GENETIC ENGINEERING OF T CELL SPECIFICITY

GENETISCHE CONSTRUCTIE VAN T CELL SPECIFICITEIT

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CHAPTER 1

General introduction

GENETIC ENGINEERING OF T CELL SPECIFICITY

INTRODUCTION

The development of new strategies for the treatment of cancer and infectious diseases have primarily been based on advances in basic biology. Technologies that evolved from our understanding of the immune system include monoclonal antibody production, isolation and cloning of immune cells, large scale tissue culture, cloning of genes encoding all kinds of functional molecules and the development of technologies to transfer these cloned genes to somatic cells of choice. To date the most effective and specific eradication of solid tumors is surgical removal, but this has not resulted in overall cure of patients because metastasis of tumors are not always accessible for the surgeons knife. Therefore T cell-based strategies that aim to selectively target and destroy cancer cells are being developed.

IMMUNE RECOGNITION

The presence of specific receptors on immune cells is fundamental to immunologically based therapeutic strategies. These receptors recognize foreign antigens such as viruses, bacteria, MHC-antigens on partially or fully mismatched organ grafts or antigens on malignantly transformed cells. All these antigens are distinct from "self" antigens, and from so-called "self" antigens that are expressed in an "aberrant" way, and thus may serve as targets for the immune cells. Soluble antigens are primarily neutralized by antibodies (Abs), but when presented by MHC, by immune cells. The idea that the immune attack leaves normal and non-infected tissues "self" intact and eliminates "non-self" was postulated at the beginning of the 20th century by Paul Ehrlich in his hypothesis of "magic bullets" [1]. At the end of the 19th century, studies were initiated that aimed to actively immunize cancer patients against their own cancerous tissue. Cancer patients were non-specifically immune-stimulated with relatively crude leukocyte extracts such as transfer factor, immune-RNA, bacterial extracts such as bacillus Calmette-Guérain or Coley's toxin or levamisol. These studies were initiated in spite of the fact that little was known about the various components of the immune system that could react against cancer and even less was known about tumor associated antigen structures.

In 1970, the immune-surveillance theory was reformulated by Burnett, emphasizing the critical role of cell-mediated immunity in the control of cancer and infectious diseases (2). The central focus in basic and applied immunology became the study of "self" versus "non-self" recognition by immune cells. Yes, the immune system continuously scans whether in the host "self" becomes "non-self" due to e.g. viral infections; to neoplastic transformation, or to aberrant "self" expression. The first line of defense we know in the body is provided by the relatively non-specific NK cells and macrophages (3).

In the 80s, the second wave of immunotherapy started, now with emphasis on the adoptive transfer of "immune-specific" cells. Clinical studies were performed and adoptive transfer of immune cells then was combined with mAbs and cytokines (4,5). The ultimate goal of all these efforts was and is to generate a specific and long lasting immune response that not only eradicates or controls the growth of tumor cells, but also provides protection for future encounters: protection through immunologic memory. In this concept, the immune intervention is thought to *in vivo* trigger the second line of the body defense, which involves macrophages, antigen presenting cells, eosinophils and the full range of NK, B and T cells. The $CD3^{POS}$ T and the $Immunoglobulin^{POS}$ B cells of the acquired immune system demonstrate exquisite antigen specificity. T cells recognize MHC-presented antigens: through the T cell receptor (TCR) (6), but B cells recognize antigens in an MHC-unrestricted fashion: through the membrane anchored immunoglobulins (Ig) (7). The T cell population comprises $CD8^{POS}$ cytotoxic T lymphocytes (CTLs), that specifically kill target cells, and T helper (T_H) cells that produce cytokines and regulate Ig synthesis. Antigen presenting cells such as macrophages, dendritic cells and others are important initiators of the immune response against foreign invaders. At any rate, all immune cells communicate with each other through membrane anchored receptors which mediate cell-cell contact, followed by intracellular and intercellular transduction of immune regulatory signals and through cytokine production.

T lymphocytes, key players in the cellular immune response.

A major focus in modern cancer research has been to explore the possibility to use the prime mediators of adaptive cellular immunity, the T

lymphocytes, to combat cancer and viral infections.

TCR on T lymphocytes specifically recognize and bind to antigens presented by MHC class I or class II molecules (6). As such T cells, like NK cells, scan the surface of body cells for the presence of peptides derived from degraded pathogens. The TCR is a multi-chain complex consisting of an Ig-like antigen binding portion: the polymorphic $\alpha\beta$ (8) or $\gamma\delta$ (9) TCRs that are non-covalently linked to non-polymorphic signal transducing elements: the CD3/$\delta\varepsilon$, CD3/$\gamma\varepsilon$, ζ and η chains (8). The $\alpha\beta$ and $\gamma\delta$ chains are disulphide-linked heterodimers or tetramers and each chain contains an invariant constant (C) region and an antigen binding variable (V) region. The exquisite antigen specificity and diversity of TCRs is the hallmark of the T cell response and is mainly created through gene rearrangements.

T lymphocyte immune functions comprise cytokine production by T_{H1} and T_{H2} cells, and lytic functions by cytotoxic T lymphocytes (CTL) to preserve the integrity of the interior milieu of an individual.

When T lymphocytes recognize antigens in the absence of (a) co-stimulatory and danger signal(s) they become tolerant or anergic (10). Although the intrinsic binding affinities of TCRs for antigen are low in comparison to Abs, already low numbers of MHC-peptides per target cell alert T lymphocytes and induce TCR signaling that triggers immune functions such as cytolysis, cytokine production, cell-cell communication, cell trafficking and homing. This is possible because the same peptide/MHC complexes can serially bind to TCRs after their disengagement from TCR following signal transduction (11,12).

Although most attention in cancer immunology has focused on $CD8^{POS}$ CTL and their lytic response to tumor cells, $CD4^{POS}$ T lymphocytes should deserve equal or even more attention because of their immune regulatory roles in e.g. the defense against cancer and viral infections. Adoptive transfer of tumor specific $CD8^{POS}$ CTL has shown effective immune specific anti-tumor responses in mouse models as well as in humans (13-17). However, $CD4^{POS}$ T lymphocytes not only provide critical help to $CD8^{POS}$ CTL, but also play a direct role in killing tumor cells. Furthermore $CD4^{POS}$ T lymphocytes are essential in the initiation and persistence of a protective immune response in viral disease (18, 19). This was demonstrated in clinical studies using human Cytomegalovirus (CMV)-specific CTL clones for the treatment of CMV reactivation following bone marrow transplantation (19). After adoptive transfer of CMV specific $CD8^{POS}$ CTL to patients, which were deficient in $CD4^{POS}$ CMV specific T lymphocytes, cellular immunity to CMV declined. Adoptive transfer of $CD4^{POS}$ tumor specific T lymphocytes therefore appears to be clinically relevant for effective anti-tumor responses. Indeed, in mice, the adoptive transfer of activated MHC class II restricted, tumor specific $CD4^{POS}$ T lymphocytes has resulted in de novo generation of tumor specific $CD8^{POS}$ T lymphocytes and effective anti-tumor responses (20).

TUMOR REJECTION ANTIGENS

Identification of tumor rejection antigens presented by MHC class I and II molecules

Clinical studies carried out with tumor-infiltrating lymphocytes (TIL) and *in vitro* stimulated peripheral blood mononuclear cells (PBMC) demonstrated that $CD8^{POS}$ T cells can specifically lyse tumor cells, or histogeneticaly unrelated tumor cells, but not normal cells, in an MHC restricted fashion (6, 21,22). The MHC class I molecules are expressed on the surface of virtually all nucleated cells, where they serve as targets for $CD8^{POS}$ T cells. An exemption are cells in testes and placenta trophoblasts, that do not express MHC molecules, and thus can not serve as tumor antigen presenting targets for $CD8^{POS}$ T cells. Investigators set out to identify MHC presented antigens recognized by tumor reactive T cells, the so called tumor rejection antigens (TRA) (reviewed in 23). To this end, cDNA or DNA cosmid pools derived from tumor cells were transiently transfected into tissue cultured target cells expressing identical MHC class I molecules. These cultured target cells expressing the MHC/TRA complexes were then assayed for their ability to specifically stimulate cytokine release from tumor reactive T cell clones via their TCR (24). Transfection of individual cDNAs or cosmids from pools that specifically stimulated the T cell clones then allowed the identification of the single TRA. The first human TRA that was identified in this way by screening a large genomic library from a melanoma patient was termed MAGE-1 (24). To identify the peptide epitope encoded by the MAGE-1 gene, that was presented by HLA-A1 molecules, synthetic peptides were synthesized. Peptides presented by MHC class I molecules are usually 8 to 10 amino-acids long, and contain two to three residues, so called anchoring residues, that fit into specific pockets of the MHC molecule (25). These synthetic peptides, that fit the HLA-A1 pockets, were then analyzed for their capacity to specifically stimulate a relevant cytotoxic T cell clone (26). This "reversed immunology" technology has since identified a large number of TRA, that are divided into distinct categories, based on their expression patterns in tumors and "normal" tissues (Table 1). Next, MHC-class I peptide binding motifs, i.e., the nature and position of pockets that

bind the anchoring residues of the TRA peptides have also been defined, allowing the screening of large numbers of potentially immunogenic TRA and other peptides (25).

The MHC class II molecules present peptides that are mainly derived from the extracellular compartment, as opposed to peptides presented by MHC class I molecules that capture endogenously processed peptides (27).

Table 2 shows MHC class II TRA peptides that have been identified. Sofar, the identification of class II binding peptides has met more problems then the identification of class I binding peptides due to (*i*) the variable length of class II bound peptides, and (*ii*) because less information is available on class II peptide anchoring residues. In fact, to date there exists no strategy for the identification of class II restricted antigens that matches the successes obtained in the identification of class I restricted antigens.

Recently, technologies have emerged that allow the generation of soluble MHC class I and MHC class II molecules containing peptides of choice, provided of course that these peptides contain the right anchoring residues (28). In spite of the fact that monomeric soluble MHC Class I molecules are poorly bound by specific TCR on T lymphocytes they do so when converted into tetrameric peptide/MHC complexes. Importantly, when relevant tetrameric peptide/MHC class I molecules are complexed with a fluorochrome they can now be used to identify and select virus or tumor specific T lymphocytes (29, 30). Fortunately, these soluble peptide/MHC complexes can also be used to select antibodies with MHC restricted specificity from large phage display libraries (31-33).

Table 1: MHC Class I presented Tumor Rejection Antigens (TRA)

Tumor antigens with mutations	Testis antigens	differentiation antigens	overexpressed in tumors
BCR-ABL	MAGE-1-10	CEA	G250
CASP-8		gp100	HER-2/NEU
Beta-catenin		MART-1	MUC-1
Cdc27		PSA	p53
CDK4		TRP-1	Hdm2
ETV6-AML1		Tyrosinase	PRAME
Hsp70-2	NY-ESO-1		RAGE
K-ras	LAGE-1		Telomerase
MUM-2	BAGE-1		
	GAGE-1-8		

Table 2: MHC Class II presented Tumor Rejection Antigens (TRA)

Tumor antigens with mutations	Testis antigens	differentiation antigens	overexpressed in tumors
CDC27/m	MAGE-1-6	Tyrosinase	
TPI/m	NY-ESO-1		
BCR-ABL			

ANTIBODIES AND TUMOR TARGETING:

Antibodies produced by B cell derived plasma cells are the key molecules of the humoral immune system. Antigens such as bacteria, allergens and possibly tumor associated antigens (TAA) expressed on tumor cells, but not presented by MHC molecules, are eliminated by NK cells, macrophages, monocytes and mast cells expressing receptors that bind to the Fc region of the antigen-coated Ab. The NK cells, macrophages, monocytes and mast cells exert antibody dependent cellular cytolysis of tumor target cells when they are targeted by TAA specific Ab (34).

Tumor Associated Antigens (TAA): tumor targets for antibodies

TAA are not presented by MHC molecules and hence can not be identified by tumor reactive CTL, but are identified by Ab obtained after immunization of the host with TAA^{POS} tumor cells. Many of these TAA are merely over- or aberrantly expressed on tumor cells when compared with expression on normal cells. Although the TAA are not "tumor specific' in the strict immunological sense, they can effectively be used to selectively target the immune system to tumors resulting in clinical anti-tumor effects (35). Like TRA, TAA are also categorized based on their expression patterns in tumors and normal tissues (Table 3). Finally, one can identify a group of antigens that are not related to tumors, with respect to expression patterns, but that are tissue specific. Antigens such as CD19 and CD20 are expressed by B cells and serve as target structures for immuno-therapeutic intervention (35).

Table 3: Tumor associated Antigens (TAA)

Antigen	Tumor	Expression
Angiogenic endothelial receptor: KDR	Tumor vasculature	overexpressed
Carcinoembryonic antigen: CEA	Colorectal, breast, lung and other cancers	overexpressed
Her-2/Neu/ERB2	Adenocarcinomas	overexpressed
TAG72	Gastrointestinal adeno-carcinoma and pan-adenocarcinoma	overexpressed
Folate receptor	Ovarian cancer	overexpressed
Vascular endothelial growth Factor: VEGF	non-small-cell lung cancer colorectal cancer	overexpressed
Epidermal-growth-factor Receptor: EGFR	colorectal cancer	overexpressed
Epithelial cellular-adhesion Molecule:EpCam	colorectal cancer	overexpressed
CD33	acute myelogenous leukaemia	tissue specific
CD20	B-cell lymphoma	tissue specific
Prostate specific membrane Antigen:PSMA	Prostate cancer	tissue specific

Phage display: a technology to produce libraries of immune receptors

Phage display involves the expression of proteins on the surface of phages fused to proteins attached to normal phage coat proteins (36). It is the physical link between the protein on the phage surface and the gene encoding it within the phage that allows the rapid evolution of protein properties on the basis of binding by a series of repetitive cycles of phage binding, elution and re-growth to amplify the phage population of choice. The phage display technology also allows enhancement of the affinity of selected Abs by mutation and further selection (37). From libraries of randomly paired combinations of Variable (V) domains of heavy and light chains, structurally formatted as single chain Abs (scFv), Fab or diabody fragments, phages can be specifically selected. Human mAbs can also be generated when the libraries are constructed from "naive" human B cells, or from cloned human gene segments (37, 38). Indeed large repertoires now have been made, with over 10 billion different mAbs, and thus mAbs to any "chosen" antigen, and with affinities ranging from moderate to high, can easily be isolated.

Because of the high affinity, Ab with the same MHC-restricted specificity as TCR i.e., recognizing peptides from tumor rejection antigens (TRA) and presented by MHC class I or II molecules, are valuable tools both for the identification of target tumor cells as well as the construction of receptors that allow specific targeting of primary human T lymphocytes. Only recently such Abs have been isolated from a large "non-immune" phage display

library, with the advent of the technology to produce soluble MHC/antigen complexes (31-33). The phage-Ab G8, specific for the MAGE-A1 antigen presented by HLA-A1 was isolated from a large "non-immune" human Fab-phage library, using soluble peptide/MHC complexes (31). The use of peptide/MHC complexes for the selection of specific phages has now resulted in the selection of mAb specific for at least 12 different HLA-A2 complexes presenting different tumor or viral peptides (32,33). These findings demonstrate that selection of anti-peptide/MHC antibodies against, in principle, any complex is feasible, and that this technology may become a reliable and routine source of viral or tumor antigen specific receptors.

IMMUNOTHERAPY OF DISEASE WITH THE ADOPTIVE TRANSFER OF IMMUNE CELLS

Tumor Infiltrating Lymphocytes: TIL

As described earlier, immune system based therapies employing tumor reactive T cells have been developed for patients with, e.g. metastatic melanoma, and have resulted in significant tumor reduction or even complete remissions in patients (15-17, 39-41). Especially tumor infiltrating lymphocytes (TILs) that were grown from murine and human tumors were effective in pre-clinical and clinical studies. In mice, TILs were shown to recognize unique antigens on murine transplantable tumors and adoptive transfer of TILs mediated rejection of established lung and liver metastasis (42).

Anti-tumor responses in melanoma patients were significant, with an objective regression of cancer in 34% of patients, irrespective whether they had earlier responded to Interleukine-2 (IL-2) treatment or not (15). These TILs were able to: 1) specifically traffic to tumor sites; 2) lyse the relevant tumor cells, and 3) produce cytokines following interaction with the tumor targets (40,41). In cancer patients, unfortunately, such TILs can only be established at low frequencies, with the exception of melanoma where a success rate of 50% could be reached (15). At this point in time the TIL approach therefore does not provide a clinically practical and reliable approach for immuno-therapy of cancer patients, with the exception of melanoma patients.

Targeting of CTL with bi-specific-mAbs

In contrast to the lack of success in reliably generating TRA-specific T lymphocytes in individual patients, a plethora of mAbs were already available with specificity for TAA that are common to a wide range of cancers (table 3). To combine the ability of these mAbs to selectively recognize and bind to tumors with the potent anti-tumor functions of T lymphocytes, bi-specific-(bs)-mAbs were designed with the aim to retarget the immune specificity of T lymphocytes to TAA (43,44). The multiple bonds created by bs-mAbs between TAA and, e.g. the CD3 complex on T cells result in crosslinking of receptors and activation of the T lymphocyte machineries for a) cytokine production; b) T cell proliferation, and c) cytolysis of target cells. The induction of human anti-mouse bs-mAb (HAMA) responses that were observed in the patients could be dramatically reduced or even avoided entirely when gene encoding Ab fragments of human origin were used for the construction of bi-mAbs (45). Such (bs)-mAbs from human origin are now readily available due to the advances made in phage display technology.

Bs-mAb targeted T lymphocytes for cancer treatment: the clinic.

We and others have exploited the capacity of bs-mAbs to induce lymphocyte activation and to trigger the lytic and cytokine producing machineries of T cells for *in vivo* preclinical and clinical studies (43-48). The first international multi-center study involved locoregional (intraperitoneal) treatment of ovarian cancer in patients with advanced disease (47). These patients were selected because of the high frequency of relapses after induction chemotherapy. This high relapse rate called for an innovative treatment approach. Patients eligible for this study underwent laparotomy in order to surgically reduce tumor load and to objectively locate and document remaining lesions. Immunotherapy consisted of two 5-day cycles of treatment of daily intraperitoneal injections of *in vitro* activated and expanded autologous T cells targeted with bs-mAbs specific for CD3 on T cells and the folate receptor which is over-expressed on ovarian carcinoma cells. Per infusion, 10^9 T lymphocytes that were targeted with bs-mAbs, were administered into the peritoneal cavity. Patients received one additional infusion of IL-2 and bs-mAbs to maintain T cell survival, lytic activity and bs-mAb dictated specificity. An impressive overall anti-tumor intraperitoneal response of 27% was observed, with 3 patients showing even complete remissions in the intraperitoneal cavity.

It is important to note that the beneficial and objective, significant anti-ovarian cancer effects of the bs-mAb-retargeted T lymphocytes were only loco-regional. Extraperitoneal lesions were not affected by this immune-therapy (47). Other clinical pilot studies that involved infusion of bs-mAb targeted T cells regarded patients with malignant ascites or plural effusions resulting from

colon, mammary, ovarian, lung- and gastric carcinomas (48). In these patients also strong biological *in vivo* effects were observed within hours after infusion. These clinical studies have provided "proof of concept" and set the stage for the further development of combined humoral and cellular anticancer treatments.

TARGETING NON-MHC RESTRICTED, TUMOR ASSOCIATED ANTIGENS.

Grafting T cells with antibody-based receptors.

The use of bs-Abs for cancer therapy is hampered for several reasons: (a) the inaccessibility of solid tumors to Ab penetration (49); (b) bs-Ab targeted T lymphocytes retain the bs-Ab on their surface for only a limited time, i.e. 48-96 hrs, due to dissociation (50); and (c) bs-Ab targeted T lymphocytes loose their signal transducing and lytic capacity following target cell recognition and lysis (51). We and others developed an alternative approach in which T lymphocytes are grafted with a permanent Ab-dictated TAA specificity (reviewed in 52-54). To this end, chimeric receptors were constructed that exert two functions: 1) antigen binding mediated through an Ab domain, and 2) T cell activation mediated through an intracellular domain derived from a signaling receptor. Chimeric receptors have been constructed from V_H and V_L domains of Abs, and fused to the constant regions of TCR α and β chains, thereby creating chimeric $V_HC\beta$ and $V_LC\alpha$ chains (55,56). These chimeric $V_HC\beta$ and $V_LC\alpha$ receptors were functionally expressed on the membrane of T cells, resulting in receptor mediated immune functions, such as target cell recognition and lysis as well as cytokine production.
At that time the necessary gene-transfer technology to simultaneously introduce two genes into large numbers of primary human T cells was not yet fully developed, for which reason single chain Ab fragments have been constructed (scFv). These molecules incorporate the V_H and V_L domains from a mAb, joined by a flexible linker, into one gene (57-59). Such scFv display similar antigen binding affinities and antigen specificities as the "parental" mAb from which they were derived (60-62). Chimeric scFv receptors were constructed by fusing the ligand-binding domain to a signal-transducing element that allows for the activation of specific immune functions (56,63). Most of the chimeric Ab-based receptors described use either Fc(ε)RI γ or CD3-ζ for signaling, and have been functionally expressed in mouse T cell hybridomas, CTLs, TILs, human NK cells and primary human T lymphocytes (57-60, 63-67). Stimulation of chimeric Ab receptor^{POS} T cells with relevant TAA^{POS} tumor cells results in specific T cell activation and specific immune functions (52-60, 63-67). There exists a functional balance between the level of chimeric receptor expression on T lymphocytes on the one hand and TAA density on tumor cells on the other hand (68,69). In example, T lymphocytes having a high density of the chimeric receptor were able to lyse tumor cells that express either high or low levels of G250 antigen, whereas T cells that have a low density of the chimeric receptor only lyse tumor cells that express high levels of G250 antigen (68). Importantly, the level of chimeric receptor expression may be controlled by the use of a tetracycline trans-activator responsive promotor, which enables fine-tuning of the T cell mediated responses (69). Next to receptor and its antigen, critical roles are played by adhesion and accessory molecules, such as CD2, CD3, CD11a, CD18 and CD28 in the activation of engineered T cells (70).

Chimeric antibody based receptors and their *in vivo* efficacy

The *in vivo* anti-tumor efficacy of T lymphocytes, equipped with chimeric Ab-based receptors, was evaluated in mouse tumor models (71-73). In one of these studies nude mice intraperitoneally implanted with human ovarian cancer were treated with murine TIL cells expressing a chimeric scFv/γ receptor recognizing the folate binding protein (FBP), present on ovarian cancer cells. Mice treated with these gene modified TIL had significantly increased survival compared to mice treated with TIL expressing an irrelevant receptor (71). A study performed by Altensmidt *et al.* demonstrated complete remission of established tumors expressing the ErbB2 antigen upon injection of syngeneic T cells transduced with the ErbB2 specific scFv/ζ receptor (72). Furthermore, Mouse T lymphocytes expressing chimeric scFv/ζ receptors specific for the carcinoembrionic antigen (CEA) were able to control and reject human colon carcinoma in *scid* mice or mouse colon adenocarcinoma in syngeneic C57BL/6 mice (73-75). So far, *in* vivo studies show partial and complete remissions of cancer as a consequence of treatment with chimeric mAb-engrafted T cells. Chimeric receptor^{POS}, CD8^{POS} T cells produce perforin and IFN-γ, that are critically involved in the anti-tumor effect (73-75). Other types of immune cells, such as natural killer, $\gamma\delta$ T cells or macrophages may be effective as anti-tumor cells when grafted with Ab-based receptors. Wang *et al.* reported that retroviral introduction of a FBP specific chimeric Ab-based receptor into mouse bone marrow cells resulted in significant anti-tumor responses (76). Importantly, in this study *in vivo* T cell depletion did not affect the observed anti-

tumor activity, suggesting an important role for other non-T cells in tumor rejection.
Adoptive transfer of human T cells genetically engineered to express human immunodeficiency virus HIV) specific chimeric CD4-ζ receptors to patients was reported to be safe (77,78). Importantly, long-term survival and specific tissue trafficking following adoptive transfer of chimeric receptor positive, HIV specific T cells in HIV infected patients was observed.

Optimal design of chimeric Ab-based receptors: introduction of co-signaling elements.

To optimize expression and function of mAb-based chimeric receptors, spacer or hinge domains can be selected and introduced into the chimeric receptor constructs (Fig 1, refs 79-81). Spacers extend the distance between the antigen binding domains and the T cell membrane, adding flexibility to the chimeric receptor construct. In fact, incorporation of a hinge domain such as the CH2-CH3 domain of the Ig heavy chain, or the CD4 transmembrane domain into chimeric Ab-based receptors enhances transgene expression and function of the T cell transductants (80,81). It is important to note that the transmembrane domain of CD3 components may negatively affect chimeric receptor-mediated responses. A study, analyzing the response of resting primary T cells derived from mice transgenic for chimeric receptors comprising the CD3-ζ transmembrane domain demonstrated that primary T cells might not respond upon cross-linking of the receptors (82). In contrast, others demonstrated efficient activation of chimeric receptor^{POS} T cells, lacking the CD3-ζ transmembrane domain upon receptor triggering (83,84). These apparently conflicting results may well be the consequence of distinct structural formats of the chimeric receptors. Upon cross-linking of chimeric/ζ receptors, the CD3-ζ but not non-TCR molecules such as the MHC class II chain may promote association of the chimeric receptor with the endogenous TCR CD3 complex, adversely affecting surface expression and responsiveness of the chimeric receptor. Chimeric receptor constructs that do support T cell activation, phosphorylation of the receptor ITAMs and recruitment of ZAP-70 to the ζ chain have been made (83).
The use of T lymphocytes equipped with antibody-based receptors for adoptive immuno therapy has raised some concerns. The high affinity of the mAb-based receptors may not allow T lymphocytes to recycle their lytic capacity (51, 85). Furthermore, the strong ligand binding capacity of the mAb-based receptor on T lymphocytes may even induce T cell apoptosis (86,87). These issues have been addressed experimentally, and co-stimulation of T cell constitutes an answer to the questions raised. Normally, resting primary T cells require two signals in order to become activated; a) one signal derived from the antigen specific TCR-peptide/MHC interaction, and b) a second co-stimulatory signal, following e.g. T cell-CD28/tumor cell-CD80, CD86 interactions (88,89). These signals stimulate T cells to proliferate and differentiate into potent effector cells. CD28 cross-linking on T cells prevents a) T cell apoptosis; b) results in increased cytokine production, and c) enhanced cytolytic activity (88,89). Hence, the combination of CD28 co-stimulatory signaling with chimeric receptor signaling leads to improved antigen-specific T cell activation (90-94). The synergistic action of two separate single chain chimeric receptors either linked to CD3ζ or CD28 resulted in the secretion of maximum levels of IL-2 (90). When introducing the CD28 domain into one single chain scFv/ζ or γ receptors, T cell transductants expressing the scFv-CD28/ζ or /γ receptors proliferated well, even without exogenous IL-2 upon stimulation with the relevant TAA^{POS} tumor cells (91-94). These T cell transductants also exhibited enhanced cytokine secretion and protection against apoptosis upon antigen stimulation, but no enhanced cytolytic capacity. Importantly, *in vivo* data have allowed a direct comparison between T lymphocytes that express chimeric receptors with or without the CD28 signaling domain in their efficacy to eliminate tumor cells (74,75). These data clearly demonstrated superior anti-tumor activities of T lymphocytes equipped with receptors that incorporate CD28 co-signaling capacity.
These pre-clinical *in vitro* and *in vivo* experiment calls for carefully designed clinical trails using patients T lymphocytes engineered to functionally express such tumor or virus specific receptors. Only a few clinical anti-cancer trails have been initiated, of which results are not yet available (Table 4). We designed a phase I clinical protocol with intend to treat patients with advanced metastatic renal cell carcinoma. Patients will be infused with autologous peripheral blood lymphocytes transduced with a renal cell carcinoma specific chimeric mAb-based receptor i.e. scFv G250-CD4/γ (95).

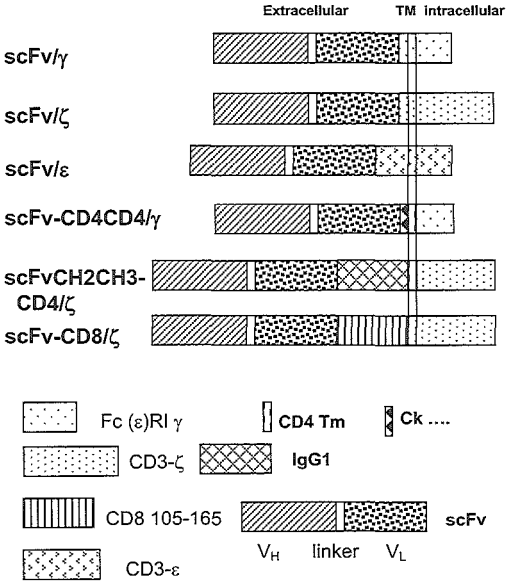

Figure 1. Chimeric scFv constructs. Schematic presentation of distinct scFv constructs, comprising one or several of the following domains: the hinge domains IgG1 CH_2CH_3 or CD8 (amino-acids 105 to 165), a short Ck fragment, the transmembrane region derived from CD4, the intracellular domain from the Fc(ε)RI γ chain or the complete CD3-ζ or CD3-ε molecules, as described in Weijtens et al [80], Patel et al [81] and Willemsen et al [112].

Table 4: Clinical trails with TAA specific mAb-based chimeric receptor engineered T lymphocytes.

Phase	Disease	Antigen	chimeric rec
I	Ovarian cancer	folate receptor	scFv-γ
I	HIV	gp120	CD4-ζ
I	Adenocarcinoma	TAG72	scFv-ζ
I	Adenocarcinoma	CEA	scFv-ζ
I	Lymphoma	CD19	scFv-ζ
I	Renal cell carcinoma	G250	scFv-CD4/γ

TARGETING MHC-RESTRICTED TUMOR REJECTION ANTIGENS

As indicated before, studies have demonstrated that $CD8^{POS}$ T cells specifically lyse tumor cells, or histo-genetically unrelated tumor cells, but not normal cells, in an MHC restricted fashion (6, 21,22). Investigators therefore set out to identify MHC presented antigens, the so called tumor rejection antigens (TRA), recognized by tumor reactive T cells (reviewed in 23). MHC presented TRA are recognized by TCR, but not classical mAbs. Therefore, in order to target the immune system to TRA or viral antigens one needs to graft T lymphocytes with genes encoding TCR or TCR-like receptors. Until 1995, one could only successfully introduce mouse TCR into mouse T cell lines or hybridomas, but not primary human T cells (96,97). Retroviral vectors were the obvious choice to introduce TCR genes into T cells at that time because they transmit genes to recipient cells as well as to their progeny in a relatively stable manner. However, retroviral vector technology was not advanced to a stage that sufficient numbers of primary human T lymphocytes could be efficiently transduced. Recently, we succeeded to adapt retroviral vectors in such a way that they efficiently and functionally facilitate expression of TCR and TCR-based receptors in primary human T lymphocytes (see below).

Concerns associated with TCR gene transfer.

Besides problems related to (retroviral) vector technology, the safe application of TCR gene transfer technology is associated with other problems (98). Transfer of complete TCR α and β genes to primary human T lymphocytes results in reciprocal pairing between introduced TCR αβ with endogenous TCR αβ chains, possibly, creating new and unknown immune-specificities. Theoretically, self-antigens may become recognized and cause an autoimmune response. In addition, pairing of introduced TCR with endogenous TCR chains dilutes the expression of functional introduced TCR αβ heterodimers. This may reduce the overall T cell avidity, immune functions and recycling capacity (99,100). Unstable TCR α surface expression in T cells may impose yet another problem to the introduction of non-modified (full-length) TCR chains (101). Unfortunately, the limited number of TCR Vα family type specific mAb available for the detection of the highly diverse TCR α chains present on T cells does not allow a detailed study of this phenomenon.

Luckily, new strategies become at hand that prevent pairing of introduced and endogenous TCR αβ chains and also resolve unstable TCR α

expression. TCR αβ heterodimers comprising specific signals into the TCR αβ chains to enhance expression and exclusive pairing may be developed. Such an alternative is presented below.

Retroviral TCR gene transfer to primary human T lymphocytes

Essential to the success of genetic engineering of T cell specificity are improved retroviral gene transfer technologies. Retroviruses are widely used for the transfer of genes to proliferating somatic cells, including tumor cells and immune cells (102). Most retroviruses only infect proliferating cells and as a consequence, immune cells first have to be activated *in vitro* to become proliferative prior to transduction. Futhermore, *In vitro* gene transfer needs to be followed by *in vitro* expansion of the gene modified T cells to reach sufficient numbers of lymphocytes for clinical application.

First generation viral vectors such as LXSN and first generation packaging cell lines were used successfully to introduce chimeric TCR-based receptors into mouse T cells (unpublished results), but had several drawbacks. First, transduction efficiencies of primary human T lymphocytes were low, usually lower than 1%, were obtained. This necessitated repeated and prolonged lymphocyte culture in media containing the appropriate selective antibiotics. Second, these vectors resulted in low membrane expression levels of the transgene, typically undetectable by flow cytometry (59). We and others developed novel retroviral vectors (80, 103-105). One of these, the pBullet vector, contains the extended Moloney Murine Leukemia Virus (MoMLV) packaging signal, donor and acceptor RNA splice sites, and cloning sites that allow the introduction of a transgene at the optimal protein translation initiation position (Figure 2A)(105). To construct the pBullet vector, the U3 region of the 5' LTR was replaced by the cytomegalovirus immediate early (CMV IE) promoter to enhance RNA synthesis in cells that express the Adenoviral E1A protein. The SV40 origin of replication present in the retroviral vector and the retroviral helper constructs i.e. *GAG/POL* and *ENV*, are responsible for high copy numbers of the constructs, in cells that contain the SV40 large T protein. Transfection of pBullet and helper constructs into 293T cells resulted in transient but high virus production: up to 10^7 infectious units/ml. Improved and safer stable packaging cell lines were also generated (106). The retroviral helper elements in these cells, *GAG/POL* and *ENV,* are now split and present on different plasmids to reduce the chance of homologous recombination, and thus prevent the generation of replication competent retroviruses (RCR). The use of distinct promotors for the expression of helper elements, as well as a reduction in sequence overlap between the individual constructs further decreased the possibility that RCR are generated. The efficiency of retroviral gene transfer to primary human T lymphocytes ultimately depends on the retroviral vector construct and type of packaging cell line used, as well as the gene transduction protocol one employs (102-107).

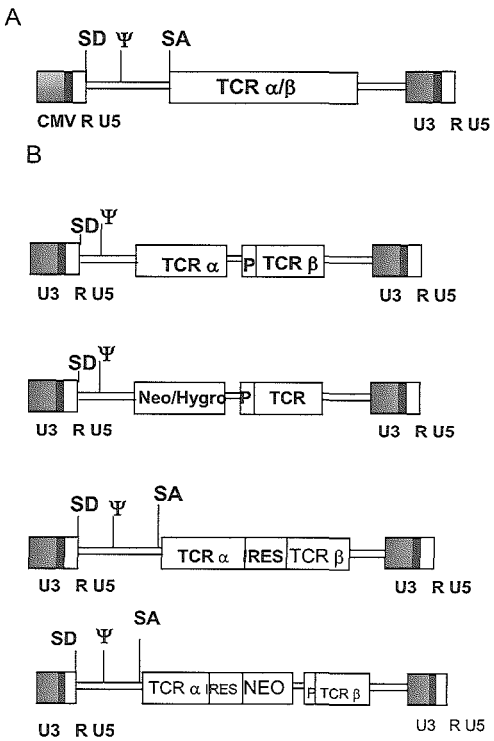

Figure 2. (A) Schematic presentation of the pBullet retroviral vector used to express full-length and chimeric TCRαβ genes in human T lymphocytes. (B) Retroviral vectors used in other TCR transfer studies. CMV = cytomegalovirus promoter; R and U5 = regions of the murine leukemia virus LTR; SD = splice donor site; SA = splice acceptor site; ψ = packaging signal; P = internal promoter; Hygro/Neo = hygromycin or neomycin resistance gene; TCRαβ = T-cell receptor α or β transgenes; IRES = internal ribosome entry site.

Transduction of primary human T lymphocytes on retronectin coated culture disks in our hands results in high transduction efficiencies of up to 90% (range 40-90%) (106,107). This high efficiency allowed us to develop a clinical applicable gene transduction protocol to transfer an Ab-based receptor into T cells, that meets GMP and GLP standards (108). In extension, advances in retroviral vector technology also enabled for the first time the efficient introduction and high-level expression of TCR-based receptors in primary human T lymphocytes.

Genetic engineering of T cell specificity: introduction of chimeric TCR αβ receptors

An alternative strategy to the transfer of full-length TCR αβ genes was developed, to avoid the risk of introducing new and possibly undesirable specificities into primary human T lymphocytes (98,105, this thesis). Based on knowledge that cross-linking of CD3ζ-linked proteins, expressed at the cell surface, induces T cell activation (109,110), chimeric two chain TCR VαCαζ and VβCβζ (tcTCR αζ / βζ), and chimeric single chain TCR VαVβCβζ (scTCR) genes were constructed. In scTCR, both TCR Vβ and Vα are present within one TCR chain, and in tcTCR αζ / βζ the presence of ζ induces the preferential formation of αζ / βζ heterodimers. TCR α and β genes were cloned from the HLA-A1-specific, MAGE-A1-restricted CTL clone MZ2-82/30 (111), were reformatted into chimeric CD3ζ-containing TCR genes and introduced into the retroviral vector pBullet (105, this thesis). It is of interest to note that retroviral introduction of one chimeric tcTCR/ζ gene (either TCRα or β) into primary human T lymphocytes did not result in its cell surface expression, proving that chimeric tcTCR αζ or βζ do not associate with endogenous TCR α or β chains. On top of that, flow-cytometric analysis of tcTCR αζ/βζ transduced T lymphocytes, stained with fluorochrome labeled TCR Vα and Vβ family type specific mAbs demonstrated a strict correlation between cellular TCR αζ and βζ expression, pointing to exclusive pairing between the introduced TCR/ζ chains. In addition, the use of chimeric TCR receptors does not show unstable TCRα surface expression since the TCR transgenes easily allow detection with TCR Vβ and Vα family-type specific mab (98,105). Flow-cytometric analysis further demonstrated that chimeric tcTCR as well as scTCR genes remain stably expressed, at high levels, in activated, bulk cultured primary human T lymphocytes for several months.

Bulk cultured primary human T lymphocytes expressing HLA-A1/MAGE-A1 specific chimeric TCR-based receptors are functionally retargeted and exert specific anti-tumor activities, i.e. ,cytolysis of cancer cells and cytokine production. Importantly, the high percentage of chimeric receptor positive cells does not necessitate selection or cloning of chimeric TCR expressing cells , required for clinical application.

