen bonden wel aan de P naar S tweevoudige az mutant gecomplexeerd met HLA-A2, ofschoon minder goed dan aan de parentale gp100 peptide/HLA-A2 complexen. Uit deze gp100 mutant analyse concluderen wij dat alle bestudeerde enkelvoudige az mutanten van het gp100 peptide functioneren als agonisten, uitgezonderd de E naar A peptide mutant, welke niet aan de TCR bindt en als non-respons ligand functioneert. De P naar S tweevoudige az mutant van het gp100 peptide gedraagt zich als een partiële agonist, en liet zien dat een verminderde TCR binding receptor-gemedieerde cytotoxiciteit van TNFα productie en NFAT activatie scheidt.

Om te onderzoeken of chimere TCR gen-transfer ook humane T lymfocyten naar virale epitopen kan herrichten, werden chimere TCR constructen specifiek voor drie verschillende Epstein-Barr virale epitopen, welke op EBV-gerelateerde tumoren geexpresseerd worden, i.e., EBNA-3A, BMLF-1, and EBNA-3B, gecloneerd (beschreven in **hoofdstuk 5**). Een receptor-specifieke activatie van NFAT werd gezien wanneer chimere TCR^{pos} Jurkat T cellen werden gestimuleerd met sterke stimuli, zoals anti-TCR mAbs and peptide/MHC complexen, maar niet wanneer ze werden gestimuleerd met antigeen-positieve target cellen. Daarbovenop, slechts 20 tot 56% van de getransduceerde T cel populaties lyseerden specifiek EBV-peptide belaadde target cellen. De dk-TCR-ζ specifiek voor EBNA-3B was de meest efficiënte TCR. Daarentegen werd er geen respons gezien ten opzichte van tumor cellen die natief EBV-tumor antigenen expresseren. Dit lag niet aan functionele anergie, want deze cellen lieten wel fosforilatie van CD3ζ en NFAT activatie zien wanneer ze gestimuleerd werden met anti-TCR mAbs en peptide/MHC liganden. Deze data suggereerden dat de EBV-specifieke chimere TCRs een sterke stimulus nodig hebben om een signaal-drempel te bereiken. Afwezigheid van lysis van natief EBV-positieve cellen kan op verschillende manieren worden verklaard. Ten eerste: de dichtheid van chimere receptoren op TCR-getransduceerde T cellen zou te laag kunnen zijn om een functionele wisselwerking aan te gaan met target cellen. Ten tweede: de dichtheid van EBV-antigeen op *in vitro* gecultiveerde target cellen zou te laag kunnen zijn om een functionele wisselwerking aan te gaan met TCR-getransduceerde T cellen. En ten derde: EBV-specifieke T cel activatie zou speciale eigenschappen van de effector cel kunnen eisen (bijvoorbeeld hogere expressie van co-stimulatoire/adhesie moleculen en signaal moleculen), aan welke bulk-gecultiveerde, TCR-getransduceerde primaire humane T cellen niet kunnen voldoen. Deze studie demonsteerde dat herrichting van humane T lymfocyten naar EBV-geïnfecteerde cellen met

chimere TCR mogelijk is. Echter de receptoren missen voldoende gevoeligheid en moeten verder geoptimaliseerd worden.

De studies beschreven in hoofdstuk 2 tot 5 worden bediscussieerd in **hoofdstuk 6**. De resultaten beschreven in hoofdstuk 2, 4 en 5 bevestigen dat de NFAT-reporter assay gebruikt kan worden voor de functionele analyse van tumor-specifieke (chimere) receptoren, en dat de assay voorziet in een goede maat voor immuun-functies gemedieerd door deze receptoren in getransduceerde primaire humane T lymfocyten. Deze assay maakt daardoor een snellere selectie van receptoren voor de immunogen-therapie van kanker en virale infecties mogelijk. Receptor-getransduceerde Jurkat T cellen kunnen ook helpen bij de verduidelijking van interacties tussen receptoren en andere T cel moleculen, en de ontrafeling van intracellulaire signaal routes.

