

Adoptive T cell Therapy Against Solid Tumors:

Success Requires Safe TCRs and
Countering Immune Evasion

André Kunert



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**Adoptive T cell Therapy Against Solid Tumors:
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Immune Evasion**

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**Behandeling van solide tumoren met T cellen:
succes bepaald door veilige TCRs en tegengaan
immuun-ontwijking**

Thesis

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TABLE OF CONTENTS

CHAPTER 1	General Introduction	9
	<ul style="list-style-type: none">• 1.1 Cancer Immune Therapies• 1.2 Current Challenges of TCR Gene Therapy• 1.3 Scope of this Thesis	10 14 18
CHAPTER 2	TCR engineered T cells meet new Challenges to Treat Solid Tumors	25
	<ul style="list-style-type: none">• 2.1 TCR Gene Therapy: Clinical Potency and Toxicities• 2.2 Choices of Target Antigen• 2.3 Fitness of T Cells• 2.4 Sensitization of the Micro Milieu for T Cell Therapy• 2.5 Future Perspectives	27 31 35 39 42
<hr/> PART I - SELECTING ANTIGENS AND TCRs		
CHAPTER 3	MAGE-C2 Specific TCRs Combined with Epigenetic Drug-Enhanced Antigenicity Yield Robust and Tumor-Selective T Cell Responses	57
CHAPTER 4	T Cell Receptors for Clinical Therapy: In Vitro Assessment of Toxicity Risk	89
<hr/> PART II – STRATEGIES TO COUNTERACT IMMUNE EVASION		
CHAPTER 5	T cell Receptors Equipped with ICOS Enhance T Cell Persistence and Mediate Sustainable Anti-Tumor Responses upon Adoptive T Cell Therapy	107
CHAPTER 6	Intra-Tumoral Production of IL-18, but not IL-12 by TCR-Engineered T Cells is Non-Toxic and Counteracts Immune Evasion of Solid Tumors	133
CHAPTER 7	General Discussion	165
	<ul style="list-style-type: none">• 7.1 Selecting Suitable Antigens• 7.2 T Cell Engineering to Counter the Immune Suppressive Tumor Micro Environment• 7.3 Suggested Improvements of TCR Gene Therapy	166 173 175

Summary/Samenvatting	183
Acknowledgements	191
List of Publications	197
PhD portfolio	201
About the Author	205



GENERAL INTRODUCTION

1.1 CANCER IMMUNE THERAPIES

Over the past decade, cancer treatment has seen the emergence of immune therapy as an effective and promising addition or alternative to surgery, chemotherapeutic agents and/or radiotherapy. The idea to treat malignant disease by utilizing the patient's own immune system has solidified itself in dozens of clinical trials and countless pre-clinical and basic research studies. Amongst the most promising and currently employed immune treatments are:

1.1.1 Targeting of immune checkpoints via antibody-based therapies

Various phase III clinical trials (1-4) revealed the potential of using antibodies to enhance T cell activity by blocking co-inhibitory receptors or their ligands on the surface of T cells. In a healthy setting, up-regulation of receptors such as Programmed Cell Death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a means to dampen an ongoing immune response – constituting a negative feedback loop following an antigen-mediated T cell response. In many instances, tumor cells have exploited this feedback loop by up-regulating co-inhibitory ligands. Antagonistic antibodies are able to inhibit interactions between co-inhibitory receptors and ligands, thus enabling the maintenance of T cells in a prolonged state of activation. Along the same principle it is possible to target co-stimulatory receptors present on T cells using agonistic antibodies, aiding in initial T cell stimulation. At the time of writing, several FDA-approved antibodies have been tested in melanoma, non-small-cell lung carcinoma, renal cell carcinoma, head-and-neck cancer and bladder cancer patients and microsatellite-instability positive tumor in general, showing impressive clinical results. Blocking of CTLA-4 using the monoclonal antibody (mAb) Ipilimumab prolonged overall survival of melanoma patients previously treated with a glycoprotein 100 (gp100) peptide vaccine (1). Also blocking of PD-1 with the mAbs Pembrolizumab or Nivolumab prolonged median overall survival and progression-free survival of patients compared to standard treatment in various tumor types (2,5,6). Targeting PD-L1 with the mAb Atezolizumab prolonged overall survival from 12,6 months compared to 9,7 months with docetaxel in patients with previously treated non-small cell lung cancer (4). Targeting the PD-1/PD-L1 axis showed better anti-tumor effects and reduced side-effects when compared to Ipilimumab. Combined therapy using Nivolumab and Ipilimumab prolonged the progression free survival of previously untreated melanoma patients and increased objective response (OR: 57,6%) compared to single treatment with Nivolumab (OR: 43,7%) or Ipilimumab (OR: 19%) (7-9). Notably, the above mentioned antibodies are only a few examples of the checkpoint inhibitor repertoire currently undergoing application or assessment.

1.1.2 Vaccination with tumor-peptides, proteins or antigen-loaded autologous dendritic cells

Vaccination studies aim at stimulating an anti-cancer immune response by providing the patient's immune system with stimulation through antigen presenting cells (APCs) such as dendritic cells (DC). These are artificially loaded with tumor lysates, tumor-derived antigens or peptides in order to inducing activation and proliferation of tumor specific T cells. This approach may rely on peptide presentation by endogenous DCs, e.g., vaccinations with the telomerase peptide GV1001 or the NY-ESO-1₁₅₇₋₁₆₅ peptide, which are expressed by many types of cancer (10,11). Alternatively, vaccinations may rely on isolation of DCs from patients via leukapheresis, loading them with a chosen antigen or with tumor lysates (12), followed by reintroducing them into the patient (13,14). Overall response rates (ORR) to vaccination therapy vary between different tumor types as well as vaccines and range from 8.5% in melanoma, over 11.5% in renal cell carcinoma to 15.6% in glioma patients (reviewed in (15)). In NSCLC patients vaccination with various antigens such as MAGE-A3 or MUC-1 has yielded no clinical benefit (16-18). Notably, the number of complete responses in vaccination studies is lower than for checkpoint inhibitors. Pre-clinical evidence suggests that a combination of both approaches may yield better outcomes, but this remains to be established in clinical studies (19,20).

1.1.3 Adoptive T cell therapy

Adoptive transfer of T cells to treat cancer patients revolves around either isolation of tumor-infiltrating lymphocytes (TILs) from tumor tissue or genetic engineering of T cells isolated from peripheral blood, and in vitro amplification of these T cells with stimulatory antibodies and/or cytokine support. After chemotherapeutic pre-treatment of the patient, these therapeutic and autologous T cells are then reinfused. Initial TIL-based therapies showed promising results with objective responses of 50% in metastatic melanoma patients and complete response rates of up to 22% (21-23). Despite these successes, TIL-therapy relies on the availability of tumor tissue for isolation and expansion of sufficient numbers of T cells, limiting it to certain tumor types and patient populations. Artificially equipping blood-derived T cells with a T cell receptor (TCR) or chimeric antigen receptor (CAR) specific for a chosen antigen is meant to circumvent this issue and make this treatment more universally applicable. Adoptive transfer of both CAR and TCR-engineered T cells have demonstrated clinical benefit, in particular the use of a TCR targeting the cancer testis antigen (CTA) NY-ESO-1 in patients with metastatic melanoma (OR:55%, CR18%), metastatic synovial sarcoma (OR: 61%) and multiple myeloma (OR: 80%) (24-26) as well as the use of a CAR targeting CD19 in patients suffering from B-cell malignancies (OR: up to 93%) (27-30).

While these treatments are diverse (see also figure 1) and many other immunotherapeutic approaches exist, such as targeting checkpoints with drugs (i.e. Indoleamine-2,3-dioxygenase; "IDO"; (31)), use of oncolytic viruses (32) and stimulation of innate immunity via TLR agonists (33), all of the above mentioned therapies have in common that their clinical success critically depends on CD8 T cells (directly in case of adoptive T cell transfer or some checkpoint inhibitors, and indirectly in case of DC vaccination) as their final effector cells to mediate anti-tumor immunity. The clinical relevance of CD8 T cells is further substantiated by observations that their presence in patients with solid tumors correlated with improved clinical outcome (34,35). Notably, beneficial effects of many standard of care treatments, such as chemotherapy or radiation, can be partially related to activation of tumor-specific T cells upon treatment-induced immunogenic cell death in malignant tissue ((36); reviewed in (37-39)).

Out of this broad spectrum of immunotherapeutic agents, this thesis focuses on TCR gene therapy, the direct modification of patient-derived T cells to generate an anti-tumor therapeutic, its challenges and different strategies to enhance the efficacy of TCR engineered T cells.

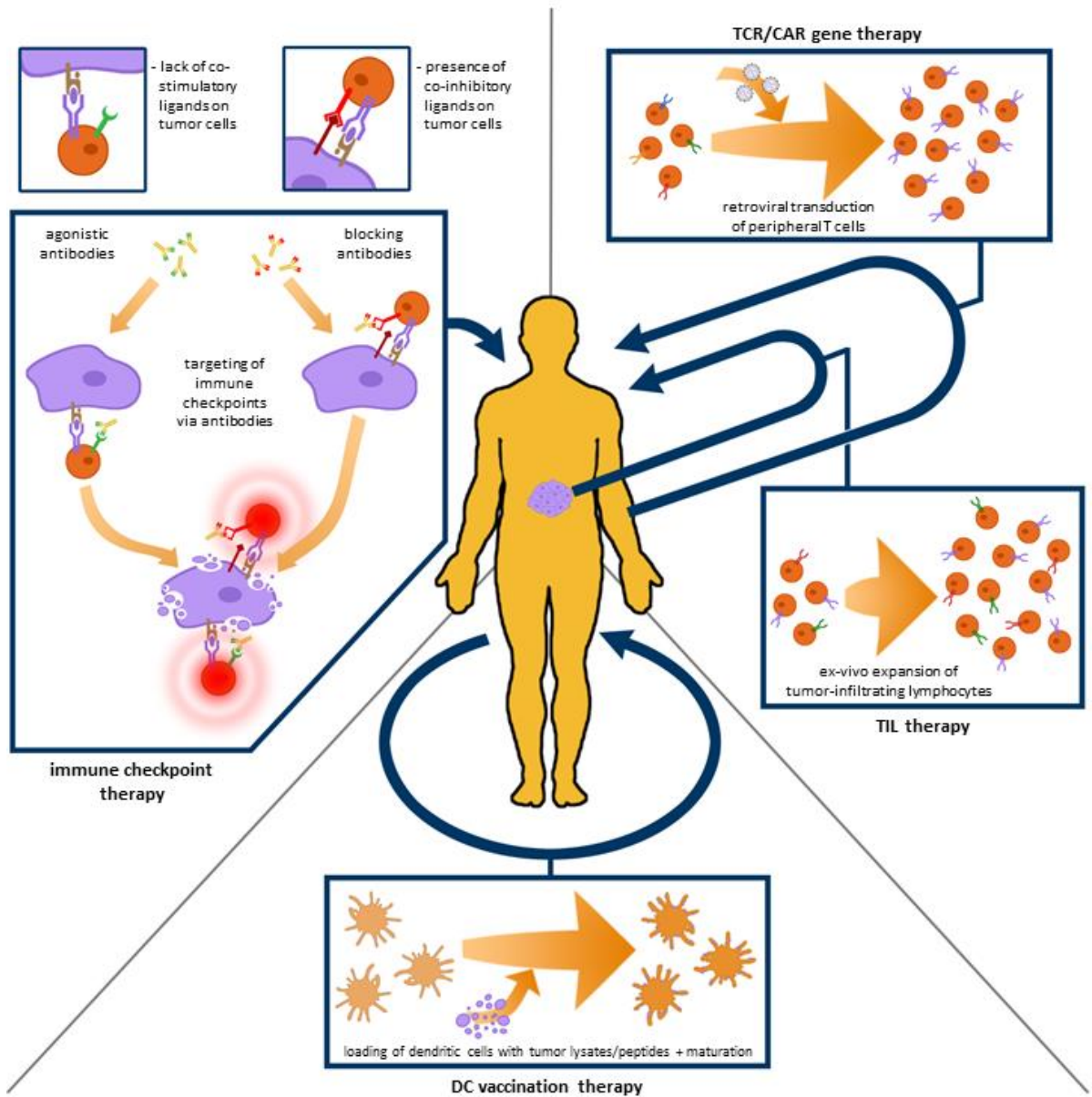


Figure 1. Main categories of cancer immune therapies

Depicted are the three most commonly applied branches of immune therapy to treat cancer patients (CAR = chimeric antigen receptor; DC = dendritic cell; TCR = T cell receptor; TIL = tumor infiltrating lymphocyte).

1.2 CURRENT CHALLENGES OF TCR GENE THERAPY

Despite the promising results of the above-mentioned clinical trials with TCR-engineered T cells, and the progress that has been made over the last few years, treatment of cancer utilizing genetically engineered T cells still faces several challenges. These can be categorized into 1) selection and validation of tumor specific target antigens and corresponding TCRs and 2) T cell engineering to enhance therapy efficacy. We argue that addressing these challenges in an integrated, multi-faceted manner will critically impact the clinical outcomes of TCR gene therapy. In this thesis we provide examples of such an approach.

So far, most therapies are limited to certain tumor types of high immunogenicity, such as melanoma. The degree of immunogenicity, meaning likelihood to elicit an immune response, is highly complex and dependent on multiple factors: e.g. accessibility of tumor tissue to immune cells; expression of immunogenic antigens (percentage of antigen-positive tumor cells as well as expression level per cell) as well as the tumor's intrinsic ability to inhibit an immune response. While it is known that some types of cancer are more sensitive to immunotherapy than others, such as solid tumors with high mutational load (40) or hematologic B cell tumors that are accessible and efficiently present antigens to therapeutic T cells, it is important to realize that even amongst the same tumor types, tumor intrinsic, environmental, but also inter-patient differences can contribute to the ultimate effectiveness of a T cell response and greatly affect the clinical outcome (41-45).

Selection of a suitable target antigen and selection of corresponding CARs or TCRs represent one approach to control the degree of immunogenicity. CARs are based on the antigen-binding domain of a monoclonal antibody, meaning they recognize their target antigen independent of MHC presentation. While this allows detection of broader patient populations, it limits CAR targets to a pool of structures naturally presented on the cell surface. TCRs recognize a specific antigen that is presented in the context of a human major histocompatibility complex (MHC), which is a highly diverse group of molecules normally reflecting the health status of a cell. So far most clinical TCR gene therapy trials utilized receptors restricted by human leukocyte antigen HLA-A1 and HLA-A2. Although among the most common restriction elements, they still only represent 15-50% of the Caucasian patient population (46).

Target antigens for T cell therapies can be divided into four groups (47,48): 1) differentiation antigens and over-expressed antigens, both of which are of particular interest due to their high expression levels; 2) retroviral antigens which are incorporated in the human genome and may become re-expressed in tumors; 3) cancer germline antigens (CGAs), of which a selected number is characterized by absent expression in healthy tissue - in particular those with strict epigenetic regulation - and 4) neo-antigens, a type of antigen derived from mutations within the tumor and whose absence in other tissues provides them with a high safety profile. In **chapter 3** we assess

the suitability of the CGA MAGE-C2 (MC2) in regard to its immunogenicity by testing four different MC2-specific TCRs.

While assessing expression of target antigen is crucial for the efficacy of immune therapy, both in regard to homogeneity within the tumor as well as quantity per individual cell, ensuring that expression is tumor-restricted is equally crucial. T cell recognition and destruction of healthy tissues that is positive for the target antigen or highly similar antigens is the main reason for therapy related toxicity. With regard to these toxicities, we need to differentiate between on-target and off-target toxicity. In case of CD19-specific CAR T cells, concomitant loss of normal B cells is exemplary for on-target toxicity. The CD19 CAR binds to its target, which is not only expressed by the malignant, but also by healthy B cells. TCR T cell trials targeting over-expressed or differentiation antigens such as gp100 and MART1 have also faced on-target toxicities, leading to inflammation of skin, eyes, ears (49) and colon (50). Off-target toxicity is defined as the recognition of healthy tissue, lacking expression of target antigen, by therapeutic T cells. It is considered to be a phenomenon related to TCRs, often with enhanced degeneracy with respect to ligand binding, that under certain circumstances bind antigens highly similar to the target antigen. Targeting certain CGAs with affinity-enhanced TCR-engineered T cells was accompanied by lethal neurological (51) and cardiological toxicities (52). Most likely explanation for this encompasses promiscuity of the TCR's recognition motif for the target antigen, e.g., allowing the binding of highly similar self-antigens ((51,52); Govers, manuscript in prep; also explained in **chapters 3 and 4**). Other TCR-related causes of toxicity such as allo-MHC reactivity or TCR mis-pairing between introduced and endogenous TCR chains (see figure 2) cannot be excluded, but lack clear clinical evidence.

To prevent occurrence of on- and off-target toxicities in clinical trials, it is crucial to establish a series of in vitro screens that determine the expression of target antigen in healthy tissues and predict the auto-reactivity of therapeutic TCRs ((52,53) and **chapters 3 and 4**). Despite a still growing panel of available antigen targets and corresponding TCRs, however, there is currently no established guideline for safety assessment of clinical TCRs. **Chapter 4** of this thesis proposes an optimized pipeline of several in vitro and in silico assays to evaluate the risks posed by either the chosen antigen or therapeutic TCR.

In many patients, T cells fail to clear the tumor completely or an initial response to TCR gene therapy is followed by tumor relapse and disease progression. Here we distinguish between tumors either inherently evading immune detection or acquiring an immune suppressive micro environment over time. Examples of such evasive mechanisms are up-regulated expression of checkpoints (e.g., PD-L1 (54)); down-regulated antigen or HLA expression (55); the tumor's ability to evade infiltration, migration and/or local activation of CD8 T cells (48,56) due to a changed expression of extracellular matrix components (reviewed in (57)), adhesion molecules and chemo attractants (58,59); and enhanced presence of immune suppressive cells like regulatory T cells (Tregs), M2-type macrophages or MDSCs ((60,61), see also figure 2). Please note that this list is

not intended to be complete and for a more detailed review, see **chapter 2** and the review by Vinay et al (62).

The exact occurrence and dominance of above-mentioned immune evasive mechanisms varies strongly between patients, tumor types, treatment history etc. As such, overcoming the immune inhibitory tumor microenvironment can be considered the most diverse and demanding challenge facing the efficacy of TCR gene therapy. However, recent findings by Charoentong et al indicate that there seem to be patterns dictating the escape mechanisms employed by tumors (45). Along these lines it may be possible to identify the dominant evasive mechanisms at play for particular patient subgroups. In an attempt to support adoptive T cell therapy, the laboratory of tumor immunology is generating integrated inventories of evasive mechanisms using state-of-the-art techniques (outside scope of current thesis). In the second part of this thesis, we have designed and tested two strategies to counter local immune suppression. First, we have generated co-stimulatory TCRs that, upon transduction, are expected to yield T cells with enhanced fitness (**chapter 5**). Second, in **chapter 6** we have created T cells that next to the TCR transgene harbor gene constructs that encode for cytokines. These are produced upon activation of these so-called smart T cells in the tumor tissue and expected to sensitize tumors for a T cell response.

failure:

continued growth (inherent resistance) or resumed growth/relapse (acquired resistance) of tumor as the result of:

- up-regulated expression of checkpoints (e.g., PD-L1) and altered expression of co-signaling ligands
- insufficient TCR-mediated activation of TILs due to low expression or cross-presentation of target antigen/MHC
- insufficient trafficking of therapeutic T cells towards and inside tumors due to altered expression of adhesion molecules, production of chemoattractants and/or presence of physical barriers (e.g., extracellular matrix)
- insufficient co-stimulation of TILs due to presence of immune-suppressive cells, and immune-suppressive chemokines and cytokines

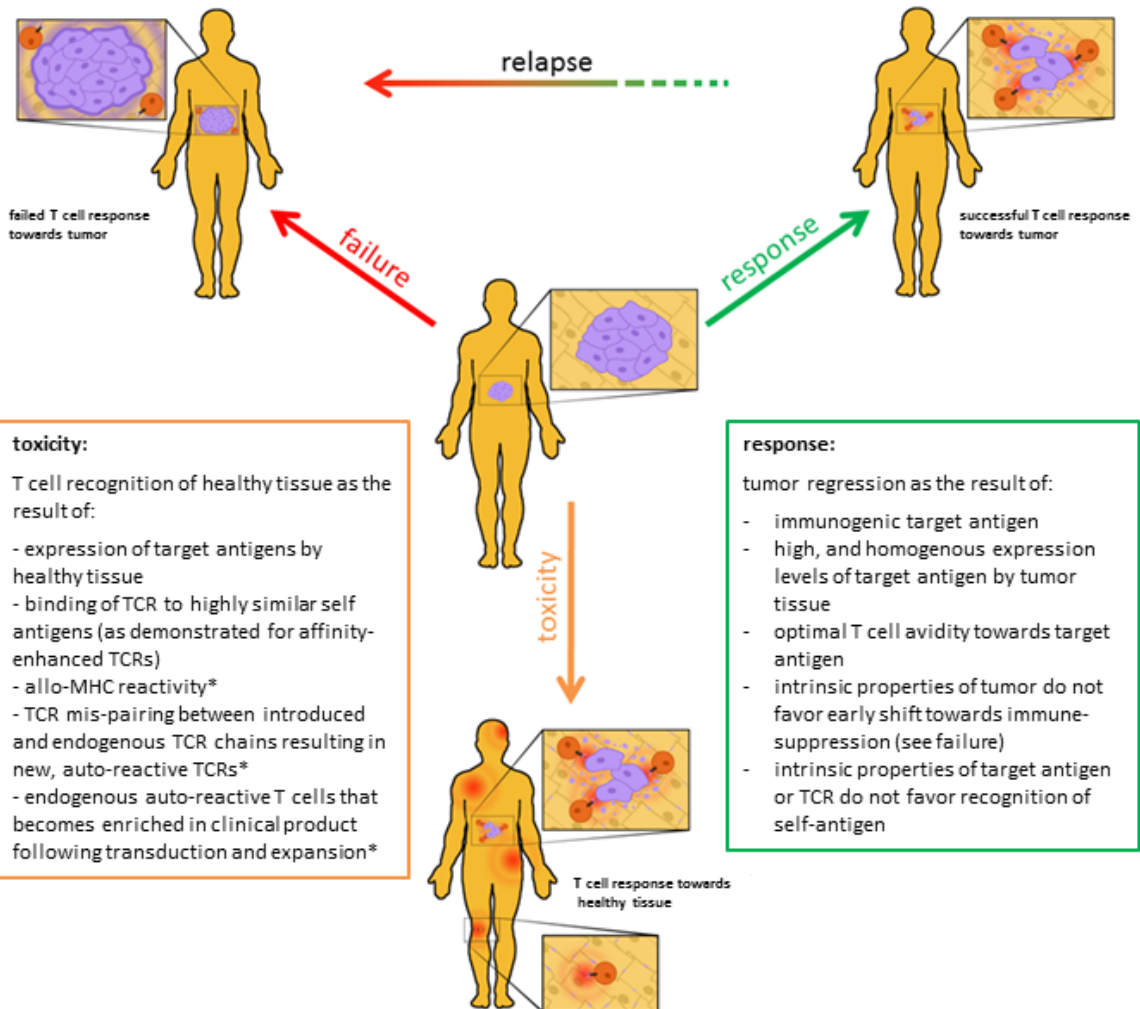


Figure 2. Clinical outcomes of TCR gene therapy and underlying mechanisms

Depicted are clinical outcomes following treatment with TCR-transduced T cells, ranging from clearance of tumor cells, treatment-related toxicities as well as immediate failure to respond to therapy or non-durability of response. For each outcome, underlying mechanisms that potentially explain the observed outcome are listed in the corresponding boxes. Mechanisms indicated with a '*' are based on preclinical data only.

1.3 SCOPE OF THIS THESIS – IMPROVING TCR GENE THERAPY REQUIRES CAREFUL SELECTION OF TARGET ANTIGENS AND TCRS AS WELL AS STRATEGIES TO COUNTER THE IMMUNE SUPPRESSIVE TUMOR MICROENVIRONMENT

As evident from the above headings, successful therapy of solid tumors requires careful consideration of multiple factors, including (and the focus of this thesis) selection of target antigen, corresponding TCR and overcoming tumor-mediated T cell evasion. **Chapter 2** provides a more detailed overview of the challenges that TCR gene therapy is facing and lists current approaches to overcome such challenges. In the subsequent chapters of this thesis, I have covered the following two main challenges:

1. Selection and validation of tumor specific target antigens and corresponding TCRs
2. T cell engineering to counteract local immune suppression.

In **Chapter 3** we chose and validated MAGE-C2 as a safe and effective target antigen for TCR gene therapy. MAGE-C2-specific T cells were able to target cell lines derived from melanoma, head-and-neck squamous cell carcinoma, triple-negative breast cancer and bladder carcinoma. TCRs were derived from patient T cells, characterized and further selected based on *in vitro* T cell performances and tumor-specific recognition. (Challenge 1)

Chapter 4 proposes a pipeline of assays to validate safety of target antigen and corresponding TCRs. MAGE-C2 antigen and selected TCRs from chapter 3 were used as examples in this chapter. (Challenge 1)

In **Chapter 5** we equipped TCRs with co-signaling elements derived from the co-stimulatory receptors CD28, OX40, ICOS, 4-1BB and CD40L. Assessment of these co-stimulatory TCRs revealed that addition of ICOS signaling cassettes in particular enhanced T cell responsiveness *in vivo* in melanoma-bearing, immune competent mice, delaying tumor recurrence and improving on complete responses to therapy. (Challenge 2)

Chapter 6 describes the generation of 'smart T cells' equipped with TCR transgenes as well as an inducible construct mediating secretion of IL-12 or IL-18 following TCR triggering. In addition to the establishment of a protocol to generate these smart T cells, we observed that therapeutic T cells that were able to release IL-18 upon target specific activation, manipulate the tumor micro-environment and resulted in enhanced therapy response and prolonged survival. (Challenge 2)

Finally, **Chapter 7** summarizes and discusses the main findings of the chapters 3 to 6, and how our findings may address the earlier mentioned challenges and potentially translate into future TCR gene therapy trials.

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**TCR-ENGINEERED T CELLS MEET NEW CHALLENGES
TO TREAT SOLID TUMORS: CHOICE OF ANTIGEN,
T CELL FITNESS AND SENSITISATION
OF TUMOR MILIEU**

**Andre Kunert^{1,2}, Trudy Straetemans^{1,2}, Coen Govers^{1,2}, Cor Lamers^{1,2},
Ron Mathijssen², Stefan Sleijfer², Reno Debets^{1,2}**

¹Laboratory of Experimental Tumor Immunology, ²Department of Medical Oncology,
Erasmus MC Cancer Institute, Rotterdam, The Netherlands

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ABSTRACT

Adoptive transfer of T cells gene-engineered with antigen-specific T cell receptors (TCRs) has proven its feasibility and therapeutic potential in the treatment of malignant tumors. To ensure further clinical development of TCR gene therapy, it is necessary to target immunogenic epitopes that are related to oncogenesis and selectively expressed by tumor tissue, and implement strategies that result in optimal T cell fitness. In addition, in particular for the treatment of solid tumors, it is equally necessary to include strategies that counteract the immune-suppressive nature of the tumor micro-environment. Here, we will provide an overview of the current status of TCR gene therapy, and redefine the following three challenges of improvement: 'choice of target antigen'; 'fitness of T cells'; and 'sensitisation of tumor milieu'. We will categorize and discuss potential strategies to address each of these challenges, and argue that advancement of clinical TCR gene therapy critically depends on developments towards each of the three challenges.

2.1 TCR GENE THERAPY: CLINICAL POTENCY AND TOXICITIES

T cells possess distinct properties such as the ability to specifically recognize tumor antigens, serially kill tumor cells, self-replicate, form memory and induce a complete tumor response. It is because of these properties that the therapeutic use of T cells in certain types of cancer may be advantageous when compared to drugs, antibodies or small molecule inhibitors.

T cell therapy intends to treat cancer by transferring autologous and *ex-vivo* expanded T cells to patients. Therapy with tumor-infiltrating T lymphocytes (TILs) preceded by non-myeloablative lymphodepletion resulted in objective responses in about 50% of metastatic melanoma patients in two different medical centers (1,2). Equally notable were the durable complete responses observed in these trials that ranged between 10 and 22% (ongoing for more than three years) (1,2). Likewise, adoptive transfer of tumor-specific T cell clones generated from autologous peripheral T cells resulted in regression of individual metastases, and responses in 8 out of 10 melanoma patients (3). In addition, co-culture of peripheral T cells with artificial antigen-presenting cells (APC) loaded with tumor antigens resulted in T cells that were clinically effective in 4 out of 7 evaluable melanoma patients (4). Response rates observed with T cell therapy are generally higher than those observed for other treatments of melanoma, such as chemotherapeutic drugs, high-dose cytokines, inhibitors of kinases or antibodies against T cell co-inhibitory molecules. See Table 1 for an overview of clinical outcomes of T cell therapies and other treatments of melanoma.

Despite its clinical successes, T cell therapy has its limitations in availability and generation of therapeutic T cells for a larger group of patients. Genetic introduction of T cell receptors (TCRs) or chimeric antigen receptors (CARs) into autologous T cells, termed gene-engineering of T cells, can provide an alternative that is more widely applicable and can potentially be extended to multiple types of cancer (5). Key preclinical achievements and clinical tests with TCR-engineered T cells, the focus of the current review, are depicted in Figures 1A and 1B, respectively. Therapeutic advances with CAR-engineered T cells is reviewed elsewhere (6). The principle of clinical TCR gene therapy is straightforward: transferral of TCR $\alpha\beta$ genes into T cells; *ex-vivo* expansion of T cells; and infusion of T cells into the patient. In this way, TCR α and β genes are used as “off the shelf” reagents to confer tumor reactivity to patients whose tumor expresses the appropriate antigen and HLA restriction element. At the moment of writing this review, eight clinical trials using TCR-engineered T cells have reported their results (see Figure 1B and Table 2 for details), and at least another ten trials using TCR-engineered T cells are open and actively recruiting patients or will recruit patients soon (www.clinicaltrials.gov).

Table 1. Overview of standard and experimental none-gene-based therapies for metastatic melanoma

Therapy	Function	Type of trial	OR (%)*	CR (%)*	Refs.
T cell therapy Tumor infiltrating lymphocytes (TILs)	Adoptive transfer of tumor-specific T cells	n.c.	52/93 (56)	20/93 (22)	(1)
		n.c.	15/31 (48)	3/31 (10)	(2) [§]
T cell clones		n.c.	8/10 (80)	n.r.	(3)
'Educated T cells'		n.c.	4/9 (44)	1/9 (11)	(4)
Standard therapy High dose IL-2	Cytokine that induces T cell growth	n.c.	43/270 (16)	16/270 (6)	(178)
Dacarbazine (DTIC)	Drug that alkylates DNA	Phase III trial	18/149 (12)	4/149 (3)	(179)
Vemurafenib (PLX-4032)	Small molecule that inhibits BRAF kinase activity	Phase III trial	106/219 (48)	2/219 (1)	(180)
Experimental therapy Dabrafenib	Small molecule that blocks BRAF kinase activity	Phase III trial	29/54 (54)	n.r.	(181)
Dabrafenib + Trametinib	Small molecules that block BRAF and MEK kinase activities	Phase III trial	41/54 (76)	n.r.	(181)
Ipilimumab (MDX-010) + vaccination	Antibody that blocks T cell CTLA4	Phase III trial	39/137 (28)	3/137 (2)	(182)
Ipilimumab + DTIC		Phase III trial	34/252 (14)	26/252 (10)	(183)
Nivolumab (MDX-1106) #	Antibody that blocks T cell PD1	Phase I trial	5/39 (13)	1/39 (3)	(184)
		Phase I trial	26/94 (28)	n.r.	(185)
Nivolumab + Ipilimumab		Phase I trial	21/53 (40)	n.r.	(186)
Pembrolizumab (MK-3475)	Antibody that blocks T cell PD1	Phase I trial	51/135 (38)	n.r.	(187)
Anti-PD-L1 (MDX-1105)	Antibody that blocks tumor cell PDL1	Phase I trial	17/135 (13)	n.r.	(188)

* OR = Objective responses, CR = Complete responses, both according to Response Evaluation Criteria for Solid Tumors (RECIST). Number of patients with responses = before dash; total number of patients treated = after dash; percentage of responses = between brackets.

[§]Dr. Jacob Schachter, Cellular Therapy of Cancer Symposium, Sept 24-27th, Montpellier, France, 2010

This study included patients with metastatic melanoma, but also patients with renal cell carcinoma, colorectal cancer, prostate cancer and non-small-cell lung cancer.

Abbreviations: BRAF = gene responsible for production of B-Raf-kinase; CTLA4 = Cytotoxic T-lymphocyte antigen 4; IL-2 = Interleukin 2; n.c. = not classified; n.r. = none reported; mAb = monoclonal antibody; MAPK = Mitogen-activated protein kinase; PD1 = Programmed cell death 1 receptor; PDL1 = Programmed cell death 1 ligand.

Most clinical TCRs tested so far were HLA-A2-restricted and directed against either melanoma-associated antigen recognized by T cells 1 (MART-1), glycoprotein (gp) 100, carcinoembryonic antigen (CEA), p53, melanoma-associated antigen (MAGE-)A3 or New York esophageal squamous cell carcinoma antigen (NY-ESO)1. Another TCR tested clinically was HLA-A1-restricted and directed against MAGE-A3. Collectively, these trials have not only demonstrated feasibility but also demonstrated significant clinical responses in patients with metastatic melanoma, colorectal carcinoma and synovial sarcoma (Table 2). Responses, although variable and tested in a cumulative number of about 80 patients (based on trials listed in Table 2), ranged from 12 to 67 %. Notably, the finding that TCR gene-engineered T cells 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 underscored the strength of T cell therapy towards metastasized and poorly-accessible tumors (7). Clinical testing, however, also clearly demonstrated that therapy is currently hampered by treatment-related toxicity and a transient nature of tumor regression. Treatment-related toxicity became evident from studies with TCRs, in particular those of high-affinity, directed against antigens that are over-expressed on tumors but also expressed on healthy cells. Toxicities included severe but treatable inflammation of skin, eyes, ears (MART-1/HLA-A2; gp100/HLA-A2) and colon (CEA/HLA-A2). In addition, lethal neurological toxicities were observed in two patients when targeting MAGE-A3/HLA-A2, and lethal cardiac toxicities were observed in three patients when targeting MART-1/HLA-A2 (another epitope as above) or MAGE-A3/HLA-A1. The transient nature of tumor regression became evident from observations that anti-tumor responses are initially significant but not sustainable and ultimately incomplete in 80 to 90% of patients. Table 2 offers an up-to-date and detailed overview of toxicities as well as clinical responses reported for TCR gene therapy trials.

Strategies that aim at preventing or limiting toxicities as well as tumor recurrences have already been developed, some of which need further preclinical testing and some of which have already been implemented in clinical trials. In this review, we have categorized these strategies along three renewed challenges, i.e., 'choice of target antigen'; 'fitness of T cells' and 'sensitisation of micro-milieu for T cell therapy', as illustrated in Figure 2. We propose and will argue that optimizations along each or combinations of these challenges will contribute most significantly to the advancement of clinical TCR gene therapy.

Table 2. TCR gene therapy trials – an update on efficacy and safety

Target antigen (epitope)	Original T cell clone/lines	Tumor Type	OR (%)	CR (%)	Toxicity (%) *	Type of Toxicity	Refs
MART-1(AAG) /HLA-A2	TIL clone DMF4 from responding patient	Metastatic melanoma	2/17 (12)	n.r.	0/17 (0)	n.r.	(189)
MART-1(AAG) /HLA-A2	TIL clone DMF5 from responding patient with high <i>in vitro</i> avidity	Metastatic melanoma	6/20 (30)	n.r.	9/36 (25)	Severe melanocyte destruction in skin, eye and ear (in some cases leading to uveitis and hearing loss)	(190)
gp100(KTW) /HLA-A2	Splenocytes from immunized mouse	Metastatic melanoma	3/16 (19)	n.r.			
CEA(IMI) /HLA-A2	Splenocytes from immunized mouse; TCR is affinity-enhanced	Metastatic colorectal carcinoma	1/3 (33)	n.r.	(3/3) (100)	Severe inflammation of colon	(191)
NY-ESO1(SLL) /HLA-A2	T cell clone 1G4 from human subject; TCR is affinity-enhanced	Metastatic melanoma	5/11 (45)	2/11 (18)	0/11 (0)	n.r.	(192)
		Metastatic synovial sarcoma	4/6 (67)	0/6 (0)	0/6 (0)		
MAGE-A3(KVA) /HLA-A2	Splenocytes from immunized mouse; TCR is affinity-enhanced	Metastatic melanoma	5/9 (55)	2/9 (22)	3/9 (33)	Changes in mental status, two patients fell into coma and subsequently died, one patient recovered	(29)
MART-1(ELA) /HLA-A2	T cell clone 1D3 from human subject; TCR is codon-optimized and murinized	Metastatic melanoma	n.r.	n.r.	1/1 (100)	Lethal cardiac toxicity in one patient	\$
MAGE-A3(EVD) /HLA-A1	T cell clone a3a from human subject; TCR is affinity-enhanced	Metastatic melanoma and multiple myeloma	n.r.	n.r.	2/2 (100)	Lethal cardiac toxicity in two patients	(30)

OR = Objective Responses, CR = Complete Responses, both according to Response Evaluation Criteria for Solid Tumors (RECIST). Number of patients with responses = before dash; total number of patients = after dash; percentage of responses = between brackets.

*Number of patients with Serious Adverse Events (toxicity grading ≥ 3 according to National Cancer Institute common toxicity criteria) and total number of patients treated are put before and after dash, respectively.

\$Dr. John Haanen, Cellular Therapy of Cancer Symposium, London, UK, Febr. 27th - March 2nd, 2013.

Abbreviations: CEA = Carcinoembryonic antigen; gp = glycoprotein; HLA = Human leukocyte antigen; MAGE = Melanoma associated antigen; MART = Melanoma antigen recognized by T Cells; n.r. = none reported; NY-ESO1 = New York esophageal squamous cell carcinoma 1.

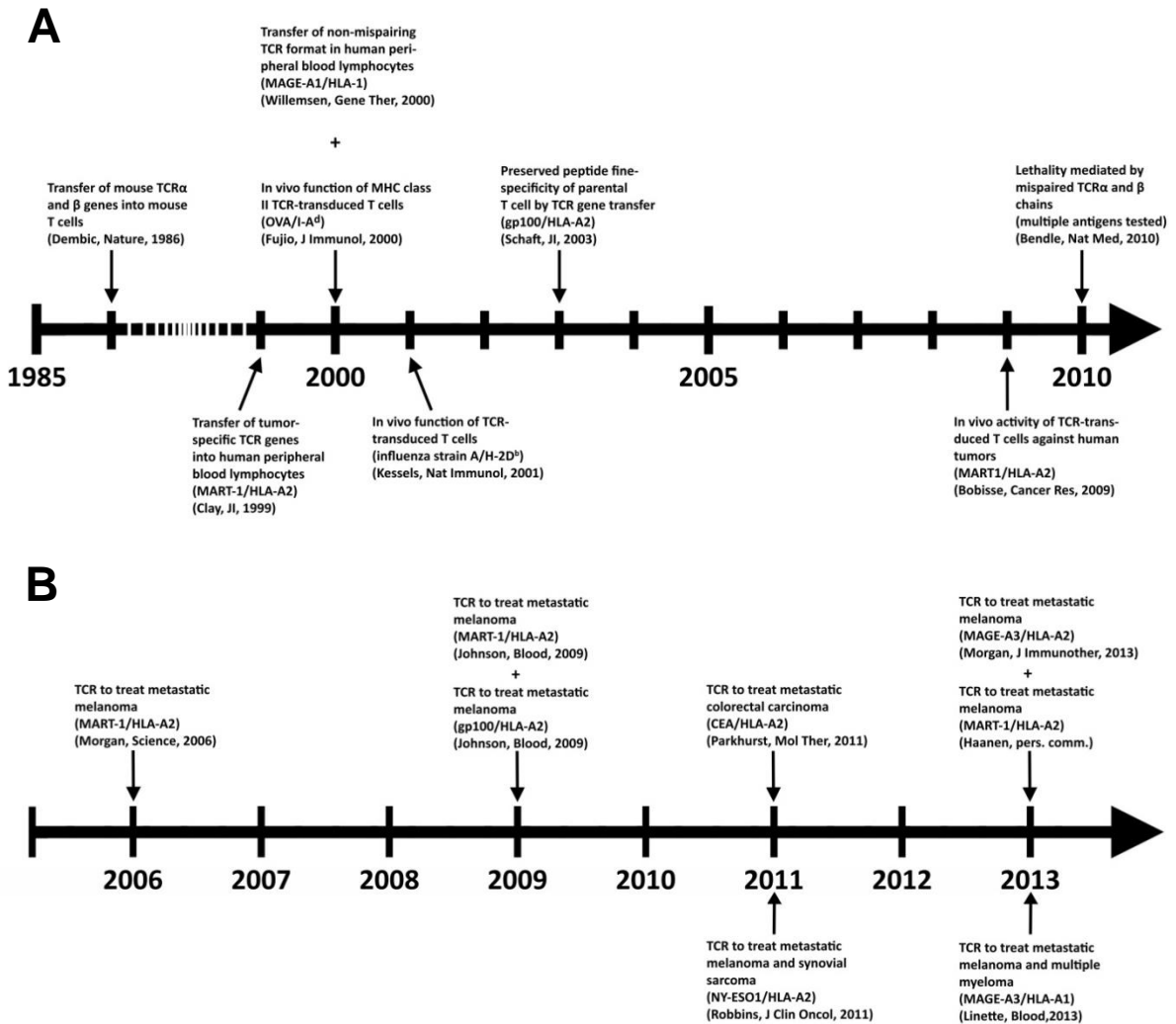


Figure 1: Key achievements in the field of TCR gene therapy directed against solid tumors
A: Timeline of selected preclinical findings that have contributed to the development of TCR gene therapy.
B: Timeline of clinical findings with TCR gene-engineered T-cells. Details with respect to clinically used TCRs can be found in Table 2.