Choice of signaling element: impact on chimeric TCR expression and function

To investigate the role of different signaling domains, chimeric scTCR constructs were generated that incorporate signaling units such as CD3-ζ or CD3-ε, derived from a human T cell clone, or the Fc(ε)RI γ chain, derived from human mast cells. These chimeric scTCR receptor constructs, VαVβCβζ, VαVβCβε or VαVβCβCD4/γ did all appear at the cell surface of primary human T lymphocytes with equal densities, but revealed differences with respect to the stability of membrane expression: VαVβCβγ being most stable and VαVβCβζ being least stable (54). These differences in stability may be caused by differences in their ability to form homodimers, or alternatively, to associate with endogenous TCR chains. ScTCR/ζ receptors may associate with endogenous CD3-ζ chains or form homodimers, whereas VαVβCβγ receptors, that include the CD4-transmembrane domain are not likely to associate with endogenous CD3 molecules or form homodimers. Down-regulation of TCR chains by for example IL-2 present in culture medium, may therefore more drastically affect scTCR/ζ receptors than for instance scTCR/γ (112). The scTCR^{POS} T lymphocytes all demonstrated the same HLA-A1 restricted MAGE-A1-specificity, identical to the CTL clone from which the TCR gene fragments were cloned. Incubation of scTCR^{POS} T lymphocytes with HLA-A1/MAGE-A1^{POS} melanoma target cells resulted in immune specific functions such as cytolysis, cytokine production and activation of the transcription factor NFAT. Membrane topology of these chimeric TCR molecules was assessed via immuneprecipitation and Fluorescence Resonance Energy Transfer studies. Importantly, such studies show that chimeric TCR-based receptors, when expressed on T lymphocytes, interact with endogenous CD3 and CD8 components following a TCR stimulus, indicating that these receptors are able to initiate the formation of an immunological synapse and proximal signaling events (Debets et al, manuscript in preparation)

In extension to chimeric Ab-based receptors that comprise the CD28 signaling domain, we also introduced the CD28 signaling domain into chimeric TCR-based receptor constructs. A direct comparison of T lymphocytes that express such "tripartite" TCR, i.e., ligand binding, CD28 and CD3ζ or Fc(ε)RI-γ domains, with those that express chimeric TCR receptors without the CD28 domain will further contribute to the development of an optimal receptor format

Introduction of non-modified, full-length TCR αβ genes

Next to chimeric TCR gene transfer, the functional transfer of full-length human TCRαβ genes specific for TRA or viral antigens to human T cell lines and primary human T lymphocytes was also a focus of intense research (113-119). However, most of these TCR αβ transduced T cells only responded to peptide pulsed target cells but not to the "native" TRA^{POS} tumor cells or virally infected cells (113-117). This lack of recognition of antigen expressing target cells may be due to the relative low expression levels of the introduced TCR αβ genes, or the absence or low expression of CD8 molecules in the T cell transductants.

The low expression levels of introduced TCR αβ genes is most likely a consequence of the type of retroviral vectors used in these studies (figure 2B). The elimination of the ENV start codon in most retroviral vectors to allow insertion of the native start codon of the transgene may have lowered expression levels. Furthermore, some of the retroviral vectors incorporate both TCR α and β genes, each having their own promoter. Promotor interference may cause lowered activity of one or both promotors. Even, when an internal ribosomal entry site (IRES) is used to express two TCR genes from the same promotor, the RNA encoding the TCR gene downstream the IRES may not be transcribed efficiently. Low transduction efficiencies and low levels of TCR αβ expression due to the applied vector technology, described above, necessitated T cell cloning for the identification of clones specific for native TRA^{POS} tumor cells (116,118).

Nevertheless, functional transfer of full-length human TCR αβ genes into primary human T lymphocytes, recognizing peptide pulsed target cells as well as TRA^{POS} tumor cells has met some success (116, 118). The use of the pBullet retroviral vector and an optimized retroviral transduction protocol (105,108) allowed successful introduction of full-length TCR αβ chains with specificities for gp100 and Hdm2 into primary human T lymphocytes (119,120). High transduction efficiencies of full-length TCR genes allowed direct analysis of the cytolytic activity of bulk cultured TCRαβ transduced human T lymphocytes. These non-enriched TCRαβ transduced T lymphocyte populations responded to peptide pulsed and more importantly to "native" TRA^{POS} tumor cells by tumor cell kill and cytokine production (119,120). The introduction of two different retroviral vectors, each containing either the TCR α or β chain into individual packaging cells results in high-level gene transfer of the two TCR genes. Such packaging cells showed efficient transfer of two distinct TCR receptor genes into individual primary human T lymphocytes (119,120).

Sources of TCR for genetic engineering of T cell specificity

Although the number of CTL clones available for cloning of TRA or viral antigen specific TCR is expanding, the induction of new CTL against TRA is cumbersome. Therefore, alternative strategies are needed to construct TCR αβ chains with predefined TRA or viral specificity (120-122). In this respect, mice, transgenic for human HLA-A2 and human CD8α, were used successfully to induce T cell responses against a human peptide/MHC complex (120). Injection of an HLA-A2 binding peptide derived from the TRA Hdm2 resulted in an effective T cell response, which then allowed isolation and cloning of mouse CTL specific for the human Hdm2/HLA-A2 complex. From these mouse CTL, full-length TCR αβ genes were isolated and introduced into the pBullet retroviral vector. Primary human T lymphocytes genetically engineered to express these mouse or humanized full-length TCR αβ demonstrated TRA specific immune functions. The Mouse-$TCR\alpha\beta^{POS}$ primary human T lymphocytes specifically lysed Hdm2/$HLA\text{-}A2^{POS}$ tumor cells. Other strategies that allow the generation of TCR with new antigen specificities as well as TCR with enhanced affinities, such as TCR display, will also contribute to the expansion of TCR molecules to be used for genetic engineering of T cell specificities (121,122).

Novel technologies: TCR-like molecules

Strategies that enable identification of TRA on tumor cells and isolation of TRA specific molecules allow the development of T cell based therapies that are not compromised by a limited availability of tumor or virus specific CTL. In this respect the phage display technology offers high throughput selection of antibodies with MHC-restricted

specificity (31). An alternative approach, immunization of mice with tumor cells and subsequent use of the hybridoma technology to isolate peptide/MHC specific mAb, did neither prove to be successful nor provided a high throughput technology for the selection of peptide/MHC specific molecules. The production of soluble peptide/MHC complexes allowed the isolation of Fab-phages from a large "non-immune" library that demonstrated exquisite HLA-A1/MAGE-A1 specificity (31). Up to now, the combined use of phage display and the production of soluble peptide/MHC complexes resulted in the selection of Abs against at least 12 different HLA-A2-based complexes, with each selection taking no more than 3 weeks (32,33). The genes encoding the Fab V_H and V_L domains of the HLA-A1/MAGE-A1 specific phage were used to construct a chimeric two-chain-Fab receptor (123, this thesis). We linked the VH as well as the VL gene fragment to the CD4 transmembrane domain and the intracellular domain of Fc(ε)RI γ. Primary human T lymphocytes transduced with chimeric Fab-CD4/γ genes specific for HLA-A1/MAGE-A1 functionally demonstrated the introduced specificity. Incubation of bulk cultured Fab-CD4/γ^{POS} lymphocytes with HLA-$A1^{POS}$, MAGE-$A1^{POS}$ melanoma cells resulted in peptide/MHC restricted tumor cell lysis and cytokine production.
Phage display derived TCR-like Ab have several advantages over full-length TCR αβ for the genetic engineering of T cell specificity: 1) the relative ease of isolation, expansion and handling of phages, 2) and the option to manipulate phage displayed Abs with respect to ligand binding affinity and format i.e. Fab or scFv. In example, we increased the ligand binding affinity of the original HLA-A1/MAGE-A1 specific Fab fragment G8 18 fold. The resulting Fab, i.e., Hyb3, was cloned and reformatted into a chimeric receptor. This high affinity chimeric FabCD4/γ receptor could be efficiently expressed in primary human T lymphocytes, and resulted in increased immune specific activities (124, this thesis). As a consequence, tumors that present low levels of peptide/MHC complexes on their membrane may not escape from recognition by such high affinity Fab transduced T cells. Indeed, recently it was demonstrated that high avidity CTL provide better protection against viral infection because they require less antigen to become activated; they initiate cell lysis more rapidly and thus are able to eliminate more infected cells (125).

SCOPE OF THIS THESIS: GENETIC ENGINEERING OF T CELL SPECIFICITY

The current thesis deals with the development of strategies to permanently graft primary human T lymphocytes with MHC-restricted, tumor-specificity. The design and functional introduction of two types of chimeric TCR-based receptors in primary human T lymphocytes is described in chapter 2. These chimeric tc-TCR/ζ and scTCR/ζ receptors provide an alternative to the introduction of to full-length TCRαβ genes into human T lymphocytes. The difference in fine-specificity of two scTCR, with identical TCR VαVβ, but with distinct transmembrane and intracellular signaling domains is described in chapter 3. Furthermore, we demonstrated that differences must exist for MHC class I molecules that are loaded intracellular with tumor peptides and MHC-class I molecules that are loaded extracellular with tumor peptides.
In chapter 4 the functional transfer of chimeric tc-TCR/ζ to $CD4^{POS}$ T lymphocytes is described. The requirement for CD8α co-expression into these $CD4^+$, tc-TCR/ζ^+ was shown to be a prerequisite for chimeric receptor mediated immune functions such as cytokine release and cytotoxicity.
The second part of this thesis deals with a unique type of receptors, based on novel MHC-restricted, tumor specific antibody fragments isolated from a large phage display library. In chapter 5 the design and functional expression of such an antibody based receptor with "TCR-like" specificity, the two chain F_{AB}G8-CD4/γ receptor, is described. Chapter 6 describes the *in vitro* affinity maturation of the MHC-restricted, tumor specific antibody fragment that was used in the experiments described in chapter 5. T lymphocytes equipped with this high affinity antibody-based receptor proved to be more responsive to tumor cells, i.e. higher levels of cytokine production were observed as well as enhanced cytolytic functions of receptor engrafted T lymphocytes.
Finally, chapter 7 describes an alternative way to improve receptor mediated functions of T lymphocytes grafted with antibody-based chimeric receptors. Incorporation of the CD28 co-signalling domain into chimeric scFv receptors resulted not only in enhanced cytokine release, but also in increased cytolytic functions of the receptor grafted T lymphocytes.

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CHAPTER 2

Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chan TCR

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Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR

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Primary human activated T lymphocytes were genetically grafted with chimeric T cell receptors (TCR). Three domain single chain (sc-) TCR as well as two chain (tc-) TCR gene constructs were derived from the melanoma-specifi ccytotoxic human T cell (CTL) clone 82/30, and linked to the CD3-ζ signaling element. Chimeric TCR α and β receptor genes were structurally designed to prevent pairing with endogenous TCR α and β chains in order to prevent the generation of unpredictable immune specifi cities.After transduction of polyclonally activated human peripheral blood lymphocytes with retroviral vectors harboring the chimeric receptor genes, genetically engineered cells specifi cally recognized and responded to MAGE-A1^{POS}/HLA-A1^{POS} cells. Importantly, each type of transduced T lymphocytes that bound specifi - cally to peptide/MHC complexes also showed specifi cantitumor reactivity as well as lymphokine production. Genetically engineered primary human T lymphocytes expressing chimeric sc- or tc-TCR therefore hold promise for disease-specific therapies. Gene Therapy (2000) **7**, 1369–1377.

Keywords: *chimeric; T cell receptor; MAGE-A1; HLA-A1; T lymphocytes*

Introduction

Adoptive transfer of MHC-restricted, antigen-specific CTL can mediate anti-tumor effects in patients with, for example, metastatic melanoma.[1] Unfortunately, most patients do not mount a strong and effective *in vivo* cytotoxic T cell response to their tumors. Moreover, isolation of tumor-specific T lymphocytes is only successful in a fraction of patients and expansion of cancer-specific, MHC-restricted human T lymphocytes to therapeutic doses presents other difficulties. Hence, the rate of success in obtaining autologous therapeutic T lymphocytes is unpredictable, and this has hampered their clinical application in adoptive T lymphocyte transfer for the treatment of cancer and viral infections. In contrast, monoclonal antibodies (mAb) with specificity for a wide range of tumor types are available. Therapeutic strategies, which combine the tumor-specific recognition capacity of mAbs with the lytic potential of CTL, have been developed by ourselves and others,[2,3] for example, ovarian tumor recognition by CTL was obtained by retargeting CTL using bispecific mAbs[4,5] (bsmAb). Also, human CTL have permanently been stably retargeted with mAb-based tumor selectivity and anti-tumor activity by transduction of genes coding for chimeric Ab-type receptors.[6–11] This genetic approach avoids loss of tumor selectivity due to dissociation of the bs-mAbs from the CTL surface.[12] However, all recently identified tumor rejection antigens (TRA), for example, MAGE, tyrosine, MART, BAGE and RAGE[13] are processed intracellularly by tumor cells and presented by MHC class I molecules on the membrane.[14] These TRA can be recognized by (human) CTL *in vivo* and *in vitro*.[14,15]

Retroviral transduction of transgenes encoding for TCR that are derived from existing MHC-restricted T cell clones specific for TRA in principle should confer TRA immune specificity to activated human T lymphocyte populations. This genetic programming of human lymphocyte specificity would bypass the requirement to isolate TRA-specific T lymphocytes from individual cancer patients and/or their *in vitro* generation using autologous tumor stimulator cells.

Indeed, the ability of chimeric tc-TCR αζ/βζ heterodimers[16] and of full-length αβ TCR heterodimers[17] to bind antigen/MHC complexes has previously been tested by exposure of either rat RBL-2H3 cells, expressing chimeric tc-TCRs, or human Jurkat cells expressing full-length αβTCRs, respectively, to target cells pulsed with relevant peptides. Following antigen-specific triggering rat RBL-2H3 cells secreted serotonin, and human Jurkat cells secreted IL-2. However, relevant native tumor antigen-positive tumor cells did not trigger any immune reactivity. Chung[18] suggested that introduction of complete TCR α and β chains in T lymphocytes may have resulted in suboptimal levels of TCR αβ cell surface expression due to instability of the exogenous TCR α chain,[18] which prohibits detection of immune functions. Lack of recognition of native tumor antigen by the Jurkat transfectants was also ascribed to the need for additional factors in

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order to allow selective discrimination of specific native peptide/MHC complexes within a more complex context in the tumor cell membrane. After all, tumor cells present native antigen amidst a multitude of irrelevant, yet highly homologous complexes of the same MHC molecules carrying a variety of 'self' or 'non-self' peptides. Activation by native antigen therefore may require simultaneous co-signaling by CD8 or need the involvement of adhesion molecules in order to activate T cells during TCR/MHC interaction.[15,17,19] Moreover, the genetically introduced TCR α and β chains may have paired with endogenous TCR α and β chains in the T lymphocytes that resulted in unpredictable, irrelevant immune specificities at the expense of the numbers of engineered relevant TCR to become expressed in the T lymphocyte membrane. Until now, attempts to introduce functional human chimeric single chain- or two chain-TCR αβ genes into primary human T lymphocytes have remained unsuccessful.

Here we describe the first successful, reproducible and functional transduction of primary human T lymphocytes with chimeric sc- and tc-TCR genes encoding MAGE-A1/HLA-A1-specific TCR. High percentages and high levels of chimeric TCR αβ expression in primary human T lymphocytes were obtained as visualized using fluorochrome-labeled TCR family typing antibodies.

Our results also show that soluble MAGE-A1/HLA-A1 complexes can be used to identify human T lymphocytes expressing chimeric TCR αβ genes and these T lymphocytes exert MAGE-A1/HLA-A1-specific anti-tumor reactivities as evidenced by tumor cell kill and lymphokine production.

Results

Chimeric sc- and tc-TCR expression on the membrane of gene-transduced primary human T lymphocytes

Chimeric sc-TCR VαVβCβζ genes and chimeric tc-TCR VαCαζ and VβCβζ genes were constructed from TCR α and β chain DNA fragments (Figure 1), derived from the MAGE-A1-specific, HLA-A1-restricted human T cell clone MZ2–82/30, that had been isolated from a melanoma patient.[14] Based on knowledge that antibody-mediated cross-linking of proteins, expressed at the cell surface, and linked to CD3-ζ, induces T cell activation,[7,20–22] the sc-TCR VαVβCβ, and the tc-TCR VαCα and VβCβ genes were ligated to the ζ gene that was cloned from a cytotoxic T cell clone, as previously described.[23] Chimeric tc-TCR VαCα-ζ and VβCβ-ζ genes were formatted to exclude pairing with endogenous TCR αβ chains on T

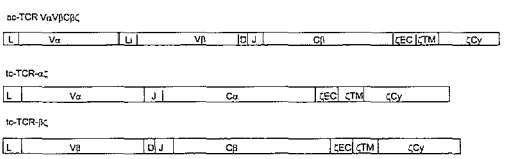

Figure 1 Chimeric-T cell receptor αβ constructs. Schematic representation of: (1) the chimeric sc-TCR VαVβCβζ gene and (2) chimeric tc-TCR VαCαζ and VβCβζ genes (L, leader; V, variable region; J, joining region; D, diversity region; C, constant region; EC, extracellular region; Tm, transmembrane region; Cy, Cytoplasmic region; Li, linker). Constructs were made as described in Materials and methods.

lymphocytes. This was accomplished by fusion of the extracellular TCR αβ domains in frame to CD3-ζ, which was shown to induce preferentially heterodimerization of the chimeric TCR αβ genes.[16,21] In chimeric sc-TCR VαVβCβζ genes, the V regions are covalently joined by a short peptide linker and hence, the problems associated with unstable TCR α expression and pairing with endogenous TCR αβ chains were avoided.[18] Chimeric tc-TCR VαCα-ζ and VβCβ-ζ genes were then cloned into the retroviral vector pStitch[23] and the chimeric sc-TCR VαVβCβζ gene was introduced into the pBullet vector, that was derived from the pStitch vector (Materials and methods). These retroviral vectors were specifically designed to transduce efficiently human T lymphocytes. To obtain 'therapeutic' numbers (approximately 10^9) of dividing T lymphocytes as well as optimal gene transduction efficacy, fresh or cryopreserved/thawed peripheral blood mononuclear cells (PBMC) derived from donors were polyclonally activated using soluble anti-CD3 monoclonal antibody (mAb) supplemented with recombinant interleukin-2 (IL-2). Activation could also be performed on immobilized anti-CD3 and anti-CD28 mAbs plus IL-2. Retroviral transduction of chimeric tc-TCR VαCαζ and VβCβζ gene constructs was then performed by cocultivating the anti-CD3 mAb activated human lymphocytes with virus-producing 293T packaging cells. The chimeric sc-TCR VαVβCβζ gene was introduced into human T lymphocytes by: (1) cocultivation with virus producing 293T cells or (2) by incubation with supernatant obtained from PG13 packaging cells that contained the chimeric sc-TCR VαVβCβζ gene.

Flow cytometric analysis of gene-transduced T lymphocytes demonstrated expression of: (1) chimeric sc-TCR VαVβCβζ and (2) chimeric tc-TCR VαCαζ and VβCβζ transgenes, in as high as 15 to 40% of primary polyclonally activated human T lymphocytes (Figure 2). Apparently, no pairing of chimeric TCR αβ molecules with endogenous TCR αβ chains occurred, as suggested by the FACS staining pattern that shows a strictly coordinated expression of the chimeric TCR Vα12.1 and Vβ1 chains (Figure 2). Moreover, no expression of either the chimeric tc-TCR VαCαζ or VβCβζ transgene was observed when only one of these genes was introduced into activated human T lymphocytes (Figure 2c and d). The level of chimeric-TCR αβ expression in the lymphocyte membrane varied over a 100-fold range. Chimeric-TCR $\alpha\beta^{POS}$, $CD4^{NEG}$ T lymphocytes were then enriched by flow sorting using fluorochrome-labeled Vα-12.1, Vβ1 family typing mAbs and anti-CD4-Cy5 mAb. FACS sorted T lymphocyte fractions where then used for functional analysis, ie specific binding of soluble MAGE-A1 peptide/MHC complexes; MAGE-A1-specific target cell cytolytic capacity, and lymphokine production during stimulation with peptide pulsed target cells or native melanoma cells (see below).

Chimeric TCR $\alpha\beta^{POS}$ T lymphocytes specifi callybind soluble MAGE-A1/HLA-A1 complexes

The MAGE-A1/HLA-A1 complexes (peptide: EADPTGHSY) and irrelevant Influenza/HLA-A1 complexes with a peptide derived from Influenza virus A nucleoprotein (peptide: CTELKLSDY) were generated and used for the identification of MAGE-A1/HLA-A1-specific engineered TCR in the membrane of bulk gene-transduced human polyclonal T lymphocytes. It

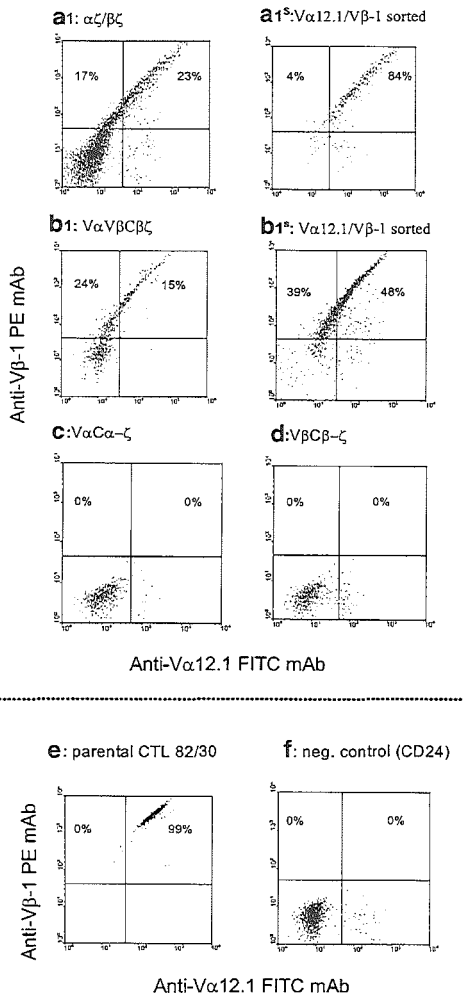

Figure 2 Cell surface expression of chimeric-TCR αβ-transduced primary human T lymphocytes. Expression of: (1) chimeric sc-TCR VαVβCβζ and (2) chimeric tc VαCαζ /VβCβζ constructs was determined by flow cytometry after gene transduction of primary human T lymphocytes. Events shown represent viable lymphocytes stained with anti-Vα12.1^{FITC} and anti-Vβ1^{PE} mAbs (>95% viable, checked by trypan blue staining). (a1) Human T lymphocytes simultaneously transduced with VαCα-ζ and VβCβ-ζ retroviral vectors. (a1^{s}) Human T lymphocytes transduced with VαCα-ζ and VβCβ-ζ retroviral vectors and followed by enrichment by flow sorting using anti-Vα12.1 FITC and anti-Vβ1 PE mAb. (b1) Human T lymphocytes transduced with VαVβCβζ retroviral vectors. (b1^{s}) Human T lymphocytes transduced with VαVβCβζ retroviral vectors and enriched by flow sorting using anti-Vα12.1 FITC and anti-Vβ1 PE mAbs. (c) Human T lymphocytes transduced with the VαCα-ζ retroviral vector. (d) Human T lymphocytes transduced with the VβCβ-ζ retroviral vector. (e) HLA-A1 restricted, MAGE-A1-specific parental CTL clone MZ2–82/30 (positive control). (f) Human T lymphocytes transduced with the CD24 gene (negative control). Data presented in a1^{s}, b1^{s}, e and f were derived from one experiment; a1, b1 and c and d were derived from two separate experiments.

appeared that indeed only relevant MAGE-A1/HLA-A1-streptavidin^{PE} complexes specifically bound to: (1) chimeric sc-TCR VαVβCβζ (Figure 3c), and (2) chimeric tc-TCR αζ/βζ (Figure 3d) expressed on the surface of human T lymphocyte transductants, ie control fluorochrome- labeled peptide/HLA-A1 complexes comprising the Influenza peptide did not bind. Moreover, human T lymphocytes that were transduced with the enhanced green fluorescence protein (EGFP) transgene did not bind the MAGE-A1/HLA-A1/streptavidin^{PE} complexes (Figure 3a).

MAGE-A1 peptide pulsed HLA-A1^{POS} target cells specifi callytrigger lysis and lymphokine production by genetically programmed CTL

To provide optimal signaling conditions for the transduced T lymphocytes we used MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cells (MEL 2A) and EBV-transformed B cell blasts (72–2 and APD) that had been pulsed with 10 μg/ml of MAGE-A1 peptide or with irrelevant Influenza virus peptide. Polyclonal T lymphocyte populations expressing either: (1) chimeric sc-TCR VαVβCβζ or (2) chimeric tc-TCR αζ/βζ on their membrane, each became specifically activated by MAGE-A1 peptide pulsed HLA-A1^{POS} MEL 2A melanoma cells or by MAGE-A1 peptide pulsed HLA-A1^{POS} B lymphoblastoid cell lines (72–2 and APD). Non-pulsed target cells, or target cells pulsed with irrelevant Influenza peptide were not lysed (Figure 4). Moreover, each type of transduced T lymphocytes also specifically responded to MAGE-A1 peptide pulsed melanoma target cells with production of TNF-α, GM-CSF and IFN-γ (Table 1). Five of five donors that were transduced with the chimeric tc- and sc-TCR were shown to lyse MAGE-A1 peptide pulsed target cells specifically (Table 2).

Native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells specifi callytrigger lysis and lymphokine production by chimeric TCR αβ^{POS} T lymphocytes

We then investigated whether chimeric sc-TCR VαVβCβζ or chimeric tc-TCR αζ/βζ expressing T lymphocytes each could also specifically recognize native MAGE-A1^{POS} /HLA-A1^{POS} melanoma cells. To this end, several MAGE-A1^{POS}/HLA-A1^{POS} melanoma cell lines as well as HLA-A1^{NEG} control cell lines were mixed with aliquots of each type of transduced T lymphocytes. As shown in Figure 5, anti-Vα12.1^{FITC} and anti-Vβ1^{PE} mAbs sorted, chimeric sc- and tc-TCR αβ^{POS} T lymphocytes were each capable of specifically lysing native MAGE-A1^{POS}, HLA-A1^{POS} melanoma cells (MZ2-MEL 3.0 and 518A2). To illustrate the specificity of lysis further, it was shown that addition of anti-MHC class I mAb resulted in significant reduction of specific tumor cell kill, whereas irrelevant mAb did not (Figure 5). Negative control HLA-A1^{POS}, MAGE-A1^{NEG} cell lines (MEL 2A, 72–2 and APD, see Figure 4) and distinct MHC-class I^{POS} (ie non-HLA-A1^{POS}) target cell lines used in this experiment were not lysed (MEL 78, BLM and SKRC17–4, data not shown). Native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells also specifically induced TNF-α, GM-CSF and IFN-γ production by the sorted chimeric TCR αβ^{POS} T lymphocytes (Table 3).

High effi cientsc-TCR VαVβCβζ gene transfer into human T lymphocytes using retronectin-coated culture plates

So far, the efficacy of retroviral gene transfer into primary human T lymphocytes for clinical application has been

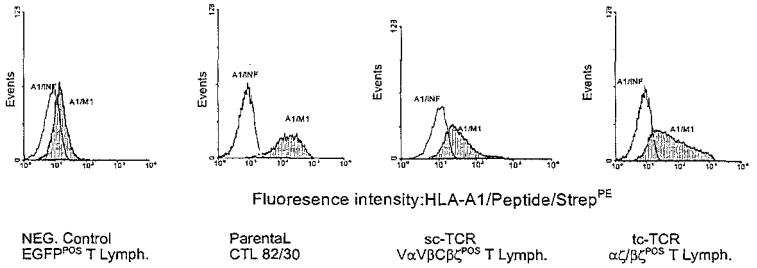

Figure 3 Peptide–MHC complex binding of chimeric-TCR αβ expressing primary human T lymphocytes. Specific binding of soluble MAGE-A1/HLA-A1/streptavidin^{PE} complexes by transduced and flow-sorted primary human T lymphocytes was determined by flow cytometry. Flow-sorted T lymphocytes were stained with soluble MAGE-A1/HLA-A1/streptavidin^{PE} complexes (A1/M1, shaded area) and soluble Influenza peptide/HLA-A1/streptavidin^{PE} complexes (A1/IFN, open area) as described in Materials and methods.

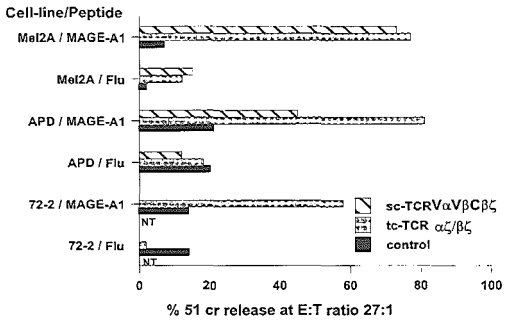

Figure 4 Peptide-pulsed target cells are lysed by chimeric-TCR αβ-transduced human T lymphocytes. MAGE-A1 peptide-pulsed HLA-A1^{pos} target cells were incubated with flow-sorted human T lymphocytes transduced with: (1) the EGFP gene, (2) chimeric tc-TCR αζ/βζ genes, and (3) the chimeric sc-TCR VαVβCβζ gene, and tested in 6 h ^{51}Cr release assays at four effector to target cell ratios (E:T). Shown are representative results obtained at E:T 27:1. ^{51}Cr-labeled MAGE-A1^{NEG}, HLA-A1^{POS}, EBV-transformed B cell lines 72–2 and APD, and ^{51}Cr-labeled MAGE-A1^{NEG}; HLA-A1^{POS} MEL 2A melanoma cells were pulsed with MAGE-A1 or Influenza virus peptides (10 μg/ml). Experiments were performed in triplicate, and the s.d. did not exceed 10%. Shown are data of one representative experiment out of three.

hampered by poor transduction efficiencies of supernatant transduction protocols. Our viral vectors[23] in combination with immobilized recombinant fibronectin fragments[24] now allow for high transduction efficiencies into primary human T lymphocytes. After bulk transduction on retronectin-coated culture plates of primary, CD4-depleted activated human T lymphocytes, derived from three donors, with the chimeric sc-TCR VαVβCβζ gene, 40–70% of the transduced populations were chimeric sc-TCR Vα12.1/Vβ1^{POS} as determined by cytofluorometric analysis (Figure 6a). The scTCR^{POS} T lymphocytes responded to MAGE-A1 peptide pulsed MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cells (Mel2A) as well as native MAGE-A1^{POS}, HLA-A1^{POS} melanoma cells (MZ2-MEL3.0) (Figure 6b), but not to the MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cells (Mel2A).

Discussion

Here, we describe the first successful and functional transduction in primary human T lymphocytes of chimeric TCR αβ genes encoding sc- (VαVβCβζ) or tc- (VαCαζ/VβCβζ) TCR with immune specificity for MAGE-A1^{POS}/HLA-A1^{POS}, native as well as peptide loaded melanoma cells. Eight out of eight polyclonally activated human T lymphocyte populations were functionally transduced, ie (1) the T cell transductants

Table 1 TNF-α, GM-CSF and IFN-γ production by chimeric-TCR αβ-transduced CTL: incubation with MAGE-A1 peptide pulsed melanoma cells

	MAGE-A1 peptide pulsed melanoma cells[a]					
	TNF-α pg/ml		*GM-CSF pg/ml*		*IFN-γ pg/ml*	
	No pept.	*+ MAGE-A1 pept.*	*No pept.*	*+ MAGE-A1 pept.*	*No pept.*	*+ MAGE-A1 pept.*
CTL 82/30	0.0	2996	0.0	426	39	6678
sc-TCR VαVβCβζ^{POS} T lymphocytes	56	105	0.0	629	34	577
tc-TCR αζ/βζ^{POS} T lymphocytes	61	380	0.0	3767	97	1954

[a] As described in Materials and methods.

Table 2 Chimeric sc- and tc-TCR-transduced T lymphocytes: FACS analysis and cytotoxicity

Donor	FACS analysis % Vα 12.1^{POS}/Vβ1^{POS} T lymph		Cytotoxicity analysis[a] % ^{51}Cr release at E:T 27:1	
	pre-sort %	post sort %	no pept. %	+ pept. %
tc-TCR VαCαζ/VβCβζ				
D2	32	>90[b]	15	73[d]
D3	26	65[c]	40	73[e]
D4	24	52[c]	28	46[e]
D5	15	48[c]	0	24[f]
D6	29	64[c]	9	67[f]
sc-TCR VαVβCβζ				
D1	23	63[c]	26	64[e]
D2	28	>90[b]	15	73[d]
D3	34	60[c]	11	40[f]
D4	26	34[c]	34	63[e]

[a] As described in Materials and methods.
[b] Enrichment by flow cytometric sorting using anti-Vα12.1^{FITC} and anti-Vβ1^{PE} mAbs.
[c] Enrichment by anti-Vβ1-coated magnetic beads.
[d] MAGE-A1 peptide loaded HLA-A1^{POS}, MAGE-A1^{NEG} MEL2A melanoma cells.
[e] MAGE-A1 peptide loaded HLA-A1^{POS}, MAGE-A1^{NEG} MZ2-MEL 2.2 melanoma cells.
[f] MAGE-A1 peptide loaded HLA-A1^{POS}, MAGE-A1^{NEG} B-LCL cells (APD).

expressed chimeric sc- and tc- αβ TCR on their membrane, (2) specifically killed native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells as well as MAGE-A1 peptide pulsed HLA-A1^{POS} target cells; and (3) were specifically triggered by these cells to produce TNF-α, GM-CSF and IFN-γ.

Table 3 TNF α, GM-CSF and IFN-γ production by chimeric-TCR αβ-transduced CTL: incubation with native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells

	Native HLA-A1^{POS}/MAGE-A1^{POS} melanoma cells[a]		
	TNF-α pg/ml	GM-CSF pg/ml	IFN-γ pg/ml
CTL 82/30	428/0[b]	54/0[b]	1159/39[b]
sc-TCR VαVβCβζ^{POS} T lymphocytes	85/56[b]	1889/0[b]	272/34[b]
tc-TCR αζ/βζ^{POS} T lymphocytes	120/61[b]	1785/0[b]	306/97[b]

[a] As described in Materials and methods.
[b] Negative control HLA-A1^{POS}, MAGE-A1^{NEG} cell line MZ2-MEL 2.2.

The ability to generate primary polyclonal human chimeric-TCR αβ^{POS} CTL with predefined immune specificities is of fundamental as well as clinical importance. Fundamental, because functional TCR gene grafting to (human) T lymphocytes allows the study of: (1) whether and how genetically introduced TCR (and other receptors) with distinct molecular configurations, and linked to distinct signaling elements, can interact with other T cell (co-) signaling/adhesion molecules, and (2) how these may integrate downstream with other intracellular signaling pathways.[25,26] Clinical, because such CTL can be used in disease-specific therapies.[27–29] This important notion is supported by recent reports that adoptive transfer of CMV-specific[30] or melanoma-specific[31] CTL have been clinically effective in the prophylaxis of CMV reactivation and tumor growth control, respectively.

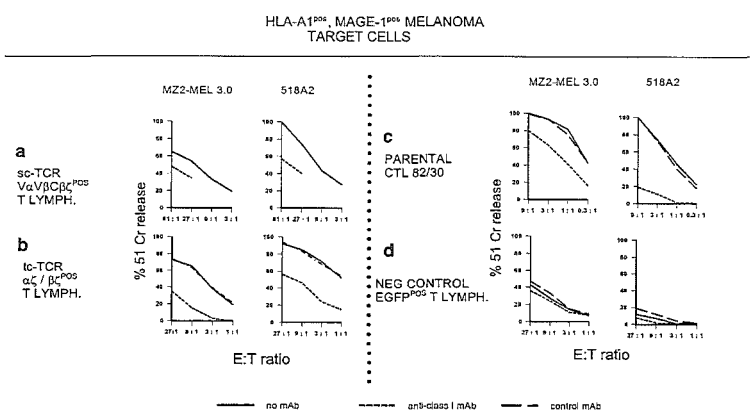

Figure 5 Native MAGE-A1^{POS}, HLA-A1^{POS} melanoma cells are susceptible to lysis by chimeric-TCR αβ transduced T lymphocytes. Cytolytic capacity of transduced and flow-sorted human T lymphocytes was determined in 6 h ^{51}Cr release assays. Left two rows show results obtained with ^{51}Cr-labeled MAGE-A1^{POS}, HLA-A1^{POS} melanoma cells (MZ2-MEL 3.0 and 518A2) incubated with sc-TCR VαVβCβζ^{POS} and tc-TCR VαCαζ/VβCβζ^{POS} T lymphocytes. The right two rows shows results obtained with ^{51}Cr-labeled MAGE-A1^{POS}, HLA-A1^{POS} melanoma cells (MZ2-MEL 3.0 and 518A2) incubated with the positive control CTL 82/30 and negative control EGFP gene-transduced T lymphocytes. Anti-HLA-ABC (10 μg/ml) or mouse Ig (10 μg/ml) was added to the melanoma target cells (MZ2-MEL 3.0 and 518A2) 15–30 min before incubation with the effector T lymphocytes. Data obtained with negative control MAGE-A1^{NEG}, HLA-A1^{POS} target cells in this experiment are shown in Figure 4. Shown are mean percentages of triplicates of per cent-specific ^{51}Cr release. Data of one representative experiment out of three are shown.

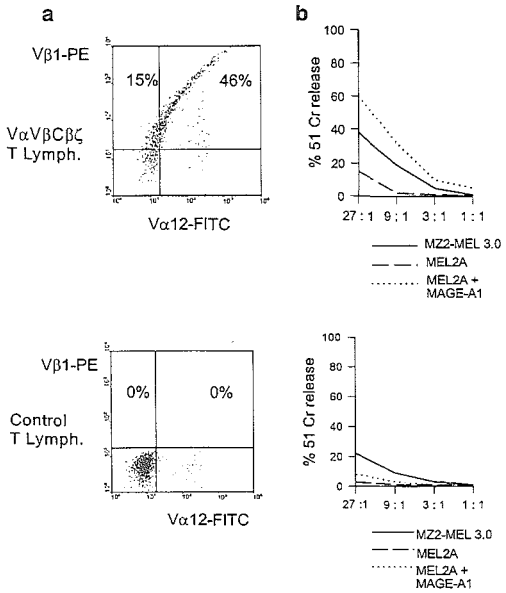

Figure 6 Human T lymphocytes transduced with the chimeric TCR VαVβCβζ on retronectin-coated culture plates show HLA-A1-restricted, MAGE-A1 specificity. (a) Expression of chimeric single chain TCR VαVβCβζ on human T lymphocytes was determined by flow cytometry. Events shown represent viable lymphocytes stained with anti-Vα12.1^{FITC} and anti-Vβ1^{PE} mAbs. (b) Negative control MAGE-A1^{NEG}, HLA-A1^{POS}, melanoma cells, MAGE-A1 peptide-pulsed HLA-A1^{POS} melanoma cells and native MAGE-A1^{POS}/HLA-A1^{POS} target cells were incubated with the indicated human T lymphocytes and tested in 6 h ^{51}Cr release assays. The data show the per cent-specific ^{51}Cr release from: (1) negative control HLA-A1^{POS} Mel2A melanoma cells, (2) HLA-A1^{POS} Mel2A melanoma cells pulsed with 10 μg/ml MAGE-A1 peptide and, (3) MAGE-A1^{POS}/HLA-A1^{POS} MZ2-MEL 3.0 melanoma cells. Cytolytic activity of γδ T lymphocytes present in the transduced polyclonal human T lymphocyte populations was blocked by addition of anti-γδ mAb (1:1000 11F2 ascites) to the effector cells. Experiments were performed in triplicate, and the s.d. did not exceed 10%. Shown are the results obtained with one representative donor transduced with the chimeric scTCR VαVβCβζ and CD24 gene from one representative experiment (one out of three).