Studies met As-gebaseerde receptoren hebben laten zien, dat individuele receptor componenten een grote invloed op receptor expressie en functie kunnen hebben, en dat verandering van deze componenten tot geoptimaliseerde receptoren kan leiden. De geïnitieerde klinische trials met T lymfocyten, die een As-gebaseerde chimere receptor expresseren, zullen ons essentiële kennis over toxiciteit en klinische efficiëntie van deze cellen geven. Bevindingen beschreven in dit proefschrift over complete keten en chimere TCR-gebaseerde receptoren, specifiek voor tumor en virale antigenen, demonstreren hun geschiktheid voor de genetische herrichting van T lymfocyten. Chimere TCR-gebaseerde receptoren kunnen voordelen t.o.v. ck-TCR hebben, i.e., introductie van ongewenste TCR specificiteiten, lage TCRα keten expressie en lage expressieniveaus van gewenste TCR$\alpha\beta$ heterodimeren worden voorkomen door het gebruik van chimere TCR. Analoog aan As-gebaseerde receptoren kunnen chimere TCR-gebaseerde receptor-formaten worden geoptimaliseerd. Naast TCR-gebaseerde MHC-gerestricteerde receptoren zijn MHC-gerestricteerde Fab-gebaseerde receptoren ontwikkeld. Deze MHC-gerestricteerde specificiteiten kunnen onafhankelijk van de beschikbaarheid van tumor-specifieke CTL clonen en hun TCR genen gegenereerd worden. Bovendien kan de ligand bindingsaffiniteit van deze Fabs verhoogd worden door affiniteitsmaturatie. Het is echter onduidelijk welk effect een verhoogde affiniteit van de Fab-gebaseerde receptoren op T cel activatie heeft.

Samengevat: de mogelijkheid primaire humane T lymfocyten te transduceren met antigeenspecifieke As-gebaseerde, TCR-gebaseerde en TCR-achtige Fab-gebaseerde (chimere) receptoren, met een optimaal receptor-ontwerp, zal ons van krachtige nieuwe instrumenten voorzien voor de behandeling van virale infecties en kanker. Preklinische studies in muizenmodellen en klinische trials met receptor-getransduceerde T lymfocyten moeten het bewijs leveren van de efficiëntie van dit type immunogen-therapie.

Dankwoord

Hoe begin je een dankwoord? Dit is een van de moeilijkste onderdelen van het proefschrift. Ik wil immers zo veel mensen bedanken die een bijdrage tot het geheel hebben geleverd en absoluut niemand vergeten.

Goed; beginnen we bij het begin: Jan-Willem Gratama en Reinder Bolhuis. Jan-Willem en Reinder, onder jullie leiding was het EBV-project gestart, en kreeg ik als tweede een kans om daarop te promoveren. Bedankt daarvoor. EBV bleek toch moeilijker dan verwacht, maar met de nodige zijsprongetjes, die nu de hoofdmoot van dit proefschrift vormen, is het toch gelukt om een mooi boekje te maken. Ralph Willemsen, bedankt voor de dagelijkse begeleiding in het begin van het onderzoek; ik heb veel van je geleerd. Gerrit Stoter, bedankt dat je mijn promotor wil zijn, dank voor de ondersteuning in deze laatste fase en de geruststellende telefoongesprekken. Natuurlijk mijn co-promotor, Reno Debets, heel erg bedankt voor de begeleiding en steun in de laatste helft van het onderzoek. Bedankt dat je keer op keer door de manuscripten bent gegaan, en ook bedankt voor alle adviezen. Zonder jou zouden er nu niet zulke mooie papers zijn, en was het boekje waarschijnlijk verre van af. Birgit, ik ben je natuurlijk niet vergeten. Naast het vele werk wat we samen hebben gedaan, vond ik de Wednesdaynight-skate heel erg gezellig. Ook jij bedankt.

