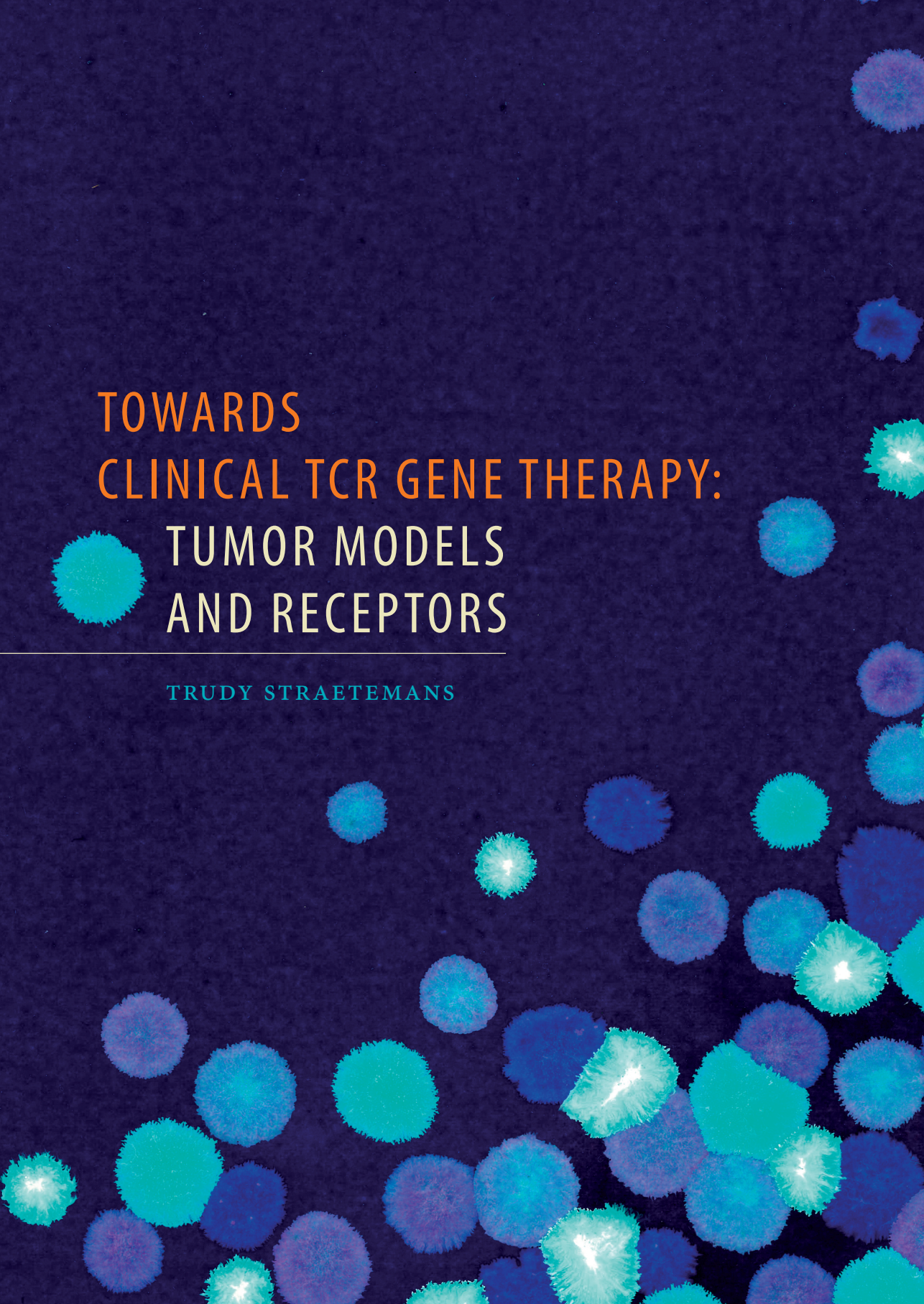
TOWARDS CLINICAL TCR GENE THERAPY: TUMOR MODELS AND RECEPTORS

TRUDY STRAETEMANS



Towards clinical TCR gene therapy: tumor models and receptors

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Towards clinical TCR gene therapy: tumor models and receptors

De weg naar klinische TCR gentherapie: tumor modellen en receptoren

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CONTENTS

Chapter 1
General Introduction

1.1 IMMUNE THERAPY FOR THE TREATMENT OF TUMORS

During the past few decades great progress has been made in the field of cancer immune therapy. There has been increased understanding on strategies tumors use to induce immune tolerance and evade immune responses, which enabled the design of clinical immune therapy trials. Two recent approaches that either oppose or circumvent immune tolerance towards tumors have successfully been tested in phase I clinical trials.

First, immune tolerance can be opposed by monoclonal antibodies that block T cell inhibitory molecules. For example, Ipilimumab and MDX-1106 block Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) and Programmed Death-1 (PD-1) receptor, respectively, and administration of these antibodies resulted in anti-tumor activities in patients with metastatic melanoma and renal cell carcinoma (1, 2). In fact, the combination of Ipilimumab and Dacarbazine (DTIC, currently the standard first-line treatment for melanoma) increased the response rates by three-fold (3). These results have encouraged further testing of dosing and combinations of these immune modulating reagents.

Second, immune tolerance can be circumvented by adoptive transfer of autologous and *ex-vivo* expanded tumor infiltrating lymphocytes (TILs). TIL therapy preceded by non-myeloablative lymphodepletion resulted in objective response rates in about 50% of advanced metastatic melanoma patients in two different medical centers (4-6), suggesting, but not yet proving anti-melanoma activity. Equally interesting were the complete responses observed in TIL therapy that ranged between 10 and 22% and seem higher than those observed for other treatments of melanoma (see Table 1). Recently, a simpler and faster method was developed to generate 'young' TILs that harbor characteristics associated with improved T cell persistence and *in vivo* survival. This young TIL culture protocol yields bulk T cells, omits the extensive *in vitro* testing of tumor reactivity, enriches for CD8+ T cells and depletes suppressive T cells (Treg cells) (4, 7). Next to TILs, adoptive transfer of tumor specific T cell clones generated from autologous peripheral T cells resulted in objective responses in 8 out of 10 metastatic melanoma patients (8). Notably, Butler and colleagues 'educated' peripheral T cells using artificial antigen-presenting cells loaded with HLA-A2-restricted tumor antigens (9). This protocol yielded tumor-specific T cells that are clinically long-lived and effective in melanoma patients. Collectively, the above-mentioned studies show the potential of immune therapy and especially the successes of adoptive therapy with tumor-specific T cells.

However, so far successes have been reported for melanoma and renal cell carcinoma only, tumor types that are suggested to be more immunogenic than other tumor types. Furthermore, most of these therapies are immune stimulatory in general and do not direct the immune response towards pre-defined tumor antigens. For those tumor types that do not allow isolation of TILs, or when it is not possible to enrich tumor-specific T cells from peripheral blood mononuclear cells (PBMCs), adoptive transfer with T cell receptor-(TCR) or chimeric antigen receptor-(CAR) engineered T cells are promising alternatives. Adoptive therapy with

Table 1. Overview of therapies for metastatic melanoma.

Treatment	OR (%)	CR (%)	Reference
Standard first-line treatment			
-DTIC, phase III trial	18/149 (12)	4/149 (3)	(30)
Experimental therapy			
-PLX4032 (BRAF inhibitor)			
Phase I trial	26/32 (81)	2/32 (6)	(31)
Phase II trial	70/132 (53)	8/132 (6)	(32)
Phase III trial	106/219 (48)	2/219 (1)	(33)
-Ipilimumab (anti-CTLA-4 mAb)			
Phase III trial	39/137 (28)	3/137 (2)	(2)
Ipilimumab + DTIC, phase III trial	34/250 (15)	26/252 (10)	(34)
-MDX-1106 (anti-PD-1 mAb)			
Phase I trial	5/39 (13)	1/39 (3)	(1)[#]
Adoptive T cell therapy			
-TIL therapy	52/93 (56)	20/93 (22)	(35)
-T cell clone therapy	8/10 (80)	-	(8)
-'Educated T cell' therapy	4/9 (44)	1/9 (11)	(9)
-TCR gene therapy[$]			
MART-1/A2, DMF-4	2/17 (17)	-	(22)
MART-1/A2, high affinity DMF-5	6/20 (30)	-	(21)
gp100/A2, murine	3/16 (19)	-	(21)
NY-ESO/A2, high affinity 1G4	5/11 (45)	2/11 (18)	(24)

Abbreviations used in this table:
OR = Objective Responses, CR = Complete Responses, both according to Response Evaluation Criteria for Solid Tumors (RECIST; (36)).
[#] This study included patients with advanced metastatic melanoma, but also patients with renal cell carcinoma, colorectal cancer, prostate cancer and non-small-cell lung cancer.
[$] TCR specificities and parental T cell clone used for the isolation of the TCR genes

receptor gene-engineered T cells is based on the ability to transfer a tumor-specific receptor into autologous T cells, expand them *ex vivo* and infuse the T cells into the patient (Figure 1). In this way the tumor immune response is directed towards a defined tumor-specific antigen of choice making this therapy applicable to tumor types of different histological origins.

This thesis focuses on adoptive T cell therapy with TCR-engineered T cells (in short TCR gene therapy). Antigen-specificity of a T cell is genetically controlled only by its TCR, a heterodimer composed of a TCRα and a TCRβ chain, which, together with CD3 molecules, constitute a TCR/CD3 complex on the cell surface (Figure 2). The TCR recognizes peptides presented by Major Histocompatibility Complex (MHC)-encoded molecules that are referred to as Human Leukocyte Antigens (HLA). Upon recognition of the appropriate peptide/MHC by the TCR, CD3 mediates signaling and initiates T cell activation. Recently, it has been implicated that for optimal TCR signaling additional di/multimerization of TCR/CD3 complexes is necessary (10, 11). Table 2 shows a timeline of key achievements in the field of engineered T cells. In the mid

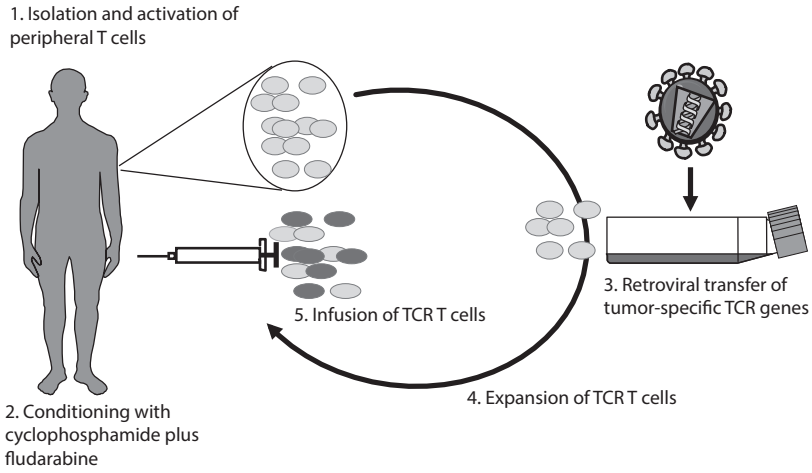

Figure 1. Schematic overview of T cell receptor gene therapy, adapted from chapter 2. Human peripheral blood mononuclear cells (PBMCs) are isolated from the cancer patient and T cells are activated *in vitro* before retroviral transfer with tumor-specific TCR genes. Following T cell expansion and validation of TCR surface expression, T cells are re-infused into the patient who is conditioned with chemotherapy.

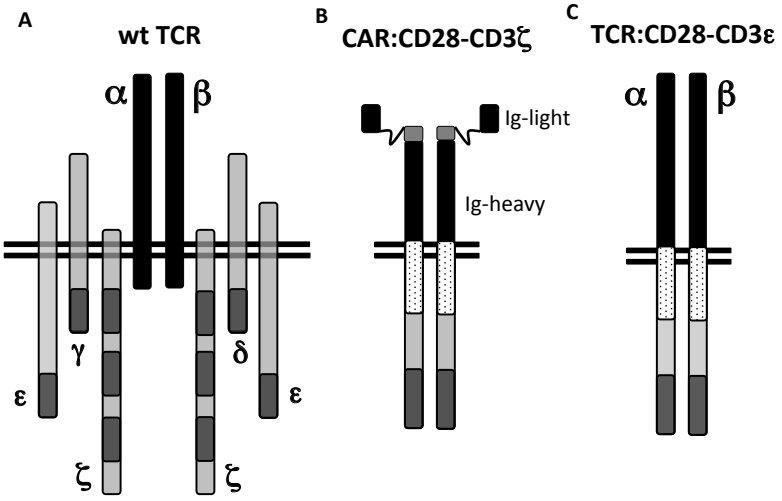

Figure 2. Schematic representation of a wild type T cell receptor (wt TCR) and examples of a Chimeric Antigen Receptor (CAR:CD28-CD3z) and a modified T cell receptor (TCR:CD28-CD3e). A. TCRα/β chains form a complex with CD3 components and determine the antigen specificity of a T cell. When the TCR binds its cognate peptide/MHC complex, immunoreceptor tyrosine-based activation motifs (ITAM domains: dark grey boxes) of CD3 $\delta\varepsilon$, $\gamma\varepsilon$, and $\zeta\zeta$ signaling dimers become phosphorylated and initiate T cell activation. Proper T cell activation is thought to require the dimerization of TCR/CD3 complexes (not indicated in this figure). **B.** CARs generally consist of an antibody-derived single chain variable fragment (scFv) composed of immunoglobulin heavy (Ig-heavy) and light chain (Ig-light) variable

domains. These domains are intervened by a linker and coupled to CD3ζ (with dark grey ITAM domains) for signaling capacities (1st generation CAR). CARs containing the CD3ζ transmembrane domain may form homodimers (as depicted in the Figure) and may associate with endogenous CD3 chains (not depicted in the Figure). CARs may include extra domains derived from co-stimulatory molecules such as CD28 or CD137 (the dotted boxes) or combinations thereof (2nd and 3rd generation CARs). **C**. A modified TCR format composed of the TCRα/β extracellular domains coupled to CD28 and CD3ε domains. This TCR is currently being characterized in our laboratory. The extracellular spacer and transmembrane domains are not indicated in **B** and **C** for simplicity reasons. The figure is adapted from Kalos, CII, 2012 (27).

Table 2. Timeline of key achievements in the field of adoptive therapy with receptor engineered T cells

Year	*Achievement*	*Reference*
1986	Redirection of T cell specificity by transfer of mouse TCRα and β genes into mouse T cells *in vitro*.	Dembic, Nature (12)
1993	Redirection of T cell specificity by transfer of a CAR in mouse T cells *in vitro*.	Esshar, PNAS (45)
1997	Anti-tumor function of CAR T cells *in vivo*.	Altenschmidt, JI (46)
1999	Redirection of T cells by transfer of human tumor specific TCR genes into human peripheral blood mononuclear cells *in vitro*.	Clay, JI (17)
2000	Targeting a Cancer Testis Antigen and testing of modified TCR formats in human peripheral blood lymphocytes *in vitro*.	Willemsen, Gene Ther (16)
2001	Function of TCR T cells *in vivo*.	Kessels, Nature (18)
2006	Clinical TCR gene therapy trial to treat metastatic melanoma patients.	Morgan, Science (22)
2006	Clinical CAR gene therapy trials and evidence for the occurrence of on-target toxicities.	Lamers, JCO; Kershaw, JCO (37, 38)
2011	Clinical TCR gene therapy trial 1) targeting a cancer testis antigen and 2) resulting in 2 out 11 complete responses in metastatic melanoma patients.	Robbins, JCO (24)
2011	Clinical trial with CAR T cells resulting in 1 partial and 2 complete responses out 3 in CLL patients.	Kalos, Science Trans Med (41) and Porter, NEJM (42)

eighties Dembic and colleagues were the first to demonstrate that antigen specificity can be transferred between T cells by introducing genes encoding the TCRα and β chains (12). Following this important finding, numerous research groups have reported that tumor reactivity of TCRs introduced in human peripheral blood lymphocytes (PBLs) is preserved and identical to the tumor reactivity of the parental tumor specific T cell (13-17). Importantly, when adoptively transferred, TCR gene-engineered T cells are antigen-specific and functional *in vivo* as evidenced by the induction of anti-tumor immune responses after antigen encounter (18-20). These preclinical studies constituted a solid basis to initiate the first clinical TCR gene therapy trials using TCRα and β genes as "of the shelf" reagents to confer tumor reactivity to T cells from patients with the appropriate HLA-restricting allele. Six clinical trials using TCR-engineered T cells have been initiated so far and performed under supervision of prof.dr. Steven Rosenberg (National Cancer Institute, National Institutes of Health, Bethesda, United States of America) and additional trials are currently being initiated in other centers. The TCRs tested so far were all HLA-A2-restricted and directed against melanoma differentiation antigens MART-I or gp100, carcinoembryonic antigen (CEA) or the cancer testis antigen NY-ESO-1. Clinical responses have been observed in patients with metastatic melanoma, colorectal and

synovial carcinoma (21-24) (Table 1). Notably, the finding that TCR gene-modified T cells (as well as TILs) were able to traffic to the central nervous system and cause complete responses of brain metastasis in patients with melanoma was not only encouraging but also underscores the strength of T cell therapy towards metastasized and poorly-accessible tumors (25). These clinical trials have demonstrated feasibility. Initial responses, although variable and based on a relatively small number of patients, are promising but generally fall behind those observed in TIL therapy. In addition, therapy with TCR-engineered T cells resulted in severe inflammation of the skin, eyes and ears (for MART-I and gp100) or colon (for CEA) (21, 26), likely due to low levels of target antigen expression in these organs. The gp100 and CEA specific TCRs were high affinity TCRs from mouse origin and the MART-I TCR in one study was a high affinity TCR from human origin (21). These TCRs may yield T cells that are more effective towards tumors, but at the same time induce on-target toxicity towards normal tissues. Hence, TCR gene therapy is considered a highly potent immune therapy against cancer, but T cells may damage healthy tissues if redirected towards an easy-accessible self-antigen. In contrast, no toxicities were observed in the most recent study using the NY-ESO-1-specific TCR, that targets a cancer testis antigen with a high degree of tumor-specific expression (24).

Besides TCRs, Chimeric Antigen Receptors (CARs) are successfully used to retarget T cells towards tumors. CAR and TCR T cells differ with respect to the following. *First*, CAR-T cells target surface antigens only, whereas TCR T cells target antigens not restricted to a certain cellular compartment. *Second*, the presentation of target antigen for TCR T cells, but not CAR T cells, is dependent on MHC expression levels and antigen processing efficiency. *Third*, TCR T cell function, but not CAR T cell function is compromised due to TCR mispairing and limited amounts of endogenous CD3 components (extensively reviewed in (28)). *Last*, CARs that contain mouse Ig sequences are potentially immunogenic (29), whereas TCRs generally contain native human sequences. CARs are fusion proteins combining the antigen-specific binding properties of an antibody with signaling properties of T cell molecules. The first generation CARs are composed of single-chain variable fragments (scFv) derived from monoclonal antibodies that recognize tumor-specific antigens and are most often linked to the intracellular signaling region of the CD3ζ (Figure 2). T cells can be engineered to functionally express these receptors, but in phase I clinical trials the first generation CARs induced no responses in patients with renal cell cancer (37) and ovarian cancer (38), and only modest responses in patients with lymphoma (39) and neuroblastoma (40). Next generation CARs contain intracellular domains derived from T cell co-stimulatory molecules such as CD28, 4-1BB (CD137), OX40 (CD134) or ICOS (CD278) or combinations thereof, to increase T cell signaling. Different CAR formats have recently been tested in phase I clinical studies targeting the B-cell antigen CD19 expressed on B-cell malignancies (41-44). CD19 may represent an interesting tumor-associated target antigen since it is also expressed on antigen-presenting cells, and as such may provide additional co-stimulation to CAR T cells, and it resides in the same physiological compartment as CAR T cells. Prof.dr. Carl June and his team (University

of Pennsylvania, Pennsylvania, United States of America) reported complete remissions in 2 out of 3 chronic lymphocytic leukemia patients treated with anti-CD19 CAR:CD137-CD3ζ T cells (41, 42). In two separate studies, patients with B cell malignancies were treated with anti-CD19 CAR:CD28-CD3ζ T cells resulting in 1 complete and 5 partial responses out of 8 patients treated in the first study (43) and 4 out of 5 partial responses in patients treated with cyclophosphamide and CAR T cells (44).

1.2 CHALLENGES OF TCR GENE THERAPY

The two main challenges of TCR gene therapy are 1) improvement of the anti-tumor response and 2) prevention/limitation of toxicity. In Table 3, I have further defined these challenges and indicated potential therapeutic strategies to address these challenges. Chapter 2 provides a detailed overview of the challenges and solutions. In short:

Challenge 1: Improve anti-tumor responses
Compromised anti-tumor responses are either related to the infused TCR T cells or the tumor. T-cell related factors include limited avidity, *in vivo* persistence, expansion, and homing and effector functions of T cells. Tumor-related factors include target antigen, such as its accessibility and level of expression, and tumor-induced immune suppression.

Challenge 2: Prevent/limit toxicities
Toxicities of TCR gene therapy can be divided into on-target and off-target toxicities. On-target toxicity represents toxicity related to the T cell target antigen, which can occur in case the antigen is expressed, even at low levels, on healthy non-tumor tissue. This type of toxicity can be prevented by choosing a target antigen of which the expression is restricted to tumor tissue, see paragraph 1.4. Off-target toxicity is a direct consequence of TCR-mispairing, i.e. the incorrect pairing of the introduced and endogenous TCR$\alpha\beta$ chains (28, 47). Consequences of TCR-mispairing are diluted expression of the introduced TCR but more problematically the occurrence of unknown TCR specificities that potentially result in auto-reactivity. Preclinical findings in a mouse model of TCR gene therapy (48) and *in vitro* systems in human T cells (49) stress the use of strategies to prevent the occurrence of TCR-mispairing. Strategies to prevent TCR-mispairing are described in chapter 2 and in (28).

1.3 MOUSE TUMOR MODELS FOR TCR GENE THERAPY

The development of cancer is the result of a cascade of mutations that makes the disease process complex and difficult to model. Animal models used to translate experimental find-

Table 3. Challenges of TCR gene therapy and their potential solutions

Challenges	Potential Solutions	References
1. IMPROVE ANTI-TUMOR TCR T CELL RESPONSES		
T cell-related factors:		
a) Low T cell avidity	-Improve retroviral vector design (pMP71)	(50, 51)
	-Use TCRβ-2A-TCRα expression cassette	(52)
	-Codon-optimize TCR transgenes	(53)
	-Modify TCR transgenes to enhance preferential pairing of introduced TCR chains	(54-57)
	-Affinity maturation of TCR-V region	(58)
	-Remove N-glycosylation site in TCR chains	(59)
	-Isolate TCR from non-tolerant T cell repertoire	(60, 61)
	-Combine introduction of TCR genes and CD3 genes	(62)
b) Low in vivo T cell expansion and persistence	-Lymphodeplete host with chemotherapy/irradiation	(63)
	-Vaccinate host after adoptive T cell transfer	(64, 65)
	-Provide cytokine support after adoptive T cell transfer	(66, 67)
	-Transfer less differentiated T cells (T_N, T_{CM}, T_{SCM})	(68-73)
	-Treat T cells with common-γ cytokines (IL-7, IL-15, IL-21)	(74-77)
e) Low T cell homing and effector functions	-Transfer less differentiated T cells	(69-73)
	-Block T cell inhibitory molecules such as CTLA-4, PD-1, TIM-3, BTLA	(78-82)
	-Genetically introduce functional molecules into T cells to enhance tumor infiltration (CCR2) or activation of T cells and innate immune cells (IL-12)	(83, 84)
	-Increase T cell extravasation in the tumor using angiogenesis inhibitors	
	-Target MHC class I and II restricted antigens simultaneously	(85)
Tumor-related factors		
a) Choice of target antigen	-Target antigens that can be cross-presented on tumor stroma	(86)
	-Target antigens that are expressed on tumor initiating cells	(63, 87)
	-Target multiple antigens simultaneously	(88)
b) Antigen expression/ presentation	-Enhance release/presentation of antigens by chemotherapy, irradiation or tyrosine kinase inhibitors	(89)
	-Epigenetically enhance antigen expression	(90-92)
c) Immune suppression	-Antagonize suppressive cytokines such as TGF-β and IL-10	(93, 94)
	-Deplete or reduce the activity of immune suppressor cells such as Tregs, type 2 macrophages and MDSCs	
	-Decrease metabolic degradation of the amino acids tryptophan and arginin	
2. PREVENT/LIMIT TOXICITY		
a) On-target toxicity	-Choose highly tumor restricted target antigens such as cancer testis antigens (MAGE, NY-ESO-1)	(24)
	-Introduce a genetic 'safety switch' into T cells	(95, 96)
b) Off-target toxicity	-Modify TCR transgenes to enhance preferential pairing of introduced TCR chains	(54-57)
	-Introduce a genetic 'safety switch' into T cells	(95, 96)
	-Silence endogenous TCR genes	(97)

Abbreviations used in this table in alphabetical order: BTLA, B- and T-Lymphocyte Attenuator; CCR2, CC Chemokine Receptor 2; CTLA-4, Cytotoxic T-Lymphocyte Antgen-4; IL-7, Interleukine-7; MDSC, Myeloid Derived Suppressor Cells; PD-1, Programmed Death-1; T_N, naïve T cells; T_{CM}, central memory T cells; T_{SCM}, stem-cell like memory T cells; TGF-β, Transforming Growth Factor-β; TIM-3, T cell Immunoglobulin Mucin-3; Tregs, regulatory T cells; .

ings into clinical testing need to recapitulate these mutations, the context of the disease and the heterogeneity of human tumors. Macaques (*Macaca Nemestrina*) have a close evolutionary relationship with humans and have proven valuable to study the effect of T cell subsets, dosing regimens and cytokine treatment in the context of adoptive T cell therapy (68, 98, 99). Disadvantages of macaques animal models include high costs, limited availability in research centers, high ethical barrier to use primates, and non-existence of a macaque tumor model (98). The mouse (*Mus Musculus*) is by far the most used animal in cancer models for many reasons including its small size, low costs, capability to breed in captivity, life-span of three years, physiological and molecular similarities to humans, complete sequenced genome and option to genetically manipulate mouse strains. Therefore the mouse represents the animal species of choice to model human cancer and test for TCR gene therapy. Mouse tumor models are used in cancer research for more than a century and range from xenograft or syngeneic graft models with tumors derived from cell lines and chemical and viral carcinogenesis or UV-induced tumors to several varieties of genetically engineered mice with inducible or spontaneous tumor growth. See Table 4 for an overview of various advantages and disadvantages of mouse models generally used to assess TCR gene therapy.

1.3.1 Genetically engineered mouse tumor models

Genetically engineered mice with constitutive gene expression

The most straightforward and widely used genetically engineered mice have constitutive expression of oncogenes or mutated onco-suppressor genes under the control of a ubiquitous or tissue-specific promoter. These mice have been informative in cancer biology and enabled studies into the mechanisms of oncogenic transformation and therapeutic responses of autochthonous tumors in an intact microenvironment (110). In these models it is possible to characterize immune responses to tumors in relation to specific cancer genotypes. Disadvantages of mouse models with engineered constitutive gene expression comprise the labor-intensive generation of these mice, which generally takes several years and often only yields tumor development in minorities of mice with variable latency (Table 4). Moreover, many genetically engineered mice are heterogeneous with respect to their tumor phenotypes, as a result of incomplete tissue-specificity of the promoter, and would need increased colony sizes to obtain statistically meaningful cohorts (111). An example of genetically engineered mice with constitutive gene expression are MT/*ret* transgenic mice expressing the RET oncogene fused to the metallothionein promoter and models development of melanoma. In this model the role of endogenous tumor-specific T cells during tumor development has been studied (100, 112). Recently, it was demonstrated with this model that myeloid derived suppressor cells (MDSC) present in the melanoma microenvironment block the anti-tumor activity of T cells (113). In mouse models with engineered constitutive gene expression, a few examples exist in which tumors express a defined T cell antigen. For instance, Tag antigen in TRAMP mice (TRansgenic Adenocarcinoma of the mouse Prostate) (101) and in a model of pancreatic

Table 4. Strengths and weaknesses of mouse tumor models to assess TCR gene therapy

Characteristics	*Advantages*	*Disadvantages*	*Examples*	*Reference*
Mouse models				
Genetically engineered *Constitutive*@	-Autochthonous tumors -Autochthonous microenvironment	-Variable tumor yield# and latency* -Long period to generate mice -High costs	-MT/*ret* model -TRAMP model	(100, 101)
Genetically engineered *Conditionally*@	-Spatiotemporal control of autochthonous tumors -Autochtonous micro-environment	-Variable tumor yield and latency -Long period to generate mice -High costs	-*K-ras$^{LSL-G12D/+}$;p53$^{fl/fl}$* model -*Tyr::CreER; Braf^{CA}; Pten$^{lox/lox}$* model	(102, 103)
Environmentally induced tumors	-Testing effect of carcinogens -Low costs	-Variable tumor yield and latency -Restricted to limited number of tumor types	-MCA-induced sarcoma model -UV-induced skin tumor model	(104, 105)
Transplanted *Xenograft tumor*	-Testing graft of human tumors and receptor-engineered T cells -Rapid tumor growth -Low costs	-Absence of host immune system -No reflection of authochtonous tumorigenesis -Graft-related inflammation	-ovarian/lung cancer models to test CAR T cell therapy	(106, 107) Chapter 3
Transplanted *Syngeneic tumor*	-Rapid tumor growth -Low costs	-No reflection of autochtonous tumorigenesis -Graft-related inflammation	-B16 mouse melanoma models -HLA-A2tg B16 mouse melanoma model	(65, 108, 109) Chapter 4

@ Genetically engineered mice with constitutive or conditional expression of the introduced gene(s).
#Tumor yield indicates the number of mice developing a relevant tumor.
*Tumor latency indicates the time period before tumor development.

cancer (114). In both mouse models cancer is induced by a viral oncogene, i.e. Simian Virus 40 large T antigen (Tag), and these mice are treated by various protocols such as vaccination (114) without or with anti-CTLA-4 antibodies (115) and TCR gene therapy (116). A sporadic tumor model has been developed in which tumors only express the Tag tumor antigen from the onset of tumor formation (so called neo-antigen) (117). These tumor models would be very attractive for testing strategies to enhance the therapeutic efficacy of TCR gene therapy.

Genetically engineered mice with conditional gene expression

In addition to tumor models with constitutive gene expression, tumor models are developed in which a gene is conditionally expressed or lost from its native promoter. Cancer can be controlled in these models by (de)activation of genes in specific tissues at desired time-points in the lifespan of a mouse. These genetic tools are used to recapitulate cancer progression and to model its stochastic nature (111). Despite increased flexibility and accuracy compared with classically gene-engineered mice with constitutive gene expression, similar engineering and breeding efforts are necessary to obtain large cohorts of mice for preclinical studies. Tumor incidence is often low. In example, a conditional melanoma model yields only 30% tumor incidence (118). Taken together, such characteristics may imply logistic and economic

barriers for the use of genetically engineered mouse tumor models in preclinical TCR gene therapy studies (Table 4). In these mice, a latent allele is phenotypically wild type until stimulated in a tissue and time-specific manner with exogenous chemicals or viruses (111). These models often rely on the use of site-specific-recombinases to control the mutation of the genome. The most commonly used system is based on bacteriophage Cre-*loxP* with Cre recombinase recognizing a pair of inverted repeat DNA elements (LoxP sites) resulting in irreversible inversion or deletion of a sequence in between LoxP sites (119). Traditionally, alleles of interest become constitutively activated or inactivated in the transgenic mice by Cre recombinase under the control of a tissue-specific promoter. To achieve conditional gene expression, Cre can be delivered via a virus administrated directly to the organ of interest where it will result in a genetic mosaic in the target tissue. An example is the use of adenoviral vector to express oncogenic K-RAS in a lung cancer model (120). Other possibilities to control gene expression are a tamoxifen-inducible Cre system (CreERT), an Flp-*FRT* system and Tet-On or Tet-Off systems. The CreERT system is based on a fusion protein of Cre and oestrogen response elements. Upon administration of tamoxifen (or analogues) to the mouse, CreERT recombinase becomes activated (121). A mouse model was developed in which a CreERT gene was introduced under control of the tyrosine promoter, and Cre became activated in melanocytes only upon application of tamoxifen. As a result of Cre activation both expression of oncogenic BRAF and deletion of the tumor suppressor PTEN were induced and mice developed malignant melanoma (102). An alternative site-specific recombinase system is the Flp-*FRT* system based on the Flp recombinase (122). In a mouse model of soft tissue sarcoma the combination of Cre-l*oxP* and Flp-*FRT* systems allowed spatiotemporal control of distinct genetic events, namely the sequential genetic mutation of common cancer genes p53 and K-RAS (123). In addition to site-specific-recombinase systems, reversible control of gene expression is achieved with Tet-On and Tet-Off systems, and as long as mice are fed with tetracycline (or analogues, often doxycycline) the gene of interest is switched on or off (124). In a doxycyline-inducible oncogenic H-RAS mouse model of melanoma, withdrawal of doxycyline and consequent inactivation of oncogenic H-RAS resulted in melanoma regression (125). There are a few conditional models in which tumors express a defined T cell antigen, such as a melanoma model expressing the mouse germ line gene P1A (118) and a lung cancer model expressing SIYRYYGL (126). Recently, DuPage and colleagues used a very elegant model of lung cancer expressing defined T cell antigens to study the interaction of the immune system with 'naturally arising' tumors (103). In these mice, lung adenocarcinoma was initiated by inhalation of lentiviral vectors expressing Cre recombinase. To induce tumors that express tumor neo-antigens, the lentiviral vector expressed SIYRYYGL and two antigens from Ovalbumin (103). Next to the study of endogenous immune responses to tumors, these models are valuable to assess and better understand parameters of TCR gene therapy.

1.3.2 Environmentally induced tumor models

Certain mouse strains develop cancer upon exposure to environmental stimuli including chemicals, viruses or irradiation. Examples of chemical agents that can initiate cellular alterations resulting in cancer are methylcholanthrene (MCA) (105) and asbestos fibers (127). Also UV irradiation is used to induce cancer in some mouse models (104). Mouse tumor models with environmentally-induced tumors have been used to identify oncogenes, tumor suppressor genes, and tumor susceptibility traits. However, such models develop a restricted set of tumors with variable tumor incidences and latencies making them less suitable for preclinical TCR gene therapy studies.

1.3.3 Transplantable tumor models

Diverse mouse or human tumor cell lines, representing a range of tumor types, are available for study and are easy to grow *in vitro* as well as in syngeneic or immune deficient mice, respectively. Tumor cells can be transplanted subcutaneously, intravenously or orthotopically to induce tumors within different microenvironments. An advantage of transplantable models is the relative short time transplanted tumor cells need to grow in order to form established tumors. Depending on the tumor cell line and mouse strain, tumor cells can grow within a few weeks into large tumors. There are, however several disadvantages of transplantable tumor models. *First*, fast tumor growth does generally not or only in part reflect the slow tumor and tumor vascular development observed in cancer patients. *Second*, injection of tumor cells involves rapid and ample cell death, and evokes acute inflammation with the generation of cytotoxic T cells in the 10-14 days upon tumor cell transplant (128, 129). It is therefore not surprising that treatment with vaccines or adoptively transferred T cells within the first two weeks after tumor cell inoculation is much more effective than treatment at later time points (129). Setting aside these above-mentioned disadvantages, important findings can be attributed to transplantable tumor models. Examples of these findings are: the identification of unique tumor-specific antigens that are effective tumor-rejection antigens (in a rat model (130)); the role for tumor stroma in tumor growth and rejection (86, 131); pro- and anti-tumorigenic activities of cytokines and the contribution of immune suppressor cells like regulatory T cells (Treg) cells to tumor formation (89).

Xenograft models

Xenograft tumor models are based on immune-deficient mice transplanted with a human tumor. Immune-deficient mouse strains lack components of the immune system either as a result of spontaneous mutations or engineered deletions in genes crucial for the development of a functional immune system. For example, the Severe Combined Immune Deficiency (SCID) mouse strain harbors a mutation in the *Prkdc* gene resulting in an impaired ability to develop T and B cells. The more stringent immune-deficient strain, NOD.SCID/il2rg$^{-/-}$ (NSG), is generated on a NOD.SCID background and lacks a functional common IL-2 receptor γ-chain

(132). As a result NSG mice lack NK cells, T and B cells and have impaired innate immunity. Immune-deficient mice not only allow the transplantation and growth of human tumor cells but also the adoptive transfer of human receptor-engineered T cells, providing these mouse models with translational value for clinical testing. Therefore, xenograft models have been extensively used in preclinical studies to test therapy with gene-engineered T cells directed against both hematological and solid tumors. Many studies focused on the assessment of different receptors and receptor formats that potentially can be used in a clinical setting. A range of CAR formats have been tested in this way resulting in the notion that genetic introduction of (domains of) co-stimulatory molecules (see Figure 2) significantly enhance anti-tumor activity and T cell persistence ((106, 107, and overviewed in (27)). In addition, xenograft models have frequently been used to image human immune cells (83, 133-135). In example, Craddock and colleagues have used human luciferase positive CAR T cells directed against diasialoganglioside GD2 that were also transduced with a chemokine receptor (CCR2). In this way the biodistribution of adoptively transferred CAR T cells directed against neuroblastoma could be visualized, showing that the genetic introduction of the chemokine receptor in CAR T cells increased their accumulation at the tumor site (136). In chapter 3, we provide an extensive literature overview of immune-deficient mouse tumor models used to test the therapeutic efficacy of gene-engineered T cells. In short, anti-tumor responses of systemically injected human TCR T cell against *solid tumors* are absent, whereas anti-tumor responses have been observed for human TCR T cells against hematological tumors. In chapter 3, we have tested and evaluated the use of different immune-deficient mouse strains for TCR gene therapy against solid tumors.

Syngeneic models

Syngeneic tumor models are based on either immune-deficient mice or immune-competent mice transplanted with a mouse tumor. These tumors are usually derived from spontaneous or environmentally induced primary mouse tumors. In some cases, these tumors are genetically engineered to express reporter genes, such as luciferase, or tumor-specific antigens. The first report on the *in vivo* efficacy of TCR gene therapy was demonstrated in an immune-deficient syngeneic mouse model with mice bearing a mouse lymphoma expressing an antigen derived from influenza virus. In this model adoptive transfer of T cells gene-modified with an influenza-specific TCR resulted in tumor regression (18). A clear advantage of immune-competent mouse models is the presence of a functional immune system, which potentially interacts with and shapes the tumor and its microenvironment (137), making these models better equipped to predict the clinical outcome of TCR gene therapy than immune-deficient models. The first reports of adoptive T cell therapy in immune-competent models made use of tumor-specific T cells that were transferred from immunized mice to tumor-bearing mice and were responsible for anti-tumor responses (138-141). These findings demonstrated that anti-tumor immunity can be transferred and further stimulated the development of

adoptive T cell therapy. Also, T cells derived from TCR transgenic mice were used to treat tumors that naturally expressed tumor antigens, and to investigate dosing and combination treatment protocols (65, 142). Furthermore, TCR gene therapy has been applied in immune-competent models targeting solid tumors with T cells engineered to express high affinity murine TCRs (143, 144). Collectively, adoptive T cell transfer in transplantable mouse tumor models demonstrated the importance of parameters such as lymphodepletion (145-147), high TCR surface expression levels, percentage of TCR positive cells within the transferred cell population (108), and a 'young' T cell phenotype for enhanced persistence and anti-tumor function *in vivo* (70-73, 148). Other strategies to enhance the therapeutic efficacy of TCR gene therapy have been explored in transplantable mouse tumor models with examples given in Table 3. Transgenic mouse strains expressing human MHC molecules, such as HLA-A2 transgenic mice (HHD mice) (149) or HLA-DP4 transgenic mice (150), allow assessment of TCR gene therapy directed against human antigens in an immune-competent setting. HLA transgenic mice bearing tumors expressing the corresponding HLA molecules can be treated with T cells modified to express human TCR-Variable genes, as described in (151). An HLA-A2 transgenic mouse model of melanoma has been used to study the kinetics and distribution of adoptively transferred TCR T cells (109). In chapter 5, we have set up and explored HLA-A2 transgenic mice to target TCR-engineered T cells towards human gp100/HLA-A2.

1.4 TARGET ANTIGENS AND TCR TRANSGENES: TOPICS OF THIS THESIS

1.4.1 Safe target antigens

Target antigens for TCR T cells must meet a number of criteria regarding therapeutic efficacy and safety. With regard to therapeutic efficacy, ideal target antigens are expressed homogeneously and at high levels by tumor cells and their expression is insensitive for down-regulation. Second, tumor antigens need to be safe targets. Tumor antigens that can be targeted in immunotherapy can be divided into 4 main classes: differentiation antigens; over-expressed antigens; antigens resulting from mutations; and shared tumor-specific antigens (reviewed in (152)).

Differentiation or over-expressed antigens are expressed at high levels by tumor cells, but not exclusively by tumor cells. The possible risk of on-target toxicity mediated by T cells directed against these antigens became clear in patient studies (Table 5). In an early clinical trial to treat metastatic renal cell cancer, CAR T cells caused reversible yet discrete cholangitis and damage to bile duct epithelium. This toxicity was a likely consequence of T cell activity against normal tissue expressing the target antigen Carbonic Anhydrase IX (CAIX) (37). In trials with CAR T cells directed against CD19 to treat B-cell leukemia, it was observed that besides a dramatic regression of malignant cells, T cells acted against normal B cells. Depletion of normal B cells resulted in low, but treatable, levels of serum antibody

Table 5. Reported toxicities in clinical trials of receptor gene therapy.

Target	Receptor	Toxicity*	Reference
CAIX	CAR:CD3ζ	Cholangitis and damage to bile duct epithelium (4/12)	(37) and personal communication Cor Lamers
HER-2/neu	CAR:CD28-CD137-CD3ζ	Case report on acute respiratory toxicity and death**	(26)
CD19	CAR:CD28-CD3ζ	Acute sepsis-like syndrome and death*** (1/10)	(44, 153)
CD19	CAR:CD137-CD3ζ	Long term depletion of normal B cells (3/3)	(41, 42)
CD19	CAR:CD28-CD3ζ	Cytokine-associated toxicities and long-term depletion of normal B cells (4/8)	(43)
MART-1	MART-1/A2 TCR, DMF4	None (0/17)	(22)
MART-1	MART-1/A2 TCR (high affinity, DMF5)	Severe melanocyte destruction in skin, eye and ear (in some cases leading to uveitis and hearing loss) (9/36)	(21)
Gp100	Gp100/A2 TCR (murine)		
CEA	CEA/A2 TCR (murine and affinity enhanced)	Severe inflammation of colon (3/3)	(23)
NY-ESO	NY-ESO/A2 TCR (1G4 affinity enhanced)	None (0/17)	(24)

*Number of patients with serious adverse events (= toxicity grading ≥ 3, National Cancer Institute common toxicity criteria) and the total number of patients treated are put between brackets.
**Toxicity was related to massive cytokine release and T cell infiltration in the lungs
***Precise cause of death is unclear and infection may have played a role.
Abbreviation used: CEA: carcinoembryonic antigen.

(42, 44). More serious was a case report of acute sepsis and death of a patient with CLL who received lymphodepletion combined with anti-CD19 CAR T cells, where infection may have been the underlying cause of death (153). Another patient with colon carcinoma treated with anti-HER-2/NEU CAR T cells died of acute respiratory toxicities, a likely result of T cell activity against HER-2/NEU expressed at low levels in the lung epithelium (26). Also, clinical studies using TCRs have shown evidence of on-target toxicities. TCRs directed against the HLA-A2-restricted antigens MART-I, gp100 and CEA resulted in mild and severe transient autoimmune side-effects (21, 23). Collectively, these studies strongly suggest that gene-engineered T cells directed against differentiation or over-expressed antigens cause (severe) side-effects and that therapy protocols should be designed to redirect T cells against antigens with a highly tumor-specific expression pattern.

Antigens that result from mutations can potentially provide safe T cell targets. When a mutation has occurred in initiating tumor cells and is transferred to daughter cells it may provide an ideal tumor target antigen for TCR gene therapy (152). Examples of such unique T cell antigens are peptides derived from mutated $BRAF^{(V600E)}$ presented by HLA-A2 and which induce specific T cell responses in melanoma patients (154). These examples, however, are exceptions and generally these mutations are unique and patient-specific, currently not making these antigens suitable for use in TCR gene therapy. In fact, targeting these antigens

would require the development of high throughput techniques to sequence tumor genomes to detect mutated genes and acquire knowledge about their immunogenicity. On the other hand, when it will turn out that mutated antigens are immune-dominant in anti-tumor immune responses, it might be worthwhile to invest in techniques that simplify such a personalized approach (155).

Cancer/Testis Antigens (CTA) are a class of shared antigens expressed in a variety of tumor types, but silenced in healthy cells, except for germ line cells and thymic medullary epithelial cells. Germ line cells are devoid of MHC molecules making CTA antigens, expressed by these cells, 'invisible' for T cells (156-158). Thymic medullary epithelial cells generally express low levels of CTA mRNA, which suggests existence of T cell tolerance towards these antigens (159). However, preclinical and clinical studies have shown the immunogenic nature of these antigens (eloquently reviewed in (156)). Already in the early nineties, Van der Bruggen and colleagues identified MAGE-A1 as the first immunogenic tumor antigen, which triggered cytotoxic T-lymphocyte responses in a cancer patient (158). So far, there are over 70 identified combinations of CTA peptides and HLA molecules recognized by T cells. Of special interest are MAGE antigens given their expression in multiple types of tumors and potential oncogenic nature. Four families of MAGE genes are located on chromosome X: *MAGE-A, B, C and D*. In somatic cells the expression of MAGE proteins is prevented by methylation of the promoter genes (160, 161), whereas MAGE genes are hypomethylated and expressed in cancer cells. Histology of these cancer cells include brain, breast, gastric, gynaecological, head and neck, haematological, lung, ovarian and prostate cancer (for a complete overview see www.cta.lncc.br). The expression of MAGE proteins is higher in more advanced stage tumors, predicts metastasis and tumor recurrence and as a result correlates with poor prognosis in patients (152, 162-167). More and more evidence indicates that MAGE proteins actively contribute to the development of malignancies. For example, MAGE proteins suppress p53-dependent apoptosis (168-170) and promote p53 degradation in cells (171). Over-expression of MAGE-A3 causes a fibronectin-controlled increase in tumor cell proliferation and thereby an increase in primary tumor size and metastatic capacity (172). Chapter 2, Table 2, summarizes advantages of MAGE antigens as targets for T cell therapy.

1.4.2 T cell receptors

With respect to TCRs, three main sources can be distinguished: 1) patient-derived material; 2) healthy donor-derived material; and 3) vaccinated HLA transgenic mice. Patient-derived materials such as tumor biopsies, tumor draining lymph nodes and peripheral blood represent commonly used sources for the isolation of TCR genes. The first TCR gene therapy studies used a MART-1-specific TCR that was isolated from a melanoma patient who responded after TIL therapy (22). An advantage of TCRs derived from responding patients is that such TCR clones often have enhanced frequencies, which facilitates detection and isolation of these clones, and are likely immune dominant in anti-tumor T cell responses. In chapter 5, TCR genes were

isolated from 2 MAGE-specific TCR clones with enhanced frequencies in peripheral blood and tumor tissue of melanoma patients who responded clinically upon vaccination with MAGE antigens (173-175). These TCR genes were characterized and used to redirect human T cells to MAGE positive tumor targets. A disadvantage of patient-derived or healthy donor-derived material as a source for tumor-specific TCR genes may be the relatively low affinity of these TCRs as a result of self-tolerance. Self-tolerance usually results in clonal deletion of high avidity T cells (i.e. T cells with a high affinity TCR) specific for self-antigens. Although options do exist to enhance the affinity of isolated TCRs (see chapter 2), the use of MHC-mismatched CTL systems allows the isolation of tumor-specific TCR genes from healthy donor-derived material and circumvents self-tolerance (176). Immunization of HLA-transgenic mice with tumor antigens would also circumvent self-tolerance. Both the gp100 and CEA-specific TCRs used in clinical trials were isolated from HLA-A2 transgenic mice (21, 23). Not completely unexpected, both responding and non-responding patients developed antibodies against epitopes derived from mouse TCRs (177). Recently, the group of prof.dr. Thomas Blankenstein (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) put great effort into the generation of a mouse in which the complete human TCR loci were introduced while the murine TCR loci were inactivated (178). This mouse model can be used to isolate complete human TCRs from a non-tolerant repertoire directed against those human peptide antigens that differ between mice and humans. An advantage of this model would be that resulting TCRs are not immunogenic in humans. Currently, these mice express HLA-A2 molecules but any desired human MHC molecule can be introduced making this model a potentially valuable source of TCRs that can be used in TCR gene therapy.

Various protocols have been described to obtain and select for TCRs for use in immune therapy and can be roughly divided into 1) the *in vitro* generation of tumor-specific T cells, and 2) the *in vitro* selection of TCR genes. Tumor-specific T cell clones can be obtained from patient- or healthy donor-derived material. For example, PBMCs from responding patients can be repeatedly stimulated with autologous irradiated tumor cells or peptides of interest and monoclonal T cells can be obtained from tumor-specific T cell cultures by limited dilution. In this way the MAGE-specific CTL clones were obtained (173) and characterized for TCR gene therapy in chapter 5. In TIL therapy studies, TILs from melanoma patients were cultured and amplified for adoptive T cell transfer. From these polyclonal T cell cultures the dominant tumor-specific TCR genes were isolated, such as the MART-specific TCR, characterized and used for TCR gene therapy studies (5, 21, 22). Another approach is based on the labeling of T cells derived from peripheral blood, tumor draining lymph nodes or tumor metastasis with tumor-specific peptide-MHC complexes without additional culture procedures. These cells can be single cell sorted and cultured until sufficient numbers of clonal T cells are obtained. These T cells were then further characterized for anti-tumor activity. Using this method, the NY-ESO/A2 TCR (derived from T cell clone 1G4) was obtained, affinity matured and successfully used in clinical TCR gene therapy (24, 1879-181). For the *in vitro* selection of TCR genes

different platforms have been developed including yeast (182), phage (58) or T cell line-display libraries (183). Using these platforms high affinity TCRs can be obtained for TCR gene transfer purposes. In addition, with an antibody phage display library antibodies that bind tumor-specific peptide-MHC complexes, i.e. 'TCR-like' antibodies, can be selected and also used to redirect T cells towards tumors (184). Recently, an elegant technique was developed that is based on high throughput peptide-MHC tetramer screening of tumor-specific T cells within therapeutic T cell populations by the group of prof.dr. Ton Schumacher (National Cancer Institute, Amsterdam) (185). This technique allows the identification of TCR specificities that play a role in anti-tumor immune responses. Results from such studies will likely support a more deliberate choice for tumor target antigens and corresponding TCRs for clinical use.

1.5 AIMS AND SCOPE OF THIS THESIS

To successfully apply clinical TCR gene therapy for cancer it is necessary to improve the response rate and prevent the occurrence of treatment-related toxicities. To this end, I defined 2 main objectives:

1) *To evaluate TCR gene therapy directed against human antigens in relevant preclinical mouse models*
2) *To characterize clinically relevant TCRs that target highly tumor-restricted antigens.*

Chapter 2 provides an overview of the lessons we have learned from preclinical and clinical TCR gene therapy studies and includes potential strategies to increase the clinical success of this form of immune therapy.

In **chapter 3** we summarize the literature on xenograft tumor models used to test gene-engineered T cells and evaluate different immune-deficient mouse strains for their use in studies with these T cells. Both human and mouse T cells gene-engineered to express a human gp100/HLA-A2 specific TCR were tested for their anti-tumor effect. We answered the following specific questions:

- *What is the value of testing human or mouse TCR gene-modified T cells in a xenograft tumor model to further develop TCR gene therapy?*
- *What may cause the low persistence of human TCR T cells in immune-deficient mice?*

Chapter 4 describes a fully immune-competent HLA-A2 transgenic mouse model in which we target human gp100 and HLA-A2 positive mouse melanoma with T cells gene modified with a human gp100/HLA-A2 TCR. We demonstrate that adoptive transfer of TCR T cells

caused regression of established tumors that was durable in 10-20% of mice. However in 80-90% of mice anti-tumor responses were transient and tumors relapsed. We answered the following specific questions:

- *What is the role of T cell antigen in the occurrence of relapsed tumors?*
- *Are relapsed tumors sensitive for a second treatment with TCR T cells?*

In **chapter 5,** an MHC class I and MHC class II-restricted human TCR were identified and characterized that target highly tumor-specific antigens: MAGE-$C2_{336-344}$/HLA-A2 (MC2/A2) and MAGE-$A3_{243-258}$/HLA-DP4 (MA3/DP4). In addition we answered the questions:

- *Can we redirect human CD8+ T cells with the MC2/A2 TCR towards MAGE-C2 positive tumor targets?*
- *Can we redirect human CD4+ T cells with the MA3/DP4 TCR towards MAGE-A3 positive tumor targets or dendritic cells?*

Finally, in **chapter 6** the main results are discussed and a scheme for a future TCR gene therapy trial is presented.

REFERENCES

1. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I & Topalian SL (2010) Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol 28(19):3167-3175.
2. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A & Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363(8):711-723.
3. Hersh EM, O'Day SJ, Powderly J, Khan KD, Pavlick AC, Cranmer LD, Samlowski WE, Nichol GM, Yellin MJ & Weber JS (2011) A phase II multicenter study of ipilimumab with or without dacarbazine in chemotherapy-naive patients with advanced melanoma. Invest New Drugs 29(3):489-498.
4. Besser MJ, Shapira-Frommer R, Treves AJ, Zippel D, Itzhaki O, Hershkovitz L, Levy D, Kubi A, Hovav E, Chermoshniuk N, Shalmon B, Hardan I, Catane R, Markel G, Apter S, Ben-Nun A, Kuchuk I, Shimoni A, Nagler A & Schachter J (2010) Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. Clin Cancer Res 16(9):2646-2655.
5. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A & Rosenberg SA (2005) Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 23(10):2346-2357.
6. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, Thomasian A, Downey SG, Smith FO, Klapper J, Morton K, Laurencot C, White DE & Rosenberg SA (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 26(32):5233-5239.
7. Dudley ME, Gross CA, Langhan MM, Garcia MR, Sherry RM, Yang JC, Phan GQ, Kammula US, Hughes MS, Citrin DE, Restifo NP, Wunderlich JR, Prieto PA, Hong JJ, Langan RC, Zlott DA, Morton KE, White DE, Laurencot CM & Rosenberg SA (2010) CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. Clin Cancer Res 16(24):6122-6131.
8. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E & Greenberg PD (2002) Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A 99(25):16168-16173.
9. Butler MO, Friedlander P, Milstein MI, Mooney MM, Metzler G, Murray AP, Tanaka M, Berezovskaya A, Imataki O, Drury L, Brennan L, Flavin M, Neuberg D, Stevenson K, Lawrence D, Hodi FS, Velazquez EF, Jaklitsch MT, Russell SE, Mihm M, Nadler LM & Hirano N (2011) Establishment of antitumor memory in humans using in vitro-educated CD8+ T cells. Sci Transl Med 3(80):80ra34.
10. Kuhns MS, Girvin AT, Klein LO, Chen R, Jensen KD, Newell EW, Huppa JB, Lillemeier BF, Huse M, Chien YH, Garcia KC & Davis MM (2010) Evidence for a functional sidedness to the alphabetaTCR. Proc Natl Acad Sci U S A 107(11):5094-5099.

11. Minguet S, Swamy M, Alarcon B, Luescher IF & Schamel WW (2007) Full activation of the T cell receptor requires both clustering and conformational changes at CD3. Immunity 26(1):43-54.
12. Dembic Z, Haas W, Weiss S, McCubrey J, Kiefer H, von Boehmer H & Steinmetz M (1986) Transfer of specificity by murine alpha and beta T-cell receptor genes. Nature 320(6059):232-238.
13. Morgan RA, Dudley ME, Yu YY, Zheng Z, Robbins PF, Theoret MR, Wunderlich JR, Hughes MS, Restifo NP & Rosenberg SA (2003) High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. J Immunol 171(6):3287-3295.
14. Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R & Adema GJ (2003) Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. J Immunol 170(4):2186-2194.
15. Stanislawski T, Voss RH, Lotz C, Sadovnikova E, Willemsen RA, Kuball J, Ruppert T, Bolhuis RL, Melief CJ, Huber C, Stauss HJ & Theobald M (2001) Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat Immunol 2(10):962-970.
16. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P & Bolhuis RL (2000) Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. Gene Ther 7(16):1369-1377.
17. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA & Nishimura MI (1999) Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. J Immunol 163(1):507-513.
18. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA & Schumacher TN (2001) Immunotherapy through TCR gene transfer. Nat Immunol 2(10):957-961.
19. Morris EC, Tsallios A, Bendle GM, Xue SA & Stauss HJ (2005) A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. Proc Natl Acad Sci U S A 102(22):7934-7939.
20. Tahara H, Fujio K, Araki Y, Setoguchi K, Misaki Y, Kitamura T & Yamamoto K (2003) Reconstitution of CD8+ T cells by retroviral transfer of the TCR alpha beta-chain genes isolated from a clonally expanded P815-infiltrating lymphocyte. J Immunol 171(4):2154-2160.
21. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM & Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114(3):535-546.
22. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA & Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314(5796):126-129.
23. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM & Rosenberg SA (2011) T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther 19(3):620-626.
24. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C & Rosenberg SA (2011) Tumor regression in patients with

metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29(7):917-924.

25. Hong JJ, Rosenberg SA, Dudley ME, Yang JC, White DE, Butman JA & Sherry RM (2010) Successful treatment of melanoma brain metastases with adoptive cell therapy. Clin Cancer Res 16(19):4892-4898.
26. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM & Rosenberg SA (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 18(4):843-851.
27. Kalos M (2012) Muscle CARs and TcRs: turbo-charged technologies for the (T cell) masses. Cancer Immunol Immunother 61(1):127-135.
28. Govers C, Sebestyen Z, Coccoris M, Willemsen RA & Debets R (2010) T cell receptor gene therapy: strategies for optimizing transgenic TCR pairing. Trends Mol Med 16(2):77-87.
29. Lamers CH, Willemsen R, van Elzakker P, van Steenbergen-Langeveld S, Broertjes M, Oosterwijk-Wakka J, Oosterwijk E, Sleijfer S, Debets R & Gratama JW (2011) Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. Blood 117(1):72-82.
30. Middleton MR, Grob JJ, Aaronson N, Fierlbeck G, Tilgen W, Seiter S, Gore M, Aamdal S, Cebon J, Coates A, Dreno B, Henz M, Schadendorf D, Kapp A, Weiss J, Fraass U, Statkevich P, Muller M & Thatcher N (2000) Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. J Clin Oncol 18(1):158-166.
31. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K & Chapman PB (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 363(9):809-819.
32. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Hersey P, Kefford R, Lawrence D, Puzanov I, Lewis KD, Amaravadi RK, Chmielowski B, Lawrence HJ, Shyr Y, Ye F, Li J, Nolop KB, Lee RJ, Joe AK & Ribas A (2012) Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. N Engl J Med 366(8):707-714.
33. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA & Group B-S (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 364(26):2507-2516.
34. Robert C, Thomas L, Bondarenko I, O'Day S, M DJ, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH, Jr., Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen TT, Humphrey R, Hoos A & Wolchok JD (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 364(26):2517-2526.
35. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE & Dudley ME (2011) Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 17(13):4550-4557.
36. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D & Verweij J (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45(2):228-247.
37. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G & Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol 24(13):e20-22.

38. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA & Hwu P (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res 12(20 Pt 1):6106-6115.
39. Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, Qian X, James SE, Raubitschek A, Forman SJ, Gopal AK, Pagel JM, Lindgren CG, Greenberg PD, Riddell SR & Press OW (2008) Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. Blood 112(6):2261-2271.
40. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, Yvon E, Weiss HL, Liu H, Rooney CM, Heslop HE & Brenner MK (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 14(11):1264-1270.
41. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A & June CH (2011) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 3(95):95ra73.
42. Porter DL, Levine BL, Kalos M, Bagg A & June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365(8):725-733.
43. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, Stetler-Stevenson M, Phan GQ, Hughes MS, Sherry RM, Yang JC, Kammula US, Devillier L, Carpenter R, Nathan DA, Morgan RA, Laurencot C & Rosenberg SA (2011) B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood.
44. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, Taylor C, Yeh R, Bartido S, Borquez-Ojeda O, Olszewska M, Bernal Y, Pegram H, Przybylowski M, Hollyman D, Usachenko Y, Pirraglia D, Hosey J, Santos E, Halton E, Maslak P, Scheinberg D, Jurcic J, Heaney M, Heller G, Frattini M & Sadelain M (2011) Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood 118(18):4817-4828.
45. Eshhar Z, Waks T, Gross G & Schindler DG (1993) Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc Natl Acad Sci U S A 90(2):720-724.
46. Altenschmidt U, Klundt E & Groner B (1997) Adoptive transfer of in vitro-targeted, activated T lymphocytes results in total tumor regression. J Immunol 159(11):5509-5515.
47. Schumacher TN (2002) T-cell-receptor gene therapy. Nat Rev Immunol 2(7):512-519.
48. Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, Jorritsma A, Kaiser AD, Pouw N, Debets R, Kieback E, Uckert W, Song JY, Haanen JB & Schumacher TN (2010) Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. Nat Med 16(5):565-570, 561p following 570.
49. van Loenen MM, de Boer R, Amir AL, Hagedoorn RS, Volbeda GL, Willemze R, van Rood JJ, Falkenburg JH & Heemskerk MH (2010) Mixed T cell receptor dimers harbor potentially harmful neoreactivity. Proc Natl Acad Sci U S A 107(24):10972-10977.
50. Engels B, Cam H, Schuler T, Indraccolo S, Gladow M, Baum C, Blankenstein T & Uckert W (2003) Retroviral vectors for high-level transgene expression in T lymphocytes. Hum Gene Ther 14(12):1155-1168.