The 'pStitch' and pBullet viral vectors we developed[23] and used in this study were critical to transduce efficiently and functionally chimeric sc-and tc-TCR gene constructs into primary human T lymphocytes activated with anti-CD3 mAb. Each type of transduced TCR αβ gene was stably expressed in the transduced human T lymphocytes, and yielded high membrane expression levels of the chimeric receptors, as visualized using fluorochrome-labeled TCR Vα12.1 and Vβ1 family typing monoclonal antibodies or specific MAGE-A1/HLA-A1 complexes. Chimeric two chain-TCR αζ/βζ gene constructs were formatted to facilitate preferential efficient heterodimerisation and membrane expression of these gene products.[16] We therefore expected that the chimeric αζ and βζ chains would not pair with endogenous full-length TCR β and α chains, respectively. Indeed, the strictly coordinated expression of chimeric TCR αβ chains illustrates that no such pairing of chimeric tc-TCR αζ/βζ chains as well as chimeric sc-TCR VαVβCβζ with endogenous TCR αβ chains occurred, avoiding the creation of unknown immune specificities. Indeed, following transfer of either chimeric TCR αζ or βζ genes alone, no chimeric αζ or chimeric βζ chains were detected on the membrane of T cell transductants that were either PCR Vα12.1 or Vβ1 positive, respectively (Figure 2).

We then used in-house synthesized MAGE-A1/HLA-A1 complexes to identify relevant chimeric sc- and tc-TCR because Altman *et al*[32] recently showed that fluorochrome-labeled peptide/MHC class I tetramers provide an extremely sensitive and practical tool to identify and isolate T lymphocytes with MHC-restricted TRA binding capacity. Moreover, Yee *et al*[27] recently demonstrated that peptide/MHC tetramers not only identify but also select high avidity melanoma reactive CTL from heterogeneous T lymphocyte populations that were generated in mixed T lymphocyte/peptide pulsed dendritic cell cultures. Importantly, the CTL with high GP100/HLA-A2 tetramer binding capacity recognized not only peptide pulsed targets but also native TRA^{POS} melanoma, in contrast they showed that CTL with low tetramer binding capacity did not recognize native melanoma. Because they found no differences in the densities of adhesion molecules or CD8 on the membrane of these T cells it was concluded that the level of tetramer binding directly reflected the level of TCR affinity of individual T lymphocytes in heterogeneous T lymphocyte populations.

Here, we also show that primary polyclonal human T lymphocytes that are genetically engineered to express chimeric sc- or tc-TCR and bind soluble HLA-A1/MAGE-A1 complexes lyse native MAGE-A1^{POS} melanoma cells and peptide pulsed target cells. However, in our system, individual T lymphocytes express the same, clonal chimeric Vα12.1/Vβ TCR, and bind MAGE-A1/HLA-A1 complexes. Thus, based on published data[27] the chimeric TCR is of high affinity. Because of the clonal nature of the chimeric TCR, differences in MAGE-A1/HLA-A1 complex binding levels (avidity) in our experiments must reflect differences in the absolute numbers of chimeric TCR expressed in individual T lymphocytes, not differences in affinities between receptors. The relatively 'low TRA/MHC complex binding' chimeric TCR^{POS} T lymphocyte fraction lacks melanoma reactivity, and this could be attributed to a combination of relatively low TRA expression on melanoma cells on the one hand and the observed low chimeric TCR Vα12.1/Vβ1 expression on the CTL on the other. Indeed, we recently demonstrated that such functional relationship between antigen receptor density on human T lymphocytes and tumor antigen density on tumor cells, respectively exists.[41]

Following gene transfer on retronectin-coated culture plates, 40–75% of the bulk cultured, CD4-depleted polyclonal human T lymphocytes were chimeric sc-TCR^{POS}, and specifically killed native HLA-A1^{POS}/MAGE-A1^{POS} melanoma cells. This makes this T cell transduction technology a promising candidate for therapeutic applications. The already large fraction of chimeric TCR αβ^{POS} transductants in bulk-transduced human T lymphocytes can be directly and readily expanded *in vitro* to therapeutic numbers and used for immuno-gene therapy, ie without the need to clone melanoma reactive T cells before expansion. Moreover, the clinical use of transduced polyclonal immune lymphocytes, each subtype having its particular lymphokine production repertoire,

proliferative and/or trafficking capacities, may prove optimal to control tumor cell growth. We have successfully transduced distinct lymphocyte populations, for example, NK cells, TCR αβ (this article) and γδ T lymphocytes, with chimeric sc- and tc-TCR αβ, that have distinct immune reactivities (manuscript in preparation). Moreover, we can now also confer multiple peptide or TRA-specific TCRs to individual T lymphocytes. The therapeutic use of multiple effector cell types, each expressing one or multiple receptor specificities may enhance anti-tumor activity by reducing the chance of tumor cells to escape from immune attack, and result in a prolonged anti-tumor response. To this end, large libraries can be composed of cDNAs encoding for any antigen/MHC specificity for which human CTL clones are or become available, they can be generated from *in vivo* isolated T lymphocytes, generated *in vitro* by specific stimulation, or created from TCR αβ or γδ display libraries.[33,34]

In conclusion, genetic engineering of T lymphocyte specificity with chimeric TCR αβ provides an efficient and reliable tool to produce MHC-restricted, antigen-specific human T lymphocytes that may prove of significant use for disease-specific immune gene therapy of, for example, cancer and viral infections.

Materials and methods

Cells and antibodies

T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere.[23] Target cell lines used in this study are: (1) the MAGE-A1^{POS}, HLA-A1^{POS} melanoma cell line MZ2-MEL.3.0; (2) the MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cell line MZ2-MEL 2.2 (kindly provided by T Boon and P Coulie, Ludwig Institute for Cancer Research, Brussels, Belgium);[14] (3) the MAGE-A1^{POS}, HLA-A1^{POS} melanoma cell line 518A2 (kindly provided by P Schrier, Leiden University Medical Center, Leiden, The Netherlands); (4) the MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cell line Mel 2A; (5) the HLA-A1^{NEG} melanoma cell line MEL 78; (6) the HLA-A1^{NEG} melanoma cell line BLM (kindly provided by G Adema, University Hospital Nijmegen St Radboud, Nijmegen, The Netherlands); (7) the HLA-A1^{NEG} renal carcinoma cell line SKR17–4 (kindly provided by E Oosterwijk, University Hospital Nijmegen St Radboud, Nijmegen, The Netherlands); and (8) the HLA-A1^{POS} EBV-transformed B cell blasts APD and 72–2 (kindly provided by P Traversari, Instituto Scientifico HS Raffaelle, Milan, Italy). The human embryonic kidney cell line 293T[35] (kindly provided by Y Soneoka, University of Oxford, Oxford, UK) was used as a packaging cell line for the pStitch VαCαζ/VβCβζ and pBullet VαVβCβζ retroviral vectors.[23] The mouse packaging cell line PG13 (ATCC CRL-10686) was used to obtain stable sc-TCR VαVβCβζ retrovirus producing cells. The CTL clone MZ2–82/30[14] was used for isolation of RNA coding for the HLA-A1-restricted, MAGE-A1-specific TCR αβ (kindly provided by T Boon and P Coulie). The mAbs used in this study were: anti-HLA-ABC (clone W6/32, Sera-Lab, Crawley Down, UK); anti-CD4^{CY-5}; anti-CD8$^{FITC/PE}$ (Becton Dickinson Biosciences, San Jose, CA, USA); the TCR Vα and Vβ family-specific mAbs anti-Vα12.1 (T Cell Diagnostics, Woburn, MA, USA); and anti-Vβ1 (Coulter-Immunotech, Marseille, France) and the anti-TCR γδ mAb 11F2.[36]

Construction of soluble MAGE-A1 peptide/HLA-A1 and Influenzavirus A nucleoprotein peptide/HLA-A1 complexes

Cloning of HLA-A1 heavy chain: The gene coding for the HLA-A101 gene was amplified by PCR from cDNA clone Vi105 (kindly provided by T Boon) using the primers GCGGCGGCGGCCATGGGCTCCCACTCCATGAGG and TTTCTGTGCATCCAGAATATGATGCAGGG ATCCGA GCTCCCATCTCAGGGT, and the product of this first PCR was used as template in a second PCR using the primers GCGGCGGCGGCCATGGGC TCCCACTCCAT-GAGG and CGGCAGGAGAGCGGCCGCTTAACG ATGATTCCACACCATTTTCTGTGCATCCAGAAT. The restriction sites *Nco*I and *Not*I used for cloning are underlined. The forward primers encode the peptide HHIL-DAQKMVWNHR recognized by the BirA enzyme used for *in vivo* biotinylation. The PCR products were ethanol-precipitated, digested with *Nco*I and *Not*I enzymes, gel-purified and ligated into the plasmid pET21d (Novagen, Madison, WI, USA) and transformed into DH5α, and clones containing an insert were sequenced. Clones with the correct sequence were transformed into BL21DE3 for protein production, together with a compatible plasmid containing the Bir A gene under the control of the tac promoter (pBirCm; Avidity, Denver, CO, USA). The plasmid pHNβ2m was used to produce the β2m.

Inclusion body purification of HLA-A1 proteins and reconstitution of peptide /HLA-A1 complexes were adapted from Garboczi *et al*[37] and Altman *et al*.[32] Soluble peptide/HLA-A1/ streptavidin^{PE} complexes were made by mixing equal volumes of streptavidin PE (5 μg/ml, Becton Dickinson Biosciences) and soluble peptide/HLA-A1 (120 μg/ml), followed by 30 min incubation on ice.

Construction of chimeric sc-TCR VαVβCβζ genes and chimeric tc-TCR VαCα-ζ/VβCβ-ζ genes

For construction of chimeric sc-TCRVαVβCβζ genes a cloning vector was designed that allows construction of these single chain molecules. The vector was made by replacement of the multiple cloning site in pBluescript (Stratagene, La Jolla, CA, USA) by insertion of the polylinker: GTACGAATTCGCAGATCTGGCTCTACTTCCG GTAGCGGCAAATCCTCTGAAGGCAAAGGTACTAGT GCGG ATCCGGCTCGAGCAGCT into the *Kpn*I and *Sac*I site. TCR Vα and TCR Vβ fragments, amplified from CTL 82/30 with primers Vα-ATG and Vα-3′ and Vβ-ATG and Vβ-3′, respectively, were cloned into this vector. The extra-cellular domain of the TCR constant β chain was amplified separately (primers Cβ-5′ and Cβ-3′) and inserted next to the single chain TCR Vα-linker-Vβ. The TCR Vα-linker-Vβ-Cβ fragment was then amplified with primers that introduced restriction sites (Vα-*Sfi*I and Cβ-cys) allowing cloning into a retroviral expression cassette containing the signal sequence derived from the mouse immunoglobulin G250 variable heavy chain. This retroviral expression cassette pBullet-Cass was made in three steps: first, two *Nco*I sites (positions 317 and 2902) and an *Xho*I site (position 2785) were deleted in the pStitch retroviral vector, followed by insertion of the linker CCATGGGTCGACGGATCCGCGGCCGCTCGCGACTC GAG into the *Nco*I and *Bam*HI sites.[23] Next, the signal sequence of the G250 variable heavy chain (CCATG GACTTCGGGCTCAGATTGATTTTCCTTGTCCTGGTTT AAAAGGTGTCCTGTGTGCGGCCCAGCCGGCC) and ζ

chain were inserted into this vector resulting in the pBullet-Cass retroviral vector. Finally, the Vα-linker-Vβ-Cβ fragment was inserted into this vector next to the human ζ chain. The ζ chain was isolated from the CTL clone D11.[38] For construction of the chimeric two chain TCR VαCαζ and VβCβζ genes, extracellular domains from the TCR α and β chain were amplified from the CTL clone MZ2-82/30, using Vα-ATG and Cα-cys or Vβ-ATG and Cβ-cys-specific primers including the appropriate restriction sites. The VαCα and VβCβ gene fragments were each ligated 5′ to the ζ gene in pBluescript. Chimeric VαCα-ζ and VβCβ-ζ receptor genes were then cloned into the pStitch retroviral vector. Correct sequences of the chimeric sc-TCR VαVβCβζ and chimeric tc-TCR VαCα-ζ/VβCβ-ζ were verified using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). The sequences of the above mentioned primers were:

Vα-ATG: 5′-GCG-AAT-TCT-ACG-TAC-CAT-GGA-CAT-GCT-GAC-TGC-CAG-C-3′, Vα-3′: 5′-GCG-GAT-CCG-GGT-TTG-ACC-ATT-ACC-CTT-G-3′,
Vα-*Sfi*I: 5′-TTA-CTC-GCG-GCC-CAG-CCG-GCC-ATG-GCC-CAG-AAG-GTA-ACT-CAA-GCG-CAG-3′, Cα-cys: 5′-GCG-GAT-CCA-GAT-CCC-CAC-AGG-AAC-TTT-CTG-GGC-TGG-GGA-AGA-AGG-TGT-CTT-CTG-G-3′,
Vβ-ATG: 5′-GCG-CCA-TGG-GCT-TCA-GGC-TGC-TCT-GC-3′,
Vβ: 5′-GCG-AAT-TCT-ACG-TAC-CAT-GGG-CTT-CAG-GCT-GCT-CTG-CTG-TGT-GGC-3′, Vβ 3′: 5′-GCG-GAT-CCG-AGC-ACT-GTC-AGC-CGG-GTG-CC-3′,
Cβ: 5′-TAC-CTC-GAG-GCA-TCG-ATG-AGC-AGG-TAC-AGG-AGA-A-3′,
Cβ-cys: 5′-GCG-GAT-CCA-GAT-CCC-CAC-AGT-CTG-CTC-TAC-CCC-AGG-CCT-CGG-CGC-TGA-CGA-TCT-GC-3′.

Retroviral chimeric-TCR αβ gene transduction into primary human T lymphocytes: in vitro expansion of transduced T lymphocytes

Activated human peripheral blood lymphocytes (PBL) (5×10^6) were transduced using the pStitch or pBullet retroviral vector with the chimeric two chain TCR VαCαζ and VβCβζ receptor genes or chimeric sc-TCR VαVβCβζ, respectively, as described earlier.[23] Briefly, anti-CD3 activated lymphocytes were incubated for 72 h with an irradiated (25 Gy) monolayer of recombinant retrovirus-producing 293T cells, using culture medium (RPMI 1640 with 25 mM Hepes, 10% human serum, 2 mM glutamine, penicillin 100 U/ml and streptomycin 100 μg/ml) supplemented with 4 μg/ml polybrene (Sigma, St Louis, MO, USA), and 360 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands). Retroviral transduction of the chimeric sc-TCR VαVβCβζ was also performed with supernatant produced by PG13 packaging cells containing the pBullet TCR VαVβCβζ vector essentially as described.[24] Expansion of transduced primary human T lymphocytes was performed in the presence of feeder cells as we have described elsewhere.[39,40]

Cytofluorometric analysis and sorting of retrovirally transduced human T lymphocytes

Human T lymphocytes (5×10^5) were stained with TCR Vα and Vβ family-type-specific anti-Vα-12.1^{FITC} (2 μg/ml) and anti-Vβ1^{PE} (2 μg/ml) mAb in a volume of 50 μl. For staining with soluble HLA-A1/peptide/streptavidin^{PE} complexes, cells (5×10^5) were incubated for 30 min on ice, with a 1:10 dilution of freshly prepared complexes in a volume of 20 μl. Before staining T lymphocyte viability was assessed by trypan blue staining: only T lymphocyte populations >95% viable were used. The dot plots show viable T lymphoblasts selected by gating on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACSCAN instrument (Becton Dickinson Biosciences). Flow sorting was performed on a FACS-Vantage instrument (Becton Dickinson Biosciences) using saturating concentrations of anti-Vα-12.1^{FITC}, anti-Vβ-1^{PE} and anti-CD4$^{Cy\text{-}5}$. Flow-sorted T lymphocytes were expanded before use in functional assays as described elsewere.[39,40]

Cytotoxicity assays

Cytolytic activity of transduced human T lymphocytes was measured in 6 h ^{51}Cr-release assays as described.[9] In experiments aimed at blocking specific cytolytic activity, mAbs were added to the T lymphocytes 15–30 min before addition of the target cells (W6/32: 10 μg/ml, or irrelevant mIg: 10 μg/ml). Peptide loading of target cells was performed by addition of MAGE-A1 nonapeptide (EADPTGHSY) or irrelevant Influenza peptide derived from Influenza virus A nucleoprotein (CTELKLSDY) (both 10 μg/ml) to the target cells before incubation with effector T lymphocytes. Percentage-specific ^{51}Cr release was calculated as follows: ((test counts − spontaneous counts)/(maximum counts − spontaneous counts)) × 100%.

TNF-α, GM-CSF and IFN-γ production

To quantify TNF-α, GM-CSF and IFN-γ production by the transduced flow-sorted human T lymphocytes after antigen-specific stimulation, 6×10^4 transduced T lymphocytes were cultured for 24 h either in the presence or absence of 2×10^4 adherent tumor cells in RPMI-1640 medium supplemented with 360 IU/ml rIL-2. At the end of culture, supernatant was harvested and levels of TNF-α, GM-CSF (Medgenix, Fleurus, Belgium) and IFN-γ (CLB, Amsterdam, The Netherlands) were measured by standard ELISA according to the manufacturer's instructions.

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CHAPTER 3

Identical scTCR$\alpha\beta$ with distinct non-variable signalling elements have distinct specificities

Submitted

IDENTICAL scTCRαβ WITH DISTINCT NON-VARIABLE SIGNALLING ELEMENTS HAVE DISTINCT SPECIFICITIES

Ralph Willemsen, Cees Ronteltap, Reno Debets, and Reinder Bolhuis.

Summary:
We investigated the antigen specific triggerability of human CTL populations genetically programmed to functionally express single chain T cell receptors (scTCRs) with: identical Variable alpha ($V\alpha_{12.1}$) and Variable beta ($V\beta_1$) chains, but distinct non-variable transmembrane/intracellular signalling domains, i.e., γ versus ζ. As targets we used cells expressing membrane bound Class I-HLA-A1/MAGE-A1 complexes produced via the intracellular versus the extracellular route. The intracellular route involved: (i) native MAGE-A1 antigen; or (ii) gene reconstituted MAGE-A1 peptide synthesis, or (iii) intracellular MAGE-A1 peptide transfection. The extracellular route involved: (i) addition of free synthetic MAGE-A1 peptides produced *in vitro* to MAGE-A1^{NEG}/HLA-A1^{POS} target cells, or (ii) immobilization of soluble MAGE-A1/HLA-A1 complexes to plastic, or generation of Class I-HLA-A1/MAGE-A1 tetramers. Both ζ-scTCR$V\alpha_{12.1}V\beta_1$ ^{POS} and γ-scTCR$V\alpha_{12.1}V\beta_1^{POS}$ CTL's specifically recognized and lysed HLA-A1^{POS}/MAGE-A1^{POS}: (i) native melanoma cells; (ii) MAGE-A1 peptide transfected B-LCL, and (iii) MAGE-1 gene reconstituted antigen lost mutant Melanoma cells. Surprisingly, only ζ-scTCR$V\alpha_{12.1}V\beta_1$ ^{POS}, but not γ-scTCR$V\alpha_{12.1}V\beta_1^{POS}$, T lymphocytes responded to Mel and B-LCL's, that expressed Class I-HLA-A1/MAGE-A1 complexes generated via the extracellular route. Noteworthy, the free MAGE-A1 peptide added extracellularly was identical to the MAGE-A1 peptide introduced intracellularly by electroporation.
We conclude that scTCR VαVβ specificity is not exclusively dictated by the variable VαVβ chains, but can be co-determined by non-variable TCR (signaling) components. Moreover, MAGE-A1/HLA-A1 complexes generated by the exogenous route are antigenically distinct from those generated via the intracellular route.

Introduction

Self- or foreign antigenic proteins are intracellularly processed into peptides that are transported into the endoplasmic reticulum and enable the formation of stable trimeric MHC class I heavy chain- microglobulin-peptide complexes (1,2). Class I MHC antigenic peptide complexes are then transported to the outer cell membrane where they are presented to CD8^{POS} CTL. T cell receptors (TCR) on CD8^{POS} cytotoxic T lymphocytes (CTL) can specifically recognize and respond to the peptides presented by Class I molecules (3,4).
We and others have developed strategies to genetically program the specificity of human T lymphocytes by retroviral transfer of genes encoding non-MHC restricted, mAb-based (5-13) or MHC restricted, TCRαβ-based (14-19) chimeric receptors. Such CTL transductants subsequently specifically respond to the relevant Ag^{POS} tumor cells by tumor cell kill and Ag-triggered cytokine production (5-19). We and others showed that the invariant domains, i.e. extracellular spacers (e.g. CD8 hinge, Ig CH2CH3), transmembrane (e.g. CD4, CD8, Fc(ε) RI-γ, CD3-ζ) and intracellular domains (e.g. Fc(ε) RI-γ, CD3-ζ or ε) significantly affect receptor stability, lymphocyte membrane density, and T lymphocyte triggerability, resulting in qualitatively and quantitatively different immune responses (9,10,13).
In the present paper we describe two types of gene-transduced CTL's that each were grafted with identical scTCR $V\alpha_{12.1}V\beta_1$, linked to distinct non-variable γ versus ζ signaling domains. Both ζ- and γ-scTCR$V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes specifically and equally well responded to native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells. It has been reported that a) differences exist in tumor antigen reactivities between human CTL (clones) generated *in vivo* or *in vitro* by stimulation with MHC Class-I^{POS} cells that present native- (intracellular route) versus synthetic peptide tumor antigens by peptide loading (extracellular route) (20,21). Moreover, complexes generated by the binding of free peptides to Class II MHC molecules can be antigenically diverse compared with those generated by intracellular processing (22,23). Hence, the ζ-scTCR$V\alpha_{12.1}V\beta_1$ ^{POS} and γ-scTCR$V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes were then used to study whether differences in antigen responses could be detected at the scTCR level, i.e., triggerability, and antigen specificity by membrane expressed Class I-HLA-A1/MAGE-A1 complexes, that were generated via the intracellular- versus the extracellular-

route. The intracellular route involved: intracellular Class I-HLA-A1 complexed with (i) intracellular "natural" MAGE-A1 peptides, (ii) intracellular MAGE-A1 peptide generated by genetic reconstitution of MAGE-A1^{NEG} antigen lost mutant melanoma cells, (iii) synthetic MAGE-A1 peptides introduced intracellularly by electroporation of B-LCL cells. These complexes then become expressed in the outer membrane of the cells. The extracellular route involved: a) already membrane expressed Class-I HLA-A1 complexed with exogenous, free MAGE-A1 peptides by peptide exchange, b) in vitro generated Class I-HLA-A1/MAGE-A1 complexes immobilized to plastic, and c) in vitro generated Class-I HLA-A1/MAGE-A1 tetramers.

At the effector cell level we clearly demonstrate that identical scTCRV$\alpha_{12.1}$Vβ_1, linked to either the γ or ζ signaling domain each recognized a distinct antigenic determinant on Class I-HLA-A1/MAGE-A1. Apparently, the scTCR specificity is not exclusively dictated by the VαVβ chains, but critically co-determined by the γ or ζ non-variable signalling chains. At the tumor target cell level, our results show that antigenic differences can arise between membrane expressed Class I/antigen (native or synthetic peptides) complexes generated via the intracellular route versus those generated via the extracellular route.

Results

ScTCRs with identical TCR V$\alpha_{12.1}$Vβ_1 chains but distinct ζversus γ signaling chains differ in their capacity to respond to membrane expressed HLA-A1/MAGE-A1 complexes generated via the extracelullar route.

We investigated the capacity of scTCR with identical V$\alpha_{12.1}$/Vβ_1, but distinct signaling elements, ζ versus γ, derived from CD3 or Fc(ε)RI-γ respectively, (Fig 1) to recognize a set of distinct HLA-A1/MAGE-A1 complexes on the membrane of target cells, generated via the intracellular- versus the extracellular route.

Human ζ-scTCRV$\alpha_{12.1}$Vβ_1 and γ-scTCRV$\alpha_{12.1}$Vβ_1 gene transduced CTL's equally well express the ζ-scTCRV$\alpha_{12.1}$Vβ_1 and γ-scTCRV$\alpha_{12.1}$Vβ_1 in the membrane, as was demonstrated with TCR Vα and Vβ family type specific mAb's (Fig 2A), albeit at lower levels than the parental TCR clone MZ2-82/30 (Fig 2A). They also specifically bound HLA-A1/MAGE-A1 tetramers (Fig 2B); specifically responded to monomeric HLA-A1/MAGE-A1 complexes immobilized to plastic with IFN-γ production (Fig 2C), and specifically lysed melanoma cells expressing native peptide: HLA-A1/MAGE-A1 complexes, (Fig 2D).

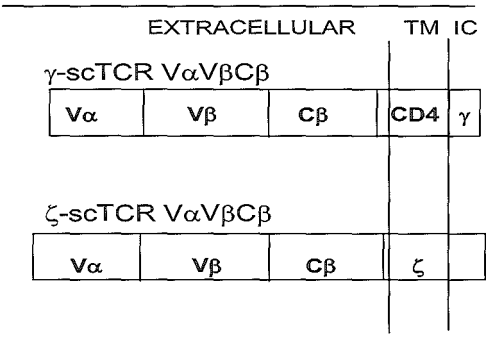

Figure 1: Chimeric single chain T cell receptor constructs: γ-scTCR V$\alpha_{12.1}$Vβ_1 and ζ-scTCR V$\alpha_{12.1}$Vβ_1. Schematic representation of chimeric γ-scTCR V$\alpha_{12.1}$Vβ_1 and ζ-scTCR V$\alpha_{12.1}$Vβ_1. (ss, signal sequence; V, variable region; C, constant region; Cκ constant Ig kappa chain fragment; CD4, CD4 transmembrane region; L, linker, γ, Fc(ε) RI-γ chain; ζ, CD3-ζ

Surprisingly, only ζ comprising scTCRV$\alpha_{12.1}$Vβ_1, but not γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} human CTL's specifically lysed melanoma target cells that express HLA-A1/MAGE-A1 complexes generated via the extracellular route by binding of exogenous synthetic peptide to membrane bound Class I-HLA-A1 by peptide exchange (Fig 2E). To exclude MAGE-A1 antigen density to be the limiting factor for the lack of γ-scTCRV$\alpha_{12.1}$Vβ_1 response, MAGE-A1 peptide pulsing of melanoma target cells was performed with increasing MAGE-A1 peptide concentrations (up to 100 μg/ml), but no triggering of lysis was observed (data not shown). Because the γ- and ζ-scTCR^{POS} CTL's share the same V$\alpha_{12.1}$Vβ_1 sheets they must recognize distinct antigenic determinants on HLA-A1/MAGE-A1 complexes in the membrane depending on the route of complex formation.

For the same reason this difference in antigen recognition pattern by the scTCRV$\alpha_{12.1}$Vβ_1^{POS} T lymphocytes must have been generated by linkage of the distinct γ versus ζ non-variable signaling structures to the scTCRV$\alpha_{12.1}$Vβ_1 chains.

These distinct antigen recognition patterns were also observed using IFN-γ, TNFα and GM-CSF production as readout systems of the T lymphocyte response (Table I and II).

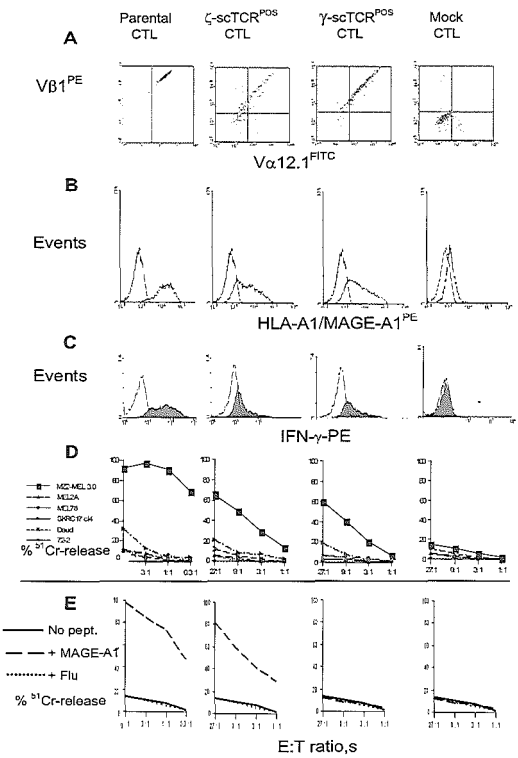

Figure 2A: Cell surface expression of γ-scTCR $V\alpha_{12.1}V\beta_1$ and ζ-scTCR $V\alpha_{12.1}V\beta_1$ on gene-transduced primary human T lymphocytes. Expression of the γ-scTCR $V\alpha_{12.1}V\beta_1$ and ζ-scTCR $V\alpha_{12.1}V\beta_1$ genes was determined by flow-cytometric analysis of retrovirally transduced primary human T lymphocytes. The T lymphocytes were stained with anti-Vα12.1^{FITC} and anti-Vβ1^{PE} mAbs. The dot plots show viable T lymphocytes selected by gating on forward (FSC) and sideward (SSC) light scatter signals. Results of a representative experiment are shown. **Figure 2B: γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes specifically bind HLA-A1/MAGE-A1 tertramers.** Transduced T lymphocytes were stained with MAGE-A1/HLA-A1 tetramers and analyzed by flow-cytometry as described in the Materials and Methods section. **Figure 2C: γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes specifically produce IFN-γ upon incubation with immobilized peptide/MHC complexes.** The production of IFNγ by γ-scTCR$V\alpha_{12.1}V\beta_1$ and ζ-scTCR$V\alpha_{12.1}V\beta_1$ transduced T lymphocytes upon incubation with immobilized M1/A1 complexes (shaded area), and INF/A1 complexes (white area), was determined by flow-cytometry as described in the Materials and Methods section. **Figure 2D: γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes specifically lyse native MAGE-A1/HLA-A1^{POS} melanoma cells.** The cytotolytic capacity of human CD8^{POS}, γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes towards various cell lines was determined in 6 h-^{51}Cr release assays. The following cell types were ^{51}Cr-labeled and used as target cells: the MAGE-A1^{POS}/HLA-A1^{POS} melanoma cell line MZ2-MEL 3.0, the MAGE-A1^{NEG} melanoma cell lines MEL 2A and MEL 78, the MAGE-A1^{NEG}/HLA-A1^{NEG} renal carcinoma cell line SCRC17 cl4, the Burkit lymphoma cell line Daudi and the EBV transformed B-LCL APD. Mean percentages of specific ^{51}Cr release of triplicate measurements are shown, with SDs not exceeding 10% of mean values . Data of one representative experiment out of three are shown. **Figure 2E: ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ CTL but not γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ CTL lyse M1 peptide pulsed target cells.** The cytolytic capacity of transduced human CTL's towards MAGE-A1 peptide pulsed MAGE-A1^{NEG}/HLA-A1^{POS} melanoma cells (MZ2-MEL2.2) was determined in 6h-^{51}Cr-release assays. The MZ2-MEL2.2 melanoma cells were pulsed with MAGE-A1 peptide (at 10 μg/ml final) 30 min prior to incubation with effector cells. Results in represented data obtained at an E:T ratio of 27:1. Experiments were performed in triplicate, and the SD did not exceed 10%. Data of one representative experiment out of three are shown.

To demonstrate that the lack of response of γ-scTCR$V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes was indeed due to lack of interaction with stimulator cells presenting Class I bound to exogenous free MAGE-A1 peptide (extracellular route), we performed cold target cell inhibition cytotoxicity assays. Native MAGE-A1^{POS} melanoma cells and MAGE-A1^{NEG} (antigen lost mutant) melanoma cells pulsed with relevant or irrelevant peptide, MAGE-A1 and Influenza, respectively, were used as

"cold" targets. Expectedly, natural MAGE-A1^{POS} melanoma cells specifically inhibited lysis of natural ^{51}Cr-labeled MAGE-A1^{POS} melanoma cells by both the ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} and γ-scTCRV$\alpha_{12.1}$Vβ_1 ^{POS} CTL's. In contrast, the MAGE-A1 peptide loaded "cold" melanoma target cells did not inhibit lysis of the native MAGE-A1^{POS} melanoma cells by γ-scTCRV$\alpha_{12.1}$Vβ_1 ^{POS} CTL, but significantly inhibited lysis by ζ-scTCR^{POS} CTL (Figure 3)
Our results thereby also demonstrate that a structural difference must exist between Class I-HLA-A1/MAGE-A1 complexes formed with natural (or synthetic, see below) peptide produced via the intracellular route versus such complexes produced via the extracellular route, i.e. by binding of exogenous free peptide to already membrane expressed class I-HLA-A1/MAGE-A1, by endogenous peptide exchange.

The conformational structures of Class I-HLA-A1/MAGE-A1 complexes formed via the intracellular- versus the extracellular route are distinct, resulting in antigenic differences.

The observed differences in the conformational structure between membrane expressed Class I-HLA-A1/MAGE-A1 complexes formed via the intracellular route versus the extracellular route could be due to differences in composition of natural versus synthetic peptides, e.g. differences in (a) amino-acid sequence (A.A.), or (b) introduction of side-chains. Because the peptide A.A. sequence of natural MAGE-A1 peptide could not be determined for technical reasons, we introduced: (i) cDNA encoding the MAGE-A1 gene into MAGE-A1^{NEG} melanoma cells (MZ2-MEL2.2) [ref], and (ii) synthetic MAGE-A1 (non-glycosilated) peptide into the cytosol of HLA-A1^{POS} EBV transformed B-LCL by electroporation. Both procedures allow for intracellular Class I-HLA-A1/MAGE-A1 complex formation, followed by their transportation to and expression in the cell membrane as determined by the relevant scTCRV$\alpha_{12.1}$Vβ_1^{POS} immune responses (Fig 4).
The same synthetic peptide that was used for peptide loading of Class I HLA-A1 by peptide exchange was also used for electroporation of HLA-A1^{POS} target cells.
Expectedly, γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} as well as ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} CTL's were specifically triggered by MAGE-A1 cDNA transfected melanoma cells synthesising "natural" peptide (figure 4A). Interestingly, synthetic MAGE-A1 peptide that was electroporated into the cytosol of HLA-A1^{POS} B-LCL, complexed with intracellular HLA-A1, and then became expressed in the membrane also specifically triggered cytokine production by both γ- and ζ-scTCR^{POS} T cell transductants (Fig 4B and C).
These data, together with the observed lack of triggering of γ-scTCRV$\alpha_{12.1}$Vβ_1 ^{POS} CTL's by synthetic peptide pulsed target stimulator cells reveal that MAGE-A1/HLA-A1 complexes generated intracellular versus extracellular assume distinct conformational structures with a distinct antigenic makeup.

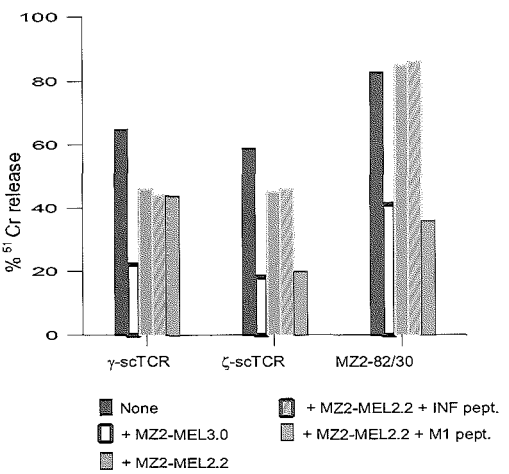

Figure 3. MAGE-A1 peptide pulsed Mel do not block the γ-scTCR V$\alpha_{12.1}$Vβ_1 mediated lysis of Mel presenting endogenously processed MAGE-A1 peptide. ^{51}Cr release of MZ2-MEL3.0 target cells without cold target cells (closed bar) and MEL3.0 cells in the presence of a 15 fold excess of (i) unlabeled MZ2-MEL3.0 cells (open bar); (ii) MZ2-MEL2.2 cells (finely hatched bar); (iii) MZ2-MEL2.2 cells pulsed with INF peptide (coarsely hatched bar); or (iv) MZ2-MEL2.2 cells pulsed with MAGE-A1 peptide (dotted bar), respectively, were measured after 6h incubations with γ-scTCR V$\alpha_{12.1}$Vβ_1^{POS}, ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} T lymphocytes and the parental CTL clone MZ2-82/30. Peptide loading of MZ2-MEL2.2 cells was performed as described in the legend of figure 2E. Data of one representative experiment out of two are shown.

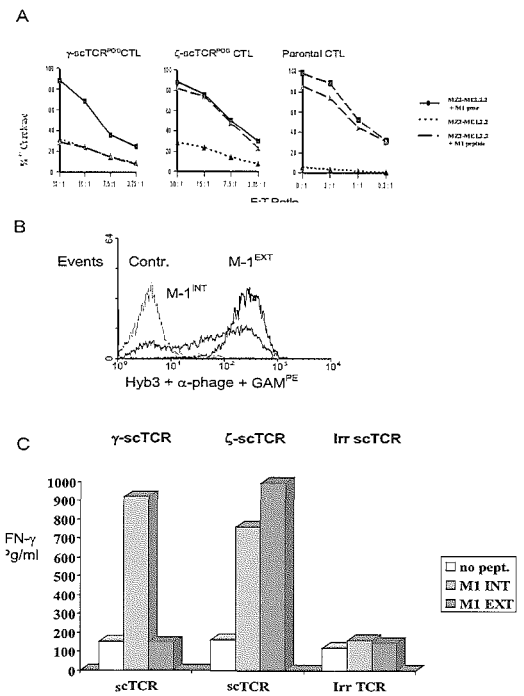

Figure 4A: Introduction of the MAGE-1 gene in melanoma cells fully restores the triggering capacity of γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ primary human T lymphocytes.
The melanoma cell line MZ2-MEL2.2 was transfected with MAGE-A1 cDNA and used as a target cell for $CD8^{POS}$, γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes in 6h-^{51}Cr-release assays. Shown are mean percentages of specific ^{51}Cr-release of triplicate measurements, with SDs not exceeding 10% of mean values. Data of one representative experiment out of two are shown. **Figure 4B: synthetic MAGE-A1 peptide complexed to intracellular HLA-A1 molecules are presented by HLA-A1 on the cell membrane.** Flow-cytometric analysis of MAGE-A1 peptide electroporated B-LCL APD, (i) immediately after MAGE-A1 peptide transfection and two washes with PBS (control) , (ii,) after MAGE-A1 peptide transfection and 18 hours incubation at 37°C, 5% CO_2 ($M1^{INT}$) , and (iii) after MAGE-A1 peptide loading and 18 hour incubation ($M1^{EXT}$).

Data of one representative experiment out of two are shown. **Figure 4C: HLA-A1/MAGE-A1 complexes generated by electroporation of free synthetic MAGE-A1 peptide into B-LCL specifically trigger γ- and ζ-scTCR^{POS} T lymphocytes to produce IFN-γ.**
γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes ($6x10^4$) were incubated for 18 hour with MAGE-A1 peptide transfected cells, (i) immediately after MAGE-A1 peptide transfection and two washes with PBS (gray bars), (ii,) after MAGE-A1 peptide transfection and 18 hours incubation at 37°C, 5% CO_2 (black bars) and after MAGE-A1 peptide loading and 18 hour incubation (white bars) Supernatants were harvested and IFN-□ production was quantified by standard ELISA (CLB, Amsterdam, The Netherlands).