2.2 CHOICE OF TARGET ANTIGEN

Ideally, target antigens are selectively expressed by tumor tissue and not healthy tissue, and hence not expected to evoke a response against self. At the same time, target antigens should have proficient immunogenicity to initiate an effective anti-tumor response.

2.2.1 Selective expression

Tumor-associated antigens (TAAs) can generally be divided into four groups (8).

- **Differentiation antigens:** cell surface proteins that are expressed at different stages of tissue development or cell activation. Expression of these antigens may discriminate tumor cells from surrounding healthy cells, but expression by healthy cells is not absent. Examples include MART-1, gp100, CEA and tyrosinase related protein (TRP)1 and 2.
- **Over-expressed antigens:** cell surface proteins that are highly, but not selectively, expressed by tumor cells when compared to healthy cells. Examples include the epidermal growth factor receptor (HER)2 or survivin.
- **Cancer Testis Antigens (CTAs):** proteins that are expressed by tumors and a limited number of healthy and adult cell types. A defined number of CTAs may not be expressed by healthy adult cell types. Examples include MAGE-A1, MAGE-C2 and NY-ESO1.
- **Neo-antigens:** proteins that result from gene mutations or aberrations in tumor cells. These proteins are uniquely expressed by tumor cells but not healthy cells. Examples include mutated protein (p)53, B-Raf kinase and cyclin-dependent kinase 4 (CDK4).

Looking at these four groups of TAAs, CTAs and neoantigens may represent the best available choices for therapy with TCR-engineered T cells. With respect to CTAs, over several hundreds of genes have been identified (see for a full description of CTAs: <http://www.cta.Incc.br>). Approximately 40 of these genes belong to multigene families that are located on the X chromosome. A selected number of mostly X-chromosome-located CTAs may be of interest for T cell therapy. *First*, these antigens are not expressed by healthy tissues except testes and placentas (determined using RT-PCR), and these latter tissues do not express Major Histocompatibility (MHC) molecules and cannot be targeted by T cells (9). *Second*, CTAs are expressed by tumor tissues of various histological origins as a result of aberrant epigenetic regulation (9), and expression of CTAs has been associated with advanced stages of disease and unfavourable patient prognosis (10). Along these lines, there is evidence that MAGE proteins are related to oncogenesis as they suppress p53-dependent apoptosis and cause fibronectin-controlled increase in tumor cell proliferation and metastasis (11-15). *Third*, CTAs are immunogenic proteins that have been reported to induce both humoral and cell-mediated immune responses in patients without the concomitant induction of toxicities (10,16,17). Undeniably, current patient studies emphasize the need for careful identification of target CTAs. In one study, Robbins and colleagues demonstrated that a TCR directed against NY-ESO1/HLA-A2 showed significant anti-tumor responses in patients with metastatic melanoma and synovial sarcoma without detectable toxicities (Table 2). Unexpectedly, in another study using a TCR directed against MAGE-A3/HLA-A2, two patients with metastatic melanoma lapsed into coma and died. These adverse events were most likely caused by T cell recognition of rare neurons that were positive for MAGE-A12 and possibly MAGE-A9 antigens, which contain shared or highly similar epitopes compared to MAGE-A3 antigen (Table 2). In a third study, in which a TCR was used directed against MAGE-A3/HLA-A1, one patient with melanoma and one patient with myeloma suffered from cardiovascular toxicity and died. This toxicity was

possibly caused by T cell recognition of a similar but not identical peptide from the muscle protein titin (so-called 'off-target' toxicity, Table 2).

With respect to neoantigens, the expression of these antigens may vary significantly among different patients, but their expression is unique to tumor tissues. In case a neo-antigen is the result of 'driver mutations', the antigen may constitute an ideal target for T cell therapy. Driver mutations are related to oncogenesis, may be linked to known genes (~400), and may provide tumors with a selective growth advantage (18,19). Nevertheless, it is important to realize that only 15% of up to 100,000 mutations that are encountered in tumor genomes are considered 'driver' mutations (18,20). Moreover, not all driver mutations may result in new immunogenic antigens. A quest for neo-antigen targets does not only require next-generation sequencing techniques to identify tumor-specific mutations (21), but also techniques to determine whether a neo-epitope can be presented by MHC and recognized by T cells (22,23).

In short, we consider epitopes from selected (non-shared) CTA and neo-antigens as potentially safe T cell target antigens. However, no matter what the antigen, it is recommended to perform stringent *in silico* analysis and preclinical testing to confirm the antigen's absence from vital organs. Strategies used to identify titin as a cross-recognized peptide, such as amino acid scanning, gene database searches and use of 3-dimensional cell cultures, are potentially helpful in this respect (24). In addition, one could consider using suicide systems to deplete self-reactive T cells prior to proceeding with clinical testing (25-28). Although suicide genes provide the option to delete TCR-transduced T cells, it is questionable whether such a switch could counteract the fast kinetics of toxicity reported in the above-mentioned trials (29,30).

2.2.2 Immunogenicity

The immunogenicity of an antigen, i.e., its ability to initiate immune responses, is determined by the level of its expression, how it is processed and presented, and how well it is recognized by T cells.

Level of expression and processing of antigens

Ideally, target antigens should be expressed at high levels by most if not all tumor cells. Such a property is generally restricted to those antigens that are related to oncogenesis and that tumors cannot easily do without (see part 2.1). It is noteworthy that the production of antigens, such as those of MAGE-A family members and NY-ESO1, is enhanced and becomes more homogeneous within tumors by treatment with demethylation agents and/or histone deacetylases (31-34). In a phase II clinical study, in which haematological malignancies were targeted and which included treatments with epigenetic drugs, it was observed that T cell responses directed against CTA were enhanced with no evidence of adverse events (35). In addition, the production of antigens may depend on immune or intermediate proteasomes, rather than standard proteasomes, and on unconventional post-translational events such as reverse splicing and deamidation of proteins (36-38). Such processing of antigens, in particular when mediated by immune proteasomes, may

benefit from local production of interferon (IFN) γ . Finally, the release and hence the availability of antigens may be enhanced via treatment-induced cell death following (co-treatments with) chemotherapy, irradiation and/or therapy with tyrosine kinase inhibitors (39,40).

Cross-presentation of antigens

Antigen cross-presentation may take part in the infiltration of antigen-specific CD8 T cells (41) and cause activation of T cells and subsequent stroma destruction, thereby preventing outgrowth of antigen-negative tumor cells. Recently, Engels and colleagues revealed that peptide:MHC affinities of 10 nM or less allowed for cross-presentation of antigens by stromal cells (42). Notably, using an experimental model in which mice transgenic for TCRs with different antigen specificities were used either as donors or recipients of T cells, they showed that the use of peptide targets that can be cross-presented result in complete anti-tumor responses. Destruction of tumor stroma, a bystander response that may put an advantage to T cells over drugs (43,44), may require optimal T cell fitness (as measured by production of IFN γ) and IFN γ -mediated preservation of Fas expression by stromal cells (45).

Robustness of antigenicity

Loss of tumor antigen expression after infusion of T cells, and its impact on the recurrence of tumors, is an important yet controversial aspect. Decreased antigen expression has been proposed to be a consequence of molecular alterations in tumor cells, such as genetic and epigenetic changes in antigen genes, MHC genes and genes related to antigen processing and presentation (46-48). Indeed, selective loss of antigen or HLA-A2 expression has been reported in primary and metastatic melanoma lesions in non-treated patients (49,50) as well as patients treated with T cells (51,52). Also, Landsberg and colleagues, using a gene-engineered model of melanoma, have eloquently demonstrated that a therapy-resistant phenotype may be directed by an inflammatory milieu and tumor necrosis factor (TNF) α 's ability to lead to epithelial dedifferentiation and decreased expression of melanoma antigens (53). In contrast to these findings, there is increasing evidence to support the view that tumors progress without loss of T cell antigens. In various preclinical models, in which either skin, lung or ovarium tumors were studied, it was observed that tumors progressed despite continued antigen expression (54-56). In these models, tumor progression was rather a consequence of reduced T cell infiltration and reduced T cell responsiveness. We postulate that in the setting of T cell therapy, loss of target antigen, whether by T cell-dependent selection or epigenetic silencing (57,58), is *not* necessarily a driving mechanism in tumor recurrence (Straetemans *et. al.*, manuscript submitted).

Target multiple antigens simultaneously

In current TCR gene therapy trials, single MHC class I-restricted antigens are targeted. Preclinical studies have suggested that the targeting of two or more antigens enhances the therapeutic potential of T cells. For example, adoptive transfer of two CD8 T cell populations to simultaneously target ovalbumine and gp100, rather than either one antigen, resulted in delayed recurrence of

tumors (59). Interestingly, treatment with viruses positive for three MHC class II-restricted antigens, i.e., neuroblastoma RAS, TRP1 and cytochrome c1, resulted in complete anti-tumor responses that were accompanied by significant CD4 T helper cell type 17 (Th17) responses (60). Since cooperation of CD4 and CD8 T cells appears important in the effector phase of an anti-tumor response and may contribute to the bystander elimination of tumor stroma (61), it may be worthwhile to simultaneously target MHC class I and II targets. With respect to human antigens, it is interesting to note that X-chromosome linked CTAs are co-ordinately expressed in tumor tissues (62), which may allow the simultaneous targeting of multiple CTAs.

2.3 FITNESS OF T CELLS

The responsiveness of T cells towards tumor antigen is generally tuned down, most likely at various levels. First, reactive T cells may be deleted during T cell development in the thymus; second, peripheral T cells may be susceptible to anergy; and third, intra-tumoral T cells may require enhanced co-stimulation (63). To overcome such T cell tolerizing mechanisms one can optimize T cell fitness. Here, we define T cell fitness according to the following three T cell properties: functional T cell avidity, T cell co-signalling and T cell differentiation.

2.3.1 Functional T cell avidity

Functional T cell avidity is considered as the ability of T cells to respond to a given concentration of cognate peptide antigen, and can be enhanced via strategies, often involving gene-engineering of TCR $\alpha\beta$ transgenes, that either increase the level of cell surface expression of TCR chains or the TCR's affinity for peptide-MHC.

Expression level of TCR transgenes

One angle to enhance the surface expression of TCR transgenes is through optimization of the TCR gene transfer methodology, including choice of gene delivery method, use of optimal vector elements, and use of transgene cassettes (reviewed in (6,64)). Another angle to enhance the surface expression of TCR transgenes is through limitation or abolishment of TCR mis-pairing. TCR mis-pairing is the formation of TCR heterodimers that comprise one transgenic TCR chain and one endogenous TCR chain, and represents a phenomenon that is inherent to the generation of TCR-engineered T cells. Importantly, TCR mis-pairing dilutes the surface expression of the transgenic TCR $\alpha\beta$ chains, and mis-paired TCRs are of unknown specificity and can yield self-reactive T cells. Although in clinical trials performed so far, no formal observations of toxicities mediated by TCR mis-pairing have been made, preclinical studies have clearly demonstrated that TCR mis-pairing has the potential to induce harmful recognition of self-antigens (65,66). Strategies to promote preferential pairing between transgenic TCR α and TCR β chains (and consequently prevent or reduce TCR mis-pairing) can be grouped according to those that depend on gene-engineering of TCR transgenes and those that do not. The first group of strategies are reviewed in (67). In short, these strategies include murinization of TCR (68), addition of cysteine amino acids to TCR (69,70),

mutations in TCR transmembrane and constant domains (71,72), and equipment of TCR with a signaling cassette that replaces TCR transmembrane and intracellular domains with the CD3 ζ accessory molecule (73,74). More recently, a limited number of murine amino acids have been identified that are responsible for enhanced expression and preferential pairing of murinized TCRs (75,76). Similar efforts to minimize the number of amino acids in a CD3 ζ signaling cassette failed, and it was observed that properties of TCRs equipped with CD3 ζ signalling cassettes are best preserved when incorporating a complete CD3 ζ molecule (77). The other group of strategies includes technologies that enhance expression levels of CD3 molecules in T cells and those that interrupt expression of endogenous TCR chains. Co-transfer of CD3 and TCR genes into T cells resulted in higher levels of TCR expression and allowed T cells to respond to lower concentrations of antigen, and to infiltrate and eliminate tumors with faster kinetics (78). RNA interference techniques have been shown to specifically down-regulate the expression of endogenous but not transgenic TCR chains (79,80). An alternative method encompasses the use of zinc finger nucleases and a sequential knock-out of endogenous TCR α and β chains, followed by introduction and sorting of TCR α and β transgenes (81). The latter method is relatively new and not yet widely or clinically applied, but holds promise to effectively address TCR mis-pairing.

Affinity enhancement of TCR $\alpha\beta$ transgenes

Affinity-enhancement of tumor specific TCRs, and its exploitation, relies on the existence of a window for optimal TCR affinities. The existence of such a window is based on observations that TCRs specific for HLA-A2-restricted pathogens have K_D values that are generally about 10-fold lower when compared to TCRs specific for HLA-A2-restricted tumor associated self-antigens (82). In support of this notion are the observations that a high-affinity MART-1/HLA-A2 TCR mediated improved objective response rates compared to a lower affinity MART-1/HLA-A2 TCR, and that an affinity-enhanced NY-ESO1 TCR mediated significant clinical responses (Table 2). Affinity-enhanced TCRs can be obtained through various routes. First, allo-reactive settings can be used to circumvent self-tolerance and yield T cells with a higher avidity when compared to T cells derived from autologous settings (= patients). Examples of such settings include *in vitro* generation of allo-HLA reactive, peptide-specific T cells (83-85), and immunization of mice transgenic for human-MHC or human TCR (86,87). Second, TCR affinities can be enhanced by rationally designed mutations of the TCR's complementarity-determining regions (CDRs) (88,89). Third, high-affinity TCR variants can be selected from a library of CDR mutants by yeast, phage or T cell display (90-92). Although the affinity of TCRs significantly contributes to the functional avidity of T cells, recent studies warrant caution when therapeutically implementing this strategy. Clinical reports suggest that CDR mutations in TCRs directed against CEA/HLA-A2, MAGE-A3/HLA-A2 and MAGE-A3/HLA-A1, but not NY-ESO/HLA-A2, were possibly related to patient toxicities (Table 2). Investigations whether defined locations and types of mutations are more prone to lead to toxicities than others would most likely benefit further development of CDR-mutated TCRs. Also, preclinical reports suggest the existence of a functional ceiling with respect to TCR affinity (93,94). In fact, studies with primary human T cells transduced with affinity-enhanced TCRs directed against NY-

ESO1/HLA-A2 (93) or gp100/HLA-A2 (Govers *et al.*, manuscript submitted) pointed to the existence of a K_D threshold of 1 to 5 μM , below which T cell function became compromised. The functional impairment of high avidity T cells in the presence of high levels of antigen, as is often the case in tumors, may be related to enhanced expression of the exhaustion marker programmed cell death (PD1) and enhanced activity of its downstream sarcoma homology domain 2 phosphatase (SHP)1 (95,96).

2.3.2 T cell co-signalling

T cell co-signaling is directed by interactions between co-stimulatory or co-inhibitory molecules and their ligands and determines, in addition to interactions between TCR and peptide-MHC, the functional outcome of T cells (reviewed by (97)). The best characterized co-stimulatory and co-inhibitory molecules expressed by T cells are CD28 and cytotoxic T-lymphocyte associated protein (CTLA)4, respectively, which both interact with CD80 and CD86 ligands expressed by APCs. More recent examples of co-stimulatory and co-inhibitory molecules include inducible T cell co-stimulation (ICOS), 4-1BB, OX40, CD40, B and T-lymphocyte attenuator (BTLA), and PD1.

Tumors provide continuous stimulation with antigen often in the absence of co-stimulatory ligands, which may result in exhausted T cells with reduced proliferative capacity, reduced effector function (such as IFN γ production) (98) and up-regulated expression of T cell co-inhibitory molecules (99). Immunotherapy with monoclonal antibodies to block the T cell co-inhibitory molecules CTLA4, PD1, PDL1, or the combination of CTLA4 and PD1 showed clear clinical successes in the treatment of advanced melanoma (see Table 1). These clinical activities have provided an impetus for the development of blocking other co-inhibitory molecules and/or stimulation of co-stimulatory molecules (100-104). The beneficial outcome of targeting T cell co-signaling most likely relies on enhancement of infiltration of T effector cells (Teff) into tumor tissue and activation of Teff, as well as depletion of intra-tumoral T regulatory cells (Treg) (103-105). We would advocate explorative studies to test the combination of blocking T cell co-inhibitory molecules and adoptive transfer of Teff. In addition to this combination of immune therapies, two other approaches to implement T cell co-signaling in protocols of T cell therapy have already been clinically tested. First, TCR transgenes can be equipped with a signalling cassette that harbors a co-stimulatory molecule. Such a signalling cassette, designed in analogy to those used in co-stimulatory CARs (6), typically introduces accessory and co-stimulatory molecules to enhance the function of T cells expressing the TCR transgene. It is noteworthy that clinical trials using CARs containing CD28 or CD137 demonstrated significant objective responses in patients with B cell leukemia (106-108), and while CARs may evoke immune responses, these were directed against murine idiotypes, but never against boundaries between genetically introduced human molecules (109). According to this rationale, single and two-chain TCR genes have been coupled to a combination of CD28 and CD3 molecules and were shown to provide T cells with improved function *in vitro* (110,111) (Govers *et al.*, manuscript submitted). Second, T cells can be stimulated *ex vivo* with human artificial APC (aAPCs) that express co-stimulatory ligands (4,112). In addition to co-stimulatory ligands, these

aAPCs are mostly engineered to express HLA-A*0201 and used to stimulate T cells in the presence of common- γ cytokines other than interleukin (IL)-2. These combined activations allow for the generation of HLA-A2-restricted, antigen-specific T cells with a less differentiated phenotype (CD45RA⁺ CD62L⁺) and superior T cell functions *in vivo* (112). In a clinical study, T cells educated with aAPC presenting CD80, CD83, and a MART-1 peptide, and cultured in the presence of IL-2 and IL-15, resulted in objective responses in patients with metastatic melanoma (Table 1). Notably, inclusion of T cell co-stimulation by either one of the two above-mentioned approaches relieved the requirement for patient preconditioning with chemotherapy and/or *in vivo* IL-2 administration (4,106).

2.3.3 T cell differentiation

The differentiation of naïve T cells into mature CD8 Teff or CD4 Th1 or Th17 cells is required for T cells to make full use of their functional attributes directed against tumor cells, such as cytotoxicity and production of IFN γ and TNF α . The differentiation of T cells is largely driven by environmental stimuli, with cytokines being well-studied examples of such stimuli (113,114). Progression of T cells into a differentiated subset is not necessarily permanent, and in particular T helper cell subsets have shown plasticity and may change into another T helper cell subset (114). Differentiation of CD8 and CD4 T cells, although occurring according to similar principles, follow different routes and show different outcomes. Strategies to manipulate T cell differentiation to advance T cell therapy are discussed separately for both T cell subsets.

CD8 T cells

Naïve CD8 T cells can differentiate, depending on the quantity and quality of the initial antigenic and co-stimulatory stimuli, into stem cell memory T cells, central memory T cells, effector memory T cells or T effector cells (115). An important observation that came from preclinical studies was the inverse relationship between CD8 T cell differentiation and proliferation, and hence the inverse relationship between CD8 T cell differentiation and *in vivo* persistence and therapeutic activity (113). Two strategies have been reported to exploit this inverse relationship and improve adoptive T cell therapy. In one such strategy, as shortly mentioned in section 3.2, T cells are exposed to common- γ cytokines other than IL-2 prior to adoptive T cell transfer. For example, treatments with either IL-7+IL-15 or IL-15+IL-21 generated gene-engineered T cells with a less differentiated CD8 T cell phenotype (i.e., central memory phenotype), prolonged peripheral persistence and potent antigen reactivity (116,117). In addition to soluble cytokines, Singh and colleagues reported on aAPC that express membrane-bound IL-15 and IL-21 and facilitate the generation of 'young' T cells (112). In other reports, the anti-tumor efficacy of T cells was enhanced either via *in vivo* administration of IL-15+IL-21 (118) or conjugation of nanoparticles, encapsulating these cytokines, to the surface of therapeutic T cells (119). In a second strategy, T cells are enriched for less differentiated T cell populations, i.e., based on CD62L expression, and subsequently used as recipient cells for gene transfer (120,121). A recently identified population of 'stem-cell memory' CD8 T cells, expressing high levels of CD95, IL2R β and demonstrating increased proliferative

potential and ability to mediate anti-tumour responses, may represent a promising subset of T cells for gene-engineering and therapeutic application (122). In fact, Cieri and colleagues have set up a protocol to obtain and gene-modify stem-cell memory CD8 T cells, which includes the use of CD3/CD28 mAbs and IL-7 and IL-15 and could potentially be translated to a clinical setting (123).

CD4 T cells

Naïve CD4 T cells can differentiate into multiple subsets, including Th1, 2, 9, 17, 22, follicular helper and various Tregs, often defined by the expression of 'signature cytokines' or typical functions, such as B cell activation or the down-modulation of T cell responses (124). With respect to anti-tumor responses, it appears that upon cell transfer Th1 and Th17 are the most potent CD4 T cell subsets (125,126). Administration of CD4 T cells, and in particular Th1 cells, has been shown to prevent exhaustion of CD8 T cells, enhance tumor infiltration of CD8 T cells and result in effective tumor eradication (125,127-130). More recently, it was discovered that adoptive transfer of Th17 cells effectively mediate rejection of TRP1-positive tumours in a TCR-transgenic mouse model (126). Furthermore, Th17 cells appear to be long-lived and their molecular signature resembles that of stem-cell memory CD8 T cells (131). Interestingly, the anti-tumour activity of Th17 cells depended on its (incomplete) differentiation and conversion into Th1 cells, resulting in a co-existence of Th17 and Th1 cells, and it may very well be this multi-potent aspect that provides a therapeutic advantage.

Collectively, these data argue in favor of a combined therapeutic use of CD8 T cells and Th1 or Th17 cells. To this end, CD4 T cells can be functionally endowed with MHC I-restricted TCR and/or CD8 via gene transfer (132-135). Alternatively, one could opt for strategies that induce *in vivo* conversion of CD4 T cells into Th1 cells, such as IL-12, IFN α , IFN γ , or blocking PD1 ligation (136-139). Also, metabolic signals, such as activation of T cell mammalian target of rapamycin (mTOR) and aerobic glycolysis can enhance differentiation towards IFN γ -producing T cells and may be exploited therapeutically (140,141).

2.4. SENSITISATION OF MICRO MILIEU FOR T CELL THERAPY

Tumors, following initial regression upon treatment with T cells, most often become resistant to T cell therapy and recur. Recent understanding suggests that, at least in some tumors, therapy resistance may be part of a negative feedback loop that is initiated once an anti-tumor CD8 T cell has occurred (142). Therapy resistance is often characterized by a dis-balance between numbers and activation state of immune effectors cells versus those of suppressor cells. Strategies to manipulate numbers and activation state of immune cells are discussed separately for effector and suppressor cells.

2.4.1 Recruitment and activation of immune effector cells

Immune effector cells that have been recognized for their contribution to an anti-tumor response are numerous and, in addition to CD4 and CD8 T cells, include natural killer (NK), natural killer T cells (NKT), macrophages and neutrophils. Here we will focus on Teff and macrophages and how manipulation of the micro-milieu may enhance their recruitment and activation.

Enhance recruitment of T effector cells

Clinical studies have demonstrated an unfavorable prognostic value of a limited CD8 T cell infiltration in melanoma, colorectal and ovarium carcinomas (143-145). Vascular changes have been reported to contribute to arrested T cell infiltration and include insufficient vascular maturation and enhanced expression of endothelin B receptor, regulator of G-protein signalling 5 (Rgs5) and/or extracellular matrix components (reviewed in (146)). Such changes may be targeted, as evidenced by angiostatic therapy in which antibodies directed against vascular endothelial growth factor (VEGF) or angiopoietin 2, or in which T cells gene-engineered with a CAR directed against VEGF receptor (VEGFR)2 resulted in enhanced T cell infiltration (147-149). In addition, drugs that inhibit angiogenesis or endothelin receptor B were able to enhance the expression of intercellular adhesion molecule (ICAM)1 on endothelial cells and to normalize T cell infiltration (150,151). In various solid tumors, T cell infiltration appears to be facilitated by vessels that closely mimic high endothelial venules (HEV) and which may be part of ectopic lymphoid structures in tumor stroma (152,153). A better understanding of the development of such HEV in tumor stroma may provide novel targets to improve T cell infiltration in tumors.

In addition to vascular changes, spontaneous cutaneous melanoma tumors in mice demonstrated a decreased mRNA expression of chemoattractants that contribute to recruitment of CD8 T cells, such as chemokine (CC motif) ligand (CCL)5 and chemokine (CXC motif) ligands (CXCL)9 and 10 (146). In a subset of patients with melanoma metastases, lack of chemoattractants coincides with limited migration of CD8 T cells and limited presence of lymphoid structures (154). Current findings from our laboratory suggest that a decreased expression of selected chemoattractants and adhesion molecules are related to a decreased infiltration of CD8 T cells and tumor relapse following T cell therapy (Straetemans et. al., manuscript submitted). Interestingly, Hong and colleagues have shown that the chemotherapeutic drugs dacarbazine, temozolomide, and cisplatin enhanced the expression of CCL5, CXCL9 and CXCL10 in patient melanoma, which in turn correlated with improved immune control of tumors (155). Vice versa, T cells when gene-engineered to express chemokine (CXC motif) receptor (CXCR)2 displayed enhanced trafficking towards tumor cells secreting the corresponding chemokine ligand CXCL1 (156). Also, in xenograft tumor models of mesothelioma and neuroblastoma, the genetic introduction of chemokine (CC motif) receptor (CCR)2 in T cells resulted in increased T cell infiltration in tumors secreting CCL2 and was associated with significantly increased anti-tumor activity (157,158). Other molecules often present in the micro-milieu that, when targeted, resulted in enhanced T cell accumulation at the tumor site are indoleamine 2,3-dioxygenase (IDO) and reactive nitrogen species. Inhibition of

IDO by a small molecule blocks tryptophan depletion, enhances T cell infiltration and delays tumor growth (159). Reactive nitrogen species induce TIL unresponsiveness (160), nitration of the TCR complex (161) and modification of the chemokine CCL2 (162). Drugs affecting the local production of reactive nitrogen species restore TIL function and improve intra-tumoral T cell migration and an anti-tumor T cell response (160,162). Taken together, the above studies show the drug-ability of molecules that are involved in T cell extravasation and T cell migration into tumor tissues, and advocate studies to combine such drugs with adoptive T cell therapy.

Enhance T cell effector functions

Early protocols of adoptive T cell therapy already demonstrated the beneficial effects of co-treatments such as chemotherapy, vaccination and/or cytokine support on T cell activation (reviewed in (64)). More recently, additional strategies that enhance anti-tumor functions of T_H1 have been reported. A first strategy became apparent from clinical success with additional T cell co-stimulation or blocking of T cell co-inhibition (see section 3.2 and Table 1). A second strategy relates to the inhibition of T cell suppressive cytokines, such as transforming growth factor (TGF) β . For example, genetic introduction of a dominant-negative TGF β receptor II in TCR-engineered T cells resulted in increased anti-tumor T cell responses in a spontaneous tumor model of prostate cancer (163). Another study tested the safety of mouse T cells engineered with this dominant-negative receptor, and could not detect spontaneous proliferation of these T cells *in vivo* (164). Genetic knockdown of negative regulators of T cell activation represents yet another strategy to enhance T cell activation. T cells with siRNA-mediated knockdown of casitas B-lineage lymphoma b (Cbl-b) displayed a lower threshold for T cell activation and, when adoptively transferred in mice with disseminated leukemia, resulted in enhanced anti-tumor effects (165). These latter findings warrant further testing of T cells with enhanced T cell activation, including tests that assess the safe use of these T cells.

Enhance recruitment and activation of macrophages

High numbers of macrophages with a tumor-promoting (M2) phenotype, but not those with a tumor-inhibiting (M1) phenotype, correlate with poor prognosis for patients with various cancers (166). When conjugated to a vascular homing peptide and targeted to tumors, TNF α resulted in a switch from M2 to M1 macrophages, which was accompanied by normalization of tumor vasculature and enhanced infiltration of CD8 T cells (167). Interestingly, T cells gene-engineered to release the cytokine IL-12 were shown to improve the therapeutic efficacy of T cells, an effect that is likely mediated by cells of the innate immune system (168,169). 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 with a prominent role for monocytes and monocyte-derived TNF α (168). Such findings are not necessarily restricted to IL-12 since IL-15, when provided locally into tumors, also enhanced the responsiveness of adoptively transferred T cells and facilitated the removal of antigen-negative tumor cells (170).

2.4.2 Reduce numbers and activity of immune suppressor cells

Tregs, M2 macrophages and myeloid-derived suppressor cells (MDSC) are among the major immune suppressive cell types in the tumor micro-milieu. Immune suppressor cells can reduce T cell infiltration into the tumor and suppress local T cell responses by: release of reactive nitrogen and oxygen species (171); expression of IDO and arginase (159,172); and production of cytokines such as TGF β , IL-4 and IL-13 (173). Despite initial removal of these cells by administration of chemotherapeutic agents, the populations of MDSCs and Tregs may recover at a faster rate than CD4 and CD8 Teff (174). Furthermore, Jensen and colleagues demonstrated that therapeutic CD4⁺ T eff can convert into a Foxp3⁺CD4⁺ Treg population (175). Various strategies have been reported to deplete or inactivate Tregs. These strategies include administration of anti-CD25 antibodies, combined intratumoral injection of anti-CTLA4 and OX40 mAbs, or blocking IDO (104,176). Interestingly, blocking IDO may induce conversion from Treg to Th17 helper cells, which can further contribute to anti-tumor T cell responses (176). With respect to MDSCs, it is of interest to note that classical chemotherapeutic agents, such as docetaxel, are able to deplete these cells. Docetaxel-mediated depletion of MDSC, when combined with adoptive T cell therapy and dendritic cell vaccination, was shown to enhance anti-tumor responses (174). Alternatively, differentiation of MDSCs into mature myeloid cells, which can be established upon administration of β -glucans (glucose monomers from cell walls), may also provide an angle to relieve immune suppression (177).

2.5. FUTURE PERSPECTIVES

By now, the feasibility of TCR gene therapy studies has been well established by the pioneering trials listed in Figure 1B, and is further enhanced by current optimizations and standardizations of protocols. TCR gene therapy, alike any cell-based therapy, requires specialized good manufacturing practice (GMP) and patient treatment facilities. Such facilities allow the generation and testing of virus batches and the gene processing and expansion of T cells, and are already integrated in multiple academic and private centers. Notably, parameters, such as time-lines and costs to manufacture a therapeutic T cell product, are considered competitive when compared to other clinical-grade products, such as antibodies. An ongoing EU project to treat metastatic oesophagus-gastric cancer and melanoma with NY-ESO1 TCR-engineered T cells, in which we participate, shows that time-lines and costs to obtain a T cell product are about two weeks and 36 k€ per patient (13.5 k€ for production, quality testing and test runs of virus batch; and 22.5 k€ for T cell processing), respectively. For comparison: estimated per patients costs of Ipilimumab (3 mg/kg every 3 weeks, 4 times) and Vemurafenib (0.96 g twice daily for 6 months), both registered treatments for metastasized melanoma in the Netherlands since 2012, are 84 k€ and 57 k€ (Association of Health Insurances (CVZ), the Netherlands). The next step, and allowing a more valid comparison, would be the testing of T cell therapy versus standard treatment of care in a randomized trial.

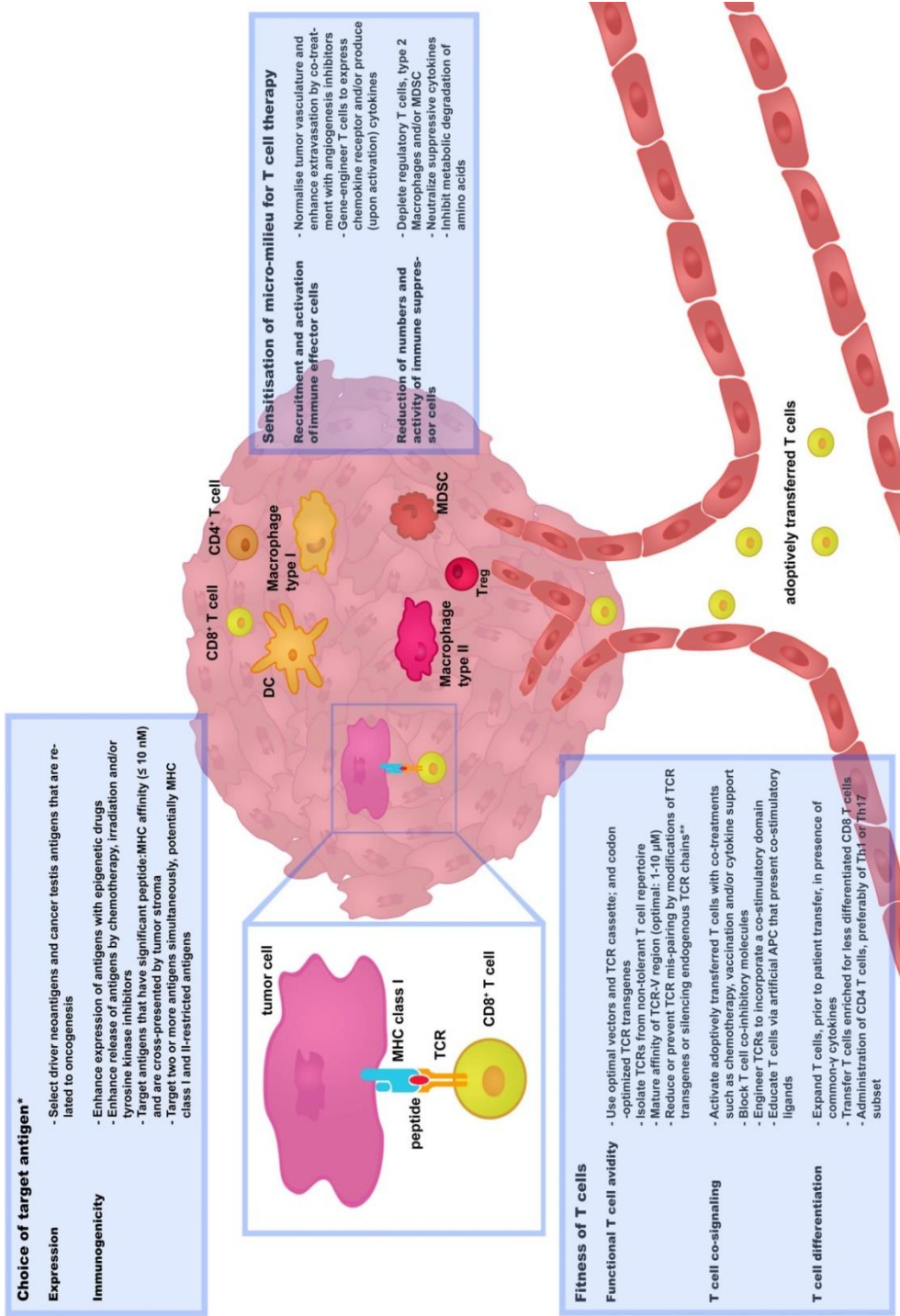


Figure 2: Three challenges that determine the success rate of TCR gene therapy

In this figure, recent and successful strategies to improve TCR gene therapy have been categorized along three renewed challenges: 'choice of target antigen'; 'fitness of T cells'; and 'sensitisation of micro-milieu for T cell therapy'. Boxes provide selected strategies that are discussed in more detail in sections 2, 3 and 4. We propose that advancement of clinical TCR gene therapy is guided by the principles of these challenges.

* Independent of choice of target antigen, it is recommended to perform stringent in silico analysis and preclinical tests to confirm that healthy cells do not express the target antigen prior to proceeding with the clinical testing of TCR-engineered T cells.

** Strategies to reduce or prevent TCR mis-pairing do not only enhance T cell avidity but also reduce the potential risk of off-target toxicity.

Abbreviations: APC = Antigen presenting cells; DC = Dendritic cells; MDSC = Myeloid-derived suppressor cells; Th = T helper cells; Treg = T regulatory cells

Clinical testing of TCR-engineered T cells, when looking at single trials, demonstrated impressive and unprecedented efficacy but at the same time is hampered by treatment-related toxicity and a transient nature of tumor regression (Table 2). There exists a multitude of strategies that are developed and tested towards advanced safety and efficacy of TCR gene therapy. Here, we have defined three challenges and have categorized recent and successful strategies along these three challenges, which have been schematically depicted in Figure 2. With respect to the first challenge, i.e., choice for target antigen, an important criterion is minimal or no expression of such an antigen by healthy tissues. In this respect, non-shared and tumor-restricted CTAs as well as neoantigens should be considered as potentially safe target antigens. Advances in the isolation and characterization of anti-tumor T cells from individual patient samples may increase the number of CTAs and neoantigens that may qualify as target antigens. T cell-based recognition of similar, but unrelated peptides should be excluded, and to this end it is strongly recommended to perform stringent *in silico* analysis and preclinical tests to confirm that cross-reactive antigens are absent in healthy tissue. In order to improve patient safety further, measures to allow directed killing of engineered T cells have been tested and should be considered, at least for novel TCRs tested in the near future. In addition to tumor-restricted expression, another criterion to choose target antigens is maximal immunogenicity. Peptide epitopes that are cross-presented or the targeting of a more than a single peptide have been reported to induce complete anti-tumor responses, and may represent examples to consider when selecting target antigens.

With respect to the second and third challenges, i.e., fitness of T cells and sensitisation of tumor micro-milieu, we would like to propose a two-step treatment protocol. The first step represents the transfer of fit T cells. T cell fitness involves optimal T cell avidity, additional T cell co-signalling, and using T cells with a preferred differentiation stage. T cell avidity can be optimized by enhancement of TCR affinity, yet reported treatment-related toxicities warrant caution when using affinity-enhanced TCRs (Table 2) and recommend further studies to define rules of TCR binding of cognate versus non-cognate peptides. With respect to T cell co-signalling, antibodies that block T cell co-inhibitory molecules and T cells gene-engineered with co-stimulatory receptors have demonstrated clinical successes. The implementation of such strategies in T cell therapy protocols holds promise for future trials. Also, developments to obtain and gene-modify early differentiation stages of CD8 T cells, including stem-cell memory CD8 T cells, are at the brim of being translated to a clinical setting. Whatever the chosen route, an important measure for T cell fitness *in vivo* is the ability of these cells, whether it be CD8 T cells or certain subsets of CD4 T cells, to produce IFN γ and TNF α . The production of these cytokines not only determines T cell responsiveness, but also to what extent innate immune cells are recruited into the tumor and become activated to further improve an anti-tumor response and potentially avoid tumor relapse. The second step represents antagonism of an immune-suppressed milieu. Various strategies, such as antibodies or drugs to mediate angiostasis, chemotherapeutic agents to enhance intratumoral T cell infiltration, and local (T cell-mediated) delivery of cytokines, have proven beneficial to enhance the local ratio between effector and suppressor immune cells. Development of such a two-step protocol, together with the

targeting of a selected antigen, is the way forward and expected to further enhance the success rate of TCR gene therapy to treat solid tumors.