51. Schambach A, Wodrich H, Hildinger M, Bohne J, Krausslich HG & Baum C (2000) Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. Mol Ther 2(5):435-445.
52. Leisegang M, Engels B, Meyerhuber P, Kieback E, Sommermeyer D, Xue SA, Reuss S, Stauss H & Uckert W (2008) Enhanced functionality of T cell receptor-redirected T cells is defined by the transgene cassette. J Mol Med 86(5):573-583.
53. Scholten KB, Kramer D, Kueter EW, Graf M, Schoedl T, Meijer CJ, Schreurs MW & Hooijberg E (2006) Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. Clin Immunol 119(2):135-145.
54. Cohen CJ, Li YF, El-Gamil M, Robbins PF, Rosenberg SA & Morgan RA (2007) Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. Cancer Res 67(8):3898-3903.
55. Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA & Morgan RA (2006) Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. Cancer Res 66(17):8878-8886.
56. Kuball J, Dossett ML, Wolfl M, Ho WY, Voss RH, Fowler C & Greenberg PD (2007) Facilitating matched pairing and expression of TCR chains introduced into human T cells. Blood 109(6):2331-2338.
57. Sebestyen Z, Schooten E, Sals T, Zaldivar I, San Jose E, Alarcon B, Bobisse S, Rosato A, Szollosi J, Gratama JW, Willemsen RA & Debets R (2008) Human TCR that incorporate CD3zeta induce highly preferred pairing between TCRalpha and beta chains following gene transfer. J Immunol 180(11):7736-7746.
58. Varela-Rohena A, Molloy PE, Dunn SM, Li Y, Suhoski MM, Carroll RG, Milicic A, Mahon T, Sutton DH, Laugel B, Moysey R, Cameron BJ, Vuidepot A, Purbhoo MA, Cole DK, Phillips RE, June CH, Jakobsen BK, Sewell AK & Riley JL (2008) Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. Nat Med 14(12):1390-1395.
59. Kuball J, Hauptrock B, Malina V, Antunes E, Voss RH, Wolfl M, Strong R, Theobald M & Greenberg PD (2009) Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. J Exp Med 206(2):463-475.
60. Stauss HJ (1999) Immunotherapy with CTLs restricted by nonself MHC. Immunol Today 20(4):180-183.
61. Theobald M, Biggs J, Hernandez J, Lustgarten J, Labadie C & Sherman LA (1997) Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. J Exp Med 185(5):833-841.
62. Ahmadi M, King JW, Xue SA, Voisine C, Holler A, Wright GP, Waxman J, Morris E & Stauss HJ (2011) CD3 limits the efficacy of TCR gene therapy in vivo. Blood 118(13):3528-3537.
63. Klebanoff CA, Khong HT, Antony PA, Palmer DC & Restifo NP (2005) Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. Trends Immunol 26(2):111-117.
64. Ly LV, Sluijter M, Versluis M, Luyten GP, van Stipdonk MJ, van der Burg SH, Melief CJ, Jager MJ & van Hall T (2010) Peptide vaccination after T-cell transfer causes massive clonal expansion, tumor eradication, and manageable cytokine storm. Cancer Res 70(21):8339-8346.
65. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA & Restifo NP (2003) Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. J Exp Med 198(4):569-580.

66. Boyman O, Kovar M, Rubinstein MP, Surh CD & Sprent J (2006) Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science 311(5769):1924-1927.
67. Stephan MT, Moon JJ, Um SH, Bershteyn A & Irvine DJ (2010) Therapeutic cell engineering with surface-conjugated synthetic nanoparticles. Nat Med 16(9):1035-1041.
68. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C & Riddell SR (2008) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest 118(1):294-305.
69. Bondanza A, Valtolina V, Magnani Z, Ponzoni M, Fleischhauer K, Bonyhadi M, Traversari C, Sanvito F, Toma S, Radrizzani M, La Seta-Catamancio S, Ciceri F, Bordignon C & Bonini C (2006) Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes. Blood 107(5):1828-1836.
70. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA & Restifo NP (2005) Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest 115(6):1616-1626.
71. Gattinoni L, Zhong XS, Palmer DC, Ji Y, Hinrichs CS, Yu Z, Wrzesinski C, Boni A, Cassard L, Garvin LM, Paulos CM, Muranski P & Restifo NP (2009) Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. Nat Med 15(7):808-813.
72. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M & Restifo NP (2011) A human memory T cell subset with stem cell-like properties. Nat Med 17(10):1290-1297.
73. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA & Restifo NP (2005) Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A 102(27):9571-9576.
74. Huarte E, Fisher J, Turk MJ, Mellinger D, Foster C, Wolf B, Meehan KR, Fadul CE & Ernstoff MS (2009) Ex vivo expansion of tumor specific lymphocytes with IL-15 and IL-21 for adoptive immunotherapy in melanoma. Cancer Lett 285(1):80-88.
75. Kaneko S, Mastaglio S, Bondanza A, Ponzoni M, Sanvito F, Aldrighetti L, Radrizzani M, La Seta-Catamancio S, Provasi E, Mondino A, Nagasawa T, Fleischhauer K, Russo V, Traversari C, Ciceri F, Bordignon C & Bonini C (2009) IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. Blood 113(5):1006-1015.
76. Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, ten Hagen T, Verweij J & Debets R (2010) Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. Cancer Immunol Immunother 59(6):921-931.
77. Pouw N, Treffers-Westerlaken E, Mondino A, Lamers C & Debets R (2010) TCR gene-engineered T cell: limited T cell activation and combined use of IL-15 and IL-21 ensure minimal differentiation and maximal antigen-specificity. Mol Immunol 47(7-8):1411-1420.
78. Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T & Gajewski TF (2004) PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 64(3):1140-1145.
79. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR & Honjo T (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192(7):1027-1034.

80. Leach DR, Krummel MF & Allison JP (1996) Enhancement of antitumor immunity by CTLA-4 blockade. Science 271(5256):1734-1736.
81. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK & Anderson AC (2010) Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. J Exp Med 207(10):2187-2194.
82. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, Hurchla MA, Zimmerman N, Sim J, Zang X, Murphy TL, Russell JH, Allison JP & Murphy KM (2003) BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 4(7):670-679.
83. Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, Powell DJ, Jr., Riley JL, June CH & Albelda SM (2011) Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. Clin Cancer Res 17(14):4719-4730.
84. Chmielewski M, Kopecky C, Hombach AA & Abken H (2011) IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. Cancer Res 71(17):5697-5706.
85. Dings RP, Vang KB, Castermans K, Popescu F, Zhang Y, Oude Egbrink MG, Mescher MF, Farrar MA, Griffioen AW & Mayo KH (2011) Enhancement of T-cell-mediated antitumor response: angiostatic adjuvant to immunotherapy against cancer. Clin Cancer Res 17(10):3134-3145.
86. Spiotto MT, Rowley DA & Schreiber H (2004) Bystander elimination of antigen loss variants in established tumors. Nat Med 10(3):294-298.
87. Blank CU, Hooijkaas AI, Haanen JB & Schumacher TN (2011) Combination of targeted therapy and immunotherapy in melanoma. Cancer Immunol Immunother 60(10):1359-1371.
88. Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, Vyas P, Cavenagh J, Stankovic T, Moss P & Craddock C (2010) Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood 116(11):1908-1918.
89. Rabinovich GA, Gabrilovich D & Sotomayor EM (2007) Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 25:267-296.
90. Allavena P, Sica A, Garlanda C & Mantovani A (2008) The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev 222:155-161.
91. Gabrilovich DI & Nagaraj S (2009) Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9(3):162-174.
92. Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 5(4):263-274.
93. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T & Van den Eynde BJ (2003) Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 9(10):1269-1274.
94. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, Gilbert J & Ochoa AC (2005) Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med 202(7):931-939.
95. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, Straathof K, Liu E, Durett AG, Grilley B, Liu H, Cruz CR, Savoldo B, Gee AP, Schindler J, Krance RA, Heslop HE, Spencer DM, Rooney CM & Brenner MK (2011) Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med 365(18):1673-1683.

96. Kieback E, Charo J, Sommermeyer D, Blankenstein T & Uckert W (2008) A safeguard eliminates T cell receptor gene-modified autoreactive T cells after adoptive transfer. Proc Natl Acad Sci U S A 105(2):623-628.
97. Ochi T, Fujiwara H, Okamoto S, An J, Nagai K, Shirakata T, Mineno J, Kuzushima K, Shiku H & Yasukawa M (2011) Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. Blood 118(6):1495-1503.
98. Berger C, Berger M, Anderson D & Riddell SR (2011) A non-human primate model for analysis of safety, persistence, and function of adoptively transferred T cells. J Med Primatol 40(2):88-103.
99. Berger C, Berger M, Hackman RC, Gough M, Elliott C, Jensen MC & Riddell SR (2009) Safety and immunologic effects of IL-15 administration in nonhuman primates. Blood 114(12):2417-2426.
100. Lengagne R, Graff-Dubois S, Garcette M, Renia L, Kato M, Guillet JG, Engelhard VH, Avril MF, Abastado JP & Prevost-Blondel A (2008) Distinct role for CD8 T cells toward cutaneous tumors and visceral metastases. J Immunol 180(1):130-137.
101. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ & Rosen JM (1995) Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 92(8):3439-3443.
102. Dankort D, Curley DP, Cartlidge RA, Nelson B, Karnezis AN, Damsky WE, Jr., You MJ, DePinho RA, McMahon M & Bosenberg M (2009) Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. Nat Genet 41(5):544-552.
103. DuPage M, Cheung AF, Mazumdar C, Winslow MM, Bronson R, Schmidt LM, Crowley D, Chen J & Jacks T (2011) Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression. Cancer Cell 19(1):72-85.
104. Kripke ML (1974) Antigenicity of murine skin tumors induced by ultraviolet light. J Natl Cancer Inst 53(5):1333-1336.
105. Stutman O (1974) Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science 183(4124):534-536.
106. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I & June CH (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A 106(9):3360-3365.
107. Song DG, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, Figini M, June CH, Coukos G & Powell DJ, Jr. (2011) In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). Cancer Res 71(13):4617-4627.
108. de Witte MA, Jorritsma A, Kaiser A, van den Boom MD, Dokter M, Bendle GM, Haanen JB & Schumacher TN (2008) Requirements for effective antitumor responses of TCR transduced T cells. J Immunol 181(7):5128-5136.
109. Koya RC, Mok S, Comin-Anduix B, Chodon T, Radu CG, Nishimura MI, Witte ON & Ribas A (2010) Kinetic phases of distribution and tumor targeting by T cell receptor engineered lymphocytes inducing robust antitumor responses. Proc Natl Acad Sci U S A 107(32):14286-14291.
110. Sharpless NE & Depinho RA (2006) The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov 5(9):741-754.
111. Heyer J, Kwong LN, Lowe SW & Chin L (2010) Non-germline genetically engineered mouse models for translational cancer research. Nat Rev Cancer 10(7):470-480.

112. Umansky V, Abschuetz O, Osen W, Ramacher M, Zhao F, Kato M & Schadendorf D (2008) Melanoma-specific memory T cells are functionally active in Ret transgenic mice without macroscopic tumors. Cancer Res 68(22):9451-9458.
113. Meyer C, Sevko A, Ramacher M, Bazhin AV, Falk CS, Osen W, Borrello I, Kato M, Schadendorf D, Baniyash M & Umansky V (2011) Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. Proc Natl Acad Sci U S A 108(41):17111-17116.
114. Otahal P, Schell TD, Hutchinson SC, Knowles BB & Tevethia SS (2006) Early immunization induces persistent tumor-infiltrating CD8+ T cells against an immunodominant epitope and promotes lifelong control of pancreatic tumor progression in SV40 tumor antigen transgenic mice. J Immunol 177(5):3089-3099.
115. Hurwitz AA, Foster BA, Kwon ED, Truong T, Choi EM, Greenberg NM, Burg MB & Allison JP (2000) Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. Cancer Res 60(9):2444-2448.
116. de Witte MA, Bendle GM, van den Boom MD, Coccoris M, Schell TD, Tevethia SS, van Tinteren H, Mesman EM, Song JY & Schumacher TN (2008) TCR gene therapy of spontaneous prostate carcinoma requires in vivo T cell activation. J Immunol 181(4):2563-2571.
117. Willimsky G & Blankenstein T (2005) Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. Nature 437(7055):141-146.
118. Huijbers IJ, Krimpenfort P, Chomez P, van der Valk MA, Song JY, Inderberg-Suso EM, Schmitt-Verhulst AM, Berns A & Van den Eynde BJ (2006) An inducible mouse model of melanoma expressing a defined tumor antigen. Cancer Res 66(6):3278-3286.
119. Lakso M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL & Westphal H (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci U S A 89(14):6232-6236.
120. Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D, Hingorani SR, Zaks T, King C, Jacobetz MA, Wang L, Bronson RT, Orkin SH, DePinho RA & Jacks T (2004) Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5(4):375-387.
121. Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P & Metzger D (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res 27(22):4324-4327.
122. Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF & Dymecki SM (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet 25(2):139-140.
123. Young NP, Crowley D & Jacks T (2011) Uncoupling cancer mutations reveals critical timing of p53 loss in sarcomagenesis. Cancer Res 71(11):4040-4047.
124. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W & Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. Science 268(5218):1766-1769.
125. Chin L, Tam A, Pomerantz J, Wong M, Holash J, Bardeesy N, Shen Q, O'Hagan R, Pantginis J, Zhou H, Horner JW, 2nd, Cordon-Cardo C, Yancopoulos GD & DePinho RA (1999) Essential role for oncogenic Ras in tumour maintenance. Nature 400(6743):468-472.
126. Cheung AF, Dupage MJ, Dong HK, Chen J & Jacks T (2008) Regulated expression of a tumor-associated antigen reveals multiple levels of T-cell tolerance in a mouse model of lung cancer. Cancer Res 68(22):9459-9468.

127. Altomare DA, Menges CW, Pei J, Zhang L, Skele-Stump KL, Carbone M, Kane AB & Testa JR (2009) Activated TNF-alpha/NF-kappaB signaling via down-regulation of Fas-associated factor 1 in asbestos-induced mesotheliomas from Arf knockout mice. Proc Natl Acad Sci U S A 106(9):3420-3425.
128. Khong HT & Restifo NP (2002) Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 3(11):999-1005.
129. Schreiber K, Rowley DA, Riethmuller G & Schreiber H (2006) Cancer immunotherapy and preclinical studies: why we are not wasting our time with animal experiments. Hematol Oncol Clin North Am 20(3):567-584.
130. Baldwin RW (1955) Immunity to methylcholanthrene-induced tumours in inbred rats following atrophy and regression of the implanted tumours. Br J Cancer 9(4):652-657.
131. Kammertoens T, Schuler T & Blankenstein T (2005) Immunotherapy: target the stroma to hit the tumor. Trends Mol Med 11(5):225-231.
132. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL & Handgretinger R (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 174(10):6477-6489.
133. Brentjens RJ, Latouche JB, Santos E, Marti F, Gong MC, Lyddane C, King PD, Larson S, Weiss M, Riviere I & Sadelain M (2003) Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. Nat Med 9(3):279-286.
134. Kruschinski A, Moosmann A, Poschke I, Norell H, Chmielewski M, Seliger B, Kiessling R, Blankenstein T, Abken H & Charo J (2008) Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. Proc Natl Acad Sci U S A 105(45):17481-17486.
135. Parente-Pereira AC, Burnet J, Ellison D, Foster J, Davies DM, van der Stegen S, Burbridge S, Chiapero-Stanke L, Wilkie S, Mather S & Maher J (2011) Trafficking of CAR-engineered human T cells following regional or systemic adoptive transfer in SCID beige mice. J Clin Immunol 31(4):710-718.
136. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM & Foster AE (2010) Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. J Immunother 33(8):780-788.
137. Dunn GP, Bruce AT, Ikeda H, Old LJ & Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3(11):991-998.
138. Granziero L, Krajewski S, Farness P, Yuan L, Courtney MK, Jackson MR, Peterson PA & Vitiello A (1999) Adoptive immunotherapy prevents prostate cancer in a transgenic animal model. Eur J Immunol 29(4):1127-1138.
139. Overwijk WW, Tsung A, Irvine KR, Parkhurst MR, Goletz TJ, Tsung K, Carroll MW, Liu C, Moss B, Rosenberg SA & Restifo NP (1998) gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. J Exp Med 188(2):277-286.
140. Rosenstein M, Eberlein TJ & Rosenberg SA (1984) Adoptive immunotherapy of established syngeneic solid tumors: role of T lymphoid subpopulations. J Immunol 132(4):2117-2122.
141. Vierboom MP, Nijman HW, Offringa R, van der Voort EI, van Hall T, van den Broek L, Fleuren GJ, Kenemans P, Kast WM & Melief CJ (1997) Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. J Exp Med 186(5):695-704.

142. Hanson HL, Donermeyer DL, Ikeda H, White JM, Shankaran V, Old LJ, Shiku H, Schreiber RD & Allen PM (2000) Eradication of established tumors by CD8+ T cell adoptive immunotherapy. Immunity 13(2):265-276.
143. Abad JD, Wrzensinski C, Overwijk W, De Witte MA, Jorritsma A, Hsu C, Gattinoni L, Cohen CJ, Paulos CM, Palmer DC, Haanen JB, Schumacher TN, Rosenberg SA, Restifo NP & Morgan RA (2008) T-cell receptor gene therapy of established tumors in a murine melanoma model. J Immunother 31(1):1-6.
144. Chamoto K, Tsuji T, Funamoto H, Kosaka A, Matsuzaki J, Sato T, Abe H, Fujio K, Yamamoto K, Kitamura T, Takeshima T, Togashi Y & Nishimura T (2004) Potentiation of tumor eradication by adoptive immunotherapy with T-cell receptor gene-transduced T-helper type 1 cells. Cancer Res 64(1):386-390.
145. Cheadle EJ, Hawkins RE, Batha H, O'Neill AL, Dovedi SJ & Gilham DE (2010) Natural expression of the CD19 antigen impacts the long-term engraftment but not antitumor activity of CD19-specific engineered T cells. J Immunol 184(4):1885-1896.
146. Cho BK, Rao VP, Ge Q, Eisen HN & Chen J (2000) Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. J Exp Med 192(4):549-556.
147. Dummer W, Niethammer AG, Baccala R, Lawson BR, Wagner N, Reisfeld RA & Theofilopoulos AN (2002) T cell homeostatic proliferation elicits effective antitumor autoimmunity. J Clin Invest 110(2):185-192.
148. Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, Sanchez-Perez L, Muranski P, Kern SJ, Logun C, Palmer DC, Ji Y, Reger RN, Leonard WJ, Danner RL, Rosenberg SA & Restifo NP (2009) Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. Proc Natl Acad Sci U S A 106(41):17469-17474.
149. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA & Perarnau B (1997) HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 185(12):2043-2051.
150. Hagihara M, Shimura T, Takebe K, Munkhbat B, Sato T, Tsuchida F, Sato K & Tsuji K (1996) Xenogeneic iso-skin graft and mixed lymphocyte reaction studies using HLA-DP transgenic mice. Transpl Immunol 4(3):220-226.
151. Pouw NM, Westerlaken EJ, Willemsen RA & Debets R (2007) Gene transfer of human TCR in primary murine T cells is improved by pseudo-typing with amphotropic and ecotropic envelopes. J Gene Med 9(7):561-570.
152. Lucas S & Coulie PG (2008) About human tumor antigens to be used in immunotherapy. Semin Immunol 20(5):301-307.
153. Brentjens R, Yeh R, Bernal Y, Riviere I & Sadelain M (2010) Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. Mol Ther 18(4):666-668.
154. Somasundaram R, Swoboda R, Caputo L, Otvos L, Weber B, Volpe P, van Belle P, Hotz S, Elder DE, Marincola FM, Schuchter L, Guerry D, Czerniecki BJ & Herlyn D (2006) Human leukocyte antigen-A2-restricted CTL responses to mutated BRAF peptides in melanoma patients. Cancer Res 66(6):3287-3293.
155. Parmiani G, De Filippo A, Novellino L & Castelli C (2007) Unique human tumor antigens: immunobiology and use in clinical trials. J Immunol 178(4):1975-1979.
156. Caballero OL & Chen YT (2009) Cancer/testis (CT) antigens: potential targets for immunotherapy. Cancer Sci 100(11):2014-2021.

157. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T & Lucas S (2001) An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res 61(14):5544-5551.
158. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A & Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643-1647.
159. Gotter J, Brors B, Hergenhahn M & Kyewski B (2004) Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. J Exp Med 199(2):155-166.
160. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F & Boon T (1996) The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A 93(14):7149-7153.
161. De Smet C, Lurquin C, Lethe B, Martelange V & Boon T (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 19(11):7327-7335.
162. Brasseur F, Rimoldi D, Lienard D, Lethe B, Carrel S, Arienti F, Suter L, Vanwijck R, Bourlond A, Humblet Y & et al. (1995) Expression of MAGE genes in primary and metastatic cutaneous melanoma. Int J Cancer 63(3):375-380.
163. Brichard VG & Lejeune D (2007) GSK's antigen-specific cancer immunotherapy programme: pilot results leading to Phase III clinical development. Vaccine 25 Suppl 2:B61-71.
164. Chen YT, Ross DS, Chiu R, Zhou XK, Chen YY, Lee P, Hoda SA, Simpson AJ, Old LJ, Caballero O & Neville AM (2011) Multiple cancer/testis antigens are preferentially expressed in hormone-receptor negative and high-grade breast cancers. PLoS One 6(3):e17876.
165. Curioni-Fontecedro A, Nuber N, Mihic-Probst D, Seifert B, Soldini D, Dummer R, Knuth A, van den Broek M & Moch H (2011) Expression of MAGE-C1/CT7 and MAGE-C2/CT10 predicts lymph node metastasis in melanoma patients. PLoS One 6(6):e21418.
166. Gure AO, Chua R, Williamson B, Gonen M, Ferrera CA, Gnjatic S, Ritter G, Simpson AJ, Chen YT, Old LJ & Altorki NK (2005) Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. Clin Cancer Res 11(22):8055-8062.
167. von Boehmer L, Keller L, Mortezavi A, Provenzano M, Sais G, Hermanns T, Sulser T, Jungbluth AA, Old LJ, Kristiansen G, van den Broek M, Moch H, Knuth A & Wild PJ (2011) MAGE-C2/CT10 protein expression is an independent predictor of recurrence in prostate cancer. PLoS One 6(7):e21366.
168. Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M & Schneider C (2006) MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. Proc Natl Acad Sci U S A 103(30):11160-11165.
169. Nardiello T, Jungbluth AA, Mei A, Diliberto M, Huang X, Dabrowski A, Andrade VC, Wasserstrum R, Ely S, Niesvizky R, Pearse R, Coleman M, Jayabalan DS, Bhardwaj N, Old LJ, Chen-Kiang S & Cho HJ (2011) MAGE-A inhibits apoptosis in proliferating myeloma cells through repression of Bax and maintenance of survivin. Clin Cancer Res 17(13):4309-4319.
170. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ & Longley BJ (2007) MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. Cancer Res 67(20):9954-9962.
171. Doyle JM, Gao J, Wang J, Yang M & Potts PR (2010) MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell 39(6):963-974.

172. Liu W, Cheng S, Asa SL & Ezzat S (2008) The melanoma-associated antigen A3 mediates fibronectin-controlled cancer progression and metastasis. Cancer Res 68(19):8104-8112.
173. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethe B, De Plaen E, Velu T, Boon T & Coulie PG (2005) High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. J Exp Med 201(2):241-248.
174. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG & Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 201(2):249-257.
175. Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, Bender A, Feuerstein B, Fritsch PO, Romani N & Schuler G (2002) Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med 195(10):1279-1288.
176. Sadovnikova E, Jopling LA, Soo KS & Stauss HJ (1998) Generation of human tumor-reactive cytotoxic T cells against peptides presented by non-self HLA class I molecules. Eur J Immunol 28(1):193-200.
177. Davis JL, Theoret MR, Zheng Z, Lamers CH, Rosenberg SA & Morgan RA (2010) Development of human anti-murine T-cell receptor antibodies in both responding and nonresponding patients enrolled in TCR gene therapy trials. Clin Cancer Res 16(23):5852-5861.
178. Li LP, Lampert JC, Chen X, Leitao C, Popovic J, Muller W & Blankenstein T (2010) Transgenic mice with a diverse human T cell antigen receptor repertoire. Nat Med 16(9):1029-1034.
179. Chen JL, Dunbar PR, Gileadi U, Jager E, Gnjatic S, Nagata Y, Stockert E, Panicali DL, Chen YT, Knuth A, Old LJ & Cerundolo V (2000) Identification of NY-ESO-1 peptide analogues capable of improved stimulation of tumor-reactive CTL. J Immunol 165(2):948-955.
180. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, Xu H, Morgan RA, Feldman SA, Johnson LA, Bennett AD, Dunn SM, Mahon TM, Jakobsen BK & Rosenberg SA (2008) Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. J Immunol 180(9):6116-6131.
181. Zhao Y, Bennett AD, Zheng Z, Wang QJ, Robbins PF, Yu LY, Li Y, Molloy PE, Dunn SM, Jakobsen BK, Rosenberg SA & Morgan RA (2007) High-affinity TCRs generated by phage display provide CD4+ T cells with the ability to recognize and kill tumor cell lines. J Immunol 179(9):5845-5854.
182. Holler PD, Holman PO, Shusta EV, O'Herrin S, Wittrup KD & Kranz DM (2000) In vitro evolution of a T cell receptor with high affinity for peptide/MHC. Proc Natl Acad Sci U S A 97(10):5387-5392.
183. Kessels HW, van Den Boom MD, Spits H, Hooijberg E & Schumacher TN (2000) Changing T cell specificity by retroviral T cell receptor display. Proc Natl Acad Sci U S A 97(26):14578-14583.
184. Willemsen R, Chames P, Schooten E, Gratama JW & Debets R (2008) Selection of human antibody fragments directed against tumor T-cell epitopes for adoptive T-cell therapy. Cytometry A 73(11):1093-1099.
185. Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw J, Hombrink P, Castermans E, Thor Straten P, Blank C, Haanen JB, Heemskerk MH & Schumacher TN (2009) Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. Nat Methods 6(7):520-526.

Chapter 2

T cell receptor gene therapy to treat melanoma: lessons from clinical and preclinical studies

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ABSTRACT

Importance of the field: Adoptive T cell therapy (ACT) with tumour infiltrating lymphocytes is currently the best treatment option for metastatic melanoma. Despite its clinical successes, ACT has its limitations in availability and generation of therapeutic T cells for a larger group of patients. Introduction of tumour-specific T cell receptors (TCR) into T cells, termed TCR gene therapy, can provide an alternative for ACT that is more widely applicable and might be extended to other types of cancer.

Areas covered in this review: The current status of TCR gene therapy studies including clinical challenges, such as on-target toxicity, compromised anti-tumour T cell responses, compromised T cell persistence, and potential immunogenicity of receptor transgenes. In addition, this review covers strategies being exploited to address these challenges.

What the reader will gain: A listing and discussion of strategies that aim at improving the efficacy and safety of TCR gene therapy. Such strategies address antigen choice, TCR mis-pairing, functional avidity and persistence of T cells, immune responses towards receptor transgenes, and the combination of ACT with other therapies.

Take home message: To ensure further clinical development of TCR gene therapy, it is necessary to choose safe T cell target antigens, and implement (combinations of) strategies that enhance the correct pairing of TCR transgenes as well as the functional avidity and persistence of T cells.

ARTICLE HIGHLIGHTS

- Clinical TCR gene therapy can offer a promising and widely applicable alternative to adoptive T cell therapy (ACT) for the treatment of tumours and viral infections.
- TCR gene therapy currently faces the following challenges: I) on-target toxicity; II) compromised anti-tumour T cell responses; III) compromised T cell persistence; and IV) immunogenicity of receptor transgenes.
- Current T cell therapy studies aim at improving the safety of TCR gene therapy by choosing safe T cell targets; TCR transgenes that are modified to minimize the chance of TCR mis-pairing; and strategies that prevent immune responses towards receptor transgenes.
- Current preclinical and clinical work is aimed at improving the efficacy of TCR gene therapy via strategies that enhance the functional avidity and persistence of gene-engineered T cells; and the combination of ACT with other therapies.

2.1 TCR GENE THERAPY TO TREAT MELANOMA: AN INTRODUCTION

The incidence of metastatic melanoma has increased dramatically over the past 40 years. Patients with metastatic disease face a dismal prognosis in view of a 10 year survival rate of less than 10% after standard systemic treatment with chemotherapy, such as dacarbazine (1). Melanoma is an immunogenic tumour that is recognized by the patient's immune system and, although melanoma only sporadically resolves due to a spontaneous immune response, it turned out feasible to isolate tumour-reactive T cells from tumour material or blood. Such tumour infiltrating lymphocytes (TIL) or CD8 T cell clones derived from peripheral T cells can be expanded to great numbers *in vitro* and after reinfusion into patients lead to a significant reduction of tumour burden and even complete remission of the tumour (2, 3). Adoptive T cell therapy (ACT) demonstrated objective clinical response rates of approximately 50% when TIL infusions were preceded by lympho-ablative patient preconditioning, and could be increased to approximately 70% when applying more stringent myelo-ablative patient preconditioning (4, 5). In contrast, other types of immunotherapy tested in melanoma patients, such as vaccines, high dose IL-2 or anti-CTLA4 antibody administration, have demonstrated maximal response rates of 15% (6, 7). Although ACT with TIL is currently the most clinically effective treatment option for metastatic melanoma, its widespread application is hindered by the laborious nature and limited success rate of isolating and expanding TILs for patient treatment. To overcome this disadvantage, and to make adoptive immune therapy more accessible for cancer patients in general, and melanoma patients in particular, a more universal strategy has been developed to generate tumour-specific T cells: T cell receptor (TCR) gene therapy. TCR gene therapy is based on the transfer of specific immunity to peripheral T cells by genetic introduction of TCRα and β genes. With a single set of TCR$\alpha\beta$ genes, it is possible to create millions of tumour-specific T cells without complicated *in vitro* selection or culture regimens.

The principle of clinical TCR gene therapy is simple: one set of TCR genes can redirect many T cells towards the same peptide/MHC target antigen expressed on diseased tissue. Already over two decades ago, a desired specificity has been successfully conferred to T cells by means of transferring TCR genes (8), and since then many efforts have been made to characterize and optimize the process of TCR$\alpha\beta$ gene transfer (9-11). Generally, genetic engineering and adoptive transfer of T cells starts with the isolation of peripheral blood lymphocytes from a patient, followed by transduction with the receptor genes of interest and expansion to numbers that are needed clinically (i.e., > $10x10^9$ T cells) within a period up to 2 weeks prior to infusion in the patient. A few years ago, the first successful clinical trial with TCR gene modified T cells directed against melanoma was conducted (12). Although not directly compared, the objective clinical responses reached in this study and a subsequent study were markedly lower than studies using TIL (12-30% vs. 51-72%) (4, 5, 12, 13). Moreover, clinical application of T cells gene-modified with antibody-based receptors (so-called chimeric antibody

receptors, CARs) to treat renal cell cancer, ovarian cancer, neuroblastoma or lymphoma has, despite some successes, generally not shown anti-tumour responses in a substantial number of patients (14-17). From the clinical receptor gene therapy trials executed so far several lessons may be learnt that are presented in Table 1 and may help in providing explanations for the limited therapeutic effectiveness of receptor-transduced T cells. These lessons represent

Table 1: Lessons from clinical receptor gene therapy and adoptive cell therapy trials

On-target toxicity	* MART-I (F5) + gp100 TCR:	Melanocyte destruction and inflammation in ears and eyes (11)
	* CAIX CAR:	Liver toxicity (12)
	* ACT studies:	Vitiligo and uveitis (4)
Anti-tumor responses	* MART-I TCR:	DMF4: 17 pts. treated: 2PR, 1MR (10) DMF5: 20 pts. treated: 6PR (11)
	* gp100 TCR	16 pts. treated: 1CR, 2PR (11)
	* CAIX and FR CAR:	No responses (12,13)
	* GD2 CAR[a]:	8 pts. treated: 1CR, 1SD, 2 tumor necrosis (14)
	* CD20 CAR[b]:	7 pts. treated: 2CR, 1PR, 4SD (15)
	* ACT studies: Chemotherapy: TBI, 2Gy: TBI, 12Gy:	Total 93 pts. treated (5): 43 pts. treated: 4CR, 17PR 25 pts. treated: 2CR, 11PR 25 pts. treated: 4CR, 14PR
T cell persistence	* MART-I TCR:	DMF4 : T cell persisted up to 12-13 months (10) DMF5: T cell persisted at least 1 month (11)
	* CAIX CAR :	T cell persisted up to 30 days (12)
	* FR CAR:	T cells persisted less than 2-3 weeks (13)
	* GD2 CAR[a]:	T cell persisted at least 6 weeks (14)
	* CD20 CAR[b]:	T cells persisted up to 9 weeks (15)
	* ACT studies:	T cells persisted up to 27 months (4)
Immunogenicity of receptor transgene	* CAIX CAR:	Humoral and cellular immune responses ((12) and Lamers et al., ms. submitted)

Evaluation of clinical trials performed with T cells retrovirally transduced with either TCR or CAR compared to TIL, the latter preceded by lympho-ablative pre-conditioning, with respect to on-target toxicity, anti-tumor reactivity, T cell persistence and immunogenicity of the transgene.

[a]In this study virus-specific T cells were used in contrast to the other receptor gene therapy studies which use polyclonal peripheral T cells.

[b]In this study peripheral T cells were electroprated in contrast to the other receptor gene therapy studies in which T cells were retrovirally transduced.

Abbreviations: TCR: T cell receptor, CAR: chimeric antigen receptor, TIL: tumor infiltrating lymphocytes, CAIX: carbonic anhydrase IX, FR: folate receptor, GD2: diasialoganglioside GD2, CR: complete response, PR: partial response, MR: minor response, SD: stable disease

major challenges, namely: 1) on-target toxicity; 2) compromised anti-tumour T cell responses; 3) compromised T cell persistence; and 4) immunogenicity of receptor transgenes, which will all be discussed, together with potential solutions, in more detail in the following sections.

2.2 FIRST CHALLENGE: ON-TARGET TOXICITY

Several ACT studies using expanded T cell populations in mice (18-21) and humans (3) as well as two recent receptor gene therapy studies (13, 14) demonstrated the occurrence of toxicities of healthy tissues expressing cognate antigen. For example, Lamers and colleagues treated renal cell carcinoma (RCC) patients with T cells engineered with an antibody-based receptor directed towards Carbonic Anhydrase IX (CAIX) and observed severe liver toxicity that was most probably due to expression of the target antigen on the large bile ducts of liver. In the study by Johnson and colleagues, healthy tissues in skin, ears and eyes were targeted by T cells engineered with a TCR$\alpha\beta$ directed against MART-I/A2. Although such toxicities can often be suppressed and even reversed, these studies strongly suggest that T cell therapy should be directed against safer target antigens of which the expression is restricted to malignant tissues. For a comprehensive review of possible suitable and non-suitable target antigens for ACT studies we refer to a recent review (22).

Antigens that show tumour-specific expression are those that belong to either mutated or shared antigens (23). Mutated or unique antigens, such as CDK-4/m, provide safe T cell targets yet the vast majority of these antigens is expressed in individual tumours making their clinical utility at present difficult (24). In contrast, shared antigens, such as 'Cancer testis Antigens' (CTA), are expressed in many tumours but silenced in normal cells except male germline cells (being devoid of MHC molecules) and thymic medullary epithelial cells (expression detected at the mRNA level) (25, 26). These qualities put CTA forward as candidate tumour antigens for T cell therapy. *In vitro* studies have already shown that gene transfer of TCR$\alpha\beta$ directed against MAGE-A1/A1, NY-ESO-1/A2 as well as NY-ESO-1/DP4 can result in effective T cell responses against tumour cells expressing cancer testis antigens (27-29). Of the group of cancer-germline genes, in particular the MAGE genes constitute attractive candidates given not only tumour-specific expression but also their role in tumour biology, expression in multiple tumours and potential to constitute effective T cell targets. There are four families of MAGE genes located on chromosome X: MAGE-A (12 genes), B (6 genes), C (4 genes) and D (2 genes). Up to now, over 50 combinations of MAGE peptides and HLA class I or class II molecules have been identified, recognized by CD8 or CD4 T cells, respectively. Several MAGE proteins have recently been recognized for their active contribution to the development of malignancies. MAGE-A, B and C antigens suppress p53-dependent apoptosis (30), whereas MAGE-A3 antigen mediates fibronectin-controlled progression and metastasis (31), and is expressed by melanoma stem cells (32). Furthermore, MAGE antigens are expressed by

Table 2. Advantages of MAGE antigens as targets for TCR gene therapy.

Oncogenic potential	Suppression of p53-dependent apoptosis Mediation of fibronectin-controlled progression and metastasis
Prevalence	MAGE-C2 presented by HLA-A2 and MAGE-A3 presented by HLA-DP4, which are the most common restriction elements among Caucasians. MAGE-C2/A2 and MAGE-A3/DP4 show a high prevalence on metastatic melanoma, being 43% and 76%, respectively
Safety	Belong to cancer testis antigens. No expression on healthy adult tissue.
Efficacy	Clinical responses to MAGE vaccination: increased numbers of MAGE-C2/A2 and MAGE-A3/DP4 specific T cells in patients. Simultaneous targeting of tumors with class I and class II restricted TCRs may counteract the development of selective antigen-loss variants and establish long-term anti-tumor activity.

An overview of potential advantages of MAGE antigens, especially MAGE-C2 and MAGE-A3, as candidate T cell targets for gene therapy trials with respect to their contribution to oncogenesis, their prevalence in the population, safety and efficacy.

multiple tumour types and their expression is associated with poor clinical outcome in these tumour types (33, 34). For instance, MAGE-C2 is expressed in 43% of metastatic melanomas, 30% of bladder carcinoma, 20% of head and neck carcinomas and 10% of non-small cell lung carcinomas (35). MAGE-A3 is expressed in 76% of metastatic melanomas (36), in up to 50% of non-small cell lung cancer (37), and in many other tumour types such as colon rectal cancer, hepatocyte cellular carcinoma, prostate cancer, multiple myeloma and breast cancer (33, 38-40). With respect to melanoma, MAGE antigens potentially constitute clinically effective T cell target antigens, as evidenced by significant and durable patient responses linked to induction and activation of in particular MAGE-C2/A2 and MAGE-A3/DP4 T cell responses (41-43). Table 2 summarizes advantages of MAGE antigens as targets for T cell therapy.

Following the choice of a candidate target antigen for ACT, one generally generates corresponding TCR chains via the 'reverse immunology' approach. With this approach MHC I or II-presented epitopes within the antigenic amino acid sequence are predicted and experimentally identified, after which T cell clones directed against such pMHC complexes are generated and tested, and finally the corresponding TCR genes are obtained and characterized. Alternatively, patient-derived T lymphocytes directed against an antigen of interest may provide a starting point, and potentially represent a more robust and reliable starting point to clone and characterize the TCR genes of interest.

2.3 SECOND CHALLENGE: COMPROMISED ANTI-TUMOUR T CELL RESPONSES

T cell responsiveness of TCR-engineered T cells is considerably less when compared with that of non-modified T cells and can be improved via various strategies, such as the use of 1) optimal vectors and transgene cassettes to increase transduction efficiency, 2) genetically modified TCR formats to augment TCR surface expression and function, or 3) simultaneous use of multiple TCR transgenes.

2.3.1. Vectors and transgene cassettes

Procedures for retroviral gene transfer have currently been optimized for transductions of murine T cells (44) and human T cells, the latter at Good Manufacturing Practice-level (45). Here, we will focus on advances that have been made with respect to the make-up of the vector backbone and transgene cassette. Most TCR gene transfer studies published to date have made use of gammaretroviral vectors for transgene delivery, and recently it has also been shown to be possible to reach high levels of TCR transgene expression using a lentiviral system (46). Clinically, there is experience with gammaretroviral MFG vectors or MFG-derived vectors (such as pSTITCH), vectors that contain Moloney Murine Leukemia Virus (moMLV) or Murine Stem Cell Virus (MSCV) Long terminal repeats (LTRs), and vectors that contain optimized splicing and start codons (12-15). There is now accumulated preclinical evidence that transduction efficiencies differ substantially between different vectors, with a major role for the viral origin of the LTRs and splice and start sequences. In this respect it is of interest to mention that the pMP71 vector, which has a Myeloproliferative Sarcoma Virus (MPSV) LTR and optimal 5' sequences, demonstrated highly improved TCR$\alpha\beta$ transduction efficiencies (47, 48).

For the simultaneous introduction of TCRα and β genes into the host T cell, several strategies can be applied. Two separate constructs, one containing the TCRα chain and the other containing the β chain can be co-transduced simultaneously. This method keeps the total size per construct relatively small, which may facilitate transduction efficiencies. However, the transduction efficiency of both constructs may differ and consequently the expression of both TCR chains may be different. To circumvent this, single constructs containing both the TCRα and β chain have been made. Such constructs consist of TCRα and β genes that are driven either by separate promoters or a single promoter with the two genes separated by an internal ribosomal entry site (IRES) to achieve promoter-independent translation of the second gene. Another method to allow more than one gene to be expressed from a single construct is to separate the genes by a 2A peptide sequence, which leads to a single mRNA that is processed into two proteins by endoproteases. Whereas IRES may result in lowered expression of the gene downstream relative to the one upstream of IRES (49), this is not the case when using 2A sequences (50, 51). In clinical studies, both IRES and 2A sequences have been used to separate TCRα and β genes (13). Last, the orientation of the TCRα and β chains

within a vector is of importance. Recent work suggests that placing the TCRβ chain in front of the TCRα chain, especially when separated by a 2A sequence, yields optimal functional TCR expression levels for most TCRs tested (52).

An important safety issue concerning retroviral gene transfer is linked to insertional mutagenesis as is evidenced by reports of leukaemia as a result of treating X-SCID patients with CD34+ progenitor cells transduced with common γ-chain (53). It is of note that mature T cells in contrast to haematopoietic progenitor cells are resistant to oncogenic transformation when followed up long-term in a preclinical transplant setting (54). Also clinically, there are currently more than 100 patients treated with high numbers of T cells providing no evidence of adverse effects of retroviral gene transfer into mature T cells. Lentiviral vectors are generally believed to constitute a safer alternative to gammaretroviral vectors since integration appears to be less biased towards transcription start sites (55). Self-inactivating (SIN) LTRs, which result in transgene expression driven from an internal promoter rather than the viral LTRs, may provide an additional safety lock. SIN vectors are less likely to disturb expression of endogenous genes in the vicinity of integration sites, although experimental data on this issue are still scant. Nevertheless, also lentiviral vectors do preferentially integrate in transcription units and gene-dense regions of the genome, suggesting that deregulation of local gene activity may occur as well, and that the increase in safety is far from absolute. In fact, a relative clonal dominance was detected in a patient with β-thalassemia major, two years after receiving hematopoietic stem cells genetically modified with a SIN lentiviral vector (56).

2.3.2 Genetically modified TCR formats

TCRαβ molecules can be genetically engineered to enhance T cell surface expression and function. To this end, multiple strategies have been reported which we have classified as 1) codon optimization; and strategies that either enhance 2) preferential TCR pairing, 3) ligand-binding affinity, or 4) receptor's signaling potency.

2.3.2.1 Codon optimization

Transgene expression may be enhanced by creating synthetic genes in which the codon usage has been altered to generate a maximal amount of transgenic protein. The genetic code is redundant in that many of the 20 amino acids are encoded by multiple codons. Highly expressed mammalian genes share a similar codon usage, suggesting that codon usage can affect protein production. In line with this, there are many examples of non-mammalian genes of which the expression can be substantially increased when the codon usage is altered to resemble that of highly expressed mammalian genes (57). It is noted that other aspects, such as the presence of secondary structures within the codon region of genes can also affect gene expression and such factors may also be taken into account when designing synthetic genes for (TCR) gene transfer (58). Recent data show that codon optimization of

TCR genes has a beneficial effect on surface expression and *in vitro* and *in vivo* function of TCR-engineered T cells (59, 60).

3.2.2.2 Strategies to enhance preferential TCR pairing

Surface expression of TCR transgenes may be compromised by the fact that introduced TCR chains not only form functional heterodimers with each other, but also with endogenous TCR chains already present in the host T cell. This so-called mis-pairing of TCR chains dilutes the number of correctly expressed introduced TCRs at the cell surface, and leads to new TCR heterodimers with unknown specificities that can potentially be auto-reactive and cause off-target toxicity. Various strategies have been developed that address TCR mis-pairing through genetic modification of the introduced TCR.

Murinization of TCR chains

Murinization of human TCRs is defined by replacement of human constant α and β domains by the corresponding murine TCR constant (TCR-C) domains. Although human and murine TCR-C domains show a high degree of homology, it is anticipated that small differences affect the stability of TCR/CD3 interactions and hence TCR surface expression levels. Evidence for a competitive advantage of murinized TCR for TCR/CD3 surface expression comes from the observation that murine TCR-C domains bind human CD3ζ more strongly than human TCR-C domains (61). TCR murinization as a means to address TCR mis-pairing was investigated using the MDM2/A2 and WT1/A2 specificities (62, 63) and more extensively using the p53/A2 and MART-1/A2 specificities (61). These studies generally demonstrated enhanced surface expression and pMHC binding of murinized TCRs. Notably, surface expression of a murinized MART-1/A2 TCR in TCR-engineered human T cells was reduced upon introduction of a second non-related human TCRαβ. This reduction was lower when compared to a non-modified TCR yet still significant and suggests that TCR murinization does address TCR mis-pairing but not to a full extent (61). Moreover, murinized WT1/A2 TCR did not reveal reduced mis-pairing compared to non-modified TCR, and the level of TCR mis-pairing appeared to be determined by sequences of the endogenous TCR variable (TCR-V) domains (63). In general, murinization leads to an increase in T cell avidity and a reduction in TCR mis-pairing, although this was not always reflected in enhanced functional responses to antigen-positive tumour cell lines (61, 63).

Cysteine-modified TCR

Introduction of cysteine amino acids at structurally favourable positions allows formation of an additional disulfide bridge and promotes correct pairing between the TCRα and β chains (64). Site-directed mutations of Cα Thre48Cys and Cβ Ser57Cys resulted in WT1/A2 TCRαβ linked by two inter-chain bonds (i.e., introduced plus endogenous cysteines) (65). Introduction of cysteine-modified TCRαβ into human CD8 T cells resulted in increased TCRβ

expression and pMHC multimer binding compared to non-modified TCRαβ, which corresponded to enhanced peptide-specific T cell cytotoxicity and IFN-γ production (63, 65). Experiments measuring TCRβ that remains surface expressed following antigen-specific TCR down-regulation, as a measure of correct TCR pairing, demonstrated that cysteine-modified TCR, but not non-modified TCR, were correctly paired with corresponding TCRα chains (65). In contrast, the ability of cysteine-modified TCR to mispair were at best modestly decreased when compared to non-modified TCRs (63, 65). When combining murinization and cysteine modification, expression and functional data look promising (61), yet to date only minimal characterization regarding TCR mis-pairing of such dual-modified TCRs has been performed.

Exclusive TCR heterodimer

To generate an exclusive TCR heterodimer, steric and electrostatic forces have been exploited to inhibit mispairing and at the same time facilitate correct pairing between TCRα and β chains. The crystal structures of murine 2C/H-2K^b and human Tax/A2 TCRs identified Ser85Arg for TCR-Cα and Arg88Gly for TCR-Cβ as mutations that were predicted to yield the required changes in electrostatic charge. These mutations were expected to generate a reciprocal 'knob-into-hole' configuration, and to minimally distort secondary and tertiary structures (66). Experimentally, mutated TCR chains did show a reduced ability to mispair, which was accomplished at the expense of tetramer binding and antigen-specific cytolysis, but not IFN-γ secretion, in primary human T cells (62).

TCR coupled to CD3ζ

TCRα and β chains both fused to a complete human CD3ζ molecule (abbreviated as TCR:ζ) endow TCR chains with CD3ζ-mediated dimerization between TCRα and β chains. In 2000 it was reported that transduction of primary human T cells with either single TCR α:ζ or TCR β:ζ does not result in cell surface expression, demonstrating that these TCR chains lack the ability to mispair with endogenous TCR chains (27). In a subsequent study, flow cytometric measurement of Fluorescent Resonance Energy Transfer (FRET) between corresponding as well as non-corresponding TCRα and TCRβ chains indicated a high preferential pairing for TCR:ζ in the absence of TCR mis-pairing (67). Immune precipitation studies revealed that TCR:ζ did not associate with CD3ε, CD3γ, CD3δ, and only marginally with CD3ζ. Consequently, TCR:ζ does not compete with endogenous TCRαβ for available CD3 molecules, and shows improved cell surface expression (67). Regarding functional assays, such as NFAT activation, cytotoxicity, and cytokine production, TCR:ζ performed as well as non-modified TCR (27, 67, 68).

Single chain TCR

A single chain (sc)TCR combines the variable domains of TCRα and β into one chain and hence its surface expression is anticipated to be less affected by TCR mis-pairing. Generally, a TCR-Vα domain is attached to a TCR-Vβ domain, interspersed by a linker sequence, and

followed by a TCR-Cβ2 domain which is partially replaced by the complete human CD3ζ to provide downstream signaling and T cell activation (27, 69) (abbreviated as scTCR:ζ). A scTCR:ζ based on a parental MAGE-A1/A1-specific TCR, derived from the CTL 82/30 (70), led to an increased surface expression but a reduced pMHC binding as well as antigen-specific T cell functions compared to two-chain TCR:ζ (27).

TCR-like antibodies

Antibody receptors, whether non-MHC- or MHC-restricted antibodies, lack TCR-V and C domains, and are not expected to be prone to mis-pairing with endogenous TCRα and β chains. General reviews on conventional non-MHC-restricted antibody receptors and their use to redirect T cell responses have been published elsewhere (71-74). Non-conventional TCR-like antibodies are derived *in vitro* from phage-displayed antibody fragments following selection with pMHC complexes and are subsequently engineered for gene transfer to enable T cells to bind and functionally respond to MHC-restricted tumour antigens (reviewed in refs (75, 76)). For example, a MAGE-A1/A1 specific human Fab fragment, termed Fab G8 (77), was engineered by separately fusing the heavy (V_HC_H) and -light chains (V_LC_L) to the human CD4 transmembrane region and the Fc(ε)RIγ intracellular domain (78). Gene transfer of Fab G8 enabled T cells to bind tetramer and functionally respond to antigen-positive tumour target cells (78). Thus far, phage-display and pMHC tetramer selections yielded more than a dozen MHC-restricted Fab molecules, especially evident for HLA-A2-restricted human antigens (76). For example, A2-restricted antibodies have been generated against gp100, TERT, MUC1, MelanA, NY-ESO1 and various viral antigens. In contrast to full-length or modified TCR, the generation of these reagents is an entirely *in vitro* procedure and does not depend on the availability of T cells making these receptors promising candidates for gene therapy.

Summary strategies to enhance preferential TCR pairing

Taken together, strategies described above affect TCR pairing, and thereby TCR surface expression, but at the same time address a potential safety issue caused by TCR mis-pairing. On top of receptor modifications to minimize TCR mis-pairing, in section 2.4.3 other strategies to address TCR mis-pairing that are based on defined host cell populations for TCR gene transfer are discussed. Despite the availability of various means to address TCR mis-pairing, one may choose for an (additional) strategy to eliminate potentially auto-reactive T cells. Such removal of T cells can be divided according to the mechanism of removal: 1) drug-induced T cell suicide; and 2) tag-mediated T cell killing. Drug-induced T cell suicide is based on genetic introduction of a suicide gene that can be activated by addition of a specific substrate or ligand, such as ganciclovir for the herpes simplex virus-thymidine kinase suicide gene (79) or FK506 dimers for the iCasp9$_M$ receptor (80, 81). Tag-mediated T cell killing is based on complement mediated clearance following antibody treatment directed at an introduced CD20 (82) or myc-epitope incorporated into a TCR chain (83). The mentioned strategies show high

potential in eradicating transduced T cells, but may pose drawbacks, such as the induction of immune responses against suicide genes or tags or the development of gene-loss variants.

2.3.2.3. Strategies that enhance the TCR's ligand-binding affinity

Tumour-specific TCRs often are at the lower affinity end of the natural TCR repertoire. The dissociation constant K_D, a biochemical measure of ligand-binding affinity and defined by the ratio between the dissociation and association rates (K_{off}/k_{on}), of these TCRs ranges from 100-1 μM. Several approaches have been developed, including the introduction of mutations in TCR-V and TCR-C domains, to enhance the ligand-binding affinity of TCR transgenes.

Mutations in TCR-V Complementary Determining Regions (CDR)

In vitro display of libraries of TCRs to select TCRs with either an altered fine specificity or an increased affinity have been reported. TCRs have been expressed on T cells (84), yeasts (85, 86) and phages (77, 87). In a recent phage display study to select high affinity HIV-1 specific T cells, the ligand binding affinity of a TCR could be improved from the nM range to a high affinity pM interaction (87). In this study, length and sequences of individual CDR loops were randomized, and phage libraries were allowed to compete for pMHC binding in multiple rounds of selection. Previously, the same phage display methodology was used for the evolution of affinity-matured antibody molecules, including TCR-like antibodies, which after expression by human T-cell transductants result in improved ligand-binding affinity. For example, the affinity-matured variant of Fab G8 (i.e., Fab Hyb3) revealed an 18-fold increase in ligand binding affinity with a K_D of 14nM, thereby improving ligand-specific T cell responses (88). In addition to phage display, mutated TCRs have also been screened via RNA-based transfections, which yielded affinity-improved MART-I/A2 and NY-ESO-1/A2 TCRs following single or dual amino acid substitutions in the CDRs (89). Such strategies may be of great benefit to affinity-mature suboptimal TCRs directed against tumour antigens. However, it should be noted that this would be especially interesting for TCRs directed against unique tumour antigens and not for TCRs against ubiquitously expressed antigens given the increased potential to develop on-target toxicities.

Mutations in TCR-C domains

Engineering TCR-C domains also affects TCR-pMHC interactions. Decreased *N*-glycosylation of the TCR has been reported to enhance functional T cell avidity as measured by cytokine release and lytic activity (90). Glycosylation of T cell proteins, such as the TCR may lead to an increased threshold for T cell activation, and deglycosylation of the TCR-C domain is anticipated to decrease this threshold, possibly as a consequence of improved membrane movement or multimerization of the TCR. This glycosylation technique was found to be effective for multiple TCRs without evidence for self-reactivity and is considered to be widely applicable in the field of TCR gene transfer.

2.3.2.4 Strategies that enhance the TCR's signaling potency

In extension to the genetic introduction of CD3ζ or Fc(ε)RIγ into receptors, as discussed for TCR:ζ, scTCR:ζ and TCR-like antibodies, other 'building blocks' constituting transmembrane and/or intracellular domains of accessory molecules, co-stimulatory molecules and kinases have been analyzed for their effect on surface expression and function of various receptors. Receptors, including scTCR, that contain the transmembrane domain of CD3ζ followed by the intracellular domain of CD28 and the co-receptor molecule Lck (i.e., scTCR:ζ-28-Lck) constitute a promising format with respect to T cell activation and peptide-specific T cell functions (91, 92). Despite constructive efforts to optimize scTCRs, it has generally been observed that these receptors were less responsive than non-modified TCR to low concentrations of antigen (27, 92). The CD28 co-stimulatory domain has also been incorporated in TCR:ζ and TCR-like antibodies, which resulted in enhanced tumour-specific cytokine production of receptor-transduced T cells (93, 94). In addition to CD28, the incorporation of other TNF receptor super family members, such as CD134 and CD137, into TCR molecules may further optimize T cell functions as suggested by scFv receptors that incorporate co-stimulatory molecules (95). For example, a recent publication describes that the incorporation of CD28 and CD137 signaling domains in scFv leads to potent anti-tumour activity and prolonged *in vivo* persistence (96).

2.3.3 Simultaneous use of multiple TCRαβ transgenes

Limited (i.e., single) antigen specificity is inherent to gene transfer of a single TCRαβ, which is in contrast to TILs that may cover a few antigen specificities. Restricted antigen reactivity of TCR-engineered T cells may have contributed to compromised clinical responses following adoptive transfer of TCR-engineered T cells versus TILs. Targeting tumours with a T cell population specific for a single antigen leads to the selective outgrowth of antigen negative tumour variants (2, 59). To reduce the risk of immune escape, one could opt to create multiple T cell pools with different specificities using a different TCRαβ per T cell pool to address this issue. Simultaneous use of both CD4 and CD8 T cells directed at the same antigen or different antigens may further enhance the efficacy of ACT.

2.4 THIRD CHALLENGE: COMPROMISED T CELL PERSISTENCE

Peripheral persistence of TCR-engineered T cells is decreased when compared to TILs following ACT, and can be improved via various strategies, such as the use of 1) less differentiated T cells, 2) CD4 T cell help, or 3) defined T cell populations.

2.4.1 Use of less differentiated TCR-engineered T cells

Clinical anti-tumour efficacy of T cells irrespective of gene-modification appears to be directly related to T cell persistence (15, 97, 98) which in turn is reported to be associated with

differentiation state and replicative history of transferred T cells (99). One way to obtain less differentiated T cells and enhance the efficacy of TCR gene therapy may be to expose T cells to common-γ cytokines other than interleukin-2 (IL-2) prior to ACT. Notably, antigen priming of CD8 T cells in the presence of IL-21, and to a lesser extent IL-15, suppresses differentiation of naive T cells into effector T cells, which in turn enhances *in vivo* persistence and antigen-specific effectiveness of T cells (100-102) IL-2, IL-15 and IL-21 all act as co-mitogens for antigen-activated effector CD8 T cells (103-105). However, in contrast to IL-2, IL-15 enhances survival of CD8 effector T cells (106) and IL-21 does neither induce activation-induced cell death nor proliferation of regulatory T cells (Tregs), which normally prevent T cells from exerting their function (107). Moreover, IL-15 and IL-21 are able to enhance the *in vivo* anti-tumour effects of CD8 T cells (102, 108-111). Notably, a recent non-human primate study has shown that administration of IL-15 after ACT leads to better survival of memory T cells and NK cells, and does not result in increased numbers of Tregs, as compared with IL-2 administration (112). Interestingly, it has been shown that administration of a complex of IL-15 and its receptor enhances the biological activity of IL-15 and promotes rapid tumour regression mediated by T cells (113). IL-21 turns out to be superior to IL-15 in inducing anti-tumour responses of endogenous as well as adoptively transferred CD8 T cells (102, 105). Moreover, combined *in vivo* treatment with IL-15 and IL-21 enhanced CD8 T cell expansion and anti-melanoma responses compared to the administration of either cytokine separately (114). Treatment of primary T cells derived from melanoma patients (115), or primary T cells that are TCR engineered (116), with a combination of IL-15 and IL-21 results in improved T cell cytotoxicity and production of IL-2 and IFN-γ. Other common-γ cytokines, in particular IL-7, may also have beneficial effects in ACT. IL-7 has been tested in HIV-infected humans and was shown to increase the numbers of circulating CD4 and CD8 T cells, predominantly of central memory phenotype (117). Moreover, a combination of IL-7 and IL-15 was demonstrated to generate gene-engineered T cells with a less differentiated CD8 T cell phenotype (i.e., central memory phenotype) that showed potent antigen reactivity and prolonged persistence in a mouse model (118). Instead of using common-γ cytokines either *in vivo* or *ex vivo*, one can also opt to use less differentiated T cell populations as recipient cells for gene transfer. A study in macaques demonstrated prolonged persistence and effective memory after the adoptive transfer of central memory T cells, possibly making them ideal candidates for TCR gene therapy studies (119). In this respect, it is of note that hematopoietic stem cells have also been used as recipient cells for TCRαβ genes, and were shown to mature into T cells when antigen-challenged following adoptive transfer in a mouse model or when *in vitro* co-cultured with OP9 stromal cells expressing a human Notch-1 ligand (120-122). TCR-engineered central memory T cells and hematopoietic stem cells potentially provide non-fully differentiated antigen-specific T cells that show long-term persistence *in vivo*.

2.4.2 CD4 T cell help

Administration of CD4 T helper cells concurrently with CD8 T cells has been shown to prevent exhaustion of infused CD8 T cells (123, 124) and to result in effective anti-tumour T cell responses (125). It has been well documented that CD4 T cells activate professional antigen presenting cells, which leads to priming of antigen-specific CD8 cytotoxic T lymphocyte (CTL) function (126). Moreover, adoptive transfer of CD4 T cells results in *de novo* generation of antigen-specific CD8 T cells, activates CD8 T cells that are already present at the site of the tumour, activates other immune cells, induces an anti-tumour humoral response, and CD4 T cells are a major source of IFN-γ (123, 127, 128). Notably, CD4 T cells producing T_H1-type cytokines, but not those producing T_H2-type cytokines, have been reported to eradicate tumours in T cell transfer studies (129, 130). In extension to these findings, Muranski and colleagues reported that adoptive transfer of T_H17-polarized CD4 T cells effectively mediate rejection of tyrosine-related-protein 1 (TRP-1)-positive tumours in a TCR-transgenic model (131). The observed anti-tumour effects depended on IFN-γ production, and assumable on *in vivo* evolution of T_H17 into T_H1-type cells. Building on the therapeutic need for high antigen-specific IFN-γ production, CD4 T cells have been studied as recipient T cells for MHC class I-restricted TCRαβ in combination with CD8α genes. Not only can CD4 T cells be functionally endowed with MHC I-restricted TCRαβ via gene transfer (132, 133), genetic co-introduction of CD8α skews TCR-engineered T cells towards an antigen-specific T_H1-type T cell response (134). However, the *in vivo* proliferation and anti-tumour responses of adoptively transferred CD4 T cells that produce high levels of IFN-γ, such as TCR/CD8α-engineered CD4 T cells, appears compromised when compared to IFN-γ^{low} CD4 T cells (135). This may suggest that *in vivo* expansion of antigen-challenged IFN-γ^{low} CD4 T cells is required prior to development of IFN-γ^{high} CD4 T cells and synergy with CD8 T cells to build a potent anti-tumour response. Further studies are needed to better understand the therapeutic benefit of various CD4 T cell subsets in ACT.

2.4.3 TCRαβ gene transfer into defined T cell populations

Introducing TCR transgenes into a T-cell population with a restricted TCR usage, such as virus-specific T cells, is expected to improve peripheral persistence of T cells due to ongoing stimulation via the endogenous TCR (136). In fact, treatment of neuroblastoma patients with EBV-specific CTL expressing a receptor directed against diasialoganglioside GD2 showed enhanced T cell survival and tumour regression in four out of eleven patients treated (16).

In addition to enhanced peripheral T cell persistence, TCR gene transfer into restricted T cell populations is anticipated to reduce the risks of auto-immune reactivities for two reasons: recipient T cells are non-self reactive, and the chance that new TCR reactivities are generated is reduced as a consequence of limited availability of endogenous TCR chains that can mispair with the exogenous TCR chains (16, 136, 137). Nevertheless, in case a self-recognizing TCR is formed, potential GvHD responses are expected to be stronger since all T-cells will

recognize such a self-antigen. Interestingly, the concept of redirecting immune cells with a limited endogenous TCR repertoire has recently been translated to γδ T cells (138, 139), pointing out that aside advantages, the *in vivo* anti-tumour efficacy may be adversely affected by choosing another T cell population.

2.5 FOURTH CHALLENGE: IMMUNOGENICITY OF RECEPTOR TRANSGENES

Introduction of non-human sequences, chimeric sequences, cryptic open reading frames and non-germline CDRs may result in immunogenic epitopes. Already in 1996, Riddell and colleagues reported that ACT with HIV-specific CD8 T cells gene modified with an antibiotic resistance gene resulted in a potent cellular immune responses, which were responsible for the loss of the therapeutic T cells (140). More recently, immunogenicity of an introduced antigen-specific receptor was reported in a renal cell carcinoma trial, in which patients received T cells genetically redirected with a CAIX-specific CAR (14). In this study, humoral responses were observed against the murine variable domains of the antibody directed against CAIX. RCC patients also developed a T cell response against the same epitopes (141). In case of modified TCRs, the TCR-V domains remain unchanged, but the extent of modifications at the level of TCR-C domains vary between the different receptor formats (see section 2.3.2.2) and may evoke immune responses. Although severity of potential immunogenicity might be estimated based on the extent of genetic modifications, for all described receptors this needs to be verified in *in vivo* studies.

Another way to address the immunogenicity of receptor-transduced T cells may be immune suppressive preconditioning of ACT patients in order to limit or delay the patient's immune response towards gene-engineered T cells. Indeed, in a nonhuman primate model it was shown that non-myeloablative preconditioning delayed the induction of a cellular immune response towards gene-engineered T cells and prolonged their *in vivo* persistence (142). In extension, in immune-suppressed human lymphoma patients, Till and colleagues have shown that treatment with T cells electroporated with a CD20-specific CAR did neither result in clear humoral nor cellular anti-scFv responses (17).