Table I: production of TNFα, GM-CSF and IFN-γ by γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ and ζ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes in response to native MAGE-A1/HLA-A1^{POS} MZ2-MEL 3.0 melanoma cells.

Cytokine response to native HLA-A1^{POS}/MAGE-1^{pos} melanoma cells *			
	TNFα pg/ml	GM-CSF pg/ml	IFNγ pg/ml
CTL MZ2-82/30	428	54	1159
scTCR/ζ^{POS} CTL	183	1889	272
scTCR/γ^{POS} CTL	392	2444	526
Mock CTL	54	12	33

* As described in materials and methods

Table II: No production of TNFα, GM-CSF and IFN-γ by γ-scTCR $V\alpha_{12.1}V\beta_1^{POS}$ T lymphocytes in response to peptide pulsed HLA-A1^{POS} MZ2-MEL 2.2 melanoma cells.

	Cytokine response to MAGE-A1 peptide pulsed melanoma cells		
	TNFα pg/ml	GM-CSF pg/ml	IFNγ pg/ml
	No pept. / + M1 PEPT	No pept. / + M1 PEPT	No pept. / + M1 PEPT
CTL MZ2-82/30	0 - 2996	0 - 426	39 - 6678
scTCR/ζ^{POS} T lymph.	56 - 105	505 - 1351	64 - 577
scTCR/γ^{POS} T lymph.	49 - 46	301 - 319	94 - 93
Mock	56 - 54	254 - 255	64 - 63

* As described in materials and methods

Discussion

We clearly demonstrate at the scTCR level that genes encoding non-variable signalling elements, γ or ζ, that were individually linked to identical CβVβVα encoding genes results in γ-or ζ-scTCRV$\alpha_{12.1}$Vβ_1, respectively, that recognize distinct antigenic determinants. γ- and ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS}T lymphocytes have distinct antigen specificities. This was demonstrated by retroviral transfer of genes encoding γ- or ζ-scTCRV$\alpha_{12.1}$Vβ_1 into aliquots of the same T lymphocyte populations.

We conclude that the antigen specificity of ζ-scTCRV$\alpha_{12.1}$Vβ_1 (15) can be chanced by exchanging the ζ domain with the CD4/γ transmembrane/signaling element. These conclusions are based on the following experimental evidence. Both γ- and ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} T cell transductants recognize Class-I HLA-A1/MAGE-A1 complexes in the outer membrane of melanoma cells when these complexes were generated via the intracellular routes: intracellular synthesized Class I-HLA-A1 complexed to 1) intracellular synthesized native MAGE-A1 peptide, 2) intracellular "native" MAGE-A1 peptide resulting from genetic reconstitution of MAGE-A1antigen lost mutant melanoma cells, 3) intracellular synthetic peptide that was introduced by electroporation into the cytosol.

In contrast γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} T cells could not respond to "already" membrane expressed Class I-HLA-A1 complexed to free synthetic MAGE-A1 peptide by peptide exchange, whereas ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} T cells did respond. The lack of response of γ-scTCR^{POS} T lymphocytes was due to lack of interaction with MAGE-A1 peptide loaded target cells as shown by cold-target cell inhibition studies.

At the target cell level our experimental design and results also allow the important conclusion that differences in expression of antigenic determinants must exist between the various HLA-A1/MAGE-A1 complexes in the membrane of stimulator target cells tested. We show that these antigenic differences depend on the route of HLA-A1/MAGE-A1 complex formation, i.e., intracellular versus extracellular, and was not due to differences in amino acids or side-chains between natural and/or synthetic MAGE-A1 peptide. The difference in antigenic profiles must therefore reflect differences in conformational structure following a single peptide/MHC interaction. This has been described for Class II by Unanue and coworkers (22,23). They propose that the distinct conformational structure and hence generation of diverse antigenic determinants is depended on whether or not the catalytic H2-DM molecule was involved in the complex formation between Class II and IAk/peptide and hence, the site of complex formation (23,24).

In our case Class I-HLA-A1/ MAGE-A1 tetramers produced *in vitro*, specifically bound to both γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} and ζ-scTCR V$\alpha_{12.1}$Vβ_1^{POS} CTL's, and both γ-scTCR V$\alpha_{12.1}$Vβ_1^{POS} as well as ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} CTL's specifically responded with cytokine production to Class I-HLA-A1/MAGE-A1 complexes immobilized to plastic, and no catalytic molecules were involved. Mixing of soluble synthetic MAGE-A1 peptides with soluble Class I-HLA-A1, and β2-M may allow direct complex formation involving all contact residues of the MHC molecule, as in the case of complex formation in the ER, whereas fewer MHC contact residues may be involved in outer membrane expressed MHC when its bound endogenous peptide is exchanged with free synthetic MAGE-A1 peptide.

As discussed, γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} CTL's specifically interact with natural MAGE-A1^{POS} melanoma cells but not melanoma cells expressing Class I-HLA-A1 presenting MAGE-A1 by peptide exchange with exogenous free MAGE-A1 peptide. Apparently, the antigenic determinant that is recognized by γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} CTL's on MHC class I complexes generated via the intracellular routes is lost on the MHC Class I-HLA-A1/MAGE-A1 complexes generated by the extracellular route. In fact, 5 out of 10 point-mutated MAGE-A1 peptides loaded on MAGE-A1^{NEG}/HLA-A1^{POS} melanoma cells were recognized by ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} CTL's, but never by γ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} CTL's. Consequently, these results allow the conclusion that γ- and ζ-scTCRV$\alpha_{12.1}$Vβ_1^{POS} T lymphocytes recognize distinct antigenic determinants and hence that the conformational structure of Class I HLA-A1/ MAGE-A1 complexes generated extracellular versus intracellular are distinct.

In conclusion, we demonstrate that the scTCR specificity can be critically changed by linking distinct non-variable signalling elements to the scTCR. Moreover, Class I-Hla-A1/MAGE-A1 complexes generated via the extracellular route can be antigenically distinct from those generated via the intracellular route.

MATERIALS AND METHODS

Cells and antibodies.

T lymphocytes derived from healthy donors were isolated and expanded as described previously (25). Cell lines used as targets in cytotoxicity and in this study are: *(i)* the MAGE-A1^{POS}, HLA-A1^{POS} melanoma cell line MZ2-MEL.3.0; *(ii)* the MAGE-A1^{NEG}, HLA-A1^{POS} antigen lost mutant melanoma cell line MZ2-MEL 2.2 (kindly provided by drs. T. Boon and P. Coulie, Ludwig Institute for Cancer Research, Brussels, Belgium (26)); *(iii)* the MAGE-A1^{NEG}/HLA-A1^{POS} melanoma cell line Mel 2A; (*iv*) the HLA-A1^{NEG} melanoma cell line MEL 78; *(v)* the HLA-A1^{NEG} renal carcinoma cell line SKRC17-4 (kindly provided by dr. E. Oosterwijk, Academic Hospital Nijmegen, Nijmegen, the Netherlands); *(vi)* the HLA-A1^{POS} EBV transformed B cell blast APD, and (*vii*) the MAGE A1^{NEG}/HLA-A1 ^{NEG} Burkit lymphoma cell line Daudi. The human embryonic epithelial kidney cell line 293T (27) (kindly provided by dr. Y. Soneoka, Oxford University, Oxford, UK) was used as a packaging cell line for the pBullet γ-scTCR and pBullet ζ-scTCR retroviral vectors (15). The CTL clone MZ2-82/30 (kindly provided by T. Boon and P. Coulie (25)) was used for isolation of RNA encoding the HLA-A1 restricted, MAGE-A1 specific TCR α and β chains. The mAbs used in this study comprise the TCR family specific anti-Vα12.1 mAb (T Cell Diagnostics, Woburn, MA, USA); and anti-Vβ1 mAb (Coulter-Immunotech, Marseille, France) mAbs, anti-Class I mAb W6/32 (Seranlab, Crawley Dawn , UK) and control mouse Ig (Nordic, Tilburg, The Netherlands). The MAGE-A1 cDNA used to restore MAGE-A1 expression in the MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cell line MZ2-MEL 2.2 was kindly provided by P. Coulie, Ludwig Institute for Cancer Research, Brussels, Belgium.

Construction of the chimeric single chain TCR genes.

The pBullet scTCR VαVβCβζ vector was constructed as described (15). To construct the scTCR-CD4/γ receptor, a VαVβCβ fragment, re-amplified to introduced *SfiI* and *NotI* sites, was inserted into the pBullet retroviral vector, comprising an expression cassette (27).A schematic presentation of the chimeric scTCR constructs is shown in figure 1.

Retroviral gene introduction of γ-scTCRV$\alpha_{12.1}$Vβ_1Cβ and ζ-scTCR V$\alpha_{12.1}$Vβ_1Cβ into primary human T lymphocytes.

Human Peripheral Blood Lymphocytes (PBL) (5 x 10^6) were activated for 2 days with anti-CD3 mAb OKT3 and transduced using the retroviral vectors pBullet γ-scTCRV$\alpha_{12.1}$Vβ_1 and pBullet ζ-scTCRV$\alpha_{12.1}$Vβ_1 as described (see ref. 15 for details). In vitro expansion of transduced primary human T lymphocytes was performed in the presence of feeder cells as we described elsewhere (28).

Flow-cytometric analysis and enrichment of retrovirally transduced human T lymphocytes.

Human T lymphocytes ($5x10^5$) were stained with TCR Vα and Vβ family-type specific anti-Vα12.1^{FITC} (4 μg/ml) and anti-Vβ1^{PE} (10 μg/ml) mAb (50 μl total volume). Tetramer staining of transduced T lymphocytes ($5x10^5$) was performed for 30 min on ice, with (20 μl total volume) (15). Depletion of CD4^{POS} T lymphocyte populations was performed with anti-CD4 mAb coated magnetic beads according to the manufacturer's instructions (Dynal, Oslo, Norway). Enrichment of Vβ1^{POS} T lymphocytes was performed in two steps. First, transduced T lymphocytes (10^7 cells) were labeled with anti-Vβ1 mAb (5 μg/ml, 50 μl total volume). Next, Vβ1-labeled T lymphocytes were incubated with goat anti-mouse mAb-coated magnetic beads (Dynal) after which Vβ1^{POS} T lymphocytes were obtained by magnetic selection. The dot plots show viable T lymphoblasts selected by gating on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACS^{CAN} instrument (Becton Dickinson Biosciences, San Jose, CA, USA).

MAGE-A1 peptide transfection of EBV transformed B-cell Blasts and staining with HLA-A1/MAGE-A1 specific phages

HLA-A1^{POS} EBV-transformed B-cell blasts (APD) were transfected with synthetic MAGE-A1 peptide using a BTX 600 electroporator. Cells were harvested, washed once with medium and resuspended in 250 μl ice-cold medium supplemented with MAGE-A1 peptide (50μg/ml final concentration). APD cells were then electroporated (settings, 1900 μF, 128 Ω, 200V) and immediately transfered to ice cold conditions were 1 ml fresh, ice cold, medium was added. Transfected cells were cultured overnight at 37°C, 5% CO_2 before further analysis or washed and used immediately for staining with HLA-A1/MAGE-A1 specific phages as described (29). The histograms represent viable cells selected by gating on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACS^{CAN} instrument (Becton Dickinson Biosciences, San Jose, CA, USA).

IFN-γ capture assay.
Induction of IFN-γ production of transduced human T lymphocytes by immobilized peptide/MHC complexes was analyzed by flow cytometry as we described elsewhere (27).

Cytotoxicity assays.
Cytolytic activity of transduced human T lymphocytes was measured in 6 hr ^{51}Cr-release assays as described previously (15). For blocking purposes, mAbs were added to the T lymphocytes, 15-30 min before addition of the target cells (W6/32: 10 μg/ml, or irrelevant mIg: 10 μg/ml final). Peptide loading of target cells was performed by adding MAGE-A1 nonapeptide (EADPTGHSY, Leiden University Medical Center, the Netherlands) or irrelevant HLA-A1-binding Influenza peptide derived from Influenza virus A nucleoprotein (CTELKLSDY, Leiden University Medical Center, the Netherlands) at indicated concentrations to the target cells 5-15 min prior to incubation with effector T lymphocytes essentially as described (15).). Cold target cell inhibition experiments were performed by addition of unlabeled target cells to ^{51}Cr-labeled target cells (30:1 ratio) prior to incubation with effector cells. Percentage specific ^{51}Cr release was calculated as follows: ((test counts - spontaneous counts)/(maximum counts - spontaneous counts)) x 100%.

TNFα, GM-CSF and IFNγ ELISA
To quantify TNFα, GM-CSF and IFNγ secreted by the transduced human T lymphocytes after antigen-specific stimulation, $6x10^4$ transduced T lymphocytes were cultured for 24 h either in the presence or absence of $2x10^4$ target cells in RPMI-1640 medium supplemented with 360 IU/ml rIL-2. At the end of culture, supernatant was harvested and were measured by standard ELISA for TNF-α, GM-CSF (both from Medgenix, Fleurus, Belgium) and IFN-γ (CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions.

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CHAPTER 4

Redirecting human $CD4^+$ T lymphocytes to MHC Class I restricted melanoma antigen expressing tumor cells by TCR$\alpha\beta$ gene transfer: requirement for CD8α

Submitted

REDIRECTING HUMAN CD4+ T LYMPHOCYTES TO MHC CLASS I RESTRICTED MELANOMA ANTIGEN EXPRESSING TUMOR CELLS BY TCRαβ GENE TRANSFER: requirement for CD8α

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Adoptive immunotherapy involving the transfer of autologous tumor or virus reactive T lymphocytes has demonstrated its effectiveness in the eradication of cancer and virally infected cells. Clinical trails and *in vitro* studies have focused on $CD8^+$ cytotoxic TCR αβ lymphocytes since these are the cells that specifically kill virally infected- and tumor cells directly via their antigen specific T cell receptor (TCR) αβ. However, increasing evidence suggests that induction of sustained immunity against cancer and viral infections depends on the presence of tumor- or virus specific $CD4^+$ T lymphocytes, which are restricted by MHC class II. Here we show that these MHC class II restricted $CD4^+$ T lymphocytes can efficiently be redirected to MHC class I restricted tumor cells by retroviral introduction of an HLA-A1/MAGE-A1 specific chimeric two chain TCR VαCαζ/VβCβζ (tcTCR/ζ). However, $CD4^+$ and tcTCR/ζ^{POS} T lymphocytes were only able to specifically bind to HLA-A1/MAGE-A1 complexes and respond to HLA-$A1^+$/MAGE-$A1^+$ melanoma cells when the CD8α gene was co-introduced. These $CD4^+$, $CD8^+$/tcTCR/ζ^{POS} T lymphocytes produce cytokines typical for CD4 T helper I cells (Th1) when specifically stimulated via the introduced tc-TCR/ζ with immobilized HLA-A1/MAGE-A1 complexes or HLA-$A1^+$/MAGE-$A1^+$ melanoma cells. Furthermore, introduction of the CD8α gene into tcTCR/ζ^{POS} T lymphocytes rendered these T lymphocytes cytotoxic for HLA-$A1^+$/MAGE-$A1^+$ melanoma cells. These results demonstrate that human $CD4^+$ T lymphocytes when genetically grafted with an HLA-A1/MAGE-A1 specific tcTCR/ζ and CD8α are induced to kill and produce cytokines upon specific interaction with the relevant melanoma cells. Hence, $CD4^+$ T lymphocytes, in addition to $CD8^+$ T lymphocytes may be critical effector cells for adoptive immuno-gene therapy to generate a sustained tumor specific immune response in cancer patients.

Introduction

Although both $CD4^+$ and $CD8^+$ T lymphocytes are involved in the immune response against cancer and viral infections, there has been a preferential attention to anti-tumor responses mediated by MHC class I restricted, $CD8^+$ T lymphocytes. This focus on $CD8^+$ T lymphocytes stems from experimental data showing that (*i*) many tumors express MHC class I but not MHC class II [1] and (*ii*) $CD8^+$ T lymphocytes are capable to directly kill tumor or virally infected cells upon recognition of MHC-presented antigenic peptides [2]. Moreover, (clinical) adoptive immunotherapy studies demonstrated that the transfer of viral or tumor reactive $CD8^+$ T lymphocytes can result in effective anti-viral and anti-tumor responses, respectively [3-7]. Finally, vaccination studies using MHC class I immuno-dominant peptides have shown that protective anti-tumor immunity can be generated [8,9]. On the other hand, the important role of $CD4^+$ T lymphocytes in the induction and persistence of effective immunity was demonstrated in studies using CMV specific CTL clones as well as CD4 knockout mice [10-15]. After adoptive transfer of CMV specific $CD8^+$ CTL to patients, which lacked $CD4^+$ CMV specific T lymphocytes, the $CD8^+$ T cell response to CMV declined, suggesting an essential role for $CD4^+$ T helper lymphocytes in the persistence of the transferred $CD8^+$ CTLs [10,11]. The efficacy of the infused $CD8^+$ CTL was improved by the co-administration of the Th1 cytokine IL-2, supporting the notion of collaborations between CD4 and CD8 T cells [11]. Indeed, mice deficient for CD4 were shown to be unable to maintain Lymphocytic Choriomeningitis Virus (LCMV) specific $CD8^+$ CTL or clear virus following a challenge with low dose LCMV [12]. Additionally, the induction of tumor specific CTL against autologous MHC class II negative effusion-associated mononuclear cell tumors was shown to be severely impaired indicating that $CD4^+$ T lymphocytes are essential for the induction of protective anti-tumor $CD8^+$ CTL [16]. Adoptive transfer of $CD4^+$ tumor specific T lymphocytes may therefore be clinically relevant for effective anti-tumor responses. Indeed, in mice, the adoptive transfer of activated MHC class II restricted, tumor specific $CD4^+$ T lymphocytes has resulted in de novo generation of tumor specific $CD8^+$ T lymphocytes and effective anti-tumor responses [17]. In addition, adoptive transfer of vesicular stomatitis virus specific

(VSV) $CD4^+$ T lymphocytes into mice was able to confer protection against infection with a vaccinia virus expressing the VSV-G protein [18].

The generation of tumor specific MHC class II restricted $CD4^+$ T lymphocytes is hampered by the lack of well defined MHC class II binding peptides. Powerful techniques, such as reversed immunology, used to identify MHC class I restricted peptide epitopes [19] are not yet available for the identification of MHC class II binding peptides. Moreover, the isolation of tumor specific MHC class II restricted $CD4^+$ T lymphocytes from individual patients is troublesome and unpredictable, and hence limits its clinical application.

We have recently demonstrated the reproducible and efficient transfer of HLA-A1-restricted, MAGE-A1-specific chimeric T cell receptor (TCR) genes to bulk cultured primary human T lymphocytes [20]. These chimeric single chain- and two chain-TCR were functionally expressed on $CD8^+$ T lymphocytes and have the advantage that they do not associate with endogenous TCR $\alpha\beta$ chains, when compared with full-length TCR$\alpha\beta$ [20,21]. Upon incubation with HLA-$A1^+$, MAGE-$A1^+$ melanoma cells these transduced T lymphocytes were specifically activated to lyse the relevant tumor cells and to produce cytokines. In this study we addressed the question whether MHC class II restricted $CD4^+$ T lymphocytes can be grafted with MHC class I restricted tumor specificity so that triggering via the tcTCR/ζ induces CD4 helper functions.

We demonstrate that also primary activated $CD4^+$ T lymphocytes can be efficiently re-targeted to HLA-A1 restricted, MAGE-$A1^+$ melanoma cells by retroviral introduction of an HLA-A1/MAGE-A1 specific chimeric tcTCR/ζ. Moreover, we demonstrate that only upon introduction of both the CD8α gene and the tcTCR/ζ genes $CD4^+$ T lymphocytes acquire the capacity to specifically bind to HLA-A1/MAGE-A1complexes.

$CD4^+$/ CD8/tcTCR/ζ^{POS} T lymphocytes, bulk cultured as well as cloned T lymphocytes, specifically respond to immobilized HLA-A1/MAGE-A1 complexes and HLA-$A1^+$/MAGE-$A1^+$ melanoma cells, i.e. they produce Th1 cytokines such as IFN-γ, TNFα and IL-2. Moreover, $CD4^+$, $CD8^+$, tcTCR/ζ^{POS} T lymphocytes also specifically killed HLA-$A1^{POS}$, MAGE-$A1^{POS}$ melanoma cells. Our results demonstrate that class II restricted human $CD4^+$ T lymphocytes grafted with a class I specific tcTCR/ζ plus CD8α exert CD4 Th1-like immune functions that are likely to be important to provide sustained anti-tumor responses in patients following their adoptive transfer.

Results

Retroviral transduction of activated primary human T lymphocytes

Bulk populations of OKT3 mAb activated primary human T lymphocytes, derived from healthy donors, were retrovirally transduced with the pSTITCH tcTCR VαC$\alpha\zeta$ and VβC$\beta\zeta$ retroviral vectors and the pBullet CD8α vector, essentially as described [20]. Following retroviral transduction, bulk cultures of T lymphocytes were expanded in media containing recombinant IL-2. To obtain $CD4^+$/tcTCR/ζ^+, $CD8\alpha^+$/tcTCR/ζ^+ and $CD4^+$/$CD8\alpha^+$/tcTCR/ζ^+ T lymphocytes transduced T lymphocytes were enriched by flow-cytometric sorting after staining with mAbs anti-$V\beta1^{PE}$, anti-$V\alpha12.1^{FITC}$ and anti-$CD8\alpha^{APC}$. Enriched T lymphocyte fractions were then analyzed for CD4 and CD8α and chimeric tcTCR/ζ expression. As shown in figure 1A, homogeneous populations of $CD8^+$, $CD4^+CD8^+$ or $CD4^+$ T lymphocytes were obtained. Furthermore these enriched T lymphocyte fractions all express the chimeric tcTCR/ζ (Fig1B).

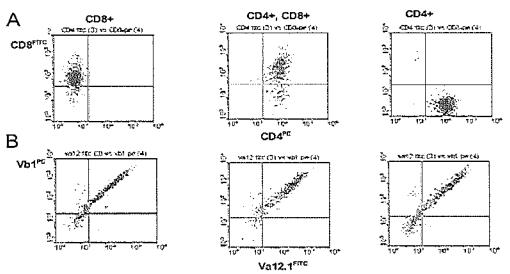

Figure 1. Expression of (A) CD4 and CD8α and (B) TCR Vα12.1 and Vβ1 on primary human T lymphocytes transduced with the chimeric tc-TCR $\alpha\zeta/\beta\zeta$ genes and the CD8α gene and sorted by flow cytometry using mAbs binding to Vβ1, Vα12.1 and CD8α.
T lymphocytes were stained and sorted as described in *Materials and Methods.*

Expression of CD8α on $CD4^+$/tcTCR/ζ^+ T lymphocytes is required for staining with MAGE-A1/HLA-A1 tetramers

Specific binding to MAGE-A1/HLA-A1 tetramers by flow-sorted $CD4^+$/tcTCR/ζ^+, $CD8^+$/tcTCR/ζ^+ and $CD4^+$/$CD8\alpha^+$/tcTCR/ζ^+ T lymphocytes was determined by flow-cytometry

analysis as described [20]. Only $CD4^+$ T lymphocytes that were transduced with both CD8α and tcTCR/ζ retroviral constructs specifically bound MAGE-A1/HLA-A1 tetramers (M1/A1). $CD4^+$ T lymphocytes that lacked CD8α did not bind tetramers (Fig. 2) $CD8^+$/ $tcTCR/\zeta^+$ T lymphocytes and the CTL clone (MZ2-82/30, data not shown) from which the TCR α and β genes were cloned [19] served as positive controls and specifically bound the M1/A1 tetramers. The simultaneous addition of anti-CD8α mAb and M1/A1 tetramers to the $tcTCR/\zeta^+$ T lymphocytes resulted in a significant reduction of binding to the M1/A1 tetramer demonstrating that CD8α is required for the TCR/MHC interaction (data not shown). Specificity of binding was demonstrated by incubating the $CD8\alpha^+$, $tcTCR/\zeta^+$ T lymphocytes with with tetramers that contained an irrelevant Influenza virus peptide derived from Influenza virus A nucleoprotein (Flu/A1)(data not shown).

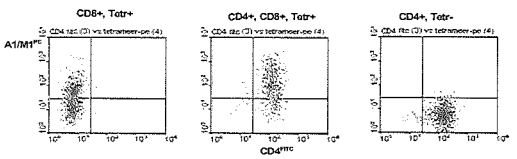

Figure 2. $CD4^+$ T lymphocytes expressing tc-TCR/ζ require co-expression of CD8α to bind HLA-A1/MAGE-A1 tetramers.
T lymphocytes were stained and sorted as described in *Materials and Methods.*

MAGE-A1 peptide loaded melanoma cells specifically activate $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes to produce TNFα

Next, we determined whether the $CD4^+$/ $tcTCR/\zeta^+$ and $CD4^+/CD8\alpha^+/tcTCR/\zeta^+$ T lymphocytes could be specifically activated by MAGE-A1 peptide loaded melanoma cells to produce TNFα. Therefore, $tcTCR/\zeta^+$ T lymphocytes were incubated for 17 hr with the $HLA\text{-}A1^+$ melanoma cell line MZ2-MEL2.2 that had been loaded with MAGE-A1 peptide prior to incubation with the transduced T lymphocytes. TNFα secretion in the culture medium was then analyzed by ELISA. As show in Figure 3, TNFα was only produced by $CD4^+CD8\alpha^+/tcTCR/\zeta^+$ T lymphocytes, when these were incubated with MAGE-A1 peptide pulsed melanoma cells and not with melanoma cells that lacked this peptide. $CD4^+/tcTCR/\zeta^+$ T lymphocytes were not stimulated to produce TNFα when incubated with the MAGE-A1 peptide pulsed melanoma cells.

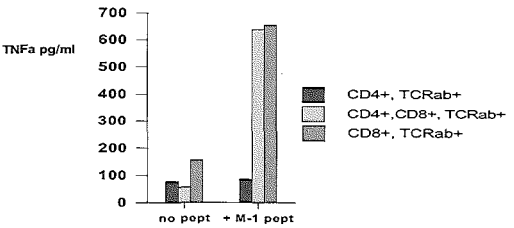

Figure 3. $CD4^+$, $tc\text{-}TCR/\zeta^+$ T lymphocytes require CD8α co-expression to produce TNF-α upon specific stimulation with MAGE-A1 (M-1) peptide loaded target cells. TNF-α production by transduced and sorted T lymphocytes was tested after stimulation for 17 hour with MAGE-A1 peptide pulsed melanoma cells. As described in *Materials and Methods.*

$CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes specifically respond to native $MAGE\text{-}A1^+$, $HLA\text{-}A1^+$ melanoma cells by TNFα, IFN-γ and IL-2 production.

We then determined whether native $MAGE\text{-}A1^+$, $HLA\text{-}A1^+$ melanoma cells could specifically activate the $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes to produce TNFα, IFN-γ and IL-2. Therefore, $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes were incubated with: (*i*) the $MAGE\text{-}A1^+$, $HLA\text{-}A1^+$ melanoma cell line MZ2-Mel3.0, (*ii*) the MAGE-A1, HLA-A1 renal cell carcinoma cell line SKRC17 cl 4, (*iii*) the HLA-A1 melanoma cell lines Mel 78 and BLM, (*iv*) the $HLA\text{-}A1^+$, MAGE-A1 melanoma cell line MEL2A and EBV transformed B cell line APD. As shown in Figure 4, only $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells specifically activated the $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes to produce produced TNFα, IFN-γ and IL-2. None of these cytokines were produced when $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes were incubated with $MAGE\text{-}A1^{NEG}$ or $HLA\text{-}A1^{NEG}$ target cell lines. Furthermore, no TNFα, IFN-γ and IL-2 was produced when $CD4^+/tcTCR/\zeta^+$ T lymphocytes were incubated with the $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells (data not shown).

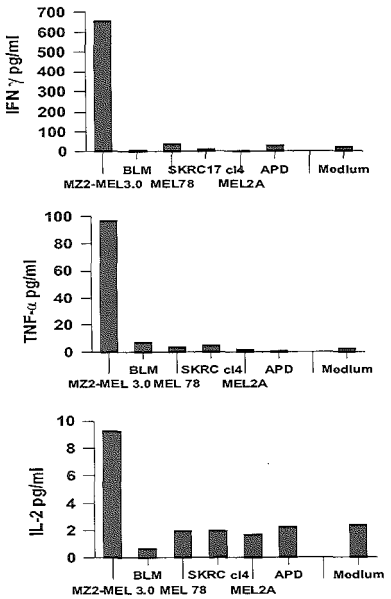

Figure 4. HLA-A1-restricted, MAGE-A1 specific cytokine production by $CD4^+/CD8^+/tcTCR/\zeta^+$T lymphocytes. To test HLA-A1/MAGE-A1 specific production of cytokines, $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes were incubated for 17 hour with a panel of cell lines positive or negative for HLA-A1 and MAGE-A1. IFN-γ, TNF-α and IL-2 production were analyzed as described in *Materials and Methods.*

Lysis of "native" $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells as well as MAGE-A1 peptide pulsed melanoma cells by $CD4^+$ T lymphocytes expressing chimeric tcTCR/ζ is depending on CD8α.

To determine whether $CD4^+$ T lymphocytes engrafted with chimeric tcTCR/ζ are capable to kill $MAGE\text{-}A1^+/HLA\text{-}A1^+$ target cells, we incubated $CD4^+/tcTCR/\zeta^+$ and $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes with ^{51}Cr-labeled $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells as well as MAGE-A1 peptide pulsed $HLA\text{-}A1^+$ melanoma cells. MAGE-A1 peptide pulsed melanoma cells and more importantly, "native" $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells were efficiently lysed by $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes (Fig 5). Melanoma cells that lack the MAGE-A1 peptide were not lysed by $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes.

Furthermore, lysis of MAGE-A1 peptide pulsed melanoma cells as well as "native" $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells depends on the co-expression of CD8α. $CD4^+/tcTCR/\zeta^+$ T lymphocytes were unable to kill these target cells. In addition, lysis of "native" $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells by $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes as well as the CTL clone MZ2-82/30 is completely blocked after addition of anti-CD8 mAb (data not shown).

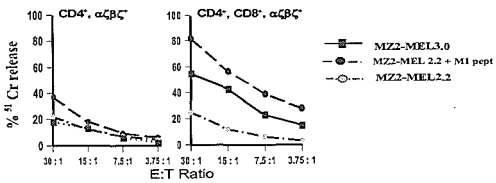

Figure 5. Lysis of native $HLA\text{-}A1^+$, $MAGE\text{-}A1^+$ and MAGE-A1 peptide pulsed melanoma cells by $CD4^+$, tc-TCR/ζ^+ T lymphocytes depends on co-expression of CD8α. The HLA-A1, MAGE-A1 specific cytolytic capacity of $CD4^+/tcTCR/\zeta^+$ and $CD4^+/CD8^+/tcTCR/\zeta^+$ T lymphocytes was analyzed in 4 hr ^{51}Cr-release assays, using $HLA\text{-}A1^+/MAGE\text{-}A1^+$ melanoma cells and MAGE-A1 peptide pulsed melanoma cells, as described in *Materials and Methods.*

Discussion

In this study, we explored the possibility to graft MHC class II restricted $CD4^+$ T lymphocytes with an MHC class I restricted tumor specificity by retroviral transfer of chimeric tcTCR $V\alpha C\alpha\zeta/V\beta C\beta\zeta$. Indeed, here we showed that $CD4^+$ T lymphocytes can be functionally endowed with chimeric TCRαβ specific for MAGE-A1 presented by HLA-A1 molecules on melanoma cells.

These $CD4^+/tcTCR/\zeta^+$ T lymphocytes were induced to produce cytokines such as TNF-α, IFN-γ and IL-2. The $CD4^+/tcTCR/\zeta^+$ T lymphocytes also exert immune specific lysis of $MAGE\text{-}A1^+/HLA\text{-}A1^+$ melanoma cells. Moreover, we also demonstrate that co-expression of CD8α molecules with tcTCR/ζ on the $CD4^+$ T lymphocytes is a prerequisite for triggering of these immune response. Attempts to generate tumor reactive human $CD4^+$ T lymphocytes have been hampered by the difficulty to identify MHC class II restricted tumor epitopes that can be used for their *in vitro* stimulation. Reproducible technologies for the identification of MHC class I presented tumor antigens have been developed [19], but

these are lacking for class II presented tumor antigens [22]. Because of fundamental differences in peptide processing and loading of the MHC class II molecules, when compared to MHC class I molecules, identification of class II presented tumor peptides awaits a technical breakthrough [22,23]. The availability of $CD4^+$ T lymphocytes may be of importance because $CD4^+$ T lymphocytes play critical roles in the generation of antigen specific T cell responses and in the induction of memory, providing sustained immunity [13-18].

Recently, we and others successfully grafted primary human T lymphocytes with MHC class I restricted specificity through introduction of full-length TCR$\alpha\beta$, chimeric single chain and two chain TCR genes. Introduction of these (chimeric) TCR conferred the T lymphocytes with a new MHC restricted tumor specificity [20, 24-26].

The physiological interaction between TCR$\alpha\beta$ on T lymphocytes and MHC class I molecules on target cells has been shown to involve the simultaneous interaction of CD8α and/or CD8β on T lymphocytes with the MHC class I molecules on target cells [27-29]. Indeed, addition of anti-CD8 mAbs generally inhibits T cell mediated immune functions [28,30] or specific binding of soluble MHC class I tetramers [31]. We addressed the question whether $CD4^+$, tcTCR/ζ^+ T lymphocytes also require CD8α to bind tetramers and become activated by relevant target cells. Therefore we introduced either the tcTCR/ζ alone or CD8α and the tcTCR/ζ simultaneously into $CD4^+$ human T lymphocytes. Only the $CD4^+/CD8^+$/tcTCR/ζ^+ T lymphocytes, but not $CD8\alpha^-$, $CD4^+$/tcTCR/ζ^+ T lymphocytes were able to bind tetramers and exerted tcTCR/ζ mediated immune functions, i.e. cytokine production and lysis of tumor cells. The cytokines produced were of the Th1 type, i.e. IL-2, IFN-γ and TNFα, and were produced only after specific stimulation with the relevant MAGE-A1/HLA-A1 expressing target cells. We conclude that triggering of the Class I restricted Th1-type immune response, normally triggered by class II restricted tumor antigens, requires the interaction between CD8α and the tcTCR/ζ.

In mice, adoptive transfer models of viral infections such as influenza and VSV have demonstrated that the transfer of CD4 T cell clones effectively protected mice from influenza or VSV induced infections [18] The $CD4^+/CD8^+$/tcTCR/ζ^+ T lymphocytes that exert Th1 immune functions may also exert help to generate $CD8^+$ CTL *in vivo* as well as memory responses [32]. In a clinical study it was demonstrated that long term persistence of adoptive transferred cytomegalovirus or human immunodeficiency virus specific $CD8^+$ CTL probably requires adequate CD4 T helper functions [11]. Taken these clinical data and data obtained from adoptive transfer studies in CD4 knockout mice together; the adoptive transfer of both $CD8^+$/tcTCR/ζ^+ T lymphocytes and $CD4^+/CD8^+$/tcTCR/ζ^+ T lymphocytes may prolong *in vivo* survival of the transferred $CD8^+$ CTL as well as help the de novo generation of patients $CD8^+$ CTL with tumor specificity.

Materials and methods

Cells and antibodies.

T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere [33]. Target cell lines used in this study are: *(i)* the MAGE-$A1^+$/HLA-$A1^+$ melanoma cell line MZ2-MEL.3.0; (*ii*) the MAGE-$A1^-$/HLA-$A1^+$ melanoma cell line MZ2-MEL 2.2 (kindly provided by T. Boon and P. Coulie, Brussels, Belgium [19]; *(iii)* the MAGE-$A1^-$/HLA-A1+ melanoma cell line Mel 2A; (*v*) the HLA-$A1^-$ melanoma cell line MEL 78; *(vi)* the HLA-$A1^-$ melanoma cell line BLM (kindly provided by G. Adema, Nijmegen, the Netherlands); (*vii*) the HLA-$A1^-$ renal carcinoma cell line SKR17-4 (kindly provided by E Oosterwijk, Nijmegen, the Netherlands); and *(viii)* the HLA-$A1^+$ EBV transformed B cell line APD. The human embryonic kidney cell line 293T [34] and phoenix-amp [35] were used for production of pStitch VαC$\alpha\zeta$/VβC$\beta\zeta$ and pBullet CD8α retroviral vectors. The mAbs used in this study were: anti-$CD4^{FITC/PE}$; anti-$CD8^{FITC/PE/APC}$ (Becton Dickinson Biosciences, San Jose, CA, USA); the TCR Vα and Vβ family specific mAbs anti-Vα12.1^{FITC} (T Cell Diagnostics, USA); and anti-V$\beta$$1^{PE}$ (Coulter-Immunotech, Marseille, France). HLA-A1/MAGE-A1 tetramers were kindly provided by P. van der Bruggen (Brussels, Belgium).

Construction of retroviral vectors and transduction of primary human T lymphocytes.

The chimeric TCR VαC$\alpha\zeta$ and VβC$\beta\zeta$ genes were derived from the CTL clone MZ2-82/30 as described previously [20]. The CD8α gene was amplified with primers that allowed introduction into the retroviral vector pBullet, from the pLCD8αSH vector (a kind gift from Jos Molenhorst, Leiden, the Netherlands).

The chimeric TCR genes and the CD8α gene were introduced into anti-CD3 mAb activated primary human T lymphocytes essentially as described [25]. After two rounds of supernatant transduction the transduced T lymphocytes were expanded in culture medium, supplemented with r-IL2 (360 IU/ml).

Flow-cytometry analysis and sorting of retrovirally transduced human T lymphocytes.

Viable human T lymphocytes ($5x10^5$) were stained with TCR Vα and Vβ family-type specific anti-Vα-12.1^{FITC} (2 μg/ml) and anti-Vβ1^{PE} (2 μg/ml) mAb in a final volume of 50 μl. Transduced T lymphocytes ($5x10^5$) were stained with MAGE-A1/HLA-A1 tetramers (10 nM final, 30 min on ice). T lymphocyte viability was assessed by trypan blue staining: only T lymphocyte populations > 95 % viable were used for flow cytometry analysis. The dot plots represent viable T lymphoblasts selected by gating on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACSCAN instrument (Becton Dickinson Biosciences, San Jose, USA). FACS sorting was performed on a FACS-Vantage instrument (Becton Dickinson Biosciences) using saturating concentrations of Vα12.1^{FITC}, Vβ1^{PE} and CD8α^{APC} FACS-sorted lymphocytes were expanded before use in functional assays as described [20].

TNF-α, IFN-γ and IL-2 production

To quantify TNF-α, IFN-γ and IL-2 production by the transduced flow-sorted human T lymphocytes, $6x10^4$ transduced T lymphocytes were cultured for 24 hr either in the presence or absence of $2x10^4$ adherent tumor cells in RPMI-1640 medium supplemented with 360 IU/ml rIL-2 ,and where indicated, with 10 μg/ml MAGE-A1 peptide. At the end of culture, supernatant was harvested and levels of TNF-α, IFN-γ and IL-2 (CLB, Amsterdam, The Netherlands) were measured by standard ELISA according to the manufacturer's instructions.