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MAGE-C2 SPECIFIC TCRs COMBINED WITH EPIGENETIC DRUG-ENHANCED ANTIGENICITY YIELD ROBUST AND TUMOR-SELECTIVE T CELL RESPONSES

**Andre Kunert¹, Mandy van Brakel¹, Sabine van Steenberghe-Langeveld¹,
Marvin da Silva¹, Pierre G. Coulie³, Cor H. J. Lamers¹,
Stefan Sleijfer², Reno Debets¹**

¹Laboratory of Tumor Immunology, ²Department of Medical Oncology, Erasmus MC Cancer
Institute, Rotterdam, The Netherlands;

³de Duve Institute, Université Catholique de Louvain, Brussels, Belgium

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ABSTRACT

Adoptive T cell therapy has shown significant clinical success for patients with advanced melanoma and other tumors. Further development of T cell therapy requires improved strategies to select effective, yet non-self-reactive TCRs. Here we isolated ten TCR sequences against four MAGE-C2 (MC2) epitopes from melanoma patients who showed clinical responses following vaccination that were accompanied by significant frequencies of anti-MC2 CD8 T cells in blood and tumor without apparent side effects. We introduced these TCRs into T cells, pre-treated tumor cells of different histological origins with the epigenetic drugs azacytidine and valproate, and tested tumor and self-reactivities of these TCRs.

Pre-treatment of tumor cells up-regulated MC2 gene expression and enhanced recognition by T cells. In contrast, a panel of normal cell types did not express MC2 mRNA, and similar pre-treatment did not result in recognition by MC2-directed T cells. Interestingly, the expression levels of MC2, but not those of CD80, CD86, programmed death-ligand 1 (PD-L1), or PD-L2, correlated with T cell responsiveness. One of the tested TCRs consistently recognized pre-treated, MC2-positive cell lines from melanoma, head-and-neck, bladder and triple-negative breast cancers, but showed no response to MHC-eluted peptides nor peptides highly similar to MC2.

We conclude that targeting MC2 antigen, combined with epigenetic drug-enhanced antigenicity, allow for significant and tumor-selective T cell responses.

INTRODUCTION

In recent years, treatment with T cells gene-engineered with T cell receptors (TCRs) has demonstrated significant clinical responses in patients with metastatic melanoma, colorectal carcinoma, synovial sarcoma and multiple myeloma (1-5). Clinical TCRs tested so far were HLA-A1 or A2-restricted and directed against the melanocyte differentiation antigens Melanoma-Associated Antigen Recognized by T cells (MART)-1, glycoprotein (gp)100, Melanoma-Associated antiGen (MAGE)-A3, New York ESophageal squamous cell carcinoma (NY-ESO)-1, Carcino-Embryonic Antigen (CEA) or p53. Collectively, these trials were performed in a cumulative number of about 80 patients and demonstrated objective responses ranging from 12 to 67% (1-3,6).

The use of TCRs directed against antigens that are expressed on tumor cells but also, albeit at a lower level, on normal cells led in some cases to severe melanocyte destruction in skin and eyes or severe inflammation of the colon (2,3). A lethal event was observed with a patient infused with TCR transduced T cells targeting MART-1/HLA-A2 (EAAGIGILTV-epitope) (7). The accompanying cytokine release syndrome in combination with semi-acute heart failure and an epileptic seizure resulted in the patient's death. In another study, targeting the cancer-germline gene-encoded MAGE-A3/HLA-A2 (KVAELVHFL-epitope) led to neurotoxicity and death of two patients due to the anti-MAGE-A3 TCR recognizing shared and highly similar MAGE-A9 and 12 epitopes (6). Also, targeting MAGE-A3/HLA-A1 (EVDPIGHLY-epitope) led to cardiotoxicity with fatal outcome in two patients (8) due to this TCR recognizing a highly similar peptide from the muscle protein Titin. In the latter two trials the TCRs were affinity-enhanced.

Here we assessed the preclinical value of targeting MAGE-C2 (MC2) antigens with TCRs that were patient-derived and that were not affinity-enhanced. We chose MC2 as a target antigen for TCR gene therapy for several reasons. First, the MC2 antigen belongs to a subfamily of cancer-germline genes encoded by the X chromosome (in more detail described at <http://www.cta.lncc.br>). It is selectively expressed in tumors but not in normal tissue, except for male germline cells (9,10). Second, MC2 expression is found in advanced tumors of different histological origins where it is associated with poor patient survival (9) and may serve as a predictor for sentinel lymph node metastasis (11). Tumor types with significant MC2 expression include metastatic melanomas (about 40% at mRNA and protein levels) (9,11), head and neck squamous cell cancers (about 20% and 10% at mRNA and protein level respectively) (12,13), ER-negative, invasive ductal breast carcinomas (about 30% at protein level) (9,14) or bladder carcinomas (15% at mRNA level) (9). Third, MC2 has been shown to contribute to carcinogenesis by suppressing p53-dependent apoptosis (15) and inducing epithelial-to-mesenchymal transition (14). Finally, one of its most promising features is that MC2-derived antigenic peptides are targeted by T cell responses in a fraction of cancer patients, without detectable toxicity (16).

MC2-derived antigenic peptides include MC2₃₃₆₋₃₄₄ (ALKDVEERV/HLA-A2, ALK/A2) (17); MC2₁₉₁₋₂₀₀ (LLFGLALIEV/A2, LLF/A2) (17); MC2₃₀₇₋₃₁₅ (SESIKKKVL/B44, SES/B44) (18) and MC2₄₂₋₅₀ (ASSTLYLVF/B57, ASS/B57) (19). Even though all 4 peptides have been identified with patient-

derived T cells (20,21), the ALK and LLF epitopes are of particular interest due to their restriction by HLA-A2*0101, the most common MHC class I allele among the Caucasian population. The processing of ALK depends on intermediate or immunoproteasomes whereas that of LLF depends exclusively on intermediate proteasomes (17,22). Exposure to IFN γ leads to the replacement of standard proteasomes by intermediate or immunoproteasomes, and activated intra-tumoral T cells secreting IFN γ are expected to increase the surface expression of these two MC2 epitopes (17,22,23).

In this study, we established a panel of ten TCR $\alpha\beta$ genes from patient-derived CD8-positive anti-MC2 T cell clones. T cell clones were obtained from melanoma patients who experienced significant clinical responses following vaccinations with MAGE-A1 and/or A3 antigens that were accompanied by enhanced frequencies of CD8 MC2-specific T cells in blood, invaded lymph node and regressing cutaneous metastases (16,20,21). We sequence-identified these TCR genes and introduced them into peripheral T cells and evaluated T cell responses towards tumor cell lines of multiple histologies. In addition, we established a pre-treatment regimen consisting of azacytidine (AZA), valproate (VPA) and IFN γ , successfully increasing responses of TCR-transduced T cells towards MC2-positive tumor lines while maintaining non-responsiveness to MC2-negative cell lines. A large panel of normal cell types did not express MC2 mRNA. Notably, one of the tested TCRs consistently recognized pre-treated, MC2-positive tumor cell lines, but showed no response to other peptides, including those that are highly similar to MC2. We conclude that targeting MAGE-C2, together with epigenetic drug treatment of tumor cells, result in safe and effective T cell responses.

MATERIALS AND METHODS

Patient-derived CD8 T cell clones and TCR genes

We acquired a total of eight CD8 T cell clones (which are listed in table I) from two melanoma patients. The first patient EB81 received vaccinations with HLA-A1-restricted MAGE-A3₁₆₈₋₁₇₆(EVD) and MAGE-A1₁₆₁₋₁₆₉(EAD) epitopes. One year after vaccination, cutaneous metastases had disappeared, and patient remained tumor-free for more than 10 years. MC2-specific T cell clones were derived by limiting dilution from co-cultures of blood CD8 T cells and autologous tumor cells. Several anti-MC2 T cell clones were present in blood and tumors at higher frequencies after vaccination (20). In particular, frequencies of the ALK-specific T cell clone 16 increased > 3000-fold in a regressing cutaneous metastasis and comprised 9% of all CD8⁺ T cells within an invaded lymph node (16). A second melanoma patient, LB2586, received vaccinations with autologous, monocyte-derived dendritic cells pulsed with MAGE-A3₁₆₈₋₁₇₆ (EVD)/HLA-A1 and MAGE-A3₂₄₃₋₂₅₈(KKL) /HLA-DP4 peptides. Vaccination led to a mixed clinical response, progressing lesions were surgically removed, and patient showed an overall survival of more than five years. The anti-MC2 T cell clone CTL A, derived as described above, was found at tumor sites with a >1,000-fold higher frequency than in blood. Messenger RNA was obtained from all T cell clones, converted into cDNA, and TCR-V encoding regions were amplified according to one of two methods: (1) PCR using either

Table I. MC2-specific T cell clones obtained from metastatic melanoma patients^a

Epitope	Amino acids (MC2 gene)	Restriction element	Clone name	Clone ID	Ref.
ALKDVEERV	336-344	HLA-A2	EB81-CTL 606 C/22.2 EB81-CTL 721 C/3.13	CTL 16 CTL 40	(16)
LLFGLALIEV	191-200	HLA-A2	EB81-CTL 606 C/19.3 EB81-CTL 606 C/21.7 EB81-CTL 606 A/16.2 EB81-CTL 721 C/3.2	CTL 4 CTL 6 CTL 11 CTL 41	(16)
SESIKKKVL	307-315	HLA-B44	LB2586- CTLA	CTL A	(21)
ASSTLYLVF	42-50	HLA-B57	EB81-CTL C/17.3	CTL 1	(16)

^a T cell clones were obtained from melanoma patients who experienced either a complete and durable response for more than 10 years following vaccination with MAGE-A1 and A3 (EB81 T cell clones) or a mixed response and an overall survival of more than five years following several injections of dendritic cells pulsed with two MAGE-A3 peptides (LB2586 T cell clone). MC2 T cell clones were derived from co-cultures between peripheral CD8 T cells and autologous tumor cells, followed by limiting dilution. See Materials and Methods for details. (HLA= human leukocyte antigen; CTL = cytotoxic T-lymphocyte)

a set of sense primers covering all TCR-V α or β segments according to the ImMunoGeneTics (IMGT) database or (2) 5' RACE PCR primers (SMARTer™ kit Clontech Laboratories, Inc., Mountain View, USA), in both cases combined with TCR-C α or β antisense consensus primers. The 5' RACE PCR was optimized to recover PCR products from low T cell numbers (~10,000 T cells). Initial products from either method were re-amplified by nested PCRs and subsequently cloned into the TOPO 2.1 vector (Invitrogen) and subjected to DNA sequencing. TCR α and β sequences were verified in at least 5 different colonies. Using the IMGT database and the HighV-QUEST tool

(<http://www.imgt.org>), the TCR V, D and J sequences were classified according to the Lefranc nomenclature. Next, TCR α - and β genes were codon optimized (GeneArt, Regensburg, Germany) and cloned into the pMP71 vector (a kind gift of Prof. Wolfgang Uckert, MDC, Berlin) using a TCR β -2A-TCR α cassette via *NotI/MluI* (TCR β genes) and *MfeI/EcoRI* (TCR α genes).

PBMC, packaging cells and reagents

PBMC from healthy human donors were isolated by centrifugation through Ficoll-Isopaque (density=1.077 g/cm³; Amersham Pharmacia Biotech, Uppsala, Sweden). T cells were cultured in RPMI 1640 medium supplemented with 25mM HEPES, 200mM L-glutamine, 6% human serum (Sanquin, Amsterdam, The Netherlands), antibiotics ('RPMI complete') and 360 U/ml recombinant human interleukin-2 (IL-2) (Proleukin; Chiron, Amsterdam, The Netherlands), and stimulated every 2 weeks with a mixture of irradiated allogeneic feeder cells as described elsewhere (24). Packaging cells 293T and Phoenix-Ampho were cultured in DMEM supplemented with L-glutamine, 10% FBS, nonessential amino acids, and antibiotics (DMEM complete).

Additional reagents included the HLA-A2-binding peptides MC2₃₃₆₋₃₄₄ (ALKDVEERV), MC2₁₉₁₋₂₀₀ (LLFGLALIEV), MAGE-B4 (MB4)₁₆₀₋₁₆₉(LVFGLALKEV), MAGE-B10 (MB10)₁₆₂₋₁₇₁ (LIFGLDLKEV) (all from Eurogentec, Maastricht, The Netherlands); a library of 114 known HLA-A2-restricted self-peptides, previously described here: (6,25) (a kind gift by Dr. Matthias Obenaus, MDC, Berlin, Germany; originally established by Stefan Stevanovic and Hans-Georg Rammensee, University of Tübingen, Tübingen, Germany), human IFN γ (Peprotech, Rocky Hill, NJ, USA), 5'-azacytidine (AZA; Sigma Aldrich, St. Louis, USA), and valproic acid (VPA; Sigma Aldrich). For flow cytometry, the following monoclonal antibodies and peptide:MHC (pMHC) reagents were used: TCR-V β 28-FITC (Beckman Coulter, Marseille, France); CD3-PerCP; CD8-APC; CD107a-PE; BB7.2-PE (all BD Biosciences, San Jose, CA); and PE-labeled pMHC multimers (dextramers; Immudex, København, Denmark).

T cell transduction

PBMC of healthy donors were activated with anti-CD3 mAb OKT3 and transduced with TCR-encoding retroviruses (TCR T cells) or empty retroviruses (mock T cells) that were produced by a co-culture of 293T and Phoenix-Ampho packaging cells as described earlier (26,27).

Tumor cell lines and pre-treatment regimens

Melanoma, head-and-neck squamous cell carcinoma (HNSCC) and bladder carcinoma cell lines were cultured in DMEM complete, while triple-negative-breast-cancer (TNBC) and esophageal cancer cell lines were cultured in RPMI 1640, L-glutamine, 10% FBS, and antibiotics. Melanoma cell lines: EB81-MEL derived from patient EB81 (MC2⁺ (determined by PCR), HLA-A2⁺ (A2⁺,determined

by PCR and flow cytometry); MEL78 (MC2⁺, A2⁺); MEL624 (MC2⁺, A2⁺); 518-A2 (MC2⁺, A2⁺); 607-B (MC2⁺, A2⁺); and MZ2-MEL (MC2⁻, HLA-A2⁻). HNSCC cell lines: SCC9 (MC2⁺, A2⁺); SCC38 (MC2⁺, A2⁺); 93VU120 (MC2⁺, A2⁺); and SCC14C (MC2⁻, A2⁻) (kind gifts by Profs. Ruud Brakenhof, VUMC, Amsterdam and Tom Ottenhof, LUMC, Leiden). TNBC cell lines: MDA-MB157 (MC2⁺, A2⁺); Sum159PT (MC2⁺, A2⁺); Sum225CWN (MC2⁺, A2⁺); and HCC1806 (MC2⁺, A2⁺). Bladder carcinoma cell lines: 1207 (MC2⁺, A2⁺); J82 (MC2⁺, A2⁺); and T24 (MC2⁺, A2⁺). Oesophageal carcinoma cell line: OEC-19 (MC2⁻, MB4⁺, A2⁺). Tumor cells were, prior to their use in T cells assays, either not treated, treated with human recombinant IFN γ (50 pg/ml for 48h) or treated with a combination of AZA (1 μ M for 72h), VPA (1mM for 48h following AZA treatment) and IFN γ (50pg/ml for 48h simultaneously with VPA).

Gene expression of MC2 antigen and T cell co-signalling ligands

Messenger RNA was obtained from tumor cell lines, reverse-transcribed and RT-PCR was performed to assess gene expression levels of MC2, in some cases MB4, HLA-A2, CD80, CD86, programmed death-ligand 1 and 2 (PD-L1, PD-L2) and GAPDH (primer sequences available upon request). PCR products were subjected to gel electrophoresis, after which intensities of products were quantified by densitometry (Quantity One v4.6.7 software), corrected for background noise and standardized for GAPDH intensities. For quantification of MC2 mRNA in healthy human tissues, qPCRs were conducted with a normal human tissue cDNA panel (OriGene Technologies, Rockville, USA) and primers and Taqman probes for MC2 (Gene ID: 51438) and GAPDH (Gene ID: 2597) (Life Technologies, Carlsbad, USA).

T cell IFN γ production

Transduced T cells (6×10^4 /well of a 96-wellsplate) were co-cultured with either T2 cells (LCLxT lymphoblastoid hybrid cell line 0.1743CEM.T2) or tumor cells (2×10^4 /well) in a total volume of 200 μ l of T cell assay medium (RPMI 1640, L-glutamine, 10% FBS, and antibiotics) for 24h at 37°C and 5% CO₂. T2 cells were pulsed with saturating (1 or 10 μ M) or titrated amounts (typically ranging from 1pM to 1 μ M) of MC2, MB4, MB10 peptides (the latter two found by NCBI Blast homology searches), or a panel of different peptides commonly restricted by HLA-A2 (list of contained peptides available upon request). Subsequently, supernatants were harvested and IFN γ levels were determined by standard ELISA (eBioscience, San Diego, USA). For TCR 6, half maximal effective concentrations of MC2 or MB4 peptides (EC₅₀) required for T cell IFN γ production were calculated using trend line approximations ($R^2 \geq 0.96$).

Flow cytometry

Transduced T cells or tumor cells (5×10^5) were washed with PBS and incubated with antibodies at 4°C or with pMHC multimers at 37°C for 30min. Following staining, cells were washed with PBS and fixed with 1% paraformaldehyde. Events were acquired on a FACS Canto flow cytometer and analysed using FCS Express 4 software (BD Biosciences).

For CD107a stainings, T cells (2×10^5 /well of 96-wellsplate) were co-cultured with either T2 cells (2×10^4 /well) or tumor cells (4×10^4 /well) in a total volume of 100µl of T cell assay medium for 2-4h at 37°C and 5% CO₂ in the presence of CD107a-PE antibody. After incubation, cells were harvested, stained with CD3-PerCP and CD8-APC antibodies, measured, and analysed for CD107a expression within CD3, CD8-positive T cells.

For pMHC titrations, cultured T cells were stained with CD8-APC antibody and titrated amounts of PE-labeled pMHC multimers. In order to determine pMHC concentrations required to achieve half maximal binding, results were analysed using GraphPad Prism to determine EC₅₀ values.

Formation of surface-expressed peptide:MHC class I

Assays to determine the stability of MC2 peptides:HLA-A2 complexes were performed as described elsewhere (28). In short, T2 cells (1×10^6 /well of a 96-wellsplate) were incubated and loaded with titrated amounts of peptide in serum-free AIM V medium at 26°C for 14–16h, and then at 37°C for another 2h before staining for HLA-A*0201 surface expression with BB7.2-PE mAb. Events were acquired on a FACS Canto flow cytometer and analyzed using FlowJo software (TreeStar, Ashland, OR). Cells were gated for viability and data are presented as Mean Fluorescence Intensities (MFI) for different peptide concentrations.

Statistical analysis

Responses of TCR T cells towards MC2-positive versus MC2-negative tumor cell lines were corrected for responses of mock T cells and tested using one-tailed, unpaired Student's t-tests. Correlations between T cell IFN γ response and gene expressions of MC2, HLA-A2, and co-stimulatory and co-inhibitory molecules were calculated using Pearson's correlation coefficients. Differences were considered statistically significant when p-values were <0.05.

RESULTS

Patient-derived MC2-specific TCRs mediate T cell recognition of cognate epitopes with varying avidities

We obtained TCR α and β genes from 8 patient-derived, MC2-specific T cell clones from two melanoma patients who experienced durable clinical responses after vaccination therapy

Table II. Sequence identification and gene classification of MC2-specific and patient-derived TCRs^a

Clone	Epitope	HLA-	TCR chain	V-GENE	J (D)-GENE	TCR
EB81-CTL 16	ALKDVEERV	A2	α	Va3*01	Ja3*01	TCR 16 ^b
			β	V β 28*01	J β 2-5*01	
EB81-CTL 40	ALKDVEERV	A2	α	Va13-1*02	Ja3*01	TCR 40
			β	V β 28*01	J β 2-5*01	
EB81-CTL 4	LLFGLALIEV	A2	α	Va12-2*01	Ja31*01	TCR 4
			β	V β 7-9*01	J β 2-1*01	
EB81-CTL 6	LLFGLALIEV	A2	α	Va12-2*01	Ja23*01	TCR 6
			β	V β 15*02	J β 2-3*01	
EB81-CTL 11	LLFGLALIEV	A2	α	Va14/DV4*01	Ja41*01	TCR 11
			β	V β 9*01	J β 2-7*01	
EB81-CTL 41	LLFGLALIEV	A2	α_1	Va8-1*01	Ja28*01	TCR 41 - I
			α_2	Va22*01		TCR 41 - II
			β	V β 9*01	J β 1-2*01	
EB81-CTL 1	ASSTLYLVF	B52	α	Va41*01	Ja54*01	TCR 1
			β	V β 7-2*04	J β 2-7*01	
LB2586-CTL A	SESIKKKVL	B44	α_1	Va12-3*01	Ja13*01	TCR A - I
			α_2	Va9-2*02	Ja37*01	TCR A -II
			β	V β 19*01	J β 1-4*01 (D β 1*01)	

^a cDNAs derived from T cell clones were PCR amplified using either a set of TCR-V α or V β sense primers and a corresponding TCR-C α or C β antisense primer or 5'RACE (rapid amplification of cDNA ends). Following nested PCRs and cloning, TCR α and β sequences were identified using www.imgt.org and classified according to the Lefranc nomenclature. See Materials and Methods for details. (HLA= human leukocyte antigen; CTL = cytotoxic T-lymphocyte; TCR = T cell receptor; V= variable gene segment; J= joining gene segment; D= diversity gene segment).

^b underlined TCRs were surface expressed and demonstrated binding to pMHC complexes upon gene transfer into primary human T cells

significantly bound cognate pMHC multimers (see supplementary figure 1). Transductions with TCRs 40, 41-I and 41-II yielded populations of T cells that showed no or very limited staining with

(16,20,21). T cell clones were directed against the ALK/A2, LLF/A2, ASS/B57 or SES/B44 epitopes (see table I), and with use of TCR-V-specific PCR techniques we obtained 10 pairs of TCR α - and β sequences (see table II). Two clones (Cytotoxic T lymphocyte (CTL) 41 and CTL A) expressed two rearranged TCR α sequences, most likely as a result of incomplete allelic exclusion. T cells from healthy donors were transduced with pairs of TCR α and β genes and assessed for TCR surface expression and binding of pMHC multimers by flow cytometry. Focussing on HLA-A2-restricted TCRs, we observed that transductions with TCR 16 (ALK) as well as TCR 4, 6 and 11 (LLF) yielded populations of T cells that

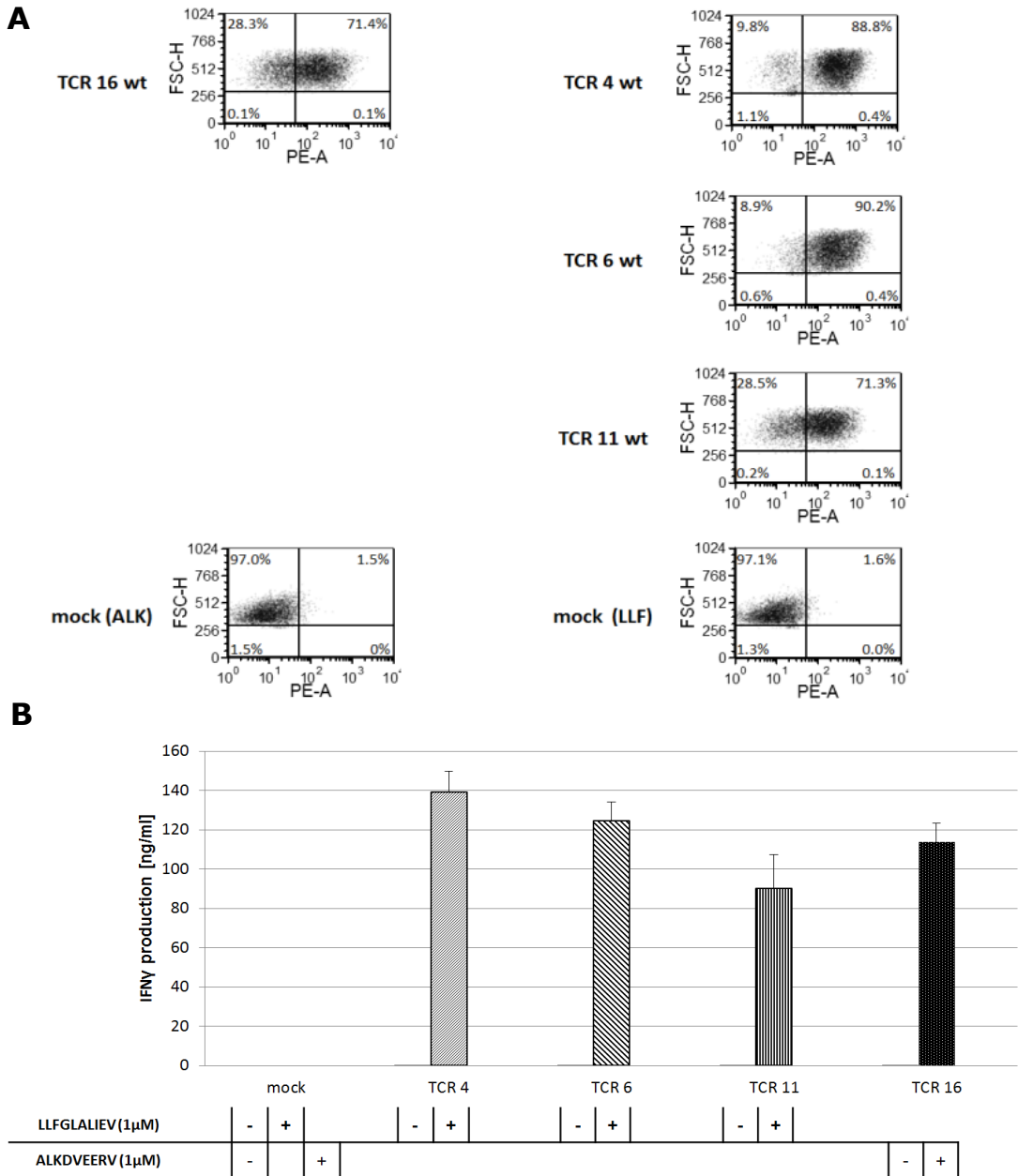


Figure 1. MC2 TCR T cells bind pMHC and produce IFN γ in response to cognate epitope.

T cells were transduced with anti-MC2 TCRs 4, 6, 11, 16 or not (mock) and FACSsorted with the corresponding pMHC multimers. **(A)** TCR-transduced and sorted T cell populations were labeled with ALK:A2-PE (TCR 16) or LLF:A2-PE (TCR 4, TCR 6, TCR11). T cells were gated for live cells and dot plots are representative of 5 different experiments in two different donors. Percentages in upper right quadrants represent fractions of T cells stained by pMHC corrected for background stainings of corresponding mock T cells. **(B)** T cells from (A) were co-cultured with T2 cells pulsed with 1 μ M of ALK (for TCR 16) or LLF (for TCR 4, 6 and 11) peptide for 24h. IFN γ levels in culture supernatants were measured by ELISA, and displayed as mean \pm SEM (n=4 experiments). IFN γ levels

from mock T cells and T cells incubated with unpulsed T2 cells ranged between 3 and 80 pg/ml. Data are from one healthy donor out of two tested with similar results.

pMHC multimers (~1%). This was not the result of compromised TCR gene transductions since TCR-V β 28-antibody staining of T cells transduced with TCR 40 (same V β -chain as TCR 16) revealed clear surface expression of the introduced TCR β chain (data not shown). For subsequent experiments, we FACS-sorted TCR-transduced T cells and obtained T cell populations with a high (>70%) and stable binding of pMHC (figure 1A) with the exception of TCR 11 T cells, which gradually lost pMHC binding capacity over time (data not shown).

To test TCR functions, transduced T cells were co-cultured with T2 cells pulsed with a saturating dose (1 μ M) of ALK (TCR 16) or LLF (TCR 4, 6 and 11) peptides. TCR-transduced but not mock T cells clearly produced IFN γ upon stimulation with peptide-loaded cells, with TCR 11 T cells producing lower amounts of IFN γ than T cells transduced with the other TCRs (figure 1B). With respect to T cell degranulation, findings were similar and TCR (but not mock) T cells up-regulated surface expression of CD107a upon stimulation with peptide-loaded cells. Again, T cells expressing TCR11 were the least responsive population (see supplementary figure 2A).

In addition to an initial test of T cell function, we have conducted pMHC titrations as a measure of T cell avidity. These studies demonstrated that TCRs can be ranked from low to high EC₅₀ to bind pMHC by CD8 T cells, as follows: TCR4<TCR6<TCR11/16 (figure 2A). When analyzing CD4 T cell populations, findings were similar but demonstrated that TCR11 and in particular TCR16 showed a significant drop in their abilities to bind pMHC (figure 2B). The observed hierarchy in T cell avidity, and in particular the CD8 dependence of TCR16, corresponded to the abilities of these T cells to up-regulate CD107a expression (see supplementary figure 2A).

Pre-treating melanoma cells with epigenetic drugs and IFN γ increases MC2 gene expression and T cell stimulation

Next, we tested T cell reactivities towards MC2-positive melanoma cell lines. As ALK and LLF peptides require processing by immune (ALK) or intermediate (ALK and LLF) proteasomes rather than standard proteasomes (22), we pre-treated melanoma cells with IFN γ to induce the expression of immune proteasome catalytic subunits. TCR T cells did not produce levels of IFN γ that exceed those of mock T cells when stimulated with untreated melanoma cells and exhibited weak to moderate IFN γ production after stimulation by IFN γ -pre-treated melanoma cells, in particular the EB81-MEL and 607B cell lines (figures 3A and B). In order to enhance the level of MC2 T cell responses, we resorted to an additional treatment with azacytidine (AZA) and valproate (VPA) for its reported effect on the expression of MAGE antigens in cancer cells and their recognition by T cells in preclinical and clinical studies (29-35). AZA is a DNA methyl transferase inhibitor that allows expression of genes that are silenced through the demethylation of CpG

islands in promotor regions. VPA is a histone deacetylase inhibitor that blocks histone deacetylation, thereby relieving chromatin condensation and increasing gene expression.

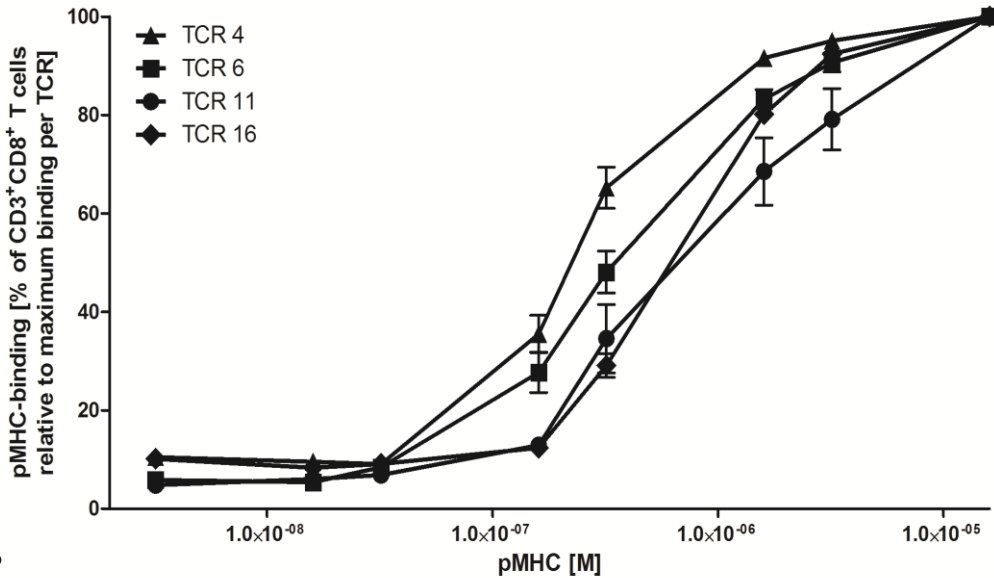
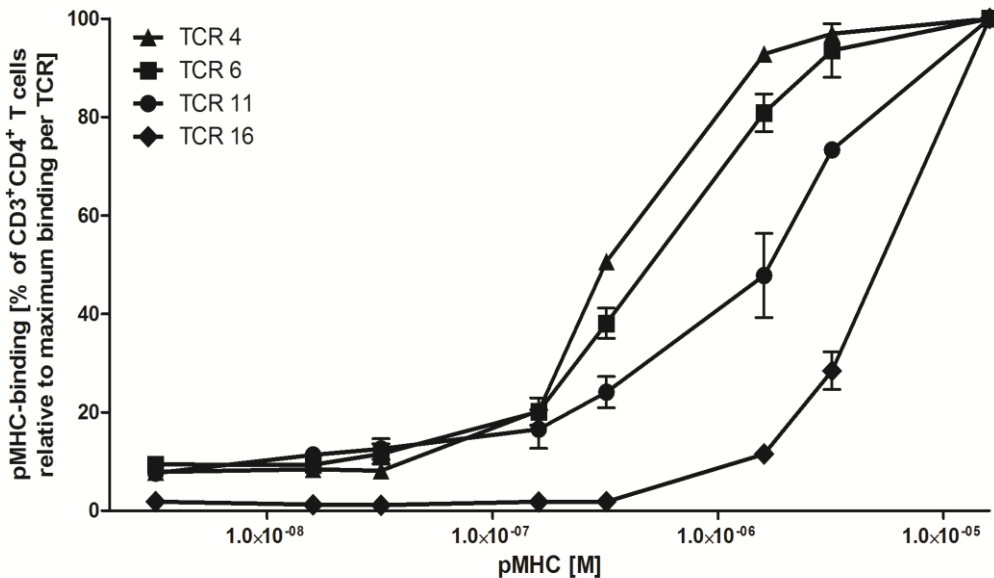
A**B**

Figure 2. MC2 TCRs provide T cells with different avidities for MC2 peptide:HLA-A2 complexes

For pMHC multimer titrations, MC2 TCR T cells were stained with either CD8-APC (**A**) or CD4-FITC (**B**) antibody and different concentrations of ALK:A2-PE (TCR 16) or LLF:A2-PE (TCR 4, TCR 6, TCR11). Maximal percentages of T cells that bound pMHC multimer were set at 100% and resulting curves and EC₅₀ values were analyzed using GraphPad Prism (CD8: TCR 4 = 2.26x10⁻⁷M, TCR 6 = 3.48x10⁻⁷M, TCR 11 = 6.67x10⁻⁷M, TCR 16 = 6.63x10⁻⁷M;

CD4: TCR 4 = $3.16 \times 10^{-7} M$, TCR 6 = $5.01 \times 10^{-7} M$, TCR 11 = $1.7 \times 10^{-6} M$, EC_{50} for TCR 16 could not be determined). Data are displayed as mean \pm SEM, n=3.

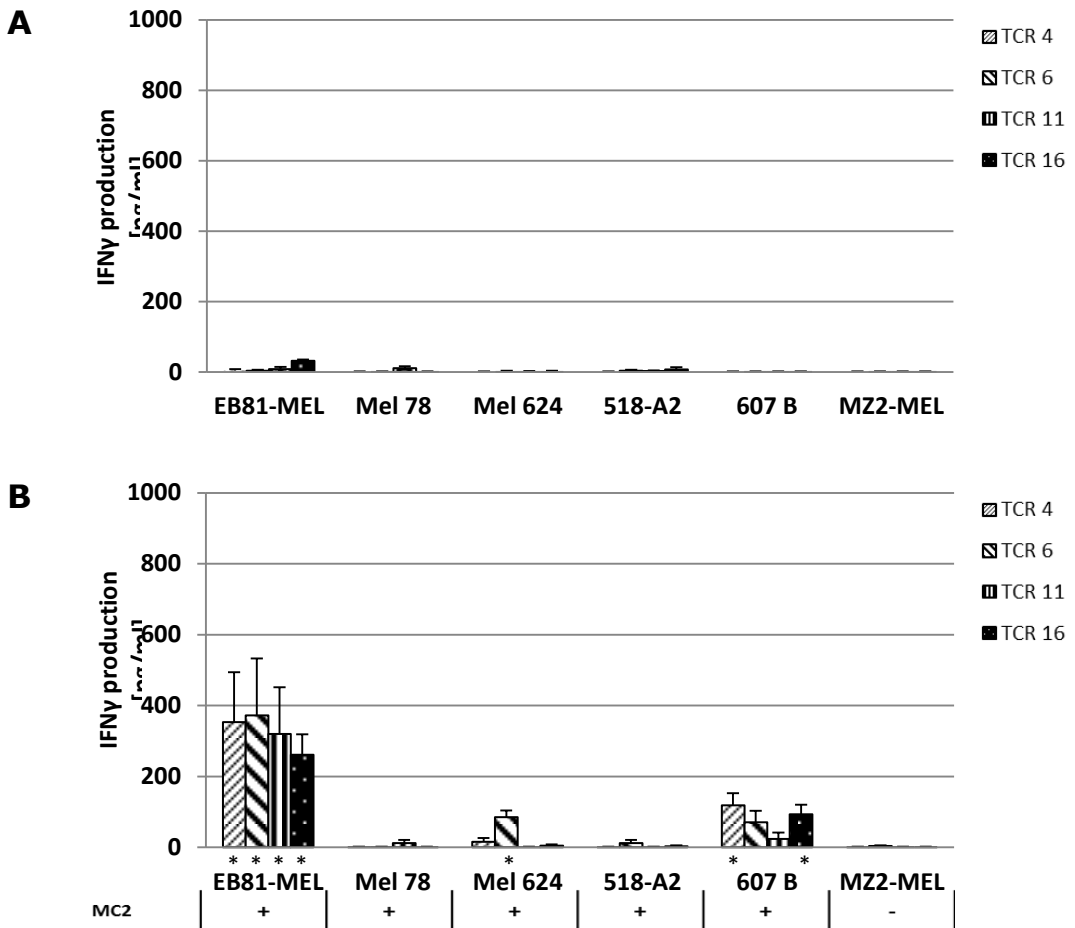


Figure 3. T cell recognition of MC2-positive melanoma cells is limited.

MC2 TCR T cells were co-cultured with tumor cells that were either positive or negative for MC2 (based on RT-PCR data, indicated as '+' or '-') at an E:T ratio of 3:1 for 24h. Melanoma cells were either untreated (A) or pre-treated with human IFN γ (50pg/ml for 48h) (B). Levels of IFN γ produced by T cells in culture supernatants were measured by ELISA, and are displayed as means \pm SEM (n=3). IFN γ production by MC2 TCR T cells was corrected for IFN γ production by mock T cells (same donor and tumor cell line); IFN γ levels produced by mock T cells ranged between 10 and 95pg/ml. Data are from one healthy donor out of two tested with similar results. Statistical significant differences between responses of TCR T cells towards MC2⁺ versus MC2⁻ target cells are calculated with Student's t-test: *p<0.05 (below bars).