2.6 COMBINATION THERAPY

Early ACT studies with high numbers of TILs (mostly > 10^{11} cells) and high dose IL-2 in metastatic melanoma resulted in an objective response rate of 31%(6). The combination of ACT/IL-2 following chemotherapy or irradiation resulted in improved objective response rates up to 51 and 72%, respectively (4, 5). Depleting the host's immune system prior to ACT may have several benefits for the infused T cells (143). First, endogenous Tregs are depleted. Second, in-

fused T cells can proliferate independently of cognate antigen recognition (i.e., homeostatic proliferation) and third, by depleting immune cells that normally consume endogenous cytokines, so-called cytokine sinks, cytokines become more readily available for the infused T cells. Last, lymphodepletion through irradiation or chemotherapy leads to mobilization and activation of antigen presenting cells (APC), increasing the anti-tumour activity of the infused T cells. Interestingly, the creation of a lymphopenic environment in combination with the activation of the innate immune system is reported to further improve T cell engraftment and anti-tumour T cell responses (144).

Removal of Tregs without lymphodepletion can also be achieved by depleting $CD25^+$ T cells *in vivo*. This strategy however, has thus far proven to be highly toxic and of a transient nature (145). More promising results were obtained in a study by Cui and colleagues, who treated mice with α-CD25 mAb and IL-7 (146). These conditions mimic lymphodepletion and lead to increased efficacy of anti-tumour T cell therapy. Alternatively, studies by both Berger and Beq and colleagues, who treated non-human primates with IL-15 and IL-7, respectively, implicated the in vivo use of cytokines to safely establish some degree of lymphodepletion (112, 147). Another way to block Tregs is to inhibit Indoleamine 2,3-dioxygenase (IDO). *In vivo* models show that inhibiting IDO not only blocks the activation of $Foxp3^+$ $CD25^+$ regulatory T cells (148), but also gives rise to T_H17 helper cells, and enhanced anti-tumour CD8 T cell responses (149).

Lastly, the potency of ACT can be enhanced when combined with vaccination strategies as has been shown in various preclinical models (18, 150, 151). In a recent study, vaccination of melanoma patients with recombinant fowlpox expressing human gp100 increased the efficacy of adoptively transferred T cells, whereas T cells in non-vaccinated patients did not elicit any objective response (152).

2.7 EXPERT OPINION

Recent advances in the field of TCR gene therapy for melanoma show promising results, but also a need to improve several therapeutic challenges, as those presented in Table 1.

Of primary importance is the choice of target antigen. Most tumour antigens are also present on normal healthy tissue, potentially giving rise to on-target toxicity when treating patients with T cells directed against such antigens. We would like to propose cancer testis antigens as the most suitable target antigens for T cell therapy, in particular MAGE antigens for reasons given in Table 2.

Second, and to enhance the avidity of TCR-engineered T cells, choices have to be made with respect to vector backbone, TCR$\alpha\beta$ transgene cassette, and TCR format. Gammaretroviral transduction is still the method that is currently best characterized preclinically and for which there is most clinical experience using validated vectors such as MFG-type vectors,

Table 3. Strategies to improve safety of TCR-engineered T cells.

On-target toxicity	Target antigens that are uniquely expressed by tumor cells.
Off-target toxicity	Modification of TCRαβ format to prevent/minimize TCR mispairing: * Murinized TCR * Cystein-modified TCR * Exclusive TCR heterodimer * TCR:ζ * scTCR: ζ * TCR-like antibodies TCRαβ gene transfer in defined cell populations to prevent/minimize TCR mispairing: * Cytotoxic T cell clone with known endogenous TCRαβ * γδ T cells
Decreased/ no immunogenicity of receptor	Potential immunogenicity of TCR-engineered T cells can be addressed by: * Choosing minimal domains to modify TCRαβ transgenes * Host pre-conditioning to delay/limit the patient's endogenous immune response
Removal of therapeutic T cells	Adoptively transferred T cells can be removed by: * Drug-induced suicide: HSV TK or iCasp9 suicide genes * Tag-mediated clearance: CD20 or c-myc tagged TCRαβ

An overview of strategies that can be employed to improve the safety of receptor gene therapy, as discussed in this paper.

Table 4. Strategies to improve the efficacy of TCR-engineered T cells.

Vector/Transgene cassette	Suitable vector for high-level gene expression, combined with optimal TCR transgene cassette.
Modification of TCR	TCRαβ genetically modified to enhance expression/function: * Codon optimization * Enhance preferential paring for high expression * Enhance ligand binding affinity * Combined incorporation of CD3 accessory and co-stimulatory domains
Gene transfer into defined T cell populations	TCRαβ gene transfer in defined cell populations to enhance survival and persistence *in vivo*: * Cytotoxic T cell clone with known endogenous TCRαβ * Simultaneous gene transfer into both CD4 and CD8 T cells
CD8 T cell differentiation status	Obtain less differentiated CD8 T cells via: * Treatment of TCR-transduced T cells with common-γ cytokines * Use central memory CD8 T cells (TCM) or hematopoietic stem cells as recipient T cells for gene transfer
Combination therapy	ACT can be combined with: * Lympho- or myelo-ablative pre-conditioning * Stimulation of innate immune system

An overview of strategies to improve the efficacy of receptor gene therapy, as discussed in this paper.

vectors that contain moMLV or MSCV LTRs, and vectors that contain optimized 5' sequences. With respect to the TCR transgene cassette, there is clinical experience with TCRα and β genes separated either by IRES or 2A. This, together with safety data based on engineered mature T cells, may favor gammaretroviral vectors, possibly even with MPSV LTRs, which contain a TCRβ-2A-TCRα cassette for clinical TCR gene therapy. The TCR format can be modified in various ways, including codon optimization, strategies that enhance preferential TCR pairing, ligand-binding affinity and/or signaling potency, all with the intent to enhance functional TCR surface expression. We consider the choice for a TCR format that is modified to improve functional surface expression, and at the same time minimizing the risk of mis-pairing with endogenous TCR chains of critical importance. Although based on *in vitro* data the murinized TCR$\alpha\beta$ and TCR:ζ may represent good candidate TCR formats, there is a need for *in vivo* data with respect to anti-tumour efficacy as well as off-target toxicity to justify the choice for any modified format.

Third, one should consider strategies to enhance persistence of adoptively transferred T cells. Currently, it appears most practical to start with bulk T cells for TCR gene transfer, after which T cells are exposed to common-γ cytokines, such as a combination of IL-15 and IL-21, to generate less differentiated T cells that demonstrate good *in vivo* performance. As an alternative strategy, one could choose to use subpopulations of CD8 T cells, such as central memory CD8 T cells, or even CD4 T cells, as recipient T cells for TCR gene transfer.

Fourth, and linked to the issue of T cell persistence, methods to reduce susceptibility of gene-engineered T cells to patient's immune responses. One way would be to minimize genetic alterations with respect to the TCR format. Another way would be to delay patient's immune responses and precede ACT with lympho-ablative preconditioning. This latter option, combining chemotherapy with T cell therapy, has several additional advantages and results in clinically more potent T cell responses, but might be at the expense of clinical complications with infections. Combining T cell therapy with other therapies, such as those stimulating the innate immune system, appears promising and awaits clinical testing.

Strategies that may enhance safety and efficacy of TCR engineered T cells for clinical application are summarized in Tables 3 and 4. Strategies or combinations of strategies that are capable of addressing each of the challenges of TCR gene therapy should ideally be implemented in a future clinical trial design. A schematic example of such a trial design according to the authors' current view is presented in Figure 1.

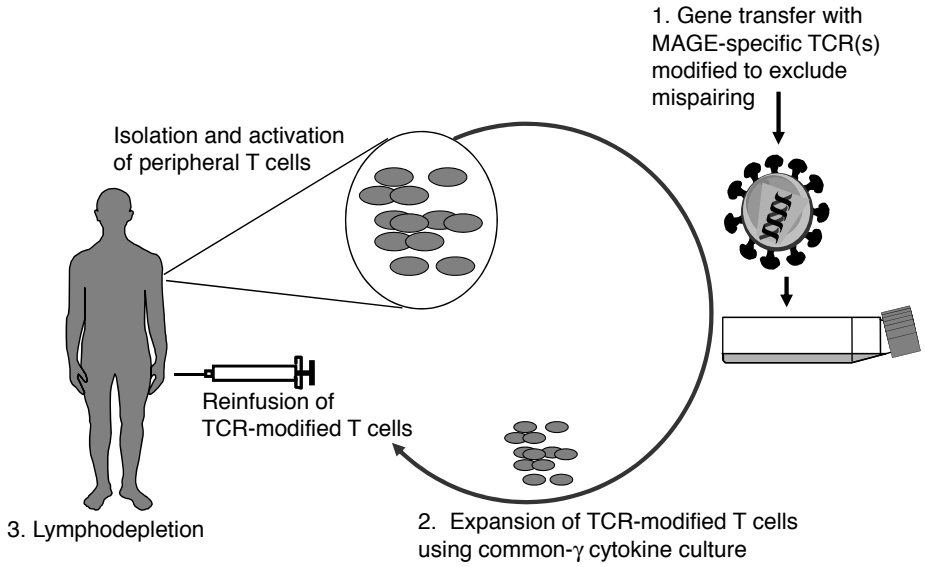

Figure 1. A schematic illustration of a TCR gene therapy trial as proposed by the authors. We propose the following ingredients for successful clinical receptor gene therapy application; (1) Gene transfer with MAGE-specific TCR(s), modified to exclude mispairing and to promote high surface expression. Preferably simultaneous gene transfer of more than one receptor, directed against different antigens, followed by (2) expansion of the transduced T cells with common-gamma cytokines, to sustain a naive phenotype of the cells. (3) Lymphodepletion prior to re-infusion of the cells, to allow for optimal grafting and expansion of the infused cells.

REFERENCES

1. Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, Urist M, McMasters KM, Ross MI, Kirkwood JM, Atkins MB, Thompson JA, Coit DG, Byrd D, Desmond R, Zhang Y, Liu PY, Lyman GH & Morabito A (2001) Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* 19(16):3622-3634.
2. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E & Greenberg PD (2002) Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* 99(25):16168-16173.
3. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE & Rosenberg SA (2002) Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298(5594):850-854.
4. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A & Rosenberg SA (2005) Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23(10):2346-2357.
5. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, Thomasian A, Downey SG, Smith FO, Klapper J, Morton K, Laurencot C, White DE & Rosenberg SA (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 26(32):5233-5239.
6. Rosenberg SA & Dudley ME (2009) Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol* 21(2):233-240.
7. Boon T, Coulie PG, Van den Eynde BJ & van der Bruggen P (2006) Human T cell responses against melanoma. *Annu Rev Immunol* 24:175-208.
8. Dembic Z, Haas W, Weiss S, McCubrey J, Kiefer H, von Boehmer H & Steinmetz M (1986) Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* 320(6059):232-238.
9. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA & Schumacher TN (2001) Immunotherapy through TCR gene transfer. *Nat Immunol* 2(10):957-961.
10. Calogero A, Hospers GA, Kruse KM, Schrier PI, Mulder NH, Hooijberg E & de Leij LF (2000) Retargeting of a T cell line by anti MAGE-3/HLA-A2 alpha beta TCR gene transfer. *Anticancer Res* 20(3A):1793-1799.
11. Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R & Adema GJ (2003) Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J Immunol* 170(4):2186-2194.
12. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA & Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314(5796):126-129.
13. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer

CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM & Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114(3):535-546.

14. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G & Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24(13):e20-22.
15. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA & Hwu P (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 12(20 Pt 1):6106-6115.
16. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, Yvon E, Weiss HL, Liu H, Rooney CM, Heslop HE & Brenner MK (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 14(11):1264-1270.
17. Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, Qian X, James SE, Raubitschek A, Forman SJ, Gopal AK, Pagel JM, Lindgren CG, Greenberg PD, Riddell SR & Press OW (2008) Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 112(6):2261-2271.
18. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA & Restifo NP (2003) Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 198(4):569-580.
19. Bos R, van Duikeren S, Morreau H, Franken K, Schumacher TN, Haanen JB, van der Burg SH, Melief CJ & Offringa R (2008) Balancing between antitumor efficacy and autoimmune pathology in T-cell-mediated targeting of carcinoembryonic antigen. *Cancer Res* 68(20):8446-8455.
20. Ugel S, Scarselli E, Iezzi M, Mennuni C, Pannellini T, Calvaruso F, Cipriani B, De Palma R, Ricci-Vitiani L, Peranzoni E, Musiani P, Zanovello P & Bronte V (2009) Autoimmune B cell lymphopenia following successful adoptive therapy with telomerase-specific T lymphocytes. *Blood*.
21. Palmer DC, Chan CC, Gattinoni L, Wrzesinski C, Paulos CM, Hinrichs CS, Powell DJ, Jr., Klebanoff CA, Finkelstein SE, Fariss RN, Yu Z, Nussenblatt RB, Rosenberg SA & Restifo NP (2008) Effective tumor treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity. *Proc Natl Acad Sci U S A* 105(23):8061-8066.
22. Offringa R (2009) Antigen choice in adoptive T-cell therapy of cancer. *Curr Opin Immunol* 21(2):190-199.
23. Lucas S & Coulie PG (2008) About human tumor antigens to be used in immunotherapy. *Semin Immunol* 20(5):301-307.
24. Parmiani G, De Filippo A, Novellino L & Castelli C (2007) Unique human tumor antigens: immunobiology and use in clinical trials. *J Immunol* 178(4):1975-1979.
25. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T & Lucas S (2001) An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* 61(14):5544-5551.
26. Gotter J, Brors B, Hergenhahn M & Kyewski B (2004) Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *J Exp Med* 199(2):155-166.

27. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P & Bolhuis RL (2000) Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther* 7(16):1369-1377.
28. Zhao Y, Zheng Z, Robbins PF, Khong HT, Rosenberg SA & Morgan RA (2005) Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. *J Immunol* 174(7):4415-4423.
29. Zhao Y, Zheng Z, Khong HT, Rosenberg SA & Morgan RA (2006) Transduction of an HLA-DP4-restricted NY-ESO-1-specific TCR into primary human CD4+ lymphocytes. *J Immunother* 29(4):398-406.
30. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ & Longley BJ (2007) MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* 67(20):9954-9962.
31. Liu W, Cheng S, Asa SL & Ezzat S (2008) The melanoma-associated antigen A3 mediates fibronectin-controlled cancer progression and metastasis. *Cancer Res* 68(19):8104-8112.
32. Sigalotti L, Covre A, Zabierowski S, Himes B, Colizzi F, Natali PG, Herlyn M & Maio M (2008) Cancer testis antigens in human melanoma stem cells: expression, distribution, and methylation status. *J Cell Physiol* 215(2):287-291.
33. Condomines M, Hose D, Raynaud P, Hundemer M, De Vos J, Baudard M, Moehler T, Pantesco V, Moos M, Schved JF, Rossi JF, Reme T, Goldschmidt H & Klein B (2007) Cancer/testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis. *J Immunol* 178(5):3307-3315.
34. Gure AO, Chua R, Williamson B, Gonen M, Ferrera CA, Gnjatic S, Ritter G, Simpson AJ, Chen YT, Old LJ & Altorki NK (2005) Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res* 11(22):8055-8062.
35. Lucas S, De Plaen E & Boon T (2000) MAGE-B5, MAGE-B6, MAGE-C2, and MAGE-C3: four new members of the MAGE family with tumor-specific expression. *Int J Cancer* 87(1):55-60.
36. Van den Eynde BJ & van der Bruggen P (1997) T cell defined tumor antigens. *Curr Opin Immunol* 9(5):684-693.
37. Jang SJ, Soria JC, Wang L, Hassan KA, Morice RC, Walsh GL, Hong WK & Mao L (2001) Activation of melanoma antigen tumor antigens occurs early in lung carcinogenesis. *Cancer Res* 61(21):7959-7963.
38. Alves PM, Levy N, Bouzourene H, Viatte S, Bricard G, Ayyoub M, Vuilleumier H, Givel JC, Halkic N, Speiser DE, Romero P & Levy F (2007) Molecular and immunological evaluation of the expression of cancer/testis gene products in human colorectal cancer. *Cancer Immunol Immunother* 56(6):839-847.
39. Peng JR, Chen HS, Mou DC, Cao J, Cong X, Qin LL, Wei L, Leng XS, Wang Y & Chen WF (2005) Expression of cancer/testis (CT) antigens in Chinese hepatocellular carcinoma and its correlation with clinical parameters. *Cancer Lett* 219(2):223-232.
40. Hudolin T, Juretic A, Spagnoli GC, Pasini J, Bandic D, Heberer M, Kosicek M & Cacic M (2006) Immunohistochemical expression of tumor antigens MAGE-A1, MAGE-A3/4, and NY-ESO-1 in cancerous and benign prostatic tissue. *Prostate* 66(1):13-18.
41. Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, Bender A, Feuerstein B, Fritsch PO, Romani N & Schuler G (2002) Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med* 195(10):1279-1288.

42. Schultz ES, Schuler-Thurner B, Stroobant V, Jenne L, Berger TG, Thielemanns K, van der Bruggen P & Schuler G (2004) Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 172(2):1304-1310.
43. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG & Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *J Exp Med* 201(2):249-257.
44. Pouw NM, Westerlaken EJ, Willemsen RA & Debets R (2007) Gene transfer of human TCR in primary murine T cells is improved by pseudo-typing with amphotropic and ecotropic envelopes. *J Gene Med* 9(7):561-570.
45. Lamers CH, Willemsen RA, van Elzakker P, van Krimpen BA, Gratama JW & Debets R (2006) Phoenix-ampho outperforms PG13 as retroviral packaging cells to transduce human T cells with tumor-specific receptors: implications for clinical immunogene therapy of cancer. *Cancer Gene Ther* 13(5):503-509.
46. Jones S, Peng PD, Yang S, Hsu C, Cohen CJ, Zhao Y, Abad J, Zheng Z, Rosenberg SA & Morgan RA (2009) Lentiviral vector design for optimal T cell receptor gene expression in the transduction of peripheral blood lymphocytes and tumor-infiltrating lymphocytes. *Hum Gene Ther* 20(6):630-640.
47. Schambach A, Wodrich H, Hildinger M, Bohne J, Krausslich HG & Baum C (2000) Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. *Mol Ther* 2(5):435-445.
48. Engels B, Cam H, Schuler T, Indraccolo S, Gladow M, Baum C, Blankenstein T & Uckert W (2003) Retroviral vectors for high-level transgene expression in T lymphocytes. *Hum Gene Ther* 14(12):1155-1168.
49. Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E & Hayakawa T (2000) IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol Ther* 1(4):376-382.
50. de Felipe P, Martin V, Cortes ML, Ryan M & Izquierdo M (1999) Use of the 2A sequence from foot-and-mouth disease virus in the generation of retroviral vectors for gene therapy. *Gene Ther* 6(2):198-208.
51. Klump H, Schiedlmeier B, Vogt B, Ryan M, Ostertag W & Baum C (2001) Retroviral vector-mediated expression of HoxB4 in hematopoietic cells using a novel coexpression strategy. *Gene Ther* 8(10):811-817.
52. Leisegang M, Engels B, Meyerhuber P, Kieback E, Sommermeyer D, Xue SA, Reuss S, Stauss H & Uckert W (2008) Enhanced functionality of T cell receptor-redirected T cells is defined by the transgene cassette. *J Mol Med* 86(5):573-583.
53. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A & Cavazzana-Calvo M (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302(5644):415-419.
54. Newrzela S, Cornils K, Li Z, Baum C, Brugman MH, Hartmann M, Meyer J, Hartmann S, Hansmann ML, Fehse B & von Laer D (2008) Resistance of mature T cells to oncogene transformation. *Blood* 112(6):2278-2286.
55. Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR & Bushman FD (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* 2(8):E234.

56. Kaiser J (2009) Gene therapy. Beta-thalassemia treatment succeeds, with a caveat. *Science* 326(5959):1468-1469.
57. Haas J, Park EC & Seed B (1996) Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol* 6(3):315-324.
58. Ross J (1995) mRNA stability in mammalian cells. *Microbiol Rev* 59(3):423-450.
59. Jorritsma A, Gomez-Eerland R, Dokter M, van de Kasteele W, Zoet YM, Doxiadis, II, Rufer N, Romero P, Morgan RA, Schumacher TN & Haanen JB (2007) Selecting highly affine and well-expressed TCRs for gene therapy of melanoma. *Blood* 110(10):3564-3572.
60. Scholten KB, Kramer D, Kueter EW, Graf M, Schoedl T, Meijer CJ, Schreurs MW & Hooijberg E (2006) Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. *Clin Immunol* 119(2):135-145.
61. Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA & Morgan RA (2006) Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res* 66(17):8878-8886.
62. Voss RH, Kuball J, Engel R, Guillaume P, Romero P, Huber C & Theobald M (2006) Redirection of T cells by delivering a transgenic mouse-derived MDM2 tumor antigen-specific TCR and its humanized derivative is governed by the CD8 coreceptor and affects natural human TCR expression. *Immunol Res* 34(1):67-87.
63. Thomas S, Xue SA, Cesco-Gaspere M, San Jose E, Hart DP, Wong V, Debets R, Alarcon B, Morris E & Stauss HJ (2007) Targeting the Wilms tumor antigen 1 by TCR gene transfer: TCR variants improve tetramer binding but not the function of gene modified human T cells. *J Immunol* 179(9):5803-5810.
64. Boulter JM, Glick M, Todorov PT, Baston E, Sami M, Rizkallah P & Jakobsen BK (2003) Stable, soluble T-cell receptor molecules for crystallization and therapeutics. *Protein Eng* 16(9):707-711.
65. Kuball J, Dossett ML, Wolfl M, Ho WY, Voss RH, Fowler C & Greenberg PD (2007) Facilitating matched pairing and expression of TCR chains introduced into human T cells. *Blood* 109(6):2331-2338.
66. Voss RH, Willemsen RA, Kuball J, Grabowski M, Engel R, Intan RS, Guillaume P, Romero P, Huber C & Theobald M (2008) Molecular design of the Calphabeta interface favors specific pairing of introduced TCRalphabeta in human T cells. *J Immunol* 180(1):391-401.
67. Sebestyen Z, Schooten E, Sals T, Zaldivar I, San Jose E, Alarcon B, Bobisse S, Rosato A, Szollosi J, Gratama JW, Willemsen RA & Debets R (2008) Human TCR that incorporate CD3zeta induce highly preferred pairing between TCRalpha and beta chains following gene transfer. *J Immunol* 180(11):7736-7746.
68. Schaft N, Lankiewicz B, Gratama JW, Bolhuis RL & Debets R (2003) Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT. *J Immunol Methods* 280(1-2):13-24.
69. Chung S, Wucherpfennig KW, Friedman SM, Hafler DA & Strominger JL (1994) Functional three-domain single-chain T-cell receptors. *Proc Natl Acad Sci U S A* 91(26):12654-12658.
70. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A & Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254(5038):1643-1647.
71. Gross G & Eshhar Z (1992) Endowing T cells with antibody specificity using chimeric T cell receptors. *FASEB J* 6(15):3370-3378.
72. Abken H, Hombach A, Reinhold U & Ferrone S (1998) Can combined T-cell- and antibody-based immunotherapy outsmart tumor cells? *Immunol Today* 19(1):2-5.

73. Sadelain M, Brentjens R & Riviere I (2009) The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 21(2):215-223.
74. Dotti G, Savoldo B & Brenner M (2009) Fifteen Years of Gene Therapy Based on Chimeric Antigen Receptors: "Are We Nearly There Yet?". *Hum Gene Ther*.
75. Denkberg G & Reiter Y (2006) Recombinant antibodies with T-cell receptor-like specificity: novel tools to study MHC class I presentation. *Autoimmun Rev* 5(4):252-257.
76. Willemsen R, Chames P, Schooten E, Gratama JW & Debets R (2008) Selection of human antibody fragments directed against tumor T-cell epitopes for adoptive T-cell therapy. *Cytometry A* 73(11):1093-1099.
77. Chames P, Hufton SE, Coulie PG, Uchanska-Ziegler B & Hoogenboom HR (2000) Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. *Proc Natl Acad Sci U S A* 97(14):7969-7974.
78. Willemsen RA, Debets R, Hart E, Hoogenboom HR, Bolhuis RL & Chames P (2001) A phage display selected fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes. *Gene Ther* 8(21):1601-1608.
79. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, Ponzoni M, Rossini S, Mavilio F, Traversari C & Bordignon C (1997) HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 276(5319):1719-1724.
80. de Witte MA, Jorritsma A, Swart E, Straathof KC, de Punder K, Haanen JB, Rooney CM & Schumacher TN (2008) An inducible caspase 9 safety switch can halt cell therapy-induced autoimmune disease. *J Immunol* 180(9):6365-6373.
81. Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, Heslop HE, Spencer DM & Rooney CM (2005) An inducible caspase 9 safety switch for T-cell therapy. *Blood* 105(11):4247-4254.
82. Serafini M, Manganini M, Borleri G, Bonamino M, Imberti L, Biondi A, Golay J, Rambaldi A & Introna M (2004) Characterization of CD20-transduced T lymphocytes as an alternative suicide gene therapy approach for the treatment of graft-versus-host disease. *Hum Gene Ther* 15(1):63-76.
83. Kieback E, Charo J, Sommermeyer D, Blankenstein T & Uckert W (2008) A safeguard eliminates T cell receptor gene-modified autoreactive T cells after adoptive transfer. *Proc Natl Acad Sci U S A* 105(2):623-628.
84. Kessels HW, van Den Boom MD, Spits H, Hooijberg E & Schumacher TN (2000) Changing T cell specificity by retroviral T cell receptor display. *Proc Natl Acad Sci U S A* 97(26):14578-14583.
85. Holler PD, Holman PO, Shusta EV, O'Herrin S, Wittrup KD & Kranz DM (2000) In vitro evolution of a T cell receptor with high affinity for peptide/MHC. *Proc Natl Acad Sci U S A* 97(10):5387-5392.
86. Shusta EV, Holler PD, Kieke MC, Kranz DM & Wittrup KD (2000) Directed evolution of a stable scaffold for T-cell receptor engineering. *Nat Biotechnol* 18(7):754-759.
87. Varela-Rohena A, Molloy PE, Dunn SM, Li Y, Suhoski MM, Carroll RG, Milicic A, Mahon T, Sutton DH, Laugel B, Moysey R, Cameron BJ, Vuidepot A, Purbhoo MA, Cole DK, Phillips RE, June CH, Jakobsen BK, Sewell AK & Riley JL (2008) Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nat Med* 14(12):1390-1395.
88. Chames P, Willemsen RA, Rojas G, Dieckmann D, Rem L, Schuler G, Bolhuis RL & Hoogenboom HR (2002) TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity. *J Immunol* 169(2):1110-1118.
89. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, Xu H, Morgan RA, Feldman SA, Johnson LA, Bennett AD, Dunn SM, Mahon TM, Jakobsen BK & Rosenberg SA (2008) Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *J Immunol* 180(9):6116-6131.

90. Kuball J, Hauptrock B, Malina V, Antunes E, Voss RH, Wolfl M, Strong R, Theobald M & Greenberg PD (2009) Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *J Exp Med* 206(2):463-475.
91. Geiger TL, Nguyen P, Leitenberg D & Flavell RA (2001) Integrated src kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes. *Blood* 98(8):2364-2371.
92. Zhang T, He X, Tsang TC & Harris DT (2004) Transgenic TCR expression: comparison of single chain with full-length receptor constructs for T-cell function. *Cancer Gene Ther* 11(7):487-496.
93. Schaft N, Lankiewicz B, Drexhage J, Berrevoets C, Moss DJ, Levitsky V, Bonneville M, Lee SP, McMichael AJ, Gratama JW, Bolhuis RL, Willemsen R & Debets R (2006) T cell re-targeting to EBV antigens following TCR gene transfer: CD28-containing receptors mediate enhanced antigen-specific IFNgamma production. *Int Immunol* 18(4):591-601.
94. Willemsen RA, Ronteltap C, Chames P, Debets R & Bolhuis RL (2005) T cell retargeting with MHC class I-restricted antibodies: the CD28 costimulatory domain enhances antigen-specific cytotoxicity and cytokine production. *J Immunol* 174(12):7853-7858.
95. Hombach A & Abken H (2007) Costimulation tunes tumor-specific activation of redirected T cells in adoptive immunotherapy. *Cancer Immunol Immunother* 56(5):731-737.
96. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I & June CH (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U S A* 106(9):3360-3365.
97. Ciceri F, Bonini C, Marktel S, Zappone E, Servida P, Bernardi M, Pescarollo A, Bondanza A, Peccatori J, Rossini S, Magnani Z, Salomoni M, Benati C, Ponzoni M, Callegaro L, Corradini P, Bregni M, Traversari C & Bordignon C (2007) Antitumor effects of HSV-TK-engineered donor lymphocytes after allogeneic stem-cell transplantation. *Blood* 109(11):4698-4707.
98. Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, Huang J, Powell DJ, Jr. & Rosenberg SA (2004) Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 173(12):7125-7130.
99. Huang J, Khong HT, Dudley ME, El-Gamil M, Li YF, Rosenberg SA & Robbins PF (2005) Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. *J Immunother* 28(3):258-267.
100. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH & Ahmed R (2003) Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4(3):225-234.
101. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA & Restifo NP (2005) Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* 115(6):1616-1626.
102. Hinrichs CS, Spolski R, Paulos CM, Gattinoni L, Kerstann KW, Palmer DC, Klebanoff CA, Rosenberg SA, Leonard WJ & Restifo NP (2008) IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 111(11):5326-5333.
103. Goldrath AW, Sivakumar PV, Glaccum M, Kennedy MK, Bevan MJ, Benoist C, Mathis D & Butz EA (2002) Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. *J Exp Med* 195(12):1515-1522.
104. Berard M, Brandt K, Bulfone-Paus S & Tough DF (2003) IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. *J Immunol* 170(10):5018-5026.

105. Moroz A, Eppolito C, Li Q, Tao J, Clegg CH & Shrikant PA (2004) IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. *J Immunol* 173(2):900-909.
106. Hsu C, Hughes MS, Zheng Z, Bray RB, Rosenberg SA & Morgan RA (2005) Primary human T lymphocytes engineered with a codon-optimized IL-15 gene resist cytokine withdrawal-induced apoptosis and persist long-term in the absence of exogenous cytokine. *J Immunol* 175(11):7226-7234.
107. Peluso I, Fantini MC, Fina D, Caruso R, Boirivant M, MacDonald TT, Pallone F & Monteleone G (2007) IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *J Immunol* 178(2):732-739.
108. Brentjens RJ, Latouche JB, Santos E, Marti F, Gong MC, Lyddane C, King PD, Larson S, Weiss M, Riviere I & Sadelain M (2003) Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med* 9(3):279-286.
109. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA & Restifo NP (2004) IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. *Proc Natl Acad Sci U S A* 101(7):1969-1974.
110. Teague RM, Sather BD, Sacks JA, Huang MZ, Dossett ML, Morimoto J, Tan X, Sutton SE, Cooke MP, Ohlen C & Greenberg PD (2006) Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. *Nat Med* 12(3):335-341.
111. Sondergaard H, Frederiksen KS, Thygesen P, Galsgaard ED, Skak K, Kristjansen PE, Odum N & Kragh M (2007) Interleukin 21 therapy increases the density of tumor infiltrating CD8+ T cells and inhibits the growth of syngeneic tumors. *Cancer Immunol Immunother* 56(9):1417-1428.
112. Berger C, Berger M, Hackman RC, Gough M, Elliott C, Jensen MC & Riddell SR (2009) Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood* 114(12):2417-2426.
113. Epardaud M, Elpek KG, Rubinstein MP, Yonekura AR, Bellemare-Pelletier A, Bronson R, Hamerman JA, Goldrath AW & Turley SJ (2008) Interleukin-15/interleukin-15R alpha complexes promote destruction of established tumors by reviving tumor-resident CD8+ T cells. *Cancer Res* 68(8):2972-2983.
114. Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, Pise-Masison CA, Radonovich MF, Brady JN, Restifo NP, Berzofsky JA & Leonard WJ (2005) Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *J Exp Med* 201(1):139-148.
115. Huarte E, Fisher J, Turk MJ, Mellinger D, Foster C, Wolf B, Meehan KR, Fadul CE & Ernstoff MS (2009) Ex vivo expansion of tumor specific lymphocytes with IL-15 and IL-21 for adoptive immunotherapy in melanoma. *Cancer Lett* 285(1):80-88.
116. Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, ten Hagen T, Verweij J & Debets R (2010) Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother* 59(6):921-931.
117. Sereti I, Dunham RM, Spritzler J, Aga E, Proschan MA, Medvik K, Battaglia CA, Landay AL, Pahwa S, Fischl MA, Asmuth DM, Tenorio AR, Altman JD, Fox L, Moir S, Malaspina A, Morre M, Buffet R, Silvestri G & Lederman MM (2009) IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. *Blood* 113(25):6304-6314.
118. Kaneko S, Mastaglio S, Bondanza A, Ponzoni M, Sanvito F, Aldrighetti L, Radrizzani M, La Seta-Catamancio S, Provasi E, Mondino A, Nagasawa T, Fleischhauer K, Russo V, Traversari C, Ciceri F, Bordignon C & Bonini C (2009) IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood* 113(5):1006-1015.

119. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C & Riddell SR (2008) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* 118(1):294-305.
120. Yang L & Baltimore D (2005) Long-term in vivo provision of antigen-specific T cell immunity by programming hematopoietic stem cells. *Proc Natl Acad Sci U S A* 102(12):4518-4523.
121. van Lent AU, Nagasawa M, van Loenen MM, Schotte R, Schumacher TN, Heemskerk MH, Spits H & Legrand N (2007) Functional human antigen-specific T cells produced in vitro using retroviral T cell receptor transfer into hematopoietic progenitors. *J Immunol* 179(8):4959-4968.
122. Zhao Y, Parkhurst MR, Zheng Z, Cohen CJ, Riley JP, Gattinoni L, Restifo NP, Rosenberg SA & Morgan RA (2007) Extrathymic generation of tumor-specific T cells from genetically engineered human hematopoietic stem cells via Notch signaling. *Cancer Res* 67(6):2425-2429.
123. Hunziker L, Klenerman P, Zinkernagel RM & Ehl S (2002) Exhaustion of cytotoxic T cells during adoptive immunotherapy of virus carrier mice can be prevented by B cells or CD4+ T cells. *Eur J Immunol* 32(2):374-382.
124. Marzo AL, Kinnear BF, Lake RA, Frelinger JJ, Collins EJ, Robinson BW & Scott B (2000) Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J Immunol* 165(11):6047-6055.
125. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, Palmer DC, Chan CC, Klebanoff CA, Overwijk WW, Rosenberg SA & Restifo NP (2005) CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* 174(5):2591-2601.
126. Schoenberger SP, Toes RE, van der Voort EI, Offringa R & Melief CJ (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393(6684):480-483.
127. Surman DR, Dudley ME, Overwijk WW & Restifo NP (2000) Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J Immunol* 164(2):562-565.
128. Ibe S, Qin Z, Schuler T, Preiss S & Blankenstein T (2001) Tumor rejection by disturbing tumor stroma cell interactions. *J Exp Med* 194(11):1549-1559.
129. Gyobu H, Tsuji T, Suzuki Y, Ohkuri T, Chamoto K, Kuroki M, Miyoshi H, Kawarada Y, Katoh H, Takeshima T & Nishimura T (2004) Generation and targeting of human tumor-specific Tc1 and Th1 cells transduced with a lentivirus containing a chimeric immunoglobulin T-cell receptor. *Cancer Res* 64(4):1490-1495.
130. Nishimura T, Iwakabe K, Sekimoto M, Ohmi Y, Yahata T, Nakui M, Sato T, Habu S, Tashiro H, Sato M & Ohta A (1999) Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med* 190(5):617-627.
131. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, Gattinoni L, Wrzesinski C, Hinrichs CS, Kerstann KW, Feigenbaum L, Chan CC & Restifo NP (2008) Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112(2):362-373.
132. Kuball J, Schmitz FW, Voss RH, Ferreira EA, Engel R, Guillaume P, Strand S, Romero P, Huber C, Sherman LA & Theobald M (2005) Cooperation of human tumor-reactive CD4+ and CD8+ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. *Immunity* 22(1):117-129.
133. Willemsen R, Ronteltap C, Heuveling M, Debets R & Bolhuis R (2005) Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. *Gene Ther* 12(2):140-146.

134. Willemsen RA, Sebestyen Z, Ronteltap C, Berrevoets C, Drexhage J & Debets R (2006) CD8 alpha coreceptor to improve TCR gene transfer to treat melanoma: down-regulation of tumor-specific production of IL-4, IL-5, and IL-10. *J Immunol* 177(2):991-998.
135. Morris EC, Tsallios A, Bendle GM, Xue SA & Stauss HJ (2005) A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. *Proc Natl Acad Sci U S A* 102(22):7934-7939.
136. Rossig C, Bollard CM, Nuchtern JG, Rooney CM & Brenner MK (2002) Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. *Blood* 99(6):2009-2016.
137. Heemskerk MH, Hoogeboom M, Hagedoorn R, Kester MG, Willemze R & Falkenburg JH (2004) Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. *J Exp Med* 199(7):885-894.
138. van der Veken LT, Hagedoorn RS, van Loenen MM, Willemze R, Falkenburg JH & Heemskerk MH (2006) Alphabeta T-cell receptor engineered gammadelta T cells mediate effective antileukemic reactivity. *Cancer Res* 66(6):3331-3337.
139. van der Veken LT, Coccoris M, Swart E, Falkenburg JH, Schumacher TN & Heemskerk MH (2009) Alpha beta T cell receptor transfer to gamma delta T cells generates functional effector cells without mixed TCR dimers in vivo. *J Immunol* 182(1):164-170.
140. Riddell SR, Elliott M, Lewinsohn DA, Gilbert MJ, Wilson L, Manley SA, Lupton SD, Overell RW, Reynolds TC, Corey L & Greenberg PD (1996) T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nat Med* 2(2):216-223.
141. Lamers CH, Willemsen R, van Elzakker P, van Steenbergen-Langeveld S, Broertjes M, Oosterwijk-Wakka J, Oosterwijk E, Sleijfer S, Debets R & Gratama JW (2011) Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. *Blood* 117(1):72-82.
142. Berger C, Huang ML, Gough M, Greenberg PD, Riddell SR & Kiem HP (2001) Nonmyeloablative immunosuppressive regimen prolongs In vivo persistence of gene-modified autologous T cells in a nonhuman primate model. *J Virol* 75(2):799-808.
143. Klebanoff CA, Khong HT, Antony PA, Palmer DC & Restifo NP (2005) Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends Immunol* 26(2):111-117.
144. Kohlmeyer J, Cron M, Landsberg J, Bald T, Renn M, Mikus S, Bondong S, Wikasari D, Gaffal E, Hartmann G & Tuting T (2009) Complete regression of advanced primary and metastatic mouse melanomas following combination chemoimmunotherapy. *Cancer Res* 69(15):6265-6274.
145. Thistlethwaite FC, Elkord E, Griffiths RW, Burt DJ, Shablak AM, Campbell JD, Gilham DE, Austin EB, Stern PL & Hawkins RE (2008) Adoptive transfer of T(reg) depleted autologous T cells in advanced renal cell carcinoma. *Cancer Immunol Immunother* 57(5):623-634.
146. Cui Y, Zhang H, Meadors J, Poon R, Guimond M & Mackall CL (2009) Harnessing the physiology of lymphopenia to support adoptive immunotherapy in lymphoreplete hosts. *Blood*.
147. Beq S, Rozlan S, Gautier D, Parker R, Mersseman V, Schilte C, Assouline B, Rance I, Lavedan P, Morre M & Cheynier R (2009) Injection of glycosylated recombinant simian IL-7 provokes rapid and massive T-cell homing in rhesus macaques. *Blood* 114(4):816-825.
148. Qian F, Villella J, Wallace PK, Mhawech-Fauceglia P, Tario JD, Jr., Andrews C, Matsuzaki J, Valmori D, Ayyoub M, Frederick PJ, Beck A, Liao J, Cheney R, Moysich K, Lele S, Shrikant P, Old LJ & Odunsi K (2009) Efficacy of levo-1-methyl tryptophan and dextro-1-methyl tryptophan in reversing indoleamine-2,3-dioxygenase-mediated arrest of T-cell proliferation in human epithelial ovarian cancer. *Cancer Res* 69(13):5498-5504.

149. Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, Mellor AL, He Y & Munn DH (2009) Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* 113(24):6102-6111.
150. de Witte MA, Bendle GM, van den Boom MD, Coccoris M, Schell TD, Tevethia SS, van Tinteren H, Mesman EM, Song JY & Schumacher TN (2008) TCR gene therapy of spontaneous prostate carcinoma requires in vivo T cell activation. *J Immunol* 181(4):2563-2571.
151. Jiang HR, Gilham DE, Mulryan K, Kirillova N, Hawkins RE & Stern PL (2006) Combination of vaccination and chimeric receptor expressing T cells provides improved active therapy of tumors. *J Immunol* 177(7):4288-4298.
152. Smith FO, Klapper JA, Wunderlich JR, Rosenberg SA & Dudley ME (2009) Impact of a Recombinant Fowlpox Vaccine on the Efficacy of Adoptive Cell Therapy With Tumor Infiltrating Lymphocytes in a Patient With Metastatic Melanoma. *J Immunother*.

Chapter 3

T cell receptor gene therapy in human melanoma-bearing immune-deficient mice: human but not mouse T cells recapitulate outcome of clinical studies

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ABSTRACT

Adoptive cell therapy of T cell receptor (TCR) engineered T cells is a clinically feasible and promising approach to target tumors, but is currently faced by compromised anti-tumor efficacies in patients. Here, we extensively validated immune-deficient mice to facilitate further development of the therapeutic potential of TCR-engineered T cells. Treatment of human melanoma-bearing SCID or NSG mice with high doses of human T cells transduced with an hgp100/HLA-A2-specific TCR did not result in anti-tumor responses irrespective of chemotherapeutic preconditioning. Imaging of human GFP-labeled T cells demonstrated significant T cell accumulation in intra-tumoral vasculature directly upon T cell transfer, which was followed by loss of T cells within 72h. Peripheral persistence of human T cells was highly compromised and appeared related to T cell differentiation. On the contrary, adoptive transfer (AT) of relatively low numbers of hgp100/HLA-A2 TCR-transduced mouse T cells resulted in rapid clearance of large established human melanomas. Unexpectedly and in contrast to reported studies with chimeric antibody receptor (CAR)-engineered T cells, anti-tumor activity and homeostatic expansion of T cells were independent of TCR transgene as evidenced in two SCID strains and using two different human melanoma cell lines. Interestingly, the xeno-reactive melanoma response of mouse T cells appeared to be dictated by $CD4^+$ tumor-infiltrating lymphocytes and did not require *in vitro* T cell activation, retroviral gene transfer or subcutaneous IL-2 support. Taken together, AT of human but not mouse T cells in human melanoma-bearing immune-deficient mice is in close accordance with clinical studies.

INTRODUCTION

Adoptive cell therapy using T cell receptor (TCR) gene-modified T cells represents an attractive immunotherapy for cancer. Generally, the endogenous repertoire of tumor-specific T cells is limited and the infusion of T cells genetically engineered with a tumor-specific TCR may provide patients with anti-tumor immunity. In this rapidly expanding field early reports have already demonstrated the successful introduction of tumor-specific TCR$\alpha\beta$ genes into T cells enabling them to specifically bind to and lyse antigen-positive tumor cells *in vitro* (1-3). In addition, immune-competent mouse models demonstrated the anti-tumor activity of TCR-engineered T cells *in vivo* (4-7). More recently, clinical trials have provided evidence of the feasibility and therapeutic potential of TCR-engineered T cells in patients (8-11). In fact, TCR-engineered T cells directed against the HLA-A2-restricted antigens MART-1, gp100, CEA or NY-ESO-1 mediated clinical responses in patients with metastatic melanoma, colorectal and synovial tumors. Although these clinical responses were variable and based on a relatively small number of patients, they generally lagged behind those observed with natural ex vivo expanded tumor-infiltrating T cells (TILs) (>50%) (12). Furthermore, the clinical use of high avidity TCR-engineered T cells directed against antigens that are over-expressed on tumors but also present on normal tissues can result in on-target toxicities with severe inflammation of skin, eyes and ears (for MART-1, gp100) or colon (CEA) (8, 10).

Clinical results with TCR-engineered T cells are promising, but improvements are necessary to further develop clinical TCR gene therapy into an effective and safe treatment for cancer patients. Immune-deficient mice transplanted with a human tumor represent a widely recognized and successfully used model with human or mouse T cells gene-engineered with either chimeric antibody receptors (CAR) or TCRs. These preclinical models potentially provide a valuable basis to test novel strategies and translate receptor gene therapy to the patient. With respect to human hematological malignancies, such as lymphoma, CAR-engineered human T cells (CAR T cells) have been reported to effectively mediate anti-tumor responses in mice with various degrees of immune deficiency, i.e., severe combined immune-deficient (SCID), non-obese diabetic SCID (NOD.SCID), SCID Beige and NOD.SCID/*il2rg*$^{-/-}$ (NSG) mice (13-19). With respect to human solid tumors, high numbers of human CAR T cells injected intratumorally (i.t.) delayed tumor growth (20) or prolonged tumor-free survival (21) in immune-deficient mice. More interestingly, intravenously (i.v.) injected human CAR T cells inhibited growth of human solid tumors when mice were pre-conditioned with either Cyclophosphamide or irradiation (22-27). Pre-conditioning appeared not required for tumor regression in NSG mice that were treated with an iv injection of T cells engineered with a CAR containing the CD137 co-stimulatory domain (19, 28, 29). In addition to human CAR T cells, in various studies mouse CAR T cells have been used successfully to target human solid tumors in nude or SCID mice without additional treatment (30-34). Despite numerous reports on the anti-tumor effects of CAR T cells, there exists only a limited number of studies on

Table 1. Treatment of Human Tumors with Receptor-Engineered *Human* T cells in Immune-Deficient Mice[a]

				T cell transfer					
Tumor cells	*Histological origin*	*Mice[b]*	*Receptor[c]*	*Route[d]*	*Dosage*	*Time points[e]*	*Additional treatment*	*Anti-tumor activitiy*	*Reference*
Lymphoma cells									
Raji cells	BL	SCID Beige	CD19 CAR:ζ	i.v.	10-20x10^6	day 6 and 7	yes	yes	(13)
NALM-6 cells	Pre-B cell ALL	SCID Beige	CD19 CAR:ζ	i.v.	10-20x10^6	day 4	no	yes[g]	
Daudi cells	BL	NOD.SCID	CD19 CAR:(28).ζ	i.p.	5x10^6	day 3	yes	yes[h]	(15)
Raji cells	BL	SCID Beige	CD19 CAR:(28).ζ	i.v.	10x10^6	day 6	no	yes	(14)
NALM-6 cells	Pre-B cell ALL	SCID Beige	CD19 CAR:(28).ζ	i.v.	10x10^6	day 2, 3, 4	no	yes[h]	
NALM-6 cells	Pre-B cell ALL	SCID Beige	CD19 CAR:(28).ζ	i.v.	10x10^6	day 2, 8, 15, 22	no	yes	
Autologous tumor cells	EBV+LCL	SCID	CD30CAR:ζ[i]	i.v.	10x10^6	between day 10 and 15	yes	yes	(18)
L428 cells	EBV-CD30+HD	SCID Beige	CD30CAR:ζ[i]	i.p.	10x10^6	day 7	yes	yes	
Raji cells	BL	SCID Beige	CD19 CAR:ζ	i.v.	10x10^6	day 6	yes	yes	(17)
Pre-B ALL	ALL	NOD.SCID-$\beta_2^{-/-}$ or NSG	CD19 CAR:(28)(BB).ζ	i.v.	1-20x10^6	between day 9 and 21	yes	yes	(16)
BV173 cells	CML	NOD.SCID	WT1-TCR	i.v.	20x10^6	day 1	no	yes	(35)
CD34+ leukemia progenitor cells	CML	NOD.SCID	WT1-SS-TCR	i.v.	20x10^6	day 1	no	yes	(36)
Solid tumors									
CWR22	PC	SCID Beige	ErbB2 CAR:28.γ	i.t.	10x10^6	day 31, 32, 33, 34, 35	yes	yes	(20)
WISH-PC14	PC	SCID Beige	ErbB2 CAR:28.γ	i.t.	50x10^6	day 14, 15, 16	yes	yes	
COLO 205 cells	CC	NOD.SCID	ErbB2 CAR:28.ζ	i.v.	10x10^6	day 0, 1	yes	yes[j]	(24)
MDA-MD-435	BC	NOD.SCID	ErbB2 CAR:28.ζ	i.v.	10x10^6	day 0, 1	yes	yes[j]	
WISH-PC14 cells	PC	SCID beige	ErbB2 CAR:28.γ	i.v.	50x10^6	day 10	yes	yes[k]	(22)
LuCaP-35 cells	PC	SCID beige	ErbB2 CAR:28.γ	i.v.	50x10^6	day 10	yes	yes[k]	
OV-CAR3 cells	OC	NOD.SCID	Le^y CAR:28.ζ	i.v.	10x10^6	day 0, 1, 2, 5, or day 7, 8, 9, 10	yes	yes	(25)
LNCaP cells	PC	SCID Beige	PSMA Pz1 CAR:ζ	i.t.	20x10^6	between day 10 and 21	no	yes	(21)
LNCaP/C4-2 cells	PC	SCID Beige	PSMA Pz1 CAR:ζ	i.t.	20x10^6	between day 15 and 30	no	yes	
M108 cells	MES	NSG	SS1 CAR:(28)(BB).ζ	i.t.	15x10^6	day 46, 53	no	yes	(29)
M108 cells	MES	NSG		i.t., i.p., i.v.	10x10^6	day 43, 49	no	yes	
BT-474 cells	BC	SCID Beige	ErbB2 CAR:(CD8)28(BB).ζ	i.v.	1.5-2x10^6	day 10	yes	yes	(23)

OV-CAR3 (MUC-CD) cells	OC	SCID Beige	MUC CD 4H11 CAR:(28).ζ	i.p., i.v.	$30x10^6$	day 2	no	yes	(26)
SK-N-AS cells	NB	NSG	GD2 CAR:28.OX40.ζ	i.v.	$10x10^6$	day 3, 10, 17	yes	yes[l]	(27)
M108 cells	MES	NSG	SS1 RNA CAR:BB.ζ[m]	i.t.	$10\text{-}15x10^6$	day 66, 70, 74, 78	no	yes	(19)
SKOV3 cells	OV	NSG	MOv19 CAR:(BB).ζ	i.t., i.p., i.v.	$8x10^6$	day 0, 5	no	yes[n]	(28)
SK-23 cells	MEL	SCID	MelanA TCR	Winn assay	$5x10^6$	day 0	yes	yes	(27)
SK-23 cells	MEL	SCID	MelanA TCR	i.t.	$10x10^6$	day 5, 10, 14	yes	yes	
SK-23 cells	MEL	SCID	MelanA TCR	i.v.	$10x10^6$	day 5, 10, 14	yes	no	

Abbreviations used in this table: ALL, Acute Lymphoblastic Leukemia; BB, CD137 (4-1BB) co-stimulatory molecule; BC, Breast Carcinoma; BD-TCR, Bi-directional TCR; BL, Burkitt Lymphoma; CC, Colon Carcinoma; CML, Chronic Myeloid Leukemia; EBV+ LCL, Epstein Barr Virus positive Large Cell Lymphoma; EBV-CD30+HD, EBV-CD30+ Hodgkin lymphoma; ErbB2 also known as HER2/Neu, Human Epidermal growth factor Recpetor 2; GC, Gastric Carcinoma; Le^y, Lewis Y antigen; Mov19, mAb clone against human folate receptor α; OC, Ovarian Carcinoma; PSMA, Prostate Specific Membrane Antigen; MelanA, Melanocyte Antigen; PC, Prostate Carcinoma; MES, Mesothelioma; MEL, Melanoma; SS1, anti-mesothelin Fv; WT1, Wilms' tumor antigen.

a) This table provides a detailed overview of studies performed with either Chimeric Antibody-based Receptor (CAR) or T cell receptor (TCR)-engineered human T cells in human tumor-bearing immune-deficient mice. The studies are compared with respect to tumor cells, mouse strains, antigen-specific receptors, T cell transfers, additional treatments and anti-tumor responses.
b) Mice strain: SCID, Severe Combined Immune Deficient mice containing the *Prkdc* mutation; SCID Beige, Mice containing the *Prkdc* and *Beige* mutation; NOD.SCID, Mice containing the *Prkdc* mutation on a Non-Obese Diabetic background; NOD.SCID-$\beta^{-/-}$, NOD.SCID mice containing a *b2m* mutation; NSG, NOD.SCID mice containing an *il2rg* mutation.
c) Receptor format: CAR receptors are chimeric receptors that consist of various 'building blocks', such as (domains of) Fc(ε)RIγ (in short γ) or CD3ζ (ζ) which are in some cases combined with co-stimulatory molecules such as CD28 (28) or 4-1BB (BB). SS-TCR is a cysteine-modified TCR.
d) Route of T cell administration: i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous.
e) Timepoint(s) of T cell transfer are after tumor transplant.
f) In case of an additional treatment details are listed here per tumor model and reference: Raij cells, T cell expansion on Artificial Antigen-Presenting Cells (AAPC) plus IL-15 (Brentjens *et al.*, 2003); Daudi cells, 2.5 Gy Total Body Irradiation (TBI) (Kowolik *et al.*, 2006); Autologous tumor cells and L428 cells, 230 cGy TBI and IL-2 support (Savoldo *et al.*, 2007); Raji cells, Cyclophosphamide (Cheadle *et al.*, 2008); pre-B ALL cells, T cell expansion on CD28 bead-based AAPC (Milone *et al.*, 2009); CWR22 and WISH-PC14 cells, IL-2 support (Pinthus *et al.*, 2003); COLO205 and MDA-MD-435 cells, 2.5 Gy TBI (Teng *et al.*, 2004); WISH-PC14 cells and LuCap-35 cells, 2 Gy TBI and IL-2 support (Pinthus *et al.*, 2004); OVCAR-3 cells, 2.5 Gy TBI (Westwood *et al.*, 2005); BT-474 cells, Cyclophosphamide and IL-2 support (Zhao *et al.*, 2009); SK-23 cells, IL-2 support (Bobisse *et al.*, 2009).
g) CD80+ but not CD80- NALM-6 tumors were rejected.
h) CD19 CAR:28ζ, but not CD19 CAR:ζ caused an anti-tumor response.
i) In this study EBV-specific cytotoxic T cells are used as host T cells for receptor gene transfer, whereas in other studies polyclonal peripheral T cells are used.
j) In case mice were not irradiated receptor-engineered T cells did not cause an anti-tumor response.
k) In case mice were irradiated or treated with cyclophosphamide receptor-engineered T cells did not cause an anti-tumor response.
l) In case mice received CAR T cells also engineered to express the chemokine receptor CCR2b, an anti-tumor response was observed.
m) In this study T cells were electroporated with CAR-encoding mRNA.
n) MOv19 CAR:BB.ζ, but not CAR:ζ caused an anti-tumor response.

Table 2. Treatment of Human Tumors with Receptor-Engineered Mouse T cells in Immune-Deficient Mice[a]

				T cell transfer					
Tumor cells	*Histological origin*	*Mice[b]*	*Receptor[c]*	*Route[d]*	*Dosage*	*Time points[e]*	*Additional treatment*	*Anti-tumor activitiy*	*Reference*
Solid Tumors									
IGROV-1 cells	OC	Nude	MOv18 CAR:γ[f]	i.p.	10-30x10^6	day 3	no	yes	(34)
COLO 205 cells	CC	SCID	CEA CAR:γ	i.v.	5x10^6	day 0, 1, 2 or 3[g]	no	yes	(33)
COLO 205 cells	CC	SCID	CEA CAR:γ or ζ	i.v.	5x10^6	day 0 and 1	no	yes	(32)
COLO 205 cells	CC	SCID	CEA CAR:(CD28)γ	i.v.	5x10^6	day 0 and 1	no	yes	(31)
COLO 205 cells	CC	SCID	ErB2 CAR:(CD28)ζ	i.v.	0.1-10x10^6	day 0, 1, and/or 3	no	yes	(30)

Abbreviations used in this table: CC, Colon Carcinoma; CEA, Carcino Embryonic Antigen; ErbB2, also known as HER2/Neu; Human Epidermal growth factor Receptor 2; Mov18, mAb clone against human folate receptor α; OC, Ovarian Carcinoma.

a) This table provides a detailed overview of studies performed with Chimeric Antibody-based Receptor (CAR)-expressing mouse T cells in human tumor-bearing immune-deficient mice. There are no *in vivo* studies reported so far with TCR-engineered mouseT cells directed towards human tumors in immune-deficient mice. The studies are compared with respect to tumor cells, mouse strains, antigen-specific receptors, T cell transfers, additional treatments and anti-tumor responses.

b) Mice strains: Nude mice, a-thymic mice due to a mutation in the foxn1 gene; SCID, Severe Combined Immune Deficient mice containing the *Prkdc* mutation.

c) Receptor format: CAR receptors are chimeric receptors that consist of various 'building blocks', such as (domains of) Fc(ε)RIγ (in short γ) or CD3ζ (ζ) which are in some cases combined with co-stimulatory molecules such as CD28 (28).

d) Route of T cell administration: i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous.

e) Timepoint(s) of T cell transfer after tumor transplant.

f) Tumor Infiltrating Lymphocytes (TIL) derived from a mouse adenocarcinoma (MC38) tumor were used as host cells for receptor gene transfer, whereas in other studies polyclonal peripheral T cells were used.

g) T cell transfers are given 1 to 4 times from day 0 till day 3.

the anti-tumor effects of TCR-engineered T cells (TCR T cells) in immune-deficient mice. Two studies by Xue and colleagues reported that i.v. injection of human TCR T cells inhibit human lymphoma engraftment in NOD.SCID mice (35, 36). Moreover, a single study by Bobisse and colleagues described that i.t. but not i.v. injection of human TCR T cells delayed growth of human melanoma in SCID mice (37). To our knowledge there are no reports of mouse TCR T cells targeting a human tumor in immune-deficient mice. See Tables 1 and 2 for an up-to-date overview of reported studies on adoptive cell therapy with human and mouse T cells, respectively, genetically directed towards human tumors in immune-deficient mice.

Here, we evaluated immune-deficient mouse models to test the anti-human melanoma efficacy of adoptively transferred human as well as mouse T cells engineered with a human gp100/HLA-A2- specific TCR. High numbers of human TCR T cells were not efficacious against human melanoma cells in SCID or NSG mice irrespective of pre-treatment with Cyclophosphamide with or without Busulfan. In contrast, mouse TCR T cells very efficiently cleared established human melanoma, but the anti-tumor responses and homeostatic expansion of mouse T cells were independent of the TCR transgene.

MATERIALS AND METHODS

T cells, packaging cells and melanoma cells

PBMC from healthy human donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm^3; Amersham Pharmacia Biotech, Uppsala, Sweden). Transduced primary human T cells were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 200 mM L-glutamine, 10% human serum, antibiotics and 360 IU/ml recombinant human IL-2 (Proleukin; Chiron, Amsterdam, The Netherlands) and stimulated every 2 weeks with a mixture of irradiated allogeneic feeder cells as described elsewhere (38). Mouse splenocytes were cultured in complete mouse medium (CMM) consisting of RPMI supplemented with 25 mM HEPES, 200 nM L-glutamine, 10% Fetal Bovine Serum (FBS; Greiner Bio-one Alphen a/d Rijn, the Netherlands), 1% MEM non-essential amino acids, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, antibiotics and 360 IU/ml IL-2. The human embryonic kidney cell line 293T and Phoenix-Ampho (Ph-A) were both used to package retroviruses carrying RNA encoding TCR$\alpha\beta$, and grown in DMEM with glutamine, 10% FBS, 1% MEM non-essential amino acids and antibiotics. The same medium and supplements were used to culture the gp100/HLA-A2^{pos} human melanoma cell lines, FM3 and BLMgp100 (BLM transfected with hgp100 cDNA).

Mice

Inbred C57BL/6 (B6), BALB/c and BALB/cJHan™Hsd-*Prkdcscid* (BALB/c SCID) mice were purchased from Harlan Laboratories (UK) and B6.CB17-*Prkdc^{scid}*/SzJ (B6 SCID) and NOD.Cg-

Prkdc^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice were housed according to the guidelines of the Erasmus Medical Center. Mice from 8 to 12 weeks of age were used in all our experiments following approval by the Experimental Animal Committee of the Erasmus Medical Center (DEC-consult) and carried out in accordance with institutional and national guidelines.

TCR transgenes and transduction of T lymphocytes

The human gp100$_{280\text{-}288}$/HLA-A2 specific (gp100/A2) TCRα and β genes were introduced separately into the retroviral vector pBullet via *Nco*I and *Xho*I (pB-TCRα and pB-TCRβ constructs) (1). To allow detection of transduced T cells *in vivo,* green fluorescent protein (GFP) DNA preceded by an internal ribosomal entry site (IRES) was inserted into the *Xho*I-digested pB-TCRα and pB-TCRβ, resulting in pB-TCRα:GFP and pB-TCRβ:GFP constructs. Human T lymphocytes of healthy donors were activated with anti-CD3 mAb and transduced with retroviruses harboring TCRα:GFP and TCRβ:GFP or GFP only (Mock). The transduction procedure was performed as described by Lamers and colleagues (39), with the only exception that TCR-encoding retroviruses were produced by a co-culture of 293T and Ph-A packaging cells. The murinized gp100/A2 TCR (hu:mo gp100/A2 TCR) was derived as described previously by Pouw and colleagues (40) and used to transduce murine T cells. Similar to the receptors used to transduced human T cells, IRES-GFP was subcloned into the *Xho*I-digested hu:mo gp100/A2 TCRα and β genes. Total mouse splenocytes were isolated and activated with concanavalinA (ConA) in CMM and transduced with the retroviral vector pBullet containing hu:mo gp100/A2 TCR$\alpha\beta$ transgenes or the pSTICH:eGFP vector as control (Mock) as described previously (40).

Flow cytometry

Gene-transduced human T cells were monitored with gp100/HLA-A2 PE-labeled tetramers (termed gp100/A2 pMHC) followed by a cell sort for gp100/A2 pMHC binding cells using a FACS-Vantage instrument (Becton Dickinson Biosciences, San Jose, CA). Surface expression of TCR transgenes by human and mouse T cells was measured using a PE-labeled anti-TCRVβ14 mAb (Arden nomenclature (41)) (clone CAS1.1.3, Beckman Coulter, Marseille, France). For adoptive T cell transfer studies we have used 3-4 week old > 90 % TCRVβ14 positive, and > 80 % gp100/A2 binding human T cells. TCR-transduced mouse T cells generally comprised about 30% TCRVβ14 positive T cells at the day of infusion. Peripheral blood, organs and tumors were analyzed for the presence of TCR-engineered T cells by flow cytometry using the anti-TCRVβ14 mAb in combination with GFP, PerCP-labeled anti-CD3ε mAb (clone 145-2C11) and APC-labeled anti-CD8α mAb (clone 53-6.7) (both from Beckton Dickinson Biosciences). We analyzed the samples on a Cytomics FC-500 flow cytometer using CXP software (Beckman Coulter) or a FACSCalibur using CellQuest software (Becton Dickinson Biosciences).