Cytotoxicity assays

Cytolytic activity of transduced human T lymphocytes was measured in ^{51}Cr-release assays as described previously [20]. Peptide loading of target cells was performed by adding MAGE-A1 nonapeptide (EADPTGHSY, Leiden University Medical Center, the Netherlands) or irrelevant HLA-A1 binding Influenza peptide derived from Influenza virus A nucleoprotein (CTELKLSDY, Leiden University Medical Center, the Netherlands) at indicated concentrations to the target cells 5-15 min prior to incubation with effector T lymphocytes, at indicated effector to target cell ratios. The incubation period of effector and target cells is indicated in the figures. Percentage specific ^{51}Cr release was calculated as follows: ((test counts - spontaneous counts)/(maximum counts - spontaneous counts)) x 100%.

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CHAPTER 5

A phage display selected Fab fragment with MHC-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes

Gene Therapy 2001, 8: 1601-1608.

A phage display selected Fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes

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The clinical benefit of adoptive transfer of MHC-restricted cytotoxic T lymphocytes(CTL) for the treatment of cancer is hampered by the low success rate to generate antitumor CTLs. To bypass the need for tumor-specific CTL, we developed a strategy that allows for grafting of human T lymphocytes with MHC-restricted antigen specificity using in vitro selected human Fab fragments fused to the Fc(ε)RI-γ signaling molecule. Retroviral introduction of a Fab-based chimeric receptor specific for MAGE-A1/HLA-A1 into primary human T lymphocytes resulted in binding of relevant peptide/MHC complexes. Transduced T lymphocytes responded to native MAGE-A1/HLA-A1^{POS} target cells by specific cytokine production and cytolysis. Therefore, peptide/MHC-specific Fab fragments represent new alternatives to TCR to confer human T lymphocytes with tumor specificity, which provides a promising rationale for developing immunogene therapies. Gene Therapy (2001) **8**, 1601–1608.

Keywords: *tumor rejection antigen; Fab; MHC; cytotoxic T lymphocyte; T cell receptor; chimeric receptor*

Introduction

T cells are equipped with unique antigen recognition units, the T cell receptors (TCR) that are composed of polymorphic TCR $\alpha\beta^1$or $\gamma\delta^2$ chains and the non-polymorphic CD3-γ, δ, ε and ζ signaling molecules.[3,4] The $CD8^+$ cytotoxic T lymphocytes (CTL) play an essential role in the eradication of virally infected cells and tumor cells, and specifically recognize and kill cells that present antigenic peptides in the context of MHC class I molecules.[5,6] Isolation and expansion of such CTL has allowed for the identification of genes that code for tumor rejection antigens (TRA), such as the melanoma-associated MAGE genes.[7,8] Peptides presented by MHC class I molecules are usually between eight and 10 amino acids long and are buried in a peptide binding groove, which consists of two antiparallel α-helices overlaying a platform of antiparallel β-strands.[9] Two or three positions in the peptide, the anchoring residues, fit into hydrophobic pockets formed by the variable residues in the peptide binding groove.[10,11] Exposed peptide residues and non-buried elements of the two α-helices are critical for the recognition of the peptide/MHCcomplex by TCR.[12]

MHC-restricted CTL with specificities for a wide range of TRA, such as tyrosinase, MART-1, gp100, p15, TRP-1, β-catenin, MAGE, BAGE and GAGE, have been identified and isolated from cancer patients or have been generated *in vitro*.[13] The identification of these peptides has triggered the development of new immune treatment strategies, aimed either at the induction of specific T cell responses by vaccination,[14,15] or the adoptive transfer of *in vitro* generated MHC-restricted CTL.[6] TRA-specific T lymphocytes, however, occur only at low frequencies in a small proportion of patients, probably because many of these TRAs represent normal differentiation antigens and are of a tolerogenic nature. This low CTL frequency together with the difficulties associated with *in vitro* generation of CTL-specific for cancers other than melanoma makes CTL-based immunotherapy impractical. In addition, it is important to note that peptide specific CTL generated *in vitro* by incubation of PBMC with peptide pulsed APC were reported to respond to peptide-pulsed target cells, but not to native antigen presenting tumor cells.[16] Moreover, CTL generated *in vivo* by vaccination with peptide-loaded antigen presenting cells (APC) do not necessarily respond to native tumor antigen-presenting tumor cells.[17,18]

Gene-based strategies were developed which aimed at transferring specificities to primary human T lymphocytes via chimeric Ig or TCR molecules.[19,20] Chimeric Ig-based receptors with specificities for tumor-associated, MHC non-restricted antigens (TAA) can be functionally expressed on human T lymphocytes.[21–24] These genetically introduced chimeric receptors do not affect the requirement of co-activation and adhesion receptors to activate T lymphocytes.[25] However, in contrast to TAA, TRA are processed intracellularly and presented as peptides in MHC molecules, and hence are not recognized by 'classical' mAbs *per se*. In addition, we recently showed that chimeric TCR αβ receptors can confer human T lymphocytes with MAGE-A1/HLA-A1 speci-

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ficity.[26] However, the applicability of T cell grafting with antitumor specificity is still limited by the availability of peptide/MHC-specific T cell clones. Methods that allow for *in vitro* selection of TRA-specific molecules from large repertoires would potentially provide a pool of tumor-reactive molecules. Thus far, several approaches have been explored to obtain molecules that bind MHC-restricted antigens on tumor cells. First, TCR libraries specific for peptide/MHC complexes were generated,[27–29] but engineering of TCR proved difficult and the TCR were of low binding affinity.[30,31] Second, monoclonal antibodies (mAbs), with MHC-restricted specificity were generated by immunizing mice with soluble peptide-MHC complexes or antigen-presenting cells. Unfortunately, these immunization protocols were rarely successful.[32–35] In this paper, we exploited the selection power of phage display that allows for the testing of tens of millions of individual clones with a high-throughput selection of Fabs with peptide/MHC complex binding capacity. Following this strategy, we recently selected a fully human Fab fragment, termed Fab-G8, specific for HLA-A1/MAGE-A1 complexes from a large non-immune phage-antibody repertoire.[36] The isolated phage specifically binds to MAGE-A1 peptide pulsed HLA-A1^{POS} B-LCL, as well as to MAGE-A1 peptide presented by HLA-A1 molecules on antigen-expressing tumor cells.

Here we report on the construction and retroviral transfer of a chimeric receptor based on this phage display selected Fab fragment, and its use to genetically program primary human T lymphocytes with an immune specificity. The Fab-G8 gene fragment was ligated via the CD4 transmembrane domain to the intracellular Fc(ε)RIγ chain signaling molecule, resulting in the Fab-G8-CD4/γ receptor, which was retrovirally introduced into human T lymphocytes. Fab-G8-CD4/γ^{POS} T lymphocytes specifically bound MAGE-A1/HLA-A1 complexes. Human T lymphocytes expressing this MHC restricted Fab-based chimeric receptor on their membrane specifically responded to both MAGE-A1 peptide pulsed HLA-A1^{POS} B cell blasts and native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells by specific tumor cell lysis, as well as cytokine production. Thus, peptide/MHC-specific chimeric Fab fragments can be used to retarget human T lymphocytes to TRA epitopes, which may provide a novel rationale for developing clinical immune-gene therapeutic modalities independent of CTL-derived TCR.

Results

Retroviral transduction of human T lymphocytes with the Fab-G8-CD4/γ receptor

The heavy and lambda light chains from the human Fab fragment G-8 specific for MAGE-A1/HLA-A1 were obtained from a non-immune human Fab library.[36] Amplification by PCR introduced *Sfi*I and *Not*I restriction sites, which enabled insertion into a retroviral expression cassette. The retroviral expression cassette used, comprises a signal sequence derived from the G250 monoclonal antibody (mAb) heavy chain, cloning sites, a short Ig constant kappa spacer (Cκ) linked to the CD4 transmembrane domain and the intracellular domain of the Fc(ε)RIγ chain. Inclusion of the CD4 transmembrane domain and the intracellular domain of the Fc(ε)RIγ chain in chimeric mAb-based receptor constructs resulted in increased membrane expression.[23] The cassette was inserted into the retroviral vector pBullet.[26] The amplified heavy and lambda chain fragments were cloned individually into this retroviral expression cassette resulting in Fab-G8-CD4/γ heavy and light chain retroviral vectors (Figure 1). The Fab-G8-CD4/γ retroviral vectors were then introduced into CD4-depleted, OKT3 mAb-activated primary human T lymphocytes by cocultivation with 293T cells, that had been transfected with both the heavy and lambda retroviral vectors and retroviral packaging constructs.[23] As a control, activated human T lymphocytes were cocultivated with 293T packaging cells that were transfected with an empty pBullet vector.

Fab-G8-CD4/γ^{POS} human T lymphocytes specifically bind MAGE-A1/HLA-A1 complexes

Membrane expression of Fab-G8-CD4/γ receptors on transduced human T lymphocytes was analyzed by incubating the cells with soluble MAGE-A1/HLA-A1/streptavidin^{PE} complexes, that had also been used to isolate the original Fab G-8 phage. As shown in Figure 2a, about 3% of the transduced human T lymphocytes specifically bound soluble MAGE-A1/HLA-A1 complexes. MAGE-A1/HLA-A1/streptavidin^{PE} complex binding T lymphocytes were enriched using anti-PE mAb coated magnetic beads as described in Materials and methods. This resulted in more than 90% Fab-G8-CD4/γ receptor^{POS} T lymphocytes (Figure 2b). Enriched Fab-G8-CD4/γ^{POS} T lymphocytes were *in vitro* expanded and used in all subsequent experiments. Nonspecific binding was not observed, neither for mock-transduced T lymphocytes using soluble MAGE-A1/HLA-A1 complexes (Figure 2d), nor for Fab-G8-CD4/γ receptor^{POS} human T lymphocytes using soluble influenza/HLA-A1/streptavidin^{PE} complexes (Flu/HLA-A1). The CTL clone MZ2-82/30 expressing an MAGE-A1/HLA-A1-specific TCR was used as a positive control (Figure 2c). FACS analysis of the Fab-G8-CD4/γ transduced T lymphocytes (ie CD4, CD8,CD56, CD16 and TCR γδ) showed that the effector populations almost exclusively consist of CD8^{POS} cells (data not shown).

Fab-G8-CD4/γ^{POS} primary human T lymphocytes specifically produce IFN-γ upon stimulation with immobilized MAGE-A1/HLA-A1 complexes

We also determined whether Fab-G8-CD4/γ^{POS} primary human T lymphocytes could be activated by immobilized MAGE-A1/HLA-A1 complexes to produce IFN-γ. To this end, we incubated transduced human T lymphocytes for 18-20 h either on immobilized MAGE-A1/HLA-A1 com-

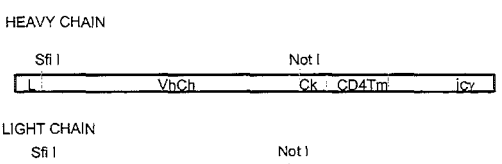

Figure 1 Fab-G8-CD4γ receptor constructs. Schematic representation of the Fab-G8-CD4/γ gene constructs: L, leader; VhCh, human heavy chain; VlCl, human lambda light chain; Ck constant Ig kappa chain fragment; CD4Tm, CD4 transmembrane region; Icγ, intracellular Fc(ε)RIγ chain region. Constructs were made as described in Materials and methods.

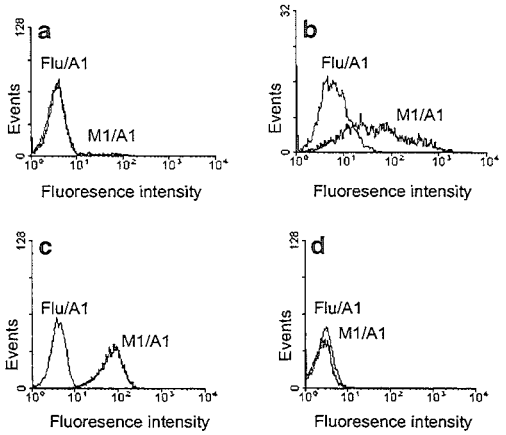

Figure 2 Cell surface expression of the Fab-G8-CD4/γ receptor on transduced primary human T lymphocytes. Cell surface expression of the Fab-G8-CD4/γ receptor was analyzed by incubation of T lymphocytes with relevant MAGE-A1/HLA-A1/streptavidin^{PE} complexes (M1/A1, bold line) and irrelevant influenza/HLA-A1/streptavidin^{PE} complexes (Flu/A1, normal line). The following cell samples were used: primary human T lymphocytes transduced with Fab-G8-CD4/γ retroviral vectors (a); human T lymphocytes transduced with Fab-G8-CD4/γ retroviral vectors, followed by selection with MAGE-A1/HLA-A1/streptavidin^{PE} complexes and anti-PE mAb-coated magnetic beads (b); the HLA-A1 restricted, MAGE-A1 specific parental CTL clone MZ2-82/30 (positive control) and mock-transduced human T lymphocytes (negative control) (c and d, respectively).

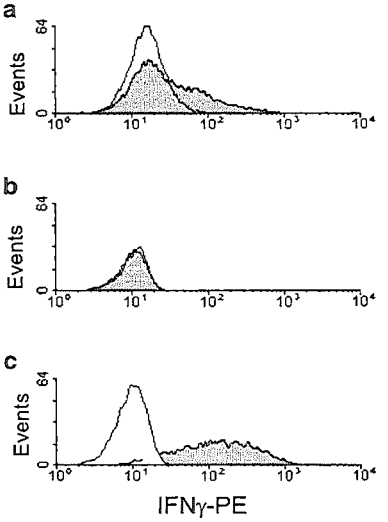

Figure 3 IFNγ production by Fab-G8-CD4/γ^{POS} human T lymphocytes upon incubation with immobilized MAGE-A1/HLA-A1 complexes. IFNγ production by the Fab-G8-CD4/γ transduced T lymphocytes upon incubation with immobilized MAGE-A1/HLA-A1 complexes (shaded area) or control influenza peptide/HLA-A1 complexes (white area) was determined by flow cytometry as described in Materials and methods. Events shown represent viable lymphocytes stained with PE labeled anti-IFNγ mAb. The following cell samples were used: (a) Fab-G8-CD4/γ transduced T lymphocytes, (b) mock transduced T lymphocytes (negative control), and (c) CTL MZ2-82/30 (positive control).

plexes or control Flu/HLA-A1 complexes and analyzed cells by IFN-γ capture assay (see Materials and methods). Only MAGE-A1/HLA-A1 complexes, but not control Flu/HLA-A1 complexes, triggered Fab-G8-CD4/γ^{POS} primary human T lymphocytes to produce IFN-γ (Figure 3). Moreover, no IFN-γ was produced when mock-transduced T lymphocytes were incubated with peptide/MHC complexes. A clear IFN-γ production was observed when CTL clone MZ2-82/30 was stimulated with MAGE-A1/HLA-A1 complexes, but not control complexes.

Fab-G8-CD4/γ^{POS} primary human T lymphocytes specifically respond to MAGE-A1 peptide pulsed HLA-A1^{POS} target cells by cytolysis and cytokine production

To analyze Fab-G8-CD4/γ^{POS} primary human T lymphocytes for their responsiveness to cellular targets expressing MAGE-A1/HLA-A1, we incubated transduced T lymphocytes, in a first set of experiments, with HLA-A1^{POS} EBV-transformed B cell blasts (B-LCL APD) which were externally loaded with MAGE-A1 peptide (at 10 μg/ml final). B cell blasts were incubated with an irrelevant influenza virus peptide to monitor nonspecific responses. Only B-LCL pulsed with MAGE-A1 peptide, but not influenza virus peptide were able to trigger target cell lysis by Fab-G8-CD4/γ^{POS} T lymphocytes (Figure 4). In addition, triggering of TNF-α and IFN-γ production was observed when Fab-G8-CD4/γ^{POS} T lymphocytes were incubated with MAGE-A1 peptide pulsed target cells, but not with irrelevant influenza peptide pulsed target cells (Figure 5). Neither target cell lysis nor cytokine production was observed when mock transduced T lymphocytes were used as effector cells. As a positive control for specific cytolysis and cytokine production the CTL MZ2-82/30 was used.

Native MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells specifically induce cytolysis and cytokine production by Fab-G8-CD4/γ^{POS} T lymphocytes

Finally, we tested whether Fab-G8-CD4/γ^{POS} T lymphocytes were able to respond to native MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells. For this purpose, we incubated Fab-G8-CD4/γ^{POS} T lymphocytes with the MAGE-A1/HLA-A1^{POS} MZ2-MEL 3.0 cell line and with MAGE-A1^{NEG} or HLA-A1^{NEG} cell lines of distinct histological origin (ie MEL 2A, MEL 78 and SKRC17 cell lines). Only MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells, but not control MAGE-A1^{NEG} or HLA-A1^{NEG} cells were specifically lysed by the Fab-G8-CD4/γ^{POS} T lymphocytes (Figure 6a), and triggered specific production of TNF-α and IFN-γ (Figure 7). MHC restriction of the observed lysis was further illustrated by the significant inhibition of Fab-G8-CD4/γ^{POS} T lymphocyte-mediated target cell lysis following addition of anti-class I mAbs (Figure 6b). Negative controls comprised addition of irrelevant mAbs, or incubation of MAGE-A1^{POS}, HLA-A1^{POS} melanoma cells with mock transduced T lymphocytes. Moreover, the cytolytic responses of the Fab-G8-CD4/γ^{POS} T lymphocytes are clearly inhibited by the addition of anti-CD8 mAb demonstrating that indeed the effector population almost exclusively consists of CD8^{POS} cells (data

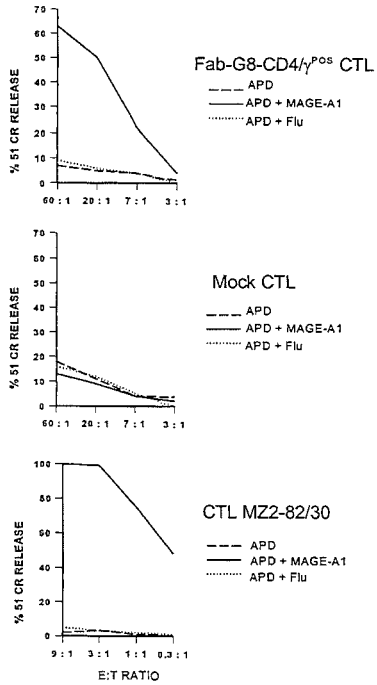

Figure 4 Peptide pulsed target cells are lysed by Fab-G8-CD4/γ transduced human T lymphocytes. MAGE-A1 peptide-pulsed target cells were incubated with Fab-G8-CD4/γ^{POS} human T lymphocytes, and tested in 6 h 51Cr release assays at four different effector to target cell ratios (E:T). 51Cr labeled MAGE-A1^{NEG} and HLA- A1^{POS} EBV transformed B cell blasts (ie APD) were used as target cells and pulsed with MAGE-A1 or influenza virus peptides (at 10 μg/ml final). As controls for specific lysis, mock transduced T lymphocytes and CTL MZ2-82/30 (positive control) were used. Experiments were performed in triplicate, and the s.d. did not exceed 10% of the mean value. One representative experiment out of three is shown.

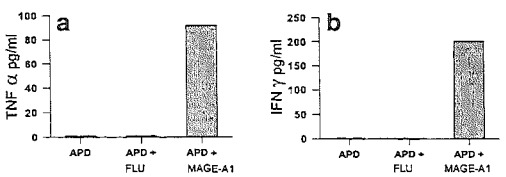

Figure 5 Fab-G8-CD4/γ^{POS} human T lymphocytes produce TNFα and IFN-γ upon stimulation with MAGE-A1 peptide pulsed target cells. Fab-G8-CD4/γ^{POS} human T lymphocytes (6 × 10^4) were incubated for 24 h with HLA-A1^{POS} EBV transformed B cell blasts (2 × 10^4) in the presence of MAGE-A1 or influenza peptide (at 10 μg/ml final). Production of TNF-α (a) and IFN-γ (b) was quantified by ELISA, as described in Materials and methods.

not shown). As a positive control again CTL MZ2-82/30 was used.

Discussion

In this study we show efficient retargeting of human T lymphocytes to tumor cells presenting the MAGE-A1 antigen in the context of the MHC class I molecule HLA-A1 by retroviral introduction of a human Ig-based chimeric receptor. The chimeric receptor was made by ligation of the anti-MAGE-A1/HLA-A1 Fab-G8 to the CD4 transmembrane domain and intracellular Fc(ε)RIγ chain, and was successfully transduced into primary human T lymphocytes. Human T lymphocytes expressing this MHC restricted Fab-G8-CD4/γ receptor specifically bound MAGE-A1/HLA-A1 complexes. Moreover, Fab-G-8-CD4/γ^{POS} T lymphocytes specifically responded to both MAGE-A1 peptide pulsed HLA-A1^{POS} B cell blasts and native MAGE-A1^{POS}/HLA-A1^{POS} melanoma cells by specific tumor cell lysis, as well as cytokine production. The MHC restriction of the observed immune reactivities was demonstrated by a significant reduction of target cell lysis by anti-MHC class I mAbs.

Antibody-based retargeting of T cells has already been achieved to tumor-associated antigens (TAA).[21–24] However, this is the first report on an Ig-based chimeric receptor conferring MHC-restricted tumor specificity to primary human T cells. Since most known TRAs are in fact MHC restricted peptides, this may have major implications for immunotherapy. Adoptive immunotherapy by clinical transfer of autologous tumor or virus-specific T cells and interleukin-2 has met success in the treatment of both hematological malignancies and melanoma,[6,37] and viral diseases caused by CMV and EBV.[5] The overall difficulty in obtaining tumor-reactive TIL from individual patients, and TIL specific for cancers other than melanoma, however, has hampered the development of clinically effective adoptive immunotherapeutic strategies. Research focussed on the engineering of chimeric receptors to transfer specificities to T lymphocytes. Our laboratory developed strategies to genetically program human T lymphocytes with non-MHC restricted (Ab-based) and MHC-restricted (TCR-αβ-based) chimeric receptors.[19,20] Chimeric single chain or double chain Ig or TCR receptor^{POS} T lymphocytes specifically respond to tumor cells expressing the relevant antigen by tumor cell kill and cytokine production.[22,26] However, in the case of TRA epitopes, this approach is still limited by the isolation of tumor-specific CTLs required for the cloning of TCR genes. A complete *in vitro* approach would allow for the selection of TRA-specific molecules, without being limited by the availability of CTL clones. The ability to produce numerous HLA complexes *in vitro*,[38] as well as the availability of large non-immune human phage-antibody libraries has made such an *in vitro* approach within reach.[36] In principle, any peptide with the right anchoring residues may be refolded *in vitro* with MHC class I heavy chain and β2 microglobulin, resulting in peptide/MHC complexes available for phage selection. Indeed, we have recently used our non-immunized library to select human antibodies against 12 HLA-A2 complexes presenting different peptides and we were able to isolate between three and nine different antibodies for each complex, some of them of high affinity, in less than 4 weeks (manuscript in preparation). This shows that it is possible to rapidly

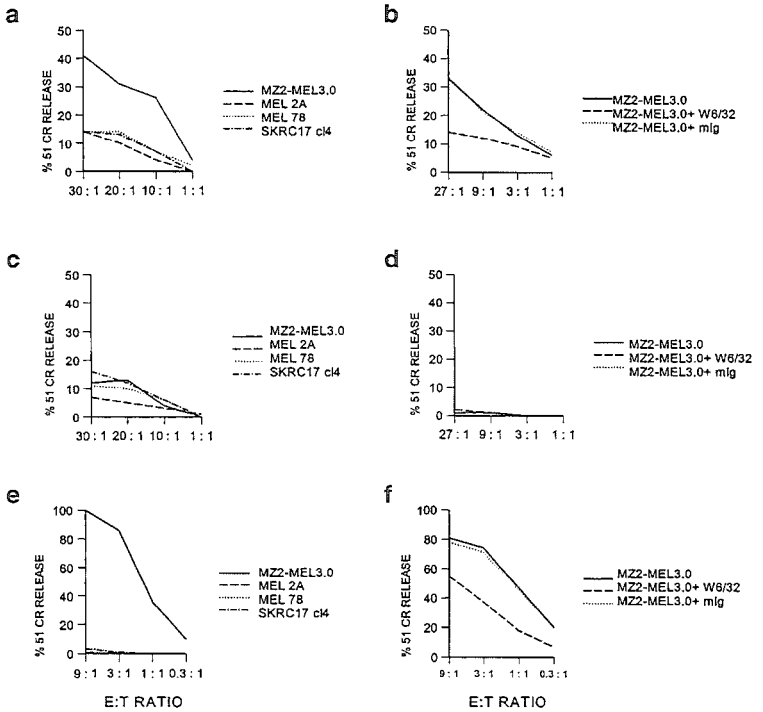

Figure 6 Native MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells are susceptible to lysis by Fab-G8-CD4/γ transduced T lymphocytes. Cytotolytic activity of human T lymphocytes versus MAGE-A1 expressing melanoma cells was determined as described in legend to Figure 4. MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells (ie MZ2-MEL 3.0) and MAGE-A1^{NEG} and/or HLA-A1^{NEG} cell lines of different histologies (ie MEL 2A, MEL 78 and SKRC17 cl4) were used as target cells. Target cells were incubated with (1) Fab-G8-CD4/γ transduced T lymphocytes (a and b); (2) mock transduced T lymphocytes (negative control, c and d); and (3) CTL MZ2-82/30 (positive control, e and f). Anti-HLA-ABC or mouse Ig (both at 10 μg/ml final) was added to the melanoma target cells (ie MZ2-MEL 3.0) 15–30 min before incubation with the effector T lymphocytes (b, d and f). Mean percentages of specific 51Cr release of triplicate measurements are shown, with s.d. not exceeding 10% of mean values. Data of one representative experiment out of three are shown.

select anti-peptide/MHC antibodies against any complex and that this technology may become a reliable source of viral or TRA-specific receptors in the near future. In contrast to TCRs, Fab fragments can be easily produced in *E. coli*, making it possible to determine and modulate the fine specificity of Fab fragments by antibody-engineering techniques before their use in retargeting experiments. Hence, we believe that the use of phage display will prove to be a flexible tool to select and modulate TRA-specific Fab molecules.

Fab-G8 has an affinity with a K_D of 250 nm, which is already high compared to TCR/peptide MHC interactions (average K_D around 10^{-5} to 10^{-6} M). We have recently improved the affinity of Fab-G8 by a factor of 18. During this process, low affinity variants have also been generated. Different mutants of Fab-G8 with the same peptide fine-specificity, but covering a large range of affinities, with K_Ds varying from 1300 to 14 nm are thus available (manuscript in preparation). The effect of receptor affinity on T cell retargeting and killing is currently being investigated. Moreover, we suggest that this Fab-CD4γ fusion may be used for comparative functional analyses between antibody-based and TCR-based T cell retargeting. Preliminary results show that it is possible to retarget T cells using a double chain TCR directed against MAGE-A1/HLA-A1 fused to Fc(ε)RIγ. The TCR-based and Fab-G8-based constructs have identical specificities and signaling moieties, providing powerful tools for investigating the requirements of additional interactions upon peptide/MHC binding, such as TCR accessory molecules.

Human T lymphocytes positive for a single chain Fv receptor with the same CD4/γ domain show CD3 independence for their cytotoxic response,[25] implying that receptors with this type of configuration do not associate with endogenous CD3. The exact membrane topology of the Fab-based receptors is currently under investigation.

In conclusion, the efficiency and reproducibility of T cell retargeting with chimeric receptor genes combined with the flexibility of antibody phage display to select TRA specific molecules hold great promise for clinical immunogene therapy.

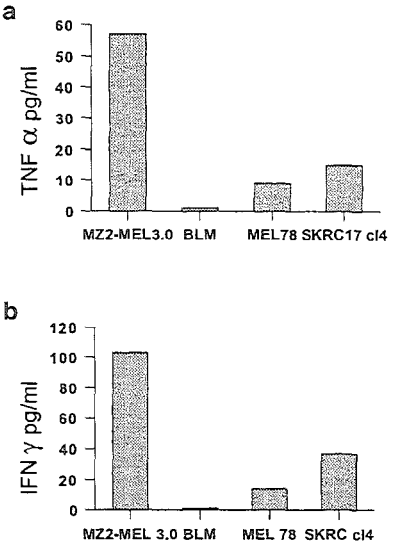

Figure 7 Native MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells stimulate production of TNF-γ and IFN-γ by Fab-G8-CD4/γ transduced T lymphocytes. Fab-G8-CD4/γ^{POS} T lymphocytes were stimulated with MAGE-A1^{POS} and HLA-A1^{POS} melanoma cells (ie MZ2-MEL 3.0) and MAGE-A1^{NEG} and/or HLA-A1^{NEG} cell lines of different histologies (ie BLM, MEL 78 and SKRC17 cl4), and analyzed for production of TNF-α (a) and IFN-γ (b) as described in the legend to Figure 5.

Materials and methods

Cells and antibodies

Human T lymphocytes were derived from healthy donors, and were isolated and expanded as described.[22] Target cell lines used in this study were: (1) the MAGE-A1^{POS} and HLA-A1^{POS} melanoma cell line MZ2-MEL3.0 (kindly provided by T Boon and P Coulie, Ludwig Cancer Research Institute, Brussels, Belgium[7]); (2) the MAGE-A1^{NEG} and HLA-A1^{POS} melanoma cell line MEL2A; (3) the HLA-A1^{NEG} melanoma cell line BLM (kindly provided by G Adema, Academic Hospital Nijmegen, Nijmegen, The Netherlands); (4) the HLA-A1^{NEG} melanoma cell line MEL78; (5) the HLA-A1^{NEG} renal carcinoma cell line SKRC17-cl4 (kindly provided by E Oosterwijk, Academic Hospital Nijmegen); and (6) the HLA-A1^{POS} EBV-transformed B cell blast APD. The human embryonic kidney cell line 293T[39] (kindly provided by Y Soneoka, University of Oxford, Oxford, UK) was used as a packaging cell line for the pBullet Fab-G8-CD4/γ retroviral vectors essentially as described.[23] The HLA-A1-restricted, MAGE-A1 specific CTL clone MZ2-82/30 (kindly provided by T Boon and P Coulie) was used as a positive control in these studies. The following mAbs were used this study: anti-CD4 mAb (Becton Dickinson Biosciences, San Jose, CA, USA), anti-class I mAb W6/32 (Seran Laboratories, Crawley Down, UK) and purified mouse Ig (Nordic, Tilburg, The Netherlands).

Construction of the chimeric Fab-G8-CD4/γ genes

The chimeric heavy chain VhChCD4/γ and lambda light chain VlClCD4/γ were generated by amplification of the Fab fragments from phage G8 DNA,[36] using the primer pairs Vh-*Sfi*I/Ch-*Not*I and Vl-*Sfi*I/Cl-*Not*I, respectively. The resulting fragments were inserted via *Sfi*I/*Not*I digests into the pBullet retroviral vector, which contains an expression cassette. The expression cassette contains: (1) the G250 variable heavy chain signal sequence, and (2) a constant kappa chain linker (Ck), the CD4 transmembrane domain and the intracellular γ chain (CD4/γ). All domains were derived from the G250 specific chimeric scFv-HKCD4/γ receptor DNA.[23]

Primer sequences were as follows:

Vh-*Sfi*I: 5′-CTC-TGG-CCC-AGC-CGG-CCA-TGG-CCG-AGG-TGC-AGC-TGG-TGG-AGT-CTG-GG-3′;
Ch-*Not*I: 5′-CTC-TGC-GGC-CGC-GCT-TGA-GAC-GGT-GAC-CGT-GGT-CCC-3′;
Vl-*Sfi*I: 5′-CTC-TGG-CCC-AGC-CGG-CCA-TGG-CCC-AGT-CTG-TGC-TGA-CTC-AGC-CAC-CCT-CG -3′, and
Cl-*Not*I: 5′-CTC-TGC-GGC-CGC-GAG-GAG-GGT-CAG-CTT-GGT-CCC-TCC-3′.

Retroviral transduction of Fab-G8-CD4/γ genes into primary human T lymphocytes and in vitro expansion of transduced T lymphocytes

CD4^{POS} T lymphocytes were depleted from human peripheral blood lymphocytes (PBL) with anti-CD4 mAb coated magnetic beads according to the manufacturer's instructions (Dynal, Oslo, Norway). The CD4-depleted human PBL (5×10^6) were activated by addition of anti-CD3 mAb and then transduced using the pBullet retroviral vectors with the Fab-G8-CD4/γ genes as described earlier.[23] Briefly, anti-CD3 mAb activated lymphocytes were incubated for 72 h with an irradiated (25 Gy) monolayer of recombinant retrovirus producing 293T cells using culture medium supplemented with 4 μg/ml polybrene (Sigma, St Louis, MO, USA) and 360 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands). Expansion of transduced primary human T lymphocytes was performed in the presence of feeder cells as described elsewhere.[40,41]

Flow-cytometric analysis of retrovirally transduced primary human T lymphocytes

Primary human T lymphocytes (5×10^5) were stained with soluble peptide/HLA-A1/streptavidin^{PE} complexes as described.[26] Briefly, T lymphocytes (5×10^5) were incubated for 30 min on ice, with a 1:10 dilution of freshly prepared complexes in a volume of 20 μl. The dot plots show viable T lymphoblasts gated on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACSCAN instrument (Becton Dickinson Biosciences, San Jose, CA, USA). Human T lymphocytes were stained with soluble peptide/HLA-A1/streptavidin^{PE} complexes and enriched via anti-PE mAb coated magnetic beads and mini-MACS columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

IFNγ capture assay

Transduced human T lymphocytes were incubated for 18-20 h on immobilized peptide/MHC complexes and were analyzed by flow cytometry for IFN-γ production. To immobilize peptide/MHC complexes, non-tissue culture 24-well plates were incubated with PBS containing streptavidin (at 1 mg/ml final) for 12–15 h at room tem-

perature (RT). Non-bound streptavidin was removed by three washes with PBS. The 24-well plates were then blocked with PBS containing 2% FCS and 2 mm EDTA for 30 min at RT. After removal of the blocking medium, soluble biotinylated MAGE-A1/HLA-A1 complexes and influenza/HLA-A1 complexes (at 10 μg/ml final) were added to the streptavidin-coated plates, and left to bind for an additional 30 min at RT. To remove non-bound peptide/MHC complexes, the plates were washed twice with PBS and once with culture medium. Finally, transduced T lymphocytes (1×10^6 cells) were then stimulated in the peptide/MHC complex-coated plates for 18-20 h at 37°C/5% CO_2 in a volume of 1 ml. IFNγ production was determined by IFNγ capture assay (Miltenyi Biotec) according to the manufacturer's protocol.

Cytotoxicity assays

Cytolytic activities of Fab-G8-CkCD4/γ transduced human T lymphocytes were measured in 6 h ^{51}Cr-release assays as described elsewhere.[22] The percentage-specific ^{51}Cr release was calculated as follows: ((test counts − spontaneous counts)/(maximum counts − spontaneous counts)) × 100%. Spontaneous ^{51}Cr release was always less than 10% of maximum release. Peptide loading of target cells was performed by addition of a MAGE-A1 nonapeptide (EADPTGHSY, Leiden University Medical Center, Leiden, The Netherlands) or an irrelevant influenza peptide derived from influenza virus A nucleoprotein (CTELKLSDY, Leiden University Medical Center) to the target cells 5–15 min before incubation with effector T lymphocytes. In blocking experiments, anti-class I mAbs were added to the target cells 15–30 min before addition of the T lymphocytes (W6/32 or irrelevant mIg, both at 10 μg/ml final).

TNFα and IFN-γ ELISA

To quantify secreted amounts of TNFα and IFN-γ, transduced human T lymphocytes (6×10^4) were cultured for 24 h either in the presence or absence of 2×10^4 adherent tumor cells in culture medium supplemented with 360 IU/ml rIL-2. Supernatants were harvested and levels of TNFα and IFN-γ, were measured by standard ELISA according to the manufacturer's instructions (CLB, Amsterdam, The Netherlands).

Acknowledgements

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CHAPTER 6

TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity

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TCR-Like Human Antibodies Expressed on Human CTLs Mediate Antibody Affinity-Dependent Cytolytic Activity[1]

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The permanent genetic programming via gene transfer of autologous T cells with cell surface receptors directed toward tumor-related Ags holds great promise for the development of more-specific tumor therapies. In this study we have explored the use of Abs directed to MHC-peptide complexes (or TCR-like Abs) to engraft CTLs with exquisite specificity for cancer cells. First, we affinity matured in vitro a previously selected TCR-like Ab, Fab-G8, which is highly specific for the peptide melanoma-associated Ag-A1 presented by the HLA-A1 molecule. A combination of L chain shuffling, H chain-targeted mutagenesis, and in vitro selection of phage display libraries yielded a Fab-G8 Ab derivative, Fab-Hyb3, with an 18-fold improved affinity yet identical peptide fine specificity. Fab-G8 and Fab-Hyb3 were expressed on primary human T lymphocytes as cell surface-anchored Fab, demonstrating that T cells expressing the high-affinity Fab-Hyb3 molecule eradicate tumor cells much more effectively. Furthermore, the gain in ligand-binding affinity resulted in a 2-log improvement in the detection of peptide/MHC complexes on melanoma-associated Ag-A1 peptide-loaded cells. In summary, an affinity-matured Ab specifically recognizing a cancer-related peptide/MHC complex was generated and used to improve the tumor cell killing capacity of human T cells. This strategy, based on engraftment of T cells with in vitro engineered Abs, is an attractive alternative to the laborious, and in many cases unsuccessful, generation of highly potent tumor-specific T lymphocytes. *The Journal of Immunology,* **2002, 169: 1110–1118.**

The recent increase in understanding of the immune responses associated with cancer and autoimmune disorders can further be advanced by recognizing the importance of the role played by peptide/MHC complexes in these diseases, and such knowledge provides various strategies for immunotherapy (1, 2). Molecules that bind specifically to these peptide MHC (pMHC)[4] complexes, which are involved in the molecular and cellular processes of Ag presentation, have a wide variety of applications, including direct visualization of the pMHC complexes (both intracellular and at the cell surface), specific masking of complexes involved in autoimmune disease, targeted delivery of toxins and drugs, and adoptive transfer of CTLs expressing pMHC-specific molecules involved in the immune response against cancer or viral infections (3). Two potent classes of reagents have been developed to bind to such complexes. Specific TCR, engineered from cloned T cells of known pMHC specificity, have been used to visualize cell surface pMHC complexes (4, 5), and MHC-restricted, peptide-specific mAb, identified by various immunization and screening schemes, have been isolated and used similarly (5–12). However, it has recently been suggested that in vitro selection of phage libraries displaying Ab fragments could be one of the most efficient ways to select specific pMHC binders (3). We have recently taken advantage of this technique to select a human Fab capable of binding an Ag of strong clinical value (1), the tumor-related pMHC complex HLA-A1/melanoma-associated Ag (MAGE)-A1 (13). Despite a moderate affinity of 250 nM, the selected Ab fragment Fab-G8 is highly specific for the HLA-A1/MAGE-A1 complex and does not bind to HLA-A1/MAGE-A3, a complex with a peptide that differs in only three residues from MAGE-A1. Nevertheless, most therapeutic applications, including targeting of toxins or cytokines and adoptive immunotherapy, are likely to demand a higher affinity. Interestingly, TCRs, the molecules that have been selected by evolution to bind pMHC complexes, always display very low affinities for their targets (10 to 0.1 μM) (14). The activity of T cells seems to be dependent on the affinity of the TCR/MHC interaction and/or the dissociation rate of the TCR from the peptide/MHC complex (15, 16). It was suggested by Valitutti et al. (17) that a single peptide/MHC complex on target cells engages multiple TCR on T cells to activate the effector T cells. This process of serial triggering of multiple TCR, essential for optimal T cell triggering, favors the interaction of low-affinity TCR with peptide/MHC complexes, because the longer dissociation times of high-affinity TCR might prevent serial triggering and thus optimal T cell activities. In this work we investigated to what extent such affinity constraints apply when T cells are artificially equipped with an Ag receptor. Indeed, there is recent evidence suggesting that in vitro affinity-matured TCRs also mediate increased peptide activation (18). While lower affinities may be acceptable in natural TCR-MHC-mediated cellular interactions, many therapeutic applications may benefit from higher-affinity TCRs or surrogate TCRs such as appropriately membrane-anchored

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[4] Abbreviations used in this paper: pMHC, peptide MHC; DC, dendritic cell; MAGE, melanoma-associated Ag; LAC, limited Ag concentration; COM, competition for binding to the Ag; SW, stringent wash; CDR, complementarity-determining region; HS, H chain-CDR3 spiking; LS, L chain shuffling; INF, influenza; B-LCL, B cell lymphoblast.