Timing and dosage of AZA and VPA are based on the work of Goodyear and colleagues (29). We tested a combination of IFN γ , AZA and VPA on 6 melanoma lines, the non-cancerous keratinocyte cell line HaCaT and primary fibroblasts. Figure 4A shows that upon treatment with AZA/VPA/IFN γ , MC2 gene expression is up-regulated in 4 out of 6 tumor cell lines, but notably not in the non-

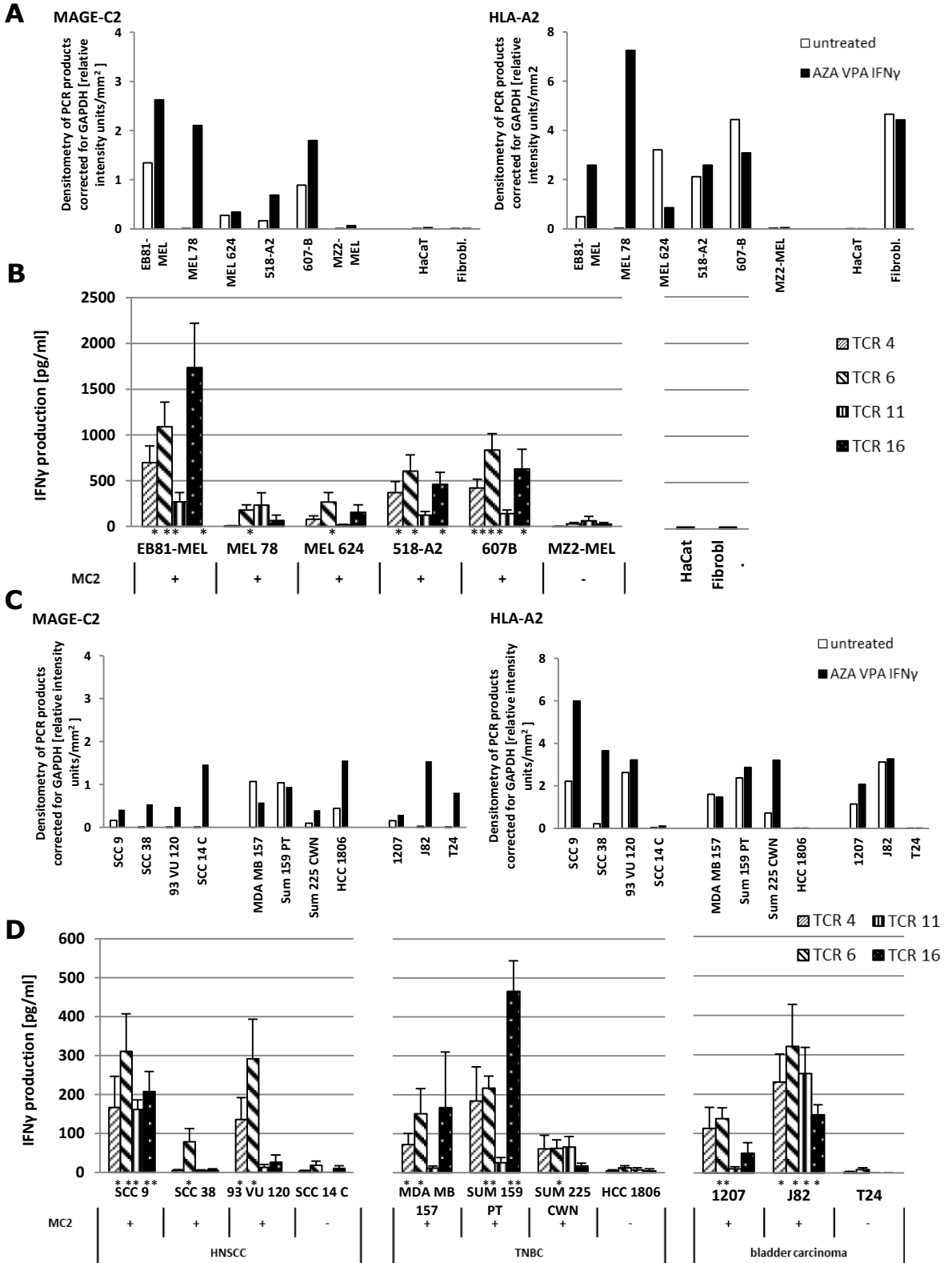
cancerous cells. Treatment variably regulates gene expression of HLA-A2, which is only up-regulated in 2 out of 6 melanoma lines. In addition, we assessed MC2 protein expression in pre-treated tumor cells via flow cytometry using the antibody LX CT10.9 that has previously been validated for immune histochemistry (36). Stainings confirmed PCR data and demonstrated up-regulated MC2 protein expression following AZA/VPA/IFN γ treatment (data not shown). Finally, melanoma and normal cells were tested for their ability to stimulate the production of IFN γ or up-regulation of surface CD107a by MC2 TCR T cells (figure 4B and supplementary figure 2B). All tested MC2-positive melanoma lines stimulated two or more of the tested TCRs. These data demonstrate that melanoma cells, but not normal cells, exhibit improved recognition by MC2 TCR T cells following treatment with AZA/VPA/IFN γ .

T cells directed against MC2 show significant responses against head and neck, breast and bladder cancer cells following pre-treatment with AZA/VPA/IFN γ

To extend our results to non-melanoma tumor cell lines, we explored the gene expression of MC2 and HLA-A2 in head and neck squamous cell carcinoma (HNSCC), triple-negative breast cancer (TNBC) and bladder carcinoma cell lines. As shown in Figure 4C, MC2 gene expression was up-regulated in the majority of tumor cell lines following treatment (9 out of 11, with the exception of the TNBC cell lines MDA157MB and Sum159PT), and HLA-A2 gene expression was up-regulated to a more variable extent in tumor cell lines following treatment (6 out of 11, with the exception of the HNSCC cell line SCC 14C; the TNBC cell lines MDA157MB and HCC1806 and the bladder cancer cell lines J82 and T24). Importantly, treatment of HNSCC, TNBC and bladder carcinoma cell lines clearly resulted in an enhancement of IFN γ production (figure 4D) and up-regulation of CD107a (data not shown) by MC2 TCR T cells. All MC2-positive tumor cell lines tested yielded a significant response by at least one of the tested TCRs. As was observed for melanomas, AZA/VPA was required in addition to the IFN γ pre-treatment to maximize MC2-specific T cell responses.

Figure 4. Treatment of melanoma, head-and-neck, bladder and breast carcinoma cells with AZA and VPA enhances MC2 gene expression and T cell recognition.

(A) Melanoma cell lines (n=6), HaCaT keratinocytes, and fibroblasts (Fibrobl.) were pre-treated or not with AZA (1 μ M for 72h), VPA (1mM for 48h) and IFN γ (50pg/ml for 48h). RNA was isolated from 1x10⁶ cells, followed by cDNA synthesis and RT-PCR with primers for detection of MC2, HLA-A2 and GAPDH transcripts. PCR products were subjected to gel electrophoresis, and intensities of MC2 and HLA-A2 bands were quantified by Quantity One (version 4.6.7), corrected for background noise and standardized for GAPDH levels, and displayed in relative intensity units/mm². In (B) MC2 TCR T cells were co-cultured with pre-treated melanoma cells from (A) at an E:T ratio of 3:1 for 24h. IFN γ levels in culture supernatants were measured by ELISA, and displayed as means \pm SEM (n=5). IFN γ production by MC2 TCR T cells was corrected for IFN γ production by mock T cells; IFN γ levels produced by mock T cells ranged between 2 and 300pg/ml. (C) Cell lines from head-and-neck squamous cell carcinoma (HNSCC) (n=4), triple-negative breast carcinoma (TNBC) (n=4) and bladder carcinoma (n=3) were pre-treated as described in (A). RNA was isolated, used for RT-PCR and subjected to analysis of MC2 and HLA-A2 expression. (D) MC2 TCR T cells were co-cultured with pre-treated tumor cell lines from (C) and analysed for IFN γ production as described in (B). IFN γ production of mock T cells ranged between 9 and 180pg/ml. Data are from one healthy donor out of two tested with similar results. Statistical significant differences between responses of TCR T cells towards MC2⁺ versus MC2⁻ target cells are calculated with Student's t-test: *p<0.05; **p<0.005 (below bars).



MC2 T cell responses are governed by expression level of antigen, but not expression of co-stimulatory or co-inhibitory molecules

Even though pre-treatment of target cells elicited enhanced responses of MC2 TCR T cells, such T cell responses were heterogeneous with respect to different TCRs and different tumor target cells. In an effort to better understand this heterogeneity, we first assessed the relationship between individual TCR and T cell IFN γ responses. When analysing the breadth of T cell responses, TCR 6 was the only TCR able to mediate a T cell response against all MC2/A2-positive tumor cell lines tested, irrespective of the tumor type (100% response, n=13 tumor cell lines), whereas TCR 4 and 16 mediated T cell responses against 50% of these target cells, and TCR 11 only against 15% of them (figure 5A). Notably, TCR 6 mediated T cell responses against all four tumor types, yet TCR 4 and 16 demonstrated a preference towards melanomas and TCR 11 against HNSCCs and bladder carcinomas (figure 5A). In addition to the breadth of response, we also analysed the quantities of IFN γ produced, which again were highest for T cells expressing TCR 6 (median IFN γ production=267pg/ml), lower for T cells expressing TCR 16 and TCR 4 (156 and 136pg/ml, respectively), and lowest for T cells expressing TCR 11 (97pg/ml) (figure 5B). Interestingly, the

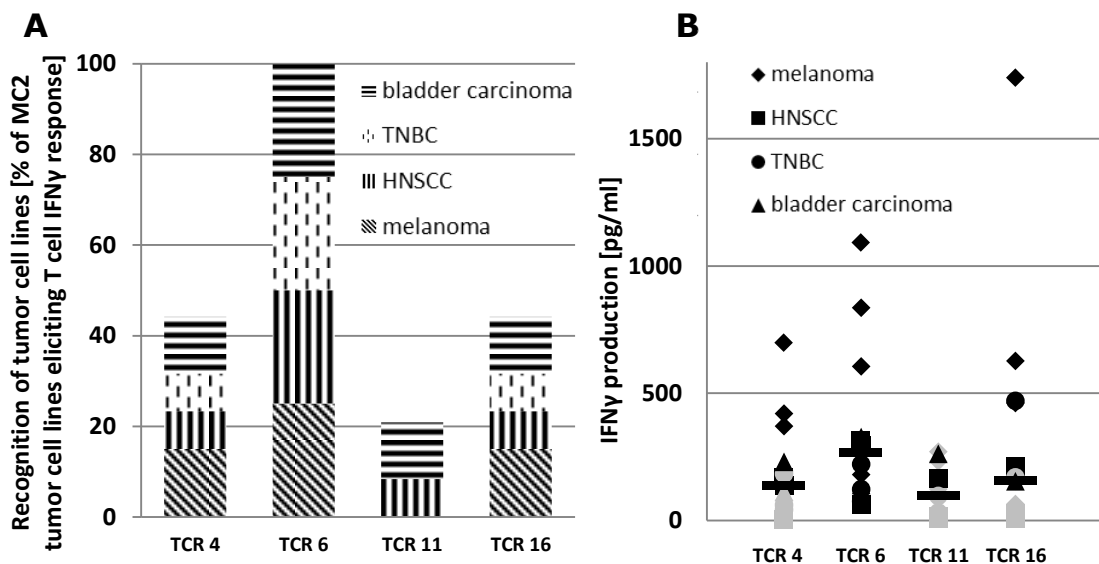


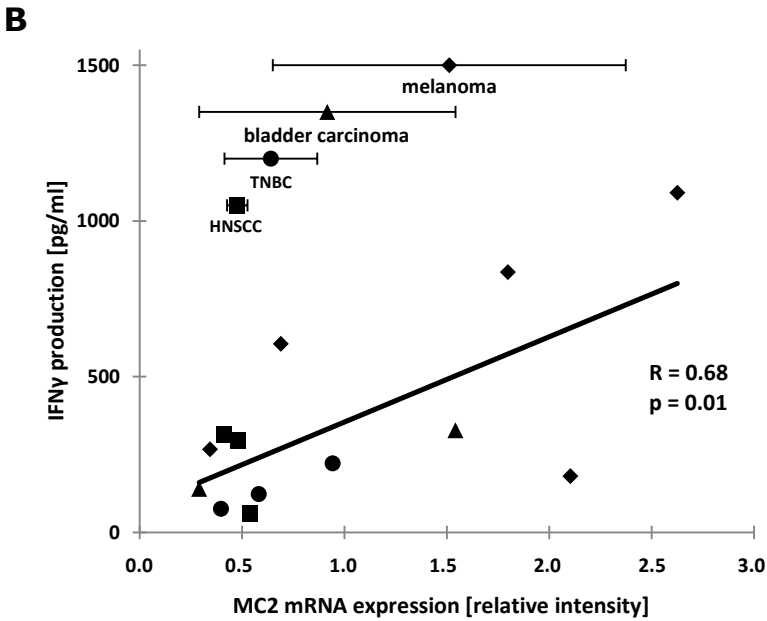
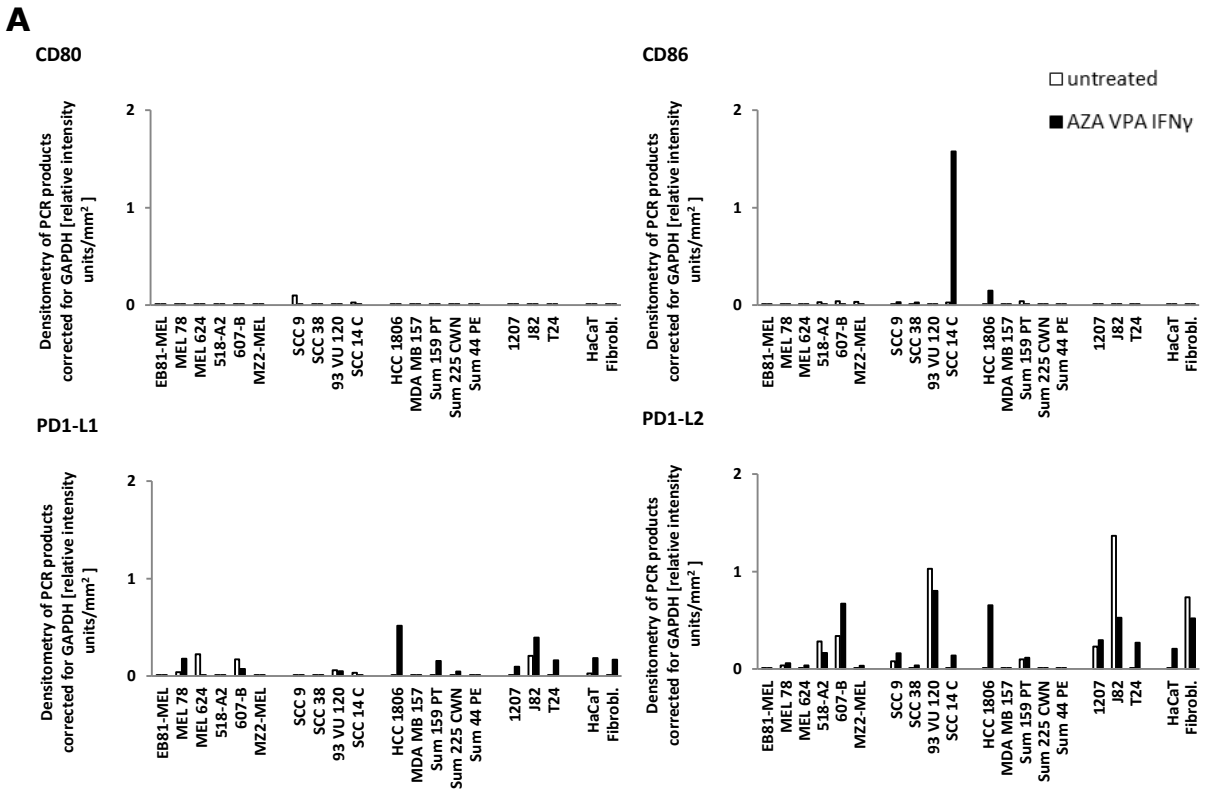
Figure 5. MC2 TCR 6 performs best with respect to tumor cell recognition.

Breadth (A) and intensity (B) of T cell responses of each TCR were quantified based on the results shown in figures 4A and 4D. Panel (A) shows the proportions of tumor lines, out of a total of 13, that resulted in significant IFN γ production by TCR-transduced T cells; maximal contribution by each individual tumor type was set to 25% (melanoma: 5 cell lines; HNSCC: 3 cell lines; TNBC: 3 cell lines; bladder carcinoma: 2 cell lines). Panel (B) shows the levels of IFN γ produced by TCR-transduced T cells following co-culture with tumor lines ($p < 0.05$ =black; $p > 0.05$ =grey). The median production per TCR is indicated as a black bar (n=5 experiments; 13 tumor lines per experiment).

highest quantities of IFN γ were found with melanoma target cells, followed by bladder carcinoma cell lines and then followed by HNSCC and TNBC lines. Collectively, these findings favour TCR 6, a TCR that provides T cells with intermediate to high avidity (figure 2), with respect to MC2-specific anti-tumor T cell responses. Secondly, we assessed the impact of the expression of genes coding for surface molecules critical for T cell stimulation, such as the MC2 antigen, HLA-A2, the co-stimulatory molecules CD80 and CD86, and the co-inhibitory molecules PD-L1 and PD-L2. CD80 and CD86, which were not or only negligibly expressed in tumor or normal cell lines, showed no change following AZA/VPA/IFN γ pre-treatment, whereas expressions of PD-L1 and PD-L2 were increased in some cell lines following pre-treatment (see figure 6A). This argues that AZA/VPA/IFN γ treatment does not generally enhance gene expression, but that the effect is restricted to genes such as the cancer germline gene MC2. Testing whether T cell responses depended on the expression levels of any of these genes, a significant correlation was only observed with expression levels of MC2 and not with any of the other investigated molecules (see figure 6B, shown for TCR 6 T cells, but observed for all TCRs, not shown). When categorizing tumor types according to the levels of MC2 expression, it appears that melanoma cells expressed the highest levels, followed sequentially by bladder carcinoma, TNBC and HNSCC (figure 6B). This result most likely explains the earlier observation that melanoma cells stimulated the highest T cell responses and warrants the use of pre-treatment of tumor cells to enhance MC2-specific T cell responses.

TCR 6 shows no on-target or off-target toxicity in vitro

To exclude possible on- and off-target toxicities, thereby enhancing the potential clinical value of MC2 TCRs, we have conducted a series of assays. First, qPCRs were performed for MC2 gene expression using a library of 48 human tissues, and revealed that the only tissue positive for MC2 gene expression was testis (figure 7A) where expression was 51-fold lower than within our positive control, the EB81-MEL cell line. Spiking experiments demonstrated a sensitivity of these MC2-specific qPCRs of 1 MC2-positive cell in 10^3 MC2-negative cells. Second, T cells transduced with TCR 6, the TCR that scored best according to our in vitro experiments, were co-cultured with T2 cells loaded with HLA-A2-eluted self-peptides. These T cell stimulations demonstrated no cross-recognition of TCR 6 against any of these peptides, with the only noticeable recognition being the one against the MC2 peptide (figure 7B). Thirdly, TCR 6-transduced T cells were co-cultured with the MAGE-B4 (MB4) peptide LVFGLALKEV and the MAGE-B10 (MB10) peptide LIFGLDLKEV that are both highly homologous to the MC2 peptide LLFGLALIEV according to NCBI blast searches (amino acids different from the MC LLF peptide being underlined). TCR 6 T cells were found to show some response to MB4 peptide, about 15% of the response seen against MC2 LLF peptide, but not to the MB10 peptide. Further titrations with MB4 peptide revealed that this peptide shows a higher EC₅₀



Gene	Effect of AZA/VPA	Correlation to T cell response
MC2	+++	0.68 (p=0.01)
HLA-A2	++	-0.11
CD80	-	0.00
CD86	-	-0.22
PD-L1	-/+	-0.13
PD-L2	-/+	-0.20

Figure 6. Expression of MC2 antigen, but not HLA-A2, co-stimulatory or co-inhibitory molecules, correlates with T cell response.

Cell lines from Melanoma, HNSCC, TNBC, bladder carcinoma and keratinocytes (HaCaT) and fibroblasts (Fibrobl.) were pre-treated or not with AZA, VPA and human IFN γ as described in legend to figure 4. In **(A)** RNA was isolated, used for RT-PCR and subjected to analysis of CD80, CD86, PD-L1, PD-L2 expression as described in legend to figure 4A. In **(B)** IFN γ response of TCR 6-transduced T cells is presented as a function of MC2 expression of pre-treated tumor cell lines of different histologies (left side). Ranges of MC2 expression levels per tumor type are indicated above the trend line (mean \pm SEM, n=4). In addition, IFN γ response of TCR 6-transduced T cells is presented as a function of either HLA-A2, CD80, CD86, PD-L1 or PD-L2 expression of pre-treated tumor cell lines (right-side). Calculations are based on Pearson's correlation coefficient. +++ = very strong effect (>75% of tested cell lines), ++ = strong effect (>50% of tested cell lines), -/+ = low effect (>5% of tested cell lines), - = no effect (<5% of tested cell lines).

value than the MC2 LLF peptide. Importantly, TCR 6-transduced T cells did not recognize target cells, such as the oesophageal cancer cell line OEC-19, expressing native MB4 (figures 7C, D and E). Collectively, these results provide clear evidence against risks for on- or off-target reactivities when targeting MC2 via TCR 6.

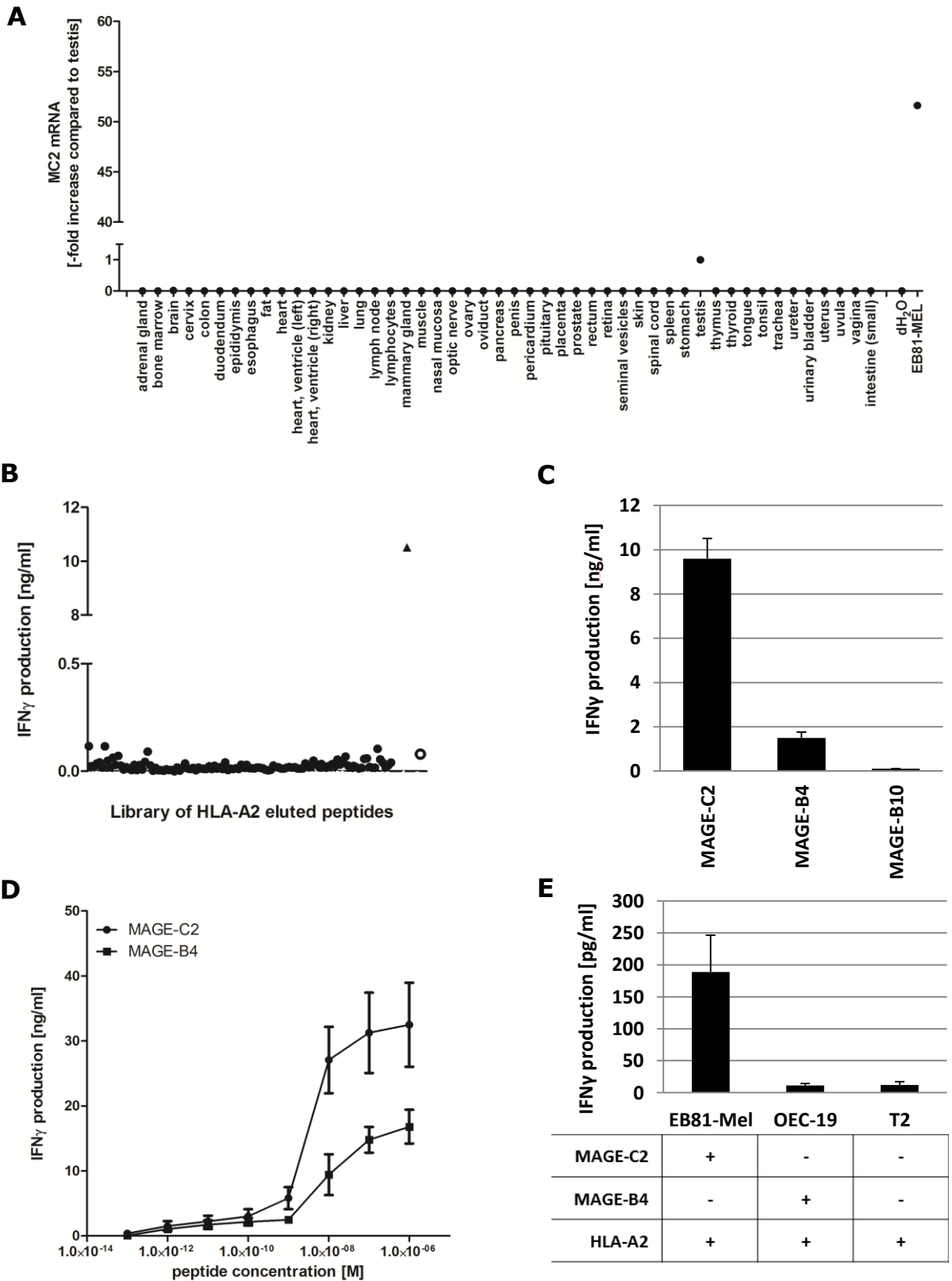


Figure 7. MC2 expression in healthy tissue is restricted to testis, and TCR 6 shows no reactivity to other, including highly similar epitopes.

(A) MC2-specific qPCR was performed using a cDNA library of 48 healthy, human tissue samples. MC2 mRNA levels are expressed as -fold increase compared to testis, the only healthy sample positive for MC2 mRNA. Fold increase was calculated based on the $\Delta\Delta C_t$ method and normalized for GAPDH expression. Patient-derived MC2-positive melanoma cell line EB81-MEL served as positive control. (B) TCR 6 T cells were co-cultured with T2 cells loaded with $10\mu\text{M}$ of 114 different HLA-A2-restricted peptides for 24h. IFN γ levels in culture supernatants were measured by ELISA, and displayed as mean (n=3). IFN γ response against the MC2 LLF peptide (positive control) is depicted as a triangle, and against no peptide (T2 cells only; negative control) is depicted as a hollow circle. (C) TCR 6 T cells were co-cultured with T2 cells loaded with 100nM of highly similar peptides of MB4 (LVFGLALKEV) and MB10 (LIFGLDLKEV) (amino acids different from the MC LLF peptide being underlined). IFN γ levels in culture supernatants were measured by ELISA, and are displayed as mean \pm SEM (n=4). IFN γ response against the MC2 LLF peptide served as a positive control. (D) Again, TCR 6 T cells were co-cultured with T2 cells but now with titrated amounts of MB4 and MC2 LLF peptide for 24h. IFN γ levels in culture supernatants were measured by ELISA, and displayed as mean \pm SEM, (n=4). Curves and EC $_{50}$ values were analyzed using GraphPad Prism (EC $_{50}$ for MC2: $3.1 \times 10^{-9}\text{M}$; EC $_{50}$ for MB4: $7.15 \times 10^{-9}\text{M}$). (E) Finally, TCR 6 T cells were co-cultured with either EB81-MEL (MC2 $^+$ /MB4 $^+$ /A2 $^+$), OEC-19 (MC2 $^+$ /MB4 $^+$ /A2 $^+$) or T2 (MC2 $^+$ /MB4 $^+$ /A2 $^+$) tumor cells. Expression of MC2, MB4 and HLA-A2 was verified or ruled out via RT-PCR. IFN γ levels in culture supernatants were measured after 24h by ELISA, and are displayed as mean \pm SEM (n=4). IFN γ production by TCR 6 T cells was corrected for IFN γ production by mock T cells; IFN γ levels produced by mock T cells ranged between 5 and 13pg/ml.

DISCUSSION

In this study, we tested the preclinical value of targeting MC2 epitopes with patient-derived and non-affinity-enhanced TCRs, in particular TCRs specific for the HLA-A2-restricted epitopes ALK and LLF. When studying HLA-A2-restricted TCRs, we observed that 3 TCRs (out of 7) did not enable transduced T cells to bind pMHC. T cells expressing the other 4 TCRs (TCR 4, 6, 11 and 16) were used to determine EC_{50} values for their binding of pMHC-multimers (figure 2). These TCRs yielded T cells with varying T cell avidities towards pMHC, and when analyzing CD8 T cells and in particular CD4 T cells, we observed that TCR 16 and 11 mediated the weakest, TCR 6 an intermediate, and TCR 4 the strongest binding of pMHC. The observed variation among TCRs, however, is in line with reported T cell avidities of the parental CTL clones obtained from patient EB81 (17). In example, CTL 16 as well as our TCR 16-transduced T cells exhibited lowest avidity amongst the tested CD8 T cell clones, while this CTL clone was present at the highest frequencies in patient blood and an invaded lymph node (16). This suggests that high affinity of a TCR is not a pre-requisite for effective *in vivo* stimulation by naturally presented tumor antigens (37,38). Other reasons for differences of pMHC binding, apart from TCR affinity, may include that certain TCRs show enhanced proneness to mispair with endogenous TCR chains, which results in dilution of surface TCR expression (39), and that certain TCRs are less dominant with respect to CD3 assembly and surface expression (40). To minimize the occurrence of TCR mis-pairing, we have introduced TCRs into the pMP71 vector using a TCR β -2A-TCR α cassette. Nevertheless, TCR mis-pairing cannot be completely excluded. Although not a primary topic of the current manuscript, we have explored additional strategies to limit TCR mis-pairing and showed that incorporation of a CD3 ζ -CD28 cassette (41) into TCR 16 maximally enhances the fraction of pMHC-binding T cells (data not shown).

Within the panel of LLF-specific TCRs, our results suggest that TCR 11 has the lowest ligand-binding affinity, which is substantiated by two other lines of evidence. First, this TCR mediates the lowest level of IFN γ production in response to MC2/A2-positive tumor cell lines (see figure 6A/B), and secondly, this TCR mediates the lowest rate of proliferation as well as most rapid drop in numbers of TCR-positive T cells during culture of T cells (data not shown). In addition, TCR 11-transduced T cells have a lowered ability to produce high levels (i.e., plateau levels) of peptide-specific IFN γ when compared to TCR 4 or 6-transduced T cells (Figure 1B). TCR 11's lower level of performance may be related to a lesser ability to serially trigger T cells (42) or to an enhanced ability to up-regulate expression of T cell PD1 (43), although we could not confirm the latter option experimentally (data not shown).

When testing MC2 TCRs towards melanoma tumor targets, whether or not pre-treated with IFN γ to stimulate immune and intermediate proteasome-dependent processing of the ALK and LLF epitopes (17,22), we observed limited T cell responses (figure 3; and (26)). Pre-treatment of melanoma cells was extended with the epigenetic drugs AZA and VPA, which resulted in selective gene and protein expression of MC2 in tumor cells, but not normal cells, and enhanced T-cell

responsiveness. Importantly, we observed similar findings in multiple tumor types, including HNSCC, TNBC and bladder carcinoma. These results extend earlier studies that showed enhanced gene expression of NY-ESO1, MAGE-A1 and MAGE-A3 in cancer cell lines pre-treated with epigenetic drugs, but not in normal respiratory epithelia or lymphocytes (31,33,44). Interestingly, in our experiments different tumor cells responded differently to pre-treatment. For example, melanoma cell lines revealed a base level of MC2 expression that was further enhanced by AZA/VPA, whereas HNSCC cell lines only expressed MC2 following pre-treatment (figure 4C). Since earlier studies reported a MC2-positivity of only 8% of primary HNSCC (13), the above finding highlights the potential to increase patient populations eligible for MC2-specific T cell therapy when combined with epigenetic drug pre-treatment. Moreover, there is a timely notion that epigenetic drugs sensitize tumors to T cells, which goes beyond enhanced gene expression of target antigens (45,46). In example, epigenetic drugs have been reported to induce interferon type I gene and related genes, which can contribute to tumor immunogenicity (47), and these drugs are able to enhance expression of chemoattractants, increase CD8 T cell infiltration, and improve the therapeutic efficacy of adoptive T cell therapy (48).

Our results with respect to drug-enhanced expression of MC2 by tumor cells are of particular interest to clinical trials with adoptive transfer of T cells since AZA and VPA already have been shown to be safe in patients with advanced hematological and solid malignancies (29,49-53). The selective effects of pre-treatment towards cancer cells versus normal cells may be related to an initiating event in the de-regulation of cancer-germline gene expression, which is the recruitment of the transcription factor BORIS (brother of the regulator of imprinted sites) to cancer-germline promoters. De-repression of NY-ESO-1, MAGE-A1, and MAGE-A3 coincides with DNA demethylation, dissociation of polycomb proteins, and presence of euchromatin marks within the respective promoters, a.o., a shift to BORIS at the cancer-germline promotor site (44). BORIS is a mammalian CCCTC-binding factor (CTCF)-paralog that is absent in normal cells, which prevents binding and function of CTCF and results in the opening and activation of DNA chromatin (54,55).

Pre-treatment of tumor cells results in significantly enhanced responses of MC2 TCR T cells, although such responses are heterogeneous. Attempting to define parameters that are critical for MC2 T cell responses, we demonstrated that MC2-specific T cell responses are most optimal in vitro with TCR 6, and benefit most from higher expression levels of MC2, but not CD80, CD86, PD-L1 or PD-L2. The superiority of TCR 6 may in part be explained by affinity, considering the possible higher affinity of TCR 4 and its weaker T cell response, it may also in part be explained by TCR structure and level of TCR expression (40). The importance of antigen expression level for MC2 TCR T cell responses builds on earlier studies (56), but antigen being a driver of MC2 T cell recognition irrespective of the expression of classical co-stimulatory and co-inhibitory molecules is a novel finding. Notably, the LLF epitope, the target of TCR 11, TCR 4 and in particular TCR 6, forms highly stable peptide:HLA-A2 complexes (see supplementary figure 3), potentially due to the LLF peptide harboring the preferred peptide-binding motif for HLA-A2, i.e., "XLXXXXXXV/L" (57,58). The EC₅₀ values for HLA-A2 binding put the LLF epitope, but not the ALK epitope, below a

critical threshold of 10nM, which has been reported to enable peptide cross-presentation by stromal cells and enhance anti-tumor T cell responses (59).

In this study we have selected MC2 as an effective and safe T cell target antigen, based on its selective expression by cancer cells. MC2 peptides not being presented by normal cells is a decisive factor with respect to the clinical value of this antigen for future studies. QPCR of a large series of human tissues demonstrated that there is no gene expression of MC2, except for testis (figure 7A). These findings confirmed earlier findings ((10,60), and www.proteinatlas.org/ENSG00000046774-MC2/tissue as well as www.cta.lncc.br). Testis tissue is immune privileged (no MHC expression) and expected to express cancer germline antigens. In fact, our findings are in line with MC2 protein stainings by Zhuang et al (36). Further safety tests included exposure of T cells expressing TCR 6 towards a peptide library as well as peptides that were found to be highly similar to the MC2 LLF peptide. We observed that TCR 6-transduced T cells demonstrated no cross-recognition of any of 114 HLA-A2-eluted peptides tested (figure 7B). When testing peptides that are highly homologous to the MC2 LLF peptide, i.e., the MB4 LVF and MB10 LIF peptides, TCR 6 T cells showed a slight response to the MB4 but not MB10 peptide. Further experiments revealed that the MB4 peptide shows a higher EC₅₀ value than the MC2 peptide, but most importantly, and in line with predicted peptide processing and MHC class I presentation (<http://www.cbs.dtu.dk/services/NetCTLpan/>), TCR 6-transduced T cells do not recognize target cells expressing native MB4 (see figures 7C, D and E). Collectively, these results provide clear evidence against risks for on- or off-target reactivities when targeting MC2 via TCR 6.

Taken together, we demonstrate that T cell therapy benefits from the combination of targeting a safe, yet effective antigen, such as MC2, and epigenetic drug-enhanced antigenicity. Currently, we are preparing a phase I/II trial with adoptive transfer of MC2 TCR T cells, in combination with administration of AZA/VPA, to treat patients with metastatic melanoma and HNSCC.

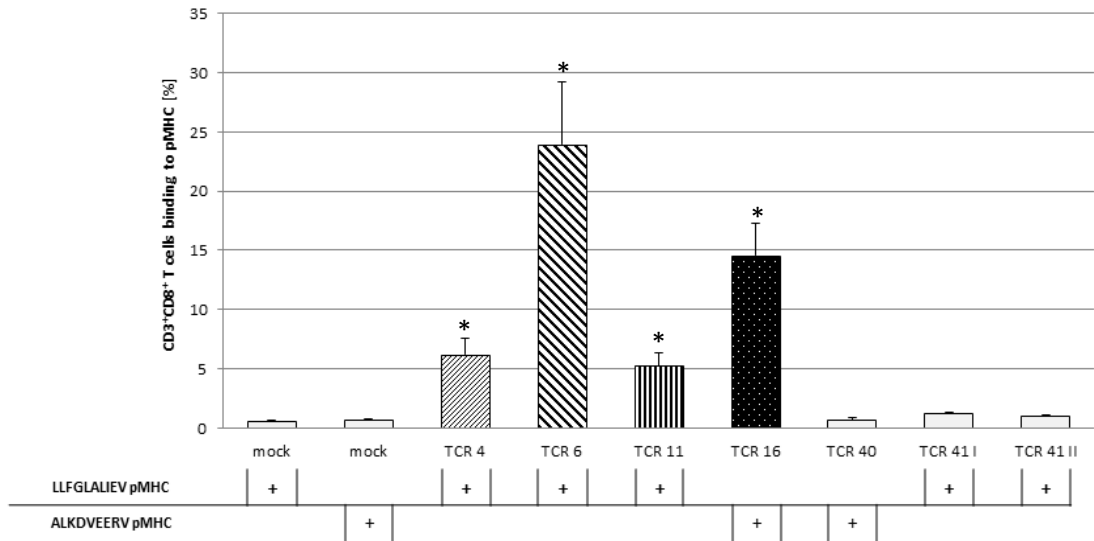
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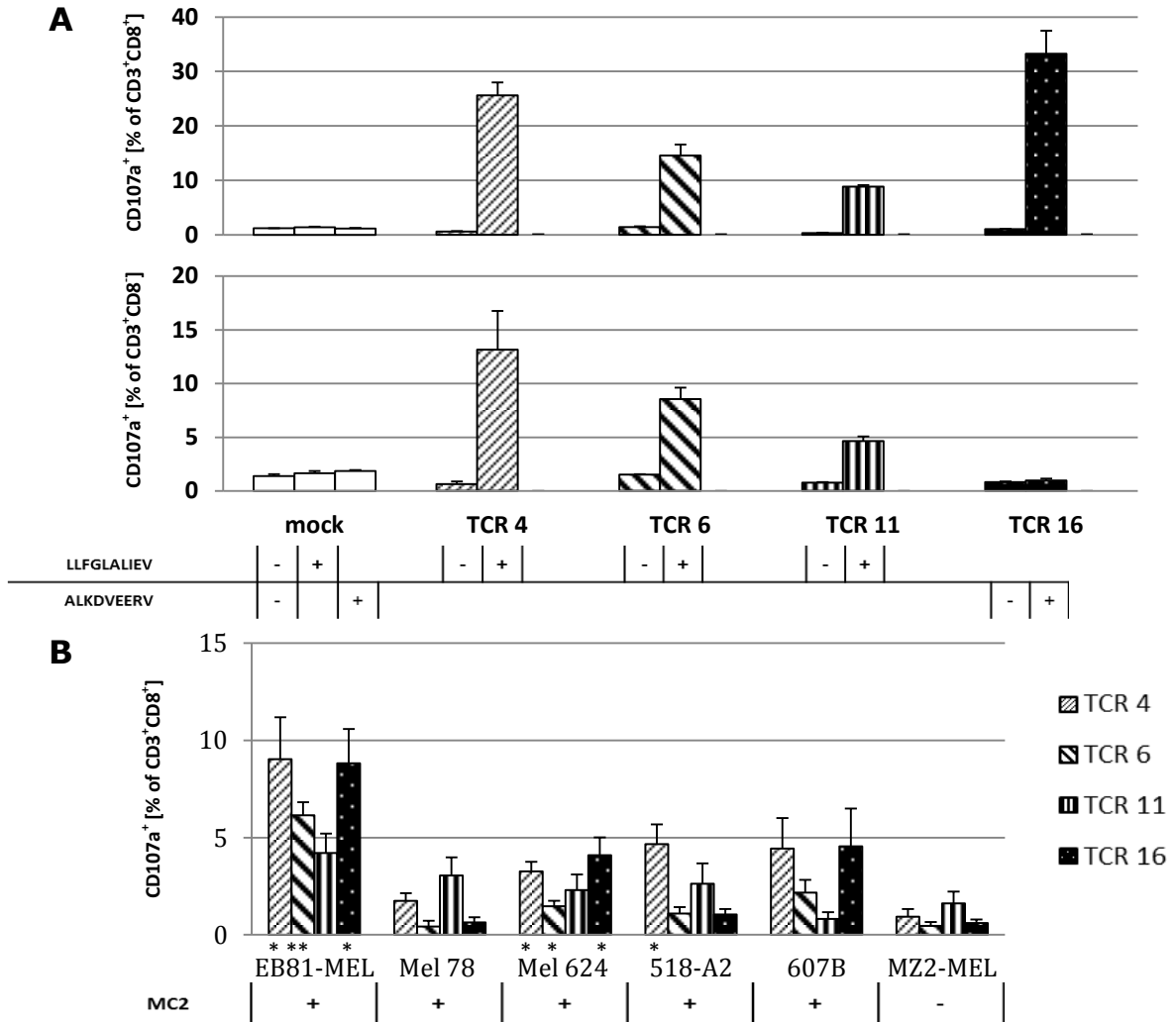
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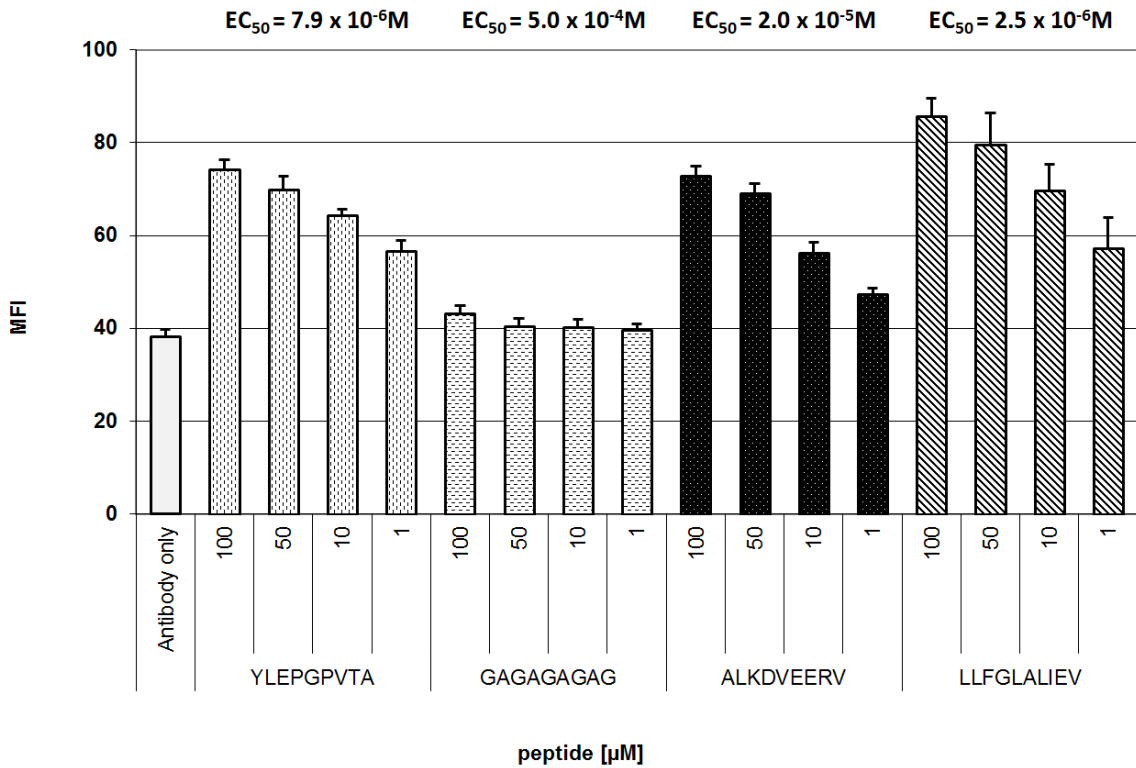
Supplementary figure 1. MC2 TCRs, when expressed by T cells, bind pMHC to varying degrees.