AT models

B6 SCID, or BALB/c SCID or NSG mice were sc transplanted in the flank either with FM3 tumor tissue (3x3x3 mm) or $1x10^6$ BLMgp100 cells. Mice bearing established tumors (5x5x5 mm) or mice having a tumor placed in a dorsal skin-fold window chamber (see below) received 2 doses of TCR-transduced human ($6\text{-}40x10^6$) or mouse ($1x10^6$) T cells administered iv at day 0 and 7. In some experiments, mice were pre-conditioned with Cyclophosphamide (200 mg/kg, Sigma-Aldrich, St Louis, MO) administered ip at day -1, preceded or not by Busulfan (16.5 μg/kg, Duchefa Farma, Haarlem, The Netherlands) administered ip at day -2 (protocol kindly provided by dr. Naomi Taylor, Montpellier, France). Human and mouse T cell infusions were followed by 5 daily sc injections of recombinant human IL-2 ($1x10^5$ IU) starting at the day of T cell transfer or as indicated otherwise. Tumor sizes were measured with a caliper along the perpendicular axes of tumors and volumes were calculated with the formula 0.4 x (A^2 x B) where B represents the largest diameter and A the diameter perpendicular to B (42). In separate experiments using mouse GFP T cells, we have assessed the individual and combined effects of T cell activation, retroviral gene transfer and sc IL-2 support on tumor growth. Blood from tail veins was collected weekly after the first T cell infusion and analyzed for T cells by flow cytometry. At indicated time points tumors and lymphoid organs were isolated. Spleens and inguinal lymph nodes were isolated and processed to obtain single cell suspensions by mechanic disruption over a cell strainer in PBS. Tumors were isolated, cut into small pieces and digested in PBS containing collagenase (1 mg/ml) for 45 min at 37°C while vortexing every 5 min. Then 1 ml 0.1 mM EDTA was added and the tumor was disrupted over a cell strainer. Erythrocytes in blood and organs were removed by incubation in erylysis buffer (154 mM $NHCl_4$) for 15 min on ice. Absolute cell counts were determined using Flow-Count Fluorospheres (Beckman Coulter).

T cell trafficking to tumor site using dorsal skin-fold chamber

We prepared dorsal skin-fold chambers according to Seynhaeve and colleagues (42). In short, mice were anesthetized with a mixture of saline, ketamine and xylazine, injected s.c.. Hair was removed from the skin on the back of the animal and the skin was dissected in a circular area leaving the fascia and opposing skin intact. Next, the skin-fold of the mouse was sandwiched between two frames and fixed with two light metal bolds and sutures. A small piece of tumor tissue was transplanted in the fascia and both sides were closed with a microscopic cover glass. During surgery the body temperature of animals was kept constant at 37 °C after which animals were housed individually at an ambient temperature of 32 °C and a humidity of 70 %. Twenty-one days after implantation mice received GFP T cells. At given time points mice were anesthetized with 3% isoflurane and fixed to a heated stage of a Leica DM-RXA microscope. To visualize the presence of T cells we used a Leica I3 filter set (excitation 450-490 and emission LP 515) and photographed the GFP T cells using a Sony 3CCD DXC 950 camera.

Immune histochemistry

Tumors were isolated, snap-frozen in liquid nitrogen, and stored at -80 °C until use. Tissue sections were cut at 5 μm and stained for T cells with the following primary antibodies: rat anti-mouse CD3ε (clone GRJ01, R&D systems, Abingdon, UK), rat anti-mouse CD4 (clone GK1.5), rat anti-mouse CD8α (clone 53-6.7) (both Biolegend, San Diego, USA) or rat IgG isotype control (clone 141945, R&D systems). Goat anti-rat IgG Alexa Fluor 488 (Molecular Probes, Invitrogen, Breda, The Netherlands) was used as a secondary antibody. Air dried cryosections were fixed with acetone for 10 min and blocked with PBS/1%BSA for 30 min prior to immune staining. Next, sections were incubated with the primary antibody for 1 h, washed and incubated with the secondary antibody for 1 h, both at room temperature. Finally, we stained the nuclei with DAPI (4′, 6-diamidino-2-phenylindole) and embedded the sections in mounting medium containing polyvinyl alcohol (MoWiol-488; Fluka, Zwijndrecht, The Netherlands). Sections were examined microscopically and photographed as described above. Recorded photographs were analyzed using Image Tool v3 (developed by Don Wilcox, University of Texas Health Science Center, San Antonio, TX) as described previously (42). The RGB images were converted to gray scale and specific fluorescence intensities (range: 0 – 255) were measured with threshold set at 20. Percentages of pixels with fluorescence intensity above threshold wer calculated and expressed as bars.

Statistical analyses

Tumor volumes, times to tumor regression and absolute cell counts were evaluated for statistical significance with the Mann-Whitney U test using SPSS v15 software, whereas immune histochemistry was evaluated for statistical significance with the Student's *t* test using GraphPad Prism v4 software. Differences with p-values ≤ 0.05 were considered significant.

RESULTS

Human TCR-engineered T cells do not control human melanoma growth in immune-deficient mice

We have assessed the *in vivo* performance of human peripheral blood-derived T cells engineered with gp100/A2 TCR, which we had previously validated *in vitro* (1). Gp100/A2 TCR T cells, used for AT, functionally expressed TCR transgenes assessed by pMHC binding and target cell-specific cytotoxicity (*Supplementary Figs. 1A and B)*. Intravenous transfer of a high dose of gp100/A2 TCR T cells (40x10^6 cells) into B6 SCID mice bearing antigen-positive human melanoma (FM3 cells) did not delay tumor growth (Fig. 1A). Pre-conditioning with Cyclophosphamide or a combination of Cyclophosphamide and Busulfan significantly depleted endogenous NK cells present in B6 SCID mice (*Supplementary Fig. 2*), but did not stimulate the anti-FM3 melanoma T-cell response (Fig. 1B). The lack of anti-melanoma response of human

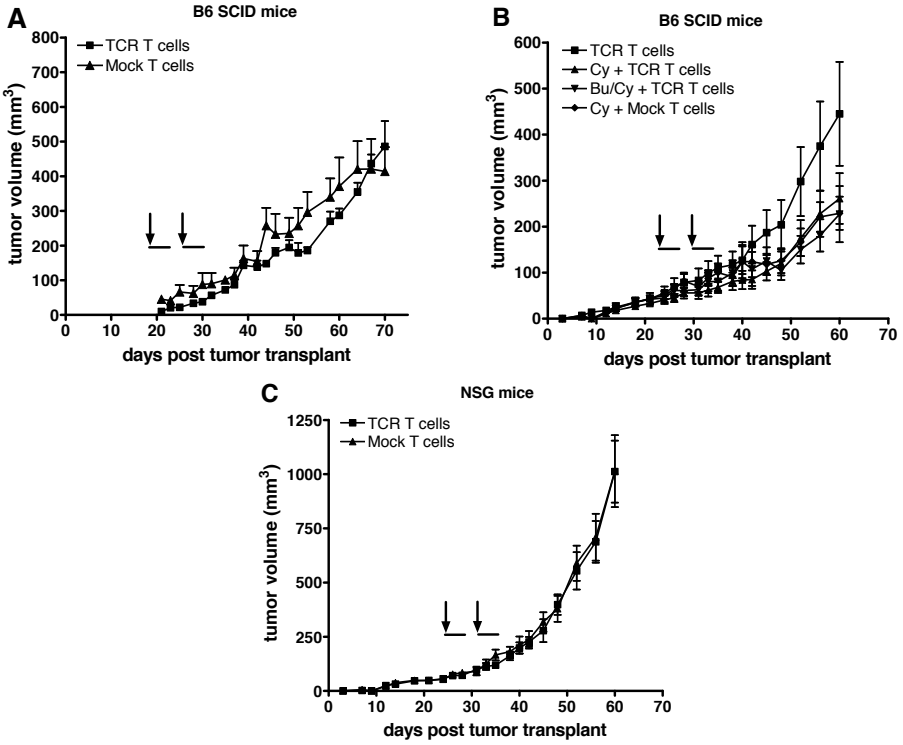

Figure 1. Systemic administration of gp100/A2 TCR transduced human T cells does not control growth of established human melanoma.

FM3 tumor tissue (gp100+/HLA-A2+) was s.c. transplanted in the right flank of B6 SCID mice **(A** and **B)**. **A.** When tumors were palpable mice received either 40x10^6 TCR or Mock-engineered human T cells i.v. (FACsorted and >90% positive for TCR-Vβ14 and GFP or GFP only) at the indicated time points (arrows) followed by s.c. IL-2 injections for 5 consecutive days (horizontal bars) (n=5 per group). **B.** Mice were pre-treated (or not) with an i.p. injection of Busulfan (16.5 μg/kg) at day 22 followed by Cyclophosphamide (200 mg/kg) or Cyclophosphamide only at day 23 after tumor transplant. At day 25 and day 32, 6-7x10^6 TCR or Mock T cells were infused i.v. followed by s.c. IL-2 injections for 5 consecutive days (n=6-7 per group). **C.** FM3 tumor cells were s.c. injected in the right flank of NSG mice. At day 20 and 27, mice were treated with 6-7x10^6 TCR or Mock T cells followed by IL-2 injections. Representative tumor growth curves are shown out of two experiments with similar results. Data is displayed as mean tumor volumes ± SEM.

T cells, whether or not in pre-conditioned mice, was supported by our inability to detect human T cells in peripheral blood by flow cytometry (*Supplementary Fig. 3A*).

To completely exclude 'leakage' of NK cells in conventional SCID mice, and their potential adverse effect on T cell engraftment, we have tested NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ mice, commonly known as NOD SCID GAMMA (NSG). The NSG mouse strain combines features of the NOD mouse, the severe combined immune deficiency mutation SCID, and IL-2R common-γ chain deficiency. Consequently, NSG mice are severely immune compromised, featuring

absence of mature T or B cells, lack of functional NK cells and deficiency in cytokine signaling. T cell engraftment was improved in NSG mice as evidenced by low but significantly higher numbers of TCR T cells in the peripheral blood at day 3 and 10 after T cell transfer (*Supplementary Fig. 3B*). However, adoptively transferred T cells did not persist beyond a time period of 2 weeks past T cell transfer and did not result in an anti-tumor response in melanoma-bearing NSG mice (Fig. 1C).

Human TCR-engineered T cells accumulate in tumor vasculature but are lost shortly after AT

We employed T cell imaging to investigate whether the observed lack of peripheral persistence and anti-tumor response of human TCR T cells was related to their inability to migrate to the tumor site. Mice bearing an FM3 human melanoma in a dorsal skinfold window chamber received $50x10^6$ gp100/A2 TCR:GFP T cells. Directly after injection of T cells, TCR T cells but not mock T cells accumulated in the tumor vasculature (Fig. 2A). Strikingly, the accumulated TCR T cells completely disappeared from the tumor site within 72 h after adoptive T cell (Fig. 2B). Notably, human T cells predominantly showed a phenotype that corresponded to effector memory and late effector T cells at the time of AT (*Supplementary Fig. 4*).

Murine T cells cause complete, yet TCR transgene-independent regression of human melanoma in immune-deficient mice

In a next series of experiments we switched to gene-engineered murine T cells, which in the setting of CARs proved effective against solid tumors in SCID mice (30-33). FM3 tumor-bearing B6 SCID mice were infused twice with $1x10^6$ murine T cells expressing gp100/A2 TCR. In these mice, tumors increased in volume until day 35 (equivalent to day 11 after 1st T cell transfer), reached a maximum average volume of 500 mm^3 before rapidly regressing (Fig. 3A). Notably, Mock T cells eradicated established FM3 tumors with the same potency and kinetics as was observed for TCR T cells. Mice remained tumor-free until the end of the experiments (i.e. day 80 after tumor transplant). The anti-tumor responses of TCR and Mock-engineered murine T cells have been confirmed with a second mouse strain (BALB/c SCID mice, Fig. 3B) and second melanoma cell line (BLMgp100 cells, Fig. 3C).

After AT of murine T cells we observed that percentages of CD3+ T cells in peripheral blood increased, which was again independent of TCR transgene (Fig. 4A). T cell expansion was not driven by the presence of tumor tissue and percentages of peripheral T cells remained high during the complete experiment (Fig. 4A). The expression of the introduced TCR measured by anti-TCRVβ14 mAb was relatively stable during the experiment and again independent of the presence of tumor tissue (Fig. 4B). Collectively, these data demonstrate effective xeno-specific eradication of large human melanoma tumors by murine T cells and homeostatic T cell expansion in classical SCID mice, independent of TCR transgene.

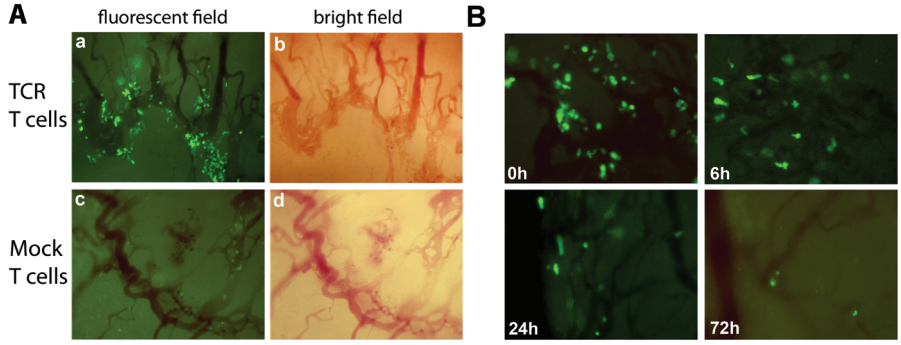

Figure 2. Gp100/A2 TCR-transduced human T cells initially accumulate in the tumor vasculature, but rapidly disappear.

Mice bearing a human melanoma tumor in a dorsal skin-fold window chamber received 50x10^6 TCR/GFP or GFP-engineered human T cells i.v. at day 21 after tumor transplantation. Immediately after T cell transfer mice were anesthetized and fixed to a heated stage of a Leica DM-RXA microscope. T cells in the tumor vasculature were visualized with a GFP filter set (excitation wavelength: 450-490 nm; and emission filter: LP 515). **A.** TCR T cells do **(a+b)** and Mock (i.e., GFP only) T cells do not **(c+d)** accumulate in the tumor vasculature directly after T cell infusion (fluorescent fields: a+c, bright fields: b+d and magnifications: 10x). **B.** The presence of T cells in the tumor vasculature was monitored at the following time-points after T cell injection in the dorsal skin-fold window chamber (n=3): o h **(a)** 6 h **(b)**, 24 h **(c)** and 72 h **(d).** Representative results of one TCR T cell treated mouse are shown (all fluorescent fields; magnification: 20x).

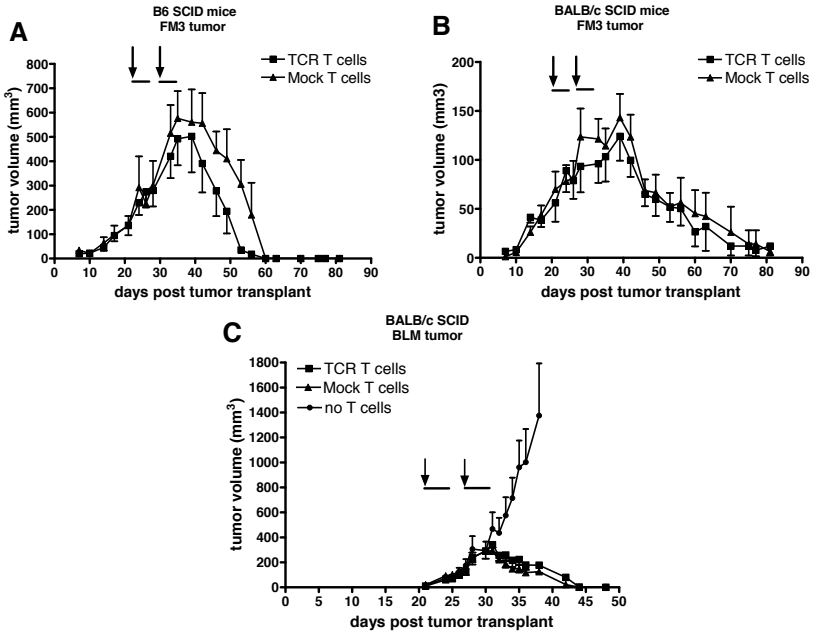

Figure 3. AT of murine T cells results in xeno-specific regression of human melanoma in SCID mice.

FM3 tumor tissue was s.c. implanted in the right flank of B6 **(A)** and BALB/c **(B)** SCID mice. On days 24 and 31 after tumor transplant groups of 6-9 mice received i.v. injections of 1x10^6 murine T cells gene-engineered with TCR/GFP or GFP (arrows). Each T cell transfer was followed by s.c. IL-2 injection as described in

Materials and Methods section (horizontal bars). **C.** In a next set of experiments BALB/c SCID mice were s.c. injected with 1x10^6 gp100+/HLA-A2+ BLM cells (i.e., BLMgp100 cells) and either received 1x10^6 TCR (n=5), Mock (i.e., GFP only) (n=7) T cells or no T cells (n=3) at days 21 and 28 after tumor transplant. Representative tumor growth curves are shown out of two experiments. Data is displayed as mean tumor volumes ± SEM.

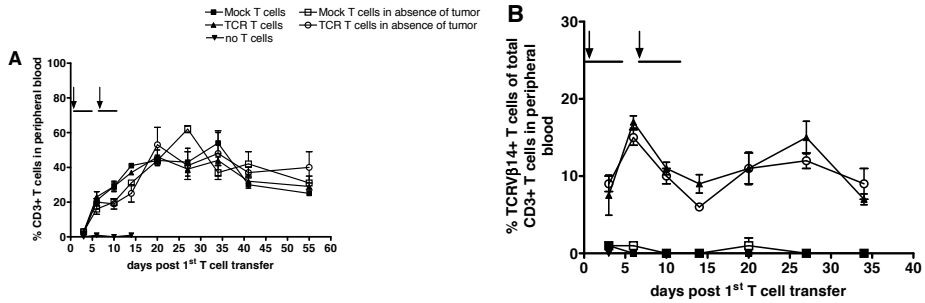

Figure 4. Homeostatic expansion of peripheral murine T cells upon AT into SCID mice.
Human BLMgp100 melanoma cells were s.c. transplanted in the flank of BALB/c SCID mice. On days 24 and 31 after tumor transplant groups of 6-9 mice received either 1x10^6 TCR/GFP or GFP-engineered murine T cells or no T cells (arrows), followed by IL-2 injections (horizontal bars). TCR and Mock (i.e., GFP only) T cells were also administrated to tumor free mice. Peripheral blood was collected at different time points and percentages of (A) CD3+ T cells and (B) TCR-Vβ14 expressing T cells were analyzed by flow cytometry. Numbers indicated are mean percentages ± SEM.

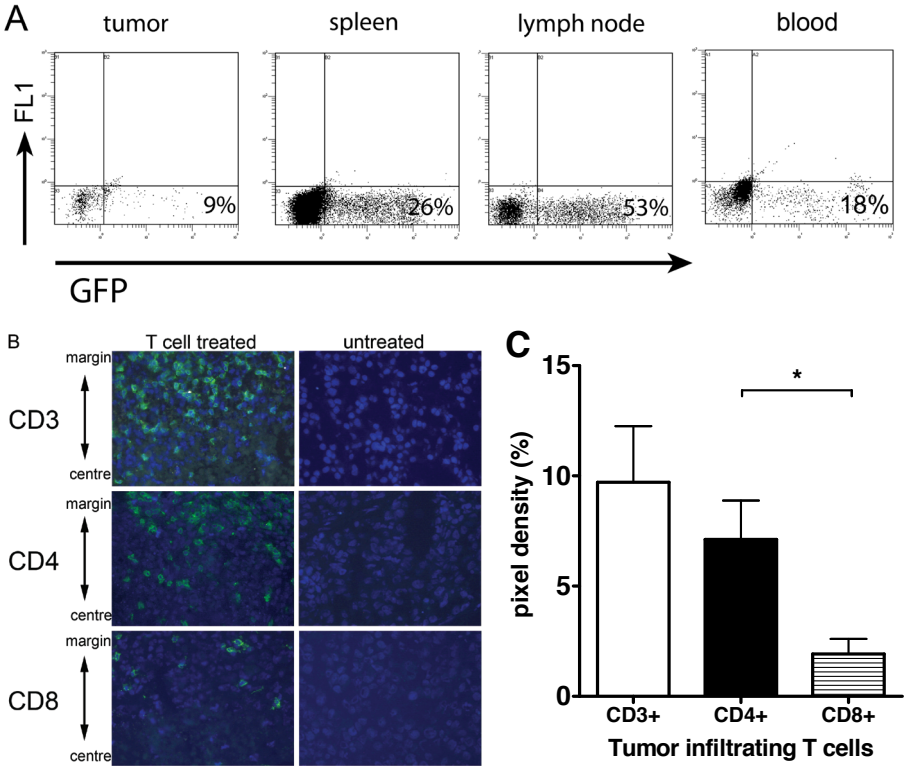

Figure 5. Adoptively transferred murine T cells are present in lymphoid organs and tumor-infiltrating lymphocytes predominantly belong to CD4+ T cell subset.
FM3 tumor-bearing BALB/c and B6 SCID mice (n=6-9) received AT of $1x10^6$ GFP-engineered murine T cells and were treated with IL-2. **A.** At different time points following T cell transfer mice were sacrificed and organs were isolated and analyzed for the presence of GFP expressing cells by flow cytometry. Representative flow cytometry data of a BALB/c mouse sacrificed at day 50 after tumor transplant is shown (out of n=3). No differences were observed between BALB/c and B6 strains. Numbers represent percentages of GFP-positive cells within the lymph gate. **B.** Human tumors (FM3 or BLMgp100) were isolated at day 53 after tumor transplant from mice treated with T cells and analyzed by immune histochemistry for the presence of CD3+, CD4+ and CD8+ T cells. Upper left panel: CD3+ T cells (green) surround the tumor cells (large blue DAPI-stained nuclei). Mid and lower left panels: CD4+ and CD8+ T cells (green) surround tumor cells (blue), respectively (magnification: 40x). The upper part of the pictures shows the margin of the tumor with many T cells, towards the tumor center the T cell numbers reduce. Data is shown from one representative mouse out of 5. No T cells were detected in untreated tumors, and no staining was observed with rat IgG isotype controls. No differences were observed either between mice treated with TCR or Mock T cells, two different SCID strains and two different human melanoma tumors (data not shown). **C.** Recorded photographs were analyzed using Image Tool v3 as described in Materials and Methods section. Fluorescent intensities of CD3+, CD4+ and CD8+ T cells were quantified and depicted graphically (mean percentages ± SEM, n=5 per staining). Student's t test was performed to assess the statistical significance of differences between percentages of different T cell subsets. * $p<0.05$. Manual counting of fluorescent cells by three independent persons (CEVS, DS and TLMTH) yielded the same results (*data not shown*).

Tumor-infiltrating T cells remain at the tumor margin, and are predominantly of the CD4 T cell subset

We have performed two additional types of experiments to further discern the potency and rapid kinetics of the observed xeno-specific response of murine T cells. First, the migratory capacity of GFP-labeled mouse T cells and their CD8 and CD4 subsets were studied by flow cytometry and immune histochemistry. Flow cytometry allowed detection of T cells in regressing tumors and peripheral blood, but their presence was most pronounced in spleens and tumor draining lymph nodes (Fig. 5A). Immune histochemical staining confirmed the presence of T cells in regressing tumor tissue (Fig. 5B). Interestingly, T cells reside predominantly in the tumor margins and not in the center of the regressing tumor. Tumor-infiltrating T cells belonged to both CD8+ and CD4+ T cell subsets, but quantification of immune histochemical stainings, summarized in Fig. 5C, revealed that numbers of intra-tumoral CD4+ T cells are about 4-fold higher than those of CD8+ T cells ($p<0.05$). We confirmed our findings on T cell migration to the tumor with a second mouse strain (i.e., BALB/c SCID mice) and a second human melanoma cell line (i.e., BLMgp100 cells).

T cell activation, retroviral transduction and IL-2 support are not necessary per se, but enhance the potency and kinetics of anti-tumor T cell responses

Second, the effects of T cell activation, retroviral gene transfer and sc IL-2 support on xeno-specific responses and peripheral persistence of murine T cells were assessed. AT of T cells

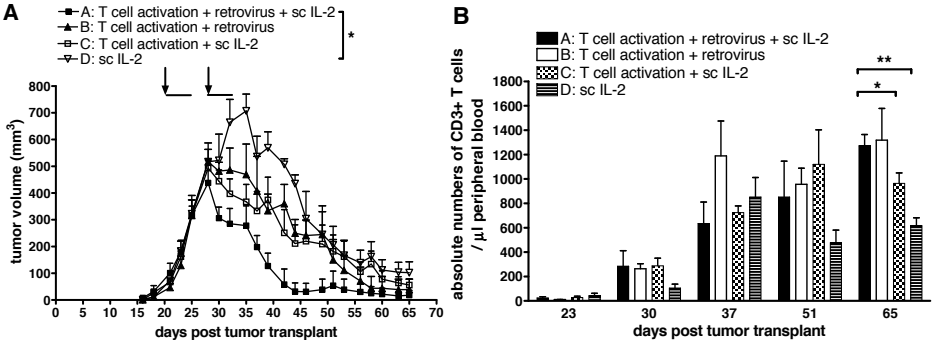

Figure 6. Effects of T cell activation, retroviral transduction and IL-2 support on the xeno-specific T cell responses and numbers of peripheral murine T cells.

A. BALB/c SCID mice were s.c. injected with $1x10^6$ BLMgp100 cells and received $1x10^6$ mouse T cells: either activated and retrovirally transduced with GFP (group A and B); activated but not GFP-transduced (group C); or freshly isolated splenocytes that were neither activated nor GFP-transduced (group D) at day 21 and 28 after tumor transplant (arrows). Mice were s.c. injected with IL-2 (horizontal bars) except for mice in group B. Results are displayed as mean tumor volumes ± SEMs (n=4 for all groups). Mann Whitney-U tests were performed to assess the statistical significance of differences in times to tumor regression between group A and the other treatment groups; *, $p<0.05$. **B.** Peripheral blood samples from the same mice described in (A) were collected at different time points after AT and the numbers of peripheral CD3 T cells were quantified by flow cytometry using FlowCount Fluorospheres (see Materials and Methods section for details). Data is expressed as mean absolute counts per µl of peripheral blood ± SEM (n=4 for all groups). The difference in absolute T cell numbers was tested with the Mann Whitney-U test between group A and the other treatment groups. * $p<0.05$; ** $p<0.01$.

that lack *in vivo* IL-2 support (Fig. 6A, group B), gene-modification (group C) or activation (group D) resulted in clearly detectable although less potent anti-tumor activity in tumor-bearing SCID mice (Fig. 6A, compare group A versus the other groups). When T cell activation was omitted, the time to tumor regression was significantly delayed (Fig. 6A, group D versus A, $p<0.05$). IL-2 support (group B) or retroviral gene transfer (group C) affected kinetics of the anti-tumor responses to a lesser extent and statistical analyses showed a trend towards a delayed onset of tumor regression (Fig. 6A). In peripheral blood, T cell numbers increased until day 37 after tumor transplant (equivalent to day 16 after 1st T cell transfer) and ranged between 600 and 1200 T cells per µl blood at the end of the experiments (Fig. 6B, day 65 after tumor transplant). Overall, T cells from the different treatment groups persisted equally well, although peripheral T cell numbers were significantly lower when T cell activation was omitted, or to a lesser extend when gene-modification was omitted (Fig. 6B group C versus A $p<0.05$ and group D versus A $p<0.01$).

DISCUSSION

Immune-deficient mice bearing a human tumor represent a well-recognized model to assess the therapeutic value of adoptively transferred receptor-engineered T cells. AT of both human and murine CAR-expressing T cells has been tested successfully against human lymphomas and solid tumors. However, only a limited number of studies assessed anti-tumor responses of TCR-expressing T cells in immune-deficient mice (Tables 1 and 2). Notably, no studies to date have reported anti-tumor activities of systemically administered TCR T cells, whether it be human or murine T cells, towards human solid tumors in immune-deficient mice. Here, we have tested the anti-tumor effect and persistence of adoptively transferred human and murine TCR-transduced T cells in immune-deficient mice transplanted with hgp100/HLA-A2 positive human melanoma.

We observed that transfer of human T cells in SCID mice with doses up to $40x10^6$ TCR T cells was not able to control growth of established tumors (Fig. 1A). This lack of anti-tumor response was neither due to an inability of gp100/A2 TCR T cells to respond towards FM3 tumors nor the immune status of the recipient mice. TCR T cells were routinely tested *in vitro* in advance of our AT experiments, and demonstrated high (> 80 % pMHC binding) and stable surface expression (> 3 months, *data not shown*) of TCR transgene, and yielded significant antigen-specific responses (FM3-specific cytotoxicity). Also, FM3 tumors which were isolated after T cell treatment expressed levels of HLA-A2 and gp100 protein that were comparable to those measured in FM3 cells cultured *in vitro*. To exclude 'leakiness' of immune reactive cells, a potential problem of SCID mice, we have tested both pre-treatment with chemotherapy and the use of more immune deficient mice. Although pre-treatment with Cyclophosphamide or irradiation may enhance anti-tumor activity of human T cells in SCIDbeige or NOD.SCID mice (22, 24, 25), we did not observe an enhanced anti-melanoma effect in SCID mice pre-treated with Cyclophosphamide (Fig. 1B). Also, a combination of Cyclophosphamide and Busulfan, which results in transient yet complete depletion of T cells, B cells, NK cells and dendritic cells in immune competent mice (personal communication, dr. Naomi Taylor, Montpellier, France; Straetemans et al., Ms submitted), did not enhance the anti-melanoma activity of TCR-engineered human T cells in SCID mice (Fig. 1B). In more immune-deficient NSG mice (43), we again observed no anti-melanoma response following treatment with TCR T cells (Fig. 1C). Notably, immediately after T cell transfer TCR but not Mock T cells accumulated in FM3 tumor vasculature but these T cells were rapidly (within 72h) lost (Fig. 2). T cell persistence in peripheral blood was negligible and not enhanced by pre-conditioning and at best only marginally enhanced in NSG versus SCID mice (*Supplementary Fig. 3*). Our data confirm a study by Bobisse and colleagues, in which TCR-engineered T cells showed anti-melanoma activity and peripheral persistence following intratumoral but not intravenous administration in immune-deficient mice (37).

Another parameter that is potentially related to T cell persistence is T cell exhaustion. Flow cytometric analysis of markers of T cell memory, activation and co-stimulation, showed that the majority of T cells at the day of AT has a late T cell differentiation phenotype (*Supplementary Fig. 4*). In fact, using two sets of definitions of T cell subsets, one including the adhesion molecule CD62L (according to (44, 45) and another including both the co-stimulatory markers CD27 and CD28 (according to (46, 47), we observed that the sum of effector memory and end stage T cells minimally represented about 90% of T cells. This finding may argue that underrepresentation of less differentiated cells (i.e., naïve and central memory T cells) relates to an inability of human T cells to persist and act against the tumor. In fact, clinical studies demonstrate that T cell differentiation is associated with T cell persistence (48), which in turn is associated with anti-tumor T cell efficacy (49). In our current study, anti-CD3 mAb stimulation and expansion with IL-2 may have created a bias towards TCR T cells with a late differentiation phenotype. Notably, in a clinical phase I trial to treat metastasized renal cell carcinoma with anti-CD3 mAb-activated and IL-2 cultured CAR T cells (50), we observed that transduced T cells of patients, who *all* showed progressive disease, consistently have a late differentiation CD8 T cell phenotype at the time of AT (Lamers, manuscript in preparation). Collectively, clinical trials with either CAR or TCR gene-transduced T cells using similar T cell activation and culture regimens together with in vivo IL-2 support demonstrate that peripheral T cell persistence, although variable, is generally limited (8-10, 50, 51). The present model, in which NSG mice with human melanoma were treated with TCR-engineered T cells, closely recapitulates the cell processing and patient treatment methods used in clinical receptor gene therapy studies and may represent a valid model to test strategies to improve T cell persistence and anti-tumor efficacy.

Two recent clinical trials provide evidence that alternative methods for T cell stimulation and cytokine support may benefit clinical receptor gene therapy (47, 52, 53). In one study, Butler and colleagues adoptively transferred MART1/A2-specific T cells that were stimulated with CD80 and CD83-expressing artificial APCs and cultured in the presence of both IL-2 and IL-15 into patients with metastatic melanoma without pre-conditioning nor *in vivo* IL-2 support (53). This study resulted in 3 mixed responses and long-lasting disease stabilizations and 1 complete response out of 7 patients. In another study, Kalos and colleagues adoptively transferred T cells that were stimulated with anti-CD3 and CD28 mAb-coated beads and gene-engineered with a CD19-specific CAR, which contained the co-stimulatory domain CD137 and the accessory domain CD3ζ, into patients with chronic lymphocytic leukemia who were pre-conditioned but did not receive IL-2 support (47). This study resulted in complete remissions in 2 out of 3 patients. Strikingly, in both studies post-infusion T cells persisted long-term in the circulation, were enriched for T cells with a central memory T cell phenotype and retained functional responses towards target antigens. Although patient numbers were low, these studies clearly demonstrate the potential value of T cell co-stimulation and exposure to IL-15 with respect to the persistence of adoptively transferred T cells. In extension to

these clinical studies, there is also ample preclinical evidence that supports the use of naïve or central memory T cell subsets to enhance T cell persistence and anti-tumor efficacy of transferred T cells (45, 54, 55). Along these lines, we are currently testing T cell stimulations with anti-CD3 and CD28 mAbs, T cell culture with IL-15 and IL-21 and gene-engineering with modified TCR formats, such as TCRs coupled to CD3ε, CD3ζ and co-stimulatory domains, for enhanced *in vivo* persistence and performance (56-58).

In a next series of AT experiments we used mouse T cells, in analogy to earlier studies with CAR-engineered mouse T cells (Table 2). Our studies show that syngeneic mouse T cells can very potently and rapidly eradicate large established human melanomas in SCID mice (Fig. 3). In addition, mouse T cells expanded and persisted *in vivo* for > 6 weeks after the 1st T cell transfer (Fig. 4). Unexpectedly and in contrast to studies with CAR T cells, the anti-tumor activity and expansion of T cells were independent of the receptor transgene and represented xeno-specific T cell responses. In fact, T cell expansions were of a homeostatic nature and independent of the cognate tumor antigen. These findings were confirmed with two SCID strains, B6 SCID and BALB/c SCID, and two human melanoma cell lines, FM3 and BLMgp100. Notably, MHC class I expression on FM3 and BLMgp100 tumor cells is high (>90% of cells in vitro and directly after in vivo isolation, data not shown) and potentially drives the anti-melanoma activity of mouse T cells. Smyth and colleagues reported that cytotoxicity of mouse T cells derived from human tumor-immunized mice can partially be blocked with an anti-human MHC class I antibody *in vitro* (59). Along these lines, MHC class I^{low} tumor cells may be considered to lower the extent of xeno-specific responses, but recognition by TCR-engineered T cells is also likely to be compromised. Alternatively, mouse T cells with a restricted or monoclonal TCR repertoire can be used as recipient cells for TCR gene transfer. This may prevent xeno-specific activity of transferred T cells, but the translational value of such a model should be considered with caution.

To further investigate and better understand the potency and kinetics of the xeno-specific effect of murine T cells, we studied T cell localization and demonstrated that mouse T cells migrate to lymph node and spleen and persist for > 6 weeks in peripheral blood (Fig. 5A). In regressing human tumors murine T cells infiltrated the tumor site and formed a ring at the tumor margin, but were not present in the center of the tumor (Fig. 5B). This finding was in line with observations reported by Hanson and colleagues (60), who showed that in regressing tumors T cells encircle the tumor site, but in progressively growing tumors T cells were scattered in patches of necrotic tumor tissue. CD4+ but not CD8+ T cells constitute the predominant T cell subset that infiltrated the regressing human tumors (Fig. 5C). Remarkably, this was found in both BALB/c and B6 SCID mice despite the fact that B6 T cells predominantly consist of CD8+ T cells (peripheral blood T cells of BALB/c and B6 mice comprise on average 30% and 80% CD8+ T cells, respectively). Although we cannot rule out an early involvement of CD8+ T cells, our immune histochemistry data suggest that rejection of human tumors in SCID mice is mediated by CD4+ T cells, confirming reports on CD4 dependence of T cell-

mediated xenograft rejections (61, 62). In another series of experiments, we observed that T cell stimulation and to a lesser extent retroviral transduction and sc IL-2 support contributed to the potency and kinetics of xeno-specific responses of mouse T cells (Fig. 6A). T cells that were not stimulated prior to T cell transfer persisted well *in vivo*, but generally lack of T cell stimulation adversely affected T cell numbers in peripheral blood (Fig. 6B). Although T cell stimulation is not necessary for the ultimate rejection of tumors and peripheral T cell persistence per se, it may be of importance in case a sub-optimal TCR is used.

In conclusion, we have demonstrated that, despite reports with CAR transgenes, immune-deficient mice have limited value to assess the therapeutic efficacy of mouse T cells engineered with TCR transgenes. With respect to TCR-engineered human T cells, stimulated with anti-CD3 mAb and cultured with and supported *in vivo* with IL-2, T cell persistence is highly compromised and anti-tumor responses are lacking in immune-deficient mice. These observations are in close accordance with clinical studies, are related to T cell differentiation, and confirm the validity of NSG mice models to test strategies to enhance T cell persistence and anti-tumor efficacy of receptor-engineered human T cells.

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REFERENCES

1. Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R & Adema GJ (2003) Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J Immunol* 170(4):2186-2194.
2. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA & Nishimura MI (1999) Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J Immunol* 163(1):507-513.
3. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P & Bolhuis RL (2000) Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther* 7(16):1369-1377.
4. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA & Schumacher TN (2001) Immunotherapy through TCR gene transfer. *Nat Immunol* 2(10):957-961.
5. de Witte MA, Jorritsma A, Kaiser A, van den Boom MD, Dokter M, Bendle GM, Haanen JB & Schumacher TN (2008) Requirements for effective antitumor responses of TCR transduced T cells. *J Immunol* 181(7):5128-5136.
6. de Witte MA, Bendle GM, van den Boom MD, Coccoris M, Schell TD, Tevethia SS, van Tinteren H, Mesman EM, Song JY & Schumacher TN (2008) TCR gene therapy of spontaneous prostate carcinoma requires in vivo T cell activation. *J Immunol* 181(4):2563-2571.
7. Morris EC, Tsallios A, Bendle GM, Xue SA & Stauss HJ (2005) A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. *Proc Natl Acad Sci U S A* 102(22):7934-7939.
8. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM & Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114(3):535-546.
9. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA & Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314(5796):126-129.
10. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM & Rosenberg SA (2011) T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* 19(3):620-626.
11. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C & Rosenberg SA (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 29(7):917-924.
12. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A & Rosenberg SA (2005) Adoptive cell

transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23(10):2346-2357.

13. Brentjens RJ, Latouche JB, Santos E, Marti F, Gong MC, Lyddane C, King PD, Larson S, Weiss M, Riviere I & Sadelain M (2003) Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med* 9(3):279-286.
14. Brentjens RJ, Santos E, Nikhamin Y, Yeh R, Matsushita M, La Perle K, Quintas-Cardama A, Larson SM & Sadelain M (2007) Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. *Clin Cancer Res* 13(18 Pt 1):5426-5435.
15. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, Gonzalez N, Smith DD, Forman SJ, Jensen MC & Cooper LJ (2006) CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res* 66(22):10995-11004.
16. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, Samanta M, Lakhal M, Gloss B, Danet-Desnoyers G, Campana D, Riley JL, Grupp SA & June CH (2009) Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Mol Ther* 17(8):1453-1464.
17. Cheadle EJ, Gilham DE & Hawkins RE (2008) The combination of cyclophosphamide and human T cells genetically engineered to target CD19 can eradicate established B-cell lymphoma. *Br J Haematol* 142(1):65-68.
18. Savoldo B, Rooney CM, Di Stasi A, Abken H, Hombach A, Foster AE, Zhang L, Heslop HE, Brenner MK & Dotti G (2007) Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30zeta artificial chimeric T-cell receptor for immunotherapy of Hodgkin disease. *Blood* 110(7):2620-2630.
19. Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, Brennan AL, Chew A, Carroll RG, Scholler J, Levine BL, Albelda SM & June CH (2010) Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res* 70(22):9053-9061.
20. Pinthus JH, Waks T, Kaufman-Francis K, Schindler DG, Harmelin A, Kanety H, Ramon J & Eshhar Z (2003) Immuno-gene therapy of established prostate tumors using chimeric receptor-redirected human lymphocytes. *Cancer Res* 63(10):2470-2476.
21. Gade TP, Hassen W, Santos E, Gunset G, Saudemont A, Gong MC, Brentjens R, Zhong XS, Stephan M, Stefanski J, Lyddane C, Osborne JR, Buchanan IM, Hall SJ, Heston WD, Riviere I, Larson SM, Koutcher JA & Sadelain M (2005) Targeted elimination of prostate cancer by genetically directed human T lymphocytes. *Cancer Res* 65(19):9080-9088.
22. Pinthus JH, Waks T, Malina V, Kaufman-Francis K, Harmelin A, Aizenberg I, Kanety H, Ramon J & Eshhar Z (2004) Adoptive immunotherapy of prostate cancer bone lesions using redirected effector lymphocytes. *J Clin Invest* 114(12):1774-1781.
23. Zhao Y, Wang QJ, Yang S, Kochenderfer JN, Zheng Z, Zhong X, Sadelain M, Eshhar Z, Rosenberg SA & Morgan RA (2009) A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *J Immunol* 183(9):5563-5574.
24. Teng MW, Kershaw MH, Moeller M, Smyth MJ & Darcy PK (2004) Immunotherapy of cancer using systemically delivered gene-modified human T lymphocytes. *Hum Gene Ther* 15(7):699-708.
25. Westwood JA, Smyth MJ, Teng MW, Moeller M, Trapani JA, Scott AM, Smyth FE, Cartwright GA, Power BE, Honemann D, Prince HM, Darcy PK & Kershaw MH (2005) Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. *Proc Natl Acad Sci U S A* 102(52):19051-19056.

26. Chekmasova AA, Rao TD, Nikhamin Y, Park KJ, Levine DA, Spriggs DR & Brentjens RJ (2010) Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen. *Clin Cancer Res* 16(14):3594-3606.
27. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM & Foster AE (2010) Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. *J Immunother* 33(8):780-788.
28. Song D, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, Figini M, June CH, Coukos G, Sr. & Powell DJ, Jr. (2011) Improved T cell survival provided by CD137 costimulatory signaling to folate receptor-redirected T cells results in tumor localization and eradication. *Cancer Res*.
29. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I & June CH (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U S A* 106(9):3360-3365.
30. Haynes NM, Trapani JA, Teng MW, Jackson JT, Cerruti L, Jane SM, Kershaw MH, Smyth MJ & Darcy PK (2002) Rejection of syngeneic colon carcinoma by CTLs expressing single-chain antibody receptors codelivering CD28 costimulation. *J Immunol* 169(10):5780-5786.
31. Haynes NM, Trapani JA, Teng MW, Jackson JT, Cerruti L, Jane SM, Kershaw MH, Smyth MJ & Darcy PK (2002) Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors. *Blood* 100(9):3155-3163.
32. Haynes NM, Snook MB, Trapani JA, Cerruti L, Jane SM, Smyth MJ & Darcy PK (2001) Redirecting mouse CTL against colon carcinoma: superior signaling efficacy of single-chain variable domain chimeras containing TCR-zeta vs Fc epsilon RI-gamma. *J Immunol* 166(1):182-187.
33. Darcy PK, Haynes NM, Snook MB, Trapani JA, Cerruti L, Jane SM & Smyth MJ (2000) Redirected perforin-dependent lysis of colon carcinoma by ex vivo genetically engineered CTL. *J Immunol* 164(7):3705-3712.
34. Hwu P, Yang JC, Cowherd R, Treisman J, Shafer GE, Eshhar Z & Rosenberg SA (1995) In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res* 55(15):3369-3373.
35. Xue SA, Gao L, Hart D, Gillmore R, Qasim W, Thrasher A, Apperley J, Engels B, Uckert W, Morris E & Stauss H (2005) Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood* 106(9):3062-3067.
36. Xue SA, Gao L, Thomas S, Hart DP, Xue JZ, Gillmore R, Voss RH, Morris E & Stauss HJ (2010) Development of a Wilms' tumor antigen-specific T-cell receptor for clinical trials: engineered patient's T cells can eliminate autologous leukemia blasts in NOD/SCID mice. *Haematologica* 95(1):126-134.
37. Bobisse S, Rondina M, Merlo A, Tisato V, Mandruzzato S, Amendola M, Naldini L, Willemsen RA, Debets R, Zanovello P & Rosato A (2009) Reprogramming T lymphocytes for melanoma adoptive immunotherapy by T-cell receptor gene transfer with lentiviral vectors. *Cancer Res* 69(24):9385-9394.
38. Van de Griend RJ, Van Krimpen BA, Bol SJ, Thompson A & Bolhuis RL (1984) Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. *J Immunol Methods* 66(2):285-298.
39. Lamers CH, Willemsen RA, van Elzakker P, van Krimpen BA, Gratama JW & Debets R (2006) Phoenix-ampho outperforms PG13 as retroviral packaging cells to transduce human T cells with tumor-specific receptors: implications for clinical immunogene therapy of cancer. *Cancer Gene Ther* 13(5):503-509.

40. Pouw NM, Westerlaken EJ, Willemsen RA & Debets R (2007) Gene transfer of human TCR in primary murine T cells is improved by pseudo-typing with amphotropic and ecotropic envelopes. *J Gene Med* 9(7):561-570.
41. Arden B, Clark SP, Kabelitz D & Mak TW (1995) Human T-cell receptor variable gene segment families. *Immunogenetics* 42(6):455-500.
42. Seynhaeve AL, Hoving S, Schipper D, Vermeulen CE, de Wiel-Ambagtsheer G, van Tiel ST, Eggermont AM & Ten Hagen TL (2007) Tumor necrosis factor alpha mediates homogeneous distribution of liposomes in murine melanoma that contributes to a better tumor response. *Cancer Res* 67(19):9455-9462.
43. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL & Handgretinger R (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 174(10):6477-6489.
44. Kaneko S, Mastaglio S, Bondanza A, Ponzoni M, Sanvito F, Aldrighetti L, Radrizzani M, La Seta-Catamancio S, Provasi E, Mondino A, Nagasawa T, Fleischhauer K, Russo V, Traversari C, Ciceri F, Bordignon C & Bonini C (2009) IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood* 113(5):1006-1015.
45. Wang X, Berger C, Wong CW, Forman SJ, Riddell SR & Jensen MC (2011) Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice. *Blood* 117(6):1888-1898.
46. Appay V, van Lier RA, Sallusto F & Roederer M (2008) Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* 73(11):975-983.
47. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A & June CH (2011) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 3(95):95ra73.
48. Huang J, Khong HT, Dudley ME, El-Gamil M, Li YF, Rosenberg SA & Robbins PF (2005) Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. *J Immunother* 28(3):258-267.
49. Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, Huang J, Powell DJ, Jr. & Rosenberg SA (2004) Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 173(12):7125-7130.
50. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G & Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24(13):e20-22.
51. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA & Hwu P (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 12(20 Pt 1):6106-6115.
52. Porter DL, Levine BL, Kalos M, Bagg A & June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 365(8):725-733.
53. Butler MO, Friedlander P, Milstein MI, Mooney MM, Metzler G, Murray AP, Tanaka M, Berezovskaya A, Imataki O, Drury L, Brennan L, Flavin M, Neuberg D, Stevenson K, Lawrence D, Hodi FS, Velazquez EF, Jaklitsch MT, Russell SE, Mihm M, Nadler LM & Hirano N (2011) Establishment of antitumor memory in humans using in vitro-educated CD8+ T cells. *Sci Transl Med* 3(80):80ra34.
54. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C & Riddell SR (2008) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* 118(1):294-305.

55. Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, Sanchez-Perez L, Muranski P, Kern SJ, Logun C, Palmer DC, Ji Y, Reger RN, Leonard WJ, Danner RL, Rosenberg SA & Restifo NP (2009) Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A* 106(41):17469-17474.
56. Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, ten Hagen T, Verweij J & Debets R (2010) Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother* 59(6):921-931.
57. Pouw N, Treffers-Westerlaken E, Mondino A, Lamers C & Debets R (2010) TCR gene-engineered T cell: limited T cell activation and combined use of IL-15 and IL-21 ensure minimal differentiation and maximal antigen-specificity. *Mol Immunol* 47(7-8):1411-1420.
58. Schaft N, Lankiewicz B, Drexhage J, Berrevoets C, Moss DJ, Levitsky V, Bonneville M, Lee SP, McMichael AJ, Gratama JW, Bolhuis RL, Willemsen R & Debets R (2006) T cell re-targeting to EBV antigens following TCR gene transfer: CD28-containing receptors mediate enhanced antigen-specific IFNgamma production. *Int Immunol* 18(4):591-601.
59. Smyth MJ, Kershaw MH & Trapani JA (1997) Xenospecific cytotoxic T lymphocytes: potent lysis in vitro and in vivo. *Transplantation* 63(8):1171-1178.
60. Hanson HL, Donermeyer DL, Ikeda H, White JM, Shankaran V, Old LJ, Shiku H, Schreiber RD & Allen PM (2000) Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity* 13(2):265-276.
61. Friedman T, Shimizu A, Smith RN, Colvin RB, Seebach JD, Sachs DH & Iacomini J (1999) Human CD4+ T cells mediate rejection of porcine xenografts. *J Immunol* 162(9):5256-5262.
62. Pierson RN, 3rd, Winn HJ, Russell PS & Auchincloss H, Jr. (1989) Xenogeneic skin graft rejection is especially dependent on CD4+ T cells. *J Exp Med* 170(3):991-996.

SUPPLEMENTARY TEXT AND FIGURES

Cytotoxicity assay

Cytotoxic activity of both gp100/A2 TCR and Mock-transduced human T cells against FM3 melanoma cells was measured in standard 5–6 h ^{51}Cr-release assays. Target cells were labelled with 100 μCi $Na_2{}^{51}CrO_4/10^6$ cells for 2 h at 37°C and 5% CO_2. Percentages of specific cytolysis, i.e., specific ^{51}Cr-release, were calculated as described previously (1).

Flow cytometric analysis and enumeration of T and NK cells present in peripheral blood

We determined the differentiation state of TCR T cells by flow cytometry stainings using anti-CD3 (clone SK7), anti-CD8 (clone SK1), anti-CD45RO, (clone UCHL-1), anti-CD45RA (clone L48), anti-CD62L (clone SK11), anti-CD27 (clone M-T271), anti-CD28 (clone CD28.2) and anti-CCR7 (clone 150503) monoclonal antibodies (mAbs). Human T cells and murine NK cells in peripheral blood were detected with anti-CD3 and NK1.1 mAbs (clone PK136), respectively (all mAbs were from BD Biosciences Pharmingen except anti-CCR7 from R&D Systems, Minneapolis, MN) and cell numbers were determined using Flow-Count Fluorospheres (Beckman Coulter, Marseille, France). Samples were analyzed on a FACSCalibur or FACSCanto II (BD Biosciences).

Statistical analysis

Statistically significant differences for T cell and NK cell numbers were calculated with the Students *t* test using GraphPad Prism4 software.

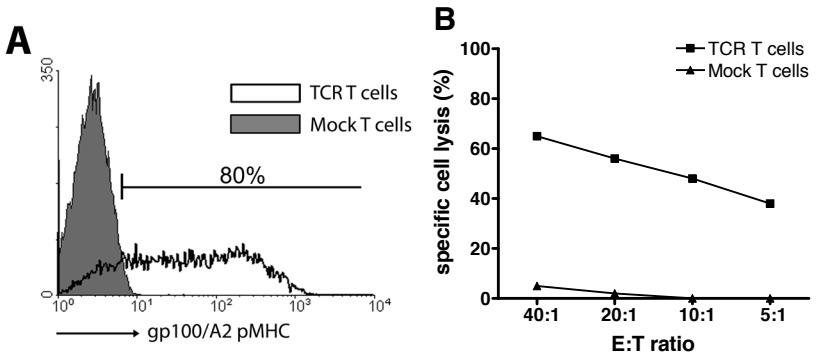

Figure S1. gp100/A2 TCR-transduced human T cells bind pMHC and mediate cytotoxicity at time of AT.

A. TCR-engineered T cells bind pMHC at time of AT. Human T cells were transduced with TCR/GFP or GFP (i.e., Mock) transgenes, FACsorted for gp100/A2 binding and GFP expression and cultured for < 4 weeks using irradiated allogeneic feeder cells (see Materials and Methods section). TCR and Mock T cells were analyzed for gp100/A2 pMHC binding. Histograms represent live-gated cells and percentages indicate

gp100/A2-positive T cells. **B.** TCR-engineered T cells mediate antigen-specific cyotoxicity at time of AT. TCR and Mock T cells were assayed for their ability to lyse FM3 melanoma target cells at different effector to target ratios (E:T ratios). Percentages of specific ^{51}Cr release are indicated in the figure. Results are from a representative experiment out of 3 individual experiments with similar results.

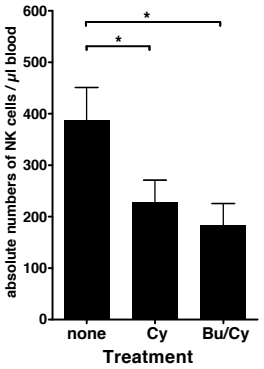

Figure S2. Cyclophosphamide depletes NK cells, that is not further enhanced by Busulfan.
FM3 tumor-bearing B6 SCID mice were pre-conditioned with Cyclophosphamide (Cy, 200 mg/kg) administered i.p. at day -1 (day 0 being the day of AT), preceded or not by Busulfan (Bu, 16.5 µg/kg) administered ip at day -2 (see Materials and Methods for details). Mice without pre-treatment served as a control. At day 0, numbers of NK cells present per µl peripheral blood were quantified by flow cytometry. Data are presented as mean number of NK cells ± SEM, n=6-7 mice per group. * p<0.05.

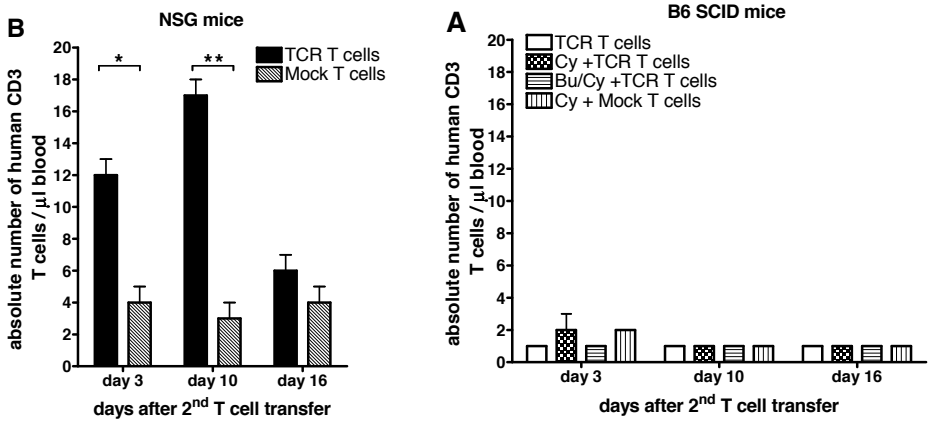

Figure S3. Anti-CD3 mAb-stimulated and IL-2 cultured TCR-transduced human T cells do not engraft in immune-deficient mice.
A. TCR-transduced human T cells do not engraft in B6 SCID mice. FM3 tumor-bearing B6 SCID mice were preconditioned or not with Cyclophosphamide (Cy) or Busulfan+Cyclophosphamide (Bu/Cy) and treated 2 and 9 days later (day 25 and 32 after tumor transplant) with TCR or Mock T cells and IL-2. See Materials and Methods for details on pre-treatment and T cell treatment. **B.** TCR-transduced human T cells do not engraft in NSG mice. FM3 tumor-bearing NSG mice were treated similarly with the exception that NSG mice were not preconditioned. At indicated time points after the 2nd T cell transfer numbers of live-gated human CD3+ T cells were enumerated by flow cytometry. Data are presented as mean numbers of T cells ± SEM, n=6-7 mice per group. * p<0.005; ** p<0.0001.

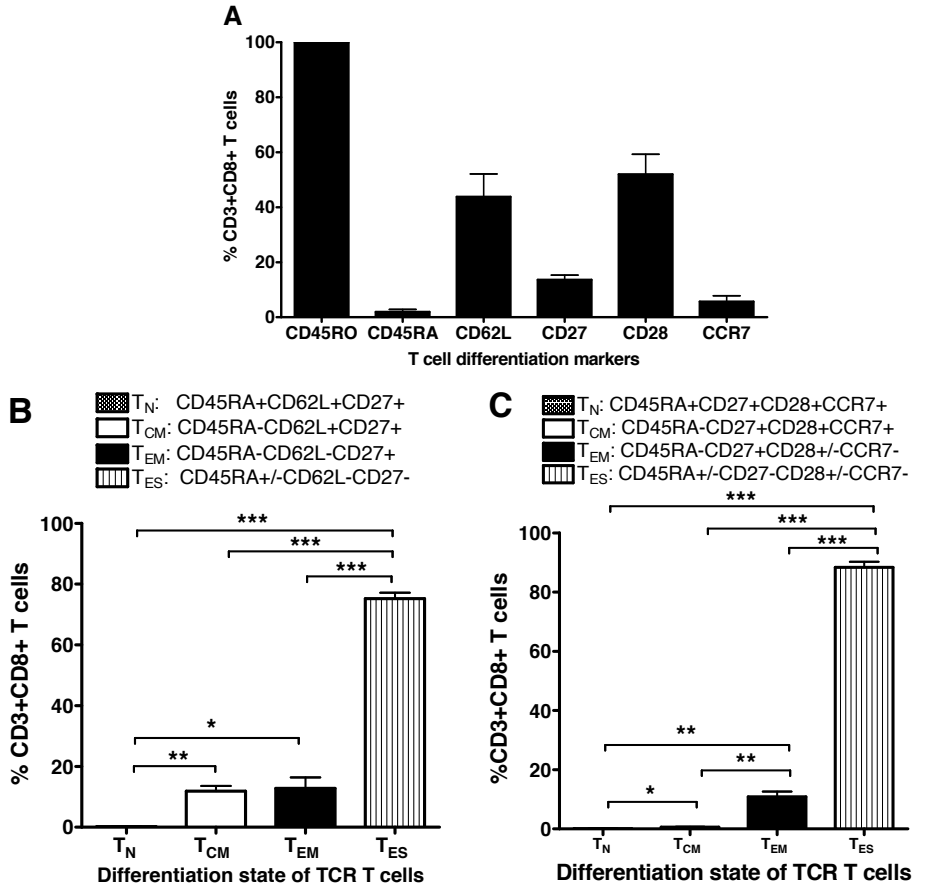

Figure S4. Phenotype of TCR-transduced human T cells corresponds to late T cell differentiation at time of AT.

A. TCR-engineered human T cells analyzed for surface expression of CD45RO, CD45RA, CD62L, CD27, CD28 and CCR7. Human T cells were transduced with TCR transgenes, sorted and cultured as described in the legend to *Supplementary Fig. S1*. Percentages of CD8+ T cells positive for single markers were assessed by flow cytometry. Data represent mean percentages of 3-6 measurements + SEM. **B.** Majority of CD8+ T cells belong to effector memory and end stage T cell subsets according to CD45RA, CD62L and CD27 markers. TCR T cells were stained for CD45RA, CD62L and CD27 and divided into naive (T_N), central memory (T_{CM}), effector memory (T_{EM}) and end stage (T_{ES}) T cell subsets according to definition in insert (2, 3). Percentages reflect live-gated CD8+ T cells and are the mean percentages of 3 experiments + SEM. **C.** The majority of CD8+ T cells belongs to effector memory and end stage T cell subsets according to CD45RA, CD27, CD28 and CCR7 markers. TCR T cells were stained for CD45RA, CD27, CD28 and CCR7 and divided into naive (T_N), central memory (T_{CM}), effector memory (T_{EM}) and end stage (T_{ES}) T cells according to definition in insert (4, 5). Percentages reflect live-gated CD8+ T cells and are the mean percentages of 3 experiments + SEM. * $p<0.05$; ** $p<0.005$; *** $p<0.0001$.

REFERENCES:

1. Weijtens ME, Willemsen RA, Hart EH & Bolhuis RL (1998) A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes. *Gene Ther* 5(9):1195-1203.
2. Kaneko S, Mastaglio S, Bondanza A, Ponzoni M, Sanvito F, Aldrighetti L, Radrizzani M, La Seta-Catamancio S, Provasi E, Mondino A, Nagasawa T, Fleischhauer K, Russo V, Traversari C, Ciceri F, Bordignon C & Bonini C (2009) IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood* 113(5):1006-1015.
3. Wang X, Berger C, Wong CW, Forman SJ, Riddell SR & Jensen MC (2011) Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice. *Blood* 117(6):1888-1898.

Chapter 4

Tumor relapse following T cell receptor gene therapy is not due to loss of immunogenicity

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Manuscript submitted

ABSTRACT

Clinical therapy with TCR-engineered T cells shows promise for cancer patients, but is currently challenged by incomplete responses and tumor relapse. The exact contribution of target antigen expression, and hence the efficacy of TCR-engineered T cells, to the occurrence of tumor relapse is unclear. Here, we treated human gp100-positive tumors with TCR-engineered T cells in immune-competent and HLA-A2 transgenic mice. TCR-engineered T cells prevented tumor growth in 70% of mice and cured tumors in 10-20% of mice, associated with the generation of memory T cells. In 80-90% of mice with established tumors, T cell treatment resulted in significant tumor regression followed by relapse. Relapsed tumors harbored variants that lacked antigen DNA which in all cases were selected from pre-treatment variants. Relapsed tumors that originated from antigen-positive cells harbored intact and non-mutated antigen genes and showed functional expression of surface antigen equal to non-treated tumors. Notably, tumor cures, although not enhanced by a second *in vivo* T cell treatment, are not associated with spread of new antigens. We conclude that tumor relapse is independent of loss or change of immunogenicity, and qualify TCR gene therapy as a valid primary therapy to target relapsed tumors.

INTRODUCTION

Adoptive therapy with autologous tumor-infiltrating T cells (TIL) preceded by non-myeloablative lymphodepletion shows significant clinical responses in melanoma patients that have been unprecedented and are long-lasting (1-3). In an effort to make T cell therapy a more universally applicable and controlled treatment rationale, T cells have been engineered to express tumor-specific T cell receptors (TCR). T cells harboring transgenic TCRs directed against the HLA-A2-restricted antigens MART-I, gp100, CEA or NY-ESO-1 have been tested in clinical trials and clinical responses have been observed in patients with metastatic melanoma, colorectal and synovial carcinoma (4-7). Clinical responses, although variable and based on a relatively small number of patients, are promising but generally lag behind those observed with TILs (2). The clinical use of high avidity TCR-engineered T cells potentially enhances response rates, but when directed against antigens that are over-expressed on tumors and also present on normal tissues results in on-target toxicities with severe inflammation of skin, eyes and ears (for MART-1, gp100) or colon (CEA) (5, 6).