Dan zijn er nog heel veel mensen die direkt of indirekt hebben bijgedragen aan resultaten, gezelligheid en vreugde in het werk. Ook allemaal bedankt! Cees, nog even volhouden en dan kan je lekker met de VUT. Brigitte, Pascal (Ajax is toch beter!), Barbara en Cor (de ananastaart wordt nu ook in Duitsland beroemd), heel veel succes met de klinische studie. Heel erg spannend allemaal; hou me op de hoogte. Emmy, ik hoop dat je veel plezier hebt in het lesgeven. Thierry (dat zeg ik...) misschien kom je nog eens stage lopen; je bent welkom. Heel veel dank aan de leden van de "scc": Mirjam, Lenard, Hans, Zsolt (Isten hozott Hollandiában, sok szerencsét!), en natuurlijk de partners+kids. De volgende bijeenkomst wordt waarschijnlijk een internationale. Mirjam en Hans, ik vind het heel leuk dat jullie mijn paranimfen willen zijn. Chris en Smiriti, ook al kennen we elkaar nog niet zo lang, ik vond het gezellig als ik weer eens langs kwam op het lab.
De ex-dames van het secretariaat, Marja en Carla, en de huidige dames van het secretariaat, Pat, Joke en Marjan, heel erg bedankt voor alle hulp bij de afronding van manuscripten en het proefschrift. En ook alle mensen van boven bedankt.

Verder wil ik de mensen van buiten het lab waarmee we hebben samengewerkt, zoals Peter Schrier en Corlien Aarnoudse (Leiden) en Gosse Adema en Jolanda de Vries (Nijmegen), hartelijk bedanken voor hun input.

Alexander, dir einen großen Dank für dein Verständnis und daß du es mir ermöglicht hast, meine Doktorarbeit ab zu schließen. Jan und Peter, auch vielen Dank für die Fortsetzung von den Experimenten während meiner Abwesendheit.

Naast de "lab-mensen" wil ik zeker ook een aantal andere mensen bedanken. Sjoerd, jammer dat je nu in de USA zit, en ik in Duitsland, want nu moet ik de computer problemen echt zelf oplossen. In ieder geval bedankt daarvoor en voor de gezellige etentjes. Slávko, děkuju moc za pomoc s vydáním téhle knížky. Mam en pap, op jullie slaat de stelling "Contact met patiënten is essentieel om een klinisch gerichte onderzoeker aan zijn doel te herinneren". Toch wil ik het niet nog eens van zo dichtbij beleven. Bedankt voor alle steun, en vergeet niet: wij zijn sterker dan kanker! Broertje bedankt voor het uitlenen van de DD (ontspanning moet er ook zijn). En niet te vergeten: Jesika. Bedankt dat je er altijd was (in goede en slechte tijden), voor de oneindige steun en peptalks! Ik hou van je!

Hoe eindig je een dankwoord?
BEDANKT.

Niels

Curriculum Vitae

Op 18 december 1974 werd ik geboren in Hoorn. Van 1987 tot 1993 volgde ik het voorbereidend wetenschappelijk onderwijs aan de OSG West-Friesland in Hoorn. In 1993 begon ik de studie Medische Biologie aan de Universiteit van Amsterdam. Tijdens deze studie liep ik stages bij de afdeling Klinische (Viro) Immunologie (Prof. F. Miedema) van het Centraal Laboratorium voor Bloedtransfusie (CLB) in Amsterdam en de afdelingen Immunologie/Gnotobiologie en Microbiologie (Prof. H. Tlaskalová en Dr. P. Šebo) van de Academie der Wetenschappen van de Tsjechische Republiek in Praag. De behandelde onderwerpen waren respectievelijk: "Bepaling van telomeer-lengte door Southern hybridisatie in HIV-negatieve individuen, en ontwikkeling van een nieuwe assay gebaseerd op Polymerase Chain Reaction" en "Recombinante adenylate cyclase toxines, die vreemde epitopen expresseren, gebruikt als een methode voor de inductie van specifieke immuun responsen". In 1997 studeerde ik af, en in 1998 begon ik als AIO bij de afdeling Medische en Tumor Immunologie van de Daniel den Hoed Kliniek op een door de Nederlandse Kankerbestrijding gefinancierd project. Het onderzoek wat ik daar heb verricht, heeft geleid tot de totstandkoming van dit proefschrift. Sinds oktober 2002 ben ik werkzaam als wetenschappelijk onderzoeker aan de Dermatologische Kliniek (Prof. G. Schuler) van de Friedrich-Alexander Universiteit Erlangen-Neurenberg in Erlangen (Duitsland).