Abs or other receptors. We decided to see whether it would be possible to increase the affinity of Fab-G8 without losing the peptide fine specificity, and to subsequently examine the effect of such an affinity increase on chimeric Fab receptor-mediated T cell functions.

In vitro affinity maturation of Abs can be conducted using phage display methods. Genetic diversity is introduced into the genes encoding the parental Ab and, from the resulting phage library; variants showing improved affinity for the specific target are selected (for review see Ref. 19). We did not know a priori the specific amino acid residues in the Ab that confer the peptide fine specificity to Fab-G8. Therefore, we used two broadly complementary affinity maturation methods for diversifying the Ab genes, including 1) chain shuffling of the intact L chain, and 2) site-directed mutagenesis of the complementarity-determining region (CDR)3 of the H chain, which for most Abs forms the heart of the Ag binding site. Variants were selected under a variety of conditions, and those with highest affinity were tested for pMHC binding specificity and for binding to the pMHC complex on the surface of APCs. Finally, we expressed Fab-G8 and higher-affinity variants as chimeric Fabs fused to the FcεRI γ-chain signaling molecule on the surface of primary human T lymphocytes, and we examined the tumor cell killing capacity of these T cells expressing these low- or high-affinity pMHC binding molecules. The increased affinity of the chimeric receptor is clearly associated with faster cytotoxic responses, increased sensitivity, and an enhanced tumor cell killing capacity. We anticipate that this higher-affinity molecule will be more effective than Fab-G8 in the eradication of cancer cells in vivo.

Materials and Methods

Library construction

Chain-shuffling library construction. To build the L chain-shuffling (LS) library, the G8 V_H gene was cloned into a vector containing a library of human Ab κ and λ L chains. The latter libraries were generated during the construction of the large nonimmune Fab library (20). Briefly, the pCES1 vector containing Fab-G8 was digested with *Sfi*I and *Bst*EII, and the fragment corresponding to G8 V_H was gel purified and extracted using the QiaEX method (Qiagen, Valencia, CA). The κ and λ libraries were similarly digested and gel purified. Large-scale ligations (using 20 μg of insert and 20 μg of vector) were performed overnight at 16°C; the mixture was ethanol precipitated and introduced into *Escherichia coli* TG1 cells by electroporation. Cells were plated on 2× TY agar plates containing 100 μg/ml ampicillin and 2% glucose. After overnight incubation at 30°C, cells were scraped from the plates and stored at −80°C in 2× TY containing 15% glycerol.

H chain CDR3 mutagenesis for H chain-CDR3 spiking (HS) library construction. To create the HS library in a one-step PCR amplification of the V_H gene, we introduced diversity in the 13 amino acid residues of the H chain CDR3 by using a primer hybridizing on the CDR3 plus FR4 region. The primer used was 5′-GCTTGAGACGGTGACCGTGGTCCCTTG GCCCCAGACGTCCATACCGTAATAGTAGTAGTGGAAACCACCACC CCTCGCACAGTAATACACAGCC-3′, with the underlined residues using 90% of the wild-type nucleotide and 10% of an equimolar mix of A, T, C, and G (purchased from Eurogentec, Liege, Belgium). The V_H fragment was amplified by PCR using the pCES1-Fab-G8 as template. This fragment was digested by *Sfi*I and *Bst*EII and cloned into the pCES1 vector containing the G8 L chain. A library was made as before. Fingerprinting analysis was performed as already described (21) using the primers pUC reverse (5′-AGCGGATAACAATTTCACACAGG-3) and fd-tet-seq24 (5′-TTT-GTCGTCTTTCCAGACGTTAGT-3′); DNA sequencing was performed by Eurogentec using pUC reverse for V_L and CH1-fw (5′-GAAG TAGTCCTTGACCAGGC-3′) for V_H.

Selection and screening procedures

Except when mentioned in *Results*, all selections were conducted as described (13). The Ag-binding specificity of individual Fabs was assessed by phage or Fab ELISA using indirectly coated complexes, as described (22). Fab were purified by immobilized metal affinity chromatography as described (13).

Surface plasmon resonance measurements

Kinetic measurements were performed by surface plasmon resonance on a BIAcore 2000 (Pharmacia Biotech, Uppsala, Sweden). PBS (pH 8) plus 0.1% Tween 20 was chosen as running buffer. A nitrilotriacetic acid chip (Pharmacia Biotech) was activated with 500 μM $NiCl_2$ for 1 min at 10 μl/min. Approximately 800 resonance units of hexahistidine-tagged Fab (20 μg/ml) was immobilized and different concentrations of pMHC complexes were subsequently injected at a flow rate of 20 μl/min to minimize rebinding effects. A blank (injection of the Ab only) was subtracted from each curve to take into account the dissociation of the Ab from the chip. The channels were regenerated by injection of 250 mM EDTA over a period of 2 min. Kinetic analysis was performed using BIAevaluation 2.0 software (Biacore, Uppsala, Sweden).

Flow cytometry

Dendritic cells (DC) were generated as described (23). In brief, PBMCs were isolated from leukapheresis by Ficoll (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation. Monocytes were isolated by plastic adherence and cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 1% plasma, 20 μg/ml gentamicin (Merck, West Point, PA), 2 mM glutamine (BioWhittaker), 800 U/ml IL-4 (Novartis Pharmaceuticals, East Hanover, NJ), and 1000 U/ml GM-CSF (Leukomax; Novartis Pharmaceuticals). At days 5–6, 2 ng/ml IL-1β (Sigma-Aldrich, St. Louis, MO), 1000 U/ml IL-6 (Novartis Pharmaceuticals), 1 μg/ml PGE_2 (Sigma-Aldrich), and 10 ng/ml TNF-α (Bender MedSystems, Vienna, Austria) were added. After an additional day of culture nonadherent cells were harvested. To demonstrate maturation of DC, FACS analysis was performed on CD80, CD83, and CD86 (all from BD PharMingen, San Diego, CA). Mature DC were >90% double positive for costimulatory molecules and CD83.

DC were then pulsed with 20 μM MAGE-A1 (EADPTGHSY) or MAGE-A3 (EVDPIGHLY), tyrosinase (KSDICTDEY), and influenza (INF) nucleoprotein (CTELKLSDY) as negative controls for 3 h. DC were washed two times in PBS (BioWhittaker) and resuspended at 10^6 cells/ml. All staining procedures were performed at 4°C. DC were incubated for 30 min with fd-Fab-Hyb3, G8, or H2, washed again, and incubated with anti-M13 mAb (Zytomed, Berlin, Germany) for additional 30 min. After two rounds of washing in PBS, DC were incubated with goat anti-mouse PE Fab (Caltag Laboratories, Burlingame, CA) for 15 min. Cells were washed again and analyzed by flow cytometry (FACScan and CellQuest software; BD Biosciences, San Jose, CA). Cultured EBV-transformed B cell lymphoblasts (B-LCL) were pulsed with MAGE-A1 peptide or irrelevant INF virus peptide (10 μg/ml final concentration) for 1 h at 37°C. Unbound peptides were removed from the B-LCL by two washes with PBS. The peptide pulsed B-LCL were then incubated with fd-Fab-Hyb3, G8, or H2 essentially as described for the DC staining.

Primary human T lymphocytes (5×10^5) were stained with soluble peptide/HLA-A1/streptavidin^{PE} complexes as described (24). Briefly, T lymphocytes (5×10^5) were incubated for 30 min on ice, with a 1/10 dilution of freshly prepared complexes in a volume of 20 μl. The dot plots show viable T lymphoblasts gated on forward and side light scatter signals. Analysis was performed on a FACScan instrument (BD Biosciences). Human T lymphocytes were stained with soluble peptide/HLA-A1/streptavidin^{PE} complexes and enriched via anti-PE mAb-coated magnetic beads and mini-MACS columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Construction of the chimeric Fab-CD4/γ receptors and retroviral gene transfer to human T lymphocytes

The chimeric Fab-G8/γ and Fab-Hyb3/γ receptors were made as recently described (24). The chimeric Fab-G8/γ and Fab-Hyb3/γ receptors were independently cloned into the pBullet retroviral vector (24) and introduced into OKT-3 mAb-activated primary human T lymphocytes using retronectin-enhanced supernatant transduction (25). The retroviral supernatants were obtained from a mixture of Phoenix packaging cells (Phoenix Pharmaceuticals, Belmont, CA) (26) and 293T cells that had been transfected 2 days before harvest of the supernatants, with the following constructs: the GAG-POL construct pHit-60 (27), the pColt-GaLV vector, the pBullet vector with the V_H-CD4/γ chain, and the pBullet vector with the V_L-CD4/γ chain. After retroviral transduction the human T lymphocytes were expanded as described elsewhere (28).

Cytotoxicity assays

Cytolytic activity of transduced human T lymphocytes was measured in ^{51}Cr release assays as described previously (29). Peptide loading of target cells was performed by adding MAGE-A1 nonapeptide (EADPTGHSY;

Leiden University Medical Center, Leiden, The Netherlands) or irrelevant HLA-A1-binding INF peptide derived from INF virus A nucleoprotein (CTELKLSDY; Leiden University Medical Center) at indicated concentrations to the target cells 5–15 min before incubation with effector T lymphocytes at indicated E:T cell ratios. The incubation period of effector and target cells is indicated in the figures. The percentage of specific ^{51}Cr release was calculated as follows: ((test counts − spontaneous counts)/(maximum counts − spontaneous counts)) × 100%.

TNF-α ELISA

To quantify the secreted amount of TNF-α, transduced human T lymphocytes (6×10^4) were cultured for 24 h in either the presence or absence of 2×10^4 adherent tumor cells in culture medium supplemented with 360 IU/ml rIL-2. Supernatants were harvested and levels of TNF-α were measured by standard ELISA according to the manufacturer's instructions (CLB, Amsterdam, The Netherlands).

Results

Construction of LS and HS libraries

In the absence of any information regarding the specific amino acid residue interactions between Fab-G8 and the HLA-A1/MAGE-A1 complex, we decided to use two complementary library generation methods in parallel. The Fab-G8 V_L gene has 18 mutations compared with the nearest germline sequence (subgroup λ III, annotated lv318 by the V base index at www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html), whereas the V_H gene has only one mutation. We decided to use V_L chain shuffling to identify germline mutations that are important for binding and to possibly generate variants with a higher affinity for the HLA-A1/MAGE-A1 complex. However, the H chain often dominates the interaction with Ag, and its CDR3 region is usually responsible for crucial interactions with the Ag. We surmised that the H chain CDR3 might provide direct contact in the G8-Ag interaction; therefore, we created a library of G8-V_H-CDR3 variants. The long length of the Fab-G8 V_H-CDR3 (13 residues) prevents a representative sampling of a library when made by complete randomization of each residue. Therefore, we decided to introduce a low percentage of mutation into each residue position using a "spiked" oligonucleotide (see *Materials and Methods*). This should result in the introduction of one to three mutations per clone, spread all along the CDR3.

Both libraries were built using standard cloning procedures (20) (see *Materials and Methods*). We obtained a library of 2×10^8 clones for the V_L chain shuffling (LS) library and 2×10^7 for the HS library. The quality of these unselected libraries was checked by fingerprint analysis using the restriction enzyme *Bst*NI, by DNA sequencing, and by ELISA. Both libraries showed a high diversity (>88%): >95% of the LS clones and 65% of the HS clones displayed a correct open reading frame (Table I).

Selection against HLA-A1/MAGE-A1

Both repertoires were independently selected against the biotinylated HLA-A1/MAGE-1 complex as previously described (13). We initially chose to carry out several rounds of selection using decreasing Ag concentration to favor selection of high-affinity binders. For both repertoires, after the first round of selection, 92 of 92 clones bound the Ag in phage ELISA. However, after three rounds of selection, most of the clones (82%) showed a high cross-reactivity with HLA-A1/MAGE-A3 complexes by phage ELISA (data not shown). Because we wanted to use the frequency of Ag positives as a readout of the stringency of the selection procedure, and we also wanted to avoid the selection of these cross-reactive clones, we decided to optimize the selection procedure by using only one round of selection. Different conditions for selection were tested, and clones were screened to find affinity variants with higher affinity while maintaining peptide fine specificity.

We compared three selection strategies based on different principles, including 1) limited Ag concentrations (LAC), 2) stringent washes (SW), favoring low dissociation rate (k_{off}), or 3) competition for binding to the Ag (COM). After selection, 40 clones derived from each selection method and each library were tested for binding to HLA/MAGE-A1 in ELISA. Clones showing the best signals were produced as Fab and screened for affinity by BIAcore. As indicated in Tables II–IV, the first two methods did not yield any significant increase of affinity and, surprisingly, the average K_d of the selected clones was above that of the parental clone value of 250 nM (see Fig. 1). The third selection process (COM) was based on competition for binding and involved adding soluble Fab-G8 directed against the same epitope. To choose a relevant concentration range for the competitor molecule, we first performed a phage-Ab ELISA mimicking the selection conditions and chose different concentrations of competitor yielding from 90% (for 40 nM) to 10% (for 5 μM) of the control experiment without competition. During the selections, increasing the competitor concentration resulted in a decrease of the output titer as well as in the number of positive clones in ELISA (see Table IV), and at 5 μM competitor none of the output phages was positive for binding to HLA-A1/MAGE-A1. Contrasting with results obtained from the other selection procedures, the affinities of all the ELISA-positive clones were better than the affinity of the parental clone, with the best affinities being 4.5-fold stronger. The best results were obtained with the HS library, with clones Com6 and Com7 showing an affinity of 55 and 60 nM, respectively (Fig. 1). In all cases, the affinity improvement was essentially due to a decrease of the k_{off}.

Construction of V_H/V_L hybrids

We next investigated whether the effects of the mutations selected in the L chain could be additive with those in the H chain. The best L chain available (from clone Lac3, 83 nM, 3-fold improvement) was combined with the H chain of clone Lac7 (85 nM, 3-fold improvement), Com7 (60 nM, 4-fold improvement), and Com6 (55 nM, <5-fold improvement) to give three new clones called Hyb1,

Table I. *Quality of the unselected repertoires*

Library	Size	Insert[a]	Diversity[b]	Open Reading Frame[c]	ELISA[d]
LS	2×10^8	40/40	35 different clones/ 40 (fingerprint)	15/15	0/92
HS	3×10^7	37/40	20 different clones/ 20 (sequencing)	13/20	3/92

[a] Number of clones presenting a full-length insert (checked by PCR).
[b] Number of different clones identified/total number of clones sequenced.
[c] Number of clones without any stop codons, deletions, insertions, or frame shifts/total number of clones examined.
[d] Number of clones positive against HLA-A1/MAGE-A1 in indirect ELISA/total number of clones examined.

Table II. *Selection results: LAC*[a]

	LS		HS	
Ag (pM)[b]	Output (10^6 CFU)	Positive (%)	Output (10^6 CFU)	Positive (%)
10,000	11	55	79	85
1,000	1.3	7	5.8	67
100	0.83	0	0.34	7
10	3	0	0.58	0
1	5.6	5	0.86	0
0.1	1.4	0	1.6	0
0.01	1.3	0	1.1	2

[a] Input for LS, 1.1×10^{12} CFU; input for HS, 1.6×10^{12} CFU.
[b] Time of incubation with the Ag, 6 h.

Hyb2, and Hyb3, respectively (see Fig. 2). The Fab encoded by these genes were produced and purified for BIAcore measurements. All three clones have K_ds well below the K_d of the parental clone (Fig. 2). At 14 nM, the K_d of the best clone, Hyb3, was 18-fold lower than that of the parental clone, thereby demonstrating a synergistic effect among the selected mutations.

Analyzing the Ag recognition fine specificity

The binding specificity of G8 and its derivatives for the HLA-A1/MAGE-A1 complex was confirmed by BIAcore analysis and by comparing binding to the HLA-A1/MAGE-A3 complex. No binding to the latter Ag could be detected for the selected clones (Lac3, Lac7, Com6, Com7, Hyb1, Hyb2, and Hyb3; data not shown). We further compared the specificity of G8 (250 nM), Lac7 (85 nM), and Hyb3 (14 nM) by sandwich ELISA using indirect coating of nine different biotinylated HLA-A1/peptide complexes via streptavidin. To choose the peptides for this study, we investigated the homology between the two MAGE peptides: MAGE-A1 differs at three positions from MAGE-A3 (V2A, I5T, and L8S). To investigate which residue is critical for G8 and Hyb3 binding, we synthesized hybrid peptides corresponding to MAGE-A3 or INF with residues from MAGE-A1 at positions 2, 5, and 8 and used them to make HLA-A1 complexes. Surprisingly, HLA-A1/INFA could not be refolded properly, as shown by the absence of binding of the conformation-specific mAb TÜ155 (13), although INFA possesses the required anchor residues for HLA-A1 binding (E3 and Y9; see Fig. 3). All the other complexes were properly refolded. As shown in Fig. 3, Fab-G8 gave a strong signal against MAGE-A1 and no signal for MAGE-A3 or INF. Interestingly, M3T was strongly recognized by G8, implying that the threonine at position 5 is crucial for binding; however, INFT with this central threonine was not recognized. The presence of a threonine in position 5 is thus necessary but not sufficient to allow G8 binding. None of the other complexes was recognized by the Abs if the threshold for binding was set at a signal lower than three times the background; when set at twice the background, some binding was seen for the peptide M3S. Clones Lac7 and Hyb3 gave an identical binding pattern, but, as expected for MAGE-A1, with higher intensities (Fig. 3). This result confirms that the peptide fine specificity of Fab-G8 was maintained during the affinity maturation process.

Cell binding of G8 wild-type and affinity variants

Clone Fab-G8 was used in our previous work to detect the complex HLA-A1/MAGE-A1 in flow cytometry, after recloning the Ab genes for display on fd particles (to increase the number of Fab per particle) (13). Fab-G8 expression on fd particles yielded a difference between control cells and MAGE-A1-pulsed cells of less than one log shift in the mean fluorescence index. To evaluate the effect of the 18-fold affinity increase on diagnostic assays, e.g., flow cytometry, Hyb3 was also recloned for multivalent display and compared with fd-Fab-G8 in staining of peptide-loaded HLA-A1$^+$ human DC or EBV-transformed B cell blasts (APD). After loading mature DC or APD with the MAGE-A1 peptide, a strong positive staining was obtained with fd-Fab-Hyb3 (Fig. 4). In accordance with our previous results, staining with fd-Fab-G8 was also positive on DC but almost two logs lower than fd-Fab-Hyb3 (Fig. 4*A*) and not detectable on APD (Fig. 4*B*). As expected, binding with control fd-Fab-H2 as well as binding of fd-Fab-Hyb3 to DC and APD loaded with a control peptide (derived from the cancer-related protein tyrosinase or derived from the INF virus, respectively) (Fig. 4), to DC loaded with MAGE-A3 (data not shown), or to cells with an irrelevant haplotype (data not shown) were all negative. Thus, fd-Fab-Hyb3 reliably visualizes HLA-A1/MAGE-A1 complexes on cells, showing its exquisite specificity and improved sensitivity over the lower-affinity variant G8.

Genetic retargeting of human T lymphocytes with affinity-matured Fab-CD4/γ receptors

To determine whether an increased affinity for the HLA-A1/MAGE-A1 complex results in enhanced chimeric Fab-based receptor-mediated T cell functions, we constructed chimeric receptors comprising either Fab-G8 or Hyb3. Retroviral transduction of

Table III. *Selection results: SW favoring low* k_{off}[a]

	LS		LS Depletion[b]		HS		HS Depletion[b]	
Washes	Output (CFU)	Positive (%)	Output (CFU)	Positive (%)	Output (CFU)	Positive (%)	Output (CFU)	Positive (%)
3 h[c]	510	80	140	2	12,000	97	260	50
Overnight[d]	2	0[e]	2	100[e]	6,500	87	87	45

[a] Input for LS library, 1.3×10^{10} CFU; input for HS library, 15×10^{10}.
[b] Depletion on immunotubes coated with HLA-A1/MAGE-A3 prior to selection.
[c] Ten times for 15 min.
[d] Three-hour washes plus a last wash overnight.
[e] Only two clones tested.

Table IV. *Selection results: COM*[a]

Fab (nM)	LS Library		HS Library	
	Output (10^3 CFU)	Positive (%)	Output (10^3 CFU)	Positive (%)
5000	2	0	16	66
1000	9	32	150	62
200	62	40	690	80
40	120	70	1600	82

[a] Concentration of the Ag: 10 nM (6-h incubation with Ag and competitor). Input: 1×10^{12} CFU.

the G8-CD4/γ receptor gene and the Hyb3-CD4/γ receptor gene into primary human T lymphocytes resulted in stable expression of the receptors on the T cell surface after enrichment using HLA-A1/MAGE-A1 tetramers and anti-PE-coated magnetic beads (Fig. 5). Enriched G8-CD4/γ^+ T lymphocytes and Hyb3-CD4/γ^+ T lymphocytes were expanded and analyzed for receptor-mediated tumor cell killing by incubation with ^{51}Cr-labeled target cells. Fig. 6*A* shows that human T lymphocytes, derived from the same donor and transduced with the G8-CD4/γ or Hyb3-CD4/γ receptor, were capable of lysing native HLA-A1/MAGE-A1$^+$ melanoma cells. However, at identical E:T cell ratios the Hyb3-CD4/γ^+ T lymphocytes demonstrated considerably higher cytolytic activity than the G8-CD4/γ^+ T lymphocytes. The kinetics of Fab receptor-mediated tumor cell lysis were analyzed in cytotoxicity experiments with a duration of 1, 2, and 4 h. As shown in Fig. 6*B*, the Hyb3-CD4/γ^+ T lymphocytes demonstrated faster kinetics with respect to their capacity to kill both peptide-loaded and MAGE-A1$^+$ tumor cells. Furthermore, we investigated the sensitivity of G8-CD4/γ^+ and Hyb3-CD4/γ^+ T lymphocytes by incubating them with target cells that had been pulsed with increasing amounts of MAGE-A1 peptide. As demonstrated in Fig. 6*C*, Hyb3-CD4/γ^+ T lymphocytes required much less peptide to be activated and kill target cells than G8-CD4/γ^+ lymphocytes. In addition, Hyb3-CD4/γ^+ T lymphocytes produced more TNF-α than G8-CD4/γ^+ T lymphocytes when incubated with HLA-A1/MAGE-A1$^+$ melanoma cells (Fig. 7).

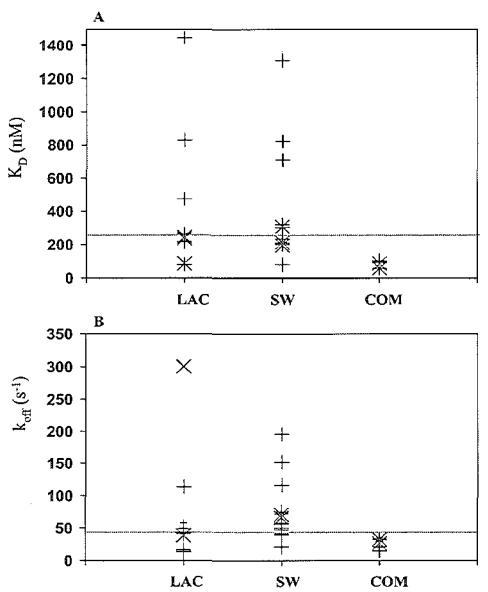
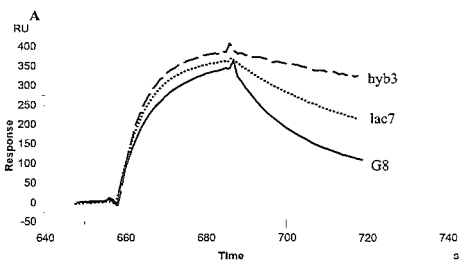


FIGURE 1. Affinity distribution of the selected clones: LAC, nine clones; SW, 15 clones; COM, eight clones. *A*, K_d ranking. *B*, k_{off} ranking. Measurements were done by surface plasmon resonance using a nitrilotriacetic acid chip (BIAcore) coated with 800 resonance units of each Fab via nickel. Different concentrations of pMHC complexes were injected at 20 μl/min. In both cases, the line shows the value corresponding to parental clone G8. The given values are the mean from three experiments.

Discussion

In this study, we investigated whether the Ab fragment G8 directed against the pMHC complex HLA-A1/MAGE-A1 could be affinity matured without loss of peptide fine specificity, and whether such genetically grafted chimeric receptors with increased affinity for HLA-A1/MAGE-A1 would accordingly result in enhanced chimeric receptor-mediated and immune-specific T cell functions such as cytotoxicity and cytokine production. In addition, we investigated whether such affinity-matured Fab could be used to efficiently detect pMHC complexes on APCs.

In the absence of any structural information about this Ab-Ag interaction, several libraries were built to generate G8 variants, and different selection techniques were compared. The first two approaches devised to select higher-affinity binders used either low



B

Clone	k_{on} ($\times 10^5$ $M^{-1}s^{-1}$)	k_{off} ($\times 10^{-3}$ s^{-1})	K_D (nM)
G8	1.8	45	250
Lac3	1.7	16	83
Lac7	1.7	14	85
Com7	2.5	15	60
Com6	3.6	20	55
Hyb1(Lac3/Lac7)	3.2	6	19
Hyb2 (Lac3/Com7)	3.1	6	20
Hyb3 (Lac3/Com6)	3.8	5	14

FIGURE 2. BIAcore sensorgram (*A*) and kinetic values (*B*) of selected and hybrid clones. BIAcore measurements were performed as described in Fig. 1. The given values are the mean from three experiments.

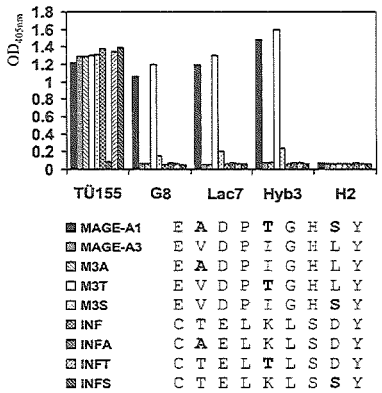


FIGURE 3. Fine specificity of the affinity-matured clones by phage-ELISA. pMHC complexes were refolded using each peptide and coated via streptavidin on an ELISA plate. Equal concentrations of phage-Abs were incubated for 1 h at room temperature. After extensive washing, bound phages were detected with an anti-M13 mAb coupled to HRP. mAb TÜ155 binds only to HLA complexes presenting a peptide and was used to demonstrate a proper conformation of the refolded complexes. H2 is an irrelevant phage-Ab (anti-Ras; Ref. 41).

Ag concentration or long washes. Unfortunately, both of these methods yielded poor binders, with some clones even showing affinities weaker than that of the parent clone G8.

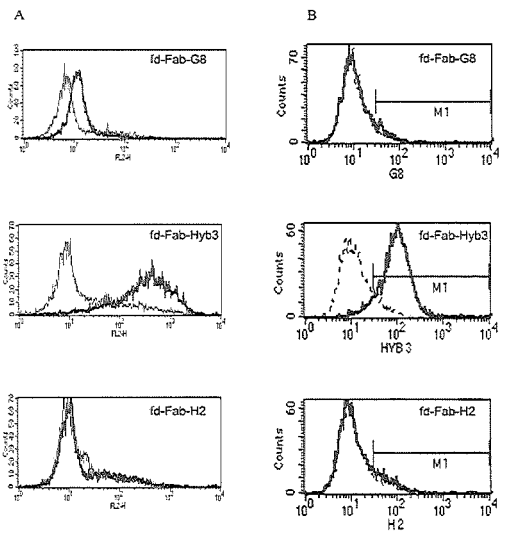


FIGURE 4. Staining of externally loaded human DC and EBV-transformed B cell blasts using fd-Fab-G8 and fd-Fab-Hyb3. *A*, DC were pulsed with 20 μM peptides, washed, incubated with phage-Abs, washed again, and incubated with anti-M13 Fab followed by anti-Fab coupled to PE. fd-Fab-H2 is an irrelevant phage-Ab. Thin line, Control peptide tyrosinase; thick line, MAGE-A1 peptide. *B*, B-LCL APD was pulsed with 10 μM peptide, washed, incubated with phage-Abs, washed again, and incubated with anti-M13 Fab followed by anti-Fab coupled to PE. fd-Fab-H2 is an irrelevant phage-Ab. Thin line, Control peptide INF peptide; thick line, MAGE-A1 peptide.

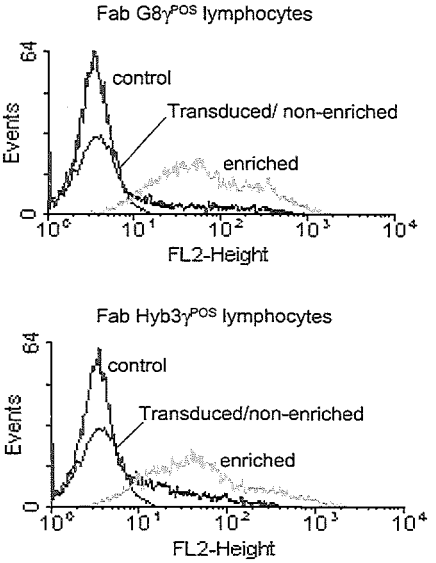


FIGURE 5. Cell surface expression of the Fab-CD4/γ receptors on transduced primary human T lymphocytes. Cell surface expression of the Fab-G8-CD4/γ and Fab-Hyb3-CD4/γ receptors on the transduced T lymphocytes was analyzed by incubation with MAGE-A1/HLA-A1/streptavidin^{PE} complexes. Results are shown as histograms. Data acquisition was restricted to viable cells.

Why such clones could compete with higher-affinity binders in such stringent conditions is unclear, but the phenomenon might be explained by a dominant avidity effect of those phage that are most tightly bound to the beads, the phage particles that display multiple Fabs on their surface (30). In a third approach, we competed off low-affinity binders during the selection with a high concentration of soluble Fab directed against the same epitope. Such competition would in principle also lower the frequency of avid interactions and may therefore also favor selection on the basis of affinity. Indeed, in contrast to the previous two methods used, this competitive selection led to binders all having higher affinities than the original Fab-G8 clone (see Fig. 1).

After sampling both libraries using this selection method, the best improvements in affinities found were only in the range of 3- to 5-fold. Sampling of the full repertoire was made difficult by the presence in the library of variants with higher affinity but altered peptide fine specificity. For this reason the selection was limited to one round only and screening of a limited set of clones from this repertoire. Instead of further selecting the separate libraries, we decided to combine the best-selected H chains with the best-selected L chain. In the best hybrid clone, Hyb3, we obtained an improvement in affinity up to 18-fold compared with Fab-G8, suggesting that the effect of the mutations was synergistic. This synergistic effect is somewhat surprising because mutations selected in parallel are usually difficult to combine in a single protein (31). Strikingly, all selected L chains originated from the same germline gene. However, it is difficult to pinpoint the crucial residues involved in binding, because most L chains have several mutations that differ from the germline.

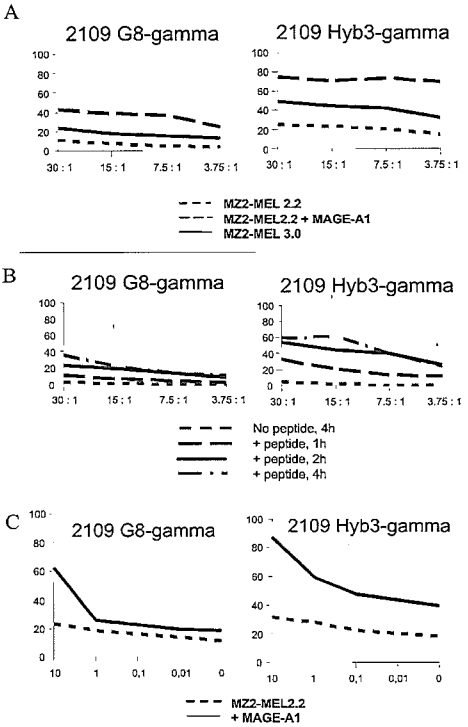


FIGURE 6. Comparison of cytolytic capacity of G8-CD4/γ^+ T lymphocytes and Hyb3-CD4/γ^+ T lymphocytes in 6-h ^{51}Cr release assays. Shown are mean percentages of specific ^{51}Cr release of triplicate measurements, with SDs not exceeding 10% of mean values. Data from one representative experiment are shown. *A*, Cytolytic capacity of G8-CD4/γ^+ and Hyb3-CD4/γ^+ T lymphocytes toward the HLA-A1$^+$MAGE-A1$^+$ melanoma cell line MZ2-MEL3.0, the MAGE-A1 Ag lost mutant MZ2-MEL2.2, and MAGE-A1 peptide-pulsed MZ2-MEL2.2 cells (10 μM final concentration). *B*, Kinetics of Fab-based chimeric receptor-mediated tumor cell kill. G8-CD4/γ^+ and Hyb3-CD4/γ^+ T lymphocytes were incubated with ^{51}Cr-labeled MAGE-A1 peptide-pulsed MZ2-MEL2.2 cells (10 μM final concentration) and nonpulsed MZ2-MEL2.2 cells and incubated for 1, 2, and 4 h. *C*, Sensitivity of Fab-based chimeric receptor-mediated tumor cell kill. G8-CD4/γ^+ and Hyb3-CD4/γ^+ T lymphocytes were incubated with ^{51}Cr-labeled MZ2-MEL2.2 cells pulsed with increasing concentrations of MAGE-A1 peptide (0.01–10 μM final concentration). Shown are the results at an E:T cell ratio of 30:1.

Sequences of clones selected from the HS library showed a small number of changes compared with G8, whereas the unselected clones showed an average of two to three mutations at the amino acid level, scattered all along the 13-residue CDR. These results suggest that most of the targeted residues either are involved in Ag binding or are necessary for the CDR structural integrity. Clones with an improved affinity share the mutation G to R at the second residue of the CDR, except one clone presenting a V to I mutation at residue 13 of the CDR. Rather than providing new direct interactions with the peptide, which would probably affect the behavior of the cross-reactivity of the Ab, these mutations likely allow a better fitting of the CDRs around the epitope, leading to higher affinity without a major change in cross-reactivity. It was indeed postulated that these types of mutations are often selected during a natural in vivo affinity maturation process and may be responsible for major affinity increases (32).

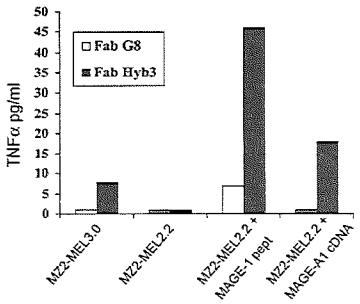


FIGURE 7. TNF-α assays using Hyb3-CD4/γ^+ and G8-CD4/γ^+ T lymphocytes. Transduced human T lymphocytes were cultured for 24 h in the presence of tumor cells. After 24 h, levels of TNF-α present in the supernatant were measured by standard ELISA. Results from one representative experiment of three are shown.

As expected, the 18-fold improvement measured by BIAcore resulted in a much stronger binding for Hyb3 compared with G8 (almost two logs), as demonstrated by flow cytometry on externally loaded DC and EBV-transformed B cell blasts. These assays used fd particles, which allow multivalent display (up to five Fabs per phage particle) (33), thereby favoring avidity effects. This increased avidity is probably responsible in part for the large increase in cell binding by Hyb3 relative to G8. Staining was shown to be specific, because HLA-A1-positive DC loaded with irrelevant peptides did not show positive staining. Staining was also negative with a control phage Ab and with DC displaying an irrelevant haplotype (data not shown). The affinity-matured Ab Fab-Hyb3 allows for the first time the direct assessment of the level of class I pMHC Ag presentation at the single cell level in the human system. This work is similar to that of Krogsgaard et al. (34), who showed class II pMHC staining. However, the quantity of peptide available for binding is thought to be much lower for class I complexes (for review see Ref. 35), making this task definitely a more challenging one.

We compared primary human T lymphocytes expressing chimeric receptors comprising Fab-G8 or Hyb3 fused to the FcεRI γ-chain signaling molecule, with respect to their tumor cell killing capacity. T cells expressing the high-affinity chimeric receptor displayed higher lytic activity and faster kinetics of cell lysis, required a much lower density of epitope to be activated, and produced more TNF-α upon incubation with target cells. These results are in full agreement with the work of Derby et al. (36), who recently demonstrated that high-avidity CTL provide better protection against viral infection for two reasons. First, they recognize lower Ag densities present earlier in the course of infection of each cell. Second, they initiate lysis more rapidly and thus more rapidly eliminate infected targets. Consequently, they prevent the accumulation of new virus particles much more efficiently than low-avidity CTL. In a cancer therapy perspective, high-affinity chimeric receptor T cells might be very advantageous. Indeed, tumor cells often express very low levels of HLA-A1, as is the case for the MZ2-MEL 2.2 cell line (data not shown), or low levels of MAGE-A1. In this case, only T cells harboring the high-affinity receptors will show a strong anti-cancer effect. Another clear advantage of chimeric receptor approach for cancer therapy is that

receptors chimerized to alternative signaling molecules other than TCRs bypass TCR-mediated proximal signaling events, which are often defective in cancer patients (37). Our results allow a direct comparison of the effect of the receptor affinity without any possible interference due to different receptor expression levels or signal transduction efficiency. Our results also show improved sensitivity and faster kinetics for cells displaying the high-affinity receptor. The results obtained by us and Holler et al. (18) are in conflict with the serial triggering model, which proposes that high-affinity TCR, with longer interaction times between TCR and peptide/MHC, results in decreased numbers of TCR interacting with the limiting numbers of specific peptide/MHC complexes on target cells. A decrease in the number of high-affinity TCR interacting with peptide/MHC complexes, according to this model, would result in decreased T cell functions such as cytotoxicity and cytokine production. On the contrary, our results clearly demonstrate that an increase in affinity of the TCR-like receptors results in enhanced T cell functions.

A further demonstration of the utility of high-affinity receptors has recently been described by Stanislawski et al. (38). The authors were able to circumvent self-tolerance of autologous T lymphocytes to universal tumor Ags by transfecting them with genes encoding a high-affinity TCR, thereby producing efficient and broad-spectrum tumor-directed CTLs.