Further development of clinical TCR gene therapy depends not only on specificity and optimization of the TCR transgene (8, 9) but also on a better understanding of the underlying cause of incomplete responses and tumor relapse. To understand incomplete T cell responses, it is of critical importance to reveal the exact contribution of target antigen expression, and its regulation, to the occurrence of tumor relapse. Currently, reports on the role of target antigen in tumor relapse are inconclusive and under debate. Loss of antigen in relapsed tumors has been reported in two separate clinical studies in which selective loss of MART-I expression was observed in relapsed and residual tumors after infusion of MART-I-specific T cells (10, 11). In addition, Dudley and colleagues reported loss of MART-I and HLA-A2 expression in relapsed tumors from patients who showed initial objective responses after treatment with adoptive T cell therapy (2). Also in non-manipulated hosts, decreased antigen expression and immune evasion of tumors may be a consequence of molecular alterations in tumor cells, such as genetic and epigenetic alterations in antigen genes, major histocompatibility complex (MHC) genes and genes related to antigen processing and presentation (12-14). Specifically, in melanoma patients, selective loss of antigen or HLA-A2 expression in primary and metastatic lesions has been described in numerous reports (15-17). Although the above-mentioned examples of antigen loss in human tumors may argue in favor of *in vivo* emergence of escape variants as a result of cancer immunoediting, as eloquently reported and reviewed by Dunn and colleagues (18, 19), formal proof against T cell selection of pre-existing tumor variants is lacking. In fact, loss of antigen in relapsed tumors is challenged in various mouse models, in which it was observed that ineffective T cell responses occured in relapsed tumors that retained expression of both antigen and MHC (20-23).

Here we present an immune-competent model of murine melanoma expressing a human $gp100_{280-288}$/HLA-A2 (gp100/A2) antigen, wherein TCR-engineered T cells are adoptively

transferred and the occurrence and origin of tumor variants are evaluated. Responses of TCR-engineered murine T cells were highly avid, directed against B16 mouse melanoma cells that stably express gp100/A2 antigen, and tested in conditioned HLA-A2 transgenic mice. Maximal T cell pressure did not prevent tumor relapse in the majority of mice, in resemblance to clinical TCR gene therapy trial. Extensive analysis of antigen gene and gene expression identified two variants of tumor cells in relapsed tumors: one that lacked antigen-encoding DNA and was selected from rare pre-treatment variants; and one that contained intact and non-mutated DNA. Antigen was surface-expressed and able to induce T cell responses *ex vivo* at levels that did not differ between relapsed and non-treated tumors. Tumor cures, observed in the minority of mice, are not accompanied by T cell responses directed to new tumor antigens. We conclude that upon T cell therapy, tumor relapse does not neither depend on changes in antigen gene or functional expression of antigen nor involvement of T cell antigens other than the target antigen.

MATERIALS AND METHODS

Cell culture

The human embryonic kidney 293T and Phoenix-Amp cell lines, both used to package retroviruses carrying RNA encoding TCR$\alpha\beta$, the mouse melanoma B16BL6 (B16WT) as well as B16:A2-YLEP and other B16 cell lines expressing gp100 antigen variants (described below and in *Supplementary text*) were grown in DMEM with 10% Fetal Bovine Serum (FBS; Greiner Bio-one Alphen a/d Rijn, The Netherlands), 200nM L-glutamine, 1% MEM non-essential amino acids and antibiotics. Mouse splenocytes were cultured in complete mouse medium (CMM) consisting of RPMI 1640 medium supplemented with 25 mM HEPES, 200 nM L-glutamine, 10% FBS, 1% MEM non-essential amino acids, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, antibiotics and 50 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands).

Mice

The HLA-A2 transgenic mice (kindly provided by prof. François Lemonnier, Paris, France), are β2m$^{-/-}$ and H-2D$^{b-/-}$ and express a chimeric HLA-A*0201 transgene (HHD, referred to as HLA-A2) composed of human β2m, the α1 and α2 domains of HLA-A*0201 and the α3 domain of H-2D^{b} (24). Mice were bred and housed at the Erasmus Medical Center. Experiments were approved by the Experimental Animal Committee of the Erasmus Medical Center and carried out in accordance with institutional and national guidelines.

TCR and antigen constructs and retroviral transduction

TCRα and β genes specific for human glycoprotein (gp)100$_{280\text{-}288}$/HLA-A2 (gp100/A2) were derived from CTL-296 as described earlier (25). TCRα and β genes were murinized (26),

codon optimized (GeneArt, Regensburg, Germany) and cloned into the pMP71 vector (kindly provided by prof. Wolfgang Uckert, Max-Delbrück Center, Berlin, Germany) with TCRα and β genes separated by an optimized T2A ribosome skipping sequence (abbreviated as pMP71:opt TCR β-T2A-α). The A2-YLEP antigen gene, a fusion between HHD (kindly provided by prof. Francois Lemonnier) and the $gp100_{280\text{-}288}$ peptide (YLEPGPVTA), was obtained by inserting DNA that covered the leader sequence of HLA-A2, the gp100 peptide sequence and a $(G_4S)_3$ linker and was flanked by *Eco*RI sites (synthesized by GeneArt) into a pLXSN vector that already contained HHD (pLXSN:A2). The above-mentioned TCR and epitope gene constructs were found to be optimal according to *in vitro* surface expression (by T cells and B16 cells, respectively) and T cell function, and *in vivo* growth kinetics of antigen-positive B16 cells (see *Supplementary Figure 1*), and were used for *in vivo* T cell transfer experiments. See *Supplementary text* for the construction of additional TCR and antigen constructs. All TCR and antigen inserts, once cloned into retroviral vectors, were sequence verified (Service XS, Leiden, The Netherlands)**.**

Total mouse splenocytes were isolated, activated with Concanavalin A and rhIL-2 in CMM, and transduced with the retroviral supernatant as described by Pouw and colleagues (26). T cells were transduced with TCR genes or empty retroviral vector (TCR and Mock T cells, respectively). B16 cells were retrovirally transduced using a similar protocol with the exception that GALV-pseudotyped viruses together with polybrene (4 μg/ml) were used and cells were incubated with the retroviral supernatant for 24h, followed by a second incubation for 8h. The B16:A2-YLEP cells (transgenes: pLXSN:A2-YLEP+pLXSH:gp100) were cultured with neomycin (1 mg/ml) (G418, Calbiochem, La Jolla, CA) and hygromicin (0.5 mg/ml, Clontech Laboratories). Following transduction with TCR and antigen constructs, surface expression levels were validated by flow cytometry and T cell IFNγ production was analyzed by ELISA (for more details see *Supplementary text*). For some *in vivo* experiments, the B16:A2-YLEP cells were single-cell sorted for high HLA-A2 expressing cells on a FACSAria cell sorter using an HLA-A2 antibody (clone BB7.2, Abcam, Cambridge, UK).

Adoptive T cell therapy models

Prevention model

HLA-A2 trangenic mice received a conditioning regimen starting at day -3 prior to tumor transplant with 4 i.p. injections of 16.5 μg/kg Busulphan ea. (Bu) (Duchefa Farma, Haarlem, The Netherlands) (day -3: injections 1 and 2, 6 h interval; day -4: injections 3 and 4, 6 h interval), followed by a single i.p. injection of 200mg/kg Cyclophosphamide (Cy) (Sigma-Aldrich, St Louis, MO) at day -1. The lymphocyte depleting effect was verified as described in *Supplementary text and illustrated in Supplementary Figure 2*. At day 0 mice were injected s.c. with $0.5x10^6$ B16:A2-YLEP cells, followed at day 1 with i.v. injection of 2.5, 7.5 or $20x10^6$ TCR or $20x10^6$ Mock T cells in a volume of 100μl. Numbers of TCR T cells reflect T cells that specifically

bind gp100/A2 pMHC, which covered 70-80 % of total T cells injected. T cell injection was followed by s.c. injections of IL-2 ($1x10^5$ IU per dose) on 4 consecutive days (day 1-4).

Curative model

At day 0 mice were injected s.c. with $0.5x10^6$ B16:A2-YLEP cells and at day 10 and 11 mice received a total of 2 or 4 Bu injections i.p. (16.5 μg/kg ea.), followed a day later by a single i.p. injection of Cy (200 mg/kg). Mice received 7.5 or $20x10^6$ TCR or Mock T cells and IL-2 at day 14 as described for the prevention model above. In some experiments, mice with relapsed tumors received a second treatment with TCR and Mock T cells (see legend to Figure 6 for details).

Tumor growth

Tumor growth was measured by caliper 3 times a week and the tumor volume was estimated with the formula $0.4 \times (A \times B^2)$ where A represents the largest diameter and B the diameter perpendicular to A (27). Animals were sacrificed once the tumor was ulcerated, the largest diameter reached 20 mm, or there was > 20 % loss in body weight.

T cell persistence and memory

Peripheral blood was drawn at day 4 post T cell transfer and at weekly intervals thereafter. Erythrocytes were removed by lysis with NH_4Cl and absolute T cell counts were determined using Flow-Count Fluorospheres (Beckman Coulter, Marseille, France). TCR T cells were monitored using a FITC-labeled anti-TCR-Vβ27 mAb (nomenclature according to www.imgt.org, clone CAS1.1.3, Beckman Coulter) and/or gp100/A2K^b PE–labeled tetramers (generated according to (28), termed gp100/A2 pMHC) in combination with PerCP-labeled anti-CD3ε (clone 145-2C11) and APC-labeled anti-CD8α mAbs (clone 53-6.7) (both from BD Biosciences Pharmingen, San Diego, CA). To detect low frequencies of memory TCR T cells, mice that remained tumor-free for > 90 days after the first tumor challenge (in a prevention setting) were re-challenged with $0.5x10^6$ B16:A2-YLEP cells. Spleens from mice that again remained tumor-free for > 90 days after tumor re-challenge were isolated and amplified for 5 days starting at $2.5x10^6$ cells/ml in the presence of $hgp100_{280-288}$ peptide (0.005 μg/ml) in CMM supplemented with 20 IU/ml IL-2. Spleens from mice that remained tumor-free after tumor regression (i.e. no macroscopic tumor) for > 75 days (in a curative setting) underwent similar procedures. T cells were harvested and stained with gp100/A2 pMHC, anti-CD3ε, anti-CD8α, APC-labeled anti-CD44 (clone IM7) and FITC-labeled anti-CD62L mAbs (clone MEL-14) (all mAb from BD Biosciences Pharmingen). In addition, T cells were stimulated O/N with B16WT and B16:A2-YLEP tumor cells in the presence of Golgi Plug (according to manufacturer instructions, BD Biosciences Pharmingen), stained with anti-CD8α mAb and analyzed for IFNγ production by intracellular cytokine staining with PE-labeled anti-IFNγ mAb (clone XMG1.2, BD Biosciences Pharmingen). All samples were analyzed on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

Molecular characterization and functional expression of target antigen

Tumors were isolated, cut into small pieces and digested in PBS containing collagenase (1 mg/ml) (Sigma-Aldrich) at 37°C for 45 min while vortexing every 10 min. Digestion was stopped by EDTA and the tumor was disrupted over a cell strainer. One to two million tumor cells were frozen down and stored for analyses of A2-YLEP transgene DNA and mRNA. Remaining tumor cells were cultured for 5-7 days, refreshing medium every 2-3 days, and analyzed for HLA-A2 expression by flow cytometry or used as target cells in a T cell stimulation assay. HLA-A2 surface expression on B16 cells was measured using a FITC-labeled anti-HLA-A2 mAb (clone BB7.2). Induction of T cell IFNγ production was measured following co-culture with TCR T cells for 20 hours, after which supernatant was harvested and analyzed for IFNγ levels by ELISA (U-CyTech, Utrecht, The Netherlands). DNA and mRNA isolation, cDNA synthesis and quantitative PCRs were performed as described previously (29, 30). For further analyses, retrovirally introduced antigen transgenes were amplified from tumor DNA (100 ng) using the pLXSN forward primer: 5'-GCCTCCGCCTCCTCTTCCTCCATC-3', the pLXSN reverse primer: 5'-GCTCAGAAGAACTCGTCAAGAAGG-3' and Phusion High-Fidelity DNA polymerase (Thermo Scientific Finnzymes, Vantaa, Finland). PCR products were analyzed by gel electrophoresis and sequencing, which allowed the design of primers and probes and performance of quantitative PCRs to distinguish between antigen transgenes with or without the gp100 peptide sequence (see legend to Figure 5 for details).

Endogenous T cell responses against B16WT

Anti-tumor responses of gp100/A2 TCR T cells that resulted in cured mice were tested for endogenous T cell responses against epitopes other than gp100/A2. To this end, splenocytes ($2x10^6$/ml) were co-cultured with irradiated (160Gy) B16:A2-YLEP tumor cells ($1x10^6$/ml) in a total volume of 1 ml CMM supplemented with 20 IU/ml IL-2 per well in a 24 well plate. After 5-7 days of T cell amplification, cells were harvested and analyzed for TCR expression by pMHC binding and IFNγ production in response to B16:A2-YLEP and B16WT by flow cytometry. Controls included T cell amplifications in the presence and absence of $hgp100_{280-288}$ peptide, and T cell stimulations in the presence and absence of Concanavalin A. T cell responses against B16:A2-YLEP and B16WT tumor cells were defined positive in case T cells show at least 10% pMHC binding and a % IFNγ staining that was at least 2.5 fold higher compared to medium-stimulated T cells (see also legend to Fig.7).

Statistical analyses

Statistical significances were calculated with the Student's *t* test and significant survival differences with the Mantel-Cox test using GraphPad Prism5 software. P-values < 0.05 were considered significant.

RESULTS

TCR T cells prevent or delay tumor growth, persist up to 3 weeks in peripheral blood and induce the generation of memory T cells

The gp100/A2 TCR had been previously validated *in vitro* in human T cells (25, 31) and mouse T cells (26, 32, 33), and T cell responses mediated by this TCR were further optimized to ensure highly avid anti-tumor T cell responses *in vivo*. First, mouse T cells were transduced with murinized gp100/A2 TCRα and β sequences (26) that were codon optimized and cloned into the pMP71 retroviral vector in a TCRβ-2A-α configuration. TCR surface expression and gp100/A2 pMHC-binding by T cells were significantly higher as compared to non-optimized and optimized TCRα and β sequences that were expressed from separate vectors (see *Supplementary text* and *Supplementary Figure 1A*). Second, in order to circumvent poor processing and presenting properties of B16 cells (34), we generated a B16 cell line with stable surface expression of HLA-A2 genetically linked to $hgp100_{280-288}$ peptide (YLEPGPVTA) (termed B16:A2-YLEP; *Supplementary Figure1B*). B16:A2-YLEP cells induced enhanced antigen recognition and IFNγ production by TCR T cells *in vitro* when compared to B16 cells engineered to express HLA-A2 and either wild type or an anchor residue-modified gp100 from separate vectors (see *Supplementary text* and *Supplementary Figure 1C*). *In vivo* growth kinetics of B16:A2-YLEP cells in HLA-A2 transgenic mice were comparable to those of B16WT cells, suggesting that the A2-YLEP antigen did not evoke an endogenous immune response (*Supplementary Figure 1D*). Third, mice were conditioned prior to T cell treatment with combined injections of Busulphan and Cyclophosphamide (Bu/Cy) (set up by VD and NT), which resulted in a clear reduction of absolute numbers of T, B, NK and dendritic cells (*Supplementary Figure 2*).

In a first series of adoptive T cell therapy experiments, we tested prevention of tumor growth in Bu/Cy conditioned HLA-A2 transgenic mice that were transplanted with B16:A2-YLEP cells and treated with various doses of TCR T cells (Figure 1A). In control groups, receiving either Mock or no T cells, the first tumors started to grow 9 days after tumor transplant and all animals developed tumors, whereas in the TCR T cell groups, the first tumors started to grow after 20 ($2.5x10^6$ TCR T cells) and 30 days (7.5 and $20x10^6$ TCR T cells) after tumor transplant (Figure 1B). A significant proportion of animals treated with TCR T cells did not develop tumors; up to 67 % in mice treated with $20x10^6$ TCR T cells. In addition, TCR T cells promoted a dose-dependent survival benefit (*Supplementary Figure 3A*). When assessing persistence of TCR T cells in peripheral blood, we observed that numbers of gp100/A2 pMHC-binding CD8 T cells were highest in mice treated with high numbers of TCR T cells, being most pronounced at early time points after T cell transfer (Figure 1C). Following a period of 3 weeks, frequencies of TCR T cells dropped below the detection level of flow cytometry. To test whether memory T cells were present at low numbers, mice that were tumor-free at day 90 were re-challenged with tumor cells. One third of re-challenged mice remained tumor-free (4 out of 13 mice; data not shown), from which splenocytes were isolated. T cells were amplified *in vitro* with gp100

peptide, after which T cells were detected that bound gp100/A2 pMHC, were predominantly CD62L-negative CD44-positve effector memory T cells and produced IFNγ upon stimulation with B16:A2-YLEP cells (Figure 1D).

Progressive tumors lack antigen DNA

Tumors that progressed despite T cell treatment were analyzed for the presence and quantities of antigen protein, mRNA and DNA. Protein expression, measured by HLA-A2 flow cytometry, was absent (≤2 % of cells were positive in 11 out of 17 mice) or significantly reduced (2 - 20 % of cells were positive in 6 out of 17 mice) on tumor cells from mice treated with TCR T cells (Figure 2A). Strikingly, mRNA and genomic DNA, both measured by quantitative and antigen-specific PCR, were not detected in tumors from mice treated with 7.5 or $20x10^6$ TCR T cells (n=6 and 7, respectively), whereas mRNA and DNA were detected in up to 50 % of mice treated with $2.5x10^6$ TCR T cells (2 out of 4 and 6, respectively) (Figure 2B and C).

TCR T cells mediate highly effective, yet transient regression of established tumors and induce the generation of memory T cells in cured mice

In a second series of adoptive T cell therapy experiments, we tested cure of tumors (average size: 300 mm^3) in mice conditioned with Bu/Cy and treated with $20x10^6$ TCR T cells (Figure 3A). In mice receiving Mock T cells tumors grew, whereas in mice receiving TCR T cells tumors rapidly regressed to volumes that were either not detectable or below 15 mm^3 (Figure 3B). In some mice tumors remained absent, whereas in the majority of mice tumors relapsed by day 30-35 post T cell transfer. TCR T cell treatment did not result in a significant increase in survival (*Supplementary Figure 3B*), which was related to severe treatment-related weight loss in 40-50% of mice and which can be alleviated by decreasing the Bu/Cy dosing (Straetemans et al., Ms in preparation). Numbers of peripheral gp100/A2 pMHC-binding CD8 T cells were increased in the TCR T cell group and were most pronounced at days 4 and 11 after T cell transfer (Figure 3C). In analogy to the preventive setting, frequencies of TCR T cells decreased below the detection level of flow cytometry 2-3 weeks after T cell transfer (Figure 3C). Again, memory T cells were detectable in mice that remained tumor-free >75 days following T cell transfer, as evidenced by detectable gp100/A2 pMHC-binding and IFNγ production upon stimulation with B16:A2-YLEP cells (Figure 3D).

Relapse of antigen-negative tumors depends on presence of antigen-negative tumor cells prior to treatment but *not* loss nor change of antigen DNA

Tumors that relapsed following T cell treatment were analyzed for the presence and quantities of antigen protein, mRNA and DNA. Quantitative analyses of the A2-YLEP antigen revealed a significantly lowered level of protein expression in relapsed tumors (HLA-A2 expression in mice treated with TCR vs Mock T cells: 18 and 48 %, respectively, Figure 4A). Lower protein levels corresponded with reduced levels and in some cases even absence of A2-YLEP-specific

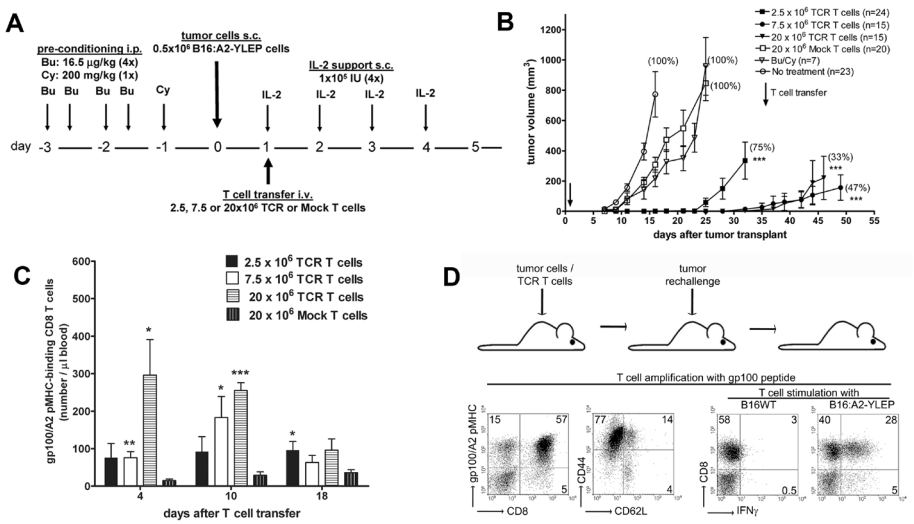

Figure 1. Treatment with gp100/A2 TCR T cells prevents or delays tumor growth and results in the generation of antigen-specific memory T cells

A. Experimental set up of tumor prevention model. HLA-A2 transgenic mice were conditioned with a total of 4 i.p. Bu injections, twice daily on 2 consecutive days, followed by a single i.p. Cy injection prior to s.c. transplantation of 0.5x10^6 B16:A2-YLEP tumor cells at day 0. At day 1 mice were either injected i.v. with gp100/A2 TCR T cells (2.5, 7.5 or 20x10^6 T cells that bind gp100/A2 pMHC), Mock T cells (number of T cells equal to T cells in the 20x10^6 TCR T cell group) or left untreated. **B.** TCR T cells prevent or delay tumor growth. Tumor sizes were measured 3 times a week with a caliper. Data are presented as mean mm^3 ± SEM. The percentages of mice that presented with a tumor are indicated in parentheses. **C.** Absolute numbers of gp100/A2 pMHC-binding CD8 T cells decline within 2-3 weeks following T cell transfer. Peripheral blood was collected from mice at the indicated time points and absolute numbers of gp100/A2 pMHC-binding CD8 T cells were determined by flow cytometry. Data are presented as mean numbers ± SEM (n=8). **D.** Prevention of tumor growth is associated with the generation of memory T cells. Mice that remained tumor-free for ^{3}90 days after tumor challenge were re-challenged with B16:A2-YLEP cells (n=13). Tumor growth was delayed in all re-challenged mice when compared to primary challenged mice and 30 % of mice remained tumor-free until the end of the experiment. Re-challenged and tumor-free mice were sacrificed and spleens were isolated to prepare single cell suspensions. Splenocytes were amplified *in vitro* in the presence of hgp100$_{280\text{-}288}$ peptide for 5 days followed by assessment of gp100/A2 pMHC-binding and expression of the CD44 and CD62L membrane markers. Next, gp100 amplified cells were stimulated O/N in the presence of B16:A2-YLEP or B16WT cells and intracellular IFNγ expression by CD8 T cells was monitored. Representative dot plots from 1 of 4 mice are shown and the % of cells in each quadrant are indicated. Statistical significances were calculated with Student's t-tests: * p<0.05, ** p<0.005, *** p<0.0001.

mRNA and DNA (Figure 4B and C). To better understand the decrease of A2-YLEP DNA levels, we PCR-amplified and sequenced retroviral integrated A2:YLEP genes. Unexpectedly, DNA sequences in 6 out of 9 relapsed tumors, but none of the tumors following treatment with Mock T cells, revealed the presence of HLA-A2 without the gp100 peptide sequence (termed A2 DNA, Figure 5A: (a) band). The A2 DNA sequences in all six samples were identical and showed no mutations other than the omission of peptide and linker sequences. In addition,

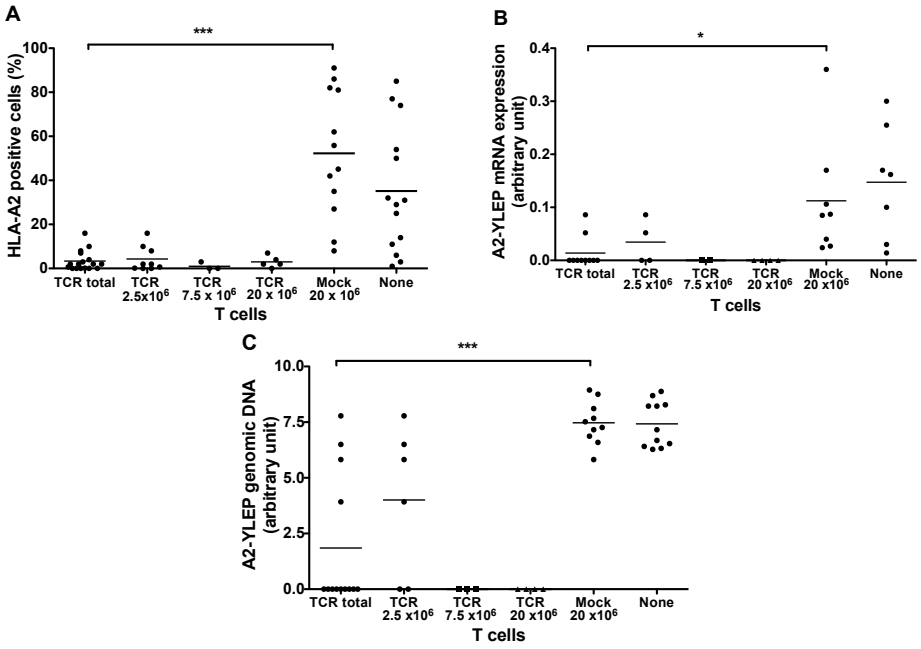

Figure 2. TCR T cells select for tumors lacking antigen DNA

HLA-A2 transgenic mice were conditioned, transplanted with tumor cells and then treated with T cells as described in the legend to Figure 1. Under conditions where tumor diameters ≥ 20 mm, mice were sacrificed, tumors were isolated and single cell suspensions were prepared. **A.** Surface expression of HLA-A2 from individual tumors. Tumors were either treated with TCR T cells (all doses together or individual doses), Mock T cells or not treated. HLA-A2 was measured by flow cytometry and data are presented as % positive cells in viable gate. Horizontal lines represent mean % in each treatment group. **B.** Levels of A2-YLEP mRNA and (**C**) genomic DNA from individual tumors. Levels of mRNA and genomic DNA were measured by quantitative PCR and data are presented relative to the endogenous reference gene TRP2, with lines representing mean levels of mRNA or DNA in each treatment group. TCR (all doses) vs Mock T cells: * $p<0.05$, and *** $p<0.0001$.

a 2nd set of quantitative PCR reagents, which specifically detected A2 without gp100 peptide (Figure 5A, lower part), revealed that levels of A2 and A2-YLEP DNA are inversely related (Figure 5B). The detection of a single variant of antigen-negative DNA, i.e., A2 DNA, with a frequency that appears to be determined by T cell selection against antigen-positive tumor cells suggest A2 tumor cells existed in pre-treated tumors. We have put this notion to the test and assessed tumors derived from B16:A2-YLEP clones, obtained from a single B16:A2-YLEP cell for tumor relapse and antigen DNA. These 'clonal' tumors relapsed with the same number and kinetics when compared with 'cell line' tumors (*Supplementary Figure 4*), but did not contain A2 DNA (Figure 5A and B). Collectively, these data show that A2 tumor cells, present in pre-treatment tumors, became exposed following T cell selection against antigen-positive tumor cells, and have not occurred due to loss or change of antigen DNA.

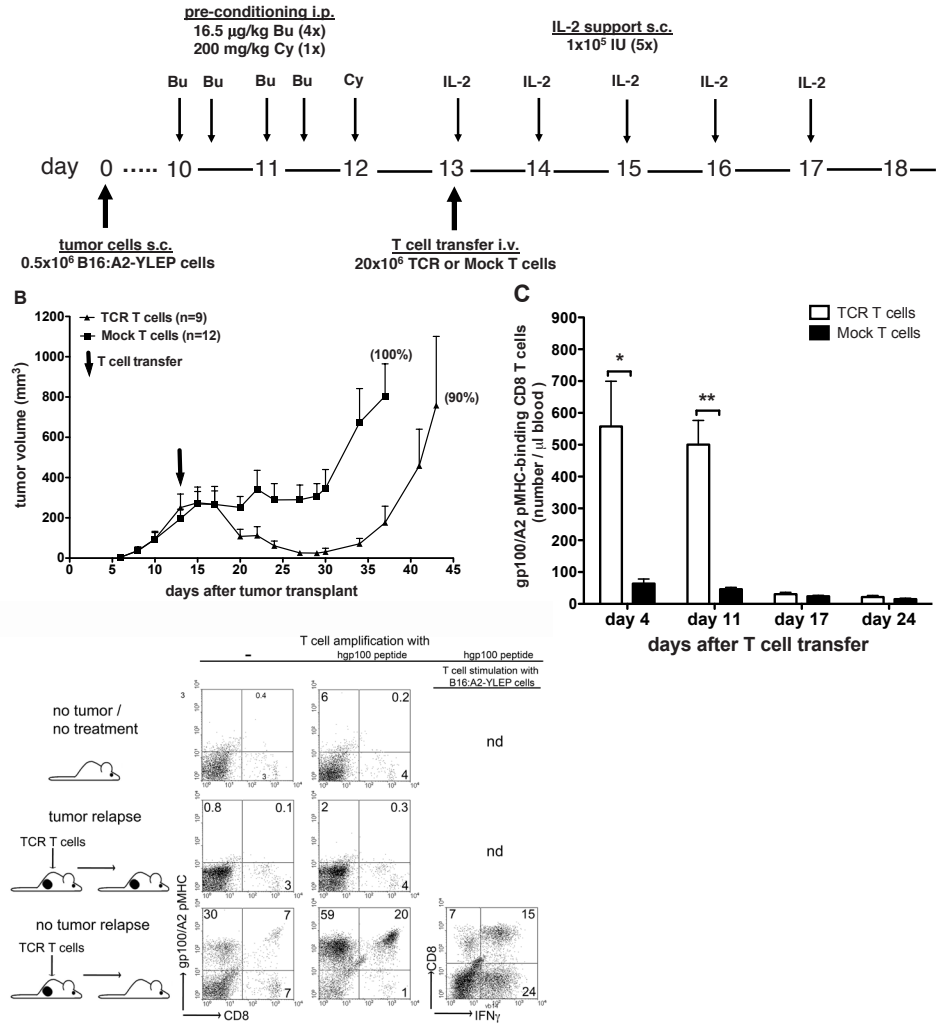

Figure 3. TCR T cells result in transient tumor regression and in the generation of antigen-specific memory T cells in case of durable regression

A. Experimental set up of the curative model. HLA-A2 transgenic mice were s.c. transplanted with 0.5x10^6 B16:A2-YLEP cells at day 0. Ten days later, mice were conditioned as described in the legend to Figure 1. At day 13, 20x10^6 T cells or Mock T cells were injected. **B.** TCR T cells mediate tumor regression. Tumor sizes were measured 3 times a week with a caliper. Data are expressed as mean mm^3 ± SEM and % of mice with tumor relapse are indicated in parenthesis. **C.** Numbers of peripheral blood TCR T cells decline within 2-3 weeks following T cell transfer. Peripheral blood was collected from mice at the indicated time points and absolute numbers of gp100/A2 pMHC-binding CD8 T cells were determined by flow cytometry. Data are presented as mean numbers ± SEM. **D.** Effective T cell responses against established tumors induce the formation of memory T cells. Splenocytes were isolated from tumor-bearing and free mice following transfer of TCR T cells and mice without tumor and not receiving T cell treatment, and cultured *in vitro* in the presence or absence of hgp100$_{280\text{-}288}$ peptide and analyzed for gp100/A2 pMHC binding and IFNγ production (see legend to Figure 1 for details). Representative dot plots showing data from 1 of 4 mice are shown and the % of cells in each quadrant are indicated. TCR vs Mock T cells: * p< 0.05, ** p < 0.005.

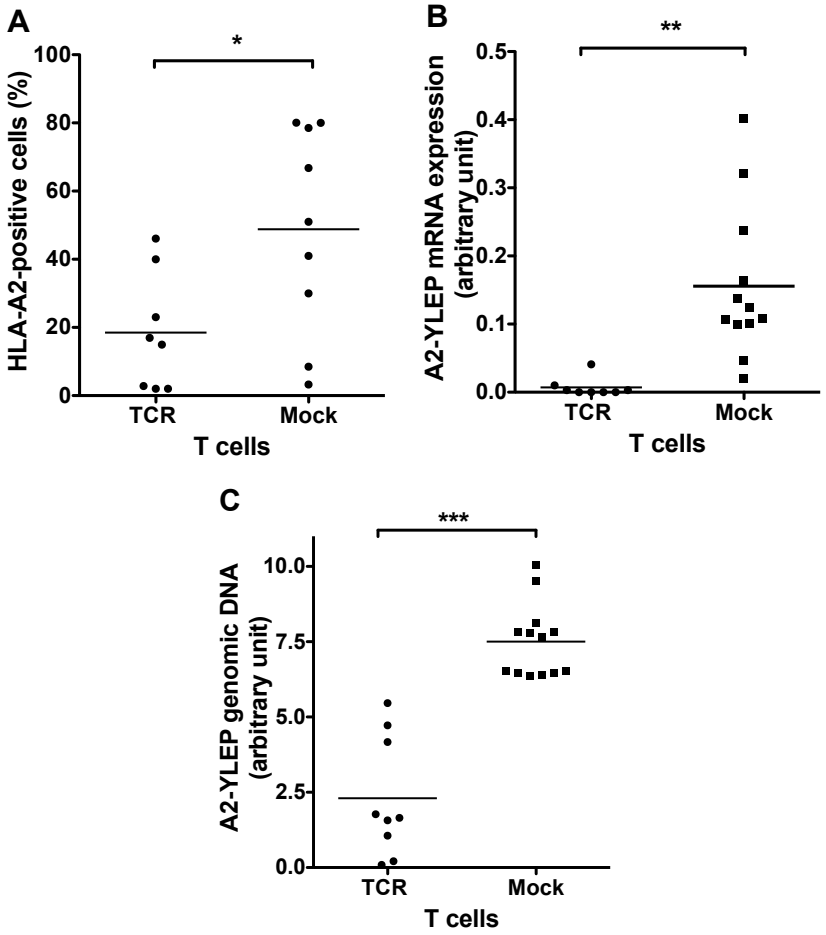

Figure 4. TCR T cell treatment results in the relapse of tumors harboring decreased levels of antigen DNA

HLA-A2 transgenic mice bearing B16:A2-YLEP tumors were conditioned and treated with T cells as described in the legend to Figure 3a. When the largest diameter of relapsed tumors ≥ 20 mm, mice were sacrificed, tumors were isolated and single cell suspensions were prepared. **A.** Surface expression of HLA-A2 from individual tumors. Tumors were either treated with TCR or Mock T cells. HLA-A2 was measured by flow cytometry and data are presented as percentage positive cells in viable gate. The horizontal lines represent mean percentages in each treatment group. **B.** Levels of A2-YLEP mRNA and (**C**) genomic DNA from individual tumors. Levels of mRNA and genomic DNA were measured by quantitative PCR and data are presented relative to the endogenous reference gene TRP2, with lines representing mean levels of mRNA or DNA in each treatment group. TCR vs Mock T cells: *$p<0.05$, **$p<0.005$, and ***$p<0.0001$. Note that individual points in Figs. 4a, b and c do not necessarily reflect identical mice and TCR and Mock T cell-treated mice should be analyzed as a group rather than individually

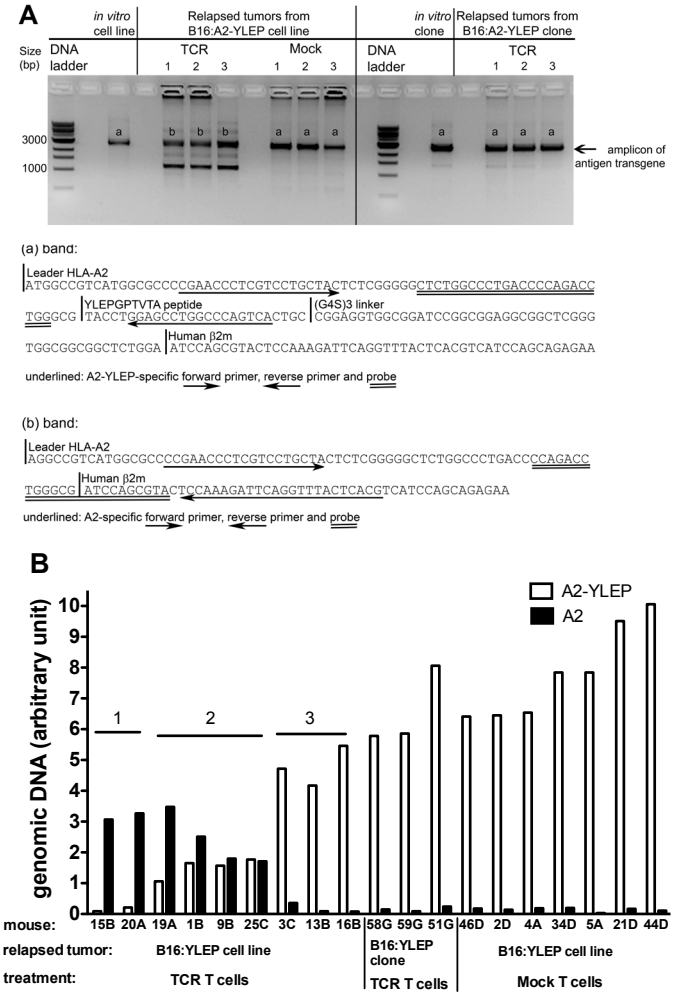

Figure 5. Detection of relapsed tumor variants that lack antigen DNA

A. Relapsed tumors derived from B16:A2-YLEP cell line contain HLA-A2 without the gp100 peptide. PCR was performed to amplify retrovirally introduced A2-YLEP DNA using primers specific for pLXSN flanking sequences (see Material and Methods section). Relapsed tumors were derived from either B16:A2-YLEP cell lines or clones. Upper part: products were subjected to gel electrophoresis and the dominant bands (termed: (a) and (b) bands, indicated by an arrow) were analyzed for their sequences. The (b) band, present in tumors derived from B16:A2-YLEP cell line contained HLA-A2 (A2) without the YLEPGPVTA peptide and $(G_4S)_3$ linker DNA (i.e., 1448 bp A2 +1329 bp retroviral sequences = 2777 bp), whereas the (a) band, present in tumors derived from B16:A2-YLEP clone contained A2-YLEP DNA (i.e., 1523 bp A2-YLEP +1329 bp retroviral sequences = 2852 bp). Tumors treated with Mock T cells or *in vitro* cultures of B16:A2-YLEP cell line or clone, all contained the (a) band with A2-YLEP. Note that the lower bands, only present in relapsed tumors derived from a B16:A2-YLEP cell line, were non-specific and did not contain A2 sequences. Shown are 3 representative samples of B16 cell line tumors treated with TCR (n=9) and Mock T cells (n=12), and samples of B16 clonal tumors treated with TCR T cells (n=3). Lower part: complete sequences of (a) and (b) bands with the different domains indicated and primers/probes, with parts underlined that distinguish between A2-YLEP and A2 DNAs. **B.** Relapsed tumors show inverse relationship

between levels of A2-YLEP and A2 DNA. Quantitative PCR for A2-YLEP and A2 DNA, using the primers/probe combination as depicted in (**A**), was performed on relapsed tumors derived from either B16:A2-YLEP cell line or clone following treatment with TCR T cells (n=9 and 3, respectively), and tumors derived from B16:A2-YLEP cell line following treatment with Mock T cells (n=7). Results identified 3 groups in relapsed tumors derived from B16 cell line. (1) tumors with no or negligible A2-YLEP DNA and harbouring A2 DNA; (2) tumors harboring both A2-YLEP and HLA-A2 DNA; and (3) tumors harboring A2-YLEP DNA and no or negligible A2 DNA.

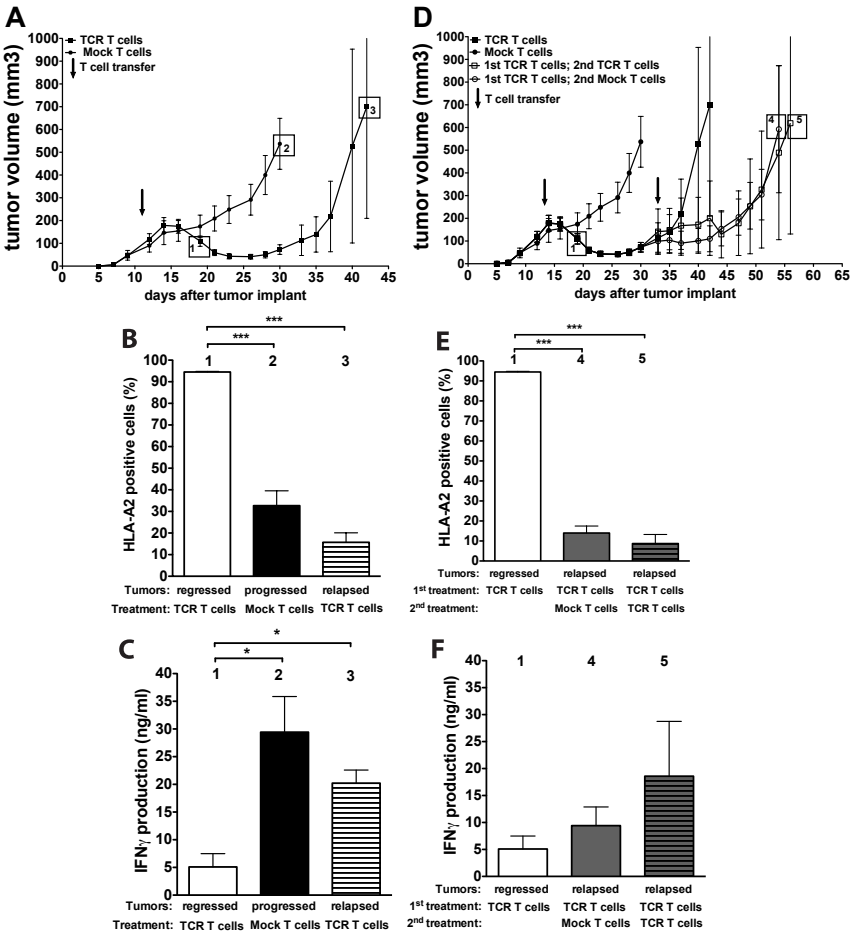

Figure 6. Tumors that relapse after T cell treatment functionally express antigen, but resist 2nd T cell treatment.

A-C. HLA-A2 transgenic mice bearing 10 days established tumors from B16:A2-YLEP clone were conditioned with a total of 2 i.p. Bu injections, on 2 consecutive days, followed by a single i.p. Cy injection and treated with 7.5x10^6 TCR or Mock T cells at day 14. **A.** Following T cell treatment, mice with regressing (TCR T cells, group 1), progressing (Mock T cells, group 2) and relapsing (TCR T cells, group 3) tumors were sacrificed at the indicated time points, tumors were isolated and single cell suspensions were prepared (n=3-5 mice per group). **B.** Tumor cell HLA-A2 surface expression is not different between progressing (Mock T cells) and relapsing (TCR T cells) tumors, but is enhanced in regressing tumors (TCR T cells). HLA-A2 surface expression was measured by flow cytometry and data are presented as mean % positive

cells in the viable gate + SEM. **C.** T cell IFNγ production is not differently induced by progressing or relapsing tumors, but is decreased by regressing tumors. Tumor cells were incubated with TCR T cells *ex vivo* and supernatants were harvested after 20 h and analyzed for ng/ml IFNγ by ELISA. Data are presented as mean ng/ml + SEM. **D-F.** HLA-A2 transgenic mice bearing B16:A2-YLEP tumors were conditioned and treated with T cells as described in the legend to (**A**), with the addition that mice with relapsed tumors were conditioned and treated with TCR T cells for a second time (at days 30-32 and 34, respectively). A second treatment with Mock T cells and no second treatment were included as controls. **D.** Following a second treatment with either Mock (group 4) or TCR (group 5) T cells, mice were sacrificed at the indicated time points, tumors were isolated and single cell suspensions were prepared (n=4-5 mice per group). **E.** HLA-A2 surface expression is not different between relapsed tumors that received a 2nd treatment with either TCR or Mock T cells or tumors that received a single treatment with TCR T cells. **F.** T cell IFNγ production is not differently induced by relapsed tumors that received a 2nd treatment with TCR or Mock T cells. Tumors were analyzed as described in legends to (**B**) and (**C**). Regressing versus progressing or relapsing tumors: *p<0.05.

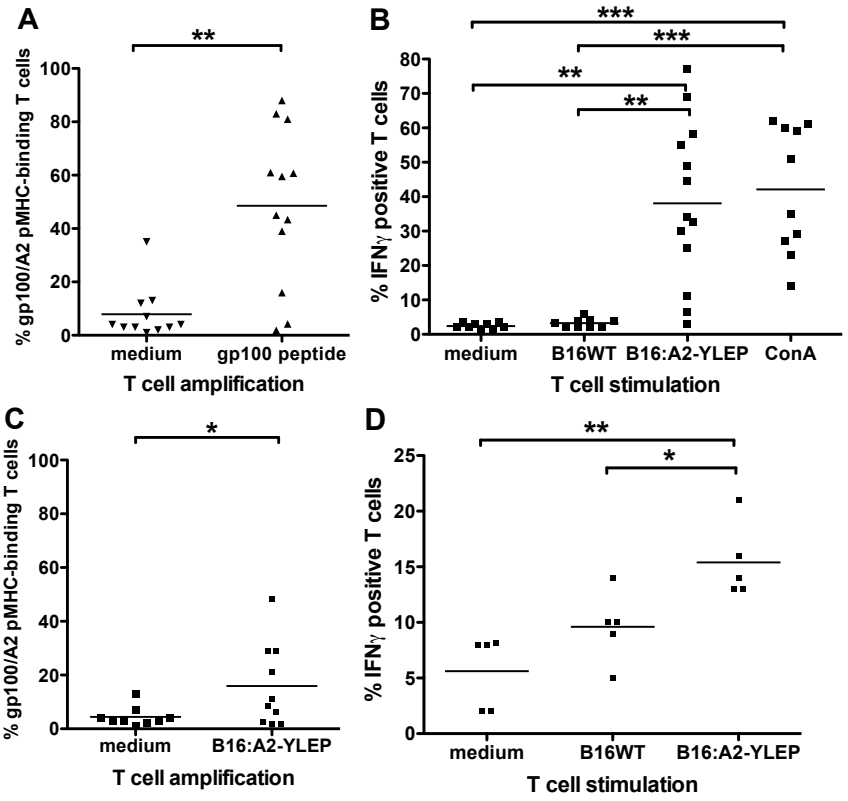

Figure 7. T cell responses in cured mice are directed against gp100/A2 but not to other tumor antigens

A-D. B16:A2-YLEP tumor bearing mice were treated as described in the legend to Figure 6. **A-B**. Gp100/A2 TCR T cells are present and functional in cured mice. **A**. Splenocytes from cured mice were cultured in the presence or absence of $hgp100_{280-288}$ peptide for 5-7 days and analyzed by flow cytometry for the percentage of gp100/A2 pMHC-binding T cells. **B.** T cells amplified in the presence of $hgp100_{280-288}$ peptide were stimulated *in vitro* with B16WT or B16:A2-YLEP for 20 h and analyzed for IFNγ staining by flow cytometry. Medium and Concavalin A were used as negative and positive controls, respectively. **C-D.**

No T cell responses were detected against B16 tumor antigens other than gp100/A2. **C.** Splenocytes from cured mice were amplified in the presence of B16:A2-YLEP cells for 7 days and analyzed by flow cytometry for the percentage of gp100/A2 pMHC-binding T cells. **D.** T cells amplified in the presence of B16:A2-YLEP were stimulated *in vitro* with B16WT or B16-A2-YLEP for 20 hours and analyzed for IFNγ staining by flow cytometry. IFNγ responses were defined positive in case T cells demonstrated at least 10% gp100/A2 pMHC binding and a % IFNγ staining that was at least 2.5 fold higher compared to medium-stimulated T cells. Concavalin A responses ranged between 35 and 60% IFNγ–positive T cells (not shown). Each dot represents an individual mouse with lines indicating mean % pMHC binding and IFNγ staining. Statistical differences were calculated with Sudent's *t* tests: *p< 0.05, **p<0.005, ***p<0.0001.

With respect to antigen-encoding DNA, our analyses identified 3 groups of relapsed tumors from cell line origin (Figure 5B). Group 1 tumors have no or negligible A2-YLEP DNA; group 2 tumors have intermediate levels of A2-YLEP DNA; and group 3 tumors have normal levels of A2-YLEP DNA (comparable to tumors treated with Mock T cells). Group 3 tumors show the same antigen profile as tumors derived from B16:A2-YLEP clones, demonstrating that tumors do relapse despite the exclusive presence of antigen DNA (Figure 5B). A2-YLEP DNAs from clonal tumors that relapsed were completely sequenced and demonstrated no gene mutations in HLA-A2 and gp100 peptide sequences. Next, we tested promoter methylation and expression of antigen gene in relapsed tumors. We observed that the methylation status of the retroviral promoter was not different between relapsed and progressed tumors (*Supplementary Figure 5A*). In addition, relapsed tumors express A2-YLEP mRNA at levels comparable to B16:A2-YLEP tumor cells *in vitro* (*Supplementary Figure 5B*) and, when cultured *ex vivo*, show surface expression of antigen protein (*Supplementary Figure 5C*). Treatment of tumor cells with Azacitidine, a demethylating agent, did not further up-regulate expression of antigen protein, whereas IFNγ treatment and extended culture periods did up-regulate expression of antigen protein. Taken the data so far, we conclude that TCR T cell treatment did not induce genetic or epigenetic changes of the antigen gene and does not compromise expression of antigen gene.

Relapsed tumors functionally express antigen at levels equal to non-treated tumors but resist treatment with TCR T cells

Next, we studied if antigen surface expression was functional in relapsed tumors derived from B16:A2-YLEP clones. Flow cytometric analyses demonstrated that relapsed tumors show equal levels of surface expressed antigen when compared to progressive tumors, i.e. tumors treated with Mock T cells (Figure 6A and B). In contrast to these two types of tumors (20-30% HLA-A2), regressing tumors revealed significantly enhanced levels of surface-expressed antigen (95% HLA-A2, Figure 6B). In addition to percentage of cells, surface-expression of antigen per cell was also significantly increased in regressing tumors (MFI, data not shown). Importantly, progressive and relapsed tumors were able to induce the production of high levels of IFNγ by TCR T cells *ex vivo* (20-30 ng/ml IFNγ), whereas regressing tumors induced the production of significantly lower levels of IFNγ (5 ng/ml, Figure 6C). Given our observation

that relapsed tumors functionally express antigen, we subjected mice with a relapsed tumor following a first T cell treatment to a second T cell treatment. A second cycle of conditioning and T cell transfer delayed tumor growth, which was independent of the TCR transgene (Figure 6D). Consequently, the observed delay in tumor growth was not antigen-specific and most likely the result of Bu/Cy treatment. After a second treatment with TCR T cells, relapsed and progressive tumors again show equal levels of surface expressed antigen (10-20% HLA-A2, Figure 6E) and induce the production of equal levels of IFNγ by TCR T cells (10-20 ng/ml IFNγ, Figure 6F). Despite functionally expressed antigen, relapsed tumors are resistant against T cell therapy.

Tumor cures are not related to T cells directed against new antigens

Besides tumor relapse, observed in the majority of mice, we also observed tumor cures in 10-20% of mice treated with TCR T cells, i.e., no occurrence of tumor relapse > 90 days. To analyze whether effective anti-tumor responses depend on endogenous T cell reactivities against tumor antigens besides the gp100 epitope, we tested splenocytes from cured mice for the presence of T cells reactive towards B16WT tumor cells. Splenocytes were cultured in the presence of gp100 peptide or B16:A2-YLEP cells, thereby amplifying the frequency of tumor-reactive T cells, which resulted in an enhanced number of T cells that bound gp100/A2 pMHC (Figure 7A and C). These T cells also produced enhanced levels of IFNγ upon stimulation with B16:A2-YLEP cells (Figure 7B and D). In contrast, when T cells were amplified in the presence of B16:A2-YLEP and stimulated with B16WT tumor cells, the number of T cells that produced IFNγ was not enhanced (Fig. 7D). From these data, we conclude that T cell responses against B16 tumor antigens other than gp100(YLEP)/A2 did not contribute to tumor cures. This conclusion was corroborated when testing B16:A2-YLEP tumor cells that were either positive of negative for hgp100 protein, i.e. allowing or not allowing cross-presentation of gp100 epitopes other than YLEP. We observed that numbers and kinetics of relapsing tumors were not affected by the presence of human gp100 protein (see *Supplementary Figure 4*).

DISCUSSION

In this study we tested TCR gene therapy in immune-competent mice directed against human gp100/A2 and questioned the contribution of target antigen expression, and its regulation, to the occurrence of tumor relapse. Highly avid TCR-engineered T cells were able to effectively prevent growth of transplanted tumors and regress established tumors, but were not able to prevent tumor relapse. Only a minority of established tumors (about 10-20%) did not re-appear after initial regression. This observation extends data from animal models reporting on tumor recurrence despite the presence of high T cell pressure (35, 36) and clinical trials with metastatic melanoma patients, where tumors relapsed after initial responses

to vaccination (17) or adoptive T cell therapy (2, 10, 11). TCR T cells were only detectable in peripheral blood up to 2 to 3 weeks after T cell transfer, when tumors reached maximal tumor regression. These findings are in agreement with a report by Charo and colleagues that shows that T cell numbers reach a critical level to achieve tumor regression after which T cell numbers contract (37). Complete anti-tumor responses were accompanied by the formation of CD4 and CD8 memory T cells (Figure 1D and 3D). A previous report from our laboratory demonstrated significant ligand-binding affinity of the gp100/A2 TCR used in this study and its ability to redirect CD4 T cells (38), which may explain the finding of both CD4 and CD8 memory T cells.

Tumors that escaped treatment with TCR T cells expressed no or significantly less antigen protein, mRNA and contained less antigen DNA when compared to tumors treated with Mock T cells (Figures 2A, B, 4A and B). Sequence analyses of retrovirally introduced antigen genes identified a single variant of antigen-negative DNA. This HLA-A2 DNA variant was non-mutated except for the lack of gp100 peptide, was found in two-third of relapsed tumors but in none of the progressed tumors and its frequency was inversely related with that of antigen-positive DNA. The existence of a single antigen-negative tumor cell variant with a frequency that appears to be determined by T cell-driven selection against antigen-positive tumor cells suggests that antigen-negative tumor cells were present in tumors prior to T cell treatment. This notion was verified by assessment of relapsed tumors with 'clonal' origins which contained complete and non-mutated antigen-positive DNA but no antigen-negative DNA (Figure 5A and B). We conclude that antigen-negative tumors are selected from rare, yet pre-existing tumor variants and their occurrence does not depend on 'de novo' gene loss or on genetic changes. In fact, extended quantitative PCRs showed that the frequency of A2 tumor cell variants, initially present in the B16:A2-YLEP tumor cell line, are between 1 in 50,000 and 500,000 cells (data not shown). T cell selection as a driving force to determine the antigen profile of tumors, although not a novel concept in itself (39), may have been underappreciated when assessing the genetic status of antigens from heterogeneous tumors or tumor cell lines. In fact, 'antigen-loss' or 'tumor escape' variants may even be misleading terms, and we would prefer to use the term 'antigen-negative' variants unless evidence is provided for active tumor-editing.

Having established that tumor relapse does not depend on changes of antigen gene, we tested gene expression and observed that relapsed tumors express antigen at mRNA and protein levels. In fact, levels of antigen surface expression and abilities to induce IFNγ by TCR T cells *ex vivo* did not differ between relapsed and progressed tumors (Figure 6A and B). Promoter methylation was similar for both types of tumor and Azacitidine, a *de*-methylating drug, was not able to further up-regulate antigen expression *ex vivo* (*Supplementary Figure 5C*). These findings argue against involvement of epigenetic mechanisms to down-regulate antigen expression. Although other studies have demonstrated that methylation of the antigen gene promoter results in progression of antigen-negative variants (36, 40), our data

are in line with previous findings by Ward and colleagues, who found in a mouse model of UV-induced skin cancer that regressor tumors regularly progressed while retaining both MHC class I and the tumor peptide (21). However, despite functional expression of target antigen, we observed that relapsed tumors do not regress and maintain functional antigen expression following a 2nd TCR T cell treatment. Importantly, our observation adds to the cancer immunoediting hypothesis, whereby tumor growth is controlled by T cells until tumor escape produces edited and detectable cancers (18, 19). We postulate that in T cell treated-tumors, loss of immunogenicity does not need to be a driving mechanism in escaping tumors.

The question arises why antigen-positive tumors progress in a setting where T cell responses are highly avid. A first explanation relates to compromised responses of TCR T cells as a consequence of the inhibitory activity of the tumor milieu (41, 42). Indeed, progression of antigen-positive tumors was found in a curative rather than preventive setting. Moreover, regressing tumors revealed a striking discrepancy between enhanced levels of surface-expressed antigen and decreased levels of induced T cell IFNγ production (Figure 6B and C). An ongoing anti-tumor response, often accompanied by an enhanced *in situ* production of IFNγ, may be responsible for increased MHC class I expression in tumor cells, whereas at the same time negative feedback mechanisms may ensue and be responsible for an impaired T cell function. It is well documented that tumors are capable to compromise an anti-tumor T cell response by several mechanisms as reviewed by Rabinovich and colleagues (43). In a model in which immunogenic autochthonous lung tumors are induced in transgenic mice, DuPage and colleagues demonstrated that despite continued antigen expression, T cell infiltration fades and tumors ultimately progress (20). In another inducible model of ovarian carcinoma, Scarlett and colleagues showed that tumors remain immunogenic at advanced stages, but T cell responsiveness weakens as a consequence of tumor-residing inhibitory dendritic cells (23). More recently, the contribution of tumor-induced immune suppression has been added to the cancer immunoediting hypothesis (44). A second explanation, in part overlapping with the first explanation, relates to responses of endogenous T cells. Durable regressions may depend on new T cell specificities, besides YLEP/A2, contributing to the anti-tumor response. Upon an initial immune response, antigen spreading and cross-presentation have both been implicated to broaden the response to tumor antigens (45-51). In tumor-bearing HLA-A2 tg mice that became tumor-free upon treatment with TCR T cells, we were unable to detect T cell responses to B16 antigens other than gp100/A2 (Fig. 7). In addition, we demonstrated that tumor cells that express the complete human gp100 protein, and thus allow cross-presentation of HLA-A2-restricted gp100 epitopes in HLA-A2 tg mice, did not respond differently to TCR T cells when compared to tumor cells that lack human gp100 protein (*Supplementary Figure 4*). Although we formally do not know whether sufficient numbers of the non-YLEP epitopes are available through the mouse antigen processing pathways, these results imply that, at least in our model, T cell specificities towards new and additional antigens do not explain tumor cure.

Relapsed tumors, similar to patient-derived tumors, contain antigen-negative and positive tumor cells. T cells select for pre-existing antigen-negative cells but T cell treatment does *not* result in the occurrence of antigen-negative tumor cells. A supporting therapy to prevent or limit relapse of antigen-negative tumor cells may include the targeting of multiple tumor antigens. In fact, clinical responses observed with adoptive therapy of TILs, containing T cells specific for multiple antigens, are generally better when compared to T cells specific for a single antigen, such as TCR T cells (4-7, 52). For future trials, one may consider to target multiple antigens that contribute to the process of oncogenesis, such as some Cancer Testis Antigens (8). Antigen-positive tumor cells, present in relapsed tumors, show intact antigen genes, no change of promoter methylation and sustained levels of functionally expressed antigen. Complete tumor eradication, observed in the minority of mice, is not accompanied by T cell responses directed to tumor antigens other than the target antigen. A supporting therapy to prevent or limit relapse of antigen-positive tumor cells may include potentiation of T cell responses. In example, clinical trials that target T cell inhibitory molecules, such as CTLA-4 and PD-1, enhanced anti-tumor T cell responses and demonstrated clinical benefit (43, 53), and such strategies may well be combined with TCR gene therapy. A model for the role of target antigen in tumor relapse after TCR T cell treatment, summarizing our observations, is illustrated in Figure 8. Overall, we have demonstrated that tumor relapse does *not* depend on loss of immunogenicity, but rather on the strength of initial T cell selection. Therefore, TCR gene therapy qualifies as a valid primary therapy to target antigen-positive tumor cells in relapsed tumors.

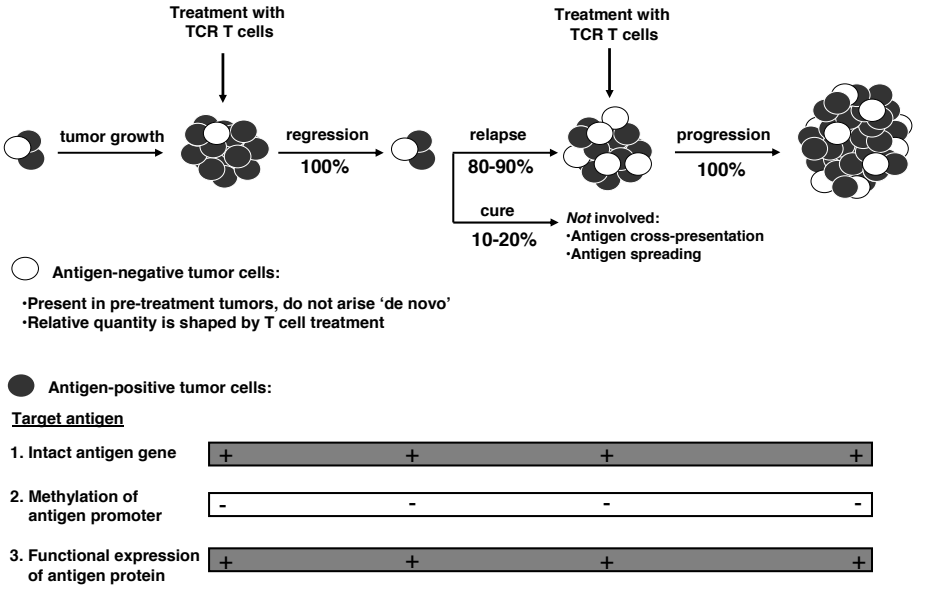

Figure 8. Expression of target antigen in relapsed tumors after TCR T cell treatment
Treatment of established tumors with TCR T cells results in rapid tumor regression. Tumor regression is durable in a small proportion of mice and is associated with the formation of memory T cells, but in most mice tumors relapse. Antigen-negative tumor cells (white cells) are present in tumor cells prior to treatment, and do not arise as a consequence of T cell treatment. The relative quantity of antigen-negative tumor cells in relapsed tumors, however, is determined by the strength of the T cell response against antigen-positive tumor cells. The relative quantity of antigen-negative tumor cells in relapsed tumors may vary between tumors, not shown for reasons of simplicity. Antigen-positive tumor cells (black cells) show continued antigen expression, which was confirmed by three sets of data: 1) antigen gene remains intact (i.e., antigen gene shows no mutations nor truncations); 2) antigen gene shows no change in methylation status; 3) antigen protein is expressed at non-changed levels and induces potent T cell responses *ex vivo*. In the present model, relapsed tumors are not cured by a second T cell treatment *in vivo*, and tumor cures are not related to T cell responses towards new tumor antigens neither as a consequence of antigen spread nor antigen cross-presentation. Overall, TCR gene therapy qualifies as a valid primary therapy to target tumor cells in relapsed tumors. Supporting therapies to prevent or limit relapse of antigen-negative and positive tumor cells may include targeting multiple tumor antigens and potentiating of T cell responses, respectively. See Discussion for details.