T cells were transduced with MC2 TCRs 4, 6, 11, 16, 40, 41 I, 41 II or not (mock) and analyzed by flow cytometry following staining either with ALK-PE pMHC (TCR 16, TCR 40) or LLF-PE pMHC (TCR 4, TCR 6, TCR 11, TCR41-I, TCR41-II). Results are displayed as bars (mean±SEM, n=3-9) of CD3, CD8-positive cells binding pMHC. Statistical significant differences of dextramer binding between TCR T cells and their respective mock control are calculated with Student's t-test: *p<0.05.



Supplementary figure 2. MC2 TCR T cells up-regulate expression of CD107a in response to peptide-loaded target cells and pre-treated melanoma cell lines

(A) MC2 TCR T cells pre-treated with epigenetic drugs (from figure 1A) were co-cultured with T2 cells pulsed with 1 μ M of ALK (for TCR 16) or LLF (for TCR 4, 6 and 11) peptide. CD107a-PE labelled antibody was added for 2h, and its binding to cells measured by flow cytometry. Results are displayed as percentages (mean \pm SEM, n=3) of viable, CD3, CD8-positive or CD8-negative cells expressing CD107a. Data are from one healthy donor out of two tested with similar results. (B) MC2 TCR T cells were co-cultured with six melanoma cell lines that were pre-treated with AZA, VPA and IFN γ (see legend to figure 4 for details) at an E:T ratio of 3:1. Tumor cell positivity for MC2 (based on RT-PCR data) is indicated below the graph. Results are displayed as percentages (mean \pm SEM, n=3) of viable, CD3, CD8-positive cells expressing CD107a corrected for CD107a expressions of mock T cells (same donor, incubation with same tumor cell line); CD107a expressions of mock T cells ranged between 0,04 and 5,27%. Data are from one healthy donor out of two tested with similar results. Statistical significant differences between responses of TCR T cells towards MC2+ versus MC2- or MC2+/HLA-A2- target cells (of same tumor type) are calculated with Student's t-test: *p<0.05; **p<0.005 (below bars).



Supplementary figure 3. ALKDVEERV and LLFGLALIEV form surface-expressed complexes with HLA-A*0201.

T2 cells were pulsed with titrated amounts of ALK or LLF peptide and formation of pMHC class I complexes on the cell surface was quantified via flow cytometry using a PE-labeled HLA-A2 mAb. The gp100 peptide YLEPGPVTA and a random peptide GAGAGAGAG were used as positive and negative peptide controls, respectively. The graphs represent Mean Fluorescence Intensities (MFI) as mean \pm SEM, n=3. EC₅₀ values of peptide: MHC class I binding (peptide concentration to yield half-maximal MFI of HLA-A2 expression) are displayed above each peptide.



T CELL RECEPTORS FOR CLINICAL THERAPY: *IN VITRO* ASSESSMENT OF TOXICITY RISK

**Andre Kunert¹, Matthias Obenaus^{2,3}, Cor H. J. Lamers¹, Thomas
Blankenstein^{2,3,4}, Reno Debets¹**

¹Laboratory of Tumor Immunology, Department of Medical Oncology, Erasmus MC Cancer
Institute, Rotterdam, The Netherlands;

²Max-Delbrück-Center for Molecular Medicine, Berlin, Germany;

³Institute of Immunology, Charité Campus Buch, Berlin, Germany;

⁴Berlin Institute of Health, Berlin, Germany

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ABSTRACT

Adoptive therapy with T cell receptor (TCR)-engineered T cells has shown promising results in the treatment of patients with tumors, and the number of TCRs amenable for clinical testing is expanding rapidly. Notably, adoptive therapy with T cells is challenged by treatment-related side effects, which calls for cautious selection of target antigens and TCRs that goes beyond their mere ability to induce high T cell reactivity. Here, we propose a sequence of in vitro assays to improve selection of TCRs, and exemplify risk assessments of on-target as well as off-target toxicities using TCRs directed against Cancer Germline Antigens. The proposed panel of assays covers parameters considered key to safety, such as expression of target antigen in healthy tissues, determination of a TCR's recognition motif towards its cognate peptide, and TCR's cross-reactivity towards non-cognate peptides.

INTRODUCTION

Therapeutic use of anti-tumor T cells has proven feasible in a multitude of trials over the last decade. Alongside the demonstration of clinical benefit and enthusiasm about the therapeutic efficacy, the occurrence of toxicities has stimulated awareness of safety pitfalls. While initial studies demonstrated recognition, and sometimes destruction of healthy tissues (1-3), later studies demonstrated lethal adverse effects in individual patients (4,5). These studies highlight the two main challenges facing safety of T cell receptor (TCR) gene therapy: on- and off-target toxicities. With a quickly expanding panel of TCRs that have generally been selected for their ability to provide T cells with high avidities towards tumor antigens, there is an urgent need for streamlining the safety assessment of these TCRs prior to clinical usage.

SELECTING TARGET ANTIGENS

An ideal target antigen for adoptive T cell therapy (AT) displays two important features: it is immunogenic and shows selective and homogenous expression in tumor tissue. Immunogenicity is best explained as an antigen's ability to be recognized by and sufficiently activate T cells, a feature that is generally well addressed when selecting a target antigen and its corresponding TCR (reviewed in (6)). In example, characteristics related to the immunogenicity of two MAGE-C2 (MC2) antigen epitopes are summarized in Table I. Selective expression in tumor tissue, and hence its absence in healthy tissues, would reduce the risk for on-target toxicities. Differentiation, over-expressed or onco-fetal antigens are not absent from healthy tissues and the targeting of these antigens, e.g. MART-1 or carcinoembryonic antigen (CEA), by TCR-transduced T cells has resulted in severe destruction of melanocytes in the eyes, ears and skin (1) or inflammation of the colon (2,3). Candidate antigens that are exclusively expressed by tumor tissues include neo-antigens and oncoviral antigens. Neo-antigens are derived from somatic DNA alterations and their identification requires analyses of mutations, gene expression, and algorithms that predict antigen processing and presentation by major histocompatibility complex (MHC) (7-9). AT studies with tumor infiltrating lymphocytes (TILs) in patients with melanoma and cholangiocarcinoma showed that clinical benefit was associated with T cell responses against neo-antigens (9-11). Current exploitation of neo-antigens in AT, however, is challenged by the uncertainty of current algorithms accurately predicting immunogenicity, and the fact that neo-antigens are usually specific per patient (12-14). Viral antigens are present in more than 10% of human cancers and are often the result of viral insertion into the genome and subsequent reactivation in tumors. AT studies using TILs reactive against either HPV or EBV have shown clinical successes in patients with cervical cancer or nasopharyngeal carcinoma, respectively (15,16). In addition to neo-antigens and onco-viral antigens, also certain cancer germline antigens (CGAs) demonstrate tumor-selective expression. In fact, CGAs are expressed in gonadal tissues and some in thymus (17), and certain CGAs are considered to be selectively de-repressed in tumor tissues (for detailed reviews, see

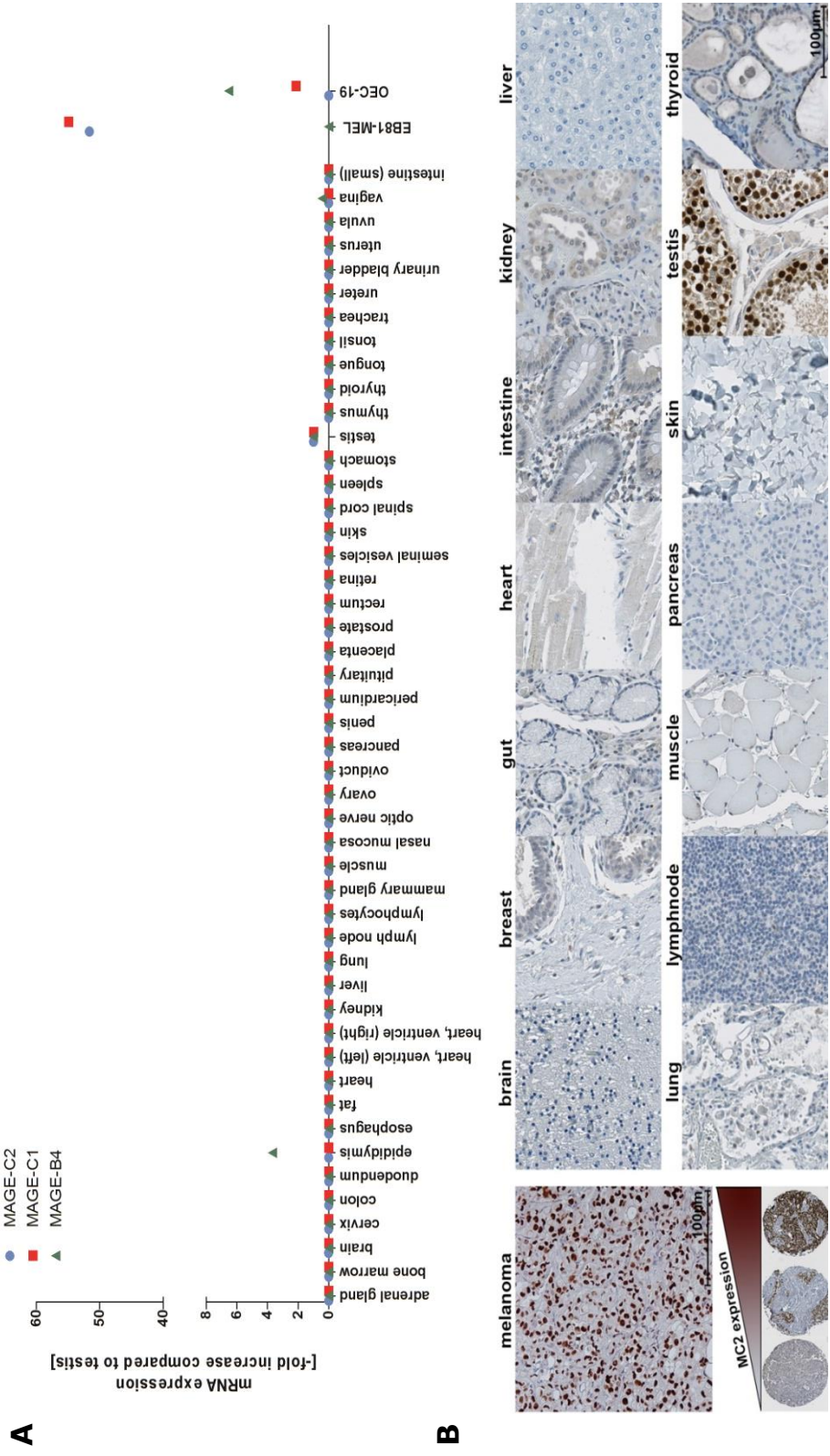


Figure 1. Target antigen is absent or shows restricted expression in healthy tissues.

(A) MC2, MC1 and MB4 qPCRs were performed on a cDNA library of 48 healthy, human tissue samples (Origene Technologies). Patient-derived melanoma cell line EB81-MEL and patient-derived esophageal cancer cell line OEC-19 served as positive controls. Spiking experiments were conducted separately to determine optimal PCR conditions for maximum sensitivity (detection limit: 1:1,000-10,000 MC2⁺ cell; MC2⁻ cells; data not shown). Relative mRNA levels are corrected for GAPDH and expressed as fold increase compared to antigen expression in the testis. Part of the displayed data is derived from (23); Copyright © 2016 The American Association of Immunologists, Inc. (B) Immune stainings of melanoma and healthy tissues were conducted using a monoclonal antibody specific for MC2 (42) on tissue micro arrays (TMA). EnVision System (Agilent Technologies) was used for signal visualization, TMAs were scanned via Nano zoomer (Hamatsu) and manually scored using Distiller (SlidePath). Exemplary tissue sections of patient-derived melanoma tissue as well as various healthy tissues obtained from the Erasmus MC pathology department are displayed with 20x magnification. MC2 positivity is scored as brown colored nuclear staining, with 3 smaller TMAs exemplifying different staining intensities.

(6,18,19)). MAGE-A3 and NY-ESO1 are examples of CGAs that have already been targeted by TCR-engineered T cells in patients with metastatic melanoma, metastatic synovial sarcoma or multiple myeloma (4,20). Although off-target toxicities were observed with the targeting of the former antigen (most likely an issue of the TCRs; see next section), the safe use of selected CGAs was suggested by the targeting of NY-ESO1 demonstrating clinical benefit without toxicities (20,21).

We recommend for any antigen, with the exception of neo-antigens, to test the antigen's absence from a large panel of healthy organs. Online databases such as the protein atlas (<http://www.proteinatlas.org/>) or the CGA database (www.cta.incc.br) combine extensive data from transcriptomic analyses and antibody stainings from numerous normal, non-cancerous cell lines and tissues. When applying these tools to assess the expression of MC2, we observed that mRNA expression is restricted to cells from cancers and testes, the latter considered to possess an immune privileged status (no MHC expression; thus no detection by T cells). The use of commercially available cDNA libraries of a large series of healthy tissues enables researchers to extend online analyses and quantify antigen expression with a laboratory assay. When performing qPCR using such a cDNA library, we demonstrated absence of MC2 mRNA in healthy tissues as illustrated in figure 1A. In case specific antibodies are available, we would recommend to follow-up qPCR with immune histochemistry. Using an MC2-specific antibody, we confirmed the presence of MC2 protein in testis and melanoma as well as its absence in multiple healthy tissues, such as brain, heart, intestine and lung (see figure 1B). However, both qPCR and immune histochemistry cannot formally exclude the presence of rare antigen-positive cells within a tissue, e.g. stem cells. For example, mRNA of certain CGAs has been detected only in medullary thymic epithelial cells but not total thymus (17). As an additional means to exclude target antigen expression, sophisticated *in vitro* cell cultures have been developed to closely mimic complete tissues or organs (commented in next section) (5,22).

SELECTING THERAPEUTIC TCRs

Once the safety of the target antigen has been assessed, one can start selecting TCRs. Procedures to obtain tumor-reactive T cells and hence TCRs can generally be divided into those that rely on tolerant and those that rely on non-tolerant repertoires of T cells. Tolerant repertoires, where deletion of T cells with avidity outside the thymic selection window has occurred, have been used to obtain T cell clones from patients following successful TIL therapy, peptide vaccination, or using *in vitro* pulsing of autologous dendritic cells (23,24). Notably, the thymic selection of T cells is most likely a trade-off between producing a self-tolerant yet sufficiently diverse and responsive TCR repertoire, and escape of self-reactive TCRs cannot be negated. Indeed, TCRs specific for over-expressed antigens and obtained from the native repertoire have been shown to initiate autoimmune side effects (1). The use of non-tolerant repertoires, with the rationale of allowing the generation of high-avidity T cells, has been applied in allogeneic *in vitro* as well as *in vivo* systems.

Table I Properties of cognate MAGE-C2 epitopes and corresponding TCRs that relate to T cell reactivity

Epitopes	LLFGLALIEV (MAGE-C2 ₁₉₁₋₂₀₀)	ALKDVEERV (MAGE-C2 ₃₃₆₋₃₄₄)
HLA-A2 binding	predicted affinity ^a : 9.9nM measured affinity ^b : 2.5μM	predicted affinity ^a : 342.2nM measured affinity ^b : 20.0μM
TCR	TCR 6	TCR 16
TCR genes ^c	α-chain: Va12-2*01 / Ja23*01 / Ca β-chain: Vβ15*02 / Jβ2-3*01 / Cβ2	α-chain: Va3*01 / Ja3*01 / Ca β-chain: Vβ28*01 / Jβ2-5*01 / Cβ2
pMHC binding ^d	EC ₅₀ CD8 ⁺ T cells: 348nM EC ₅₀ CD4 ⁺ T cells: 501nM	EC ₅₀ CD8 ⁺ T cells: 663nM EC ₅₀ CD4 ⁺ T cells: n.d.
functional T cell avidity ^e	EC ₅₀ : 3.19nM	EC ₅₀ : 73.8nM
tumor cell recognition ^f	EB81-MEL: 1090pg/ml 518-A2: 605pg/ml 607-B: 835pg/ml SCC-9: 311pg/ml 93-VU-120: 292pg/ml SUM-195-PT: 217pg/ml	EB81-MEL: 1738pg/ml 518-A2: 461pg/ml 607-B: 626pg/ml SCC-9: 207pg/ml 93-VU-120: 26pg/ml SUM-195-PT: 466pg/ml

^a according to <http://www.cbs.dtu.dk/services/NetCTLpan/>.

^b T2 cells were pulsed with titrated amounts of ALK or LLF peptide and formation of pMHC class I complexes on the cell surface was quantified via flow cytometry using PE-labeled HLA-A2 mAb (23).

^c cDNAs derived from patient-derived T cell clones were PCR amplified using either a set of TCR-Vα or Vβ sense primers and a corresponding TCR-Cα or Cβ antisense primer or 5'RACE (rapid amplification of cDNA ends). Following nested PCRs and cloning, TCRα and β sequences were identified using www.imgt.org and classified according to the Lefranc nomenclature (see (23) for details).

^d TCR T cells were pulsed with titrated amounts of PE-labeled pMHC multimer, and binding was quantified via flow cytometry.

^e TCR T cells were antigen-presenting cells that were pulsed with titrated amounts of cognate peptide, and functional T cell avidity was quantified via ELISA measurements of IFNγ production.

^f TCR-transduced T cells were co-cultured with 3 melanoma, 2 head-and-neck carcinoma and 1 triple-negative breast cancer cell lines all positive for HLA-A2 and MC2 (determined by qPCR) at an E:T ratio of 3:1 for 24h. Cell lines were treated with epigenetic drugs Azacytidine and Valproate as well as IFNγ prior to co-culture (see (23) for details). IFNγ levels in 24h culture supernatants were measured by ELISA; displayed values are means of five experiments. Note that epigenetic drugs induce enhanced and tumor-selective expression of MC2 *in vitro* (23) as well as enhanced tumor immunogenicity *in vivo* (18).

n.d. = not determined.

For example, HLA-mismatched antigen-presenting cells (25) or artificial antigen-presenting cells pulsed with peptides of interest facilitate the *in vitro* generation of tumor-reactive T cells (26). *In vivo*, mice transgenic for human HLA as well as mice transgenic for human TCR and HLA-A2 genes (27) have been immunized and used as a source of TCRs. After having obtained antigen-reactive T cells, sequences of the TCRα and β chains can be determined by molecular techniques such as 5' RACE (23), enhanced PCR methods, capturing and indexing of genomic DNA-encoding TCR chains (28), or sequencing and pairing of TCR chains based on combinatorial algorithms (29).

It is important to note that TCRs derived from above-mentioned repertoires, in particular the non-tolerant repertoire, have not been selected in the presence of all patient MHC alleles and in the case of mice neither against human peptides present in the thymus, and may show allo- and non-cognate reactivity. Moreover, TCRs, even though obtained from highly tumor-reactive T cells, have an inherent degeneracy for peptide recognition and are able to recognize more than a single peptide. This dynamic flexibility in antigen recognition is in part accredited to the bending ability of the TCR-CDR domains (30,31), but also to the dominant interaction of the TCR with a restricted number and order of amino acids present in the MHC-presented peptide. To minimize the risk of selecting a TCR that recognizes non-cognate self-peptides, we recommend a series of assays that assess the risk of this so-called off-target toxicity. These assays are illustrated with two patient-derived MC2-specific TCRs 6 and 16 with details and evaluation of anti-tumor T cell responses mediated by TCR6 and TCR16 summarized in Table I.

Recognition of random epitopes

Initial assessment of a TCR's self-reactivity can be done by testing the responsiveness of TCR-transduced T cells towards random peptides known to be presented by the respective HLA restriction allele. Mathematical projections indicate that amongst a pool of $\sim 10^{12}$ peptides, a single TCR may react with $>10^6$ peptides, supporting the notion of TCR degeneracy for peptide recognition, which potentially contributes to a more diverse TCR repertoire (32). We co-cultured TCR6 and TCR16 T cells with antigen-presenting cells loaded with saturating concentrations of >100 common, HLA-A2-eluted self-peptides (4). As depicted in figure 2, both T cell populations mediated a T cell response to their respective cognate peptides, but to none of the other peptides. Importantly, these data hint to lack of cross-reactivity of these two TCRs, but we cannot fully exclude recognition to random peptides. Another assessment of self-reactivity can be conducted by testing TCR-transduced T cells towards allogenic HLA molecules. In order to exclude activation of T cells upon recognition of foreign HLA molecules, panels of lymphoblastoid B cell lines with various HLA allotypes have proven valuable (33,34).

Recognition of cognate epitope via critical amino acids

Further assessment of a TCR's self-reactivity, and a key assay in this communication, is the testing of a TCR's intrinsic capacity to recognize peptides highly homologous to its cognate epitope (35,36). To this end, one can determine the recognition motif, i.e. the position and sequence of amino acids within the cognate epitope that are crucial for binding to the TCR. This motif is unique per TCR and can be considered a surrogate measure for the extent of cross-reactivity of TCRs (22,33). The importance to assess such motifs became apparent from two recent clinical trials

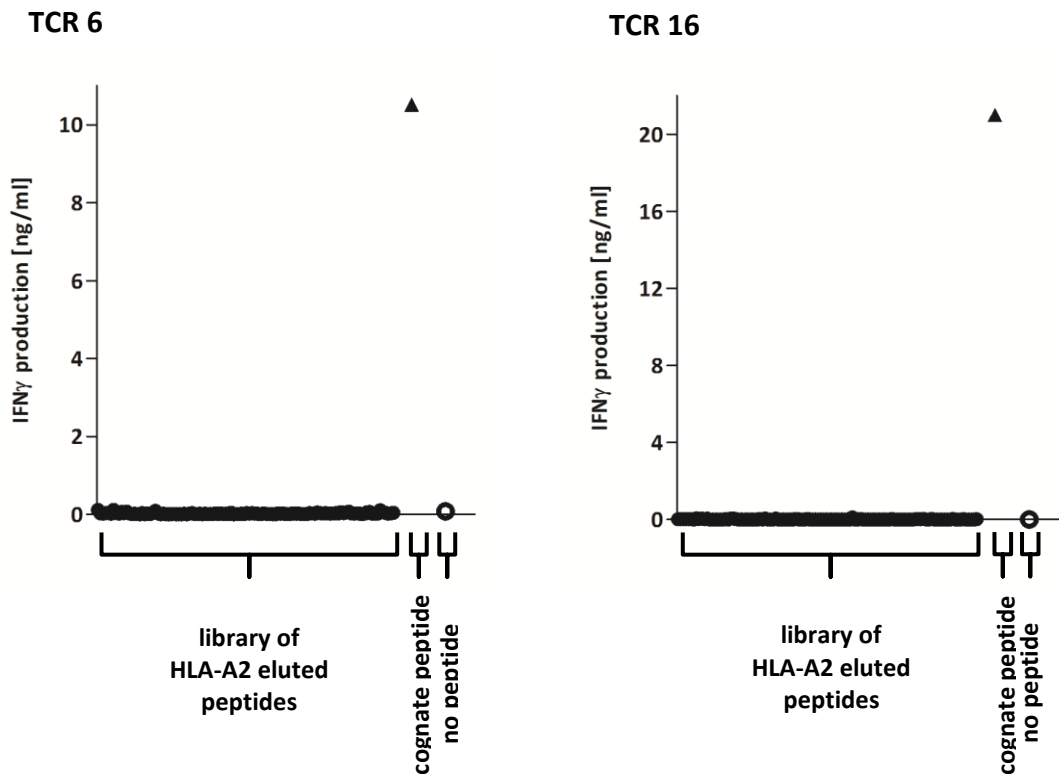


Figure 2. TCRs do not recognize random peptides eluted from the restricting MHC allele.

TCR6 or TCR16 T cells were co-cultured with T2 cells loaded with 1 μ M of 114 different HLA-A2-eluted peptides (4) for 24h. Cognate MC2 peptides (TCR6: LLFGLALIEV; TCR16: ALKDVEERV) served as positive controls. IFN γ levels in 24h culture supernatants were measured by ELISA and are displayed as mean \pm SEM (n=3). Left-hand panel is adapted from (23); Copyright © 2016 The American Association of Immunologists, Inc.

using AT with TCR-engineered T cells. The first trial targeting MAGE-A3 and A9 (MA3/9) in the context of HLA-A2 utilized the TCR 9W11 and reported neurological toxicities in two patients with metastatic melanoma (4). The second trial targeting MA3 in the context of HLA-A1 utilized the TCR a3a and reported cardiac toxicities in one patient with metastatic melanoma and one patient with multiple myeloma (5). Both TCRs were affinity enhanced in vitro and mediated toxicity by recognizing peptides highly similar to the cognate peptide, namely peptides derived from MAGE-A12 and Titin present in brain and heart tissue, respectively. These studies clearly underline the need to assess recognition motifs, and the search for T cell reactivities against homologous self-peptides, prior to clinical application. The importance of recognition motifs is timely and its assessment has only occurred for a limited number of TCRs to date, which is summarized in Table II.

Table II Recognition motifs of TCRs utilized in clinical and pre-clinical research

TCR name	target antigen	HLA -	cognate epitope	recognition motif ^a	number of antigens with motif ^b	number of epitopes with high MHC affinity (<10 μ M) ^c	refs.
a3a	MAGE-A3	A1	EVDPIGHLY	E x DPI xxx Y	5	0	(22)
9W11	MAGE-A3	A2	KVAELVHFL	-xx E -x H - ^d	-	-	(43)
T1367	MAGE-A1	A2	KVLEYVIKV	xxx EY x IK x	62	6	(33)
s24-TCR	survivin	A2	ELTLGEFLKL	x LT x GEFLK x	1	1	(44)
gp100 wt	gp100	A2	YLEPGPVTA	x LEPGP xx A	4	4	Govers, Ms. submitted
fl-MPD	gp100	A2	YLEPGPVTA	Y x EP xxxxx	>1000	>500	(45)
fl-296	gp100	A2	YLEPGPVTA	Y x E xxxxxx	>1000	>500	(45)
TCR 4	MAGE-C2	A2	LLFGLALIEV	xx FGL xLxxx	260	122	(23)
TCR 6	MAGE-C2	A2	LLFGLALIEV	L x F x L x L x E x	28	9	(23)
TCR 11	MAGE-C2	A2	LLFGLALIEV	xx FGL xLx E x	21	17	(23)
TCR 16	MAGE-C2	A2	ALKDVEERV	x LKDVEER x	2	2	(23)

^aRecognition motifs are defined through T cell IFN γ production in response to alanine scanned cognate epitopes.

^bnumber of human proteins containing matching recognition motif according to ScanProSite (<http://prosite.expasy.org/scanprosite/>).

^cnumber of proteins containing matching recognition motif (according to ^b) and a predicted affinity value <10 μ M for binding of the peptide to its respective MHC; affinity calculations according to NetMHCpan (<http://www.cbs.dtu.dk/services/NetMHCpan/>).

^drecognition motif incomplete, amino acids on positions 2-4 and 6-7 are based on stimulation assays conducted by Chinnasamy et al. with a panel of MAGE-peptides with highly similar sequences (43); '-' indicates amino acids with unknown relevance to the recognition motif.

Using a set of altered peptide ligands (APLs), peptides containing individual alanine replacements at every single position in the cognate peptide (in case of an endogenous alanine \rightarrow glycine), we conducted stimulation assays with TCR6 and 16 T cells. Critical amino acids are defined as those that, when testing the respective APLs, result in a drop of the T cell response (generally using IFN γ production as a readout) of > 50% when compared to the response toward the cognate peptide. Following the determination of these motifs, as exemplified in figure 3A, we used ScanProSite (<http://prosite.expasy.org/scanprosite/>) (37) and identified target antigens, listed in figure 3B, that harbor the recognition motif and represent potential cross-reactive targets for TCRs 6 and 16. Subsequently, these self-peptides were tested for their ability to induce T cell responses towards HLA-A2-positive antigen presenting cells loaded with saturating concentrations (1 μ M) of peptide. These experiments yielded a short list of self-peptides that are actually recognized by the TCRs under study, defined as those that resulted in at least a T cell IFN γ response > 2.5% of the response to cognate peptide (figure 3B: underlined antigens).

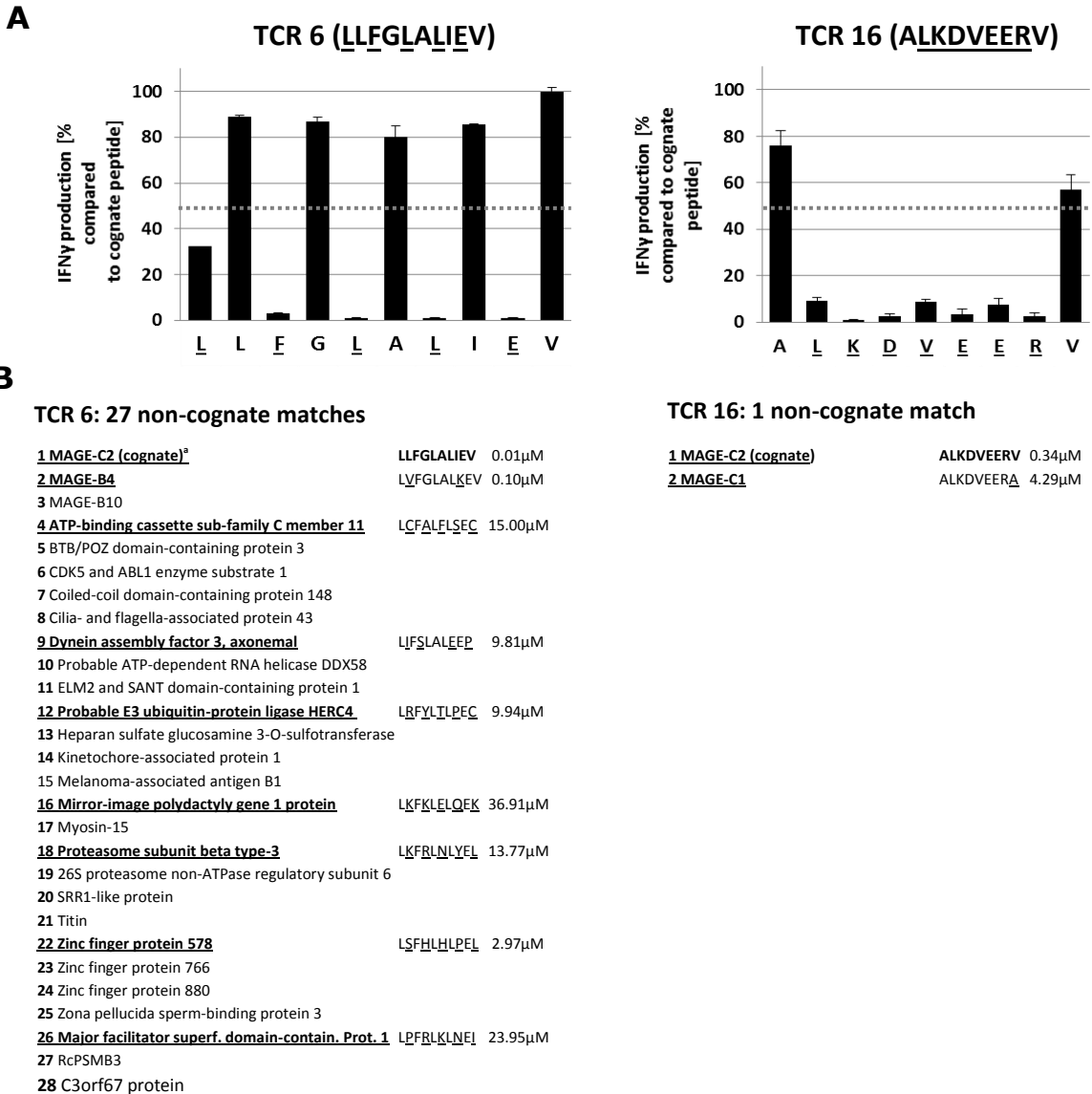


Figure 3. TCRs recognize cognate peptide via unique or restricted motif.

(A) TCR6 or TCR16 T cells were co-cultured with T2 cells loaded with 1μM of cognate peptides or peptides with a single alanine replacement (Altered Peptide Ligands (APL), in case of alanine in original peptide: glycine). IFN γ levels in 24h culture supernatants were measured by ELISA. IFN γ response to APLs is displayed as mean % relative to response to cognate peptides \pm SEM (n=4). Responses < 50% (dashed line) were indicative of amino acids critical for TCR recognition (recognition motif: underlined amino acids). Left-hand plot is adapted from (23). (B) Homologous motifs from (A) were queried against a human protein database using ScanProSite. This yielded 27 and 1 non-cognate matches for TCR6 and TCR16, respectively. Subsequently, TCR6 or TCR16 T cells were co-cultured with T2 cells loaded with 1μM of these 27 and 1 peptide(s), and IFN γ levels were measured in 24h culture supernatants by ELISA (n=4). Underlined peptides induced a T cell IFN γ response > 2.5% of the response to cognate peptide. Homology to cognate peptide (diverging amino acids underlined) as well as peptide-MHC affinity (IC₅₀ calculations according to NetMHCpan; <http://www.cbs.dtu.dk/services/NetMHCpan/>) are indicated for those peptides with a detectable IFN γ response.

Recognition of non-cognate epitopes that contain recognition motif

Once self-peptides that can be recognized by TCR T cells have been identified, we recommend to execute two additional tests to more stringently assess the risk for self-reactivity. These tests aim to provide measures for T cell avidity as well as efficiency of cellular processing and presentation. Towards the first test, one can titrate amounts of non-cognate peptides and determine the concentration of these peptides that elicits 50% of the maximal T cell response (EC_{50}). For both TCR6 and 16 T cells, we found that only a single self-peptide, namely a peptide from the antigen MAGE-B4 (MB4) or MAGE-C1 (MC1; see figure 3B for homology and predicted peptide-HLA binding), respectively, revealed detectable EC_{50} values that were only 2-5 fold lower than those of the cognate peptides (figure 4). Extent of homology and predicted peptide-MHC binding of peptides that induce T cell IFN γ are listed in figure 3B, which shows that loss of homology and peptide-MHC affinity was least affected for MB4 and MC1 peptides. All other self-peptides revealed no T cell reactivities at titrated doses, or at the very best 5 log scales lower compared to cognate peptide. These were considered not recognizable by the TCRs under study and excluded from further assays. Towards the second test, one can predict whether self-peptides are the result of antigen processing and presentation to enable T cell recognition *in vitro*. NetCTLpan (<http://www.cbs.dtu.dk/services/NetCTLpan/>) (38) takes proteasomal C terminal cleavage, TAP transport efficiency, and peptide MHC class I binding of peptides into account, and can be employed to obtain an initial score for antigen processing and presentation. Analysis of the MB4 peptide, but not MC1 peptide, yielded a high score according to this web-based tool. Such predictions may not be fully accurate and should be verified using cells known to express the antigen or antigen presenting cells (e.g. dendritic cells) transfected with antigen-encoded RNA followed by co-cultivation with TCR-transduced T cells. To this end, we stimulated TCR T cells with the esophageal cancer cells line OEC-19, which natively expresses the MB4 and MC1 antigens, but is devoid of the MC2 antigen, and observed that both TCRs failed to initiate T cell activation against either MB4 or MC1 (see figure 4B). When using cell lines that natively express the MC2 antigen, as a control for the processing and presentation of MAGE antigens, we observed that both TCRs did initiate T cell activation. It is noteworthy that standard tissue culture systems may not always accurately reflect antigen processing and presentation. This was evidenced by the recognition of Titin by the TCR $\alpha 3a$ that could only be observed in more elaborate tissue culture systems, such as 3D cultures of beating cardiomyocytes derived from induced pluripotent stem cells (22). In case the above two assays do not exclude self-reactivity of TCR T cells, one could pursue assessment of the tumor-selective expression of such new antigens. In case expression of new antigens is not selective for tumors, the corresponding TCR should be excluded. Using online tools (39) as well as qPCR (figure 1A), MB4 showed expression within epididymis and vagina, whereas MC1 showed no expression in any of the healthy non-gonadal tissues. These data highlight the stringent safety profile of TCR16, the TCR selected for a clinical trial to treat melanoma and head-and-neck carcinoma, currently prepared at Erasmus MC.

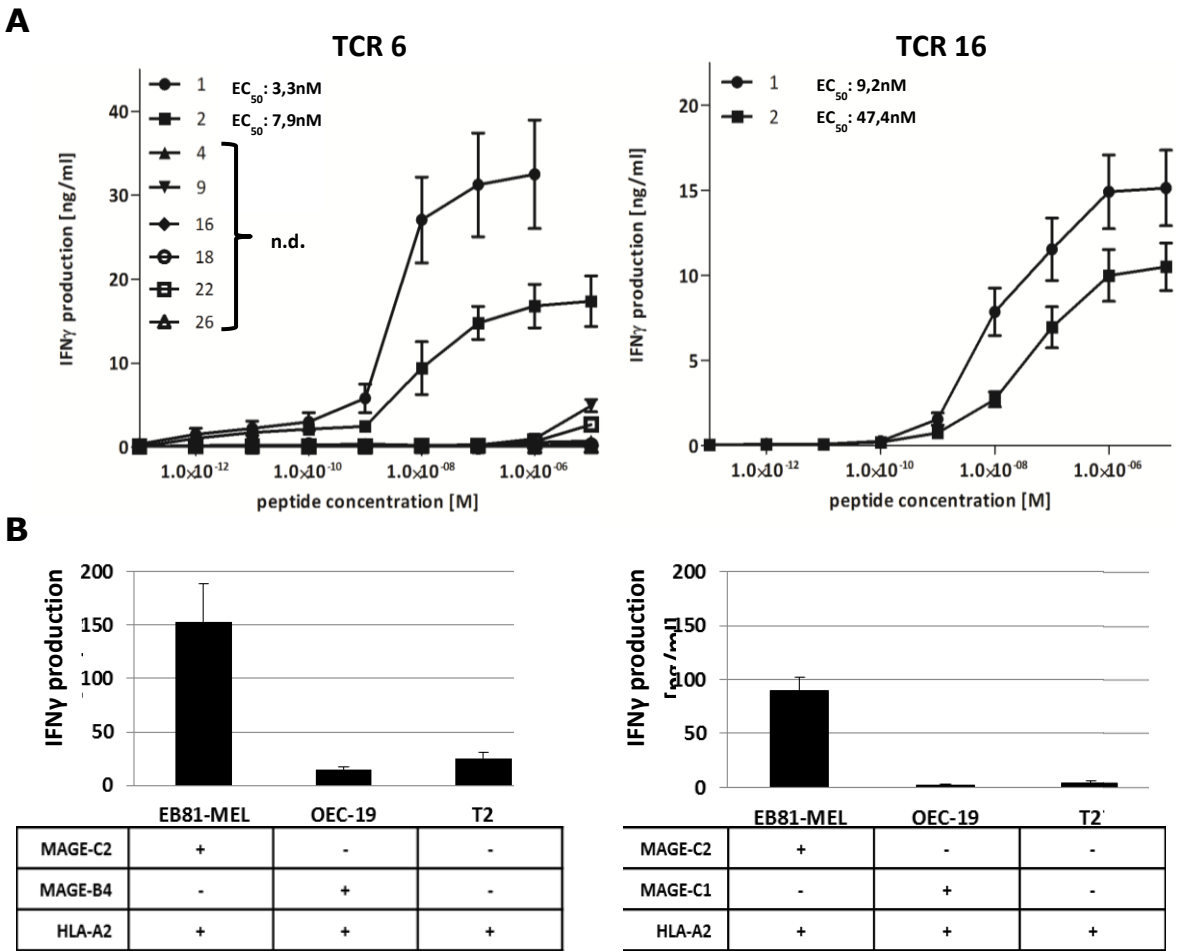


Figure 4. TCRs mediate negligible T cell avidity nor recognize natively presented non-cognate peptides that harbor recognition motif.