As shown by these last works and our results, high-affinity TCRs are highly desirable. Using phage display, we have recently isolated Abs against 5 different HLA-A2-based complexes, most of them displaying affinity in the 10–50 nM range for their particular peptide complex (39, 40). This suggests that it is possible to select such molecules against any pMHC complexes in <3 wk. This efficient selection of high-affinity, specific pMHC binders, together with the versatility of Abs in terms of protein engineering, should make anti-pMHC Fab very attractive tools in a variety of virus-related and cancer applications, including diagnosis with the Fab or its engineered variants directly, or in therapy, as chimeric Fab-based TCRs.

Acknowledgments

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CHAPTER 7

T cell retargeting with MHC class I restricted antibodies: the CD28 costimulatory domain enhances antigen specific cytotoxicity and cytokine production

Submitted

T CELL RETARGETING WITH MHC CLASS I-RESTRICTED ANTIBODIES: THE CD28 COSTIMULATORY DOMAIN ENHANCES ANTIGEN SPECIFIC CYTOTOXICITY AND CYTOKINE PRODUCTION.

Ralph Willemsen, Cees Ronteltap, Reno Debets and Reinder Bolhuis

Abstract

T cells require both primary and co-stimulatory signals for optimal activation. The primary antigen-specific signal is delivered by engagement of the T cell receptor. The second antigen-independent co-stimulatory signal is mediated by engagement of the T cell surface co-stimulatory molecule CD28 with its target cell ligand B7. However, many tumor cells do not express these co-stimulatory molecules. We previously constructed phage display derived F_{AB}, G8 and Hyb3, antibodies with identical specificity but distinct affinities for HLA-A1/MAGE-A1, i.e. 'TCR-like' specificity. These chimeric receptors comprised the Fc(ε)RI-γ signalling element. We analysed whether linking the CD28 costimulation structure to it (γ+CD28) could affect the levels of MHC-restricted cytolysis and/or cytokine production. Human scFv $G8^{POS}$ T lymphocytes comprising the γ+CD28 versus the γ signalling element alone produced substantially more IL-2, TNF-α and IFN-γ in response to HLA-A1/$MAGE\text{-}A1^{POS}$ melanoma cells. Also a drastic increase in cytolytic capacity of scFv $G8^{POS}$ T cells, equipped with CD28+γ versus the γ-chain alone was observed.

Introduction

Tumor associated antigens (TAA) [1] or MHC class I presented tumor rejection antigens (TRA) [2,3] can be targeted by cytotoxic T lymphocytes (CTL) expressing (chimeric) single chain (sc) or two chain (tc) mAbs [4-10] or TCR [11-16], respectively. Most immuno-therapeutic strategies targeting TRA aim to induce and enhance the number of tumor-specific T lymphocytes in patients by peptide vaccination, since such cells can have the capacity to lyse (native) tumor cells [17,18], and the transfer of such tumor specific T lymphocytes to patients can have clinical significance [19-22]. However, the isolation of tumor-specific CTL and their expansion to significant numbers for clinical application, on an individual basis is cumbersome and the outcome unpredictable.

We and others developed strategies to permanently graft primary human T lymphocytes with MHC-restricted tumor specificity via retroviral introduction of e.g. chimeric or non-modified TCR αβ genes [11-16] or mAb-based receptors [23,24]. Engineered primary human T lymphocytes expressing (chimeric) TCRs or two chain 'TCR-like' mAbs, display the antigen specificity dictated by the introduced TCR or 'TCR-like' mAb, i.e., they specifically lyse relevant tumor cells and produce cytokines. To become independent of the availability of host-derived or *in vitro* stimulated tumor specific CTL (clones) as a source of TCR αβ genes but still target TRA, we generated phage display derived human mAbs with MHC-restricted, 'TCR-like' antigen specificities to genetically retarget human T cells to their tumors [23,24]. TCR as well as 'TCR-like' Abs clearly differ from classical Abs not only with respect to their MHC restriction of antigen recognition, but also with regard to ligand binding affinity, which is generally at least a log phase higher for classical mAb versus 'TCR-like' Abs. We took advantage of the relative ease to adopt phage display libraries to allow for *in vitro* affinity maturation of these peptide/MHC specific mAbs. Indeed, affinity maturation of the HLA-A1/MAGE-A1 specific Fab G8 resulted in a hyb3 variant, which displayed an 18 fold higher ligand binding affinity [24]. Primary human T lymphocytes expressing this affinity matured chimeric two chain Fab receptor showed enhanced *in vitro* immune functions, i.e. showed significant higher levels of antigen triggered production of TNFα, IFN-γ and IL-2, but also tumor cell lysis [24]. Here we explored whether specific immune responses of T cells expressing chimeric single chain 'TCR-like' (scFv) receptors could be further enhanced by linking the CD28 costimulatory domain to the γ-chain signalling element in both low and high affinity 'TCR-like' $scFv^{POS}$ T lymphocytes. To this end we used scFv- rather than two chain 'TCR-like' receptor genes because this approach allows the introduction of scFv- versus two chain-TCR-like genes with higher efficiency. Primary human T lymphocytes with CD28+γ signalling elements versus γ alone showed a significantly enhanced specific anti-tumor response, also at low TRA densities on tumor cells, and produce higher levels of the cytokines TNF-α, IFN-γ and IL-2.

Results

ScFvG8 (low affinity) and scFvHyb3 (high affinity) comprising either γ+CD28 or γ signalling elements can be functionally expressed on primary human T lymphocytes.

Genes encoding the chimeric receptors scFvG8-γ+CD28, scFvG8-γ, scFvHyb3-γ+CD28 and scFvHyb3-γ, specific for HLA-A1/MAGE-A1, were retrovirally introduced into OKT3 activated PBL (Figure 1). Cell surface expression of the chimeric low affinity scFvG8-γ+CD28 and scFvG8-γ on the one hand and high affinity scFvHyb3-γ+CD28 and scFvHyb3-γ 'TCR-like' receptors on the other was demonstrated by phycoerythrin-labeled HLA-A1/MAGE-A1 tetramer staining. Over 45 % of enriched T cell transductants specifically bound the MAGE-A1/HLA-A1 tetramers (Figure 2A). Expression of the endogenous CD28 co-receptor on anti-CD3 activated gene-transduced T lymphocytes was lacking (Figure 2B), as reported for specific antigen activated T lyphocytes (25).

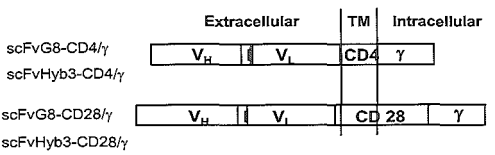

Figure 1: Schematic presentation of the low and high affinity scFvG8-γ or scFvHyb3-γ and scFvG8-γ+CD28 or Hyb3-γ+CD28 constructs. V_H: Variable heavy domain; I: linker; V_L: variable light chain domain; CD4 Tm: CD4 transmembrane domain amino acids 395 to 419; CD28: CD28 amino acids 135 to 215; γ: Fc(ε) RI-γ amino acids 45 to 88.

ScFvG8-γ+CD28^{POS} T lymphocytes produce higher amounts of TNFα, IFN-γ and IL-2 in response to melanoma target cells than scFvG8-γ^{POS} T lymphocytes.

TNFα, IFN-γ and IL-2 production by scFvG8-γ+CD28^{POS} and scFvG8-γ^{POS} T lymphocytes was measured following 24 hr incubation with stimulator cells: a) native HLA-A1^{POS}/MAGE-A1^{POS} melanoma cell line MZ2-MEL3.0; b) MAGE-A1 peptide loaded melanoma cell line MZ2-MEL2.2; and c) HLA-A1^{POS}/MAGE-A1^{NEG} MZ2-MEL2.2 melanoma cells. The low affinity -CD28+γ^{POS} T cell transductants showed increased and specific cytokine production in response to MAGE-A1^{POS} melanoma cells in comparison with -γ^{POS}T lymphocytes. High affinity-CD28+γ^{POS} T cell transductants also showed higher cytokine production levels in response to MAGE-A1^{POS} melanoma cells. However, this enhanced cytokine response was in part non-specific because MAGE-A1^{NEG} antigen lost mutant melanoma cells triggered IFN-γ and TNF-α production, but again this cytokine production in response to MAGE-A1^{POS} melanoma cells was still higher (Figure 3B). Control MAGE-A1^{NEG} target cells that were loaded with an irrelevant peptide did not induce cytokine production by scFvG8-γ+CD28^{POS}, scFvG8-γ^{POS} and scFvHyb3-γ^{POS} T lymphocytes T lymphocytes (data not shown).

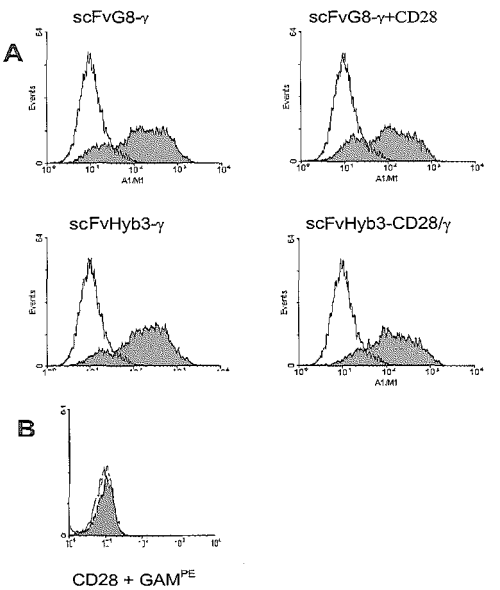

Figure 2: **(A)** Low affinity scFvG8-γ, scFvG8-γ+CD28 and high affinity scFvHyb3-γ, scFvHyb3-γ+CD28 transduced primary human T lymphocytes specifically bind HLA-A1/MAGE-A1 tetramers. Cells surface expression of the scFv-γ and scFv-γ+CD28 receptors on transduced T lymphocytes was analyzed via HLA-A1/MAGE-A1 tetramers (A1/M1; filled histogram). As a control for specific binding HLA-A1/Flu tetramers were used, which contain a peptide derived from Influenza nucleoprotein A (A1/Flu; open histogram). **(B)** absence of CD28 expression on OKT-3 mAb-activated T lymphocytes.

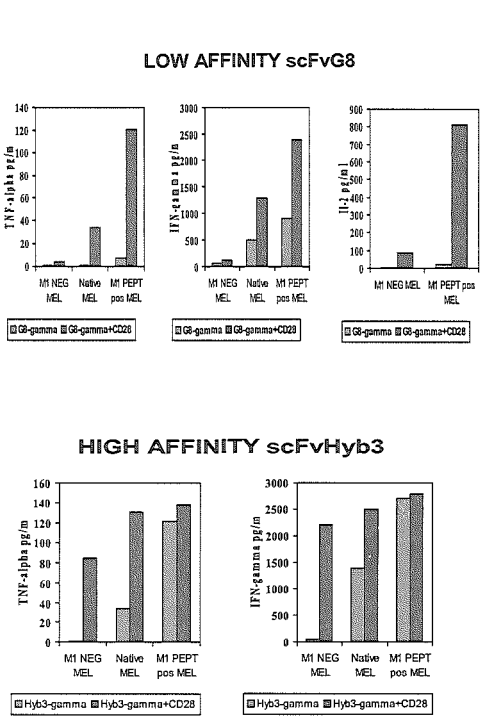

Figure 3: Tumor specific TNFα, IFN-γ and IL-2 production by: **(A)** low affinity scFvG8-γ+CD28^{POS} and scFvG8-γ^{POS} T lymphocytes, and **(B)** high affinity scFvHyb3-γ+CD28^{POS} and scFvG8-γ^{POS} T lymphocytes. Transduced T lymphocytes were cultured for 24 h in the presence of the following tumor cells (i) A1^{+}/M1^{-} MZ2-MEL2.2, (ii) A1^{+}/M1^{+} MZ2-MEL3.0; (iii) MZ2-MEL 2.2 + MAGE-A1 peptide (10 μg/ml final). After 24 h, levels of TNFα, IFN-γ and IL-2, present in the supernatant were measured by standard ELISA. Results from one representative experiment out of three are shown.

ScFv-γ+CD28^{POS} CTL mediate higher levels of MAGE-A1^{POS} melanoma cell lysis than scFv-γ^{POS} CTL

The cytotoxic responses of scFvG8-γ+CD28^{POS}; scFvG8-γ^{POS}; scFvHyb3-γ+CD28^{POS}, and scFvHyb3-γ^{POS} T lymphocytes were measured following a 4 hour incubation with ^{51}Cr-labeled target cells: a) HLA-A1^{POS}/MAGE-A1^{POS} MZ2-MEL3.0 melanoma cells; b) MAGE-A1 peptide loaded MZ2-MEL2.2 melanoma cells; c) HLA-A1^{POS}/MAGE-A1^{NEG} MZ2-MEL2.2 cells; d) control HLA-A1^{POS} B-LCL APD, and e) control K562 myeloid leukemia cells. Significant higher levels of specific cytolysis were obtained when HLA-A1^{POS}/MAGE-A1^{POS} melanoma cells and MAGE-A1-peptide^{POS} melanoma target cells were incubated with low affinity scFvG8-γ+CD28^{POS} T lymphocytes when compared to scFvG8-γ^{POS} T lymphocytes (Figure 4A).

Remarkably, significant higher levels of specific target cell lysis by high affinity scFvHyb3-γ+CD28^{POS} T lymphocytes were observed, not only of relevant HLA-A1^{POS}/MAGE-A1^{POS} melanoma cells, but also of irrelevant K562 and APD target cells, as well as MAGE-A1^{NEG} antigen lost mutant melanoma cells (Figure 4B).

When MAGE-A1^{NEG} antigen lost mutant MZ2-MEL 2.2 melanoma target cells were loaded with increasing concentrations of MAGE-A1 peptide (ranging from 1 nM to 10 μM) and used as specific target cells, the low affinity scFvG8-CD28+γ^{POS} versus scFvG8-γ^{POS} T lymphocytes showed a significant increased specific lyic capacity. ScFvG8-CD28+γ^{POS} versus scFvG8-γ^{POS} T lymphocytes required 10 fold less MAGE-A1 antigen at the target cell than scFvG8-γ^{POS} T lymphocytes for triggering of equal levels of Ag-specific cytolytic activity (Figure 4C).

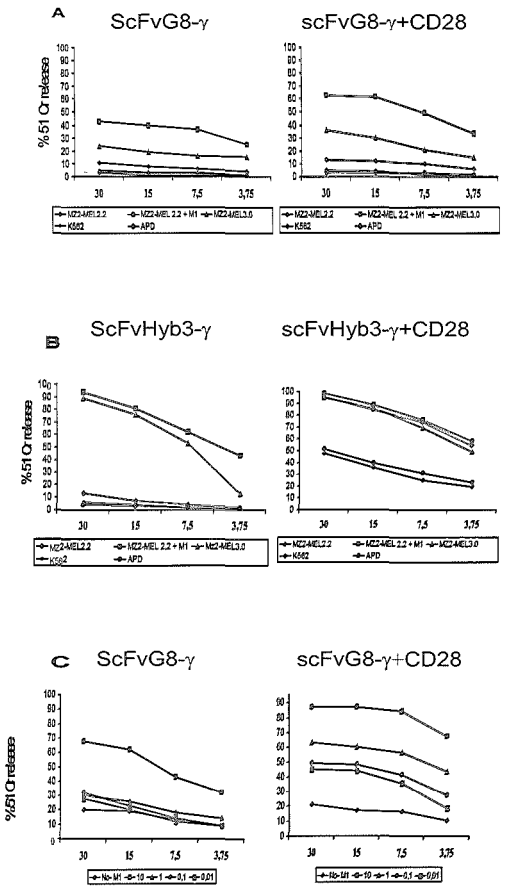

Figure 4: scFv receptors comprising γ+CD28 mediate a more potent cytolytic T cell response.

(A) Low affinity ScFvG8-γ+CD28^{POS} and scFvG8-γ^{POS} primary human T lymphocytes and **(B)** high affinity ScFvHyb3-γ+CD28^{POS} and scFvHyb3-γ^{POS} primary human T lymphocytes were incubated for 4 hours, at indicated effector to target cell ratio's, with the following ^{51}Cr-labeled melanoma target cells: (i) A1^{+}/M1^{-} MZ2-MEL 2.2; (ii) MZ2-MEL 2.2 + MAGE-A1 peptide (10 μM final); (iii) A1^{+}/M1^{+} MZ2-MEL3.0.

(C) ScFvG8-γ+CD28^{POS} T lymphocytes mediate cytolysis with a higher sensitivity to antigen density. ScFvG8-γ+CD28^{POS} and scFvG8-γ^{POS} primary human T lymphocytes were incubated for 4 hours, at indicated effector to target cell ratio's, with ^{51}Cr-labeled MZ2-MEL 2.2 cells loaded with increasing concentrations of MAGE-A1 peptide (M1, ranging from 0,01 to 10 μM final concentration).

Shown are mean percentages of specific ^{51}Cr release of triplicate measurements, with SD,s not exceeding 10% of mean values. Data from one representative experiment (out of three) are shown.

Discussion.

We have *in* vitro generated HLA-A1/MAGE-A1 specific human CTL's by genetically programming primary human T lymphocytes with low affinity G8 and high affinity Hyb3 receptors comprising the γ chain signalling element (23,24) These phage display-derived G8 and Hyb3 receptors display a 'TCR-like' specificity i.e., they recognize MAGE-A1 in the context of Class I-HLA-A1 Here we describe the immune response enhancing effects of genetically linking the CD28 co-stimulation structure to the γ chain in low and high affinity receptors (G8 and Hyb3 mAbs, respectively). This was done because first, human tumor cells reportedly lack the B7 ligand for CD28 (26); second, we discovered that virtually all human T lymphocytes lack functional expression of the critical co-stimulatory CD28 molecule following T lymphocyte activation *in* vitro by anti-CD3 mAb (OKT3), which is required for subsequent gene transduction; third, classic scFv mAb-based receptor^{POS} T lymphocytes, i.e. with MHC non-restricted antigen recognition, comprising γ or ζ + CD28 signalling structures show significant enhanced cytokine production but no enhanced cytolytic capacity (27-39).

The immune responses of phage display-derived Ab-receptor^{POS} CTL's with 'TCR-like' specificity comprising the combined γ+CD28 signalling elements were significantly enhanced, not only at the level of cytokine production triggered by TRA^{POS} stimulator cells, but surprisingly also at the level of TRA specific target cell lysis. In contrast, human T lymphocyte transductants expressing classic scFv mAb-based receptors comprising CD28+ζ or ζ only showed increased cytokine production capacity but no increased lytic capacity (32,37,39). The immune responses of these low and high affinity receptor^{POS} T cell transductants with the γ+CD28 signalling element, that lack functional expression of CD28 themselves, are efficiently reconstituted by incorporation of the CD28 co-stimulatory element into the γ-scFv receptor. Because the CD28 co-stimulatory element is now build into the γ-scFv receptor the frequent lack of the B7 ligand expression on tumor cells is bypassed.

T lymphocytes expressing low affinity scFvG8 comprising the combined γ+CD28 signalling element display significant enhanced production of TNFα, IFN-γ and IL-2 in response to relevant MAGE-A1^{POS} stimulator cells. The enhanced cytokine production levels may also have relevant consequences in adoptive immuno-genetherapy because it has been reported that classic MHC non-restricted scFv Ab-based receptor^{POS} T cell transductants comprising γ+CD28 or ζ+CD28 provided animals with increased reduction of tumor mass that appeared IFN-γ dependent (30,31,33). The tumor-specific induction of e.g. IL-2 production by scFvG8-γ+CD28^{POS} T lymphocytes may therefore bypass the need for exogenous Il-2 in clinical trials to obtain enhanced anti-tumor activity as well as prolonged life span (21,22,40). Reportedly, the life span of human CMV reactive CTL and adoptively transferred MART1/MelanA or gp100 specific T cell clones was indeed prolonged by the administration of exogenous IL-2 (21,40).

The level of immune responses of low affinity scFvG8-γ+CD28^{POS} T lymphocytes was comparable to that of high affinity scFv Hyb3-γ "alone" receptor^{POS} T lymphocytes and were HLA-A1/MAGE-A1 specific. However, when the high affinity receptor was engineered to comprise the combined γ+CD28, we discovered that they also were triggered by i.e., non-specific MAGE-A1^{NEG} cells, to produce cytokines and lyse the target cells. For example, these high affinity scFvHyb3-γ+CD28^{POS} T lymphocyte also lysed MAGE-A1^{NEG} melanoma cells, APD B-LCL, and K562 myeloid leukemia cells, albeit at lower levels (Figure 4B). Like TCR's, 'TCR-like antibodies may display affinities for Class I molecules presenting irrelevant peptides (41,42). However, the rate of dissociation of non-specific TCR/MHC complexes is sufficiently high that dissociation always occurs before the non-specific interaction can activate the TCR expressing lymphocytes. The difference in dissociation constant between Hyb3 and non-specific HLA-A1 complexes versus Hyb3 and HLA-A1 molecules presenting MAGE-A1 may be reduced when compared to the dissociation constants of the G8 Ab/HLA-A1 complexes. The consequently prolonged interaction of the Hyb3 receptor with the non-specific HLA-A1 in combination with the higher triggerable γ+CD28 signalling structure results in the "non-specific" cytolysis and cytokine production.

The affinity maturation of the 'TCR-like' Ab scFvG8 allows the generation of a library of CTL clones with a range of affinities from low to high, based on the use of differential V_HV_L genes and hence mimics the natural polymorphic T lymphocyte response. The enhancement of the overall immune responses by either affinity maturation of the receptor, or linkage of distinct signalling elements together, or combinations thereof will produce T lymphocytes transductants that display distinct TRA triggerabilities: e.g. cytokine production levels and profiles; target cell lytic capacities; tumor mass penetration abilities. However, as shown here maximum trigger ability may result in loss specificity of the immune response and hence unwanted kill of innocent bystander cells, thereby triggering autoimmune reactions. Therefore, it is mandatory to test these T cell transductants for exquisite antigen specificity before use in clinical therapy protocols.

Materials and methods

Cells and antibodies.

T lymphocytes derived from healthy donors were isolated and expanded as described (42). Target cell lines used in this study are: *(i)* the native MAGE-A1^{POS}, HLA-A1^{POS} melanoma cell line MZ2-MEL.3.0; (*ii*) the MAGE-A1^{NEG}, HLA-A1^{POS} melanoma cell line MZ2-MEL 2.2 (kindly provided by T. Boon and P. Coulie, Brussels, Belgium (44); *(iii)* the HLA-A1^{POS} B-LCL APD, and (iv) the erytroid leukemia cell line K562. The human embryonic kidney cell line 293T (45) (kindly provided by Y. Soneoka, Oxford, UK,) and phoenix-amph. (46) (Kindly provided by G. Nolan, Boston, USA), were used as packaging cell lines for the pBullet scFv-CD4/γ and scFv-γ+CD28 retroviral vectors. Antibodies that were used in this study were: anti-CD3 mAb OKT3 (ortho-diagnostics) and anti-CD28 (CLB, Amsterdam, The Netherlands).

Construct synthesis and retroviral gene transfer.

Constructs containing chimeric receptors (figure 1) were generated in two steps. First, the genes encoding the Fab G8 or Hyb3 heavy and light chain fragments [23,24) were subjected to PCR to introduce restriction sites that allow gene insertion into the pBlue-212 vector (12). Second, the scFv G8 and scFv Hyb3 were introduced into the pBullet retroviral vector (12), either 5' to a γ+CD28 fragment which was derived from the scFv-SP6CD28/γ construct (29), or 5' to a fragment comprising the CD4 transmembrane domain and the intracellular domain of Fc (ε) RI γ (23) resulting in the low affinity receptor vectors pBullet scFvG8-γ+CD28 and high affinity receptor vectors pBullet scFvG8-γ, and pBullet scFvHyb3-γ+CD28, pBullet scFvHyb3-γ, respectively.

These low and high affinity scFv receptor genes were introduced into OKT3 activated primary human T lymphocytes by retronectin enhanced supernatant transduction, essentially as described (23). Chimeric receptor expressing T cells were then expanded in medium supplemented with recombinant IL-2 (43).

Flow cytometry and enrichment of receptor expressing T cells

To assess the expression of low and high affinity scFv-γ+CD28 and scFv-γ receptors on gene-transduced primary human T lymphocytes, $0,5x10^6$ cells were stained for 30 min at 4°C, with saturating concentrations (11 nM final) of phycoerythrin (PE) labeled HLA-A1/MAGE-A1 tetramers, as described (12). The dot plots show viable T lymphoblast gated on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACSCAN instrument (Becton Dickinson Biosciences, San Jose, USA) To enrich for chimeric receptor expressing cells, transduced lymphocytes were first incubated with PE labeled HLA-A1/MAGE-A1 tetramers, followed by anti-PE coated magnetic beads. Tetramer-binding T cells were subsequently obtained by magnetic separation using Miltenyi miniMacs collums (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufactures instructions. Recombinant IL-2 (10 IU/ml) was present during the entire enrichment procedure.

Cytotoxicity assays.

Cytolytic activities of low and high affinity scFv-γ+CD28 or scFv-γ transduced human T lymphocytes were measured in ^{51}Cr-release assays at indicated times, as described elsewhere (12). Peptide loading of target cells was performed by addition of MAGE-A1 nonapeptide (EADPTGHSY, Leiden University Medical Center, Leiden, the Netherlands) or an irrelevant Influenza peptide derived from Influenza virus A nucleoprotein (CTELKLSDY, Leiden University Medical Center) to target cells 5-15 min prior to incubation with effector T lymphocytes. The percentage specific ^{51}Cr release was calculated as follows: ((test counts - spontaneous counts)/(maximum counts - spontaneous counts)) x 100%.

TNFα, IFN-γ and IL-2 ELISA.

To quantify secreted TNFα, IFN-γ, and IL-2 transduced human T lymphocytes ($6x10^4$) were cultured for 24 h either in the presence or absence of $2x10^4$ adherent tumor cells. When TNFα and IFN-γ production was assessed, culture medium was supplemented with 360 IU/ml rIL-2, whereas IL-2 production was assessed in medium without exogenous IL-2. Levels of TNF-α, IFN-γ and IL-2 production were measured by standard ELISA according to the manufacturer's instructions (CLB, Amsterdam, the Netherlands).

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CHAPTER 8

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION.

IMMUNO-THERAPY

The ultimate goal of cancer immuno-therapy is to eliminate malignant cells using the patient's immune system. Research has mainly focused on the generation of effective antigen specific T cell responses because of the general belief that T cell immunity is essential in controlling tumor growth and protection against viral infections. An important milestone in tumor immunology was the cloning of the MAGE-1 gene [1] and the subsequent characterisation of the first T-cell-defined antigenic epitope, presented by a MHC class I molecule [2]. This discovery led to the initiation of vaccination studies with tumor antigenic peptides derived from differentiation antigens, such as gp100 and MART 1, and cancer testis antigens such as MAGE-3. However, 10 years later, it appeared that results from clinical trials have been for the most part disappointing, since vaccine protocols designed to elicit potent anti-tumor T cell activity have failed to result in tumor eradication and enhanced patient survival [3-5]. The disappointing results from these peptide vaccination trials may in the first place be explained by an inability to brake "immunologic tolerance" against the 'self'-antigens that were targets in these studies. Tumors have evolved multiple mechanisms for evading the immune response, including antigen loss, down regulation of MHC and production of immuno-suppressive factors. Furthermore, tumors often lack expression of co-stimulatory molecules critical for the initial activation of naïve T cells. Additionally, peptide vaccines generally did not stimulate $CD4^{POS}$ T helper cells. Without T helper cells, that recognize class II presented epitopes, an effective anti-tumor response may not be mounted [6]. Other factors that might have contributed to this limited success include: the use of adjuvant or not, the use of dendritic cells for effective antigen presentation or not, and intravenous vs. intra-dermal injection of antigenic peptides. Last but not least, presentation of externally loaded peptides might have induced a peptide specific T cell response that does not show cross-reactivity with native antigen expressing tumor cells [7,8, and also discussed in chapter 3].

An alternative to vaccination to enhance the number of antigen specific T cells in patients is adoptive immunotherapy, i.e. the isolation, expansion and re-infusion of tumor specific T cells from individual patients. Results obtained with Tumor Infiltrating Lymphocytes (TIL) and cloned tumor specific T cells, isolated from individual melanoma patients demonstrated that the adoptive transfer of *ex vivo* activated and expanded TIL and cloned T cells may indeed be effective in the eradication of tumors [9,10]. Although promising, the widespread clinical practise of adoptive transfer of autologous tumor specific T cells is limited by several factors, such as: the difficulty to isolate T cells with the desired specificity from individual patients and the laborious and time-consuming expansion process. Therefore, the genetic programming of T cell specificity through introduction of antibody-based or TCR-based receptors into patient's primary human T lymphocytes has a lot of appeal. Most studies performed so far targeted non-MHC-restricted tumor associated antigens (TAA) (Table 3, **chapter 1**) through introduction of antibody-based chimeric receptors into T cells. These target antigens are not tumor-specific, but over- or aberrantly expressed on tumor cells. Therefore, it remains to be determined whether adoptive transfer of TAA specific T cells can be safely applied in the clinic, i.e. without severe side effects as a result of the destruction of TAA-positive healthy tissue by receptor modified T cells. To evaluate the safety of TAA specific T cells we recently started a phase I study with therapeutic intent of the treatment of metastatic renal cell cancer with autologous gene-modified T lymphocytes [11]. Renal cell cancer patients will be treated with autologous gene-transduced T lymphocytes expressing the renal cancer G250 antigen specific receptor scFvG250-CD4/γ. *In vitro,* primary human T lymphocytes retrovirally transduced with this scFvG250-CD4/γ receptor demonstrate high levels of G250 antigen specific tumor cell kill and cytokine production [12].

As opposed to TAA, tumor rejection antigens (TRA) such as the MAGE genes are considered to be truly tumor specific as these antigens are not expressed in normal cells, except in the testis were MHC molecules are lacking. In addition, TRA serve as target antigens for the immune system, demonstrated by the induction of tumor regression in melanoma patients after vaccination with an HLA-A1 binding MAGE-3 peptide [13]. The adoptive transfer of T cells that acquired a predefined TCR-specificity through transfer of

TCR or 'TCR-like' genes may therefore be a safe treatment modality for cancer patients. The requirements that limit the application of TCR gene transfer is that patients must express the MHC molecule that presents the antigenic tumor peptide. However, already 20-50% of individuals express HLA-A201 and with a panel of "off the shelf" TCR or 'TCR-like' reagents with specificities for different MHC restriction elements, it should be possible to treat most patients. In this thesis, strategies to introduce MHC class I restricted, tumor specificity into primary human T lymphocytes are described. The first part is devoted to the expression of HLA-A1-restricted, MAGE-A1 specific TCR αβ-based chimeric receptors in primary human T lymphocytes, whereas the second part deals with HLA-A1-restricted, MAGE-A1 specific "TCR-like" antibody-based chimeric receptors.

Chimeric TCR

In **chapter 2** the construction and functional expression of chimeric single chain and two-chain TCR genes, scTCR/ζ and tc-TCR/ζ, respectively, in primary human T lymphocytes is described. These chimeric scTCR and tc-TCR genes provide an alternative to non-modified, full-length TCR αβ genes to transfer MHC-restricted specificity to T lymphocytes. We and others (Greenberg PD., Nishimura MI, Falkenburg JH) have described the introduction of full-length TCR genes, of human origin, into primary human T lymphocytes. However, only a limited number of examples of successful transfer of full-length TCRαβ genes exist [14-17]. In fact, TCR αβ gene transfer may not always give rise to T lymphocytes that are capable of recognizing and responding to tumor cells that present endogenously processed tumor antigen or to virally infected cells [18,19]. This inability to respond to relevant target cells might result from: a) the formation of heterologous TCR dimers consisting of introduced and endogenous TCRαβ chains, resulting in a lowered TCR αβ expression of the desired specificity [20] or b) unstable and low TCRα expression, possibly as a result of difficulties in the post-translational assembly process of a second TCR or as a result of a tightly controlled TCR α expression process [21, 22]. The formation of heterologous TCR αβ dimers may theoretically result in unknown and possibly dangerous (e.g. autoimmune) reactivity. Although, in a mouse model no autoimmune reactivity was observed after the adoptive transfer of mouse TCR αβ transduced T cells [23], additional *in vivo* studies with a variety of TCRαβ transduced T cells are required, especially in humans. These problems and concerns are generally acknowledged by researches in the field (Greenberg PD, ref 18, Clay TM, ref 24), and chimeric TCRαβ genes are considered to provide an alternative.

To create the chimeric scTCR and tc-TCR genes, scTCR VαVβCβ and tc-TCR VαCα/VβCβ gene fragments were linked to CD3-ζ because experimental data had showed that antibody-mediated cross-linking of proteins expressed at the cell surface and linked to CD3-ζ induces T cell activation [25]. Flow-cytometry analysis using TCR α and β specific mAbs showed that the tc-TCR/ζ and scTCR/ζ molecules expressed in the T cell membrane did not associate with endogenous TCR αβ chains, thereby avoiding the problems associated with transfer of full-length TCRαβ genes [26]. In fact, pairing of introduced chimeric TCRαβ with endogenous TCRαβ chains was excluded by a strictly coordinated expression pattern of the chimeric genes. In addition, introduction of chimeric tcTCR VαCαζ/VβCβζ genes did not result in surface expression of the chimeric genes. Primary human T lymphocytes transduced with chimeric scTCR/ζ and tcTCR/ζ genes demonstrated the exquisite MHC-restricted, tumor peptide specificity of the introduced receptors. Transduced T lymphocytes specifically bound HLA-A1/MAGE-A1 tetramers and responded to MAGE-A1 peptide pulsed melanoma cells as well as native antigen positive melanoma cells by cytokine production and tumor cell kill. Importantly, the gene transduction protocol used, in combination with the retroviral vector that expresses the chimeric TCR genes, allowed direct functional analysis of bulk gene transduced T lymphocyte populations. This is in contrast to studies that used other retroviral vectors and protocols, and that required selection or cloning of the TCR expressing T lymphocytes [14,15]. Thus, chimeric TCRαβ gene transfer provides a basis to endow patients T lymphocytes with MHC-restricted, tumor specificity on a clinical scale.

Antigen specificity of chimeric TCR

In **chapter 3** we demonstrated that T lymphocytes expressing chimeric scTCR with identical TCR Vα and Vβ domains, but with distinct non-variable transmembrane and intracellular

signalling domains, i.e., CD3-ζ v.s. CD4 Tm and Fc(ε) RI-γ, differentially respond to MAGE-A1 peptide loaded target cells. Although both scTCR^{POS} T lymphocytes specifically recognised and responded to tumor target cells that present MAGE-A1 peptides in the context of HLA-A1 molecules that were loaded intracellular, only scTCR/ζ^{POS} T lymphocytes specifically responded to HLA-A1^{POS} target cells that presented externally loaded MAGE-A1 peptides (Figure 1, chapter 3). The non-responsiveness of scTCR/γ^{POS} T lymphocytes could not be explained by low levels of antigenic peptide present on target cells, or by the use of a synthetic peptide that was unable to stimulate scTCR/γ^{POS} T lymphocytes. Indeed, scTCR/γ^{POS} T lymphocytes did not respond to peptide loaded target cells at any of the peptide concentrations tested (range 0,01 to 100 μg/ml). More importantly, the peptide that was used for extracellular loading of HLA-A1 molecules on target cells was also introduced intracellular by transfection and resulted in a peptide specific immune response by scTCR/γ^{POS} T lymphocytes. The same synthetic peptide was also used for the generation of HLA-A1/MAGE-A1 tetramers, which specifically bound both scTCR/ζ^{POS} and scTCR/γ^{POS} T lymphocytes. We concluded that: (1) the difference in reactivity of the two scTCR must be due to differences in signalling elements and therefore differences in intracellular signalling events. Therefore (sc)TCR specificity is not solely dictated by the TCR VαVβ chains (2) complexes between HLA-A1 and MAGE-A1 peptide formed by intracellular processing are antigenically distinct from HLA-A1/MAGE-A1 complexes formed by extracellular peptide loading, and 3) tetramers and membrane bound MHC complexes formed by intracellular binding of MAGE-A1 to HLA-A1 are conformationally identical. In extension, these findings allows us to conclude that *in vitro* as well as *in vivo* peptide loading, used for the induction of peptide/MHC specific T-cells, yields peptide specific CTLs that may not always recognize TRA positive tumor cells. Therefore, vaccination strategies that facilitate intracellular peptide loading of MHC class I molecules are more likely to result in the generation of antigen-specific T-cells that are capable of recognising and eliminating antigen positive tumor cells. Identical results were obtained by our collaborator Dr. Pierre van der Bruggen, for a CTL clone obtained from a melanoma patients. These T cells only responded to MAGE-A3 positive tumor cells and not to MAGE-A3 peptide loaded target cells (personal communication)

CD4$^+$ T lymphocytes

The *in vitro* anti-tumor efficacy of antigen specific T-cells and the *in vivo* efficacy of adoptively transferred tumor and virus specific T lymphocytes has been studied primarily for CD8$^+$ T lymphocytes. CD8$^+$ T lymphocytes were considered to be the prime mediators of T-cell anti-tumor activity, at least because CD8$^+$ cells are able to kill tumor cells or virally infected cells directly. The role of CD4$^+$ T lymphocytes in tumor eradication has been thought to be indirect, primarily through provision of T-cell help. CD4$^+$ T lymphocytes are nowadays considered to play an important role in the initiation and persistence of an immune response against cancer and viral infections.

The induction of anti-tumor responses against MHC class II negative tumors and the induction of anti-viral T-cell responses in CD4^{NEG} mice were shown to be ineffective or at least severely hampered [27,28]. Furthermore, the persistence of adoptively transferred CMV-reactive CD8$^+$ T lymphocytes in patients that suffered from CMV reactivation, and the persistence of LCMV virus specific CD8$^+$ T lymphocytes in mice, was shown to depend on the presence of CD4$^+$ T lymphocytes or alternatively the addition of exogenous IL-2 [29,30].

Of special interest is the central role for CD4$^+$ T lymphocytes in the initiation of protective immunity against cancer. This was elegantly demonstrated in experiments using tumor reactive CD4$^+$ T lymphocytes that recognised a MHC class II presented tumor epitope. The adoptive transfer of these tumor specific CD4$^+$ T lymphocytes induced *de novo* generation of tumor reactive CD8$^+$ T lymphocytes [31]. These studies provided us with the arguments to include TCR transduced CD4$^+$ T lymphocytes in our studies. We anticipate that the introduction of MHC class I restricted specificity in CD4$^+$ T lymphocytes circumvents the lack of expression of MHC class II molecules on tumor cells. Since optimal T-cell activation requires that CD8 co-receptors and TCR on T cells bind to the same peptide/MHC molecule [32,33], we transduced CD4$^+$ primary human T lymphocytes with both chimeric tc-TCR/ζ genes and the CD8α gene (**chapter 4**). CD4$^+$ primary human T lymphocytes expressing the HLA-A1/MAGE-A1 specific chimeric tc-TCR/ζ genes

only bond to HLA-A1/MAGE-A1 tetramers and responded to HLA-A1/MAGE-A1$^+$ melanoma cells when these cells co-expressed the introduced CD8α gene. The simultaneous expression of tcTCR/ζ genes and CD8α in primary human T lymphocytes resulted not only in antigen specific production of cyokines such as IFN-γ, TNF-α and IL-2, but also converted these CD4$^+$ T lymphocytes into cells that were able to lyse HLA-A1/MAGE-A1$^+$ melanoma cells. Thus, TCR gene transfer to CD4$^+$ primary human T lymphocytes may provide not only critical helper cells, but also potent tumor specific effector cells for use in adoptive immunotherapy.