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REFERENCES

1. Besser MJ, Shapira-Frommer R, Treves AJ, Zippel D, Itzhaki O, Hershkovitz L, Levy D, Kubi A, Hovav E, Chermoshniuk N, Shalmon B, Hardan I, Catane R, Markel G, Apter S, Ben-Nun A, Kuchuk I, Shimoni A, Nagler A & Schachter J (2010) Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 16(9):2646-2655.
2. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A & Rosenberg SA (2005) Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23(10):2346-2357.
3. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE & Dudley ME (2011) Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* 17(13):4550-4557.
4. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA & Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314(5796):126-129.
5. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM & Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114(3):535-546.
6. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM & Rosenberg SA (2011) T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* 19(3):620-626.
7. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C & Rosenberg SA (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 29(7):917-924.
8. Coccoris M, Straetemans T, Govers C, Lamers C, Sleijfer S & Debets R (2010) T cell receptor (TCR) gene therapy to treat melanoma: lessons from clinical and preclinical studies. *Expert Opin Biol Ther* 10(4):547-562.
9. Govers C, Sebestyen Z, Coccoris M, Willemsen RA & Debets R (2010) T cell receptor gene therapy: strategies for optimizing transgenic TCR pairing. *Trends Mol Med* 16(2):77-87.
10. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E & Greenberg PD (2002) Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* 99(25):16168-16173.

11. Mackensen A, Meidenbauer N, Vogl S, Laumer M, Berger J & Andreesen R (2006) Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. *J Clin Oncol* 24(31):5060-5069.
12. Algarra I, Cabrera T & Garrido F (2000) The HLA crossroad in tumor immunology. *Hum Immunol* 61(1):65-73.
13. Marincola FM, Jaffee EM, Hicklin DJ & Ferrone S (2000) Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* 74:181-273.
14. Seliger B, Maeurer MJ & Ferrone S (2000) Antigen-processing machinery breakdown and tumor growth. *Immunol Today* 21(9):455-464.
15. Maeurer MJ, Gollin SM, Martin D, Swaney W, Bryant J, Castelli C, Robbins P, Parmiani G, Storkus WJ & Lotze MT (1996) Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. *J Clin Invest* 98(7):1633-1641.
16. Wang Z, Marincola FM, Rivoltini L, Parmiani G & Ferrone S (1999) Selective histocompatibility leukocyte antigen (HLA)-A2 loss caused by aberrant pre-mRNA splicing in 624MEL28 melanoma cells. *J Exp Med* 190(2):205-215.
17. Khong HT, Wang QJ & Rosenberg SA (2004) Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother* 27(3):184-190.
18. Dunn GP, Bruce AT, Ikeda H, Old LJ & Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 3(11):991-998.
19. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ & Schreiber RD (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450(7171):903-907.
20. DuPage M, Cheung AF, Mazumdar C, Winslow MM, Bronson R, Schmidt LM, Crowley D, Chen J & Jacks T (2011) Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression. *Cancer Cell* 19(1):72-85.
21. Ward PL, Koeppen HK, Hurteau T, Rowley DA & Schreiber H (1990) Major histocompatibility complex class I and unique antigen expression by murine tumors that escaped from CD8+ T-cell-dependent surveillance. *Cancer Res* 50(13):3851-3858.
22. Cheung AF, Dupage MJ, Dong HK, Chen J & Jacks T (2008) Regulated expression of a tumor-associated antigen reveals multiple levels of T-cell tolerance in a mouse model of lung cancer. *Cancer Res* 68(22):9459-9468.
23. Scarlett UK, Rutkowski MR, Rauwerdink AM, Fields J, Escovar-Fadul X, Baird J, Cubillos-Ruiz JR, Jacobs AC, Gonzalez JL, Weaver J, Fiering S & Conejo-Garcia JR (2012) Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. *J Exp Med*.
24. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA & Perarnau B (1997) HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. *J Exp Med* 185(12):2043-2051.
25. Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R & Adema GJ (2003) Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J Immunol* 170(4):2186-2194.
26. Pouw NM, Westerlaken EJ, Willemsen RA & Debets R (2007) Gene transfer of human TCR in primary murine T cells is improved by pseudo-typing with amphotropic and ecotropic envelopes. *J Gene Med* 9(7):561-570.

27. Seynhaeve AL, Hoving S, Schipper D, Vermeulen CE, de Wiel-Ambagtsheer G, van Tiel ST, Eggermont AM & Ten Hagen TL (2007) Tumor necrosis factor alpha mediates homogeneous distribution of liposomes in murine melanoma that contributes to a better tumor response. *Cancer Res* 67(19):9455-9462.
28. Wooldridge L, van den Berg HA, Glick M, Gostick E, Laugel B, Hutchinson SL, Milicic A, Brenchley JM, Douek DC, Price DA & Sewell AK (2005) Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. *J Biol Chem* 280(30):27491-27501.
29. Lamers CH, Gratama JW, Pouw NM, Langeveld SC, Krimpen BA, Kraan J, Stoter G & Debets R (2005) Parallel detection of transduced T lymphocytes after immunogene therapy of renal cell cancer by flow cytometry and real-time polymerase chain reaction: implications for loss of transgene expression. *Hum Gene Ther* 16(12):1452-1462.
30. Strijbos MH, van Krimpen BA, Debets R, Kraan J, Sleijfer S, Gratama JW & Lamers CH (2010) mRNA levels of CD31, CD144, CD146 and von Willebrand factor do not serve as surrogate markers for circulating endothelial cells. *Thromb Haemost* 104(2):318-326.
31. Willemsen RA, Sebestyen Z, Ronteltap C, Berrevoets C, Drexhage J & Debets R (2006) CD8 alpha coreceptor to improve TCR gene transfer to treat melanoma: down-regulation of tumor-specific production of IL-4, IL-5, and IL-10. *J Immunol* 177(2):991-998.
32. Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, ten Hagen T, Verweij J & Debets R (2010) Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother* 59(6):921-931.
33. Pouw N, Treffers-Westerlaken E, Mondino A, Lamers C & Debets R (2010) TCR gene-engineered T cell: limited T cell activation and combined use of IL-15 and IL-21 ensure minimal differentiation and maximal antigen-specificity. *Mol Immunol* 47(7-8):1411-1420.
34. Seliger B, Wollscheid U, Momburg F, Blankenstein T & Huber C (2001) Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. *Cancer Res* 61(3):1095-1099.
35. Bai XF, Liu JQ, Joshi PS, Wang L, Yin L, Labanowska J, Heerema N, Zheng P & Liu Y (2006) Different lineages of P1A-expressing cancer cells use divergent modes of immune evasion for T-cell adoptive therapy. *Cancer Res* 66(16):8241-8249.
36. Goldberger O, Volovitz I, Machlenkin A, Vadai E, Tzehoval E & Eisenbach L (2008) Exuberated numbers of tumor-specific T cells result in tumor escape. *Cancer Res* 68(9):3450-3457.
37. Charo J, Perez C, Buschow C, Jukica A, Czeh M & Blankenstein T (2011) Visualizing the dynamic of adoptively transferred T cells during the rejection of large established tumors. *Eur J Immunol* 41(11):3187-3197.
38. Varela-Rohena A, Molloy PE, Dunn SM, Li Y, Suhoski MM, Carroll RG, Milicic A, Mahon T, Sutton DH, Laugel B, Moysey R, Cameron BJ, Vuidepot A, Purbhoo MA, Cole DK, Phillips RE, June CH, Jakobsen BK, Sewell AK & Riley JL (2008) Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nat Med* 14(12):1390-1395.
39. Khong HT & Restifo NP (2002) Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 3(11):999-1005.
40. Kmieciak M, Knutson KL, Dumur CI & Manjili MH (2007) HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses. *Eur J Immunol* 37(3):675-685.
41. Willimsky G & Blankenstein T (2005) Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature* 437(7055):141-146.

42. Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 5(4):263-274.
43. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I & Topalian SL (2010) Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 28(19):3167-3175.
44. Schreiber RD, Old LJ & Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331(6024):1565-1570.
45. Corbiere V, Chapiro J, Stroobant V, Ma W, Lurquin C, Lethe B, van Baren N, Van den Eynde BJ, Boon T & Coulie PG (2011) Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res* 71(4):1253-1262.
46. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, Jungbluth A, Gnjatic S, Thompson JA & Yee C (2008) Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 358(25):2698-2703.
47. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG & Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *J Exp Med* 201(2):249-257.
48. Ribas A, Butterfield LH, Glaspy JA & Economou JS (2003) Current developments in cancer vaccines and cellular immunotherapy. *J Clin Oncol* 21(12):2415-2432.
49. Schietinger A, Philip M, Liu RB, Schreiber K & Schreiber H (2010) Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *J Exp Med* 207(11):2469-2477.
50. Spiotto MT, Rowley DA & Schreiber H (2004) Bystander elimination of antigen loss variants in established tumors. *Nat Med* 10(3):294-298.
51. Thomas DL, Kim M, Bowerman NA, Narayanan S, Kranz DM, Schreiber H & Roy EJ (2009) Recurrence of intracranial tumors following adoptive T cell therapy can be prevented by direct and indirect killing aided by high levels of tumor antigen cross-presented on stromal cells. *J Immunol* 183(3):1828-1837.
52. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, Thomasian A, Downey SG, Smith FO, Klapper J, Morton K, Laurencot C, White DE & Rosenberg SA (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 26(32):5233-5239.
53. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A & Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363(8):711-723.

SUPPLEMENTARY TEXT AND FIGURES

Additional TCR and antigen constructs

Murinized gp100 specific TCRα and β genes (gp100/A2 TCR) have been previously described by Pouw and colleagues (1), were codon optimized (GeneArt), and cloned separately in the pBullet retroviral vector (2) (vectors are abbreviated as pB:TCRα+β and pB:optTCRα+β). These two TCR-containing pB vectors, together with pMP71:optTCRβ-T2A-α (see Materials and Methods section) were tested in mouse T cells for TCR surface expression and TCR-mediated functions *in vitro* (see *Supplementary Figure 1a, c*). HHD cDNA (kindly provided by prof. François Lemonnier, Paris, France) and human gp100 cDNA (kindly provided by prof. Gosse Adema, Nijmegen, The Netherlands) were subcloned as *Xho*I fragments into the retroviral vectors pLXSN and pLXSH respectively (Clontech Laboratories Inc., Mountain View, CA) (termed pLXSN:A2 and pLXSH:gp100). In addition to our standard used B16:A2-YLEP cell line (transduced with pLXSN:A2-YLEP+pLXSH:gp100, see Materials and Methods section), we have transduced B16 cells with pLXSN:A2-YLEP but not pLXSH:gp100 (termed B16A2-YLEP w/o gp100) that were used in some *in vivo* experiments. We obtained a pLXSH:gp100AV variant containing an altered $gp100_{280\text{-}288}$ peptide ligand with improved HLA-A2 binding (3) by replacing the peptide's anchor alanine residue by a valine in by site-directed mutagenesis (QuikChange II kit, Stratagene, Cedar Creek, TX). B16:A2/gp100 (transgenes: pLXSN:A2+pLXSH:gp100) and B16:A2/gp100AV cells (transgenes: pLXSN:A2+pLXSH:gp100AV) were cultured in the presence of both neomycin and hygromycin. These HLA-A2 and gp100-containing B16 cell lines, together with B16:A2-YLEP cells (see Materials and Methods section) were tested for antigen expression and induction of T cell functions *in vitro* (see *Supplementary Figure 1b, c*). HLA-A2 surface expression was detected using an anti-HLA-A2 mAb (clone BB7.2) and intracellular hgp100 was detected using an anti-hgp100 mAb (clone NKI-beteb, Abcam) followed by staining with a PE-labeled goat anti-mouse Ig (H+L) (SouthernBiotech, Birmingham) and analysed by flow cytometry. IFNγ, produced by TCR T cells after 20h incubation with the different B16 cell lines, was measured in the supernatant by ELISA.

Testing lymphodepleting potential of Bu/Cy conditioning

HLA-A2 transgenic mice were Bu/Cy conditioned as described in the Materials and Methods section. At day 4 after the start of treatment blood was drawn from conditioned and unconditioned control mice. Following lysis of erythrocytes with NH_4Cl absolute cell numbers of lymphocyte subsets were determined using Flow-Count Fluorospheres (Beckman Coulter). Cells were stained with the following mAbs: CD3-PerCP, CD8-APC, CD4-FITC (clone L3T4), CD19-PE (clone 1D3), NK1.1-PE (clone PK136) and CD11c-APC (clone HL3) (all BD Pharmingen) and analyzed by flow cytometry (see *Supplementary Figure 2)*.

Assessment of promoter methylation

Genomic DNA was isolated from relapsed (TCR T cell treated) and progressed (Mock T cell treated) tumors as described in the Materials and Methods section and treated with sodium bisulphite (EZ DNA Methylation, Zymo research; Irvine, CA) according to the manufacturer's protocol. Methylation-specific PCR was performed on treated DNA using primers specific for either unmethylated (U) or methylated (M) DNA sequences of the 5' LTR promoter of the pLXSN provirus, upstream of the antigen gene. Primer sequences were: U forward primer, 5'-TGTGTTTTATTTGAATTAATTAATTAGTTT-3'; U reverse primer, 5'-CACAATCTATCAAAAAAC-TAACACC-3'; M forward primer, 5'- TTTTATTTGAATTAATTAATTAGTTCGT-3'; and M reverse primer, 5'- GCAATCTATCGAAAAACTAACGC-3'. PCR products analyzed by gel electrophoresis (see *Supplementary Figure 5a*).

Antigen mRNA and genomic DNA

Quantitative PCRs were performed to assess levels of antigen mRNA and genomic DNA. Levels were calculated using the formula $2^{-\Delta CT}$, in which C_T is defined as the cycle number at which the amplified fluorescence signal crosses a pre-set threshold and ΔC_T is defined as the difference between the C_T value of the A2-YLEP gene and that of an endogenous reference gene, namely TRP2. Sequences of primers and probes used to quantify levels of A2-YLEP and A2 DNAs are provided in Figure 5a. Sequences of primers and probe to quantify TRP2 DNA were: (forward primer) 5'-TAATTGTGGAGGCTGCAAGTTC-3'; (reverse primer) 5'-AGGATGGCC-GGCTTCTTC-3'; and (probe) FAM-5'-CTGGACCGGCCCCGACTGTAATC-3' (Applied Biosystems, Foster City, CA). Detection was monitored with an MX3000P real-time PCR System using MX Pro data analysis (Stratagene, La Jolla, CA).

Ex vivo treatments of tumor cells to up-regulate antigen expression

Relapsed tumors were isolated, single cell suspensions were prepared and cells were cultured *ex vivo* for 5-7 days (see Materials and Methods section). Tumor cells were subjected to 3 different treatments. First, Azacitidine (AZA) (Sigma-Aldrich) was added to the culture media at day 4 for 3 consecutive days (final concentration 2-10μM). Second, mouse IFNγ (100 Units/ml) (Sanquin) was added at day 5 and tumor cells were left untreated for 48. Third, tumor cells were cultured for extended periods of 14-21 days. Following treatment, tumor cells were analyzed for HLA-A2 expression by flow cytometry (see *Supplementary Figure 5c*).

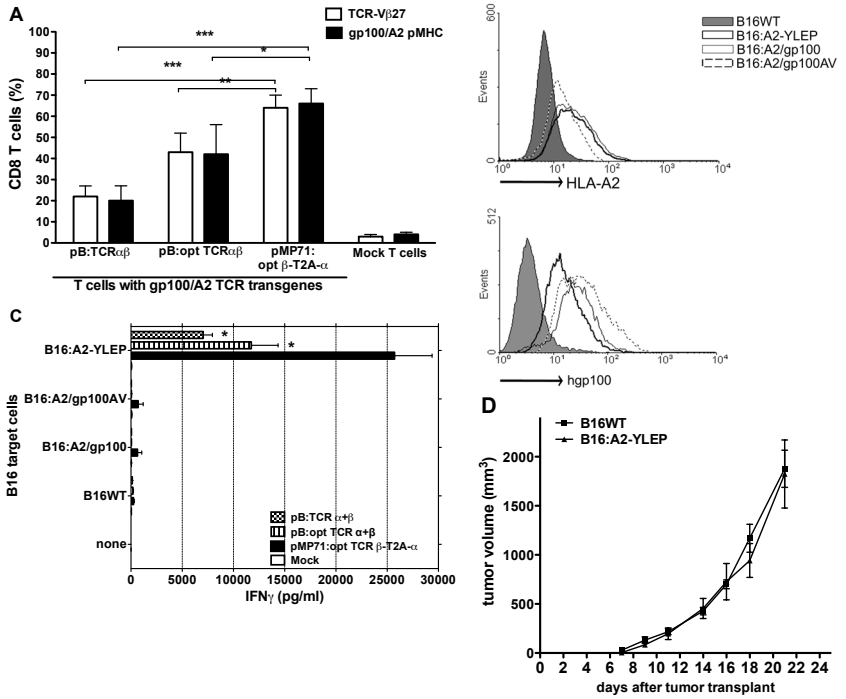

Figure S1. Validation of TCR and antigen gene constructs

A. Murine splenocytes gene-engineered to express gp100/A2 TCRαβ. Murine splenocytes were transduced with the following gp100/A2-specific TCR constructs: pB:TCRα+β (two separate pB vectors with wild type sequences for TCRα and TCRβ); pB:optTCRα+β (two separate pB vectors with codon optimized sequences for TCRα and TCRβ); and pMP71:optTCR β–T2A-α (single pMP71 vector with codon optimized TCRαβ in a β-T2A-α cassette). See Materials and Methods section and *Supplementary text* for details on the construction of TCR constructs. At day 5 following T cell activation CD8 T cells were analyzed for TCR-Vβ27 expression (white bars) and gp100/A2 pMHC binding (black bars) by flow cytometry. Data represent mean % ± SEM, n=5 separate transductions. **B.** B16 melanoma cells gene-engineered to express HLA-A2 and human gp100. B16 cells were transduced with the following gp100/A2 antigen constructs: pLXSN:A2+pLXSH:gp100 (B16:A2/gp100 cells, dashed histogram); pLXSN:A2+pLXSH:gp100AV (B16:A2/gp100AV cells, black histogram) and pLXSN:A2-YLEP+pLXSH:gp100 (B16:A2-YLEP cells, bold black histogram). See Materials and Methods section and *Supplementary text* for details on the construction of antigen constructs. Tumor cells were stained with a FITC-labeled anti-HLA-A2 or anti-hgp100 mAb followed by a PE-labeled anti-mouse Ig and analyzed by flow cytometry. Non-transduced B16WT cells were used as a negative control (grey filled histogram). **C.** IFNγ production by TCR-engineered T cells in response to gp100/A2-positive B16 cells *in vitro*. TCR-transduced splenocytes (from S1a) were incubated with different populations of B16 target cells (from S1b) and supernatants were harvested after 20 hours and analyzed for murine IFNγ. Data are presented as mean pg/ml IFNγ + SEM, n=5. **D.** B16 cells that express the A2-YLEP antigen are not immunogenic *in vivo*. B16:A2-YLEP or B16WT cells ($0.5x10^6$) were injected s.c. into HLA-A2 transgenic mice. Tumor volumes were measured 3 times a week with a caliper and data are presented as mean mm^3 ± SEM, n=5. No significant differences were observed between the *in vivo* growth kinetics of B16 cell lines with or without A2-YLEP. Statistical differences were calculated with Student's *t* tests: *p<0.05, **p<0.01, *** p<0.0001.

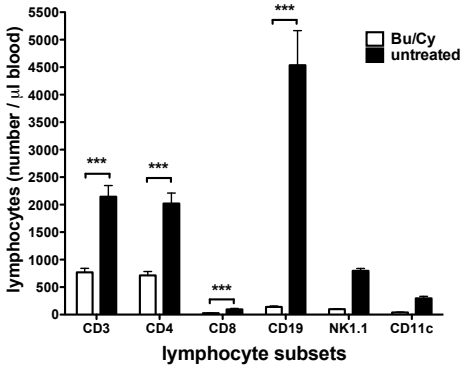

Figure S2. Busulphan and cyclophosphamide conditioning results in effective lymphodepletion *in vivo*

HLA-A2 transgenic mice were conditioned with Bu/Cy as described in the Materials and Methods section (n=24 mice). As a control, HLA-A2 transgenic mice were left untreated (n=8). Peripheral blood was collected one day after conditioning and analyzed for different lymphocyte subsets by flow cytometry (for NK1.1 and CD11c, n=2). Data are displayed as mean numbers ± SEM. Statistical differences were calculated with Student's *t* tests. ***p<0.0001.

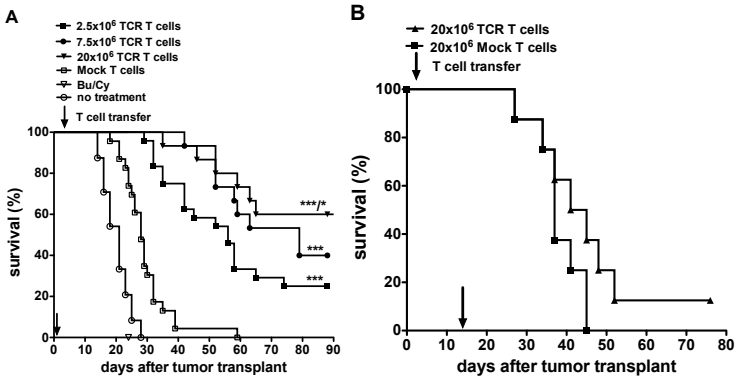

Figure S3. TCR T cells provide a survival advantage in a preventive but not a curative setting

A. Gp100/A2 TCR T cells significantly enhance survival in a preventive model. HLA-A2 transgenic mice were treated as described in the legend to Figure 1a. **B.** Gp100/A2 TCR T cells did not result in a survival advantage in a curative model. Mice were treated at day 13 with $20x10^6$ TCR T cells as described in the legend to Figure 3a. In both **A** and **B**, mice were sacrificed when the largest tumor diameter reached ≥ 20 mm, when tumors started to bleed or when mice lost 20% of the initial body weight. Statistical significances between TCR and Mock T cells and among different doses of TCR T cells (the latter preceded by /) were calculated with the Mantel-Cox test. *** p<0.0001; $20x10^6$ vs $2.5x10^6$ TCR T cells: * p<0.05; and $20x10^6$ vs $7.5x10^6$ TCR T cells: not significant.

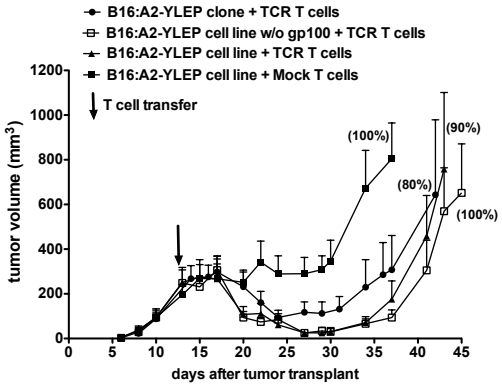

Figure S4. Numbers and kinetics of tumor relapse are not affected by presence of antigen-negative tumor variants nor cross-presentation of gp100 antigens.

HLA-A2 transgenic mice were transplanted s.c. at day 0 with $0.5x10^6$ cells of either a B16:A2-YLEP cell line that expresses wild type gp100 protein (B16:A2-YLEP), a B16:A2-YLEP cell line and clone that do not express wild type gp100 protein (i.e., B16:A2-YLEP w/o gp100 or B16:A2-YLEP clone, respectively). Once tumors were established at day 10, mice were conditioned and received $20x10^6$ TCR or Mock T cells at day 14 (see legend to Figure 3a for details). Tumor sizes were measured 3 times a week with a caliper. Data are expressed as mean $mm^3 \pm$ SEM, n=8, and % of mice with tumor relapse are indicated in parentheses.

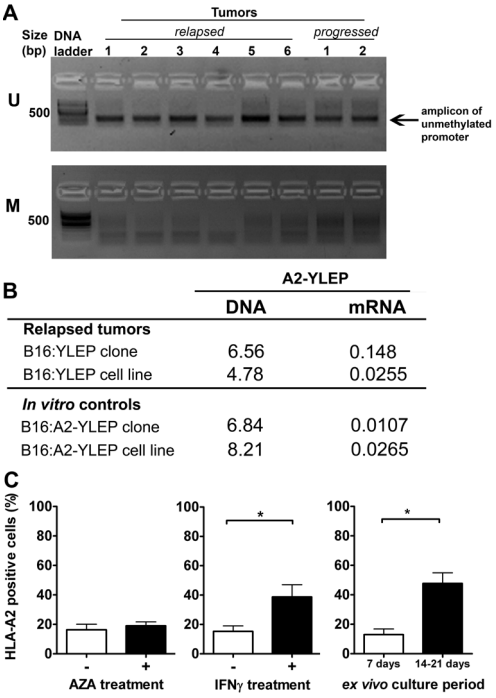

Figure S5. In relapsed tumors there was no methylation of antigen gene or further up-regulation of antigen protein expression by Azacitidine.

HLA-A2 transgenic mice bearing B16:A2-YLEP tumors were preconditioned and treated with TCR or Mock T cells as described in the legend to Figure 3a. **A.** Relapsed and progressed tumors derived from B16:A2-YLEP clones were analyzed for methlyation of the antigen gene promoter. See *Supplementary text* for details on assessment of promoter methylation. Following methylation-specific PCR, products were analyzed by gel electrophoresis. U and M denote PCR products specific for unmethylated and methylated promoter sequences, respectively. Only in case of U, the expected amplicon of 120 bp is detected in both relapsed and progressed tumors. **B.** Relapsed tumors derived from either B16:A2-YLEP clone or cell line (the latter corresponding to group 3, Fig. 5b) were analyzed for levels of A2-YLEP mRNA and DNA as described in the legend to Figure 5. Levels of mRNA and DNA of relapsed tumors were comparable with those of *in vitro* cultured B16:A2-YLEP clone or cell line. mRNA and DNA levels are presented relative to the endogenous reference gene TRP2, with n= 3-4 per group. **C.** Surface antigen expression of freshly isolated tumor cells was up-regulated by *ex vivo* treatment with IFNγ (n=11) or prolonged culture times (n=4), but not by the de-methylating agent Azacitidine (AZA) (n=9). See *Supplementary text* for details on ex vivo treatments. HLA-A2 was measured by flow cytometry and data are presented as mean % positive cells in viable gate + SEM. Statistical differences were calculated with Student's *t* tests: * $p < 0.05$.

REFERENCES

1. Pouw NM, Westerlaken EJ, Willemsen RA & Debets R (2007) Gene transfer of human TCR in primary murine T cells is improved by pseudo-typing with amphotropic and ecotropic envelopes. J Gene Med 9(7):561-570.
2. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P & Bolhuis RL (2000) Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. Gene Ther 7(16):1369-1377.
3. Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA & Kawakami Y (1996) Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J Immunol 157(6):2539-2548.

Chapter 5

TCR gene transfer: MAGE-C2/HLA-A2 and MAGE-A3/HLA-DP4 epitopes as melanoma-specific immune targets

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ABSTRACT

Adoptive therapy with TCR gene-engineered T cells provides an attractive and feasible treatment option for cancer patients. Further development of TCR gene therapy requires the implementation of T cell target epitopes that prevent 'on-target' reactivity towards healthy tissues and at the same time direct a clinically effective response towards tumor tissues. Candidate epitopes that meet these criteria are MAGE-$C2_{336-344}$/HLA-A2 (MC2/A2) and MAGE-$A3_{243-258}$/HLA-DP4 (MA3/DP4). We molecularly characterized TCR$\alpha\beta$ genes of a MC2/A2-specific CD8 and MA3/DP4-specific CD4 T cell clone derived from melanoma patients who responded clinically to MAGE vaccination. We identified MC2/A2 and MA3/DP4-specific TCR-Vα3/Vβ28 and TCR-Vα38/Vβ2 chains and validated these TCRs in vitro upon gene transfer into primary human T cells. The MC2 and MA3 TCR were surface expressed and mediated CD8 T cell functions towards melanoma cell lines and CD4 T cell functions towards dendritic cells, respectively. We intend to start testing of these MAGE-specific TCRs in a phase I clinical trial.

INTRODUCTION

Adoptive therapy with antigen-specific T cells has shown clinical successes in the treatment of viral infections and tumors (1-5). Receptor gene therapy, in which patients are treated with T cells gene-engineered with either chimeric antigen receptors (CAR) or T cell receptors (TCR), provides an attractive alternative to provide therapeutic immunity. Clinical application of gene-engineered T cells to treat various tumor types, such as renal cell cancer, ovarian cancer, neuroblastoma, lymphoma, melanoma, colorectal and synovial cancers proved feasible but did, despite some successes, generally not show anti-tumour responses in a substantial number of patients (6-13). Notably, in an early clinical trial to treat metastatic renal cell cancer with CAR-engineered T cells, with total T cell doses as low as $2x10^8$ T cells, we observed reversible yet discrete cholangitis and damage to bile duct epithelium as a likely consequence of T-cell localization and expression of the taget epitope Carbonic Anhydrase IX (CAIX) on normal tissue (6). Subsequent trials with CARs directed against Her2/Neu and CD19 and TCRs directed against the HLA-A2-restricted antigens MARTI, gp100 and CEA have confirmed this notion (11, 12, 14, 15). Collectively, these studies underscore the need for T cell target epitopes that are expressed on malignant tissue in a highly restricted manner and are able to initiate a clinically effective T cell response.

Cancer testis antigens (CTA) are immunogenic proteins expressed in many tumors but silenced in normal cells except for male germline cells, placenta and thymic medullary epithelial cells (16, 17). *In vitro* studies have provided initial proof that gene transfer of TCR$\alpha\beta$ directed against MAGE-A1/HLA-A1, MAGE-A3/HLA-A2, NY-ESO-1/HLA-A2 as well as NY-ESO-1/HLA-DP4 result in effective and CTA-specific T cell responses (18-21). Of the group of CTA, in particular the MAGE antigens constitute attractive candidates for immune therapy given not only tumour-specific expression but also their role in tumour biology, expression in multiple tumours and potential to constitute effective T cell targets. Four families of MAGE genes are located on chromosome X: *MAGE-A (12 genes), B (6 genes)*, *C (4 genes) and D (2 genes)*. Up to now there are over 50 identified combinations of MAGE peptides and HLA class I or class II molecules, recognized by CD8 or CD4 T cells, respectively (see for an overview: cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). We propose the MAGE-$C2_{336-344}$ /HLA-A2 (MC2/A2) peptide ALKVDVEERV and MAGE-$A3_{243-258}$/HLA-DP4 (MA3/DP4) peptide KKLLTQHFVQENYLEY as candidate T cell targets for the following reasons. *First*, MC2 and MA3 proteins actively contribute to the development of malignancies. MC2 suppresses p53-dependent apoptosis, thus prolonging tumor survival (22, 23), whereas MA3 mediates fibronectin-controlled progression and metastasis (24), and is expressed by melanoma stem cells (25, 26). *Second*, MC2 and MA3 are expressed in multiple tumor types and their expression is associated with poor clinical outcome in these tumor types (27-32). MC2 is expressed in 43% of metastatic melanomas, 33% of head and neck squamous cell cancers, 30% of bladder cancers, and 10% of non-small cell lung cancers (28). MA3 is expressed in 76% of metastatic melanomas (27),

in up to 50% of non-small cell lung cancer (29), and in many other tumor types such as colon rectal, hepatocyte cellular, prostate and breast cancers and haematological

l malignancies such as multiple myeloma (30, 33-36). Furthermore, HLA-A2 and HLA-DP4 are the most frequent MHC class I and II alleles among Caucasians, i.e., representing 44 and 75% of the general population, respectively. And *third*, MC2 and MA3 potentially constitute clinically effective T cell target epitopes, as evidenced by induction of enhanced numbers of anti-MAGE T cells that paralleled significant and durable clinical responses (37, 38).

The clinical potential of MC2-specific T cells is exemplified by a high frequency of MC2/A2-specific CTL (10^{-4} of CD8 T cells) observed in the blood of a melanoma patient whose tumors regressed after vaccination with MAGE-A1 and A3 peptides, whereas in the same patient the frequency of anti-vaccine CTLs was low ($3x10^{-6}$ of CD8 T cells) (37). A CTL clone recognizing this epitope (EB81-CTL16) was isolated that demonstrated the most pronounced increase in frequency not only in blood but also in a regressing cutaneous metastasis (> 100 and 1000-fold, respectively). Interestingly, the same patient also showed increased frequencies in blood and a regressing metastasis (up to 200-fold) of other T cell clones specific for the same and other MC2 epitopes (39). In a second melanoma patient who showed tumor regression upon MAGE vaccination, the most frequent anti-tumor CTL clone was again directed against a MC2 epitope (40). With respect to MA3, various trials have been performed substantiating its clinical potential as a T cell target. A phase II clinical trial with highly purified MA3 protein in non-small cell lung cancer showed a significant reduction in relative risk of cancer recurrence following surgery in vaccinated versus placebo-treated patients (41). This MA3 vaccine provided B cell responses, CD8 T cell responses as well as HLA-DP4-restricted CD4 T cell responses against the MA3 KKL epitope in lung cancer patients (42, 43). Recently, a phase III trial started to investigate the efficacy of MA3 antigen vaccination after tumor resection in lung cancer patients (44). Also in melanoma patients, MA3 protein vaccinations using either protein or MA3-expressing PBMC initiate antigen-specific immune responses (45). Vaccinations with dendritic cells loaded with MA3/DP4 peptide rapidly induced peptide-specific T helper cell responses in melanoma patients. Median survival in vaccinated patients was longer than in untreated control patients and showed no signs of major toxicities due to vaccination (46) and personal communication (Gerold Schuler, Erlangen, Germany).

In this study, we chose $MC2_{336-344}$/A2 and $MA3_{243-258}$ /DP4 as T cell epitopes, and cloned and characterized the corresponding TCR$\alpha\beta$ genes of CD8 and CD4 T cell clones derived from two metastatic melanoma patients who responded clinically to MAGE-vaccination. TCR$\alpha\beta$ genes were then introduced into primary human T cells, and tested for surface expression and MAGE-specific CD8 and CD4 T cell functions *in vitro*.

MATERIALS AND METHODS

Melanoma patients EB81 and R12 and patient-derived T cell clones

In a vaccination study metastatic melanoma patient EB81 received cutaneous vaccinations with recombinant canarypox (ALVAC) virus, carrying a minigene encoding antigenic MAGE-A1and A3 peptides that are presented by HLA-A1. These were followed by vaccinations with the same peptides. One year after the first vaccination all cutaneous metastases had disappeared, and the patient remained tumor-free for 3 years (37). CTL-606C/22.2 (EB81-CTL 16) is a cytotoxic CD8 T cell clone derived from EB81 whose increase in frequency is most pronounced in various body compartments upon vaccination with MAGE; and it recognizes $MC2_{336-344}$/HLA-A2 (ALKVDVEERV) (37). Melanoma patient 12 was included in a clinical trial in which mature monocyte-derived dendritic cells loaded with multi-HLA class I and II peptides (including MAGE-$A3_{243-258}$ peptide) were administered subcutaneously (38). CD4 T cell clone R12-C9, recognizing $MA3_{243-258}$/HLA-DP4 (KKLLTQHFVQENYLEY), was derived from PBMC from melanoma patient 12, after in vitro stimulation with $MA3_{243-258}$/DP4 peptide and sorting on IFNγ secreting CD4+ T cells by FACSVantage™ flow cytometer (BD Biosciences) as described earlier (46). CTL clones 16 and R12-C9 were cultured in IMDM with 10% human serum, glutamine and antibiotics**.**

Other cells and general reagents

PBMC from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm^3; Amersham Pharmacia Biotech, Uppsala, Sweden). Transduced primary human T cells were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 200 mM L-glutamine, 10% human serum, antibiotics and 360 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands) and stimulated every 2 weeks with a mixture of irradiated allogeneic feeder cells as described elsewhere (47). The human embryonic kidney cell line 293T and Phoenix-Ampho both used to package retroviruses carrying RNA encoding TCRαβ, were grown in DMEM with 10% Fetal Bovine Serum (FBS; Greiner Bio-one Alphen a/d Rijn, The Netherlands), glutamine, antibiotics and 1% MEM non-essential amino acids. The same medium plus supplements was used to grow the melanoma cell lines EB-81-MEL-2 (MC2/A2^{pos}) and MZ2-MEL43 (MA3/DP4^{pos}). An MC2^{neg}/A2^{pos} and MA3^{neg}/DP4^{pos} B lymphoblast cell line (BSM) and an EBV transformed HLA-DP4^{pos} B cell line (i.e. EBV-MAGJ) transduced with retrovirus encoding Ii-MA3 cDNA as described in (48) (i.e. EBV-MA3) were cultured in RPMI supplemented with glutamine, antibiotics and 10% FBS. The melanoma cell line EB-81-MEL-2 and the B cell line EBV-MAGJ were derived from the same patients from who the T cell clones were derived (melanoma patient EB-81 and patient 12, respectively). In some cases target cells were pre-treated with 50 pg/ml human recombinant IFNγ (Peprotech, Rocky Hill, NJ) for 48h prior to functional T cell assays.

MC2/A2 peptide MHC (pMHC) complexes were ordered from Proimmune (Oxford, UK). MA3/DP4 pMHC complexes were produced in S2-drosophila insect cells, essentially as described previously (46). We used the following mAbs: anti-CD4 (clone 13 B8.2, BD Biosciences, Erembodegem, Belgium), anti-CD8 (clone SK1, BD Biosciences) and anti-TCR-Vβ2 mAbs (clone MPB 2D5, Immunotech, Marseille, France). Other reagents used were the HLA-A2 binding peptides $MC2_{336-344}$ (ALKVDVEERV) and (as a control) $gp100_{280-288}$ (YLEPGPVTA), the HLA-DP4 binding peptide $MA3_{243-258}$ (KKLLTQHFVQENYLEY) (all three from Eurogentec, Maastricht, The Netherlands), Phytohemaglutinin (PHA) (Remel Ltd, Lenexa, KS), Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, St. Louis, MO), GM-CSF, IL-4, TNFα (all three from PeproTech) and PGE2 (Sigma-Aldrich).

MAGE-A3 protein

MA3 protein was expressed by the Des insect cell expression system (Invitrogen, Breda, The Netherlands). To this end, MA3 cDNA was cloned into the pMT/BiP/V5-His vector and, together with the pCoHygro vector, introduced into S2-insect cells by nucleofection (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's guidelines. MA3 protein expression by transfected S2 cells, at a density of $3x10^6$/ml, was induced by Copper Sulfate (500μM). Five days after induction of protein expression, culture medium was harvested and soluble MA3 protein was purified by FPLC (Acta, GE Healthcare, Zeist, The Netherlands) using a histrap column, followed by size exclusion on a sephadex 75 column.

Genes encoding TCRαβ specific for MC2/A2 and MA3/DP4

RNA was isolated from T cell clones EB81-CTL16 and R12-C9 and reverse transcribed with Superscript III (Invitrogen) according to the manufacturer's instructions. The TCR-Vα and Vβ regions were amplified and family-typed using a set of sense primers, covering all variable segments, in combination with either a TCR-Cα or Cβ antisense consensus primer. Nested PCR was performed on TCR-Vα and Vβ products before gel-electrophoresis. Primers specific for Cβ1 or Cβ2 were used to discriminate between both Cβ genes. Positive PCR products were cloned, and plasmid DNAs from at least 5 independent colonies were sequenced. Specific primers were then used to amplify full length (fl) TCRα and β DNAs from CTL-derived cDNA. In some cases (i.e., MA3/DP4 TCRβ) primers were also used to amplify control TCR DNAs from a spleen cDNA library. Standard primers were used to amplify the TCRα and TCRβ DNAs and will be provided upon request. TCRα and β genes were cloned as wild type TCRs into two separate pBullet retroviral vectors (49) (abbreviated as pB:TCRα+β) or as codon optimized TCRs in a TCRβ-2A-TCRα cassette in a single pMP71 vector (abbreviated as pMP71:optTCRβ-2A-TCRα, see *Supplementary text and figures*). The strategy we employed to clone MAGE-specific TCRαβ genes and to test their surface expression and function following TCR gene transfer is depicted in Figure 1A.

Transduction of human T lymphocytes

Human T lymphocytes of healthy donors were activated with anti-CD3 mAb and transduced with retrovirus harboring either MAGE-specific or control TCRα and β transgenes. The trans-

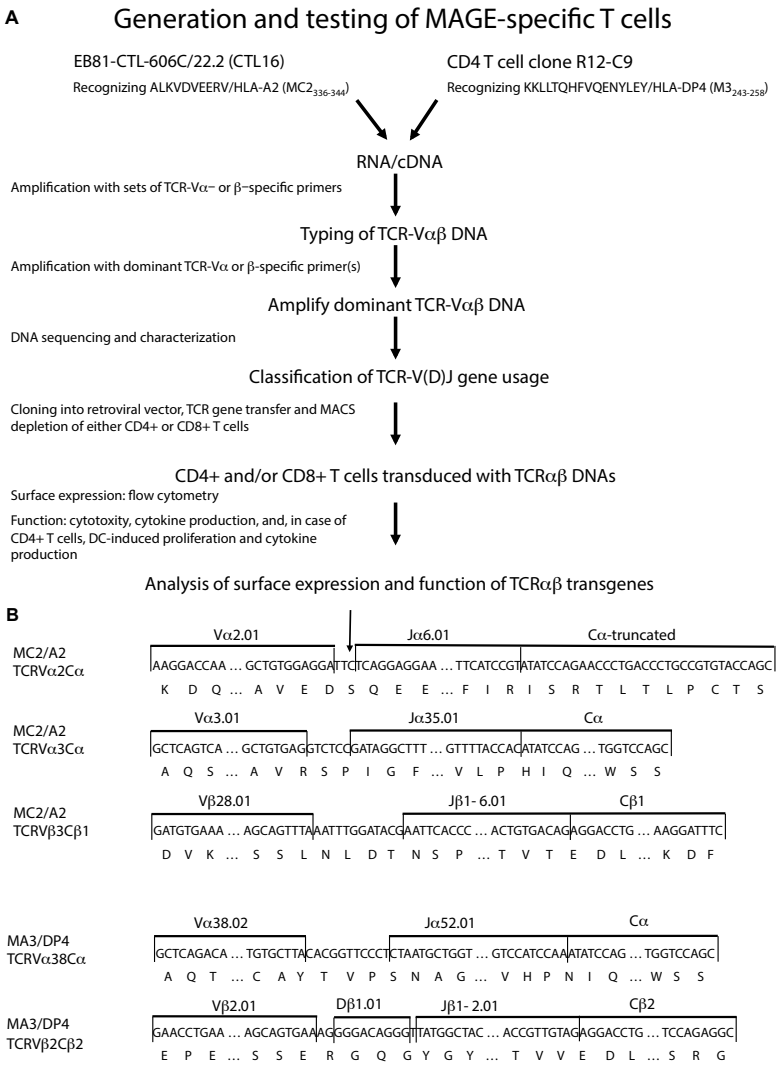

Figure 1. Cloning and validation of MC2/A2 and MA3/DP4 TCRab genes. A. Schematic representation of how TCR DNAs have been cloned, typed for TCR-V(D)J gene usage, and tested in T cells following gene transfer. **B.** TCR-V(D)J and C classification of the TCRα and β chains expressed by EB81-CTL16 and R12-C9 according to http://imgt.org. The arrow before the Jα6.01 indicates a frame shift preventing surface expression of this TCR-Vα2 chain. Sequence data for human TCR-Vα2, Vα3 and Vβ28 of EB81-CTL16-derived TCR genes are available from GenBank under accession nos. **EU427373**, **EU427374** and **EU427375**, respectively; and sequence data for human TCR-Vα38 and Vβ22 of R12-C9-derived TCR genes are available from GenBank under accession nos. **EU427376** and **EU427377**, respectively.

duction procedure was described by Lamers and colleagues (50) except that in the current study TCR-encoding retroviruses were produced by a co-culture of 293T and Phoenix-Ampho packaging cells. T cells were FACSorted using the corresponding p/MHC multimer prior to functional assays. For some experiments the MA3/DP4 TCR transduced T cells were depleted either for CD4 or CD8 T cells using anti-CD4 or CD8 mAb-coated and PE-labeled magnetic beads, and MACS columns (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Flow cytometry of TCR-transduced T lymphocytes

MC2/A2 TCR-transduced T cells were analyzed for TCR expression by flow cytometry using PE-labeled MC2/A2 pentamers (10 nM). MA3/DP4 TCR-transduced T cells were analyzed for TCR expression by PE-labeled anti-TCR-Vβ2 mAb, PE labeled MA3/DP4 tetramers (50 nM) and anti-CD4 mAb. For immunostaining, 0.5 x 10^6 transduced T cells were washed with PBS and incubated with MC2/A2 pentamer (or antibodies) at 4°C for 30 min or with MA3/DP4 tetramer at 37°C for 2h. Upon completion of the immunostainings, cells were washed and fixed with 1% paraformaldehyde. Events were acquired and analyzed on a Cytomics FC-500 flow cytometer with CXP software (Beckman Coulter, Mijdrecht, The Netherlands).

Cytotoxicity assay

Cytotoxic activity of T cells was measured in a standard 6 h ^{51}Cr-release assay as described previously (51). Peptide loading of target cells was performed by addition of either MC2, gp100 (control) or MA3 peptide (final concentrations at 10μM) for 15 min at 37 °C and 5% CO_2 prior to co-cultivation with effector T cells.

Cytokine production

To quantify the production of cytokines after antigen-specific stimulation, 6x10^6 T cells were cultured in the presence of 2x10^6 target cells for 18h at 37 °C and 5% CO_2. As a positive control, T cell transductants were stimulated with PHA and PMA. Supernatants were harvested and levels of IFN-γ and TNF-α were determined by standard ELISA (Sanquin, Amsterdam, The Netherlands).

CD4 T cell assay

CD4 T cell assays were based on dendritic cell:CD4 T cell co-cultures. To generate autologous dendritic cells (DC), we used PBMC from the same HLA-DP4-positive donor that had been used to generate MA3/DP4 TCR-transduced CD4 T cells. PBMC were MACS-enriched using CD14 microbeads (Miltenyi Biotech), seeded at 10^6 cells/ml in RPMI 1640 medium without HEPES and supplemented with glutamine, 10% FBS, 10 μg/ml gentamycine and the cytokines GM-CSF (1000 IU/ml) and IL-4 (200 IU/ml). At day 6, cells were used as a source of immature DC and incubated with MA3 protein (25 μg/ml) either in the absence or presence of TNFα

(200 IU/ml) and PGE2 (5 μM) for an additional 2 days resulting in immature or mature MA3-positive DC, respectively. DC maturation state was confirmed by flow cytometric analysis of surface expression of CD80, CD86 and HLA-DR.

Immature or mature MA3-positive DCs were washed and added at $2x10^4$ per round-bottomed microwell to $2x10^5$ CD4 T cells in 200 μl T cell medium. After 4 days of DC:T cell co-culture, supernatants were harvested and cytokine production was determined in culture supernatants with Cytokine Bead Array (Th1/Th2 CBA kit; BD Biosciences) according to the manufacturer's instructions.

RESULTS

Sequences of TCRαβ genes from MAGE-specific T cell clones

CD8 T cell clone EB81-CTL16 and CD4 T cell clone R12-C9, which were established from melanoma patients following MAGE vaccinations, were used to obtain genes encoding for MC2/A2 and MA3/DP4-specific TCRαβ's. Sequence characterization revealed that EB81-CTL 16 harbored genes encoding TCR-Vα2.01/Jα6.01/Cα, Vα3.01/Jα35.01/Cα and Vβ28.01/Jβ1-6.01/Cβ1, whereas R12-C9 harbored genes encoding TCR-Vα38.02/Jα52.01/Cα and Vβ2.01/Dβ1.01/Jβ1-2.01/Cβ2 (Figure 1A). We found that the TCR-Vα2.01/Jα6.01/Cα contained a frame shift in the Jα region (Figure 1B). As a result there was a premature stop codon in the constant domain and no surface expression of this TCRα chain (see Figure 2A). Figure 1B shows the exact nucleotide and amino acid sequences of the various MAGE TCR chains and their corresponding TCR-V(D)J and C classifications (according to www.imgt.org).

TCR-Vα3/Vβ28 chains confer T cells with the ability to bind $MC2_{336-344}$/A2 ligands

Retroviral transduction of human primary T cells with the TCR-Vα3Cα and Vβ28Cβ1 chains but not with irrelevant TCRα and β chains (i.e., mock TCR) resulted in TCR surface expression and binding to multimers of recombinant HLA-A2 molecules folded with $MC2_{336-344}$ peptide (Figure 2A). Enrichment of TCR-transduced T cells (TCR T cells) with MC2/A2 multimers by FACSort resulted in higher proportions of T cells expressing the MC2/A2 TCR (30% versus 65% pMHC binding prior and post sort; Figure 2B). TCR surface expression was stable for at least three months (data not shown).

TCR-Vα3/Vβ28-transduced primary human T cells show antigen-specific functions in vitro

To assess the antigen-specific cytolytic function of MC2/A2 TCR T cells, T cells were co-cultured with the MC2/A2-positive tumor cell line, EB81-MEL-2. Figure 3A shows that if these tumor cells were pre-treated with IFNγ they were lysed by the TCR T cells. MC2 peptide-loaded HLA-A2 positive B cells (BSM) were lysed very efficiently, whereas gp100 peptide-loaded B cells

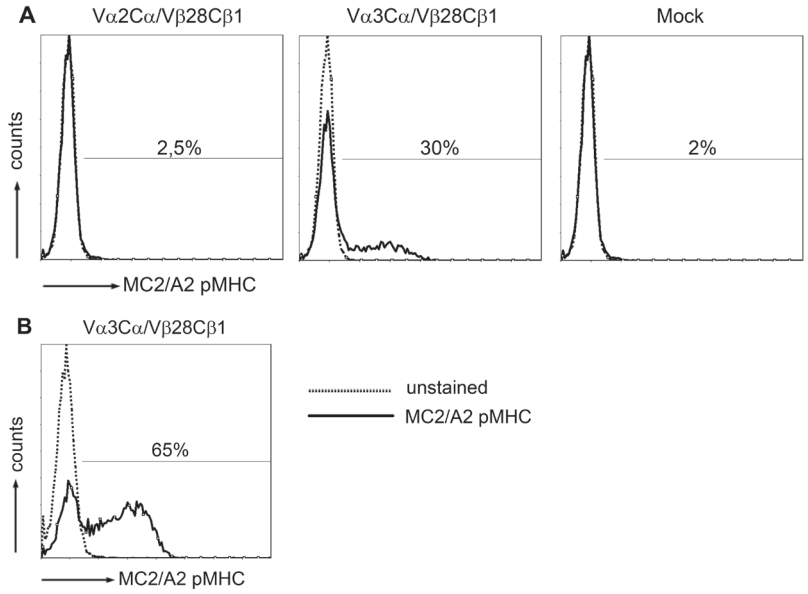

Figure 2. Primary human T cells transduced with TCR-Va3/Vb28 genes bind MC2/A2 pMHC.
The MC2/A2 TCR T cells were labeled with PE-conjugated $MC2_{336-344}$/A2 pentamers before flow cytometric analysis (solid lines). **A.** T cells transduced either with TCR-Vα2Cα/Vβ28Cβ1, Vα3Cα/Vβ28Cβ1 or control TCRαβ genes (Mock), and not sorted for MC2/A2 binding. **B.** T cells transduced with TCR-Vα3Cα/Vβ28Cβ1 genes and FACSorted with MC2/A2 pentamer. Results are from a representative transduction out of 6 transductions of PBMC from 2 donors with similar results.

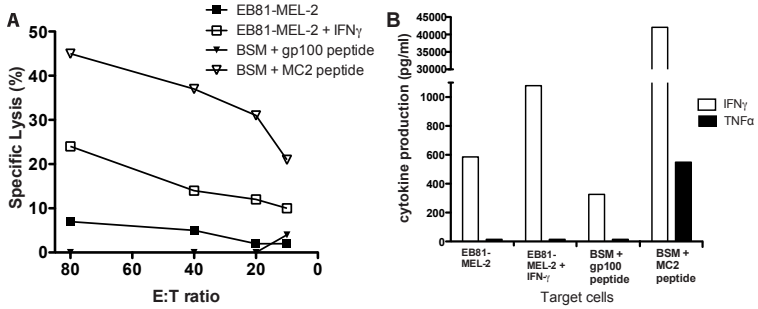

Figure 3. MC2/A2 TCR is functionally expressed by primary human T cells.
A. MC2/A2 TCR T cells lyse MC2/A2 positive target cells. TCR T cells were tested in a 6 h ^{51}Cr-release assay. The following target cells were used: MC2/A2-positive EB81-MEL-2 melanoma cells (derived from the same patient from who the MC2 TCR was derived), pre-treated or not with IFNγ, and A2-positive BSM EBV-B cells, pulsed either with gp100 or MC2 peptide (both at 10μM final). Mock T cells did not lyse MC2/A2-positive target cells (data not shown). Effector-to-target cell ratios are indicated on the X-axis and specific ^{51}Cr-releases are indicated on the Y-axis. **B.** MC2/A2 TCR T cells produce cytokines upon co-culture with MC2/A2-positive target cells. T cell production of IFNγ and TNFα (in pg/ml) were measured by ELISA in supernatants harvested after an 18 h co-culture between T cells and the target cells described in legend to Figure A. No cytokines were produced by T cells only or Mock T cells co-cultured with MC2-positive target cells (data not shown). Measurements were performed in triplicate, and expressed as mean values corrected for medium values. Data shown are from representative experiments out of 4 experiments from 2 donors with similar results.

were not recognized. Additionally, TCR T cells produced IFNγ but not TNFα in response to IFNγ pre-treated EB81-MEL-2 cells, although T cells produced both IFNγ and TNFα in response to MC2 peptide loaded cells (Figure 3B). No IFNγ was produced by MC2/A2 TCR T cells in response to $MC2^{pos}/A2^{neg}$ tumor cells (*Supplementary Figure 1B*).

TCR-Vα38/Vβ2 chains provide T cells with the ability to bind $MA3_{243-258}$/HLA-DP4 ligands

MA3/DP4 TCR-transduced T cells, whether or not depleted for either the CD4 or CD8 T cell subset, expressed high levels of TCR-Vβ2 (Figure 4A). Sorting the T cells after gene transfer for high pMHC binding resulted in expressions that improved by a factor of two (21 and 26% pMHC binding prior to sorting versus 48 and 44% post sorting for CD4 and CD8-depleted T cells, respectively) (see Figure 4B). Mock T cells did not bind MA3/DP4 pMHC multimers. Similar to the MC2/A2 TCR, MA3/DP4 TCR expression was stable for at least three months (data not shown).

MA3/DP4 TCR T cells recognize antigen-positive B cells but not melanoma cells natively expressing MA3 antigen

MA3/DP4 TCR T cells specifically lysed MA3/DP4 positive EBV B cells (EBV-MA3) (Figure 5A). Depleting MA3/DP4 TCR T cells for CD8 T cells resulted in CD4 T cells with a cytotoxic capacity similar to that of non-depleted T cells (mainly being of the CD8 T cell subset). T cells transduced with the TCRβ chain of the MA3/DP4 TCR and a TCRα chain from a human spleen cDNA library served as a negative control (referred to as Mock T cells) and did not lyse MA3-positive B cells (Figure 5A). MA3/DP4 TCR T cells did not lyse MZ2-MEL43 melanoma cells, which naturally express the MA3/DP4 antigen (Figure 5B). Pre-treatment with IFNγ did not, but addition of MA3 peptide did enhance killing of the MZ2-MEL43 melanoma cells by MA3/DP4 TCR T cells (Figure 5C and D). Next, we showed that MA3/DP4 TCR T cells, but not Mock T cells, produced IFNγ and to a lesser extent TNFα in response to EBV-MA3 cells, with CD4 T cells as the predominant source of both cytokines (Figure 6A). It is noteworthy that TCR CD4 T cells, but not the parental R12-C9 T cell clone, produced more IFNγ than TNFα (Figure 6A). Responses of TCR T cells towards EBV-MA3 B cells were blocked with an anti-TCR Vβ2 antibody, whereas those towards $MA3^{pos}/DP4^{neg}$ tumor cells (*Supplementary Figure 2B*) and $MA3^{neg}/DP4^{pos}$ B cells were always negative (data not shown). T cells expressing MA3/DP4 TCR (but not Mock) were able to respond to MZ2-MEL43 melanoma cells only when target cells were pre-loaded with MA3 16mer peptide: this demonstrates that these melanoma cells can be sensitized to peptide-specific T cell functions (i.e., cytotoxicity: data not shown; production of IFNγ and TNFα: Figure 6B).

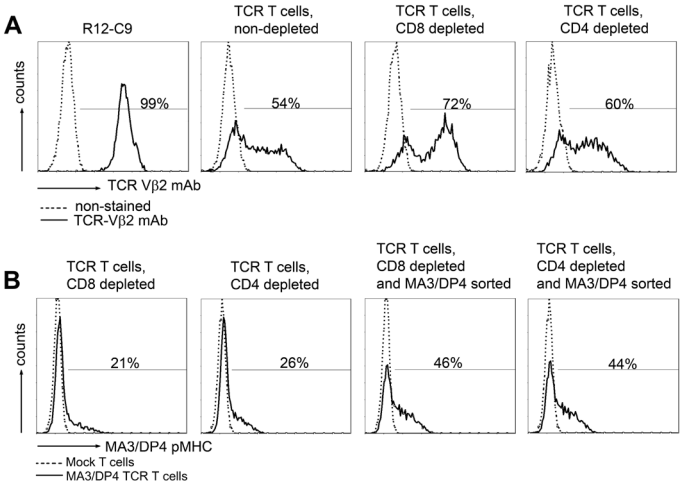

Figure 4. Surface expression of MA3/DP4 TCR on human primary T cells following gene transfer.
Human primary T cells transduced with MA3/DP4 TCRαβ genes were stained with TCR-Vβ2 mAb (in which case non-stained MA3/DP4 TCR T cells served as a negative control since control TCRαβ genes also comprise the TCR-Vβ2 chain) **(A)** or MA3/DP4 tetramer **(B)** prior to analysis by flow cytometry. **A.** The following T cells were analyzed: parental CD4 T cell clone R12-C9; TCR T cells, non-depleted (bulk); and TCR T cells depleted for either CD8 or CD4 T cells. These T cell populations are not FACSorted. **B.** TCR-transduced T cells, depleted for either CD8 or CD4 T cells, and non-sorted or FACSorted with MA3/DP4 tetramer were analyzed. Results are from a representative transduction out of 4 transductions of PBMC from 2 donors with similar results.

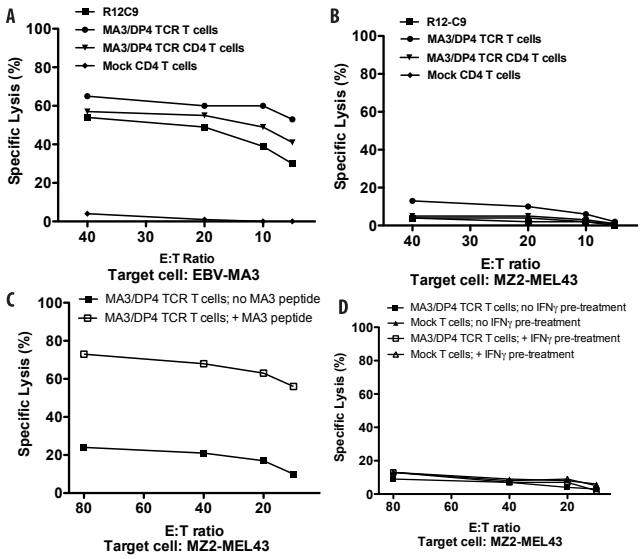

Figure 5. MA3/DP4 TCR T cells specifically lyse MA3-transduced or peptide-loaded B cells, but not MA3-positive melanoma cells.
A. MA3/DP4 TCR T cells specifically lyse DP4-positive B cells transduced with MA3-encoding cDNA. Human T cells were tested in a 6 h ^{51}Cr-release assay using EBV-MA3 target cells. The following effector T cells were used: CD4 T cell clone R12-C9; MA3/DP4 TCR T cells, non-depleted T cells; MA3/DP4 TCR T cells depleted

for CD8 T cells; or Mock T cells depleted for CD8 T cells. MA3-negative, DP4-positive B cells (BSM) were not recognized by MA3/DP4 TCR T cells (data not shown). **B.** MA3/DP4 TCR T cells do not lyse MZ2-MEL43 melanoma cells, natively expressing MA3 and DP4. Effector T cells used were those described in legend to Figure A. **(C)** MA3/DP4 TCR T cells do not lyse MZ2-MEL43 melanoma cells that are pre-treated with IFNγ. Target cells were MZ2-MEL43 cells that were either pre-treated with IFNγ or not, and effector T cells were MA3/DP4 TCR or Mock T cells. (D) MA3/DP4 TCR T cells lyse MZ2-MEL43 melanoma cells that are pulsed with MA3 peptide. Target cells were MZ2-MEL43 cells that were either pulsed with MA3 peptide or not, and effector T cells were MA3/DP4 TCR T cells. Measurements were performed in triplicate, and expressed as mean values corrected for medium values. Data are from representative experiments out of 3 experiments with similar results.

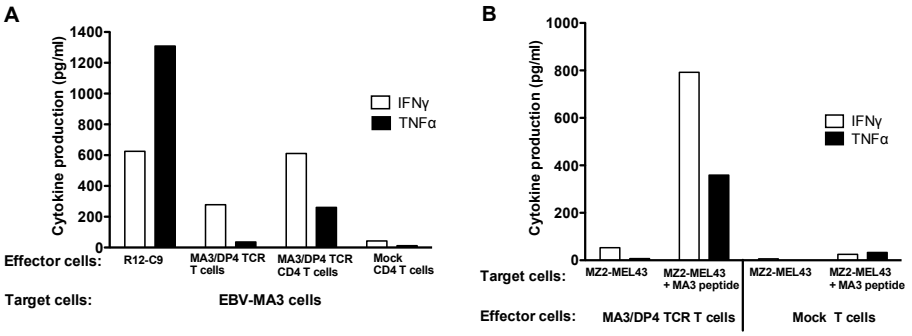

Figure 6. MA3/DP4 TCR T cells specifically produce IFNg and TNFa upon co-culture with MA3-transduced or peptide-loaded B cells, but not MA3-positive melanoma cells.

Cytokine production is determined in supernatants of T cells after an 18 h co-culture with **(A)** DP4-positive B cells transduced with Ii-MA3 cDNA (EBV-MA3) or **(B)** MZ2-MEL43 cells loaded with MA3 peptide or not. **A.** Effector T cells were: the CD4 T cell clone R12-C9; MA3/DP4 TCR or Mock T cells, either non-depleted or depleted for CD8 T cells. MA3-negative, DP4-positive B cells (such BSM) were not recognized by MA3/DP4 TCR T cells (data not shown). **B.** MA3/DP4 TCR or Mock T cells, non-depleted, were used as effector T cells. Supernatants were harvested and analyzed for IFNγ and TNFα by ELISA. Measurements were performed in triplicate, and expressed as mean values corrected for medium values. Data are from representative experiments out of 3 experiments with similar results.