(A) Non-cognate peptides underlined in figure 3B were titrated from 10 μ M (10⁻⁵M) to 1pM (10⁻¹² M) and tested for T cell IFN γ response as described in previous legends and displayed as mean \pm SEM (n=4). EC₅₀ values for cognate and selected non-cognate peptides were calculated in GraphPad, using non-linear regression; n.d. = not detectable. (B) TCR6 or TCR16 T cells were co-cultured with tumor cell lines or T2 cells (as a negative control). Expression status of MC2, MC1, MB4 and HLA-A2 for these cells was assessed via qPCR and indicated below plots with plus or minus. IFN γ levels in 24h culture supernatants were measured by ELISA and are displayed as mean \pm SEM (n=3). Left-hand plots are adapted from (23); Copyright © 2016 The American Association of Immunologists, Inc.

Future perspective of *in vitro* assays assessing risk of TCR-mediated toxicities

The proposed collection and sequence of *in vitro* assays to assess risks for toxicities are presented in figure 5. We advocate this testing for TCRs with clinical intent, in particular those TCRs reactive against a self-peptide and derived from a non-tolerant repertoire and/or following gene-enhancement. In extension to gene-enhancement, introduction of TCR-CDR mutations has been a commonly used tool to generate high-affinity TCRs (2,4,5,20). While such gene-enhanced TCRs recognize target peptides at increased affinities when compared to the corresponding wild-type TCRs, consequently such TCRs are also at risk to recognize non-cognate peptides. To test whether affinity enhancement led to an increase in degeneracy for peptide recognition, we made use of a panel of 8 TCRs specific for the same cognate peptide gp100₁₈₁₋₁₈₈:HLA-A2) but harboring 2-3 mutated amino acids in either their CDR2 β , CDR3 α or CDR3 β domains (Govers, Ms submitted). Upon assessment of the recognition motifs and search for motif-harboring self-peptides, it became apparent that enhanced affinity was accompanied by drastic increase in the TCR's ability to recognize self-peptides (Table III). These data extend earlier findings regarding a correlation between affinity enhancement and loss of TCR specificity (40), and warrant caution when trying to change the TCR-CDR structure as it compromises the stringent recognition of cognate peptide (31,41).

Taken together, here we propose a platform of *in vitro* assays that in combination with available online-databases and tools allows for optimal toxicity risk-assessment for target antigens and TCRs currently under consideration for clinical trials.

Table III TCR affinity enhancement and its effect on off-target recognition^a

TCR name	K _D [μ M]	target antigen	cognate epitope	recognition motif ^a	number of antigens with motif ^b	number of epitopes with high MHC affinity (<10 μ M) ^c
gp100 wt	18.5	gp100/HLA-A2	YLEPGPVTA	xLEPGPxxA	4	4
gp100 TCR 1	7.9	gp100/HLA-A2	YLEPGPVTA	xLExGPxxA	23	13
gp100 TCR 2	4.0	gp100/HLA-A2	YLEPGPVTA	xLExGPxxx	240	97
gp100 TCR 5	1.1	gp100/HLA-A2	YLEPGPVTA	xLExGPxxx	240	97
gp100 TCR 8	0.026	gp100/HLA-A2	YLEPGPVTA	xLxxxxxxx	>10000	>10000

^aGovers, Ms, submitted.

^bnumber of human proteins containing matching recognition motif according to ScanProSite (<http://prosite.expasy.org/scanprosite/>).

^cnumber of human proteins containing matching recognition motif (according to ^b) and a predicted affinity value of <10 μ M for binding of the peptide to its respective MHC; affinity calculations according to NetMHCpan (<http://www.cbs.dtu.dk/services/NetMHCpan/>).

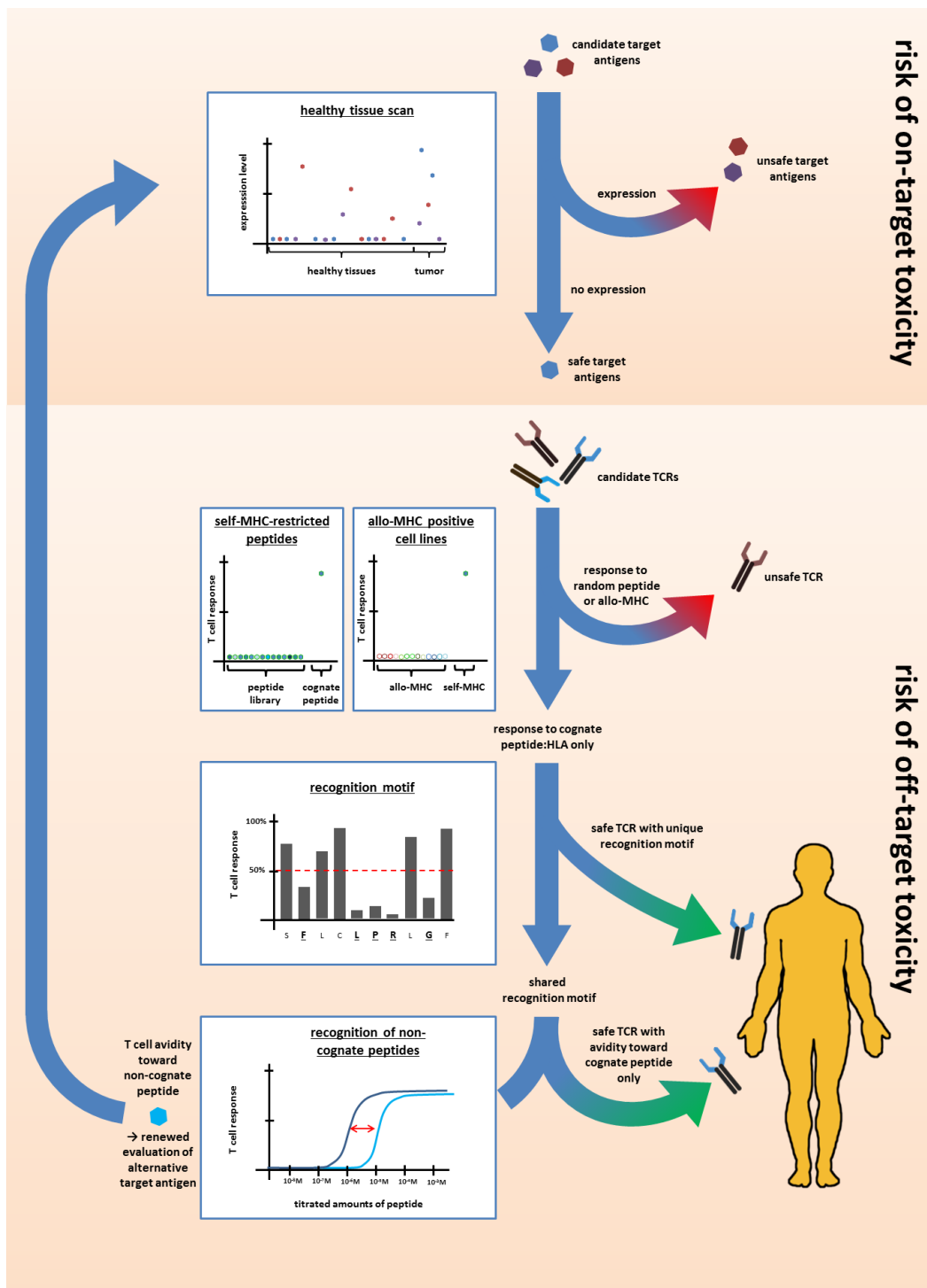


Figure 5. Platform of *in vitro* assays to select antigens and TCRs with limited risk for *in vivo* toxicity.

Flowchart proposes a series and sequence of *in vitro* techniques, exemplified in figures 1 to 4 and explained in detail in text. Such a sequence of assays would facilitate selections of potentially safe target antigens and TCRs prior to their use in clinical trials

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**T CELL RECEPTORS EQUIPPED WITH ICOS ENHANCE T
CELL PERSISTENCE AND MEDIATE
SUSTAINABLE ANTI-TUMOR RESPONSES UPON
ADOPTIVE T CELL THERAPY**

**Andre Kunert¹, Cor Berrevoets¹, Rebecca Wijers¹, Marlies Peters¹,
Rachid Bouzid¹, Stefan Sleijfer², Reno Debets¹**

¹Laboratory of Tumor Immunology, ²Department of Medical Oncology, Erasmus MC Cancer
Institute, Rotterdam, The Netherlands

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ABSTRACT

Adoptive therapy with T cell receptor (TCR)-engineered T cells constitutes a promising treatment approach for patients with solid tumors. However, this therapy is currently challenged by non-durable responses, and accumulating evidence suggests that numbers and activity of intra-tumoral T cells as well as persistence of peripheral T cells is not optimal. Here, we addressed this challenge by gene-engineering TCRs to incorporate co-stimulatory molecules derived from CD28, OX40, ICOS, 4-1BB or CD40L. T cells expressing co-stimulatory TCRs with CD28, OX40 or ICOS showed enhanced pMHC binding when compared to wt TCR, but showed lowered functional avidity following antigen exposure *in vitro*. Notably, adoptive transfer of T cells expressing these co-stimulatory TCRs into immune competent, melanoma-bearing mice enhanced anti-tumor responses and prolonged overall survival. In fact, TCR:ICOS T cells resulted in 50% complete responses at day 115 and tumor recurrences at >40 days after T cell transfer (wt TCR: 0% and 20 days, respectively). In mice treated with TCR:ICOS T cells, TCR transgene-positive T cells demonstrated enhanced numbers of CD8 T cells in the blood stream and in tumor tissue, and the latter showed an enhanced frequency of CD40L-positive T cells. In conclusion, we show that TCRs with an ICOS signaling domain improve accumulation and activation of intra-tumoral CD8 T cells as well as the peripheral persistence of CD8 T cells following adoptive transfer, and significantly aid to tumor clearance.

INTRODUCTION

Adoptive therapy with T cell receptor (TCR-)engineered T cells has demonstrated its feasibility in patients suffering from a broad spectrum of malignancies. Trials targeting melanoma antigen recognized by T cells 1 (MART-1), glycoprotein (gp)100, carcinoembryonic-antigen (CEA), melanoma-associated antigen 3 (MAGE-A3) or NY-ESO-1 have shown promising objective response rates in metastatic melanoma, colon carcinoma, synovial sarcoma and multiple myeloma, ranging from 12 to 90% (1-6). Despite initial responses to treatment, ongoing and complete responses occurred only in about maximally 20% of patients while the majority of patients initially responding to treatment eventually suffer from tumor relapse. Recurrence of tumor cells is considered a consequence of mechanisms utilized by the tumor to evade T cell infiltration, migration and/or local activation. And although diverse in nature, these mechanisms are generally related to changes in: antigen processing and presentation; lack of T cell effector cells; immune-suppressor cells; and/or checkpoint inhibitors (for an overview, please refer to (7,8)). Recently, the expression of co-inhibitory receptors by tumor-infiltrating lymphocytes, such as PD-1, LAG3 or TIM3, has gained interest with respect to immune evasion. Binding of these receptors by their ligands triggers a negative feedback loop that limits accumulation and activation of intra-tumoral T cells. In fact, clinical use of so called checkpoint-inhibitors such as pembrolizumab, nivolumab (α -PD1) or ipilimumab (α -CTLA4) has demonstrated significant enhancement of endogenous T cell responses against solid tumors in a fraction of patients (9-13).

Even though a variety of escape mechanisms exists, proficient T cell co-stimulation ultimately constitutes a prerequisite to enable sufficient T cell accumulation and activation within tumor tissue. An eloquent example along these lines is that the rescue of T cell activation with anti-PD-1 antibody depends on the availability of CD28 signaling (14,15). Tumor cells exploit the requirement of T cell co-stimulation by reducing the expression of co-stimulatory ligands and enhancing the expression of co-inhibitory ligands, thus preventing successful T cell recruitment and activation within the tumor microenvironment (16,17). In an attempt to counter suboptimal T cell co-stimulation, one can incorporate co-signaling domains into TCRs or chimeric antigen receptors (CARs), thus converging both antigen-specific as well as co-stimulatory signaling pathways into a single receptor. In CARs such efforts have already been shown to enhance tumor regression in a therapeutic setting (18-20). We have shown previously that incorporation of a signaling cassette consisting of CD28 and CD3 ϵ into TCRs results in enhanced responses of therapeutic T cells directed against EBV or melanoma target antigens (21,22). Here, we have generated and tested the therapeutic effect of TCRs equipped with OX40 (CD134), ICOS (CD278), 4-1BB (CD137) or CD40L (CD154) in vitro as well as in a mouse melanoma model. We assessed these novel co-stimulatory TCRs with regard to their surface expression, T cell function and phenotype in vitro. Following adoptive T cell therapy, we assessed T cells expressing these co-stimulatory TCRs for their ability to improve tumor clearance and survival in melanoma-bearing mice, and for peripheral persistence, accumulation in tumor tissue and expression of co-signaling receptors.

MATERIALS AND METHODS

Cell culture and reagents

The human embryonic kidney 293T and Phoenix-Amp cell lines, both used to package retroviruses carrying RNA encoding TCR $\alpha\beta$, were grown in DMEM with 10% (v/v) Fetal Bovine Serum (FBS; Greiner Bio-one Alphen a/d Rijn, The Netherlands), 200nM L-glutamine, 1% MEM non-essential amino acids and antibiotics (DMEM complete). Clones derived from mouse melanoma cell lines B16BL6 (B16 wt) and B16:A2-YLEP (expresses a fusion protein between the human glycoprotein (gp)100₂₈₀₋₂₈₈ epitope (YLEPGPVTA) and the HHD molecule (23)) were cultured using DMEM complete, which in case of B16:A2-YLEP cells contained neomycin (1 mg/ml G418, Calbiochem, La Jolla, CA). T2 cells (LCLxT lymphoblastoid hybrid cell line 0.1743CEM.T2) were cultured in RPMI 1640 medium supplemented with L-glutamine, 10% FBS, and antibiotics. Mouse splenocytes were cultured in mouse T cell medium 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 μ M β -mercaptoethanol, antibiotics and 180 IU/ml human recombinant (rh)IL-2 (Proleukin; Chiron, Amsterdam, The Netherlands). For flow cytometry the following antibodies and pMHC multimers were used: anti- TCR-V β 14-FITC (Beckman Coulter, Fullerton, CA); CD3-BV510, CD8-APC-Cy7 CD4-BV650, 4-1BB-BV421, LAG3-BV421 (all BD Biosciences, San Jose, CA); TIM3-APC, BTLA-APC, PD-1-PE-Cy7, OX40-APC (all Biolegend, San Diego, CA), OX40-PE, CTLA4-PE, 4-1BB-PE, CD40L-PE, CD4-Qdot605, LAG3-APC, ICOS-APC, ICOS-PE-Cy7, CD14-PerCP (all eBiosciences, San Diego, CA); and gp100/A2K^b pMHC tetramer-PE (a kind gift by Dr. David Cole, Cardiff University, UK).

Construction of costimulatory TCRs

TCR α and β genes specific for the human gp100₂₈₀₋₂₈₈ epitope presented by HLA-A2 (gp100/A2) were derived from clone CTL-296 (24), murinized and codon-optimized as described earlier (25). Subsequently, TCR genes were cloned into the pMP71 vector (kindly provided by prof. Wolfgang Uckert, Max-Delbrück Center, Berlin, Germany) with TCR α and β genes separated by an optimized T2A ribosome skipping sequence. TCR:28 ϵ was generated as described in (22). Murine OX40, ICOS, 4-1BB and CD40L intracellular signaling domains were ordered via Genart (Life Technologies, Carlsbad, USA) and introduced into the TCR:28 ϵ format (thereby replacing the intracellular CD28 domain) via overlap PCR using Q5 High Fidelity DNA Polymerase (New England Biolabs, Ipswich, UK). The resulting TCRs: TCR:28 ϵ ; TCR:28-OX40 ϵ ; TCR:28-ICOS ϵ ; TCR:28-41BB ϵ ; and TCR:28-CD40L ϵ are schematically depicted in Figure 1. Primer sequences used for the generation of the costimulatory cassettes are available upon request. The TCR constructs were verified by sequencing, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI sequence analyzer.

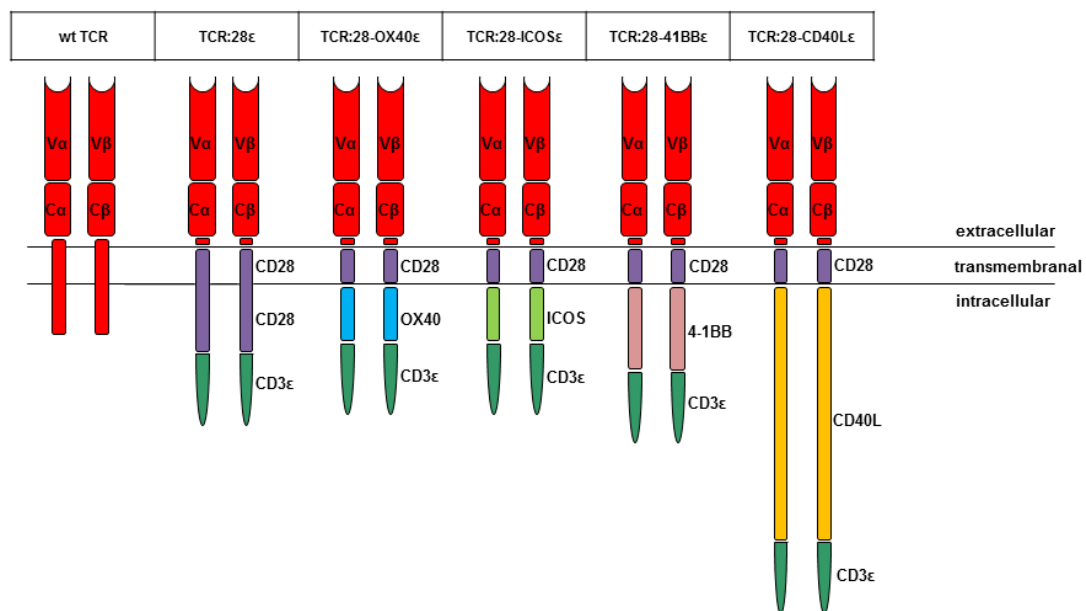


Figure 1. Schematic illustration of co-stimulatory TCRs.

Figure depicts a line-up of co-stimulatory TCRs in which transmembrane and intracellular domains of the wt TCR are replaced by the transmembrane domain of CD28, the intracellular domains of either CD28, OX40, ICOS, 4-1BB or CD40L, each followed by the intracellular domain of CD3ε. See Materials and Methods for a detailed description of origin and introduction of separate components into co-stimulatory TCRs.

T cell transduction

Total mouse splenocytes were isolated, activated with Concanavalin A (0.5µg/ml) and rhIL-2 in mouse T cell medium, and transduced with retroviral supernatant from a co-culture of TCR-transfected 293T and Phoenix-Amp cell lines as described by Pouw and colleagues (25). Control (mock) T cells were transduced with empty retroviral vector.

Flow cytometry

T cells (5×10^5) were washed with PBS and incubated with antibodies at 4°C or with pMHC tetramer at 37°C for 30 min. Following staining, T cells were washed again and fixed with 1% paraformaldehyde. Absolute T cell counts in mouse blood samples were determined using Flow-Count Fluorospheres. Stainings for co-stimulatory and co-inhibitory receptors were performed using either TCR-transduced T cells that were 24h exposed to B16:A2-YLEP or B16 wt cells, or using tumor infiltrating lymphocytes (TILs) harvested from regressing or relapsing tumors (see description of adoptive T cell therapy for time points of tumor collection). Events were acquired on a FACS Canto flow cytometer and analysed using FCS Express 4 software (BD Biosciences).

T cell stimulation assays

TCR-transduced T cells were stimulated as described above. In order to enhance HLA-A2 transgene expression, B16 cells were pre-treated with 100 IU/ml murine IFN γ for 24 h prior to in vitro assays. TCR-transduced T cells were tested at 60.000 T cells and 20.000 B16 cells per well of tissue culture-treated 96-well round-bottom plates. T cells with medium and T cells with Concanavalin A served as negative and positive controls, respectively. In some experiments, T2 cells were loaded with gp100 peptide concentrations ranging from 1 pM to 100 μ M immediately prior to co-culture with T cells. After 24h at 37°C, 5% CO $_2$, culture supernatants were harvested, and assessed for the presence of IFN γ by ELISA (eBioscience, San Diego, USA). To determine peptide concentrations required to achieve half-maximal binding, results were analyzed using GraphPad Prism.

Adoptive T cell therapy

Experiments with mice were approved by the Experimental Animal Committee of the Erasmus MC Cancer Institute and carried out in accordance with institutional and national guidelines. For adoptive T cell therapy studies we used HLA-A2 transgenic mice that express a chimeric HLA-A*0201 transgene (HHD, referred to as HLA-A2 (26)). Adoptive transfer of T cells was done as described before (22,23). In short, at day -12, mice were injected subcutaneously with 0.5×10^6 of a B16:A2-YLEP clone, and at days -4 and -3 mice received a total of two Busulfan injections intraperitoneally (16.5 μ g/kg each) and a single Cyclophosphamide injection intraperitoneally (200 mg/kg), respectively. Mice were treated with T cells at day 0 and grouped according to one of the following treatments: mock T cells (number of T cells equal to wt TCR T cell group); wt TCR T cells; TCR:28 ϵ T cells; TCR:28-OX40 ϵ T cells or TCR:28-ICOS ϵ T cells (in all TCR groups, 6×10^6 transduced T cells were administered). Tumor growth was measured by caliper 3 times a week and tumor volumes were estimated with the formula $0.4 \times (A \times B^2)$ where A represents the largest diameter and B the diameter perpendicular to A. Mice were monitored up to 115 days after administration of T cells and anti-tumor responses were classified as follows: complete responses showed no palpable tumor; partial responses showed a minimum of 30% tumor regression followed by relapse; non-responses did not reach 30% tumor regression. The day of relapse was defined as the first day with detectable size-increase of tumor that took place over the course of at least 3 days, preceded by at least 30% tumor regression. Survival was monitored daily during the same time period. Peripheral blood was collected at day 7 after T cell transfer and at weekly intervals thereafter, and used to determine numbers of CD3 $^+$ T cells. Tumor tissues of regressing tumors were collected at day 5 after T cell transfer and tumor tissues of relapsing tumors were collected when mice were sacrificed due to tumor burden exceeding 1400mm 3 . All tissues were snap-frozen in liquid nitrogen and stored at -80°C.

Isolation of TILs

Resected tumor tissue was cut up into small fragments, incubated with collagenase (Sigma Aldrich, St Louis, USA) and passed through a 70 μ m cell strainer to produce a single cell suspension. Cells were subsequently cultured in mouse T cell medium supplemented with rhIL-2 (180 IU/ml) at a concentration of 50 000 cells/ml. For flow cytometric analysis, cells were harvested after 72 h.

Statistical analyses

The different TCR treatment groups were compared with the Student's t-tests, or the Mantel-Cox test in case of survival data using GraphPad Prism5 (GraphPad Software, La Jolla, CA). P values < 0.05 were considered statistically significant.

RESULTS

Co-stimulatory TCRs mediate lowered T cell avidity in vitro

We designed a set of five co-stimulatory TCRs specific for human gp100₂₈₀₋₂₈₈ epitope, all harboring a three-party fusion of the transmembranal (tm) domain of CD28, the intracellular (ic) domains of either CD28, OX40, ICOS, 4-1BB or CD40L, and the ic domain of CD3 ϵ (see figure 1). When assessing the surface expression of these novel TCRs after transduction into mouse splenocytes, it became apparent that T cells transduced with wt TCR showed TCR V β 14-specific antibody staining in 55% of cells (see figure 2A, upper left). In contrast, T cells transduced with TCR:28 ϵ , TCR:28-OX40 ϵ and TCR:28-ICOS ϵ showed antibody staining in 22-26% of cells, while T cells transduced with TCR:28-41BB ϵ or TCR:28-CD40L ϵ showed antibody staining in 0-6% of cells. Staining of T cells with pMHC was in line with these observations (figure 2A, upper right). Although inclusion of tm CD28 into these new TCRs is based on the format of TCR:28 ϵ with reported surface expression and function ((22); and this manuscript), we did test inclusion of the autologous tm domain (i.e., TCR:41BB ϵ), but were not able to rescue surface expression (Supplementary figure 1). Due to the lack of functional expression of TCR:28-41BB ϵ and TCR:28-CD40L ϵ , both constructs were omitted from further experiments. Notably, while co-stimulatory TCRs showed expression in a lowered fraction of T cells, expression levels of individual TCR molecules per cell were enhanced on both CD4⁺ and CD8⁺ T cells (see MFI of pMHC stainings, figure 2A, lower panels).

Next to expression levels, we assessed the ability of surface expressed co-stimulatory TCRs to mediate T cell activation upon stimulation with cognate epitope. When stimulating T cells using titrated amounts of gp100 peptide, we observed that TCR:28 ϵ , TCR:28-OX40 ϵ and TCR:28-ICOS ϵ T cells produced lower amounts of IFN γ compared to wt TCR T cells (see figure 2B). EC₅₀ values, which equals the amount of peptide necessary to result in half-maximal IFN γ production, revealed the following hierarchy with respect to functional T cell avidity: wt TCR (EC₅₀: 1.3nM), TCR:28 ϵ (18.7nM), TCR:28-ICOS ϵ (23.9nM) and TCR:28-OX40 ϵ (47.7nM). In addition to cytokine production, we also performed flow cytometric analysis of TCR-transduced T cells following co-culture with antigen-positive tumor cells. These analyses did not reveal significant differences among TCRs with respect to antigen-induced expression of co-signalling receptors in either CD4⁺ or CD8⁺ T cells (see supplementary figure 2).

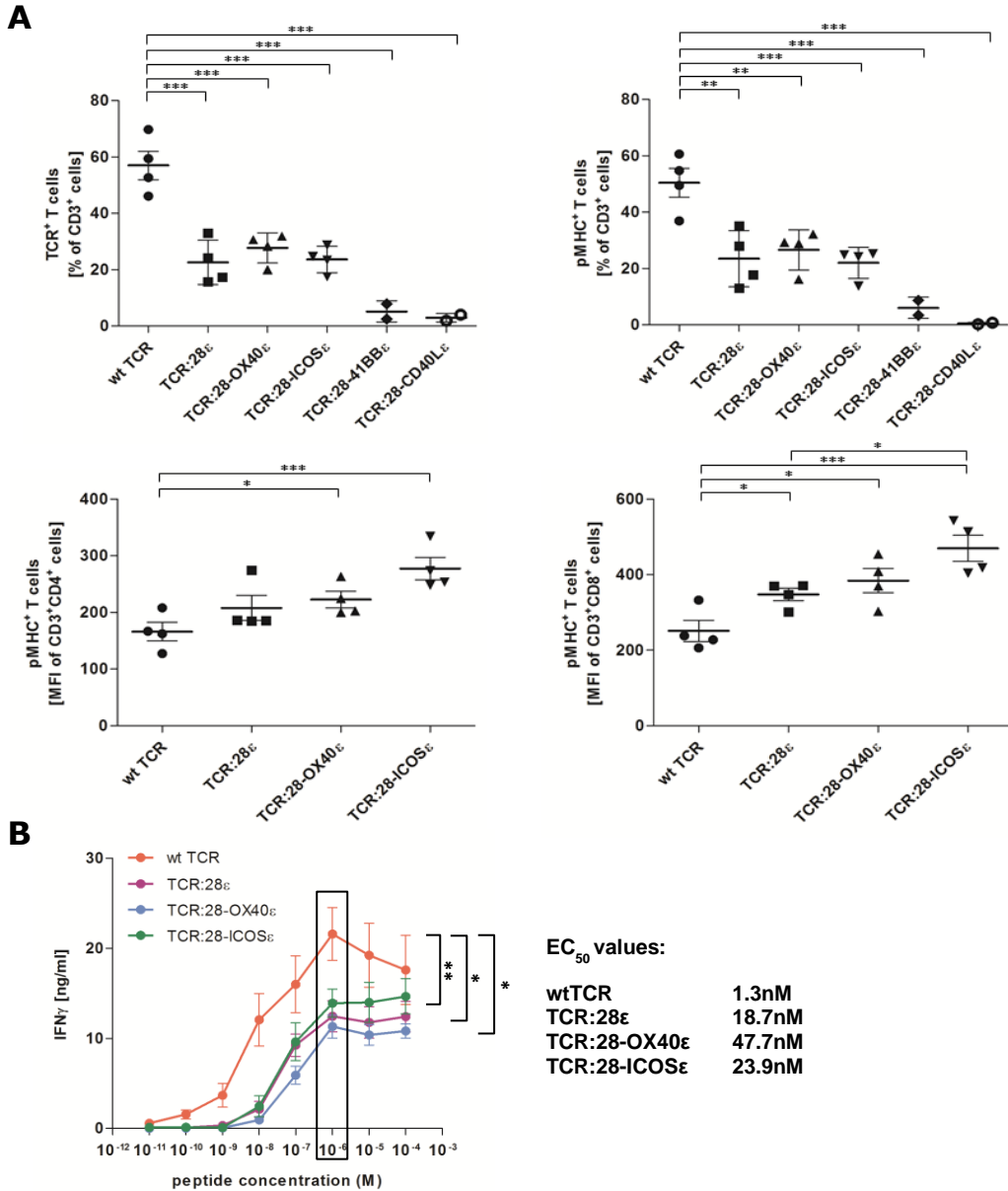


Figure 2. Co-stimulatory TCRs mediate lowered peptide responsiveness in vitro.

(A) T cells were transduced with wt TCR, TCR:28ε, TCR:28-OX40ε, TCR:28-ICOSε, TCR:28-41BBε or TCR:28-CD40Lε (all TCRs specific for human gp100/A2) and analysed by flow cytometry following staining with TCR-specific Vβ14 antibody or a gp100/A2 tetramer in combination with CD3, CD4 and CD8 antibodies. Results are displayed as mean % of T cells positive for TCR transgene or MFI of T cells binding for pMHC (±SEM, n=3-5). (B) T cells from (A) were co-cultured with T2 cells pulsed with a titrated amount from 100μM to 10pM of human gp100 (YLEPGTVPA) peptide for 24h. IFNγ levels in culture supernatants were measured by ELISA, and displayed as mean (±SEM, n=3-5). EC₅₀ values were calculated as mentioned in Materials and Methods, and listed in figure. Statistical significant differences between wt and co-stimulatory TCR T cells were calculated with Student's t-test: *p<0.05; **p<0.005; ***p<0.005.

T cells expressing co-stimulatory TCRs, in particular those with OX40 or ICOS, delay tumor recurrence and prolong survival

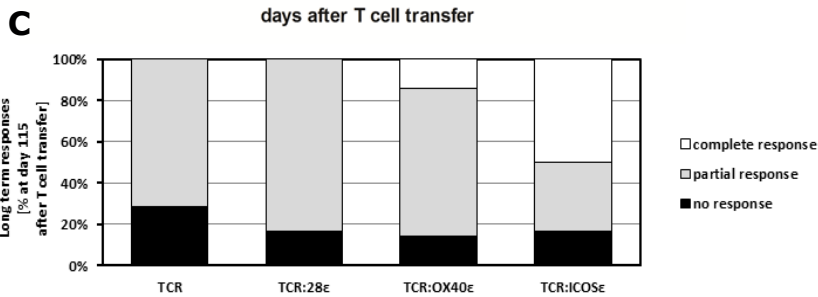
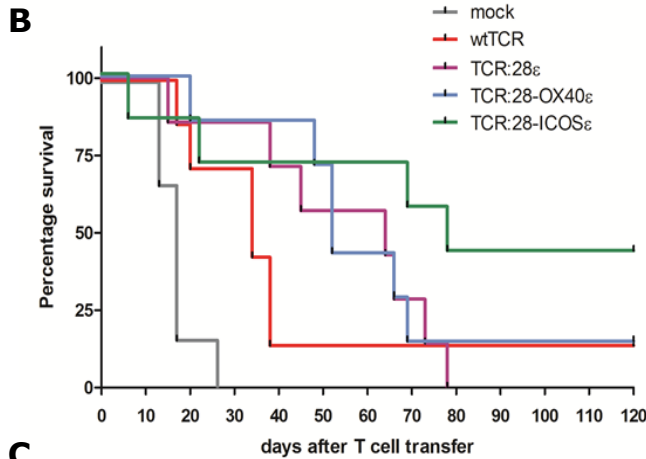
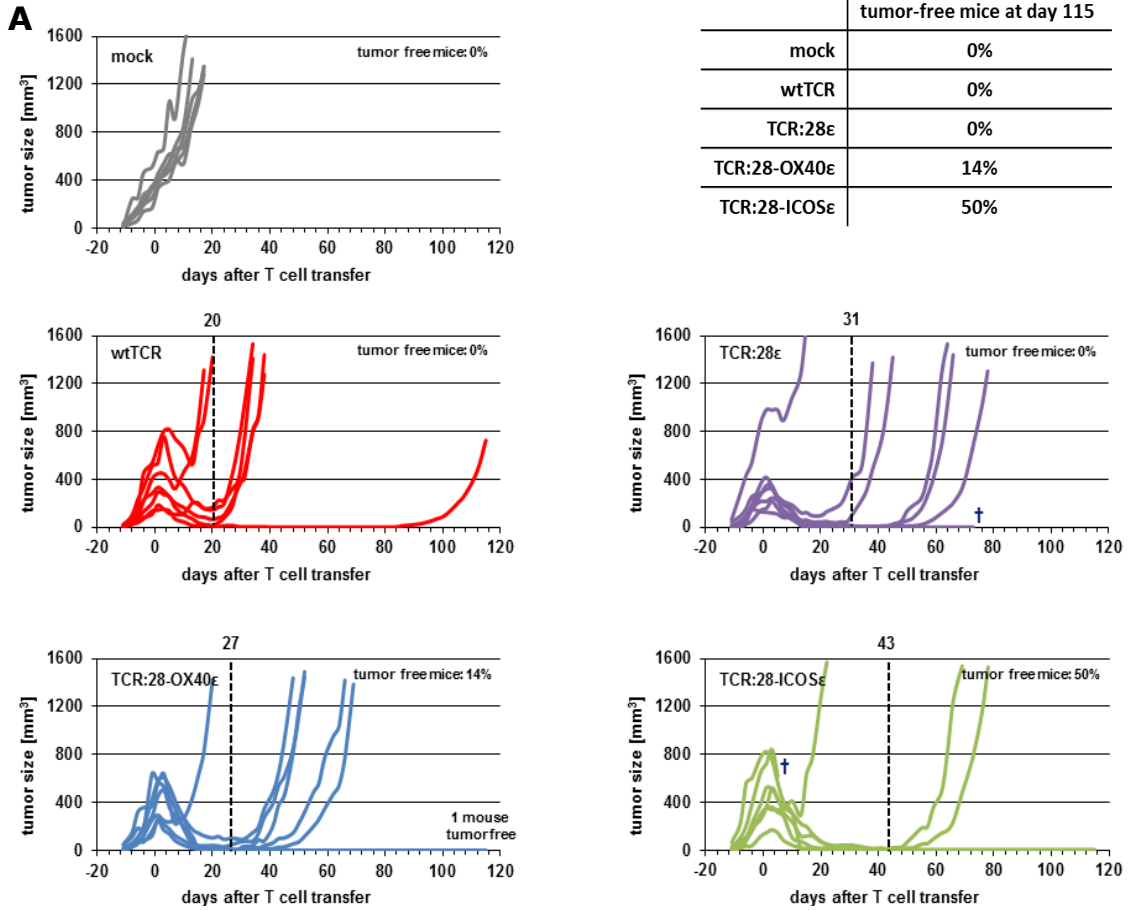
HLA-A2 transgenic mice with palpable B16:A2-YLEP tumors ($\sim 400\text{mm}^3$) received chemotherapy and a single transfer of either 6×10^6 wt TCR, TCR:28 ϵ , TCR:28-OX40 ϵ , TCR:28-ICOS ϵ or mock T cells (see Materials and Methods for details). While mice treated with wt TCR T cells showed initial regression upon treatment, all mice showed tumor relapse generally around day 20 after T cell transfer (median day of relapse, see figure 3A). Tumor relapse did not occur or was significantly delayed in all groups treated with co-stimulatory TCR T cells. Notably, 50% of mice receiving TCR:28-ICOS ϵ T cells remained tumor free till the end of the experiment (day 115 after start of T cell transfer) and the other 50% of treated mice demonstrated tumor relapse at day 43. In case of treatments with TCR:28-OX40 ϵ T cells or TCR:28 ϵ T cells, percentages of tumor-free mice at end of experiment were 14% and 0%, respectively, and days of tumor relapse were 27 and 31, respectively. Moreover, when analysing survival and responsiveness to treatment, it became apparent that the TCR:28-ICOS ϵ T cells mediated highest survival (see figure 3B) which coincided with the highest rates of complete and partial responses (see figure 3C).

OX40 and ICOS TCRs result in enhanced persistence in blood and accumulation of T cells in tumor tissue, in particular of CD8 T cells

Weekly peripheral blood measurements of treated mice revealed that co-stimulatory TCR T cells were present in larger numbers throughout therapy when compared to wtTCR T cells (see figure 4A). T cell numbers usually peaked at day 14 after transfer, coinciding with the time point of maximal tumor regression in all treated groups. The number of wt TCR T cells stabilized at 65 per μl over the following weeks, whereas those of TCR:28 ϵ , TCR:28-OX40 ϵ and TCR:28-ICOS ϵ T cells stabilized at an average of 100, 160 and 265 per μl , respectively. Remarkably, the ratio of CD8 $^+$ to CD4 $^+$ T cells shifted in T cells expressing co-stimulatory TCRs. While wt TCR T cells demonstrate a ratio of 0.12, TCR:28 ϵ , TCR:28-OX40 ϵ and TCR:28-ICOS ϵ T cells demonstrate ratios of 0.9, 2.2 and 1.3, respectively (see figure 4B). Notably, we observed that the total number of TCR $^+$ T cells one week after transfer correlated to the day of tumor relapse, and in fact served as a predictive marker for therapy efficacy (see figure 5C).

Figure 3. Adoptive T cell transfer with TCRs equipped with OX40 or ICOS domains delay tumor recurrence and enhance overall survival of melanoma-bearing mice

HLA-A2 transgenic mice bearing established tumors derived from inoculated B16:A2-YLEP clone (day -12) were conditioned with Busulfan and Cyclophosphamide (days -4 and -3) and treated with 6×10^6 mock T cells or T cells transduced with wt TCR, TCR:28 ϵ , TCR:28-OX40 ϵ or TCR:28-ICOS ϵ (day 0). Figure (A) depicts tumor growth curves of individual mice ($n=6-7$ per group). Tumor sizes were measured 3 times a week with a caliper. Dashed lines indicate median day of tumor relapse per treatment group (definition of tumor relapse is described in Materials and Methods). Percentages of mice being tumor-free at day 115 after T cell transfer are listed in each panel. '+' indicates death of mouse unrelated to tumor outgrowth. Figure (B) presents overall survival curves of treatment groups. In figure (C) stacked bars represent % of mice with different anti-tumor responses to therapy at day 115 after T cell transfer as defined in Materials and Methods.