Antibodies with MHC-restricted specificity

MHC class I-restricted specificity may also be introduced into primary human T lymphocytes by the transfer of 'TCR-like' antibody-based receptors. These antibodies with 'TCR-like'-specificity were generated by our collaborators Dr. Hennie Hoogenboom and Dr. Patrick Chames through selection of a large non-immune phage display F_{ab} library on *in vitro* generated HLA-A1/MAGE-A1 molecules [34]. These phage display-derived antibody fragments with MHC-restricted specificity may provide valuable tools not only to transfer T-cell specificity, but also as a diagnostic tool to identify tumor antigen presentation on tumor biopsies. A large number of F_{ab} fragments have already been isolated, with distinct peptide/MHC specificities and with relative ease [34-37]. Importantly, the ease of isolation of F_{ab} fragments with MHC restricted specificity allows us to become completely independent of available T-cell clones, required for the isolation of TCR αβ genes. These F_{ab} molecules are thus likely to become a valuable source for 'off the shelf' reagents for transfer of T-cell specificity.

Chapter 5 describes the construction and functional expression of a chimeric F_{ab} receptor on primary human T lymphocytes. The F_{ab}-based receptor was derived from the F_{ab}G8 fragment, having specificity for HLA-A1/MAGE-A1 [38]. Heavy and light chain gene fragments from the F_{ab} G8 were each linked to the transmembrane domain of CD4 and intracellular domain of the Fc(ε)RI-γ chain, inserted into the retroviral vector pBullet, and transduced into activated primary human T lymphocytes. TheF_{ab}G8-CD4/γ receptor was functionally expressed on primary human T lymphocytes as demonstrated by the specific binding to HLA-A1/MAGE-A1 tetramers and responsiveness to HLA-A1/MAGE-A1$^+$ tumor cells. Primary human T lymphocytes transduced with the chimeric F_{ab}G8-CD4/γ receptor genes specifically responded to HLA-A1/MAGE-A1$^+$ tumor cells by cytokine production and tumor cell kill. These results demonstrated that antibodies with MHC-restricted tumor-specificity can serve as an alternative to TCRαβ chains for the genetic programming of T-cell specificity

Affinity matured receptors.

A combination of L chain shuffling, H chain-targeted mutagenesis, and *in vitro* selection of phage display libraries yielded a F_{ab} G8 derivative, termed Hyb3, with an 18-fold improved ligand binding affinity yet identical peptide fine-specificity (**chapter 6**) [39]. Chimeric receptors based on these low and high affinity F_{ab}s were functionally expressed on primary human T lymphocytes and compared for their efficacies to mediate antigen-specific immune responses. Primary human T lymphocytes expressing the high-affinity F_{AB}Hyb3-CD4/γ receptor displayed higher lytic activities and faster kinetics of cell lysis, required a much lower density of the antigenic epitope to become activated, and produced more TNF-α upon incubation with relevant target cells. These data are of interest because high-avidity CTL were shown to provide better protection against viral infection due to their ability to recognise lower antigen densities present earlier in the course of infection of each cell, and to initiate target cell lysis more rapidly, thereby eliminating more infected cells [40]. Fore these reasons, chimeric high-affinity receptors might be advantageous in cancer therapy. Tumor cells expressing low levels of antigen and/or MHC molecules might be recognized and eliminated more efficiently by high-affinity receptor expressing T lymphocytes relative to low affinity receptor expressing T lymphocytes.

Receptors providing co-stimulatory activity

The absence of co-stimulatory molecules, such as CD80 and CD86, on many tumor cells creates an unfavourable condition for the survival and activation of tumor-reactive T lymphocytes [41]. The engagement of the TCR on naïve T-cells with MHC-restricted antigens is necessary to activate T-cells, but is insufficient to achieve full

activation. Co-stimulation through the T-cell surface receptor CD28, which interacts with CD80 and CD86 on tumor cells induces signals that result in expansion of activated T-cells, and prevents apoptosis of these activated T-cells. Primary human T lymphocytes are prone to cell death when depleted of IL-2 or in the presence of tumor cells that lack co-stimulatory molecules [42,43]. In an attempt to overcome chimeric receptor-induced cell-death upon encounter of CD80 and CD86 negative tumor cells, CD28 co-signalling capacity was linked directly to the antigen binding moiety of classic TAA specific chimeric antibody receptors [44 and reviewed in 45]. These chimeric antibody-based receptors that comprised a tumor antigen specific scFv, together with CD28 and CD3-ζ or Fc(ε) RI-γ signalling domains (e.g. tripartite receptors) were functionally expressed on primary (human) T lymphocytes, i.e. the expression resulted in enhanced cytokine production and proliferation upon incubation with antigen positive tumor cells. Of special interest is the boost in IL-2 production by these receptor engrafted T-cells, because persistence of adoptively transferred tumor-specific T lymphocytes in cancer patients required administration of exogenous Il-2. In fact, CD28 co-stimulation was shown to be required for efficient IL-2 production [46]. Although production of other cytokines tested, such as TNF-α and IFN-γ was not depended on CD28 co-stimulation, their production was dramatically increased when T-cells expressed receptors that included the CD28 co-signalling domain. The superior activity of T lymphocytes expressing chimeric tripartite receptors was obvious in animal tumor models [47]. Although T lymphocytes expressing chimeric 'tripartite' receptors or receptors that lack the CD28 co-stimulatory domain displayed equal cytolytic activity *in vitro*, T lymphocytes that express the tripartite receptor had a greater capacity to inhibit growth of established tumors and metastases. Importantly, the enhanced anti-tumor activity of T lymphocytes expressing receptors with co-signalling capacity was dependent on the production of IFN-γ.

These results illustrate the advantages of engineering chimeric antibody-based receptors that include CD28 co-signalling capacity. However, these tripartite receptors were all directed against non-MHC restricted tumor antigens, TAA. The effect of CD28 co-signalling on TCR grafted T cells was not known. Therefore we determined whether or not the inclusion of a CD28 co-signalling domain into chimeric TCR or 'TCR-like' receptors might enhance the anti-tumor activity of primary human T lymphocytes.

In **chapter 7** experiments are described using low affinity scFv G8-based chimeric receptors as well as high affinity Hyb3 based receptors that were coupled to the Fc(ε)RI-γ signalling element alone, or in combination with the CD28 signalling element, low affinity scFvG8-γ and scFvG8-γ+CD28 and scFvHyb3-γ and scFvHyb3-γ+CD28, respectively. In parallel to classical antibody-based receptors that recognize non-MHC restricted antigens, low affinity 'TCR-like'receptor^{POS} T lymphocytes with CD28 co-signalling capacity produce more cytokines, such as IL-2, TNF-α and IFN-γ, than T lymphocytes that express the low affinity receptor with γ alone. However, in contrast to expression of classical antibody-based receptors, expression of the scFvG8-CD28/γ receptor resulted in a dramatic increase in cytolytic capacity of the transduced T lymphocytes. High affinity receptor^{POS} T lymphocytes that comprised CD28 co-signalling capacity demonstrated to our surprise "non-specific" immune responses when incubated with irrelevant MAGE-A1^{NEG} Target cells. Due to the enhanced dissociation constant of the high affinity Hyb 3 receptor and the combined CD28 and γ signalling elements, interactions of the scFvHyb-γ+CD28^{POS} T lymphocytes with non-specific HLA-A1/peptide complexes may result in intracellular signalling events before dissociation occurs.

Perspectives of genetic T-cell retargeting .

With the technology and the scientific advances made today, combined with the cytokines and drugs available, it is now possible to treat cancer patients with active immune cells. Immune cells from the T, NK, or any other myeloid-lymphoid lineage can be engineered with any immune specificity for which antibodies or TCR have been identified, MHC restricted or not. New technologies such as the production of soluble peptide/MHC complexes now allow selection of TCR and TCR-like antibodies that could not be obtained before. Chimeric receptors can be generated for both MHC restricted as well as non-MHC restricted antigens, which does not restrict treatment to tumors for which MHC presented antigens have been identified or that have down modulated these complexes.

Grafting primary human T-cells with chimeric receptors is rather technology oriented than disease oriented, and therefore may be instrumental for the clinical control not only of cancer but also for the elimination of viral infections, autoimmune disease and Graft-versus-host disease. The chimeric antibody-based receptor approach has demonstrated its *in vivo* efficacy in mouse tumor models and no adverse events have been reported so far in the clinical trails. Adoptive transfer of human T-cells genetically engineered to express human immunodeficiency virus (HIV)-specific chimeric CD4-ζ receptors to patients was shown to be safe [48].

In spite of these promising results, several questions remain to be addressed to increase both the efficacy and safety of genetically retargeted T-cells for adoptive immunotherapy:

A: Will chimeric Ab-based receptors expressed on T-cells induce an immune response? Fundamental to this question is the origin of the immunoglobulin heavy and light chain domains that were used to clone the scFv fragment; mouse or human. Immunoglobulins of mouse origin may indeed induce a human anti-mouse antibody (HAMA) response as observed in clinical trials with bi-specific antibodies [49]. However, it is unlikely that the low amount of mouse immunoglobulin that is exposed to the immune system on receptor engrafted T-cells will result in a HAMA response. In contrast to an antibody mediated response, the possibility that peptides derived from the chimeric construct, antibody-based as well as TCR-based, will be presented by MHC molecules on the transduced T-cells, thereby eliciting an effective anti-receptor T-cell response may not be excluded.

B: Do ex vivo gene transduced T lymphocytes home to the tumor site? Published data obtained in clinical studies with receptor modified T lymphocytes are restricted to a single study [50]. In this study "prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous $CD4^+$ and $CD8^+$ T-cells in human immunodeficiency virus-infected subjects" was obtained. Adoptive transfer of *ex* vivo selected and expanded tumor specific T-cell clones or TIL for the treatment of patients with metastatic melanoma has demonstrated *in vivo* persistence, migration and antitumor effects [9,10]. Applying the same culturing conditions for the expansion of genetically engineered T lymphocytes cultured may yield T lymphocytes with identical homing capacities. Finally, also data obtained with syngeneic mouse tumor models demonstrated that the adoptive transfer of chimeric scFv receptor transduced mouse splenocytes can result in the rejection of established tumors and metastases, indicating efficient homing to the tumor sites [47].

C: Do chimeric receptor grafted T-cells persist in patient, and/or do they require administration of exogenous IL-2 or may receptor grafted $CD4^+$ T-cells provide this T-helper function? As indicated before $CD4^+$ T lymphocytes play a central role in the initiation and persistence of an immune response. It may therefore be expected that receptor engrafted $CD4^+$ T lymphocytes support the persistence and function of $CD8^+$ T lymphocytes that are equipped with chimeric receptors. $CD4^+$ T lymphocytes genetically engineered to express TCR or 'TCR-like' receptors may even facilitate the de novo generation of tumor specific $CD8^+$ T-cells as has been described for $CD4^+$ T lymphocytes with specificity for MHC class II presented tumor epitopes [31]. In addition, $CD4^+$ T lymphocytes may induce T-cell memory [51]. These speculations on the *in vivo* function of receptor transduced $CD4^+$ T lymphocytes are supported by data obtained both in clinical trials, as well as in animal models, were $CD4^+$ T lymphocytes turned out to be essential for the initiation and persistence of tumor and virus specific T-cell immunity [29-31].

Other factors that may contribute to the homing capacity and persistence of genetically retargeted T lymphocytes are: expansion of gene-transduced T-cells in IL-15 containing medium together with cells that express the relevant antigen and co-stimulatory molecules [43], inclusion of co-stimulatory domains in chimeric receptors to bypass the absence of co-stimulatory molecules on tumor cells, and the inclusion of an inflammatory stimulus (CD40 activation) during the treatment with adoptively transferred T-cells [52].

Expansion of receptor transduced T lymphocytes with OKT3 and high dose IL-2 was shown to yield highly cytolytic T-cells *in vitro,* but resulted in rapid cell elimination and poor anti-tumor activity *in vivo* In contrast, expansion of transduced T-cells in the presence of antigen, costimulation and IL-15 resulted in more than 1000 fold expansion and these T-cells were capable to travel to tumor sites without prematurely succumbing to apoptosis [43].

Inclusion of the costimulatory CD28 domain in chimeric receptors results in an enhanced

proliferative capacity upon incubation with tumor cells, whereas T-cells that express receptors lacking the CD28 domain were unable to proliferate after antigen encounter [46].
In the murine *listeria* model antigen specific T-cells were only able to provide protection against infection by the bacterium for the first days after infusion of the T-cells. Although the transferred $CD8^+$ T-cells were still present a week after infusion, they were deleted in response to infection. The administration of anti-CD40 monoclonal antibodies, before CTL infusion, and at the time of infection, a week later, restored the ability of the infused CTLs to control infection [52]. However, it should be noted that CD40 stimulation could actually lead to the deletion of tumor-specific CTL in certain circumstances [53].

D:Are TAA on tumor cells bona fide targets for adoptively transferred chimeric receptor transduced T lymphocytes? To answer this question one should realise that cytotoxic T lymphocytes are very potent killer cells that may in fact eliminate complete organs. TAA that are merely over-expressed on tumor cells, and that are also present on healthy tissue in essential organs may indeed be a target for genetically retargeted TAA specific T lymphocytes. Although clinical studies with bi-specific antibodies proved to be safe, the proliferative capacity of T-cells permanently engrafted with TAA specificity may result in uncontrolled expansion and damage of healthy tissue. Carefully controlled clinical trials with receptor engrafted T-cells that target TAA on tumor cells are therefore required to evaluate these risks. The ability to eliminated the receptor engrafted T lymphocytes at will through introduction of suicide genes may reduce or even eliminate these any risks. Alternatively, TRA such as the MAGE genes, which may be considered truly tumor specific are more suitable to retarget T-cells to cancer tissues.

Introduction of non-modified "full-length" TCR genes into primary human T lymphocytes also raises questions that have to be addressed before this technology can be safely and successfully applied in the clinic:
E: Can we avoid the association of introduced non-modified TCR, chains with endogenous TCR chains, thereby avoiding the risk of introducing new specificities and possibly autoimmune responses.
Several possibilities are at hand to modify the TCR α and β chain in such a way that it induces specific heterodimerisation upon simultaneous expression in T-cells. One could imagine the introduction of additional cysteine residues in both TCR chains or even the inclusion of specific dimerisation domains. However, all modifications of the complete TCRα and β chain will have an effect on the expression and stability of these TCR chains and possibly have a negative effect on the T-cell mediated immune functions.
The problem might also be dealt with in another way: as described for antibody-based receptors that target TAA on tumor cells, the inclusion of a suicide gene into the receptor modified T-cells will allow us to eliminate the adoptively transferred T-cells upon any sign of auto-reactivity. This approach allows us to use non-modified TCR $\alpha\beta$ genes for the transfer of T-cell specificity, which in our hands displayed superior activity, when compared to chimeric scTCR or tcTCR transduced T lymphocytes. Therefore, non-modified full-length TCR $\alpha\beta$ genes are the most obvious choice to be used in the clinic for the adoptive transfer of genetically retargeted T-cells, unless modifications of the chimeric receptors improve the T-cell mediated functions up to the level of full-length TCR.
A low efficiency of retroviral transduction was considered to be a limitation for the successful transfer of two transgenes, i.e. TCR α and β genes, into primary human T lymphocytes at a clinical scale. However, simultaneous highly efficient transfer of both TCR α and β genes can be achieved using an optimized retroviral production and transduction protocol [39,54]. In fact, this procedure resulted in the expression of functional TCR$\alpha\beta$ in up 60% of the transduced primary human T lymphocytes. Therefore, the requirements to transduce TCR or TCR-like receptors on a clinical scale are met by this transduction procedure.

Future prospects of T-cell specificity transfer

The chimeric TCR approach offers advantages over the transfer of full-length TCR to patients T lymphocytes. The possible introduction of new unwanted specificities into patients lymphocytes can be avoided by the chimeric TCR approach. Furthermore, data obtained with TCR-like antibodies demonstrate that immune functions of receptor grafted T-cells can be improved by optimization of the receptors ligand binding affinity. The demonstration that high-avidity CTL provide better protection against viral infections [40] makes us believe that high-affinity chimeric receptors might be advantageous for adoptive immune

therapy of cancer. Firstly, because tumor cells often express low levels of MHC class I molecules or tumor antigen. Secondly, because chimeric receptor mediated signalling bypasses TCR-mediated proximal signalling events, which might be defective in cancer patients [55]. In addition to the optimization of receptor binding affinity inclusion of co-receptor and/or co-stimulatory functions into one chimeric receptor also improves the function of genetically retargeted T-cells. This approach is currently being pursued for TCR-based receptors, which might eventually result in an 'optimal' TCR format. However, there may be limits to this approach as demonstrated for receptors that comprise both the high affinity 'TCR-like' antibody and the CD28 co-signalling domain (chapter 7). Finally, genes encoding 'TCR-like' antibodies provide an alternative to TCR αβ genes, which may not be available due to the poor growth characteristics of many CTL clones

The effectivity of adoptively transferred T-cells may be improved in several ways: 1) by the protocol used to expand the transduced T lymphocytes [43], 2) by introduction of genes encoding secreted pro-apoptotic or anti-angiogenic molecules into the T-cells, 3) by co-administration of e.g. anti-CD40 monoclonal antibodies [52], 4), or by pre-conditioning of the cancer patients by myeloablative therapy [9] . In addition, primary human T lymphocytes may be retargeted to more than one tumor rejection antigen or more than one MHC molecule. Indeed the adoptive transfer of tumor-reactive T-cells with only one specificity was shown to be ineffective as a result of the down-regulation of MART-1 expression in melanoma cells [9]. The infusion of T-cells with a variety of MHC-restricted, tumor-specificities might therefore be more effective. Furthermore, next to targeting MHC-restricted tumor antigens, targeting of the tumor endothelium through e.g. expression of a chimeric receptor comprising vascular endothelial growth factor, might also contribute to the eradication of tumors [57]. Taken together, the improvements made in chimeric receptor design and retroviral transfer technology calls for the initiation of clinical trials for the treatment of patients suffering from cancer or life-threatening viral infections

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Samenvatting

SAMENVATTING

Het doel van immuno-therapie bij kanker is het elimineren van kwaadaardige cellen door gebruik te maken van het immuun systeem van de patiënt. Studies richten zich daarom veelal op de mogelijkheid om een effectieve tumors specifieke T-cel respons te genereren aangezien algemeen word aangenomen dat T cellen verantwoordelijk zijn voor zowel de controle als eliminatie van tumor cellen en viraal geïnfecteerde cellen. Een belangrijke mijlpaal voor de tumorimmunologie was de ontdekking van het kanker testis antigen MAGE-1 en de karakterisering van een MAGE-1 peptide dat door MHC klasse I moleculen op de tumor cel membraan aan T cellen word gepresenteerd. Deze ontdekking, alsmede de identificatie van een groot aantal MHC-gepresenteerde tumor antigeen peptiden leidde tot de initiatie van klinische peptide vaccinatie studies. Nu 10 jaar later moeten we echter constateren dat deze klinische studies niet het gewenste resultaat hebben opgeleverd. Alhoewel in deze studies een verhoging van het aantal tumor peptiden specifieke T cellen werd gevonden, werd er geen vermindering van het aantal tumoren en dus geen verhoogde overleving van patiënten bereikt. Deze teleurstellende resultaten zijn mogelijk het gevolg van een onvermogen om de zogenaamde 'immunologische tolerantie' voor lichaamseigen antigenen te breken. Tumoren hebben diverse mechanismen ontwikkeld om een immunologische aanval te weerstaan. Zo kan de antigeen- en/of MHC expressie zijn verlaagd, alsmede de expressie van diverse co-stimulatoire moleculen. Een andere reden voor dit teleurstellende resultaat is de afwezigheid van peptiden in deze vaccins die $CD4^{POS}$ T helper cellen activeren. Zonder deze $CD4^{POS}$ T helper cellen kan er geen effectieve immuun response worden gegenereerd.

Een alternatief voor de verhoging van het aantal tumor specifieke T cellen door peptide vaccinatie kan adoptieve immunotherapie zijn. Hierbij worden tumor antigeen specifieke T cellen uit de patiënt geïsoleerd of *in vitro* gegenereerd, vervolgens tot klinisch relevante hoeveelheden opgegroeid en tenslotte in de patiënt met kanker ingespoten. Deze vorm van therapie heeft in een aantal gevallen geleid tot vermindering van het aantal tumoren en verlengde overleving van de patiënten. Echter, de isolatie en expansie van deze tumor specifieke T cellen is niet in alle gevallen succesvol gebleken en is daarom niet op grote schaal te gebruiken. Een alternatieve methode is door ons en andere laboratoria ontwikkelt die het mogelijk maakt om grote hoeveelheden tumor specifieke cellen in handen te krijgen. De T cellen van patiënten kunnen van een nieuwe specificiteit worden voorzien n.l.: tumor specificiteit, door introductie van genen die coderen voor tumor specifieke receptoren. Deze receptoren kunnen worden onderverdeeld in twee groepen: zij die zogenaamde tumor geassocieerde antigenen (TAA) herkennen, de antilichamen, en zij die zogenaamde MHC gepresenteerde tumor rejectie antigenen (TRA) herkenen, T cel receptoren (TCR) en op TCR gelijkende antilichamen. Een overzicht van de ontwikkeling van deze receptoren wordt weergegeven in **hoofdstuk 1**.

De TAA die door antilichamen worden herkend zijn niet tumor specifiek. De expressie van deze TAA is verhoogd op tumor cellen ten opzichte van gezond weefsel en kan daarom leiden tot destructie van gezond weefsel. De veiligheid van de behandeling van kanker patiënten met TAA specifieke T cellen wordt momenteel in een fase 1 klinische trial door ons laboratorium getest. T lymfocyten uit patiënten met nier kanker worden hiertoe voorzien van een nierkanker antigeen (G250) specifieke receptor, de enkelketen receptor scFvG250-CD4/γ. Wanneer deze receptor in primaire humane lymfocyten wordt geïntroduceerd krijgen de cellen een nieuwe specificiteit n.l. G250 antigeen specifiek, en zijn vervolgens in staat om G250 antigeen positieve cellen te doden.

In tegenstelling tot TAA worden TRA zoals het MAGE antigeen wel als tumor specifiek beschouwt omdat deze antigenen niet in normaal weefsel voorkomen, met uitzondering van de testis. Op het testis weefsel zijn echter geen MHC moleculen aanwezig zijn die MAGE peptiden aan T cellen presenteren.

Vanwege deze specificiteit is het gebruik van TRA specifieke T cellen voor de behandeling van kanker patiënten waarschijnlijk veiliger dan het gebruik van TAA specifieke T cellen. Doordat de reagentia voor het introduceren van TRA specificiteit voorhanden zijn kunnen bijna alle patiënten met deze TRA specifieke T cellen behandeld worden.

Het doel van het onderzoek zoals hier beschreven was het genereren van TRA specifieke T cellen door constructie en retrovirale introductie van chimere- T cel receptoren en op T cel receptor gelijkende antilichaam receptoren.

In hoofdstuk 2 word de constructie en functionele expressie van chimere enkelketen en dubbelketen TCR genen beschreven: scTCR/ζ en tc-TCR/ζ. Deze chimere ketens

werden gegenereerd omdat het gebruik van niet gemodificeerde TCR αβ ketens niet zonder problemen is. Alhoewel wij en anderen met succes deze niet gemodificeerde ketens in primaire T lymfocyten hebben geïntroduceerd, blijkt n.l. uit een aantal studies dat dit niet altijd leidt tot de generatie van T cellen die ook daadwerkelijk tumor cellen kunnen herkennen en doden.
De chimere scTCR en tcTCR genen werden gegenereerd door scTCR, bestaande uit de variabele alfa en bèta ketens afkomstig uit een HLA-A1/MAGE-A1 specifieke T cel kloon, aan het CD3-ζ signalering element te koppelen. Deze benadering werd gekozen omdat eerder was aangetoond dat eiwitten die aan CD3-ζ waren gekoppeld preferentieel dimeriseren. Hierdoor kan dus vermeden worden dat de chimere TCR alfa en bèta ketens met reeds in de cel aanwezige TCR alfa en bèta ketens associëren en er een nieuwe onbekende specificiteit in de T cellen word geïntroduceerd. Introductie van de chimere scTCR/ζ en tcTCR/ζ genen in primaire humane T lymfocyten leidde tot de functionele expressie van deze genen: de T lymfocyten verkregen de door de chimere receptor gedirigeerde HLA-A1/MAGE-A1 specificiteit. Melanoom cellen die zowel het betreffende HLA-A1 molecuul als het MAGE-1 molecuul tot expressie brengen werden door deze gen-getransduceerde T lymfocyten gedood. Tevens initieerden deze tumor cellen de productie van cytokinen zoals TNFα, IFNγ en GM-CSF. Het is van belang te vermelden dat de retrovirale gen transfer technologie zoals door ons ontwikkeld voor deze specifieke toepassing er toe leidde dat de immuun response van bulk gen-getransduceerde T lymfocyt populaties direct geanalyseerd kon worden, zonder gebruik te maken van intensieve klonering strategieën.
In **Hoofdstuk 3** beschrijven wij een studie naar de antigeen specificiteit van twee scTCR receptoren. Primaire humane T lymfocyten die deze receptoren met identieke variabele TCR alfa en bèta ketens, doch verschillende signalering elementen, CD3-ζ versus Fc(ε)RI-γ, tot expressie brengen responderen beiden op melanoom cellen die positief zijn voor HLA-A1 en MAGE-1. Echter, tot onze verbazing reageerden de scTCR/γ^{POS} T lymfocyten niet op doelwit cellen die met synthetisch MAGE-A1 peptide waren beladen, en scTCR/ζ^{POS} T lymfocyten wel. Het feit dat deze scTCR/γ^{POS} T lymfocyten niet op peptide beladen cellen reageerden kon niet worden verklaard door aan te nemen dat het synthetische peptide niet identiek is aan het endogeen geproduceerde peptide. Indien het synthetische peptide door middel van electroporatie in doelwit cellen werd geïntroduceerd werd het op de cel membraan door HLA-A1 moleculen gepresenteerd. Deze complexen werden vervolgens wel door de scTCR/γ^{POS} T lymfocyten herkend en leidde tot de productie van IFN□. Het synthetische peptide was ook gebruikt voor de generatie van oplosbare HLA-A1/MAGE-A1 complexen die of werden gebruikt als stimulator moleculen door ze te immobiliseren op plastic, of werden gebruikt voor flow-cytometrische bepaling van de antigeen binding capaciteit. In beide gevallen werden de HLA-A1/MAGE-A1 complexen herkend door scTCR/ζ^{POS}
en scTCR/γ^{POS} T lymfocyten. Geïmmobiliseerd op plastic leidde dit tot IFNγ productie en gekoppeld aan een fluorochroom leidde dit tot specifieke binding door beide T lymfocyt populaties. De hoeveelheid antigeen die op de cel membraan van peptide beladen cellen aanwezig is tijdens de stimulatie studies was ook niet van invloed op de response van scTCR/γ^{POS} T lymfocyten aangezien bij geen enkele geteste peptide concentratie een response gemeten kon worden. Studies waarbij gekeken werd of de specifieke interactie tussen melanoom cellen die zowel HLA-A1 als MAGE-1 tot expressie brengen en scTCR/γ^{POS} T lymfocyten kon worden geblokkeerd door een overmaat aan peptide beladen cellen toe te voegen aan HLA-A1 en MAGE-A1 positieve cellen lieten zien dat scTCR/γ^{POS} T lymfocyten niet in staat zijn om aan peptide beladen cellen te binden. Wij concluderen daarom ook dat 1) de TCR specificiteit niet alleen afhankelijk is van de TCR alfa en bèta ketens, maar ook van de geïntroduceerde signalering elementen. Daarnaast concluderen wij ook dat er een verschil moet zijn tussen HLA-A1 moleculen die in de cel worden beladen met MAGE-A1 peptide en HLA-A1 moleculen die op de cel membraan worden beladen met MAGE-A1 peptide. Tenslotte kan geconcludeerd worden dat oplosbare HLA-A1/MAGE-A1 complexen identiek zijn aan intracellulair gevormde HLA-A1/MAGE-A1 complexen.
Aangezien $CD4^{POS}$ T cellen een grote rol spelen bij de initiatie en persistentie van een immuun respons werd door ons onderzocht of deze cellen ook konden worden uitgerust met een TRA specificiteit. In **hoofdstuk 4** wordt de functionele introductie van chimere tcTCR/ζ genen in primaire $CD4^{POS}$ T lymfocyten beschreven. Echter, de $CD4^{POS}$ T lymfocyten die de tcTCR/ζ receptor tot expressie brengen waren alleen in staat om a) HLA-A1/MAGE-A1 complexen te binden en om b) te responderen op HLA-A1 en MAGE-A1 positieve cellen als in deze cellen ook het CD8α gen was geïntroduceerd. Na introductie van het CD8α gen respondeerden de $CD4^{POS}$ T

lymfocyten specifiek op HLA-A1/MAGE-A1 positieve melanoom cellen door ze te doden en cytokinen te produceren. Wij concluderen dat de introductie van MHC klasse I specificiteit in MHC klasse II specifieke $CD4^{POS}$ T lymfocyten van belang kan zijn voor de toepassing van adoptieve immuun therapie aangezien vele tumoren geen MHC klasse II moleculen tot expressie brengen en gezien de centrale rol die $CD4^{POS}$ T lymfocyten spelen in de immunologische controle van tumoren en virale infecties.

Het tweede deel van het proefschrift beschrijft studies met een uniek type receptoren, n.l. receptoren gebaseerd op antilichamen die TRA herkennen in de context van MHC klasse I moleculen. Deze antilichamen werden door Dr. Patrick Chames in het laboratorium van Dr. Hennie Hoogenboom geïsoleerd m.b.v. de "phaag-display" technologie. Het belang van deze technologie wordt duidelijk als men begrijpt dat de isolatie, klonering en expansie van TRA specifieke T cellen niet altijd succesvol is en dus de TCR genen niet beschikbaar zijn voor gen transfer. Daarnaast laat de flexibiliteit van de phaag-display technologie het toe om de antilichamen te modificeren.

In **hoofdstuk 5** word de constructie van een chimere antilichaam F_{AB} receptor beschreven. De genen coderend voor het HLA-A1/MAGE-A1 specifieke F_{AB}G8 fragment werden gekoppeld aan het Fc(ε)RI-γ signalering element en d.m.v retrovirale transductie in primaire humane T lymfocyten geïntroduceerd De lymfocyten brengen de chimere F_{AB}G8-γ receptor tot expressie zoals bleek uit flow-cytometrische analyse met fluorochroom gelabelde HLA-A1/MAGE-A1 complexen. F_{AB}G8-γ^{POS} T lymfocyten waren in staat om HLA-A1/MAGE-A1 positieve melanoom tumor cellen te doden. Tevens respondeerden de F_{AB}G8-γ^{POS} T lymfocyten met cytokine productie na stimulatie met deze melanoom cellen. Deze resultaten laten zien dat deze antilichamen als alternatief kunnen dienen voor TCR genen voor de transfer van T cel specificiteit.

Een combinatie van technieken zoals "lichte keten shuffling", plaats gebonden mutagenese van de zware keten en *in vitro* selectie van faag display bibliotheken leverde een variant van F_{AB}G8 op met een 18 maal hogere affiniteit, n.l. Hyb3. In **hoofdstuk 6** beschrijven wij de constructie en functionele expressie van chimere F_{AB}G8-γ en F_{AB}Hyb3-γ receptoren. Uit een directe vergelijking van primaire humane T lymfocyten die de laag affiniteit receptor G8-γ of de hoog affiniteit receptor Hyb3-γ tot expressie brengen bleek dat hoog affiniteit receptor Hyb3-γ^{POS} T lymfocyten 1) in staat zijn om meer HLA-A1/MAGE-$A1^{POS}$ tumoren te doden gedurende de assay tijd, in vergelijking met G8-γ^{POS} T lymfocyten; 2) een hogere productie van cytokinen laten zien in response op HLA-A1/MAGE-$A1^{POS}$ tumor cellen, in vergelijking met G8-γ^{POS} T lymfocyten, en 3) tumor cellen met minder antigeen op de celmembraan beter herkennen en doden dan met G8-γ^{POS} T lymfocyten. T lymfocyten die met deze hoog affiniteit receptoren zijn uitgerust kunnen mogelijk ook *in vivo* meer tumor cellen doden. De affiniteit maturatie van deze antilichamen met MHC gerestricteerde specificiteit kan daarom een belangrijke bijdrage leveren aan de optimalisatie van de effectiviteit van T cel retargeting.

De afwezigheid van co-stimulatoire moleculen zoals CD80 en CD86 (B7.1 en B7.2) kunnen er voor zorgen dat primaire T lymfocyten niet of onvoldoende worden geactiveerd. De interactie tussen CD28 op T lymfocyten en Cd80 op tumor cellen is naast de interactie tussen TCR en MHC complexen noodzakelijk voor optimale activatie na antigeen binding. Uit studies met TAA specifieke antilichaam receptoren is gebleken dat de incorporatie van het CD28 signalering domein in de enkelketen antilichaam receptoren leidt tot verhoogde productie van cytokinen, echter niet tot een verhoogde capaciteit om tumor cellen te doden. Tevens is uit deze studies gebleken dat tumoren in muizen beter werden bestreden door T lymfocyten die waren uitgerust met receptoren die CD28 co-signalering capaciteit bevatten. Gezien deze opmerkelijke verbetering ten opzichte van receptoren die geen CD28 signalering element bevatten werd ook door ons een CD28 signalering element in laag affiniteit scFvG8-γ en hoog affiniteit scFvHyb3-γ receptoren ingebouwd (**hoofdstuk7**). Om de bijdrage van het CD28 signalering element te bestuderen werden de scFvG8 en scFv Hyb3 receptoren met alleen het γ signalering element in T lymfocyten van dezelfde donor geïntroduceerd. In vergelijking met de scFvG8-γ^{POS} T lymfocyten werd voor scFvG8-γ+$CD28^{POS}$ T lymfocyten na incubatie met HlA-A1/MAGE-$A1^{POS}$ melanoom cellen een verhoogde cytokine productie waargenomen. Opmerkelijk was ook de waarneming dat scFvG8-γ+$CD28^{POS}$ T lymfocyten ten opzichte van scFvG8-γ^{POS} T lymfocyten een verhoogde tumor dodende capaciteit lieten zien, dit in tegenstelling tot T lymfocyten die een TAA specifieke receptor met het CD28 signalering element tot expressie brengen. De expressie van de hoog affiniteit scFvHyb3 receptor met CD28 signalering element, scFvHyb3-γ+CD28 in humane T lymfocyten resulteerde tot onze

verbazing in een tumor cel dodende capaciteit die niet MAGE-A1 specifiek was. Ook MAGE-A1 en HLA-A1 negatieve cellen werden door de scFvHyb3-γ+CD28^{POS} T lymfocyten gedood. Onder normale omstandigheden wordt door T lymfocyten het oppervlak van cellen afgetast op aanwezigheid van lichaamsvreemde antigenen die door MHC moleculen worden gepresenteerd. De aspecifieke reactie door scFvHyb3-γ+CD28^{POS} lymfocyten wordt veroorzaakt door het feit dat de T lymfocyten al worden geactiveerd voordat de receptoren dissociëren van met lichaamseigen gevulde MHC complexen. Het is daarom van groot belang de specificiteit van T lymfocyten met deze receptoren uitgebreid te testen.
De in dit proefschrift gepresenteerde technologieën maken het mogelijk om op grote schaal tumor specifieke T lymfocyten te genereren. De retrovirale transductie van primaire humane T lymfocyten is momenteel n.l. zo efficiënt dat selectie van receptor positieve cellen overbodig is.

Ook is het mogelijk om receptoren met verschillende antigeen specificiteit tegelijk in T lymfocyten te introduceren. Dit heeft als voordeel dat tumoren die de expressie van een antigen verloren hebben toch nog herkend en opgeruimd kunnen worden. De mogelijkheid om chimere receptoren te construeren met een divers scala van signalering elementen zal er in de toekomst zeker toe leiden dat een zogenaamde optimale receptor structuur gevonden wordt. Als antigeen bindende structuren zullen de antilichamen met MHC gerestricteerde specificiteit een grote bijdrage kunnen leveren, zeker in het geval wanneer tumor specifieke T cel klonen ontbreken. Door genetische manipulatie kunnen antilichamen met een verhoogde affiniteit verkregen worden die mogelijk voor verdere verbetering van de antitumor effectiviteit zorgen. Kortom, deze technologieën zijn de voorboden van nieuw te initiëren klinische studies met TRA specifieke receptoren.

Curriculum Vitae

De schrijver van dit proefschrift werd 41 jaar geleden geboren in Terneuzen, op 2 februari 1962. Na de middelbare school in Terneuzen begon het studie avontuur, allereerst in Tilburg, gevolgd door Amsterdam, Nijmegen en wederom Amsterdam. De studie Medische Biologie werd gestart in 1984 aan de Universiteit van Amsterdam. Stages bij de afdeling Medische Biochemie en Virologie resulteerden in het enthousiasme voor het medische onderzoek.
Na het beëindigen van de studie in 1988 werd een baan geaccepteerd bij het biotechnologie bedrijf "Innogenetics s.a." in België. Echter, eind 1991 werd de voorkeur voor een andere werkomgeving zo duidelijk dat dit uiteindelijk resulteerde in de aanstelling op een NKB project bij de afdeling Medische en Tumor Immunologie onder leiding van Prof.dr. Reinder Bolhuis. Na aanvankelijk gestart te zijn met de introductie van een melanoom specifieke antilichaam receptor in primaire T lymfocyten, werd vervolgens in het laboratorium van Prof. Dr. Zelig Eshhar (Rehovot, Israël) de basis gelegd voor het onderzoek met chimère T cel receptoren. De goede samenwerking met dr. Hennie Hoogenboom en dr. Patrick Chames, toen nog in Maastricht, heeft geresulteerd in een uniek onderdeel van het onderzoek, MHC-gerestricteerde antilichaam receptoren. Het onderzoek word momenteel gecontinueerd binnen de afdeling Interne Oncologie waar de schrijver sinds 2001 als staf functionaris werkzaam is.

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Nawoord

Zo, dit was het dan, de laatste pagina van het proefschrift en de laatste woorden die op papier gezet worden.
De totstandkoming van het proefschrift was natuurlijk niet mogelijk geweest zonder de bijdrage van een groot aantal mensen. Allereerst Reinder Bolhuis, de initiator van het onderzoek en (misschien de enige persoon) die net als ik bleef geloven in de mogelijkheid om chimère T cel receptoren functioneel in primaire humane T lymfocyten tot expressie te brengen. Reinder bedankt voor "ALLES". Dan Cees; zonder zijn inzet en overwinnen van een hoop "kweekfrustratie" zouden vele proeven niet tot een goed einde gekomen zijn. Cees ook jij hartelijk dank! Mirjam, en Reno jullie zijn wat later bij het onderzoek betrokken geraakt maar ook jullie bijdrage aan de totstandkoming van het proefschrift is essentieel geweest, bedankt. Vervolgens een reeks collega,s die ieder op hun manier hebben bijgedragen: Letitia, Mo, Anke, Emmy, Cor, Jan Willem, Jako, Rienier, Wim, en nog vele anderen. Allen hartelijk dank!!