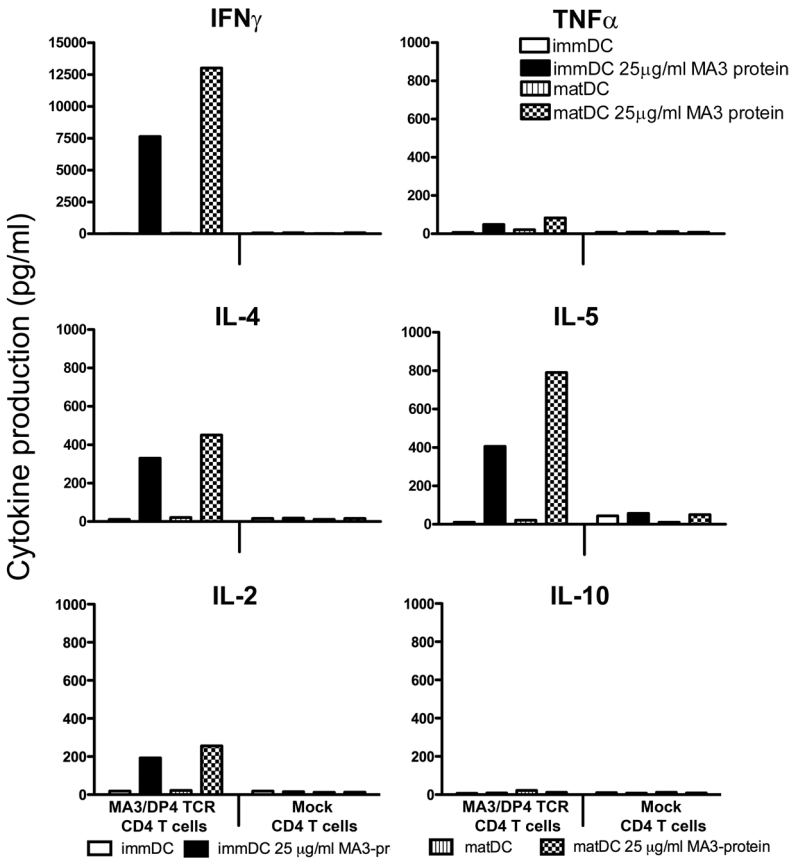

Figure 7. MA3/DP4 TCR CD4 T cells produce cytokines upon co-culture with MA3 protein-loaded autologous dendritic cells.

MA3/DP4 TCR and Mock CD4 T cells were cultured with immature or mature autologous dendritic cells that were either loaded with 25 μg/ml MA3 protein or not. After 4 days, supernatants were harvested and analyzed for cytokine production by cytokine bead arrays. Cytokine production was not detected in case T cells were cultured without dendritic cells (data not shown). Measurements were performed in duplicate, and expressed as mean values. Data are from a representative experiment out of 2 experiments with similar results.

MA3/DP4 TCR CD4 T cells produce cytokines upon co-culture with MA3 loaded autologous monocyte-derived DC

Since MA3/DP4 TCR T cells are unable to directly recognize antigen-positive melanoma cells, an ability that is generally expected only for anti-tumor CD8 T cells, we analyzed a more typical CD4 T cell response that is based on (cross)presentation of tumor antigens by DC. To this end, MA3/DP4 TCR and Mock CD4 T cells were co-cultured with immature or mature DC derived from autologous monocytes using two different MA3 protein concentrations for DC uptake. After 4 days, the production of various cytokines was determined in supernatants of

the DC:T cell co-cultures. Upon co-culture with the MA3-protein-loaded DC, MA3/DP4 TCR CD4 T cells (but not Mock CD4 T cells) produced significant amounts of IFNγ (up to 1300 pg/ml) and to a lesser extent TNFα, IL-2, IL-4 and IL-5 (Figure 7). MA3-specific production of IL-10 was negligible. T cell cytokine production was negligible or absent when either non-protein-loaded immature or mature DC or Mock T cells were used.

DISCUSSION

Redirection of T cells towards tumor-specific yet clinically safe antigens holds great promise for the treatment of melanoma and other tumor types. In the current manuscript, we have studied MAGE-C2/HLA-A2 (MC2/A2) and MAGE-A3/HLA-DP4 (MA3/DP4) as targets of TCR T cells; besides being prevalent in the patient population, these antigens are uniquely expressed by tumors and have proven value in initiating clinically effective CD8 and CD4 T cell responses (23, 27, 28, 46).

MC2/A2 and MA3/DP4 TCRαβ genes were derived from T cell clones obtained from MAGE vaccinated patients and were subsequently characterized following TCR gene transfer (see Figure 1). The CD8 T cell clone EB81-CTL 16 expressed the MC2/A2 specific TCR-Vα3 and TCR-Vβ28 chains. Upon gene transfer, primary human T cells bound pMHC and demonstrated MC2-specific T cell functions. TCR T cells killed and produced IFNγ and TNFα upon co-culture not only with MC2 peptide-pulsed HLA-A2-positive target cells but also native MC2-positive, HLA-A2-positive melanoma cells (see Figures 2 and 3). T cell responsiveness towards native MC2-positive, HLA-A2-positive melanoma cells (i.e., EB81-MEL-2 cells) was enhanced by IFNγ pre-treatment, which promotes antigen processing and surface expression of MHC and adhesion molecules. In fact, unlike other antigenic peptides, the MC2 epitope ALKDVEERV (i.e. $MC2_{336-344}$) requires immune proteasomes for proper processing and presentation to T cells (52), supporting the value of MC2/A2 as a target for T cell therapy. The effective dose of MC2/A2 peptide at which CD8 T cells demonstrate a half-maximal lytic response (i.e., ED50) is 0.75 nM (53). This value represents a measure of T cell avidity and lags somewhat behind in comparison to reported values for T cells expressing other MHC class I TCRs (range: 30-100 pM) (54, 55), suggesting a lower to intermediate ligand-binding affinity of this TCR. Experiments with mutated pMHC complexes that either prevent or enhance CD8α binding (according to (56, 57); kindly provided by prof.dr. Andrew Sewell, University of Cardiff, Wales) confirm the CD8-dependency of this MC2 TCR (data not shown).

The CD4 T cell clone R12-C9 expressed the MA3/DP4 specific TCR-Vα38 and TCR-Vβ2 chains, which, upon gene transfer in primary human T cells, resulted in pMHC binding (see Figure 4). In addition, MA3/DP4 TCR T cells, containing both CD8 and CD4 T cells (i.e., non-depleted), specifically lysed and produced IFNγ and TNFα upon co-culture with B cells either transduced with MA3 antigen (Figures 5A and 6A) or loaded with MA3 peptide (data not shown). The

extent of lysis, a typical measure for CD8 T cell function, was lowered when testing MA3/DP4 TCR CD4 T cells (i.e., depleted for CD8-positive T cells). The responsiveness of MA3/DP4 TCR T cells towards MZ2-MEL43 cells, generally weak and not reproducible, was not enhanced by IFNγ pre-treatment of target cells, whereas T cells were clearly able to recognize melanoma cells following loading with the MA3 16-mer peptide but not a core 12-mer peptide (TQG-FVQENYLEY, i.e., $MA3_{247-258}$) (Figure 5B, C, D and 6B). Collectively, these data argue that MA3, like other nuclear proteins, may be inefficiently presented by tumor cells, and T cell responses directed to tumor cells natively expressing MA3, such as those reported for T cell clones 22 and R12-57 (58, 59), are rare and difficult to reproduce. In fact, when screening a panel of 23 T cell clones including many patient R12-derived T cell clones, we were unable to identify a single T cell clone that responded towards MZ2-MEL43 (data not shown). In this respect, it is noteworthy that R12-derived CD4 T cell clones show a polyclonal response towards MA3/DP4, with 50% of clonotypes sharing TCR-Vβ12 gene (46). Thus, MA3/DP4 TCR T cells are able to lyse antigen-positive target cells, but lysis becomes suboptimal in case (low levels of) antigen is presented by tumor cells. The ED50 value of MA3/DP4 peptide in a CD4 T cell IFNγ assay is 30 nM (59). This value is in accordance with reported values for T cells expressing other MHC class II TCRs (range: 40-200 nM) (20, 60). Notably, functional expressions of MHC class II TCRs, such as reported for NY-ESO-1/DP4 TCR, may depend on the presence of the CD4 co-receptor (20) and are assessed by typical CD4 T cell assays, such as T cell proliferation and cytokine production. In case of MA3/DP4 TCR, we also observed that antigen-specific IFNγ production is higher in T cells depleted for CD8 T cells (i.e., CD4 T cells) when compared to non-depleted T cells (i.e., CD4+CD8 T cells, Figure 6A).

Anti-tumor responses more typical for CD4 T cells are induced by professional antigen-presenting cells, such as DC (reviewed in (61)). DC capture and process tumor antigens, and cross-present MHC class II-restricted antigens to CD4 T cells. Following activation, these CD4 T cells provide signals to DC that enhance antigen presentation and co-stimulation (via cross-linking of CD40) and lead to priming of antigen-specific CD8 CTL function (62). Importantly, activated CD4 T cells are a major source of IFNγ, an effector cytokine with potent tumor regressing activity via inhibition of tumor-induced angiogenesis or activation of tumor-infiltrating macrophages (63-65). When analyzing DC-induced T cell responses, we observed significant production of cytokines when immature or mature DC were loaded with MA3 protein and used to stimulate TCR T cells (Figure 7). Decreasing the MA3 protein concentration during maturation of the DC from 25 to 5 μg/ml resulted in only slightly lower but almost comparable cytokine responses (data not shown). These findings extend the observations with two other MHC class II-restricted TCRs specific for human antigens, i.e., NY-ESO1/DP4 and DBY/DQ5 (20, 66). MA3/DP4 TCR-transduced T cells produced high amounts of IFNγ, whereas TNFα, IL-2, IL-4 and IL-5 were produced to a lesser extent. IL-10 represents the only cytokine with production levels being low (< 20 pg/ml) and not different between TCR and Mock-transduced T cells (Figure 7). In addition, co-culture with MA3-positive DC resulted

in up-regulated expression of T cell activation markers such as CD25 (IL-2Rα chain) as well as enhanced T cell proliferation (data not shown). Analysis of DC phenotype and function after co-culture with MA3/DP4 TCR T cells was not possible, since DC died at day 1 and 2 after start of co-culture and were completely lost at day 4, which was evidenced by light microscopy and lack of IL-12 production and suggested direct killing of DC by TCR T cells. Our observation that MA3/DP4-specific CD4 T cells recognize MA3-protein-loaded DC rather than MZ2-MEL43 melanoma cells implies that these CD4 T cells yield anti-tumor activity in vivo following cross-presentation by professional antigen presenting cells. The therapeutic benefit of antigen-specific IFNγ production have initiated studies in which CD4 T cells were used as recipient T cells for MHC class I-restricted TCR. Not only can CD4 T cells be functionally endowed with MHC I-restricted TCR via gene transfer (19, 67, 68), genetic co-introduction of CD8α skews TCR-engineered T cells towards an antigen-specific Th1-type T cell response (69). Vice versa, the introduction of a MHC class II TCR and CD4 co-receptor in CD8 T cells may lead to the generation of T cells with combined helper and effector T cell functions (66).

In extension to our results with wild type MC2 and MA3 TCRs, we have tested gene optimization, a transgene cassette and another retroviral vector to enhance functional expression of TCR transgenes (70-72). To this end, we have cloned codon-optimized MC2 and MA3 TCRs in TCRβ-2A-TCRα cassette-containing pMP71 vectors, and demonstrated significant TCR surface expression and MAGE-specific IFNγ production by CD3 mAb-activated and transduced PBMC (note that results with pMP71:optTCRβ-2A-TCRα reflect bulk, non-sorted T cells, *Supplementary Figures 1 and 2*). In preparation of clinical studies, we propose the following additional strategies to enhance the therapeutic efficacy of T cells gene-engineered with MC2 and MA3 TCRs. *First*, administration of common-γ cytokines, such as a combination of IL-15 and IL-21, to cultures of TCR T cells prior to patient infusion will yield T cells that show limited T cell differentiation and are better equipped to persist and function in vivo ((73); Lamers, Manuscript in preparation). *Second*, we propose pre-conditioning of patients that, apart from non myelo-ablative treatment with cyclophosphamide and fludarabine (1), includes treatment with the DNA hypomethylating agent 5-AZA-CdR. Such treatment, already used clinically, is reported to enhance expression of MAGE antigens and HLA in melanoma (26, 74). And *third*, co-treatment with MC2 TCR-transduced CD8 and MA3 TCR CD4 T cells may be of particular interest to boost anti-tumor immunity and counteract selected growth of epitope-negative tumor variants. In fact, we have recently demonstrated that single-epitope targeting of melanoma by TCR-engineered T cells results in highly effective but transient regression in HLA-A2 transgenic mice and that more effective strategies likely require multi-epitope targeting (Straetemans, Manuscript submitted). The proposed dual-epitope targeting approach may prove especially effective for CTA epitopes because of their co-regulated expression pattern in tumor cells, with the vast majority of tumor cells expressing two or more CTAs (75). Testing of cell lines derived from tumors other than melanoma have started in our laboratory, and

may provide a preclinical rationale to extend the proposed treatment to non-melanoma tumors.

In short we have cloned and *in vitro* validated two MAGE-specific TCRs that warrant clinical testing in TCR gene therapy in melanoma patients and in other patients with cancers expressing the MC2 and MA3 antigens.

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REFERENCES

1. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A & Rosenberg SA (2005) Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23(10):2346-2357.
2. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E & Greenberg PD (2002) Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* 99(25):16168-16173.
3. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED & Riddell SR (1995) Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 333(16):1038-1044.
4. Savoldo B, Heslop HE & Rooney CM (2000) The use of cytotoxic t cells for the prevention and treatment of epstein-barr virus induced lymphoma in transplant recipients. *Leuk Lymphoma* 39(5-6):455-464.
5. Butler MO, Friedlander P, Milstein MI, Mooney MM, Metzler G, Murray AP, Tanaka M, Berezovskaya A, Imataki O, Drury L, Brennan L, Flavin M, Neuberg D, Stevenson K, Lawrence D, Hodi FS, Velazquez EF, Jaklitsch MT, Russell SE, Mihm M, Nadler LM & Hirano N (2011) Establishment of antitumor memory in humans using in vitro-educated CD8+ T cells. *Sci Transl Med* 3(80):80ra34.
6. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G & Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24(13):e20-22.
7. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA & Hwu P (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 12(20 Pt 1):6106-6115.
8. Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, Qian X, James SE, Raubitschek A, Forman SJ, Gopal AK, Pagel JM, Lindgren CG, Greenberg PD, Riddell SR & Press OW (2008) Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 112(6):2261-2271.
9. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, Yvon E, Weiss HL, Liu H, Rooney CM, Heslop HE & Brenner MK (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 14(11):1264-1270.
10. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA & Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314(5796):126-129.
11. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM & Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114(3):535-546.

12. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, Davis JL, Morgan RA, Merino MJ, Sherry RM, Hughes MS, Kammula US, Phan GQ, Lim RM, Wank SA, Restifo NP, Robbins PF, Laurencot CM & Rosenberg SA (2011) T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* 19(3):620-626.
13. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C & Rosenberg SA (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 29(7):917-924.
14. Brentjens R, Yeh R, Bernal Y, Riviere I & Sadelain M (2010) Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Mol Ther* 18(4):666-668.
15. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM & Rosenberg SA (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 18(4):843-851.
16. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T & Lucas S (2001) An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* 61(14):5544-5551.
17. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A & Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254(5038):1643-1647.
18. Chinnasamy N, Wargo JA, Yu Z, Rao M, Frankel TL, Riley JP, Hong JJ, Parkhurst MR, Feldman SA, Schrump DS, Restifo NP, Robbins PF, Rosenberg SA & Morgan RA (2011) A TCR targeting the HLA-A*0201-restricted epitope of MAGE-A3 recognizes multiple epitopes of the MAGE-A antigen superfamily in several types of cancer. *J Immunol* 186(2):685-696.
19. Willemsen R, Ronteltap C, Heuveling M, Debets R & Bolhuis R (2005) Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. *Gene Ther* 12(2):140-146.
20. Zhao Y, Zheng Z, Khong HT, Rosenberg SA & Morgan RA (2006) Transduction of an HLA-DP4-restricted NY-ESO-1-specific TCR into primary human CD4+ lymphocytes. *J Immunother (1997)* 29(4):398-406.
21. Zhao Y, Zheng Z, Robbins PF, Khong HT, Rosenberg SA & Morgan RA (2005) Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. *J Immunol* 174(7):4415-4423.
22. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ & Longley BJ (2007) MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* 67(20):9954-9962.
23. Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M & Schneider C (2006) MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. *Proc Natl Acad Sci U S A* 103(30):11160-11165.
24. Liu W, Cheng S, Asa SL & Ezzat S (2008) The melanoma-associated antigen A3 mediates fibronectin-controlled cancer progression and metastasis. *Cancer Res* 68(19):8104-8112.

25. Gedye C, Quirk J, Browning J, Svobodova S, John T, Sluka P, Dunbar PR, Corbeil D, Cebon J & Davis ID (2009) Cancer/testis antigens can be immunological targets in clonogenic CD133+ melanoma cells. *Cancer Immunol Immunother* 58(10):1635-1646.
26. Sigalotti L, Covre A, Zabierowski S, Himes B, Colizzi F, Natali PG, Herlyn M & Maio M (2008) Cancer testis antigens in human melanoma stem cells: expression, distribution, and methylation status. *J Cell Physiol* 215(2):287-291.
27. Van den Eynde BJ & van der Bruggen P (1997) T cell defined tumor antigens. *Curr Opin Immunol* 9(5):684-693.
28. Lucas S, De Plaen E & Boon T (2000) MAGE-B5, MAGE-B6, MAGE-C2, and MAGE-C3: four new members of the MAGE family with tumor-specific expression. *Int J Cancer* 87(1):55-60.
29. Jang SJ, Soria JC, Wang L, Hassan KA, Morice RC, Walsh GL, Hong WK & Mao L (2001) Activation of melanoma antigen tumor antigens occurs early in lung carcinogenesis. *Cancer Res* 61(21):7959-7963.
30. Condomines M, Hose D, Raynaud P, Hundemer M, De Vos J, Baudard M, Moehler T, Pantesco V, Moos M, Schved JF, Rossi JF, Reme T, Goldschmidt H & Klein B (2007) Cancer/testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis. *J Immunol* 178(5):3307-3315.
31. Gure AO, Chua R, Williamson B, Gonen M, Ferrera CA, Gnjatic S, Ritter G, Simpson AJ, Chen YT, Old LJ & Altorki NK (2005) Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res* 11(22):8055-8062.
32. Shigematsu Y, Hanagiri T, Shiota H, Kuroda K, Baba T, Mizukami M, So T, Ichiki Y, Yasuda M, Takenoyama M & Yasumoto K (2010) Clinical significance of cancer/testis antigens expression in patients with non-small cell lung cancer. *Lung Cancer* 68(1):105-110.
33. Alves PM, Levy N, Bouzourene H, Viatte S, Bricard G, Ayyoub M, Vuilleumier H, Givel JC, Halkic N, Speiser DE, Romero P & Levy F (2007) Molecular and immunological evaluation of the expression of cancer/testis gene products in human colorectal cancer. *Cancer Immunol Immunother* 56(6):839-847.
34. Peng JR, Chen HS, Mou DC, Cao J, Cong X, Qin LL, Wei L, Leng XS, Wang Y & Chen WF (2005) Expression of cancer/testis (CT) antigens in Chinese hepatocellular carcinoma and its correlation with clinical parameters. *Cancer Lett* 219(2):223-232.
35. Hudolin T, Juretic A, Spagnoli GC, Pasini J, Bandic D, Heberer M, Kosicek M & Cacic M (2006) Immunohistochemical expression of tumor antigens MAGE-A1, MAGE-A3/4, and NY-ESO-1 in cancerous and benign prostatic tissue. *Prostate* 66(1):13-18.
36. Otte M, Zafrakas M, Riethdorf L, Pichlmeier U, Loning T, Janicke F & Pantel K (2001) MAGE-A gene expression pattern in primary breast cancer. *Cancer Res* 61(18):6682-6687.
37. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG & Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *J Exp Med* 201(2):249-257.
38. Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, Bender A, Feuerstein B, Fritsch PO, Romani N & Schuler G (2002) Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med* 195(10):1279-1288.
39. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethe B, De Plaen E, Velu T, Boon T & Coulie PG (2005) High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. *J Exp Med* 201(2):241-248.

40. Carrasco J, Van Pel A, Neyns B, Lethe B, Brasseur F, Renkvist N, van der Bruggen P, van Baren N, Paulus R, Thielemans K, Boon T & Godelaine D (2008) Vaccination of a melanoma patient with mature dendritic cells pulsed with MAGE-3 peptides triggers the activity of nonvaccine anti-tumor cells. *J Immunol* 180(5):3585-3593.
41. Brichard VG & Lejeune D (2007) GSK's antigen-specific cancer immunotherapy programme: pilot results leading to Phase III clinical development. *Vaccine* 25 Suppl 2:B61-71.
42. Atanackovic D, Altorki NK, Stockert E, Williamson B, Jungbluth AA, Ritter E, Santiago D, Ferrara CA, Matsuo M, Selvakumar A, Dupont B, Chen YT, Hoffman EW, Ritter G, Old LJ & Gnjatic S (2004) Vaccine-induced CD4+ T cell responses to MAGE-3 protein in lung cancer patients. *J Immunol* 172(5):3289-3296.
43. Atanackovic D, Altorki NK, Cao Y, Ritter E, Ferrara CA, Ritter G, Hoffman EW, Bokemeyer C, Old LJ & Gnjatic S (2008) Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming. *Proc Natl Acad Sci U S A* 105(5):1650-1655.
44. Tyagi P & Mirakhur B (2009) MAGRIT: the largest-ever phase III lung cancer trial aims to establish a novel tumor-specific approach to therapy. *Clin Lung Cancer* 10(5):371-374.
45. Vantomme V, Dantinne C, Amrani N, Permanne P, Gheysen D, Bruck C, Stoter G, Britten CM, Keilholz U, Lamers CH, Marchand M, Delire M & Gueguen M (2004) Immunologic analysis of a phase I/II study of vaccination with MAGE-3 protein combined with the AS02B adjuvant in patients with MAGE-3-positive tumors. *J Immunother (1997)* 27(2):124-135.
46. Zhang Y, Renkvist N, Sun Z, Schuler-Thurner B, Glaichenhaus N, Schuler G, Boon T, van der Bruggen P & Colau D (2005) A polyclonal anti-vaccine CD4 T cell response detected with HLA-DP4 multimers in a melanoma patient vaccinated with MAGE-3.DP4-peptide-pulsed dendritic cells. *Eur J Immunol* 35(4):1066-1075.
47. Van de Griend RJ, Van Krimpen BA, Bol SJ, Thompson A & Bolhuis RL (1984) Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. *J Immunol Methods* 66(2):285-298.
48. Zhang Y, Chaux P, Stroobant V, Eggermont AM, Corthals J, Maillere B, Thielemans K, Marchand M, Boon T & Van Der Bruggen P (2003) A MAGE-3 peptide presented by HLA-DR1 to CD4+ T cells that were isolated from a melanoma patient vaccinated with a MAGE-3 protein. *J Immunol* 171(1):219-225.
49. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P & Bolhuis RL (2000) Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther* 7(16):1369-1377.
50. Lamers CH, Willemsen RA, van Elzakker P, van Krimpen BA, Gratama JW & Debets R (2006) Phoenix-ampho outperforms PG13 as retroviral packaging cells to transduce human T cells with tumor-specific receptors: implications for clinical immunogene therapy of cancer. *Cancer Gene Ther* 13(5):503-509.
51. Weijtens ME, Willemsen RA, Hart EH & Bolhuis RL (1998) A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes. *Gene Ther* 5(9):1195-1203.
52. Chapiro J, Claverol S, Piette F, Ma W, Stroobant V, Guillaume B, Gairin JE, Morel S, Burlet-Schiltz O, Monsarrat B, Boon T & Van den Eynde BJ (2006) Destructive cleavage of antigenic peptides either by the immunoproteasome or by the standard proteasome results in differential antigen presentation. *J Immunol* 176(2):1053-1061.

53. Ma W, Germeau C, Vigneron N, Maernoudt AS, Morel S, Boon T, Coulie PG & Van den Eynde BJ (2004) Two new tumor-specific antigenic peptides encoded by gene MAGE-C2 and presented to cytolytic T lymphocytes by HLA-A2. *Int J Cancer* 109(5):698-702.
54. Rubinstein MP, Kadima AN, Salem ML, Nguyen CL, Gillanders WE, Nishimura MI & Cole DJ (2003) Transfer of TCR genes into mature T cells is accompanied by the maintenance of parental T cell avidity. *J Immunol* 170(3):1209-1217.
55. Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R & Adema GJ (2003) Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J Immunol* 170(4):2186-2194.
56. Choi EM, Chen JL, Wooldridge L, Salio M, Lissina A, Lissin N, Hermans IF, Silk JD, Mirza F, Palmowski MJ, Dunbar PR, Jakobsen BK, Sewell AK & Cerundolo V (2003) High avidity antigen-specific CTL identified by CD8-independent tetramer staining. *J Immunol* 171(10):5116-5123.
57. Wooldridge L, Clement M, Lissina A, Edwards ES, Ladell K, Ekeruche J, Hewitt RE, Laugel B, Gostick E, Cole DK, Debets R, Berrevoets C, Miles JJ, Burrows SR, Price DA & Sewell AK (2010) MHC class I molecules with Superenhanced CD8 binding properties bypass the requirement for cognate TCR recognition and nonspecifically activate CTLs. *J Immunol* 184(7):3357-3366.
58. Schultz ES, Lethe B, Cambiaso CL, Van Snick J, Chaux P, Corthals J, Heirman C, Thielemans K, Boon T & van der Bruggen P (2000) A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4+ cytolytic T lymphocytes. *Cancer Res* 60(22):6272-6275.
59. Schultz ES, Schuler-Thurner B, Stroobant V, Jenne L, Berger TG, Thielemanns K, van der Bruggen P & Schuler G (2004) Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 172(2):1304-1310.
60. Fujio K, Misaki Y, Setoguchi K, Morita S, Kawahata K, Kato I, Nosaka T, Yamamoto K & Kitamura T (2000) Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex. *J Immunol* 165(1):528-532.
61. Wang RF (2001) The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. *Trends Immunol* 22(5):269-276.
62. Schoenberger SP, Toes RE, van der Voort EI, Offringa R & Melief CJ (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393(6684):480-483.
63. Mumberg D, Monach PA, Wanderling S, Philip M, Toledano AY, Schreiber RD & Schreiber H (1999) CD4(+) T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-gamma. *Proc Natl Acad Sci U S A* 96(15):8633-8638.
64. Qin Z & Blankenstein T (2000) CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity* 12(6):677-686.
65. Corthay A, Skovseth DK, Lundin KU, Rosjo E, Omholt H, Hofgaard PO, Haraldsen G & Bogen B (2005) Primary antitumor immune response mediated by CD4+ T cells. *Immunity* 22(3):371-383.
66. van der Veken LT, Hoogeboom M, de Paus RA, Willemze R, Falkenburg JH & Heemskerk MH (2005) HLA class II restricted T-cell receptor gene transfer generates CD4+ T cells with helper activity as well as cytotoxic capacity. *Gene Ther* 12(23):1686-1695.
67. Morris EC, Tsallios A, Bendle GM, Xue SA & Stauss HJ (2005) A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. *Proc Natl Acad Sci U S A* 102(22):7934-7939.

68. Kuball J, Schmitz FW, Voss RH, Ferreira EA, Engel R, Guillaume P, Strand S, Romero P, Huber C, Sherman LA & Theobald M (2005) Cooperation of human tumor-reactive CD4+ and CD8+ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. *Immunity* 22(1):117-129.
69. Willemsen RA, Sebestyen Z, Ronteltap C, Berrevoets C, Drexhage J & Debets R (2006) CD8 alpha coreceptor to improve TCR gene transfer to treat melanoma: down-regulation of tumor-specific production of IL-4, IL-5, and IL-10. *J Immunol* 177(2):991-998.
70. Engels B, Cam H, Schuler T, Indraccolo S, Gladow M, Baum C, Blankenstein T & Uckert W (2003) Retroviral vectors for high-level transgene expression in T lymphocytes. *Hum Gene Ther* 14(12):1155-1168.
71. Jorritsma A, Gomez-Eerland R, Dokter M, van de Kasteele W, Zoet YM, Doxiadis, II, Rufer N, Romero P, Morgan RA, Schumacher TN & Haanen JB (2007) Selecting highly affine and well-expressed TCRs for gene therapy of melanoma. *Blood* 110(10):3564-3572.
72. Leisegang M, Engels B, Meyerhuber P, Kieback E, Sommermeyer D, Xue SA, Reuss S, Stauss H & Uckert W (2008) Enhanced functionality of T cell receptor-redirected T cells is defined by the transgene cassette. *J Mol Med (Berl)* 86(5):573-583.
73. Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, ten Hagen T, Verweij J & Debets R (2010) Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother* 59(6):921-931.
74. Coral S, Sigalotti L, Colizzi F, Spessotto A, Nardi G, Cortini E, Pezzani L, Fratta E, Fonsatti E, Di Giacomo AM, Nicotra MR, Natali PG, Altomonte M & Maio M (2006) Phenotypic and functional changes of human melanoma xenografts induced by DNA hypomethylation: immunotherapeutic implications. *J Cell Physiol* 207(1):58-66.
75. Lucas S & Coulie PG (2008) About human tumor antigens to be used in immunotherapy. *Semin Immunol* 20(5):301-307.

SUPPLEMENTARY TEXT AND FIGURES

Additional tumor cell lines

Breast carcinoma cell line EVSA-T (MC2^{pos}/A2^{neg}) and esophagus carcinoma cell line TE-4 (MA-3^{pos}/DP4^{neg}) served as negative control cell lines in T cell assays, and were cultured with DMEM with 10% Fetal Bovine Serum, glutamine, antibiotics and 1% MEM non-essential amino acids.

Additional TCR constructs

MC2/A2 and MA3/DP4 TCRα and β genes were codon optimized (GeneArt, Regensburg, Germany) and cloned into the pMP71 vector (kindly provided by prof. Wolfgang Uckert, Max-Delbrück Center, Berlin, Germany), with TCRβ and TCRα genes separated by an optimized T2A ribosome skipping sequence (abbreviated as pMP71:opt TCRβ-T2A-α). TCRs were introduced into T cells and analyzed for surface expression, pMHC binding and TCR-mediated IFNγ production (*Supplementary Figures 1 and 2*). MC2/A2 TCR surface expression was measured with a FITC-labeled anti-TCRVβ28 mAb (clone CH92, Beckman Coulter) or MC2/A2 PE-labeled pMHC tetramers in combination with either anti-CD3 mAb or a combination of anti-CD3 and CD8 mAbs. MA3/DP4 surface expression was measured with a PE-labeled anti-TCRVβ2 mAb or MA3/DP4 PE-labeled pMHC tetramer in combination with either anti-CD3 mAb or a combination of anti-CD3 and CD4 mAbs.

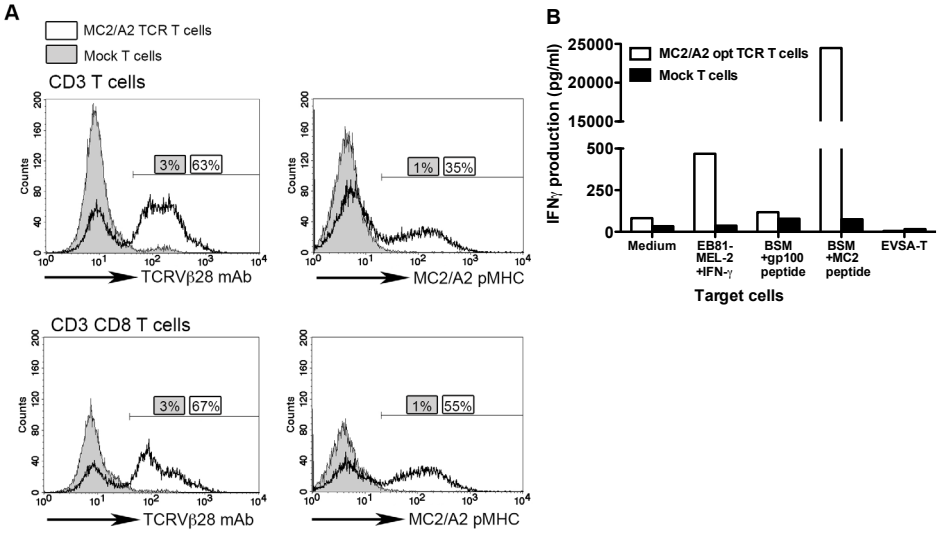

Figure S1. Codon-optimized MC2 TCR genes are functionally expressed in T cells.
A. Surface expression of codon-optimized MC2 TCR genes in PBMC. PBMCs were activated by anti-CD3 mAb and transduced with pMP71:opt MC2/A2 TCRβ-2A-α or control genes (i.e., pSTITCH: CAIX CAR:CD4γ (1), termed Mock T cells). CD3 and CD3, CD8-positive T cells were analyzed for TCRVβ28 expression and MC2/A2 pMHC binding by flow cytometry. **B.** MC2/A2-specific IFNγ production by PBMC transduced with codon-optimized MC2 TCR genes. Target cells were: MC2-positive, A2-positive EB81-MEL-2

melanoma cells; MC2^{pos}/A2^{neg} EVSA-T breast carcinoma cells, both pre-treated with IFNγ; or BSM B cells loaded with gp100 or MC2 peptide. Effector T cells were MC2/A2 TCR or Mock T cells (as in Figure S1A). T cells only were included as an additional control. Supernatants were harvested after 20 h co-culture between effector T cells and target cells, and analyzed for the presence of IFNγ by ELISA. Data are from representative experiments out of 15 experiments from 5 donors with similar results.

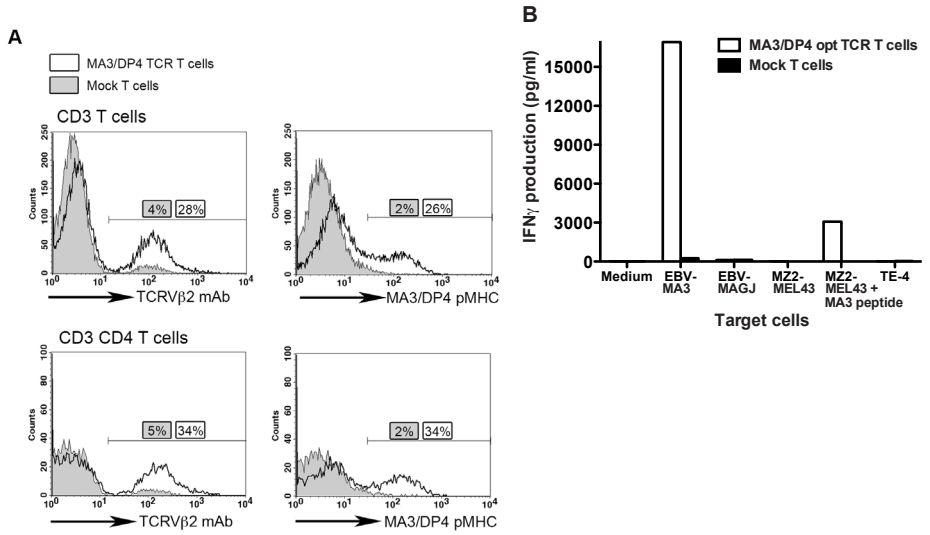

Figure S2. Codon-optimized MA3 TCR genes are functionally expressed in T cells.
A. Surface expression of codon-optimized MA3 TCR genes in PBMC. PBMCs were activated by anti-CD3 mAb and transduced with pMP71:opt MA3/DP4 TCRβ-2A-α or control genes (i.e., pMP71:opt MC2/A2 TCRβ-2A-α, termed Mock T cells). CD3 and CD3, CD4-positive T cells were analyzed for TCRVβ22 expression and MA3/DP4 pMHC binding by flow cytometry. **B.** MA3/DP4-specific IFNγ production by PBMC transduced with codon-optimized MA3 TCR genes. Target cells were: MA3-positive B cells (EBV-MA3); MA3-negative B cells (EBV-MAGJ); MZ2-MEL43 melanoma cells loaded with MA3 peptide or not (all HLA-DP4-positive cells) and MA3^{pos}/DP4^{neg} TE-4 esophagus carcinoma cells. Effector T cells were MA3/DP4 TCR or Mock T cells (as in Figure S2A). T cells only were included as an additional control. Supernatants were harvested after a 20 h co-culture between effector T cells and target cells, and analyzed for the presence of IFNγ by ELISA. Data are from representative experiments out of 6 experiments from 2 donors with similar results.

REFERENCES

1. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G & Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24(13):e20-22.

Chapter 6
General Discussion

Currently, TCR gene therapy is challenged by a lowered therapeutic efficacy compared to adoptive TIL therapy and treatment-related toxicity. In an effort to address these challenges, this thesis describes the assessment of TCR gene therapy directed against human antigens in relevant pre-clinical mouse models (chapters 3 and 4) and the characterization of clinically relevant TCRs that target highly tumor-restricted antigens (chapter 5). In paragraph 6.1 to 6.3 the major conclusions of chapters 3 to 5 are discussed and put into a clinical perspective. In paragraph 6.4, I propose how potential therapeutic strategies can be implemented in a future clinical trial of TCR gene therapy.

6.1 IMMUNE-DEFICIENT MOUSE MODELS FOR TCR GENE THERAPY

Chapter 3 presents an overview of immune-deficient mouse tumor models for the evaluation of receptor gene therapy. To our knowledge, successful treatment of human solid tumors with TCR-engineered T cells (TCR T cells) administered systemically has not yet been reported in immune-deficient mice. To obtain an immune-deficient melanoma mouse model for TCR gene therapy that provides translational value for future clinical trials, we targeted human melanoma tumors with both human and mouse T cells.

6.1.2 Human TCR T cells in immune-deficient mice

We used human TCR T cells that were processed according to protocols currently used in clinical TCR gene therapy trials. Different parts of the protocol were tested for their effect on the anti-tumor T cell response, including T cell dose and the degree of immune-deficiency. To this end, we have performed tests in both conventional SCID mice that were conditioned by different regimens and in NOD.SCID/*il-2rg*$^{-/-}$ (NSG) mice that are more deficient with respect to their immune system.

MAJOR FINDINGS CHAPTER 3, PART 1

In immune-deficient melanoma-bearing mice, human TCR T cells:

- Migrate to and accumulate at the tumor site immediately after transfer.
- Disappear gradually and are completely absent from the tumor site 72h after transfer.
- Do not persist in the peripheral blood of conventional SCID mice treated with different conditioning regimens, nor in NSG mice.
- Do not affect tumor growth in conventional SCID mice treated with different conditioning regimens, nor in NSG mice.
- Exhibit pre-dominantly (>90% of infused CD8+ T cells) a late differentiation T cell phenotype, a property that is related with compromised T cell persistence and diminished function *in vivo*.

Peripheral persistence of tumor specific T cells after adoptive transfer is the net result of proliferation and cell death of transferred T cells *in vivo* and is correlated with tumor regression in patients with melanoma (1-4). T cell proliferation and rescue from cell death depend on T cell activation and co-stimulation, and transferred T cells that persist in responding patients express high levels of the T cell co-stimulatory molecules CD27 and CD28 (1). T cell proliferation without co-stimulation can result in enhanced T cell differentiation, which is inversely related with T cell persistence (5-7). Notably, systemic treatment of solid tumors with Chimeric Antibody Receptor engineered T cells (CAR T cells) in immune-deficient mice (that were not pre-treated) proved successful and T cells. When comparing successful studies with CAR T cells with our results, one can identify parameters of interest that may improve future studies with TCR T cells. *First*, studies with CAR T cells were performed with lentivirally transduced T cells cultured for up to 10 days until transfer (8, 9), whereas our study was performed with retrovirally transduced T cells cultured for 3 to 4 weeks. The shorter T cell transduction and culture protocol resulted in less differentiated T cells with longer telomeres at the time of adoptive transfer (8). Second, CARs incorporate co-stimulatory molecules, such as CD28 and TNF receptor superfamily members CD134 (OX-40) and CD137 (4-1BB), generally in tandem with CD3ζ (8, 10-13), wheras TCRs are generally used as wild type transgenes that lack co-stimulatory molecules. Since tumor cells usually lack co-stimulatory ligands, addition of these molecules provides CARs to mediate co-stimulatory signals upon tumor recognition. Co-stimulatory CARs induced regression of solid tumors in immune-deficient mice whereas the same receptors without the co-stimulatory domains did not or significantly less (8, 9, 14-16). In particular, CARs containing CD137 proved to increase the persistence of T cells in NSG mice, which was demonstrated for three different antigens, namely the folate receptor-α in ovarian cancer (9), mesothelin in mesothelioma (8), and CD19 in leukemia (17). In addition to pre-clinical studies, a recent clinical study of CAR T cell therapy has used co-stimulatory CAR T cells to target the CD19 antigen on B cell leukemia in patients with high tumor burden and refractory to standard treatment. CAR T cell treatment resulted in complete clinical responses and long-term persistence of CAR T cells in 2 out of 3 patients (18, 19). This study made use of a CD19-specific CAR:CD137-CD3ζ, whereas other studies targeting B cell leukemia also reported encouraging clinical responses using CD19-specific CAR:CD28-CD3ζ (20, 21).

Based on above mentioned studies, it is fair to state that strategies that limit T cell differentiation and/or enhance T cell co-stimulation are expected to increase persistence and anti-tumor responses of TCR T cells. Below I have described 2 of such strategies that are developed and tested in our laboratory.

1) T cell processing methods

One way to obtain less differentiated T cells is to minimize T cell activation necessary for retroviral gene transfer (22, 23). Along this line, we have showed that activation of primary

murine T cells with soluble anti-CD3/CD28 monoclonal antibodies (mAbs) halted T cell differentiation and improved antigen-specificity of T cell responses compared to activation with immobilized anti-CD3 and CD28 mAbs (24). A second way to obtain a preferred T cell phenotype and function may be to expose T cells to common-γ cytokines other than IL-2. Recent studies suggest that cytokines such as IL-7, IL-15 and IL-21 play important roles in T cell differentiation (25-27). We observed that short-term exposure of primary murine T cells to IL-21, and to a lesser extent a combination of IL-15 and IL-21, resulted in a significant enrichment of T cells with a more naïve T cell phenotype (24). These T cells express high levels of the lymph node homing molecules CD62L and CCR7, low levels of the T cell activation marker CD44, and intermediate levels of the co-stimulatory molecule CD27. Moreover, IL-15 plus IL-21 enhanced antigen-specific T cell cytotoxicity and accelerated the kinetics and levels of T cell IFNγ (24). In extension to data with primary murine T cells, experiments with PBMC from healthy human donors demonstrated that T cell activation using soluble CD3 and CD28 mAbs, and addition of IL-15 plus IL-21 from the start of T cell activation induces T cells with a preferred phenotype and function *in vitro* (Lamers, manuscript in preparation). These T cells demonstrated enhanced binding of pMHC, contain an enhanced proportion of CD8+, CD27+, CD62L+, CD45RA+ T cells, and a lowered proportion of CD4+, CD25+, CD127- T cells and mediated enhanced T cell cytotoxicity.

2) TCR format incorporating co-stimulatory domains

With respect to modifications of the TCRαβ format, our preliminary work demonstrates that a cassette constituting the transmembrane and intracellular domains of CD28 coupled to the intracellular domain of CD3ε (i.e. TCR:CD28-CD3ε) prevents TCR mispairing and significantly enhances antigen-specific T cell function.

Results in chapter 3 were in accordance with the limited success rate of clinical studies using a comparable protocol for the production of receptor gene engineered T cells (28-30). Granted that observed anti-tumor responses of TCR T cells are disappointing, we conclude that immune-deficient mouse models may represent valid models to test strategies *to enhance* persistence and anti-tumor effects of *human TCR T cells*.

6.1.3 Mouse TCR T cells in immune-deficient mice

We have tested whether mouse TCR T cells were able to affect melanoma tumor growth in conventional SCID mice, as has been reported for CAR T cells (10, 31-34).

MAJOR FINDINGS CHAPTER 3, PART 2

In immune-deficient melanoma-bearing mice, mouse TCR T cells:

- Mediate a xeno-specific anti-tumor response resulting in clearance of established human melanoma (with volumes up to 700 mm3) independent of the presence of the TCR transgene.
- Undergo homeostatic proliferation and persistence.
- Are present at the margins of regressing melanoma tumors and are predominantly (but not exclusively) of the CD4+ T cell subset.
- Show improved T cell responses upon T cell activation, retroviral transduction or IL-2 support, although these T cell manipulations are not required per se for effective T cell responses.

High MHC class I expression levels on human tumors, necessary for efficient TCR T cell recognition and anti-tumor responses, may be responsible for the xeno-reactive response of mouse T cells (35). In contrast to TCR T cells, CAR T cells are not dependent on high MHC expression and using tumor cells with low MHC class I expression creates a window of opportunity for the use of mouse CAR T cells. Overall, and shown here for two different SCID strains and two tumor cell lines, we conclude that adoptive transfer of mouse TCR T cells in immune-deficient mouse models does *not* represent a valid pre-clinical model for TCR gene therapy.

6.2 IMMUNE-COMPETENT MOUSE MELANOMA MODEL FOR TCR GENE THERAPY

In **chapter 4** we describe an immune-competent, HLA-A2 transgenic (tg) mouse model to assess TCR gene therapy directed against a human melanoma antigen. Our TCR gene therapy protocol combined chemotherapeutic conditioning with Busulfan and Cyclophosphamide (Bu/Cy) with adoptive transfer of gp100/HLA-A2 (gp100/A2) TCR-engineered T cells directed against mouse B16 melanoma cells expressing human gp100/HLA-A2. Using this model, we determined the anti-tumor efficacy of TCR T cells and addressed the exact contribution of target antigen expression to the occurrence of tumors relapse.

6.2.1 Major findings of the HLA-A2tg melanoma mouse model

MAJOR FINDINGS CHAPTER 4

In immune-competent HLA-A2tg mice, TCR T cells:

- Prevent tumor growth in up to 70% of mice in a dose-dependent manner.
- Cure tumors in 10-20% of mice.
- Caused near to complete tumor regression followed by tumor relapse in 80-90% of mice.
- Are present at detectable levels in peripheral blood until 2-3 weeks after T cell transfer.
- Generate functional memory T cells in case of tumor prevention or cure.
- Do *not* result in 'de novo' generation of antigen-negative tumor cells, on the contrary, antigen-negative tumor cells are selected from pre-treatment variants by TCR T cells.
- Do *not* induce genetic or epigenetic changes of the antigen gene nor affect functional expression of surface antigen.
- Do *not*, when administered as a second treatment against relapsed tumors, enhance tumor cures.
- Do *not* induce a spread of new immunogenic antigens.

Notably, percentages of cured mice are comparable with complete response rates that have been reported in recent TCR gene therapy trials treating patients with metastatic melanoma (36). In our model, we observed that besides tumor cures, tumors mostly relapse. These relapsed tumors resist a second T cell treatment. Clinical TCR gene therapy is also faced with tumor relapse and resistance, hurdles that need to be taken before long-term cure of these patients becomes feasible. The immune-competent HLA-A2 tg mouse melanoma model, described in this thesis, provides a fair representation of the clinical setting and may constitute an important tool to test additional strategies or treatments to increase the efficacy of TCR gene therapy. Next, I will first discuss the characteristics of our mouse model. Then I will discuss how antigen-negative and positive tumor variants have escaped T cell treatment and how supportive treatments may lead to more durable responses.

6.2.2 Characteristics of HLA-A2tg melanoma mouse model

Development of an immune-competent mouse model for TCR gene therapy with highly avid T cell responses against a human melanoma antigen was based on the following three features (see Figure 1):

First, a B16:A2-YLEP mouse melanoma cell line has been engineered to express a chimeric molecule composed of the α1 and α2 domains of HLA-A2 and the α3 domain of a mouse MHC class I molecule, H2-Kb. This HLA-A2/Kb fusion molecule was genetically linked to the human $gp100_{280\text{-}288}$ epitope (and referred to as A2-YLEP) to constitute a target antigen for T

cell treatment. This approach intended to circumvent the poor antigen processing and presenting machinery of B16 melanoma cells (37). Indeed, B16:A2-YLEP cells induced enhanced antigen recognition and IFNγ production by TCR T cells *in vitro* when compared to B16 cells with HLA-A2 and either wild type or an anchor residue-modified gp100 expressed from separate vectors. Numerous B16:OVA cell lines that are gene-engineered to express complete ovalbumin protein have been reported to evoke robust responses by high affinity OTI/II TCR T cells *in vitro* and *in vivo* (38, 39). Funtional antigen recognized by TCR T cells implies that OVA antigens can be processed and presented by mouse MHC molecules. Possibly, B16 cells, despite the expression of tg HLA-A2 have more difficulty to correctly process and present human antigen. Importantly, *in vivo* growth kinetics of B16:A2-YLEP cells in HLA-A2 transgenic mice were comparable to those of B16WT cells, suggesting that the A2-YLEP antigen did not evoke an endogenous immune response.

Second, the gp100/HLA-A2 specific human TCR (from clone 296,(40)) has been murinized (i.e. the human TCR variable domains coupled to murine TCR constant domains) to enable expression by mouse T cells. Murinized gp100/A2 TCRα and β sequences (41) were codon optimized and cloned into the pMP71 retroviral vector in a TCRβ-2A-α configuration. TCR surface expression and gp100/A2 pMHC-binding by T cells were significantly higher when compared to non-optimized or optimized TCRα and β chains that were expressed from separate vectors. The use of a human TCR targeting a human tumor epitope provides translational value and allows validation of clinically relevant TCRs in an immune competent setting. In example, a model presented by Koya and colleagues was used to study the biodistribution of adoptively transferred T cells directed against human tyrosinase in HLA-A2tg mice and demonstrated the therapeutic efficacy of a combination of T cells, dendritic cells and IL-2 (42). It is of note that the human variable domains of the TCR may induce immune responses in immune-competent mice resulting in limited T cell persistence and function as observed for

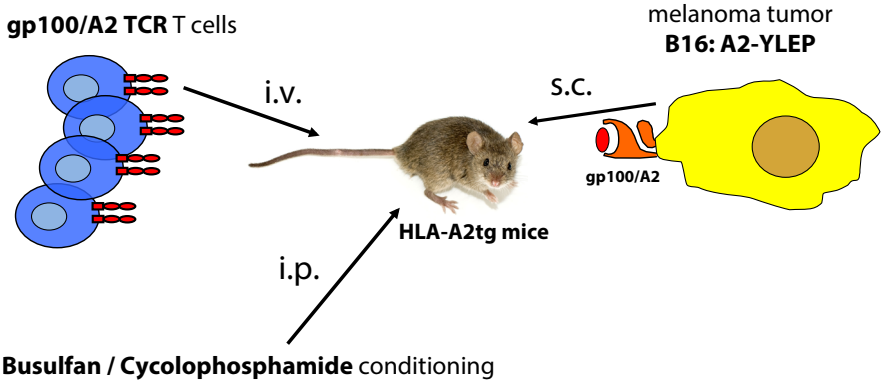

Figure 1. Schematic overview of the immune competent HLA-A2tg melanoma model
s.c.= subcutaneous, i.v.= intravenous, i.p.=intraperitoneal injections

mouse antibody sequences in patient studies with CAR T cells (43). Although our studies in chapter 4 do formally not proof lack of an immune response against the human sequences in the TCR transgene, the inclusion of chemotherapy and the detection of functional memory TCR T cells in cured mice argue against such a response.

Third, injections of Busulphan and Cyclophosphamide (Bu/Cy) were included to condition mice before T cell transfer. Bu/Cy treatment resulted in a clear, but transient, reduction of absolute numbers of T, B, NK and dendritic cells and enhanced anti-tumor T cell responses. This is in line with enhanced anti-tumor responses reported in both pre-clinical models and clinical trials, where adoptive T cell transfer was preceded by irradiation or chemotherapy (3, 38, 44). Mechanisms that contribute to the role of conditioning have been extensively studied and include: 1) elimination of cytokine 'sinks', i.e., those cell types that consume homeostatic cytokines including IL-7 and IL-15, which increases the availability of these cytokines for adoptively transferred T cells; 2) depletion of suppressor cells, such as T regulatory T cells (Treg); 3) activation of antigen presenting cells (45, 46); 4) general activation of the immune system caused by the release of 'danger signals' such as microbial translocation and the release of lipopolysaccharide following damage to the gastrointestinal tract (47); and 5) direct cytotoxic effects on tumor cells which increase their immunogenicity and result in release of antigens from dying tumor cells (48-50).

6.2.3 Tumor relapse

Antigen-negative tumor variants

As described in chapter 4, antigen-negative tumors are selected from rare, yet pre-existing tumor variants and their occurrence does not depend on 'de novo' gene loss or genetic changes. Frequencies of antigen-negative tumor cells in pre-treatment tumors as low as 0.0002% (i.e. to 1 in 500,000 cells) can increase up to 100% in relapsed tumors following T cell treatment. The significant driving force of T cell selection, although not novel in itself, is generally not taken into account when assessing the genetic status of antigens from heterogeneous tumors. Therefore, we would prefer to use the term 'antigen-negative' variants rather than 'antigen-loss' or 'tumor escape' variants unless evidence is provided for active cancer immune-editing.

Antigen-positive tumor variants

Also described in chapter 4, antigen-positive tumor cells, present in relapsed tumors, show intact antigen genes, no change of promoter methylation and sustained levels of functionally expressed antigen. These observations extend the cancer immuno-editing hypothesis, whereby tumor growth is controlled by T cells until tumors escape and become edited and grow into large cancers (51, 52). Originally, this hypothesis proposed the loss of immunogenicity by tumor cells as a fundamental driver of accelerated tumor growth. In fact, our data

argue that in T cell-treated tumors, loss of immunogenicity does not need to be a driving mechanism in escaping tumors.

6.2.3.1 Potential explanations for tumor relapse

Antigen spreading and cross-presentation

Durable anti-tumor T cell responses may need involvement of endogenous T cell specificities directed against the tumor, besides gp100 YLEP/A2, referred to as antigen spreading. Also, durable anti-tumor T cells responses may rely on presentation of the tumor target antigen on non-malignant cells, i.e. the tumor stroma, referred to as cross-presentation. Upon an initial immune response, antigen spreading and cross-presentation have both been implicated to broaden the response to tumor antigens (53-59). In example, antigen spreading has been reported in a patient with metastatic melanoma in which transfer of NY-ESO-1 specific CD4+ T cells resulted in durable clinical remission. In addition to NY-ESO-1 specific T cells, T cells directed against melanoma antigens MART-1 and MAGE-A3 weredetected in the patient's blood (54). Also, Spiotto and colleagues demonstrated complete T cell-mediated eradication of tumor cells in case sufficient antigen is released from tumor cells, taken up and cross-presented by tumor stromal cells (58). Interestingly, in the latter model, also antigen-negative tumor cells were eradicated through indirect bystander killing. However, in tumor-bearing HLA-A2tg mice that became tumor-free upon treatment with TCR T cells, we were unable to detect T cell responses to B16 antigens other than gp100/A2. We were also unable to demonstrate that tumor cells that express the complete human gp100 protein, and thus allow cross-presentation of HLA-A2-restricted gp100 epitopes, evoke an enhanced anti-tumor T cell response when compared to tumor cells that lack human gp100 protein. Although the T cell repertoire of the HLA-A2 tg mice may be biased and we formally do not know whether sufficient numbers of the non-YLEP epitopes are available through the mouse antigen processing pathways, these results imply that, at least in our model, antigen spreading and cross-presentation do not explain tumor cure.

Immune suppressive mechanisms induced by tumors

Another potential explanation for tumor relapse may relate to the T cell inhibitory activity of the tumor milieu (60, 61). There is an increasing body of evidence that tumor cells are very well capable of avoiding an immune response or disabling effector T cells, both resulting in progressive tumor growth (62-64). In fact, tumors employ a wide array of mechanisms to evade T cell responses, which include but are not limited to the following:

- Recruitment or induction of immune suppressive cells such as Treg cells (65) and myeloid-derived suppressor cells (MDSC) (66, 67). Immune suppressor cells can reduce T cell infiltration into the tumor and suppress local T cell responses by: 1) release of reactive nitrogen and oxygen species (68, 69); 2) by expression of indoleamine 2,3-dioxygenase

(IDO) and arginase that depletes amino acids such as tryptophan and L-arginine (70-72); and 3) production of cytokines such as TGF-β, IL-4 and IL-13 (73).

- Cognate interactions between tumor cells and tumor infiltrating T cells may result in decreased proximal TCR signaling by a limited expression of CD3ζ and the $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases in T cells (63, 74).
- Expression of T cell inhibitory molecules that activate signaling pathways such as CD80/CD86-CTLA-4 (Cytotoxic T-Lymphocyte Antigen-4), PD-L1/PD-1 (Programmed Death (Ligand) 1), galectin-9/TIM-3 (T cell Immunoglobulin Mucin 3), MHC II/LAG-3 (Lymphocyte-Activation Gene 3) and HVEM/BTLA (Herpes Virus Entry Mediator / B and T-lymphocyte Attenuator) (75-80).

What lines of evidence would support local immune suppression as an explanation for tumor relapse? In our melanoma mouse model, progression of tumors was found in a curative rather than in a preventive setting. Moreover, regressed tumors showed enhanced levels of surface-expressed antigen, yet induced decreased production of T cell IFNγ (see chapter 4). It is generally accepted that anti-tumor immune responses coincide with local production of cytokines, such as IFNγ, TNFα by cytotoxic CD8 T cells, T helper-1 CD4 T cells, and NK cells (81, 82), which result in increased antigen presentation by tumor cells. At the same time, tumor regression may initiate negative feedback mechanisms (as listed above) that are responsible for an impaired T cell function and tumor relapse. Notably, relapsed tumors have remained their immunogenicity and ability to induce T cell IFNγ *ex vivo*, yet are not sensitive for a second T cell treatment *in vivo*. These data suggest that relapsed tumors in contrast to regressed tumors have a microenvironment that limits T cell infiltration rather than T cell functions, a hypothesis that is currently under investigation. Our results with respect to preservation of tumor immunogenicity are in line with two other models in which tumors remain immunogenic at advanced stages, one with inducible autochthonous lung tumors and another with inducible ovarian carcinoma (83, 84). These two models demonstrate that T cell responsiveness weakens as a consequence of T cell exhaustion and local immune suppression. The contribution of tumor-induced immune suppression has recently been added to the cancer immuno-editing model (85).

6.2.3.2 Supportive treatments to prevent or limit tumor relapse

Chapter 1.2, Table 3, provides an extensive list of strategies that manipulate T cell and tumor-related factors to enhance the therapeutic efficacy of TCR gene therapy and prevent or limit tumor relapse. Some of these strategies have already been applied in our HLA-A2tg mouse model, such as optimal design of vector, TCR transgenes and chemotherapeutic conditioning regimen. Other strategies and supportive treatments that are considered promising and referred to in paragraph 6.3 and 6.4 include: T cell processing protocols that yields T cells of a preferred phenotype (see also paragraph 6.1); affinity maturation of TCR-variable domains to increase T cell avidity; the use of epigenetic drugs to enhance the expression of target antigens, such as MAGE antigens. Here, I will expand on such strategies and supportive

treatments that enhance either the effector function of TCR T cells or target the immune suppressive tumor environment.

Induction of tumor cell death

Irradiation and chemotherapeutic conditioning induces not only lymphodepletion but also tumor cell death and increased availability of tumor antigens for presentation and activation of the immune system. Along this line, enhanced anti-tumor effects may be obtained in case TCR gene therapy will be preceded by targeted therapy for cancer using small-molecules such as BRAF-inhibitors (PLX4032 and others) (reviewed in (86)). These molecules have recently been reported to specifically target signaling routes in melanoma and induce fast clinical responses (87, 88). Although, treatment with BRAF-inhibitors results in only moderate increases in tumor free survival, the induction of tumor cell death and tumor antigen presentation with BRAF-inhibitors prior to transfer of TCR T cells may improve durable clinical responses.

Enhance tumor recruitment and activation of innate immune cells

An immune suppressive tumor milieu may shift to an immune potentiating milieu by the recruitment and local activation of innate immune cells. In addition to tumor-specific receptor genes, T cells can be engineered with genes encoding effector molecules and used as gene delivery vehicles for these molecules. Recently, studies have shown that CAR T cells gene-engineered to release the cytokine IL-12 enhanced the therapeutic efficacy of CAR T cells (89, 90). Notably, it has been reported that CAR T cells that express IL-12 under the control of the Nuclear Factor of Activated T cell (NFAT) promoter, and deliver IL-12 locally in the tumor environment upon encounter of cognate antigen, induce destruction of antigen-negative cancer cells (89). The increased anti-tumor effect is likely the result of a reversion of the suppressive tumor milieu to a more acute inflammatory milieu that stimulates T cell effector functions (91). In particular, locally delivered IL-12 recruits and activates innate immune cells (i.e. monocytes) (89). Induced (i)IL-12 CAR T cells result in only transient toxicity, whereas CAR T cells that constitutively deliver IL-12 result in systemic toxicity (90). To improve TCR gene therapy it may be worthwhile to investigate the inducible T cell expression of other cytokines that, such as IL-12, induce a T helper type-1 response.

Enhance tumor recruitment of T cells

The immune suppressive tumor environment inhibits efficient trafficking and infiltration of tumor-specific T cells. T cells engineered to express chemokine receptor, CXCR2, show enhanced trafficking towards tumor cells secreting the corresponding chemokine ligand, CXCL1 (92). In xenograft tumor models of mesothelioma and neuroblastoma the genetic introduction of CCR2 in CAR-engineered T cells resulted in increased T cell infiltration in tumors secreting CCL2 and was associated with significantly increased anti-tumor activity (14, 93). In addition to chemokines, one can also target IDO or reactive nitrogen species (RNS) to

enhance T cell accumulation at the tumor site. Inhibition of IDO blocks tryptophan depletion, enhances T cell accumulation at the tumor site and delays tumor growth (71). RNS induce TIL unresponsiveness (94), nitration of the TCR complex (95) and modification of the chemokine CCL2 (69). Drugs affecting the local production of RNS restore TIL function and improve intratumoral T cell migration (69, 94).

Enhance T cell effector functions

T cell effector functions can be enhanced along the following lines: *Firs*t, selective blocking of (a combination of) T cell inhibitory molecules such as PD-1, CTLA-4, BTLA-1 or TIM-3 may increase therapeutic efficacy. In example, clinical trials that target T cell inhibitory molecules, such as CTLA-4 and PD-1, enhanced anti-tumor T cell responses and demonstrated clinical benefit (96, 97). In contrast to systemic administration of blocking antibodies, more specific targeting of TCR T cells with PD-1 siRNA (98) may represent a more elegant approach. *Second*, the local delivery of (a combination of) T cell activation molecules may enhance anti-tumor responses. In example, delivery of cytokines using T cells coated with IL-15 and IL-21 containing liposomes enhanced anti-tumor T cell responses (99). *And finally*, inhibition of T cell suppressive cytokines, such as TGF-β may be another interesting strategy. The genetic introduction of a dominant-negative TGF-β receptor II in TCR T cells has increased anti-tumor T cell responses in a spontaneous tumor model of prostate cancer (G. Bendle, personal communications).

Target multiple antigens simultaneously

The simultaneous activation of CD4 and CD8 T cells may result in enhanced anti-tumor T cell responses. In current TCR gene therapy trials, single MHC class I restricted tumor antigens are targeted, which results in mono-specific CD8 T cell responses. Clinical responses observed with adoptive therapy of TILs, containing T cells specific for multiple antigens, are generally better when compared to clinical trials with mono-specific TCR T cells (see chapter 1.1 Table 1). For future trials, one may consider to target multiple tumor-specific antigens, preferably a combination of MHC class I and II restricted antigens, and as such to involve both CD8 *and* CD4 T cells. CD4 T cells target MHC class II-restricted antigens and play a role in priming the immune response and the formation of long-lived CD8 memory T cells (reviewed in (100) and discussed in chapter 2). In fact, administration of CD4 T cells has been shown to prevent exhaustion of infused CD8 T cells (101, 102). Recently, it became clear that CD4 T cells are also essential in the effector phase of the anti-tumor immune response and enable the elimination of tumors via targeting of tumor stroma (57). See paragraph 6.3 for examples of class I and II-restricted TCRs to redirect CD8 and CD4 T cells, respectively.