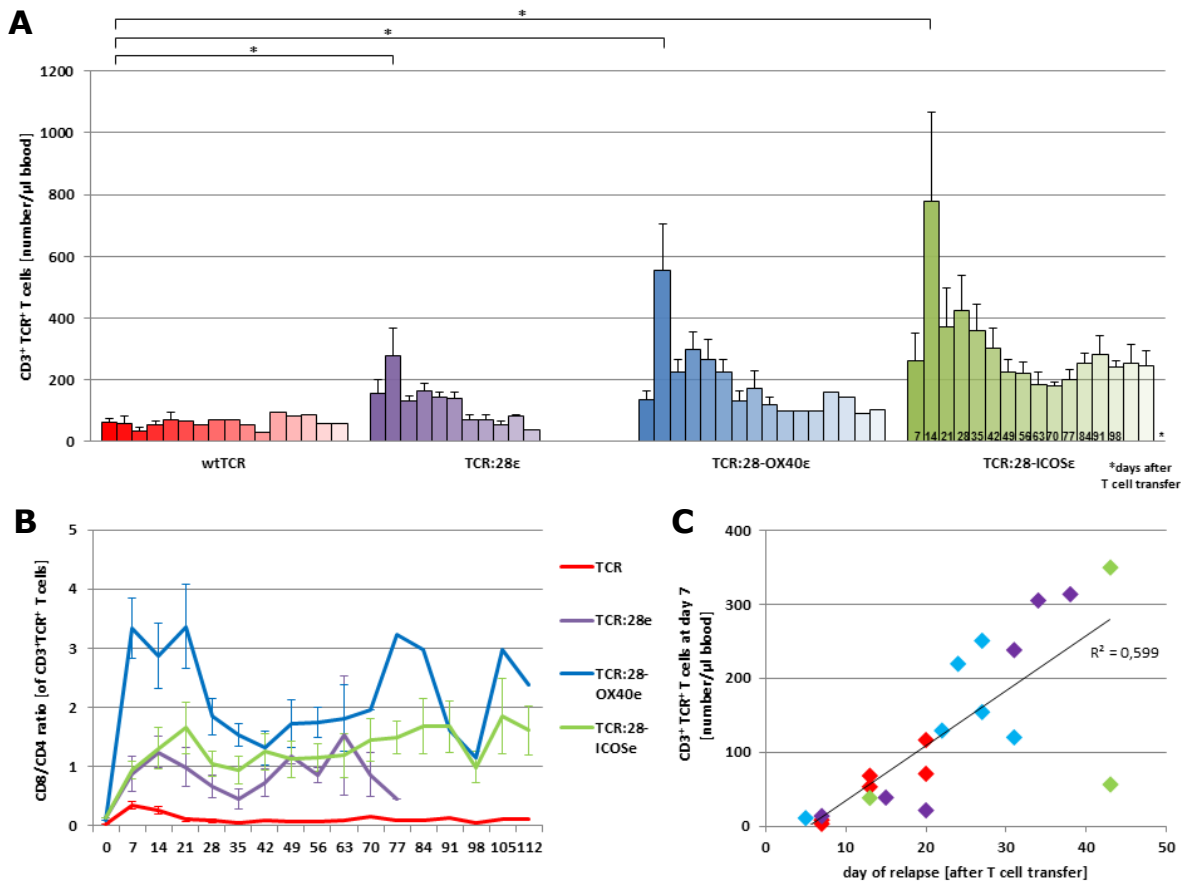


Figure 4. T cells expressing OX40 or ICOS-containing TCR show enhanced persistence in blood following adoptive transfer and are enriched for CD8 T cells

HLA-A2 transgenic mice bearing established B16:A2-YLEP tumors were conditioned with chemotherapy and treated with T cells as described in legend to figure 3. **(A)** Peripheral blood was collected from mice at the indicated time points after T cell transfer and absolute numbers of TCR⁺CD3⁺ T cells were determined by flow cytometry using Flow-Count Fluorospheres. Data are presented as mean numbers per μl blood ±SEM (n=1-7, dependent on survival of mice). Statistically significant differences between T cell numbers of mice treated with wt TCR T cells and co-stimulatory TCR T cells on day 14 after transfer were calculated with Student’s t-test: *p<0.05. **(B)** Data are presented as mean ratio of CD8⁺:CD4⁺ T cells from (A). At the time of transfer, CD8⁺:CD4⁺ T cell ratios were as follows: wt TCR = 0.03; TCR:28ε = 0.14; TCR:28-OX40ε = 0.1 and TCR:28-ICOSε = 0.14. **(C)** Day of relapse (defined in Materials and Methods) was correlated with total number of TCR⁺CD3⁺ T cells in blood at day 7 after T cell transfer. Color scheme for individual mice is identical to (A) and (B).

Our T cell analyses in peripheral blood were supported by the analyses done in tumors. After isolation of TILs from regressing tumors, we observed that TCR⁺ T cells were present at higher frequencies following treatment with TCR:28-OX40ε and TCR:28-ICOSε T cells (~12% of total CD3⁺ T cells), whereas TCR⁺ T cells were present at lower frequencies following treatment with wt TCR and TCR:28ε T cells (~4% of total CD3⁺ T cells) (see figure 5A). The frequencies of TCR⁺ T cells in relapsing tumors dropped ~10-fold in all treatment groups when compared to regressing tumors, with the exception of the TCR:28-ICOSε group. The frequency of TCR⁺ T cells was 0.1% following treatment with wtTCR T cells, whereas the frequencies of TCR⁺ T cells increased to 0.6%

and 1.8% following treatment with TCR:28ε and TCR:28-OX40ε T cells, respectively. In case of treatment with TCR:28-ICOSε T cells, relapsing tumors were marked by a substantially increased frequency of 13% TCR⁺ T cells. Again, the ratios of CD8⁺:CD4⁺ TILs were inverted in all co-stimulatory TCR T cell-treated mice when compared to wt TCR (see figure 5B). Next, we investigated whether tumor recurrence was associated with differential expression of co-signalling receptors in CD8⁺ TILs. Flow cytometry analyses revealed that CD8⁺ TILs of mice treated with TCR:28-ICOSε showed an enhanced fraction of T cells positive for CD40L (see figure 6A), while CD8⁺ TILs from mice treated with any co-stimulatory TCR displayed a trend of increased frequencies of T cells positive for PD1, LAG3 or TIM3 (see figure 6A).

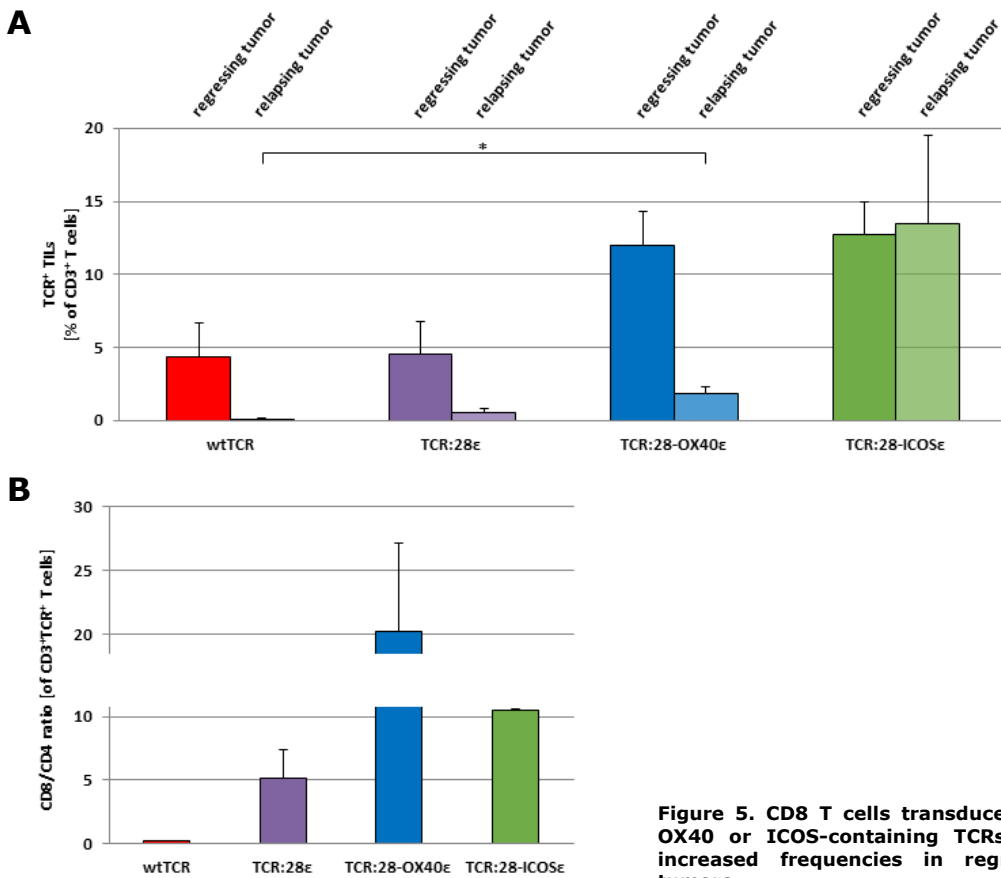


Figure 5. CD8 T cells transduced with OX40 or ICOS-containing TCRs show increased frequencies in regressing tumors

Mice were treated as described in legend to figure 3 and tumor tissues of regressing tumors were collected from 2 mice per treatment group at day 5 after T cell transfer. Tumor tissues of relapsing tumors were collected following outgrowth of tumors (>1400mm³) (n=2-5 per group). TILs, obtained as described in Materials and Methods and defined as CD3⁺ fraction of cells isolated from tumor tissue, were analysed via flow cytometry following staining with CD3, CD4, CD8 and TCR-specific Vβ14 antibodies. Figure (A) displays percentages of TCR⁺ T cells found within short-term cultures of CD3⁺ T cells of regressing and relapsing tumors, while figure (B) shows the ratios of CD8⁺/CD4⁺ T cells within TCR⁺CD3⁺ T cells of regressing tumors. Statistical significant differences between wt and co-stimulatory TCR T cells were calculated with Student's t-test: *p<0.05.

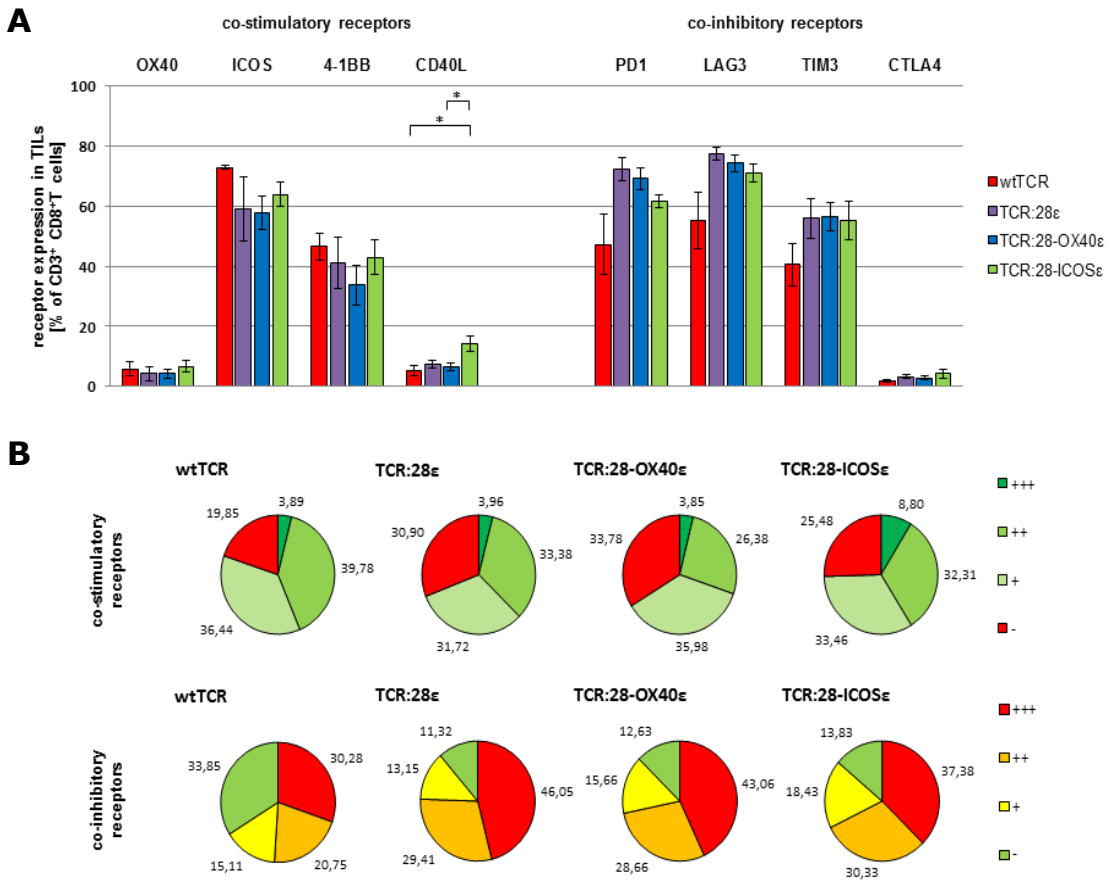


Figure 6. TILs expressing TCR:28-ICOS ϵ show enhanced % of CD40L-positivity

TILs as described in Figure 5 underwent flow cytometric analysis of the expression of co-stimulatory as well as co-inhibitory receptors in the CD8⁺ TILs. **(A)** This figure depicts the mean % of CD8⁺ TILs positive for the indicated receptors (\pm SEM, n=2-6 per group) in relapsing tumors. Statistical significant differences between wt and co-stimulatory TCR T cells were calculated with Student’s t-test: *p<0.05; **p<0.005; ***p<0.005. **(B)** this figure displays the distribution (in mean %) of CD8 TILs (n=2-6 per group) that are either negative for co-stimulatory receptors (as in A) or positive for 1, 2 or 3 co-stimulatory receptors (upper panel). The same analysis has also been performed for co-inhibitory receptors (lower panel).

DISCUSSION

In this study we have successfully engineered novel co-stimulatory TCRs equipped with the intracellular domains of either CD28, OX40, ICOS, 4-1BB or CD40L. This approach has been previously employed in chimeric antigen receptors (second or third generation CARs). These co-stimulatory CARs were shown to enhance tumor regression in a therapeutic setting (19,20,27). Similar modifications, i.e., inclusions of heterologous domains into TCR chains, proved challenging given the complex mode of surface expression, antigen binding and function of TCRs. Re-iterating design and experimental testing of TCRs containing CD28 and CD3 ϵ (22) allowed us to define non-autologous amino acid boundaries that minimally curtail TCR surface expression and function, providing a basis for the *in vitro* and *in vivo* evaluation of these new TCRs.

T cell transductions revealed that levels of surface expression of TCR:28 ϵ , TCR:28-OX40 ϵ and TCR:28-ICOS ϵ per CD8⁺ T cell were enhanced up to 2-fold, whereas the fractions of T cells positive for these co-stimulatory TCRs were about half that of T cells positive for wt TCR (25 vs 50% expression). The enhanced per-cell expression levels are most likely due to reduced mis-pairing of endogenous and introduced TCR chains directed by the tm CD28 domain, as evidenced by Govers and colleagues for TCR:28 ϵ (22). The reduced fractions of T cells expressing the co-stimulatory TCRs may be due to multiple reasons. For one, introduction of non-autologous sequences into a TCR may carry the risk of changing the structure of the TCR in a way that impedes surface expression. In a related manner, lack of surface expression may be linked to an inverse effect of size of the total transgene. This is supported by the complete lack of expression of TCR:28-CD40L ϵ , a transgene that is 1.5 to 2-fold larger in size than wt TCR. This finding is in extension to the reported negative impact on surface expression by increased size of 3rd generation CARs that were equipped with multiple intracellular domains (18). Second, the natural dimeric arrangement of TCR α - and β -chains may interfere with proper trimer formation that is naturally required for surface expression and function of the TNFR superfamily members OX40 and 4-1BB (28). Co-stimulatory molecules derived from the immunoglobulin superfamily members CD28 and ICOS normally form dimers, and although speculative, may be a better fit for the TCR format. Along these lines, the successful incorporation of OX40 or 4-1BB domains into CARs, and other domains such as CD28 or ICOS for that matter as well (20,29,30), may be due to the monomeric nature and thus intrinsic flexibility of CARs. To resolve the intrinsic inflexibility of TCRs, we have conducted experiments with TCR:4-1BB variants, but could not rescue the expression of this TCR despite inclusion of tm 4-1BB (and restoring non-autologous boundaries) (Supplementary figure 1). Functional assessment of T cells expressing co-stimulatory TCRs revealed a lowered functional avidity as determined by EC₅₀ values of peptide-induced IFN γ production. This lowered avidity may be in part the result of the structural restraints between the extracellular TCR and the CD28 tm domains (22) as mentioned above. However, the EC₅₀ differences between TCR:28-OX40 ϵ and TCR:28-ICOS ϵ T cells, despite similar surface expression levels, also suggest distinct signalling downstream of these TCRs. Production of other cytokines, such as IL-2 (31), is expected to be enhanced in T cells equipped with additional CD28, while ICOS signalling was shown to induce only

a minor increase in IL-2 production (32-34). In addition, we expect a reduced production of IL-10 by TCR:28-OX40 ϵ T cells, as was observed in CARs equipped with such a co-stimulatory domain (35). Besides, cytokine production, we have also assessed the expression of co-signaling receptors following antigen-stimulation, and found no significant differences with respect to surface expression of these receptors *in vitro* (see supplementary figure 2).

When testing co-stimulatory TCRs in an adoptive therapy setting, we observed significant delay of tumor recurrence and, in particular in the case of TCR:28-ICOS ϵ T cells, complete and long-term tumor remissions were observed in half of the mice. Similar results with regard to tumor clearance were observed in CARs equipped with ICOS signalling when treating mice suffering from glioma and mesothelioma (33,36). TCR:28-OX40 ϵ and TCR:28 ϵ performed similarly with respect to delaying tumor relapse when compared to wt TCR, but lagged behind TCR:28-ICOS ϵ T cells with respect to complete responses. In an effort to explain this superior response *in vivo*, we have assessed peripheral persistence and accumulation within tumor tissues of T cells expressing co-stimulatory TCRs. All co-stimulatory TCRs under study stood out for their enhanced persistence in the blood, with TCR:28-ICOS ϵ T cells again performing best with >200 TCR⁺ T cells/ μ l blood being detectable even 115 days after T cell transfer. In addition, the CD8⁺:CD4⁺ ratio of peripheral T cells was significantly enhanced up to 18-fold when comparing co-stimulatory TCRs versus wt TCR.

While sufficient numbers of CD4⁺ T helper cells are required to maintain and support tumor rejection, it noteworthy that sufficient numbers of cytotoxic CD8⁺ T cells are critical to ultimately result in effective tumor rejection. The increases in absolute numbers of both CD4⁺ and CD8⁺ T cells, together with the relative increase in CD8 T cells, are indicative of an ongoing and effective immune response in our model. Moreover, total numbers of TCR⁺ T cells measured in blood at day 7 after T cell transfer correlated with start of relapse and were in fact indicative of non-responsiveness to therapy in case of very low TCR⁺ T cell frequencies. Survival and persistence of adoptively transferred T cells in the blood stream have been rendered crucial factors for durable anti-tumor responses in clinical studies (37,38).

Our observations in peripheral blood of enhanced numbers of TCR⁺ T cells were mirrored in regressing tumors. Regressing tumors of mice treated with TCR:28-OX40 ϵ or TCR:28-ICOS ϵ T cells showed higher percentages of TCR⁺ T cells within TILs, and all groups treated with co-stimulatory TCR T cells showed an enhanced ratio of CD8⁺:CD4⁺ TILs when compared to wt TCR T cells. These findings extend earlier reports where enhanced CD8 to CD4 TIL ratios have been identified as beneficial prognostic factor in preclinical and clinical studies (39,40). The observed CD8 T cell dominance also matches studies in which ICOS engagement was found to promote effector status, function and persistence of CD8⁺ T cells (41,42). Indeed, flow cytometric assessment of CD8⁺ TILs within relapsing tumors revealed that treatment with co-stimulatory TCRs enhanced the frequencies of T cells expressing co-inhibitory receptors, indicative of enhanced frequencies of T cells in an activated state (reviewed in (43)). The enhanced performance of both TCR:28-OX40 ϵ or TCR:28-ICOS ϵ T cells compared to TCR:28 ϵ may be explained by the CD28-independent signaling of the former two TCRs (14,15). As CD8⁺ TILs are generally marked by enhanced PD-1

expression, and PD-1 inhibits CD28 signaling (14,15), TCR:28-OX40 ϵ or TCR:28-ICOS ϵ T cells may be less susceptible to PD-1-mediated T cell inactivation. Notably, the expression of CD40L is significantly up-regulated in CD8⁺ TILs following treatment with TCR:28-ICOS ϵ T cells, which suggests that additional ICOS-signalling aids to their activation state (44). CD40L may favourably affect DC:T cell interactions, and these interactions are generally considered beneficial to the outcomes of adoptive T cell therapy (45,46). The exact contribution of the CD4⁺ T cells towards the enhanced therapeutic effect of TCR:28-ICOS ϵ T cells deserves further studies. Along these lines, it is noteworthy that enhanced frequency of CD4⁺ICOS^{high} T cells was accompanied by enhanced tumor reactivity in studies utilizing CTLA-4 blockade (47,48). On the other hand, ICOS signalling is reported to be of particular importance in the development of CD4⁺ T helper 17 (T_{H17}) and T regulatory cells (49-54).

The enhanced T cell numbers within tumor tissues may also be linked to a change in metabolism mediated by TCRs with additional co-signalling elements. Indeed, Cao and colleagues showed that upon activation, CD8⁺ T cells up-regulated their energy production through means of glycolysis in order to cope with the increased demands in proliferation and effector function (55). Interestingly, triggering of ICOS as well as OX40 have been reported to favor a switch towards a glycolytic metabolism via the PI3K-Akt-mTOR pathway at the expense of oxidative phosphorylation (56-58). Indeed, Kawalekar and colleagues demonstrated that CARs equipped with co-stimulatory signaling cassettes may show differential metabolic exhaustion (59). To this end, we are currently studying the effects of co-stimulatory TCRs on transcriptomics and metabolomics.

Taken together, we show that equipping TCRs with co-stimulatory domains, in particular ICOS, significantly delays tumor recurrence, results in enhanced numbers of peripheral and intra-tumoral CD8 T cells, and mediates complete responses, providing a rationale for further studies into this TCR format with respect to adoptive T cell therapy.

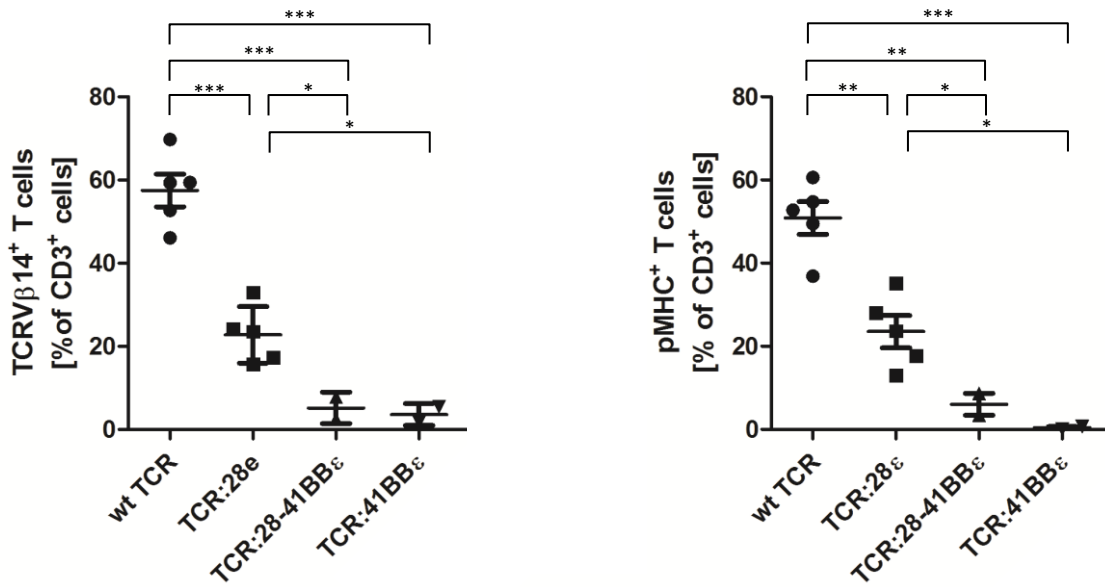
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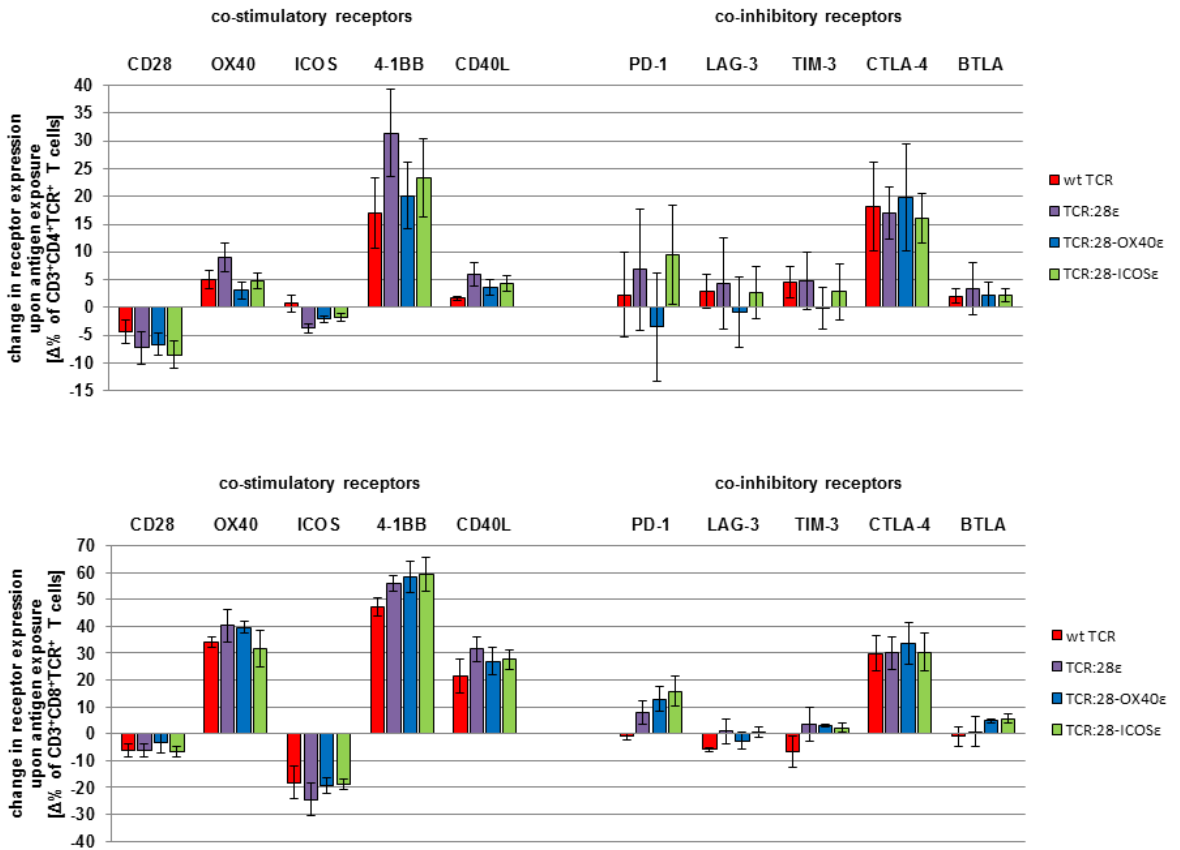
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Supplementary Figure 1: Surface expression and pMHC binding of TCRs that incorporate 4-1BB is not rescued by the autologous transmembranal domain of 4-1BB.

T cells were transduced with the following TCR variants: wt TCR, TCR:28ε, TCR:28-41BBε, TCR-41BBε (with tm 4-1BB). TCR-transduced T cells were stained with (A) anti-Vβ14 antibody or (B) gp100/A2 pMHC, and analyzed by flow cytometry as described in legend to Figure 2. Results are displayed as mean % of T cells positive for TCR or T cells that bind pMHC (±SEM, n=2-5). Statistical significant differences between wt and co-stimulatory TCR T cells were calculated with Student's t-test: *p<0.05; **p<0.005; ***p<0.005.



Supplementary Figure 2. Co-stimulatory TCRs do not mediate differential expression of co-signaling receptors following antigen exposure in vitro

T cells transduced as described in legend to figure 2A were co-cultured with either B16:A2-YLEP or B16wt cells for 24h. Subsequently, T cells were analyzed by flow cytometry for expression of co-stimulatory (CD28, OX40, ICOS, 4-1BB and CD40L) and co-inhibitory receptors (PD-1, LAG-3, TIM-3, CTLA-4 and BTLA) in both CD4⁺ (upper panel) and CD8⁺ (lower panel) subsets of TCR⁺ T cells. Bars depict the mean change in % (\pm SEM, n=3) upon antigen stimulation compared to non-antigen stimulation (the latter set to 100%). Statistical significant differences between wt and co-stimulatory TCR T cells were assessed with Student's t-test

65

INTRA-TUMORAL PRODUCTION OF IL-18, BUT NOT IL-12 BY TCR-ENGINEERED T CELLS IS NON-TOXIC AND COUNTERACTS IMMUNE EVASION OF SOLID TUMORS

**Andre Kunert¹, Markus Chmielewski², Rebecca Wijers¹, Cor Berrevoets¹,
Hinrich Abken², Reno Debets¹**

¹Laboratory of Tumor Immunology, Department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands;

²Department I of Internal Medicine; University Hospital Cologne and Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany

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ABSTRACT

Adoptive therapy with engineered T cells shows promising results in treating patients with malignant disease, but is challenged by incomplete responses and tumor recurrences. Here, we aimed to direct the tumor microenvironment in favor of a successful immune response by local secretion of interleukin (IL-) 12 and IL-18 by administered T cells. To this end, we engineered T cells with a melanoma-specific T cell receptor (TCR) and murine IL-12 and/or IL-18 under the control of a nuclear-factor of activated T-cell (NFAT)-sensitive promoter. These T cells produced IL-12 or IL-18, and consequently enhanced levels of IFN γ , following exposure to antigen-positive but not negative tumor cells. Adoptive transfer of T cells with a TCR and inducible (i)IL-12 to melanoma-bearing mice resulted in severe, edema-like toxicity that was accompanied by enhanced levels of IFN γ and TNF α in blood, and reduced numbers of peripheral TCR transgene-positive T cells. In contrast, transfer of T cells expressing a TCR and iIL-18 was without side effects, enhanced the presence of therapeutic CD8⁺ T cells within tumors, reduced tumor burden and prolonged survival. Notably, treatment with TCR+iIL-12 but not iIL-18 T cells resulted in enhanced intra-tumoral accumulation of macrophages, which was accompanied by a decreased frequency of therapeutic T cells, in particular of the CD8 subset. In addition, when administered to mice, iIL-18 but not iIL-12 demonstrated a favorable profile of T cell co-stimulatory and inhibitory receptors. In conclusion, we observed that treatment with T cells engineered with a TCR and iIL18 T cells is safe and able to skew the tumor microenvironment in favor of an improved anti-tumor T cell response.

INTRODUCTION

Up to now, dozens of clinical trials have demonstrated the effectiveness of adoptive therapy with T cells that are gene-engineered with T cell receptors (TCRs) or chimeric antigen receptors (CARs). Therapeutic successes are most prominent for the treatment of hematological malignancies (1-6), whereas in the majority of patients with solid tumors, initial regressions are challenged by rapid tumor recurrences.

Patient responsiveness to immune therapies appears to be related to numbers and location of tumor infiltrating lymphocytes (TILs) (7,8). Tumors that have low or negligible numbers of TILs may demonstrate intrinsic changes that result in limited T cell infiltration, movement and activation, and as such pose barriers to T cell treatments when compared to tumors with high numbers of TILs (9,10). In fact, predictive value towards response to clinical T cell therapies has recently been attributed to determinants of immunogenicity, such as extent of antigen presentation, composition of immune infiltrates, and presence of inhibitors of T cell activity (11). Expectedly, intervention with such determinants has the ability to enhance the sensitivity of tumors for T cell treatment (12).

Here, we investigated to what extent local depositions of IL-12 and IL-18 in the tumor tissue would enhance the efficacy of adoptive T cell therapy. IL-12 is a heterodimer that consists of the subunits p35 and p40 and, although primarily produced by antigen-presenting cells, it is also a product of T cells (13,14). Initial studies showed IL-12's ability to enhance the cytolytic activity of T cells (15), aid in the recruitment of macrophages (16), and induce a TNF α -mediated shift towards M1-type macrophages (17,18). IL-18 is a pro-inflammatory IL-1-like cytokine that is primarily produced by macrophages and is able to drive T and NK cell maturation (reviewed in (19)). Both cytokines enable an immunological shift towards Th1-type T cell responses. Notably, IL-12 increases the proportion of T cells expressing the IL-18 receptor 1 subunit (IL-18R1), resulting in further enhancement of IFN γ production by IL-12 plus IL-18-stimulated T cells (20,21). Despite IL-12's beneficial impact on anti-tumor immune responses in preclinical models, which also holds in early-phase trials, systemic administration of recombinant IL-12 has been reported to result in hepatotoxicity and high systemic levels of IFN γ (22,23). In contrast, administration of IL-18 revealed no such toxicities in studies with melanoma and renal cell cancer patients, however, did not reveal clinical responses either (24-26). We argue that delivery of these two cytokines directly at the site of the tumor, either alone or together, will minimize toxic effects and at the same time maximize immune-stimulatory effects. In earlier studies, we have shown that the use of CAR-engineered T cells with inducible IL-12 (iIL-12) enhanced tumor killing without toxicity by increasing the numbers and activation of tumor infiltrating macrophages (18). In the current study, we have extended these findings and exploited TCR-engineered T cells to provide antigen-specific and intra-tumoral release of IL-12 and IL-18, and investigated the impact of these enhanced T cells on tumor growth, toxicity, T cell phenotype and function.

MATERIALS AND METHODS

Cell culture and reagents

The human embryonic kidney 293T and Phoenix-Amp cell lines, both used to package retroviruses carrying RNA encoding TCR α and/or iIL-12 or iIL-18, were grown in DMEM with 10% (v/v) Fetal Bovine Serum (FBS; Greiner Bio-one Alphen a/d Rijn, The Netherlands), 200nM L-glutamine, 1% (v/v) MEM non-essential amino acids and antibiotics (DMEM complete). Clones derived from mouse melanoma cell lines B16BL6 (B16WT) and B16:A2-YLEP (gene-engineered to express a fusion protein between the human glycoprotein (gp)100₂₈₀₋₂₈₈ epitope (YLEPGPVTA) and the HHD molecule (27)) were cultured using the same medium, which in case of B16:A2-YLEP cells contained neomycin (1 mg/ml) (G418, Calbiochem, La Jolla, CA). In order to enhance HLA-A2 transgene expression, B16 cells were pre-treated with 100 IU/ml murine IFN γ (Sanquin, Amsterdam, The Netherlands) for 24 hours prior to in vitro assays. Mouse splenocytes were cultured in mouse T cell medium 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 μ M β -mercaptoethanol, antibiotics and 50 IU/ml human recombinant IL-2 (Proleukin; Chiron, Amsterdam, The Netherlands). For flow cytometry the following antibodies and pMHC multimer were used: anti-TCR-V β 14-FITC (Beckman Coulter, Fullerton, CA); CD3-PE, CD3-BV510, Ly6G-PE-Cy7, CD8-APC-Cy7, F4/80-APC-Cy7, CD4-BV650, 4-1BB-BV421, LAG3-BV421 (all BD Biosciences, San Jose, CA); TIM3-APC, BTLA-APC, PD-1-PE-Cy7, OX40-APC (all Biolegend, San Diego, CA); CD45-FITC, OX40-PE, CTLA4-PE, 4-1BB-PE, CD40L-PE, CD4-Qdot605, LAG3-APC, ICOS-APC, ICOS-PE-Cy7, CD14-PerCP, CD335-eF660 (all eBiosciences, San Diego, CA); and gp100/A2Kb pMHC tetramer-PE (a kind gift by Prof. Andrew Sewell, Cardiff University, UK). For immunofluorescent stainings, the following primary and secondary antibodies were used: rat anti-mouse CD3 (clone 17A2), rat anti-mouse CD335 (clone 29A1.4), rat anti-mouse Ly6G (clone RB6-8C5) (all three from eBiosciences) and rat anti-mouse CD68 (clone FA-11; BioLegend); and donkey anti-rat IgG Alexa Fluor 488 (Life Technologies, Carlsbad, California, USA).

TCR and inducible IL-12 and IL-18 constructs

TCR α and β genes specific for the human gp100₂₈₀₋₂₈₈ epitope presented by HLA-A2 (gp100/A2) were derived from CTL-296 clone (28), murinized and codon-optimized as described earlier (29). Subsequently, TCR genes were cloned into the pMP71 vector (kindly provided by prof. Wolfgang Uckert, Max-Delbrück Center, Berlin, Germany) with TCR α and β genes separated by an optimized T2A ribosome skipping sequence. Sequences of murine IL-12 (a linked dimer consisting of p35 (NM_000882) and p40 (NM_002187)) and the processed form of IL-18 (NM_001562) were ordered from Geneart (not codon-optimized) and cloned into a pSIN vector, which contains 6 repeats of the NFAT response element and a minimal mIL-2 promoter, as described in (18). The plasmids with iIL-12 and iIL-18 contain a neomycin resistance gene to allow for selection of transduced T cells.

T cell transduction

Total mouse splenocytes were isolated, activated with Concanavalin A (0.5µg/ml) and rhIL-2 in mouse T cell medium, and transduced with retroviral supernatant from a co-culture of single construct (either TCR, iIL-12 or iIL-18) transfected 293T and Phoenix-Amp cell lines as described by Pouw and colleagues (29). To generate T cells that express TCR and either iIL-12 or iIL-18, T cells were simultaneously incubated with two retroviral supernatants in a 1:1 ratio (yielding TCR+iIL-12 or TCR+iIL-18 T cells). To obtain T cell populations that provide an inducible source for both cytokines, used in some experiments, TCR+iIL-12 and TCR+iIL-18 T cells were mixed in a 1:1 ratio (yielding TCR+iIL-12+iIL-18 T cells). Control (mock) T cells were transduced with empty retroviral vector. TCR and mock T cells were cultured as described above, whereas T cells harboring (in addition to the TCR) iIL-12 or iIL-18 underwent selection with 1mg/ml of G418 starting at 24h after transduction and lasting for 72h (for a detailed flowchart of the T cell activation, transduction and selection procedure, please see figure 1A and supplementary figure 1A, B).

Adoptive T cell therapy

Experiments with mice were approved by the Experimental Animal Committee of the Erasmus MC Cancer Institute and carried out in accordance with institutional and national guidelines. For adoptive T cell therapy studies we used HLA-A2 transgenic (tg) mice that express a chimeric HLA-A*0201 transgene (HHD, referred to as HLA-A2) (30). Adoptive transfer of T cells was done as described before (27). In short, at day -15, HLA-A2 tg mice were injected subcutaneously with 0.5×10^6 of a B16:A2-YLEP clone and at days -4 and -3 mice received a total of two Busulfan injections intraperitoneally (16.5 µg/kg ea.), followed a day later by a single Cyclophosphamide injection intraperitoneally (200 mg/kg). In some experiments, the mice were not transplanted with tumor cells, but followed the same conditioning and T cell treatment protocol. Mice were treated with T cells at day 0 and grouped according to one of the following treatments: mock T cells (number of T cells equal to TCR T cell group); TCR T cells (total number corrected for 7.5×10^6 transduced T cells); TCR+iIL-12 T cells; TCR+iIL-18 T cells; and TCR+iIL12+18 T cells). Tumor growth was measured by caliper 3 times a week and tumor volumes were estimated with the formula $0.4 \times (A \times B^2)$ where A represents the largest diameter and B the diameter perpendicular to A. Tumor regression or response to treatment was defined as a >30% reduction in size compared to day 0, while relapse was defined as a size-increase of the tumor over the course of at least 3 days preceded by response to treatment. Survival was monitored daily for up to 45 days after administration of T cells. Body weight and edema like symptoms were recorded starting at the day of tumor inoculation and every third day thereafter. Peripheral blood was collected at day 6 after T cell transfer and at weekly intervals thereafter and used to determine numbers of administered T cells, whereas plasma was collected at days 16 and day 21 and used to measure cytokine levels. Tumor tissues of regressing tumors were collected at day 5 after T cell transfer and snap-frozen in liquid nitrogen and stored at -80°C for downstream assessment of cytokines in tumor lysates and

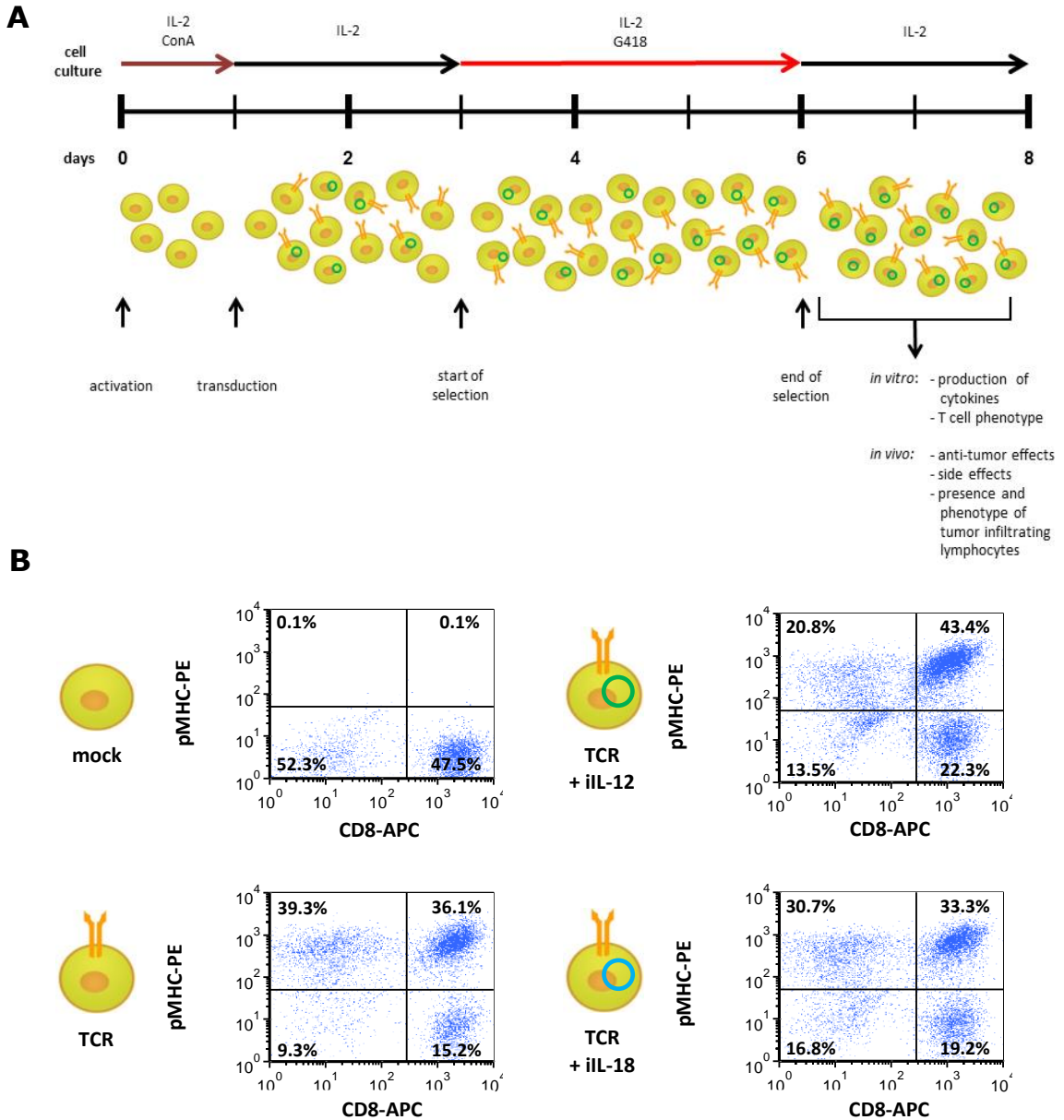


Figure 1. Protocol to generate T cells equipped with a TCR and an inducible cytokine construct

(A) Timeline for transduction procedure that yields TCR- and induced (i) cytokine-positive T cells. Optimization of individual aspects of the procedure are described in materials and methods and supplementary figure 1. (B) At the end of T cell selection (day 7 after T cell activation) T cells that were transduced with TCR and iIL-12 or TCR+iIL-18 were labeled with CD8-APC antibody and gp100/HLA-A2 tetramer-PE. Mock and TCR-only transduced T cells were stained as controls. T cells were gated for live cells and dotplots are representative of three different experiments. Percentages in upper right quadrants represent fractions of CD8⁺ T cells binding to tetramer.

performance of in situ immune fluorescence. Another part of tumor tissue was directly processed into TIL suspensions. To this end, tissue was cut into smaller fragments, incubated with collagenase (1mg/ml; Sigma, St. Louis, MI) for 45 minutes at 37°C, checked microscopically and washed prior to flow cytometry.