List of publications

Wolthers KC, Bea G, Wisman A, Otto SA, de Roda Husman AM, **Schaft N**, de Wolf F, Goudsmit J, Coutinho RA, van der Zee AG, Meyaard L, Miedema F. (1996) "T cell telomere length in HIV-1 infection: no evidence for increased $CD4^+$ T cell turnover." *Science; 274(5292):1543-7.*

Sebo P, Moukrim Z, Kalhous M, **Schaft N**, Dadaglio G, Sheshko V, Fayolle C, Leclerc C. (1999) "In vivo induction of CTL responses by recombinant adenylate cyclase of Bordetella pertussis carrying multiple copies of a viral $CD8^+$ T-cell epitope." *FEMS Immunol. Med. Microbiol.; 26(2):167-73.*

Schaft N, Willemsen RA, De Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R, Adema GJ. (2003) "Peptide Fine Specificity of Anti-Glycoprotein 100 CTL Is Preserved Following Transfer of Engineered TCRalphabeta Genes Into Primary Human T Lymphocytes." *J. Immunol.; 170(4):2186-94.*

Schaft N, Lankiewicz B, Gratama JW, Bolhuis RL, Debets R. (2003) "Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT." *J. Immunol. Methods; in press.*

Schaft N, Lankiewicz B, Knoop C, Willemsen RA, De Vries JM, Adema GJ, Debets R. "An altered gp100 TCR peptide ligand shows a decreased binding to TCR-transduced human T lymphocytes and dissects cytotoxicity from TNFα production and NFAT activation." *Submitted.*

Schaft N, Lankiewicz B, Moss DJ, Levitsky V, Bonneville M, Lee SP, McMichael AJ, Willemsen RA, Gratama JW, Bolhuis RL, Debets R. "Epstein-Barr virus-specific retargeting of primary human T lymphocytes following transfer of chimeric TCR genes." *Submitted.*

Stellingen bij het proefschrift

"Redirecting human T cells to tumors via transfer of T cell receptor genes: a study of tumor-specific T cell responses and peptide fine-specificity"

1. Activatie van NFAT is een functionele maat voor antigeen-specifieke T cel responsen gemedieerd door genetisch geïntroduceerde anti-tumor receptoren.
   Dit proefschrift

2. Jurkat T cellen, waarin (chimere) anti-tumor receptoren via gen-transfer zijn geïntroduceerd, voorzien in een gemakkelijke en gevoelige methode voor de functionele validatie van deze receptoren.
   Dit proefschrift

3. T-cel receptoren, afkomstig van tumor-specifieke cytotoxische T lymfocyten, kunnen middels gen-transfer functioneel geïntroduceerd worden in primaire humane T cellen zonder verlies van antigeen-reactiviteit en peptide fijn-specificiteit.
   Dit proefschrift

4. Een gp100 peptide mutant, welke zich als een partiële agonist gedraagt, initieert in T cellen receptor-gemedieerde cytotoxiciteit en TNFα productie, maar niet NFAT activatie.
   Dit proefschrift

5. In tegenstelling tot melanoma-specifieke chimere T cel receptoren, zijn EBV-specifieke chimere T cel receptoren niet in staat humane T cellen aan te zetten tot het doden van antigeen-positieve doelwit cellen.
   Dit proefschrift

6. Vaccinatie met messenger-RNA-getransfecteerde autologe dendritische cellen is geen gentherapie.

7. De wereld is klein; de wetenschappelijke wereld is nog veel kleiner.

8. Hoewel de werking van het dendritische-cel-systeem zeer complex is, vormen dendritische cellen een aantrekkelijke target voor therapeutische manipulatie van het immuunsysteem, zowel voor het verhogen van ontoereikende immuun reacties, als voor het verzwakken van buitensporige immuun reacties.
   J. Banchereau et al., Cell 106, 2001

9. Contact met patiënten is essentieel om een klinisch gerichte onderzoeker aan zijn doel te herinneren.

10. Een mens is van hetzelfde spul gemaakt als een mus of een salamander; het verschil zit hem niet in de materie maar in de vorm.
    M. Dekkers, De Larf, over kinderen en metamorfose, 2002

11. Met een extra hoge belasting voor werklozen zou Duitsland onmiddellijk uit het economische dal herrijzen.

Niels Schaft

Rotterdam, 5 juni 2003