In conclusion, we have demonstrated in chapter 4 that tumor relapse does not depend on loss of immunogenicity, but rather on the strength of initial T cell selection. Therefore, TCR

gene therapy qualifies as a valid primary therapy and combined with promising supportive treatment(s) may develop into a durable therapy for cancer.

6.3 MHC CLASS I AND II RESTRICTED MAGE-SPECIFIC TCRS

In **chapter 5** we have characterized two patient-derived MAGE-specific TCRs for their potential use in TCR gene therapy.

MAJOR FINDINGS CHAPTER 5, MAGE-C2/HLA-A2 (MC2/A2) TCR

- The MC2/A2 TCR is composed of a TCR-Vα3 and TCR-Vβ28 chain.
- MC2/A2 TCR gene-engineered CD8+ T cells produce IFNγ and display cytolytic activity in response to HLA-A2^{pos}MC2^{pos} tumor cells.
- MC2/A2 TCR surface expression was enhanced due to the use of codon optimized TCR transgenes introduced into the retroviral vector pMP71 in a TCRβ-2A-TCRα cassette.

MAJOR FINDINGS CHAPTER 5, MAGE-A3/HLA-DP4 (MA3/DP4) TCR

- The MA3/DP4 TCR is composed of a TCR-Vα38 and TCR-Vβ2 chain.
- MA3/DP4 TCR-engineered CD4+ T cells produce IFNγ, IL-4, IL-5 and low amounts of IL-2 in response to MA3-protein loaded dendritic cells.
- MA3/DP4 TCR surface expression was enhanced due to the use of codon optimized TCR transgenes introduced into the retroviral vector pMP71 in a TCRβ-2A-TCRα cassette.

6.3.1 MAGE specific TCRs towards clinical application

MAGE T cell epitopes represent attractive targets for anti-tumor T cell therapy, because of their highly tumor-restricted expression and contribution to tumorigenesis as described in chapter 1. The results of the NY-ESO-1/A2 TCR gene therapy trial in melanoma and synovial sarcoma patients together with available pre-clinical data (chapter 5) justify the pursuit of a clinical trial with MAGE TCRs. However, before the MC2/A2 and MA3/DP4 TCRs are tested in clinical trials, the following parameters are currently assessed and discussed below.

Response towards non-melanoma tumors

The expression of MAGE proteins in a wide range of cancer types (see for an overview of cancer testis protein expression www.cta.lncc.br) make MAGE-specific TCRs potential candidates for T cell treatment of tumors other than melanoma. In light of this view, we are currently testing a panel of tumor cells from different histological origins for recognition by the MAGE TCR-engineered T cells.

MAGE surface expression levels

The expression levels of cancer testis antigens (CTA) are heterogeneous, even within tumors (103) and generally lower than those of tumor associated differentiation antigens such as gp100 or MART-1. *In vitro* studies suggest that epigenetic modifications affect the expression of CTAs and MHC molecules (104-107). In addition, *in vivo* treatment with the DNA demethylating agent 5-aza-2-deoxycytidine (Decitabine) has been reported to enhance the expression and immunogenicity of different CTAs and HLA class I molecules on melanoma cells (108). Also treatment with another demethylating agent, Azacitidine, was found to increase CTA expression and T cell recognition of cell lines of renal cell carcinoma, neuroblastoma, multiple myeloma and others (109-114). Both Decitabine and Azacitidine are currently used as cytotoxic agents in phase II/III clinical trials to treat hematopoietic malignancies (115-118). The effect of Decitabine on CTA expression and its immunogenicity was further enhanced by other epigenetic modifying agents such as inhibitors of histone methyltransferases (119). Collectively, these studies underscore the potential value of epigenetic drugs to enhance the therapeutic efficacy of adoptive transfer of TCR T cells. Although CTA expression is highly tumor-restricted, careful monitoring of their expression pattern in the adult body is advised when using epigenetic drugs.

TCR ligand-binding affinity

TCR affinity is an important factor that defines the functional avidity of T cell (120). The MAGE-specific TCRs described in chapter 5 display low to moderate affinities for their cognate antigen. As a surrogate assay for ligand-binding affinity of the MC2/A2 TCR, the effective dose of MC2/A2 peptide at which parental cytotoxic CD8+ T cells show a half-maximal lytic response (i.e., ED50) was determined, and was observed to be 0.75 nM (121). This measure of T cell avidity lags behind those reported for T cells expressing other MHC class I TCRs (range: 30-100 pM) (40, 122). The ED50 value of MA3/DP4 peptide in a IFNγ assay of parental T cells is 30 nM (123). This value is more or less in accordance with reported values for T cells expressing other MHC class II TCRs (range: 40-200 nM) (124, 125). Affinity-enhancement of TCRs may increase the functional responses of CD4 and CD8 T cells (126, 127). Notably, a successful clinical TCR gene therapy trial has been performed with an affinity-enhanced 1G4 TCR: NY-ESO-1/A2 (K_D of 730nM, 13-fold higher than the WT TCR) (36). Binding of the MC2/A2 TCR to its peptide MHC ligand is dependent on the CD8 co-receptor (non-published data in collaboration with prof. Andrew Sewell, University of Cardiff, UK) and affinity-enhancement of this TCR is expected to increase functional responses of CD4 and CD8 T cells. Also, affinity enhancement of the MA3/DP4 TCR may result in increased functional expression of this TCR by both subsets of T cells and increase for example responses of CD4 T towards melanoma cells. Affinity-maturation of both TCRs, using methodologies as described in chapter 2, is considered a worthwhile investment prior to the clinical testing of these TCRs. Validation of such

affinity-enhanced TCRs should be performed thoroughly and take into account a potential upper limit of TCR affinity to exclude self-reactivity ((126, 128, 129) and Govers, manuscript, in preparation).

6.4 TCR GENE THERAPY: A PERSONALIZED, COMBINED THERAPY FOR CANCER

A constant cross-talk between clinical TCR gene therapy trials and laboratory studies is of vital importance to be able to address questions that arise from patient studies in appropriate pre-clinical models and, vice versa, to test new therapeutic strategies from pre-clinical models in patients. In this paragraph, and based on current knowledge obtained from preclinical and clinical reports, I have made an effort to design a TCR gene therapy trial that would result in safe and improved clinical responses.

Personalized therapy
In the last 10 years the development of TCR gene therapy has been quite impressive, from first *in vitro* (130, 131) and *in vivo* (132) anti-tumor responses of TCR T cells to clinical responses in patients (36). Increased understanding of tumor immunology and its recognition as a hallmark of cancer (133) will further boost the development of TCR gene therapy for the treatment of cancer. It is anticipated that in the future a collection of suitable TCRs becomes available and that such a collection of TCRs will be tested as 'off the shelf' reagents in patients with a wide range of tumor types. Notably, successful TCR gene therapy will incorporate strategies to counteract multiple hallmarks of cancer development to improve complete response rates (as also discussed in paragraph 6.2).

Choice of target antigen(s) and TCR
MC2/A2 and MA3/DP4 represent target antigens of choice and, when targeted simultaneously, combine MHC class I and II antigens and hence allow exploitation of both CD4 and CD8 T cells. In case of a low natural affinity of the chosen TCR, affinity maturation of the TCR may be important for good clinical responses.

T cell activation, TCR gene transfer and T cell culture protocols:
A clinical batch of TCR T cells must contain T cells with a least differentiated T cell phenotype and high functional expression of TCR genes. To this end the following steps are included:

- Retroviral gene transfer with codon-optimized TCR gene sequences in a pMP71 retroviral vector in a TCRβ-2A-α configuration.
- T cell activation using soluble anti-CD3 and CD28 mAb.
- Following retroviral transduction, T cells are cultured in the presence of IL-15 and IL-21.

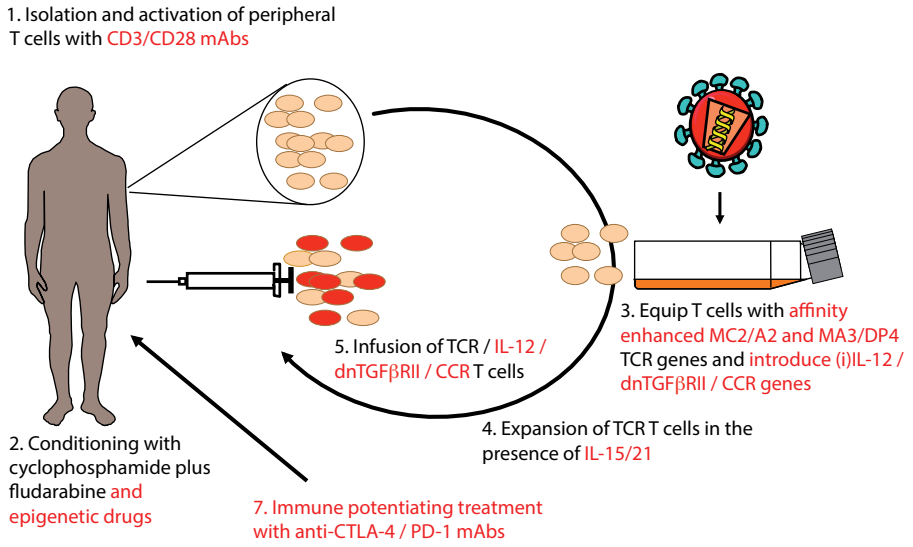

Figure 2. Design of a future clinical TCR gene therapy trial.
In red are indicated the parameters that have not yet been tested in clinical TCR gene therapy

Lentiviral-based gene transfer protocols have been reported to yield T cells with a less differentiated T cell phenotype compared to retroviral transduction protocols and are successfully applied in CAR T cell trials (19). In addition, IL-2 support following T cell transfer appears not to be necessary for tumor regression in mice (our non-published results) and men (19). Therefore, lentiviral T cell transduction and omission of *in vivo* IL-2 are validated alternatives for future clinical trials.

Patient conditioning

Prior to adoptive transfer of TCR T cells patients should be conditioned, for example with a non-myeloablative lymphodepletion regimen composed of cyclophosphamide and fludarabine. It would be worthwhile to study whether a combination of chemotherapy and demethylating drugs such as Azacitidine or Decitabine would provide improvement of patient conditioning prior to T cell therapy.

Additional supportive treatments

The following additional treatments (or combinations of treatments) may further support responses of TCR T cells by direct T cell potentiating or counteracting the immune suppressive tumor environment:

- Introduction of inducible IL-12 genes into TCR T cells.
- Introduction of a double-negative TGF-β receptor (dnTGFβRII) into TCR-engineered T cells.
- Introduction of a chemokine receptor (CCR) into TCR T cells.
- Administration of anti-PD-1 or CTLA-4 mAbs following transfer of TCR T cells.

Taken together, I would like to propose the following scheme for a future TCR gene therapy trial in Figure 2.

REFERENCES

1. Powell DJ, Jr., Dudley ME, Robbins PF & Rosenberg SA (2005) Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. *Blood* 105(1):241-250.
2. Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, Huang J, Powell DJ, Jr. & Rosenberg SA (2004) Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 173(12):7125-7130.
3. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, Citrin DE, Restifo NP, Robbins PF, Wunderlich JR, Morton KE, Laurencot CM, Steinberg SM, White DE & Dudley ME (2011) Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* 17(13):4550-4557.
4. Zhou J, Dudley ME, Rosenberg SA & Robbins PF (2005) Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. *J Immunother* 28(1):53-62.
5. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C & Riddell SR (2008) Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* 118(1):294-305.
6. Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, Sanchez-Perez L, Muranski P, Kern SJ, Logun C, Palmer DC, Ji Y, Reger RN, Leonard WJ, Danner RL, Rosenberg SA & Restifo NP (2009) Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A* 106(41):17469-17474.
7. Huang J, Kerstann KW, Ahmadzadeh M, Li YF, El-Gamil M, Rosenberg SA & Robbins PF (2006) Modulation by IL-2 of CD70 and CD27 expression on CD8+ T cells: importance for the therapeutic effectiveness of cell transfer immunotherapy. *J Immunol* 176(12):7726-7735.
8. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I & June CH (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U S A* 106(9):3360-3365.
9. Song DG, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, Figini M, June CH, Coukos G & Powell DJ, Jr. (2011) In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). *Cancer Res* 71(13):4617-4627.
10. Haynes NM, Trapani JA, Teng MW, Jackson JT, Cerruti L, Jane SM, Kershaw MH, Smyth MJ & Darcy PK (2002) Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors. *Blood* 100(9):3155-3163.
11. Imai C, Mihara K, Andreansky M, Nicholson IC, Pui CH, Geiger TL & Campana D (2004) Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* 18(4):676-684.
12. Moeller M, Haynes NM, Trapani JA, Teng MW, Jackson JT, Tanner JE, Cerutti L, Jane SM, Kershaw MH, Smyth MJ & Darcy PK (2004) A functional role for CD28 costimulation in tumor recognition by single-chain receptor-modified T cells. *Cancer Gene Ther* 11(5):371-379.
13. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM & Brenner MK (2005) A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Mol Ther* 12(5):933-941.

14. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM & Foster AE (2010) Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. *J Immunother* 33(8):780-788.
15. Pinthus JH, Waks T, Kaufman-Francis K, Schindler DG, Harmelin A, Kanety H, Ramon J & Eshhar Z (2003) Immuno-gene therapy of established prostate tumors using chimeric receptor-redirected human lymphocytes. *Cancer Res* 63(10):2470-2476.
16. Teng MW, Kershaw MH, Moeller M, Smyth MJ & Darcy PK (2004) Immunotherapy of cancer using systemically delivered gene-modified human T lymphocytes. *Hum Gene Ther* 15(7):699-708.
17. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, Samanta M, Lakhal M, Gloss B, Danet-Desnoyers G, Campana D, Riley JL, Grupp SA & June CH (2009) Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Mol Ther* 17(8):1453-1464.
18. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A & June CH (2011) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 3(95):95ra73.
19. Porter DL, Levine BL, Kalos M, Bagg A & June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 365(8):725-733.
20. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, Taylor C, Yeh R, Bartido S, Borquez-Ojeda O, Olszewska M, Bernal Y, Pegram H, Przybylowski M, Hollyman D, Usachenko Y, Pirraglia D, Hosey J, Santos E, Halton E, Maslak P, Scheinberg D, Jurcic J, Heaney M, Heller G, Frattini M & Sadelain M (2011) Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 118(18):4817-4828.
21. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, Maric I, Raffeld M, Nathan DA, Lanier BJ, Morgan RA & Rosenberg SA (2010) Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* 116(20):4099-4102.
22. Bondanza A, Valtolina V, Magnani Z, Ponzoni M, Fleischhauer K, Bonyhadi M, Traversari C, Sanvito F, Toma S, Radrizzani M, La Seta-Catamancio S, Ciceri F, Bordignon C & Bonini C (2006) Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes. *Blood* 107(5):1828-1836.
23. Duarte RF, Chen FE, Lowdell MW, Potter MN, Lamana ML, Prentice HG & Madrigal JA (2002) Functional impairment of human T-lymphocytes following PHA-induced expansion and retroviral transduction: implications for gene therapy. *Gene Ther* 9(20):1359-1368.
24. Pouw N, Treffers-Westerlaken E, Kraan J, Wittink F, ten Hagen T, Verweij J & Debets R (2010) Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother* 59(6):921-931.
25. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA & Restifo NP (2005) Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* 115(6):1616-1626.
26. Hinrichs CS, Spolski R, Paulos CM, Gattinoni L, Kerstann KW, Palmer DC, Klebanoff CA, Rosenberg SA, Leonard WJ & Restifo NP (2008) IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 111(11):5326-5333.
27. Kaneko S, Mastaglio S, Bondanza A, Ponzoni M, Sanvito F, Aldrighetti L, Radrizzani M, La Seta-Catamancio S, Provasi E, Mondino A, Nagasawa T, Fleischhauer K, Russo V, Traversari C, Ciceri

F, Bordignon C & Bonini C (2009) IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood* 113(5):1006-1015.

28. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA & Hwu P (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 12(20 Pt 1):6106-6115.
29. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G & Oosterwijk E (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24(13):e20-22.
30. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA & Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314(5796):126-129.
31. Darcy PK, Haynes NM, Snook MB, Trapani JA, Cerruti L, Jane SM & Smyth MJ (2000) Redirected perforin-dependent lysis of colon carcinoma by ex vivo genetically engineered CTL. *J Immunol* 164(7):3705-3712.
32. Haynes NM, Snook MB, Trapani JA, Cerruti L, Jane SM, Smyth MJ & Darcy PK (2001) Redirecting mouse CTL against colon carcinoma: superior signaling efficacy of single-chain variable domain chimeras containing TCR-zeta vs Fc epsilon RI-gamma. *J Immunol* 166(1):182-187.
33. Haynes NM, Trapani JA, Teng MW, Jackson JT, Cerruti L, Jane SM, Kershaw MH, Smyth MJ & Darcy PK (2002) Rejection of syngeneic colon carcinoma by CTLs expressing single-chain antibody receptors codelivering CD28 costimulation. *J Immunol* 169(10):5780-5786.
34. Hwu P, Yang JC, Cowherd R, Treisman J, Shafer GE, Eshhar Z & Rosenberg SA (1995) In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res* 55(15):3369-3373.
35. Smyth MJ (1997) Fas ligand-mediated bystander lysis of syngeneic cells in response to an allogeneic stimulus. *J Immunol* 158(12):5765-5772.
36. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C & Rosenberg SA (2011) Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 29(7):917-924.
37. Seliger B, Wollscheid U, Momburg F, Blankenstein T & Huber C (2001) Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. *Cancer Res* 61(3):1095-1099.
38. de Witte MA, Jorritsma A, Kaiser A, van den Boom MD, Dokter M, Bendle GM, Haanen JB & Schumacher TN (2008) Requirements for effective antitumor responses of TCR transduced T cells. *J Immunol* 181(7):5128-5136.
39. Goldberger O, Volovitz I, Machlenkin A, Vadai E, Tzehoval E & Eisenbach L (2008) Exuberated numbers of tumor-specific T cells result in tumor escape. *Cancer Res* 68(9):3450-3457.
40. Schaft N, Willemsen RA, de Vries J, Lankiewicz B, Essers BW, Gratama JW, Figdor CG, Bolhuis RL, Debets R & Adema GJ (2003) Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J Immunol* 170(4):2186-2194.
41. Pouw NM, Westerlaken EJ, Willemsen RA & Debets R (2007) Gene transfer of human TCR in primary murine T cells is improved by pseudo-typing with amphotropic and ecotropic envelopes. *J Gene Med* 9(7):561-570.

42. Koya RC, Mok S, Comin-Anduix B, Chodon T, Radu CG, Nishimura MI, Witte ON & Ribas A (2010) Kinetic phases of distribution and tumor targeting by T cell receptor engineered lymphocytes inducing robust antitumor responses. *Proc Natl Acad Sci U S A* 107(32):14286-14291.
43. Lamers CH, Willemsen R, van Elzakker P, van Steenbergen-Langeveld S, Broertjes M, Oosterwijk-Wakka J, Oosterwijk E, Sleijfer S, Debets R & Gratama JW (2011) Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. *Blood* 117(1):72-82.
44. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu Z, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA & Restifo NP (2003) Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 198(4):569-580.
45. Klebanoff CA, Khong HT, Antony PA, Palmer DC & Restifo NP (2005) Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends Immunol* 26(2):111-117.
46. Muranski P, Boni A, Wrzesinski C, Citrin DE, Rosenberg SA, Childs R & Restifo NP (2006) Increased intensity lymphodepletion and adoptive immunotherapy--how far can we go? *Nat Clin Pract Oncol* 3(12):668-681.
47. Paulos CM, Wrzesinski C, Kaiser A, Hinrichs CS, Chieppa M, Cassard L, Palmer DC, Boni A, Muranski P, Yu Z, Gattinoni L, Antony PA, Rosenberg SA & Restifo NP (2007) Microbial translocation augments the function of adoptively transferred self/tumor-specific CD8+ T cells via TLR4 signaling. *J Clin Invest* 117(8):2197-2204.
48. Lake RA & Robinson BW (2005) Immunotherapy and chemotherapy--a practical partnership. *Nat Rev Cancer* 5(5):397-405.
49. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, Okoye FI & Jaffee EM (2001) Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res* 61(9):3689-3697.
50. Zitvogel L, Kepp O, Senovilla L, Menger L, Chaput N & Kroemer G (2010) Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. *Clin Cancer Res* 16(12):3100-3104.
51. Dunn GP, Bruce AT, Ikeda H, Old LJ & Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 3(11):991-998.
52. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ & Schreiber RD (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450(7171):903-907.
53. Corbiere V, Chapiro J, Stroobant V, Ma W, Lurquin C, Lethe B, van Baren N, Van den Eynde BJ, Boon T & Coulie PG (2011) Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res* 71(4):1253-1262.
54. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, Jungbluth A, Gnjatic S, Thompson JA & Yee C (2008) Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 358(25):2698-2703.
55. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG & Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *J Exp Med* 201(2):249-257.
56. Ribas A, Butterfield LH, Glaspy JA & Economou JS (2003) Current developments in cancer vaccines and cellular immunotherapy. *J Clin Oncol* 21(12):2415-2432.
57. Schietinger A, Philip M, Liu RB, Schreiber K & Schreiber H (2010) Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *J Exp Med* 207(11):2469-2477.

58. Spiotto MT, Rowley DA & Schreiber H (2004) Bystander elimination of antigen loss variants in established tumors. *Nat Med* 10(3):294-298.
59. Thomas DL, Kim M, Bowerman NA, Narayanan S, Kranz DM, Schreiber H & Roy EJ (2009) Recurrence of intracranial tumors following adoptive T cell therapy can be prevented by direct and indirect killing aided by high levels of tumor antigen cross-presented on stromal cells. *J Immunol* 183(3):1828-1837.
60. Willimsky G & Blankenstein T (2005) Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature* 437(7055):141-146.
61. Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 5(4):263-274.
62. Drake CG, Jaffee E & Pardoll DM (2006) Mechanisms of immune evasion by tumors. *Adv Immunol* 90:51-81.
63. Rabinovich GA, Gabrilovich D & Sotomayor EM (2007) Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 25:267-296.
64. Zitvogel L, Tesniere A & Kroemer G (2006) Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 6(10):715-727.
65. Orentas RJ, Kohler ME & Johnson BD (2006) Suppression of anti-cancer immunity by regulatory T cells: back to the future. *Semin Cancer Biol* 16(2):137-149.
66. Kusmartsev S & Gabrilovich DI (2006) Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. *Cancer Metastasis Rev* 25(3):323-331.
67. Serafini P, Borrello I & Bronte V (2006) Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* 16(1):53-65.
68. Grohmann U & Bronte V (2010) Control of immune response by amino acid metabolism. *Immunol Rev* 236:243-264.
69. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V & Viola A (2011) Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med* 208(10):1949-1962.
70. Munn DH, Sharma MD, Hou D, Baban B, Lee JR, Antonia SJ, Messina JL, Chandler P, Koni PA & Mellor AL (2004) Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest* 114(2):280-290.
71. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T & Van den Eynde BJ (2003) Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med* 9(10):1269-1274.
72. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, Gilbert J & Ochoa AC (2005) Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med* 202(7):931-939.
73. Vesely MD, Kershaw MH, Schreiber RD & Smyth MJ (2011) Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 29:235-271.
74. Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW & Ochoa AC (1992) Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 258(5089):1795-1798.
75. Leach DR, Krummel MF & Allison JP (1996) Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271(5256):1734-1736.

76. Murphy TL & Murphy KM (2010) Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol* 28:389-411.
77. van Elsas A, Hurwitz AA & Allison JP (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190(3):355-366.
78. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E & Chen L (2002) Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8(8):793-800.
79. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE & Rosenberg SA (2009) Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 114(8):1537-1544.
80. Ngiow SF, von Scheidt B, Akiba H, Yagita H, Teng MW & Smyth MJ (2011) Anti-TIM3 antibody promotes T cell IFN-gamma-mediated antitumor immunity and suppresses established tumors. *Cancer Res* 71(10):3540-3551.
81. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ & Schreiber RD (1998) Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A* 95(13):7556-7561.
82. Qin Z & Blankenstein T (2000) CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity* 12(6):677-686.
83. DuPage M, Cheung AF, Mazumdar C, Winslow MM, Bronson R, Schmidt LM, Crowley D, Chen J & Jacks T (2011) Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression. *Cancer Cell* 19(1):72-85.
84. Scarlett UK, Rutkowski MR, Rauwerdink AM, Fields J, Escovar-Fadul X, Baird J, Cubillos-Ruiz JR, Jacobs AC, Gonzalez JL, Weaver J, Fiering S & Conejo-Garcia JR (2012) Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. *J Exp Med*.
85. Schreiber RD, Old LJ & Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331(6024):1565-1570.
86. Blank CU, Hooijkaas AI, Haanen JB & Schumacher TN (2011) Combination of targeted therapy and immunotherapy in melanoma. *Cancer Immunol Immunother* 60(10):1359-1371.
87. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA & Group B-S (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364(26):2507-2516.
88. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K & Chapman PB (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 363(9):809-819.
89. Chmielewski M, Kopecky C, Hombach AA & Abken H (2011) IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Res* 71(17):5697-5706.
90. Kerkar SP, Muranski P, Kaiser A, Boni A, Sanchez-Perez L, Yu Z, Palmer DC, Reger RN, Borman ZA, Zhang L, Morgan RA, Gattinoni L, Rosenberg SA, Trinchieri G & Restifo NP (2010) Tumor-specific

CD8+ T cells expressing interleukin-12 eradicate established cancers in lymphodepleted hosts. *Cancer Res* 70(17):6725-6734.

91. Kerkar SP, Goldszmid RS, Muranski P, Chinnasamy D, Yu Z, Reger RN, Leonardi AJ, Morgan RA, Wang E, Marincola FM, Trinchieri G, Rosenberg SA & Restifo NP (2011) IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors. *J Clin Invest* 121(12):4746-4757.
92. Kershaw MH, Wang G, Westwood JA, Pachynski RK, Tiffany HL, Marincola FM, Wang E, Young HA, Murphy PM & Hwu P (2002) Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum Gene Ther* 13(16):1971-1980.
93. Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, Powell DJ, Jr., Riley JL, June CH & Albelda SM (2011) Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. *Clin Cancer Res* 17(14):4719-4730.
94. Bronte V & Zanovello P (2005) Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* 5(8):641-654.
95. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J & Gabrilovich DI (2007) Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 13(7):828-835.
96. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I & Topalian SL (2010) Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 28(19):3167-3175.
97. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A & Urba WJ (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363(8):711-723.
98. Borkner L, Kaiser A, van de Kasteele W, Andreesen R, Mackensen A, Haanen JB, Schumacher TN & Blank C (2010) RNA interference targeting programmed death receptor-1 improves immune functions of tumor-specific T cells. *Cancer Immunol Immunother* 59(8):1173-1183.
99. Stephan MT, Moon JJ, Um SH, Bershteyn A & Irvine DJ (2010) Therapeutic cell engineering with surface-conjugated synthetic nanoparticles. *Nat Med* 16(9):1035-1041.
100. Castellino F & Germain RN (2006) Cooperation between CD4+ and CD8+ T cells: when, where, and how. *Annu Rev Immunol* 24:519-540.
101. Hunziker L, Klenerman P, Zinkernagel RM & Ehl S (2002) Exhaustion of cytotoxic T cells during adoptive immunotherapy of virus carrier mice can be prevented by B cells or CD4+ T cells. *Eur J Immunol* 32(2):374-382.
102. Marzo AL, Kinnear BF, Lake RA, Frelinger JJ, Collins EJ, Robinson BW & Scott B (2000) Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J Immunol* 165(11):6047-6055.
103. Simpson AJ, Caballero OL, Jungbluth A, Chen YT & Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 5(8):615-625.
104. Coral S, Sigalotti L, Gasparollo A, Cattarossi I, Visintin A, Cattelan A, Altomonte M & Maio M (1999) Prolonged upregulation of the expression of HLA class I antigens and costimulatory molecules on melanoma cells treated with 5-aza-2'-deoxycytidine (5-AZA-CdR). *J Immunother* 22(1):16-24.

105. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F & Boon T (1996) The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. *Proc Natl Acad Sci U S A* 93(14):7149-7153.
106. De Smet C, Lurquin C, Lethe B, Martelange V & Boon T (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* 19(11):7327-7335.
107. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M & Maio M (2002) Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. *J Immunother* 25(1):16-26.
108. Coral S, Sigalotti L, Colizzi F, Spessotto A, Nardi G, Cortini E, Pezzani L, Fratta E, Fonsatti E, Di Giacomo AM, Nicotra MR, Natali PG, Altomonte M & Maio M (2006) Phenotypic and functional changes of human melanoma xenografts induced by DNA hypomethylation: immunotherapeutic implications. *J Cell Physiol* 207(1):58-66.
109. Coral S, Sigalotti L, Altomonte M, Engelsberg A, Colizzi F, Cattarossi I, Maraskovsky E, Jager E, Seliger B & Maio M (2002) 5-aza-2′-deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinoma: immunotherapeutic implications. *Clin Cancer Res* 8(8):2690-2695.
110. Bao L, Dunham K & Lucas K (2011) MAGE-A1, MAGE-A3, and NY-ESO-1 can be upregulated on neuroblastoma cells to facilitate cytotoxic T lymphocyte-mediated tumor cell killing. *Cancer Immunol Immunother* 60(9):1299-1307.
111. Moreno-Bost A, Szmania S, Stone K, Garg T, Hoerring A, Szymonifka J, Shaughnessy J, Jr., Barlogie B, Prentice HG & van Rhee F (2011) Epigenetic modulation of MAGE-A3 antigen expression in multiple myeloma following treatment with the demethylation agent 5-azacitidine and the histone deacetlyase inhibitor MGCD0103. *Cytotherapy* 13(5):618-628.
112. Weiser TS, Guo ZS, Ohnmacht GA, Parkhurst ML, Tong-On P, Marincola FM, Fischette MR, Yu X, Chen GA, Hong JA, Stewart JH, Nguyen DM, Rosenberg SA & Schrump DS (2001) Sequential 5-Aza-2 deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. *J Immunother* 24(2):151-161.
113. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV & Schrump DS (2005) Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. *Cancer Res* 65(17):7763-7774.
114. Wargo JA, Robbins PF, Li Y, Zhao Y, El-Gamil M, Caragacianu D, Zheng Z, Hong JA, Downey S, Schrump DS, Rosenberg SA & Morgan RA (2009) Recognition of NY-ESO-1+ tumor cells by engineered lymphocytes is enhanced by improved vector design and epigenetic modulation of tumor antigen expression. *Cancer Immunol Immunother* 58(3):383-394.
115. Daskalakis M, Nguyen TT, Nguyen C, Guldberg P, Kohler G, Wijermans P, Jones PA & Lubbert M (2002) Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2′-deoxycytidine (decitabine) treatment. *Blood* 100(8):2957-2964.
116. Leone G, Teofili L, Voso MT & Lubbert M (2002) DNA methylation and demethylating drugs in myelodysplastic syndromes and secondary leukemias. *Haematologica* 87(12):1324-1341.
117. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, Klimek V, Slack J, de Castro C, Ravandi F, Helmer R, 3rd, Shen L, Nimer SD, Leavitt R, Raza A & Saba H (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer* 106(8):1794-1803.

118. Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP, Garcia-Manero G, Rios MB, Shan J, Andreeff M, Keating M & Talpaz M (2003) Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer* 98(3):522-528.
119. Rao M, Chinnasamy N, Hong JA, Zhang Y, Zhang M, Xi S, Liu F, Marquez VE, Morgan RA & Schrump DS (2011) Inhibition of histone lysine methylation enhances cancer-testis antigen expression in lung cancer cells: implications for adoptive immunotherapy of cancer. *Cancer Res* 71(12):4192-4204.
120. van der Merwe PA & Davis SJ (2003) Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol* 21:659-684.
121. Ma W, Germeau C, Vigneron N, Maernoudt AS, Morel S, Boon T, Coulie PG & Van den Eynde BJ (2004) Two new tumor-specific antigenic peptides encoded by gene MAGE-C2 and presented to cytolytic T lymphocytes by HLA-A2. *Int J Cancer* 109(5):698-702.
122. Rubinstein MP, Kadima AN, Salem ML, Nguyen CL, Gillanders WE, Nishimura MI & Cole DJ (2003) Transfer of TCR genes into mature T cells is accompanied by the maintenance of parental T cell avidity. *J Immunol* 170(3):1209-1217.
123. Schultz ES, Schuler-Thurner B, Stroobant V, Jenne L, Berger TG, Thielemanns K, van der Bruggen P & Schuler G (2004) Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 172(2):1304-1310.
124. Zhao Y, Zheng Z, Khong HT, Rosenberg SA & Morgan RA (2006) Transduction of an HLA-DP4-restricted NY-ESO-1-specific TCR into primary human CD4+ lymphocytes. *J Immunother* 29(4):398-406.
125. Fujio K, Misaki Y, Setoguchi K, Morita S, Kawahata K, Kato I, Nosaka T, Yamamoto K & Kitamura T (2000) Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex. *J Immunol* 165(1):528-532.
126. Holler PD, Lim AR, Cho BK, Rund LA & Kranz DM (2001) CD8(-) T cell transfectants that express a high affinity T cell receptor exhibit enhanced peptide-dependent activation. *J Exp Med* 194(8):1043-1052.
127. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, Xu H, Morgan RA, Feldman SA, Johnson LA, Bennett AD, Dunn SM, Mahon TM, Jakobsen BK & Rosenberg SA (2008) Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *J Immunol* 180(9):6116-6131.
128. Palmer E & Naeher D (2009) Affinity threshold for thymic selection through a T-cell receptor-co-receptor zipper. *Nat Rev Immunol* 9(3):207-213.
129. Schmid DA, Irving MB, Posevitz V, Hebeisen M, Posevitz-Fejfar A, Sarria JC, Gomez-Eerland R, Thome M, Schumacher TN, Romero P, Speiser DE, Zoete V, Michielin O & Rufer N (2010) Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *J Immunol* 184(9):4936-4946.
130. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA & Nishimura MI (1999) Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J Immunol* 163(1):507-513.
131. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P & Bolhuis RL (2000) Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther* 7(16):1369-1377.
132. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA & Schumacher TN (2001) Immunotherapy through TCR gene transfer. *Nat Immunol* 2(10):957-961.
133. Hanahan D & Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646-674.

Summary
Samenvatting

SUMMARY

To date, adoptive T cell therapy seems one of the most promising therapies for patients with metastatic melanoma. Adoptive transfer of autologous Tumor Infiltrating T cells (TILs), cultured and expanded *ex vivo*, yields impressive objective response rates of 50% in non-controlled trials in patients with metastatic melanoma. T cell receptor gene therapy (TCR gene therapy) lifts adoptive T cell therapy to another level in an effort to make T cell treatment more universal with respect to tumor target antigens and suitable for the treatment of tumors of different histological origins. TCR gene therapy is based on the transfer of genes encoding for a tumor-specific TCR into autologous T cells, rapid expansion *ex vivo* and the re-infusion of T cells into the patient. In contrast to TIL therapy, TCR gene therapy is not dependent on the presence of TILs and the isolation of tumor material from the patient.

The aims of the research presented in this thesis are all centered on target antigens and include:

- Setting up melanoma mouse models to target human antigens with TCR-engineered T cells (TCR T cells) and assessing the value of these models for the development of TCR gene therapy;
- Understanding the contribution of target antigen in the development of tumor relapse; and
- Identifying and characterizing TCRs directed against highly tumor-restricted and clinically relevant target antigens.

Chapter 1 provides a short overview of clinical TCR gene therapy and other promising experimental therapies for the treatment of metastatic melanoma in relation to Dacarbazine, currently being the standard first-line treatment of metastatic melanoma. The first-in-man studies have demonstrated the feasibility and potential of TCR gene therapy, but at the same time demonstrated the need to further improve its therapeutic efficacy and safety. An up to date summary of challenges of TCR gene therapy, together with a listing of promising therapeutic solutions, is provided. Key points to improve therapeutic efficacy have been classified according to T cell and tumor-related factors. T cell-related factors include compromised avidity, *in vivo* persistence, homing and effector functions of TCR-engineered T cells, whereas tumor-related factors involve the type, location and expression level of target antigen, and tumor-induced immune suppression. Points to improve therapeutic safety have been classified into on- and off-target toxicities related to treatment. On- target toxicities are the result of TCR$\alpha\beta$ transgenes that target healthy, non-tumor tissue that express (low levels of) cognate antigen. Off-target toxicities occur as a consequence of pairing between transgenic TCR and endogenous TCR chains that target unknown self-antigens.

In **chapter 2,** these challenges and potential therapeutic strategies are reviewed in more detail. This chapter concludes that further clinical development of TCR gene therapy would require the targeting of highly tumor-restricted antigens and the implementation of strategies that enhance correct pairing of TCR transgenes, functional avidity and persistence of TCR T cells.

Mouse models of cancer have been indispensible for the development of adoptive therapy with receptor engineered T cells. Tumor-specific TCRs and Chimeric Antibody Receptors (CARs) expressed on human T cells have been tested in xenograft models for their *in vivo* anti-tumor activity. In **chapter 3** we have described and summarized the existing literature with respect to immune-deficient mouse models that were used to assess receptor gene therapy. A number of studies demonstrate the therapeutic efficacy of intravenously injected *CAR*-engineered T cells (CAR T cells) against hematological and solid tumors. In contrast, *TCR* T cells injected intravenously have only been reported to be effective against hematological tumors but not against solid tumors. In a first set of experiments, we have extended these findings and demonstrated that human TCR T cells that have been generated and processed according to current clinical protocols (anti-CD3 mAb T cell activation, retroviral gene transfer and high IL-2 *in vivo* support) and injected intravenously did not affect growth of human melanoma in immune-deficient SCID mice. Following injection, TCR T cells accumulated in the tumor vasculature, but were only short-lived. Similar results were obtained with additional chemotherapeutic conditioning in SCID mice or in an alternative, more immune-deficient NSG mouse strain. We showed that the observed compromised T cell persistence was associated with T cells that were differentiated in late effector T cells, a well-documented characteristic of impaired *in vivo* T cell function. We have concluded that the presented xenograft model provides added value to test strategies to improve engraftment and persistence and consequently the therapeutic efficacy of human TCR T cells. In a next set of experiments, we demonstrated that mouse T cells effectively regress large human tumors, but do so independently of the TCR transgenes. These findings were perhaps expected, but in sharp contrast to reported findings with mouse CAR T cells directed against solid human tumors. In fact, mouse T cells were xeno-reactive in two different immune-deficient mouse strains, using two different human melanoma cell lines and were found to be generally *not* appropriate to test TCR gene therapy in xenograft tumor models.

Transplantable syngeneic models for cancer are widely available and used to test different parameters that are critical for TCR gene therapy. In **chapter 4**, we describe an HLA-A2 transgenic and immune-competent melanoma mouse model that we optimized to test the therapeutic efficacy of TCR gene therapy directed against a human tumor antigen (gp100/HLA-A2). Adoptive transfer of TCR T cells prevented tumor growth in a preventive setting and resulted in regression of large established tumors (average volume of 200-300 mm^3). In 10-

20% of mice durable remissions were observed, but in most mice (80-90%) tumor regression was followed by tumor relapse. Importantly, the observed response rates resemble those reported for clinical TCR gene therapy. In our model we have charted the status of target antigen at the level of DNA integrity and expression of gene and protein in relapsing and mock-treated tumors. It was found that T cells selected for antigen-negative tumor variants only if antigen-negative tumor cells were present before treatment. Relapsed tumors that originated from antigen-positive cells harbored intact and non-mutated antigen genes and showed functional expression of surface antigen equal to non-treated tumors. Relapsed tumors, despite the fact that they functionally express antigen protein, resist a second treatment with TCR T cells directed against the same antigen. In addition, we found that neither cross-presentation of human gp100 epitopes nor spreading of new antigens contributed to tumor cure in this model. We conclude that TCR gene therapy does *not* actively alter the antigen gene nor its expression and represents a valid primary treatment to target relapsed tumors. Supporting therapies to enhance the therapeutic efficacy of TCR gene therapy, such as the use of multiple target antigens and immune-potentiating strategies, are discussed in chapters 4 and 6.

On-target toxicity has been reported in clinical TCR and CAR gene therapy studies as the result of choosing receptors that target antigen expressed, although at low levels, on healthy tissue and underscore the need to target highly tumor-restricted antigens. Antigens that belong to the family of Cancer Testis Antigens (CTA) meet such a criterion. CTA are a class of shared antigens expressed in a variety of tumor types, but mostly silenced in healthy cells. MAGE antigens belong to the family of CTAs and are of special interest because of their role in tumorigenesis and expression in a variety of tumor types. **Chapter 5** describes the isolation and characterization of MAGE-specific TCR genes from T cells derived from melanoma patients who responded clinically to MAGE vaccination. We identified and characterized MAGE-C2/HLA-A2 TCR$\alpha\beta$ genes that redirect CD8 T cells and MAGE-A3/HLA-DP4 TCR$\alpha\beta$ genes that redirect CD4 T cells towards melanoma cell lines or dendritic cells, respectively. The combination of MHC class I and II-restricted TCRs to redirect T cells would make use of the synergy between CD4 and CD8 T cells and expectedly result in more effective anti-tumor responses and prevent or limit relapse of antigen-negative tumors. These TCRs are intended for evaluation in a phase I clinical TCR gene therapy trial.

In **chapter 6**, the results of chapters 3, 4 and 5 are discussed and put into perspective of recent developments in the TCR gene therapy field. In addition, I put TCR gene therapy forward as a personalized primary therapy for the treatment of cancer, and I propose supportive strategies and treatments to further improve future TCR gene therapy trials. In short, TCR gene therapy trials can be improved by (the combination of) the following elements:

1. Activate patient-derived T cells with soluble anti-CD3 and CD28 antibodies.
2. Condition patients with a combination of cyclophosphamide and fludarabine and demethylating drugs.
3. Simultaneous usage of multiple TCRs specific for highly tumor-restricted antigens, preferably a combination of MHC class I and II restricted TCRs such as affinity enhanced MC2/A2 and MA3/DP4 TCRs.
4. Gene-engineer T cells, in addition to TCR genes with an inducible IL-12 gene, a double-negative TGF-β receptor gene and/or a chemokine receptor gene to increase the therapeutic effect.
5. Culture gene-engineered T cells in the presence of a combination of IL-15 and IL-21 cytokines to obtains the preferred T cell phenotype for *in vivo* anti-tumor effect.
6. Combine T cell transfer with administration of molecules that block T cell inhibition such as anti-CTLA-4 or PD-1 antibodies.

SAMENVATTING

Momenteel is adoptieve T-cel therapie de meest succesvolle behandeling voor patiënten met uitgezaaid melanoom. Adoptieve T-cel therapie is gebaseerd op de isolatie van een type witte bloedcel, de zogenaamde T-cel, uit tumoren van patiënten en de vermenigvuldiging van T-cellen in het laboratorium. Grote aantallen T-cellen, die een antigeen op de tumor herkennen, kunnen op die manier aan de patiënt worden teruggeven. Deze therapie resulteert, in niet-gecontroleerde studies, in klinische responsen bij 50% van de patiënten met uitgezaaid melanoom. Een alternatieve vorm van adoptieve T-cel therapie is de behandeling met T-cel receptor (TCR) gemodificeerde T-cellen (TCR gentherapie). TCR gentherapie is een meer universele T-cel therapie voor kanker geschikt voor verschillende soorten tumoren. Deze therapie is gebaseerd op de introductie van genen coderend voor een tumor-specifieke T cel receptor (TCR) in perifere T-cellen afkomstig van de patiënt, die ook worden vermenigvuldigd in het laboratorium voordat ze worden teruggegeven aan de patiënt. TCR gentherapie is niet afhankelijk van de eventuele aanwezigheid van T-cellen in de tumor en kan toegepast worden bij een veel groter aantal patiënten, zonder hiervoor tumoren operatief te verwijderen.

De doelstellingen van het onderzoek, die worden gepresenteerd in dit proefschrift, hebben allen betrekking op de ontwikkeling van TCR gentherapie en richten zich op het tumorantigeen:

- Het opzetten van melanoom muismodellen waarin TCR gentherapie gericht is tegen humane tumor antigenen. Hierin kunnen parameters worden getest voor de therapeutische verbetering van TCR gentherapie.
- Het verkrijgen van inzicht in de rol van het antigeen in tumoren die recidiveren na TCR gentherapie.
- Het identificeren en karakteriseren van TCRs gericht tegen klinisch relevante antigenen met een hoge mate van tumorspecifieke expressie.

In **hoofdstuk 1** wordt een overzicht gegeven van klinische TCR gentherapie en andere veelbelovende experimentele therapieën gericht tegen gemetastaseerd (uitgezaaid) melanoom. Het resultaat van deze studies worden vergeleken met Dacarbazine, de huidige standaard eerstelijns behandeling. Uit de eerste klinische studies blijkt dat TCR gentherapie een reële en veelbelovende behandeling is, maar ook dat de effectiviteit en veiligheid verbeterd moeten worden. De belangrijkste uitdagingen van TCR gentherapie en de mogelijke therapeutische oplossingen worden besproken. Verbeterpunten voor therapeutische effectiviteit kunnen worden onderverdeeld in factoren die gerelateerd zijn aan TCR-gemodificeerde T-cellen (TCR T-cellen) en aan de tumor. Tot de factoren die gerelateerd zijn aan de TCR T-cellen behoren: suboptimale T-cel binding aan het tumorantigeen, verminderde T-cel overleving ná infusie en suboptimale T-cel migratie naar de tumor. Factoren die gerelateerd zijn aan de tumor zijn

het type, de lokalisatie en het expressie niveau van het doelwitantigeen en het immuunsuppressieve milieu dat geïnduceerd wordt door de tumor. Verbeterpunten voor therapeutische veiligheid kunnen worden onderverdeeld in de volgende twee therapie-gerelateerde bijeffecten, nl: 'on-target' en 'off-target' toxiciteit. On-target toxiciteit is het resultaat van de mogelijkheid dat de geïntroduceerde TCR gezond weefsel herkent dat lage hoeveelheden antigeen tot expressie brengt. Off-target toxiciteit treedt op als gevolg van het combineren van een endogene en een geïntroduceerde TCR keten dat leidt tot een nieuwe TCR welke onbekende antigenen van gezond weefsel herkent.

In **hoofdstuk 2** zijn deze verbeterpunten en mogelijke therapeutische oplossingen nader beschreven. Voor verdere ontwikkeling van klinische TCR gentherapie is het noodzakelijk om strategieën te implementeren die 1) correcte paring van geïntroduceerde TCR ketens bevorderen; en 2) de functionaliteit en persistentie (dwz de overleving *in vivo*) van TCR gemodificeerde T-cellen verhogen. Tenslotte is het van groot belang dat de doelwitantigenen een hoge mate van tumorspecifieke expressie laten zien.

Pre-klinische muis tumormodellen zijn onontbeerlijk in de ontwikkeling van succesvolle adoptieve T-cel therapie met receptor gemodificeerde T-cellen. De *in vivo* anti-tumor activiteit van humane T cellen gemodificeerd met tumorspecifieke TCRs en Chimere Antistof Receptoren (CARs) is volop getest in xenograft tumormodellen. Xenograft modellen zijn gebaseerd op immuun-deficiënte muizen waarin humane tumorcellen kunnen groeien. In **hoofdstuk 3** beschrijven we deze xenograft modellen en de verschillende parameters die in deze modellen zijn getest, waaronder het formaat van de receptor, het type tumor, de wijze van T-cel toediening en eventuele ondersteunende behandelingen (zogenaamde conditionering) voorafgaand aan T-cel behandeling. Een aantal studies laten therapeutische activiteit zien van intraveneus toegediende Chimere Antigeen Receptor gemodificeerde T-cellen (CAR T-cellen) gericht tegen hematologische én solide tumoren. Daarentegen, zijn intraveneus toegediende TCR T-cellen alléén effectief tegen hematologische tumoren, maar niet tegen solide tumoren. Wij hebben deze bevindingen uitgebreid en aangetoond dat humane TCR T-cellen, die zijn gegenereerd volgens de huidige klinische protocollen van TCR gentherapie en intraveneus zijn toegediend, géén invloed hebben op de groei van humaan melanoom in immuun-deficiënte SCID (Severe Combined Immune Deficient) muizen. TCR T-cellen accumuleren weliswaar in het tumorvaatbed, maar blijken *in vivo* slechts kortlevend. Vergelijkbare resultaten werden verkregen wanneer SCID muizen geconditioneerd werden met chemotherapeutica en in alternatieve, meer immuun-deficiënte, NSG (NOD.SCID $gc^{-/-}$) muizen. Deze suboptimale T cel persistentie *in vivo* bleek te zijn geassocieerd met een ver gedifferentieerd T cel fenotype op het moment van T cel infusie. Het is bekend dat ver gedifferentieerde T cellen suboptimaal functioneren *in vivo*. We concluderen dat het hier beschreven xenograft model mogelijk een toegevoegde waarde heeft voor het testen van strategieën die de persistentie en effectiviteit verhogen van *humane TCR T-cellen*. In verdere

experimenten laten wij zien dat het toedienen van *muizen TCR T-cellen* leidt tot een heel efficiënte regressie van grote humane tumoren. Deze tumorregressie is echter onafhankelijk van de geïntroduceerde TCR genen. Deze bevindingen zijn niet geheel verassend, maar niet in overstemming met gerapporteerde resultaten met muizen CAR T-cellen die gericht zijn tegen solide humane tumoren. De geobserveerde 'xeno-specifieke' T-cel responsen zijn aangetoond in twee verschillende muizenstammen en tegen twee verschillende tumorcellijnen, en wij concluderen derhalve dat muizen T-cellen in het algemeen *niet* geschikt zijn voor de pre-klinische validatie van TCR gentherapie in xenograft tumormodellen.

Zogenaamde syngene transplantatiemodellen zijn gebaseerd op de implantatie van muizen tumoren in een immuun-competente muis en worden eveneens gebruikt voor het testen van TCR gentherapie. In **hoofdstuk 4** beschrijven we een HLA-A2 transgeen en immuuncompetent muismodel voor TCR gentherapie tegen melanoom. Verschillende parameters zijn getest ter verbetering van de therapeutische effectiviteit van TCR gentherapie gericht tegen een humaan tumor antigeen (gp100/HLA-A2). Adoptieve transfer van TCR T-cellen voorkòmt groei van geïnjecteerd tumorcellen (preventieve setting) en resulteert in regressie van grote tumoren met een gemiddeld volume van 200-300 mm^3 (curatieve setting). In 10-20% van de behandelde muizen is de tumorregressie duurzaam en blijven de muizen tumor-vrij tot het einde van het experiment. In het grootste deel van de muizen (80-90%) recidiveren de tumoren na eerdere regressie. Van belang is op te merken dat de responsen in dit model overeenkomstig zijn met die van klinische TCR gentherapie studies, hetgeen de translationele waarde van dit model voor klinische toepassingen versterkt. In tumoren die recidiveren na TCR gentherapie is het tumor antigeen geanalyseerd op verschillende niveaus, hetgeen vergeleken is met het tumor antigeen in tumoren die behandeld zijn met controle T-cellen. Bij T-cel behandeling ontstaan recidieven van antigeen-negatieve tumoren, alleen wanneer er antigeen-negatieve tumorcellen aanwezig zijn voorafgaand aan T-cel behandeling. Dit duidt erop dat T-cel selectie het mechanisme is achter de uitgroei van antigeen-negatieve tumoren. Recidiverende tumoren, afkomstig van antigeen-positieve tumorcellen bevatten geheel onveranderd en ongemuteerd antigeen DNA. In deze tumoren komt het antigeen functioneel tot expressie op de tumorcellen, overeenkomstig met tumoren die behandeld zijn met controle T-cellen. Tumoren die recidiveren, zijn, ondanks het feit dat het doelwitantigeen op het oppervlak tot expressie komt, resistent tegen een tweede behandeling met TCR T-cellen gericht tegen hetzelfde antigeen. In het geval van een duurzame tumorregressie is aangetoond dat de T-cel respons niet is uitgebreid naar tumorantigenen anders dan het doelwitantigeen. In dit hoofdstuk concluderen wij dat TCR gentherapie *niet* resulteert in verandering van het gen of de expressie van het doelwitantigeen en daarom een geschikte eerste therapie is om tumoren te behandelen. In hoofdstuk 4 en 6 worden aanvullende strategieën en behandelingen besproken die de anti-tumor effectiviteit van TCR gentherapie verhogen, zoals het gebruik van verschillende tumorspecifieke TCRs om de immuun respons te verbreden of strategieën om de T-cel activiteit te verhogen.

On-target toxiciteit is gerapporteerd in klinische TCR en CAR gentherapie studies en is het gevolg van een T-cel respons gericht tegen een doelwitantigeen dat ook aanwezig is, ook al is het in lage mate, op gezond weefsel. Deze studies benadrukken de noodzaak om T-cel therapie uitsluitend te richten op antigenen met een hoge mate van tumorspecifieke expressie. Antigenen die behoren tot de familie van Cancer Testis Antigenen (CTAs) voldoen aan dit criterium. CTAs komen tot expressie in verschillende soorten tumoren, maar expressie is vrijwel afwezig in gezond weefsel. Melanoma AntiGEnen (MAGE antigenen) behoren tot de familie van CTAs en zijn interessante doelwitantigenen voor TCR gentherapie vanwege hun rol in de ontwikkeling van tumoren. **Hoofdstuk 5** beschrijft de isolatie en karakterisatie van twee MAGE-specifieke TCRs afkomstig van T-cellen van patiënten met uitgezaaide melanoom, waarin de tumorgroei werd geremd na vaccinatie met MAGE antigenen. Wij hebben MAGE-C2/HLA-A2 en MA3/HLA-DP4 TCR$\alpha\beta$ genen geïdentificeerd. Na retrovirale gentransfer komt de MAGE-C2/HLA-A2 TCR tot expressie op CD8+ T-cellen (cytotoxische T-cellen) die vervolgens melanoomcellen herkennen. De MAGE-A3/DP4 TCR komt tot expressie op CD4+ T-cellen (helper T-cellen), die vervolgens MAGE-A3 positieve dendritische cellen herkennen. Het combineren van MHC klasse I en II TCRs zal mogelijk resulteren in een synergistisch effect van CD8+ en CD4+ T-cellen en leiden tot een effectieve anti-tumor respons en het voorkòmen of beperken van recidiverende antigeen-negatieve tumoren. In een fase I TCR gentherapie studie zal het gebruik van deze TCRs worden getest in patiënten.

In **hoofdstuk 6** worden de resultaten van hoofdstukken 3, 4 en 5 kort samengevat en geplaatst in het perspectief van recente ontwikkelingen rondom TCR gentherapie.

Veelbelovende strategieën en aanvullende behandelingen worden besproken die, naar mijn mening, bijdragen aan de ontwikkeling van een efficiënte patiëntgebonden TCR gentherapie voor kanker. Een toekomstige klinische trial voor TCR gentherapie zou onder andere de volgende (combinatie van) elementen moeten bevatten:

1. Activatie van perifere T-cellen met oplosbare anti-CD3 en CD28 antistoffen.
2. Voorbehandeling van patiënt met een combinatie van cyclofosfamide and fludarabine en demethylerende medicijnen.
3. Gelijktijdig gebruik van verschillende tumorspecifieke TCRs met een voorkeur voor een combinatie van MHC I en MHC II-gerestricteerde TCRs zoals MAGE-C2/HLA-A2 en MAGE-A3/HLA-DP4 TCRs waarvan de affiniteit is verhoogd.
4. De combinatie van de introductie van TCR genen met een induceerbaar IL-12 gen, een dubbel-negatieve TGF-β receptor en/of chemokine receptorgenen.
5. Het vermenigvuldigen van TCR T-cellen in aanwezigheid van IL-15 en IL-21.
6. De combinatie van T-cel infusie met het toedienen van moleculen die T-cel activiteit verhogen zoals anti-CTLA-4 of anti-PD-1 antistoffen.

Curriculum Vitae
List of publications
PhD portfolio
Dankwoord

CURRICULUM VITAE

Trudy Straetemans was born on December 16th, 1980, in The Hague, The Netherlands. She grew up in Wassenaar as daughter of a tax consultant and a painter. In 1999, she obtained her senior high school diploma (Gymnasium) at the Adelbert College Wassenaar. In the same year she moved to Wageningen to study Animal Sciences at Wageningen University (WUR). During her studies Trudy was active in the Animal Sciences student community for which she contributed to the '19th Zootechnical Almanac'. Also, she learned horse carriage driving, passed the exam for four-in-hand driving and was active as a board member and instructor in the driving association 'De Paardengroep' in Wageningen. During her studies she did an internship for three months at the University of Queensland, Gatton Campus, Australia. In the meantime, Trudy became fascinated by the complexity and potency of the immune system and studied the polymeric immunoglobulin receptor in the ontogeny of the avian immune system under the supervision of dr. Aart Lammers in the group of dr. Henk Parmentier. Next, she studied T cell responses against *Mycobacterium tuberculosis* and *Mycobacterium leprae* antigens under the supervision of dr. Annemieke Geluk in the group of Prof.dr. Tom Ottenhoff at the Leiden University Medical Center (LUMC). In 2005, she obtained her MSc degree and worked for four months at the Rikilt-Institute of Food Safety in Wageningen. In 2006, she started her PhD research in the laboratory of Experimental Tumor Immunology, supervised by dr. Reno Debets, in the department of Medical Oncology, chaired by prof.dr. Jaap Verweij at the Erasmus Medical Center in Rotterdam. Her research was mainly focused on mouse tumor models for the development and fine-tuning of T cell receptor gene therapy against melanoma. Besides her PhD studies, Trudy was a member of the PhD committee of the Erasmus Postgraduate School Molecular Medicine (Mol Med). From April 2012 onwards, she continued her research in the T cell receptor gene therapy field as a postdoctoral fellow in the group of dr. Jürgen Kuball, department of Immunology/Hematology at the University Medical Center Utrecht. Trudy is married to Jan Schrijver and together they live in Delft, enjoy the sport of rowing at DDS and to spend time with family and friends.

LIST OF PUBLICATIONS

Trudy Straetemans, Cor Berrevoets, Miriam Coccoris, Elike Treffers-Westerlaken, David Cole, Valerie Dardalhon, Andrew Sewell, Naomi Taylor, Jaap Verweij and Reno Debets. "Tumor relapse following T cell receptor gene therapy is not due to loss of immunogenicity." *Manuscript submitted.*

Trudy Straetemans, Miriam Coccoris, Cor Berrevoets, Elike Treffers-Westerlaken, Csilla E.V. Scholten, Debby Schipper, Timo L.M. ten Hagen and Reno Debets. "T cell receptor gene therapy in human melanoma-bearing immune-deficient: human but not mouse T cells recapitulate outcome of clinical studies." *Human Gene Therapy 2011; 23(2):187-202*.

Trudy Straetemans, Mandy van Brakel, Sabine van Steenbergen, Marieke Broertjes, Joost Drexhage, Joost Hegmans, Bart Lambrecht, Cor Lamers, Pierre van der Bruggen, Pierre G. Coulie, Reno Debets. "TCR gene transfer: MAGE-C2/HLA-A2 and MAGE-A3/HLA-DP4 epitopes as melanoma-specific immune targets." *Clinical and Developmental Immunology 2012 (2012), Article ID 586314*.

Miriam Coccoris, **Trudy Straetemans**, Coen Govers, Cor Lamers, Stefan Sleijfer and Reno Debets. "T cell receptor (TCR) gene therapy to treat melanoma: lessons from clinical and preclinical studies." *Expert Opinion on Biological Therapy 2010; 10(4):547*.

Aart Lammers, Willemien H. Wieland, Leo Kruijt, Arne Jansma, **Trudy Straetemans,** Arjen Schots, Gerco den Hartog, Henk K. Parmentier. "Successive immunoglobulin and cytokine expression in the small intestine of junevile chicken." *Developmental and Comparative Immunology 2010; 34(12):1254*

PHD PORTFOLIO

PhD Portfolio	
Name:	Trudy Straetemans
Research School:	Molecular Medicine
Period:	March 2006 - February 2012
Supervisor/co-promotor:	dr. Reno Debets
Promotor:	prof. dr. Jaap Verweij

1. PhD training **1** ECTS = 28h workload		
	Year	**Credits**
1.1 General academic skills		
• Biomedical English writing and Communication	2008	4
• Research Integrity	2008	1
• Research management for PhD students and postdocs	2010	1
• PCDI retreat for last year PhDs and postdocs	2012	1
1.2 Research skills		
• Statistics, SPSS basic course	2009	0.5
1.3 In-depth courses		
• Molmed course "Basic and Translational Oncology"	2006	1
• Molmed course "Biomedical Research Techniques"	2006	0.5
• Annual course "Molecular Medicine"	2006	1
• "Animal Imaging Workshop" by AMIE	2008	1
• Course "Molecular Immunology", SMBWO Theoretical Immunology exam passed	2009	5
1.4a Oral presentations		
• Annual Dutch Tumor Immunology Meeting, Breukelen, The Netherlands	2010	1
• Cellular Therapy of Cancer Symposium, ATTACK, Montpellier, France	2010	1
• Science day, Dept. Medical Oncology, Erasmus Medical Center	2010 2010/2011	1 2
• Annual meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2011	0.5
• Molecular Medicine Day, Erasmus Medical Center	2007-2011	3
• JNI scientific research meetings, Erasmus Medical Center	2011	0.5
• T cell consortium, Erasmus Medical Center		2

1.4b Poster presentations	2007/2008	
• Annual meeting of the European Society for Cellular and Gene Therapy, Rotterdam and Brugge Belgium	2008	1
• Cold Spring Harbor Laboratory Meeting, Models and Mechanisms of Cancer, New York, US	2007-2010	4
• Molecular Medicine Day, Erasmus Medical Center	2009	3
• Cellular Therapy of Cancer Symposium, ATTACK, Milano, Italy		
1.5 International conferences		
• Cellular Therapy of Cancer Symposium, ATTACK, Montpellier, France	2010	1
• Cellular Therapy of Cancer Symposium, ATTACK, Milano, Italy	2009	1
	2007/2008	2
• European Society for Gene and Cellular Therapy, Rotterdam and Brugge, Belgium	2008	1
• Cold Spring Harbor Laboratory Meeting, Models and Mechanisms of Cancer, New York, US		
1.6 National conferences		
• Annual meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2006-2011	2
• Annual Dutch Tumor Immunology Meeting, Breukelen	2006/2007/2010	2
1.7 Seminars and Workshops		
• Symposium and master classes Post-Infectious diseases	2007	1
	2007	0.5
• Workshop "Gene transfer technologies"		
2. Teaching activities		
2.1 Lecturing		
• Annual Biomedical Research Technique course	2008-2011	2
2.2 Supervising thesis		
• Bachelor student	2008	3
• Bachelor student	2010-2011	3
2.3 Other		
• Member of PhD committee of the ErasmusMC Postgraduate School Molecular Medicine, amongst others organisation of the 'Get Out of your Lab Days' and 'MolMed Day'	2006-2011	5
Total Credits		**59**

DANKWOORD

Na veel leren, onderzoeken, analyseren, pipetteren, vallen, opstaan, bestuderen, schrijven, discussiëren, groeien, experimenteren, luisteren en presenteren is dit mijn proefschrift. Ik wil graag iedereen bedanken die heeft bijgedragen aan de totstandkoming van mijn proefschrift en een aantal mensen in het bijzonder.

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