Flow cytometry

T cells or TILs (5×10^5) were washed with PBS and incubated with antibodies at 4°C or with pMHC multimers at 37°C for 30 min. Following staining, T cells were washed again and fixed with 1% paraformaldehyde. Absolute T cell counts in mouse blood samples were determined using Flow-Count Fluorospheres. Events were acquired on a FACS Canto flow cytometer and analysed using FCS Express 4 software (BD Biosciences).

Measurement of cytokines

T cells (6×10^4 /well of 96-wellsplate) were co-cultured with either antigen-positive or negative B16 cells (2×10^4 /well) in a total volume of 200 μ l of T cell assay medium (RPMI 1640, L-glutamine, 10% FBS, and antibiotics) for 24h at 37°C and 5% CO₂. Stimulation with medium was used as a control. Subsequently, supernatants were harvested and used to determine cytokine levels by standard ELISAs (IL-2, IL-10, IL-12, IFN γ , TNF α : eBioscience, San Diego, CA; IL-18: MBL, Nagoya, Japan). Plasma levels of selected cytokines were measured using the ProcartaPlex Mouse Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (eBioscience) according to manufacturer's instructions. Tumor lysates were generated following sonication (3 cycles of 10s using SoniPrep 150, MSE, London, UK) of frozen tissue suspended in PBS with protease inhibitors (Protease inhibitor cocktail tablets, Roche), and subsequently analyzed for concentrations of IL-12 and IL-18 via ELISA.

In situ immune fluorescence

Tissue sections were cut at 5 μ m and fixed with acetone for 10 min, dried, washed, and blocked with PBS/10% donkey serum/0.3% Triton for 30 min prior to immune staining. Next, sections were incubated with the primary antibody overnight at 4°C, washed and incubated with the donkey anti-rat IgG Alexa Fluor 488 for 2h at room temperature in the dark. Sections were covered with Vectashield, and kept subsequently at room temperature for 2 h and at 4°C overnight. Sections were examined microscopically (Leica, DM IL, 200x magnification) and photographed (Leica DFC 3000G camera and LAS4 software). Recorded photographs were analyzed using Fiji software (31). The number of pictures from each tumor ranged between 12 and 16, and using an in-house developed algorithm, the mean number of positively stained cells was determined, and normalized for percentages of nucleated cells (DAPI staining).

Statistical analyses

The different treatment groups were compared with the Student's t-tests, or the Mantel-Cox test in case of survival data, using GraphPad Prism5 (GraphPad Software, La Jolla, CA). P values < 0.05 were considered statistically significant.

RESULTS

TCR+iIL-12 T cells, and to a lesser extent TCR+iIL-18 T cells, produce enhanced levels of IFN γ

We developed a protocol that yielded high numbers of T cells transduced with both the gp100/HLA-A2-specific TCR and iIL-12 or IL-18. Such inducible T cells showed maximal responsiveness towards antigen-positive tumor cells (see figure 1A and supplementary figure 1A+B for details). Surface expression of TCR genes and binding to cognate pMHC ranged between 65-75%, irrespective of the presence of iIL-12 or iIL-18 (figure 1B). TCR+iIL-12 T cells produced $>5.5\text{ng}/10^6$ cells of IL-12, and TCR+iIL18 T cells produced $>75\text{pg}/10^6$ cells of IL-18 upon co-culture with antigen-positive cells (see figure 2A). Co-culturing TCR+iIL-12 T cells and TCR+iIL-18 T cells at a 1:1 ratio (TCR+iIL-12+iIL-18) demonstrated an expected drop in the antigen-specific production of IL-12 and IL-18. Notably, T cells that harbor iIL-12 produced minor amounts of IL-12 independent of TCR engagement, which was not the case for T cells harbouring iIL-18 (see figure 2A). When testing antigen-specific production of IFN γ by these T cell populations, we observed a significant increase in case T cells harbored iIL-12, iIL-18 or both (see figure 2B) when compared to TCR T cells. TCR+iIL-12 and TCR+iIL-12+iIL-18 T cells produced more than twice as much IFN γ than TCR+iIL-18 T cells. Furthermore, testing for antigen-specific production of IL-10 revealed that both TCR+iIL-12 and TCR+iIL-12+iIL-18 T cells produced significantly increased levels of this cytokine. While the various T cell populations also produced IL-2 as well as TNF α upon co-culture with antigen-positive B16 cells, their levels of were not affected by either iIL-12, IL-18 or both (see figure 2B).

Treatment of melanoma-bearing mice with TCR+iIL-18 T cells results in more cures, yet treatment with TCR+iIL-12 T cells adversely affects survival

We treated HLA-A2 transgenic mice with palpable B16:A2-YLEP tumor ($\sim 400\text{mm}^3$) either with 7.5×10^6 TCR, TCR+iIL-12, TCR+iIL-18, TCR+iIL-12+iIL-18 or mock T cells (see Materials and Methods for details). In the TCR T cell group, 66% of mice showed tumor regression, whereas in the TCR+iIL-12, TCR+iIL-18 and TCR+iIL-12+iIL-18 T cell groups these percentages were 66, 100 and 78, respectively (see figure 3A; for individual tumor growth curves, see supplementary figure 2). Moreover, treatment with TCR T cells resulted in complete responses (i.e., no detectable tumor) until the end of the experiment (day 45 after T cell transfer) in 11% of mice, a result that was not improved by treatment with TCR+iIL-12 T cells (see figure 3B). In contrast, treatment with TCR+iIL-18 or TCR+iIL-12+iIL-18 T cells resulted in complete responses in 33% of mice. In addition, in the group treated with TCR+iIL-12 T cells, mice suffered from tumor recurrence at earlier time points than mice in the group treated with TCR+iIL-18 T cells (see supplementary figure 2). Figure 3 B provides an overview of the fractions of complete, partial and no responses among the different treatment groups. When assessing survival, 29% and 14% of mice treated with TCR or TCR+iIL-12 T cells were alive at the end of experiment, respectively, whereas 57%

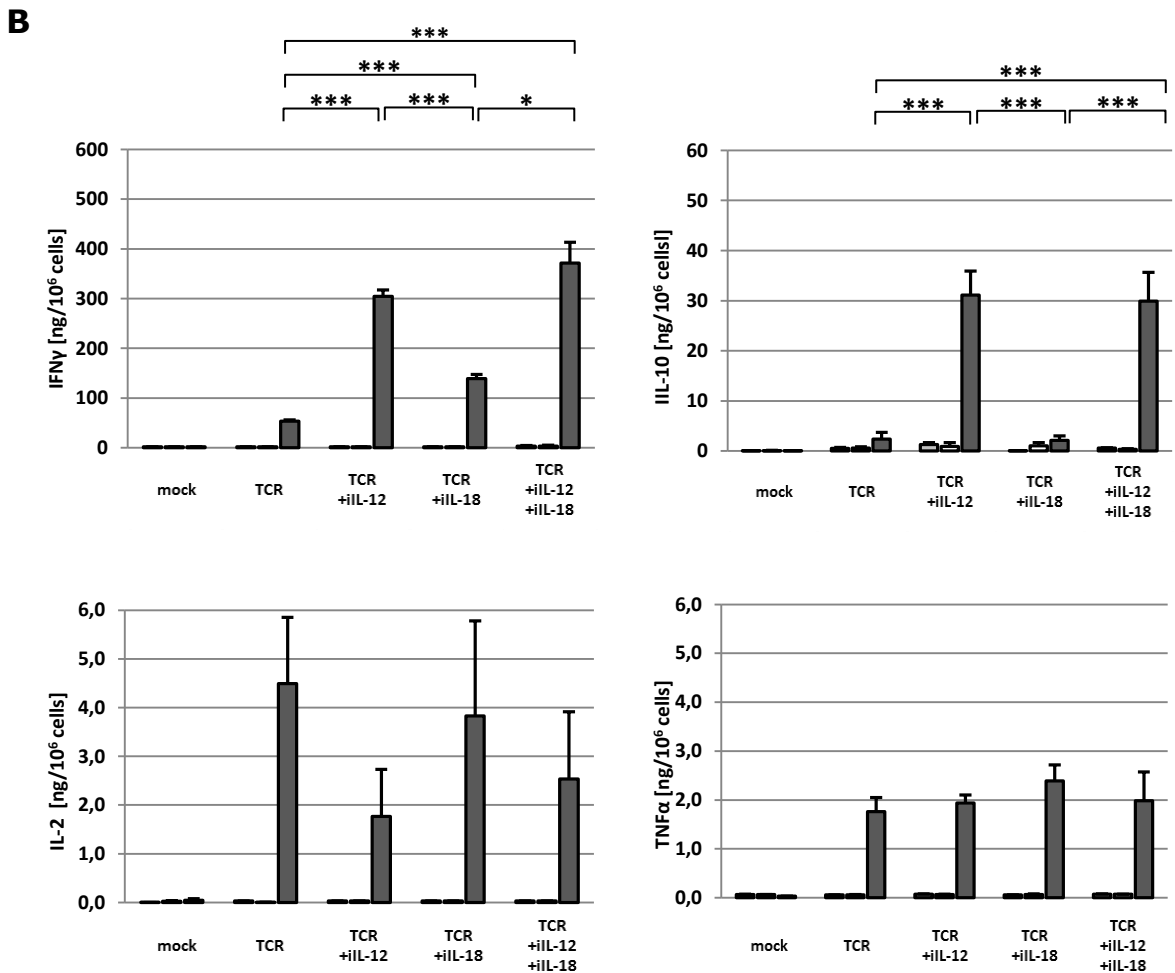
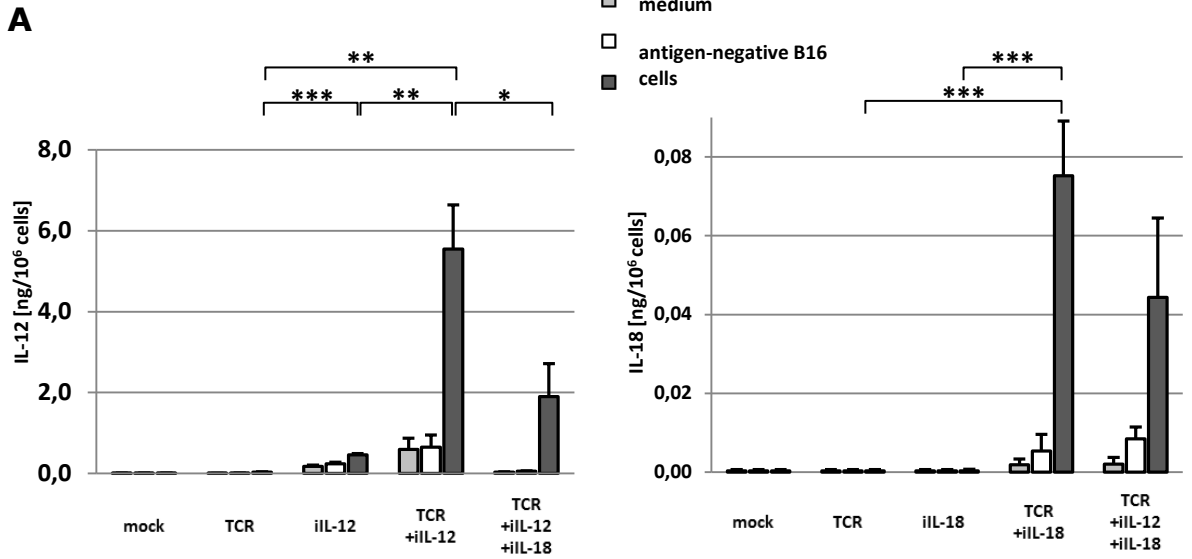


Figure 2. Inducible T cells produce IL-12 and IL-18 upon recognition of cognate antigen, which results in enhanced production of IFN γ

T cells were transduced with one or a combination of the following constructs: empty retroviral vector (mock); gp100 TCR (TCR); gp100 TCR and iIL-12 (TCR+iIL-12); gp100 TCR and iIL-18 (TCR+iIL-18). In an additional set of experiments, TCR+iIL-12 and TCR+iIL-18 T cells were mixed at a 1:1 ratio (TCR+iIL-12+iIL-18). These T cell populations were co-cultured with B16 melanoma cells that were either positive or negative for the gp100 target antigen at an effector:target (E:T) ratio of 3:1 for 24h. As a control, T cells were cultured in medium only. **(A)** Levels of IL-12 and IL-18 in culture supernatants were measured via ELISA, and displayed as mean cytokine production per 10^6 T cells \pm SEM (n=5). **(B)** Levels of IFN γ , IL-10, IL-2 and TNF α in culture supernatants were measured via ELISA, and displayed as mean cytokine production per 10^6 T cells \pm SEM (n=5). Statistically significant differences between T cell populations that were co-cultured with antigen-positive B16 cells were calculated with Student's t-test: *p<0.05; **p<0.01; ***p<0.005.

and 43% of mice treated with TCR+iIL-18 or TCR+iIL-12+iIL-18 T cells were alive at the end of experiment (see figure 3C). Treatment-related mortality, defined as death of mice after T cell treatment not coinciding with tumor outgrowth, occurred in TCR+iIL-12 and TCR+iIL-12+iIL-18 T cell-treated mice, with percentages of mice that died within 14 days after T cell transfer being 33 and 22, respectively. Treatment-related mortality was absent in the TCR and TCR+iIL-18 T cell-treated mice (see figure 3B). Along these lines, we observed enhanced loss of weight in 56-78% (see supplementary figure 3) as well as edema-like toxicities in 29% of the treatment groups with iIL-12. Table I provides details on observed therapy-related effects.

Administration of TCR+iIL-12 T cells results in compromised T cell persistence and enhanced plasma levels of inflammatory cytokines

As peripheral T cell persistence has been directly linked to clinical anti-tumor efficacy (32), blood samples were taken at various time points after T cell transfer and analyzed by flow cytometry for the presence of TCR-engineered T cells. We observed increased numbers of pMHC-binding T cells in blood of TCR+iIL-18 and the TCR+iIL-12+iIL-18 T cell-treated mice at day 6 after T cell transfer when compared to TCR T cell-treated mice (see figure 4A). Numbers of pMHC-binding T cells were decreased in the blood at day 6 after treatment with TCR+iIL-12 T cells. It is noteworthy, that the ratio of CD8 to CD4 positive T cells was significantly enhanced in the treatment groups that received treatment with iIL-18 when compared to the TCR+iIL-12 group (see figure 4B). These findings are in line with our in vitro observation that cultures of TCR+iIL-12 T cells yielded lower cell numbers when compared to cultures of TCR+iIL-18 or TCR T cells (see supplementary figure 1A, lower panel). In addition to whole blood, we also collected plasma samples on day 16 after T cell transfer, which were analyzed via multiplex assay for the presence of cytokines (see figure 5). While plasma levels of IL-12 were low in all treatment groups (<7pg/ml), plasma levels of IL-18 were considerably high in all groups (>700pg/ml) and highest in

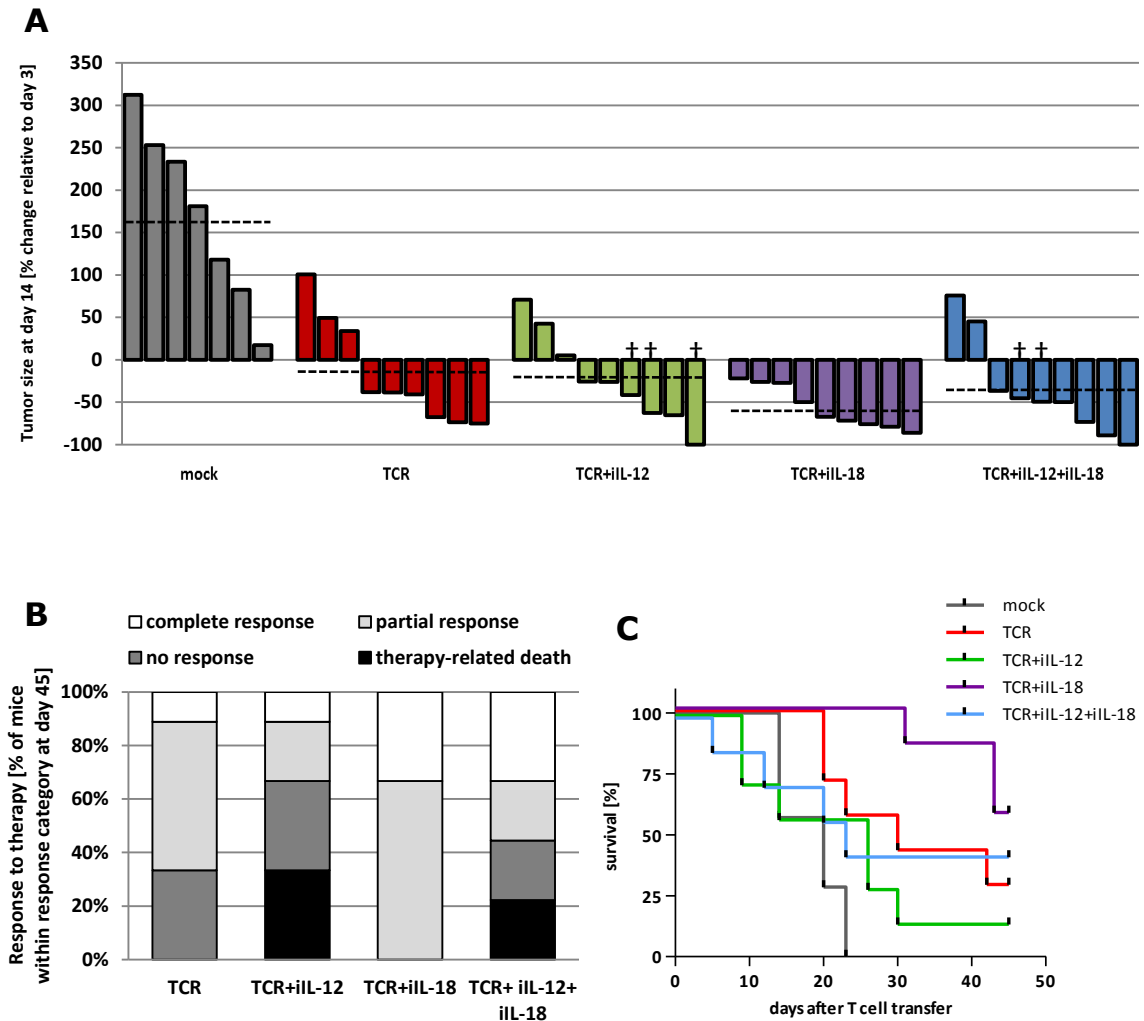


Figure 3. Treatment with TCR+iIL-18 T cells reduces tumor growth and prolongs survival

HLA-A2 transgenic mice bearing established tumors derived from inoculated B16:A2-YLEP cells were conditioned with Busulfan and Cyclophosphamide and treated with 7.5×10^6 T cells that were mock transduced or transduced with TCR, TCR+iIL-12, or TCR+iIL-18; or mice were treated with a mixture of 3.75×10^6 T cells transduced with TCR+iIL-12 and 3.75×10^6 T cells transduced with TCR+iIL-18 (TCR+iIL-12+iIL-18). **(A)** Waterfall graph of percentage change in tumor size between days 3 and 14 after T cell transfer, with each bar representing a single mouse. Tumor sizes were measured three times a week with a caliper. Dashed lines indicate average tumor size per treatment group. '+' indicates death of mouse, in which case latest record of tumor size has been used. **(B)** Stacked bars representing percentages of mice with different responses to therapy at day 45 after T cell transfer. Complete response is defined as absence of a palpable tumor at the end of the experiment (day 45); partial response is defined as $\geq 30\%$ tumor regression followed by relapse; no-response is defined as $< 30\%$ tumor regression; and therapy-related death is defined as death not coinciding with tumor progression ($n=9$ per group). **(C)** Survival curves of treatment groups. Two mice of each group were sacrificed at day 5 after T cell transfer to collect tumors and are omitted from this figure ($n=7$ per group).

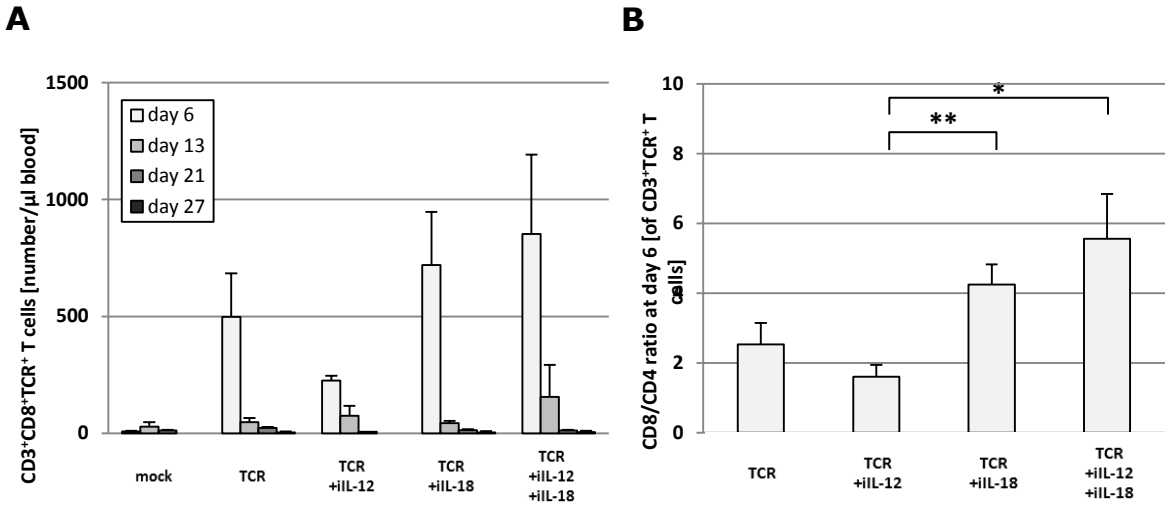


Figure 4. Treatment with TCR+iIL-12 T cells results in lowered numbers of CD8 and TCR transgene-positive T cells in blood

HLA-A2 transgenic mice bearing established B16:A2-YLEP tumors were conditioned and treated with T cells as described in legend to figure 3. **(A)** Peripheral blood was collected from mice at the indicated time points after T cell transfer and absolute numbers of gp100/HLA-A2 pMHC-binding CD8 T cells were determined by flow cytometry. Data are presented as mean numbers per μ l blood \pm SEM (n=4-6). **(B)** depicts the mean ratio of CD8/CD4 positive therapeutic T cells at day 6 after T cell transfer \pm SEM (n=4-6). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05; **p<0.01.

6

Table I. Therapy-related side-effects of T cells gene-engineered with TCR and iIL-12 and/or iIL-18

treatment	maximum weight loss ^a	edema ^b	therapy-related death ^c	overall survival ^d
mock	57%	0%	0%	0%
TCR only	11%	0%	0%	29%
TCR+iIL-12	78%	29%	43%	14%
TCR+iIL-18	0%	0%	0%	57%
TCR+iIL-12+iIL-18	56%	29%	29%	43%

^a percentage of mice showing a loss of weight exceeding 10% of starting weight (day -15)

^b percentage of mice developing edema; mice were scored positive for edema according to both, visual evaluation by a pathologist and increase in body weight of >3.0% per day over a period of 7 or more days

^c percentage of mice dying within the first 10 days after T cell administration while no tumor growth was detected

^d percentage of mice alive at day 45

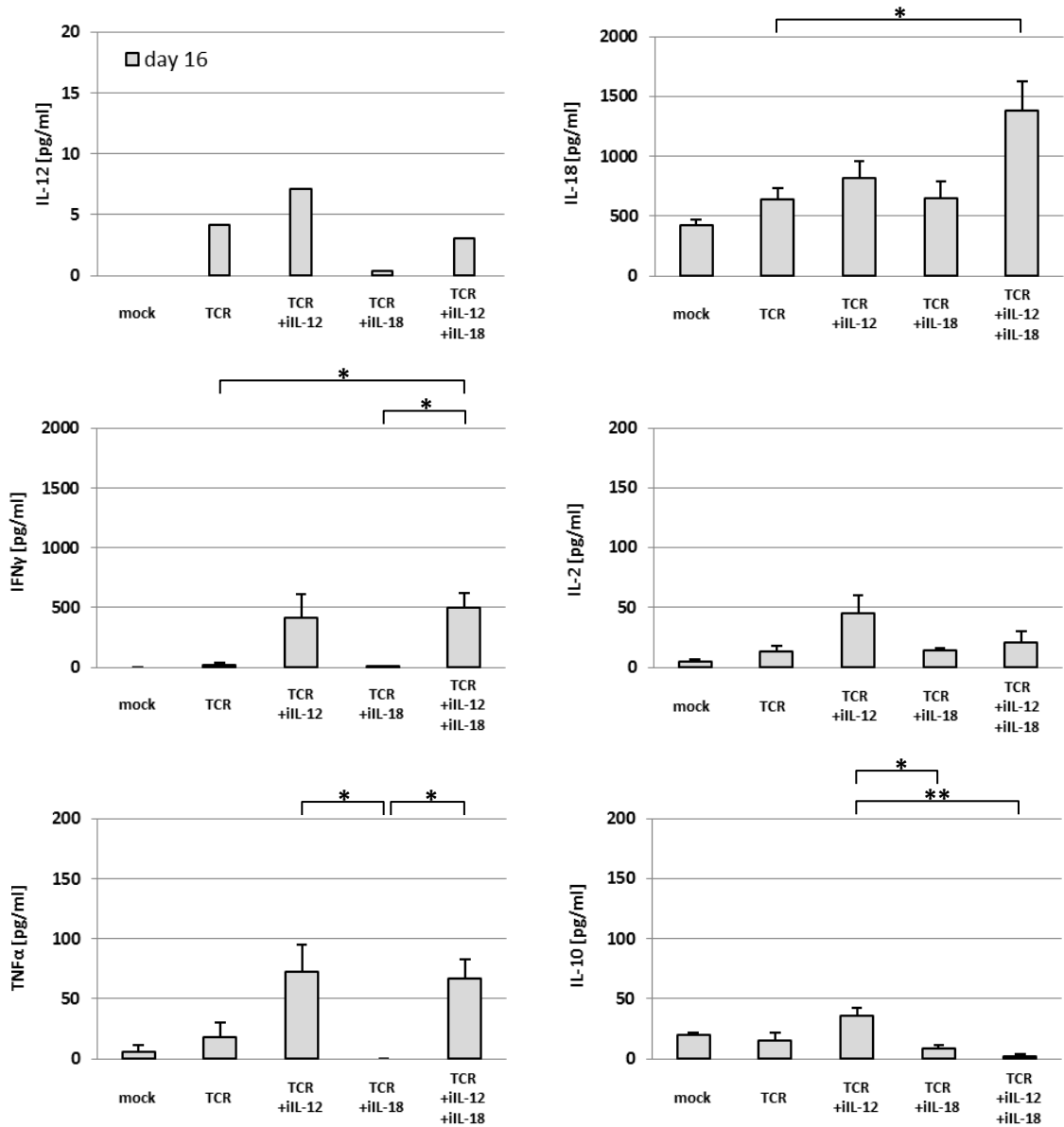


Figure 5. Treatment with TCR+iIL-12 T cells results in increased plasma levels of inflammatory cytokines

Plasma samples were collected from mice on days 16 and 21 after T cell transfer and were screened for the presence of multiple cytokines via multiplex assay ("ProcartaPlex Mouse Th1/ Th2/ Th9/ Th17/ Th22/ Treg Cytokine Panel (17 plex)" by eBioscience). Concentrations of IL-12, IL-18, IFN γ , IL-2, TNF α and IL-10 on day 16 are presented as mean \pm SEM (n=3). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05; **p<0.01; ***p<0.005.

mice treated with TCR+iIL-12+iIL-18 T cells (>1300pg/ml). Of interest, in particular in relation to the above-mentioned toxicities, is the observation that plasma levels of IFN γ and TNF α were significantly higher in the TCR+iIL-12 and TCR+iIL-12+iIL-18 T cell groups (and negligible in the TCR and TCR+iIL-18 T cell groups). IL-2 plasma levels showed no significant difference among the tested groups. Plasma levels of IL-10 were increased in mice treated with TCR+iIL-12 T cells compared with those mice treated with TCR+iIL-18 and TCR+iIL-12+iIL-18 T cells.

Treatment with TCR+iIL-18 T cells results in enhanced accumulation of CD8 and TCR-transgene-positive T cells within the tumor

To better understand the in vivo behavior of TCR+iIL-12 and TCR+iIL-18 T cells, we assessed how the different treatments affect T cell infiltration into solid tumors and the phenotype of intra-tumoral T cells. To this end, we have performed flow cytometry analyses of TILs and observed an enhanced frequency of CD8⁺TCR⁺ T cells amongst CD3⁺ TILs upon treatment with TCR+iIL-18 T cells when compared to TCR+iIL-12 T cells (39 vs 9%) (see figure 6A, left graph). Looking into the ratio of CD8⁺ and CD4⁺ T cells, it became apparent that treatment with TCR+iIL-18 but not with iIL-12 T cells results in an enrichment of CD8⁺ TILs (~ 7 vs 0.5, respectively; see figure 6A, right graph). Next, we studied the phenotype of TILs, in particular with respect to co-stimulatory as well as co-inhibitory receptor expression. The analysis of percentages of individual co-stimulatory or co-inhibitory receptors by CD8⁺ TILs, nor analysis of their co-expression by these cells, revealed no significant difference between treatment groups (see figure 6B and C). Also, phenotypic differences were not observed when analyzing CD4⁺ TILs or analyzing mean fluorescence intensities (data not shown).

Mice treated with TCR+iIL-12 T cells demonstrate extensive intra-tumoral infiltration of macrophages

In addition to numbers and phenotype of intra-tumoral T cells, we also assessed the cellular composition of the immune infiltrate in more detail. TILs from the different treatment groups were assessed by flow cytometry for the presence of T cells (CD3⁺), macrophages (CD68⁺), NK cells (CD335⁺) and neutrophils (Ly6G⁺). Strikingly, we observed that frequencies of macrophages were increased in mice receiving TCR+iIL-12 T cells, an observation that went hand in hand with a decrease in frequencies of T cells (see figure 7). Neither NK cell nor neutrophil numbers showed significant differences between treatment groups. To substantiate these findings we assessed the presence of IL-12 and IL-18 in lysates from the same tumor, and showed that the concentrations of IL-12 were higher in tumors from the groups treated with TCR+iIL-12 or TCR+iIL-12+iIL-18 T cells, whereas the concentrations of IL-18 were higher in tumors from the groups treated with TCR+iIL-18 when compared to treatment with TCR T cells. Endogenous levels of IL-18 ranged between 400 and 1000pg/ml, while those of IL-12 were below 100pg/ml (see supplementary figure

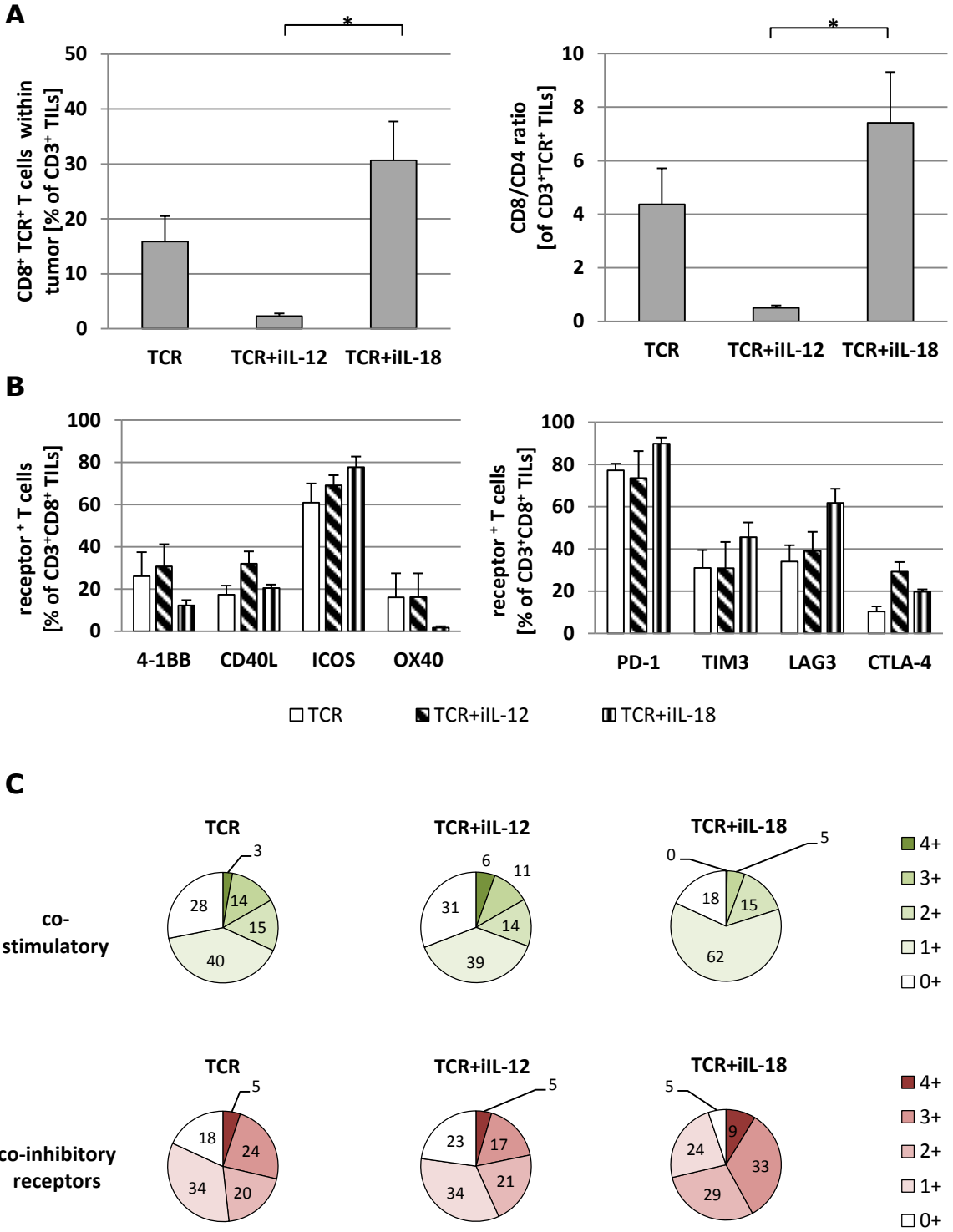


Figure 6. Upon treatment with TCR+iIL-18 T cells, numbers of CD8-positive TILs are enhanced without a change in expression of co-signaling receptors

Mice with regressing tumors (treated with TCR T cells with or without inducible cytokines) were sacrificed (n=4 per group). TILs were isolated as described in Materials and Methods and analyzed via flow cytometry for the expression of CD3, CD4, CD8, TCR, 4-1BB, CD40L, ICOS, OX40, PD-1, TIM3, LAG3 and CTLA-4. (A) depicts the percentage of CD8⁺TCR⁺ T cells amongst CD3⁺ TILs (left panel) and the CD8/CD4 ratio of TCR⁺ T cells within CD3⁺ TILs. Bars in (B) show the mean expression of individual co-stimulatory (left panel) and co-inhibitory (right panel) receptors of CD3⁺CD8⁺ TILs. (C) provides an overview of the degree of co-expression of co-stimulatory receptors (upper charts) as well as co-inhibitory receptors (lower charts). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05.

4A). Furthermore, we also assessed the composition of the immune infiltrate using in situ immune fluorescence, and quantified the various immune cell types per area tumor tissue. The numbers of T cells (CD3⁺), macrophages (F4/80⁺), NK cells (CD335⁺) and neutrophils (Ly6G⁺) that were found in the tumors from the different treatment groups confirmed the findings of the flow cytometric analysis (see supplementary figure 4B and C). It is noteworthy that NK cells and neutrophils were readily detectable by flow cytometry, while staining for the same markers in tissues slices yielded no to negligible detection of either cell type. This discrepancy between both techniques most likely relates to the use of different antibodies in different applications.

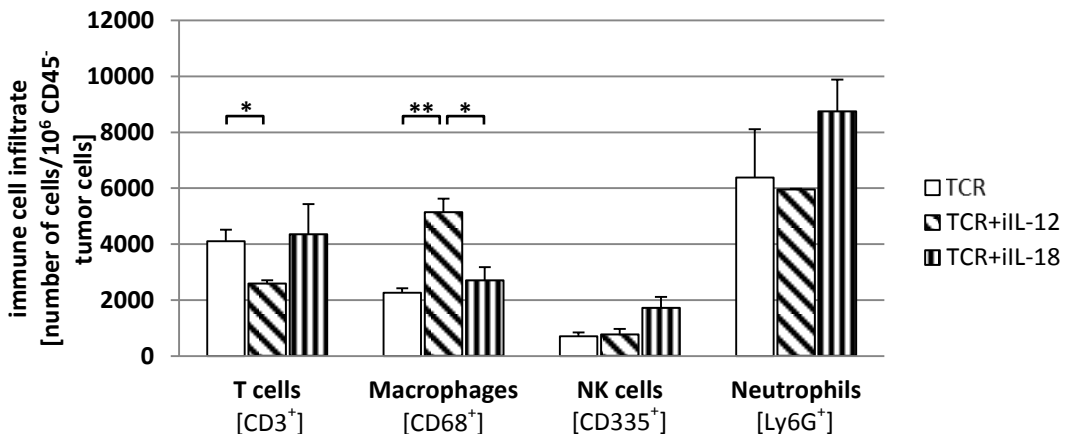


Figure 7. Mice treated with TCR+iIL-12 T cells show an enhanced number of tumor-infiltrating macrophages

Following T cell treatment, regressing tumors were collected at day 5 after T cell transfer. Tumors were dissociated into a single-cell suspension, stained with antibodies directed against CD3 (T cells), CD68 (macrophages), CD335 (NK cells) or Ly6G (neutrophils), and analyzed by flow cytometry. Figure depicts the mean number for each cell type ±SEM (n=4). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05; **p<0.01

DISCUSSION

In this study, we compared the therapeutic impact of IL-12 and/or IL-18 secretion at the tumor site by antigen-triggered T cells. To this end, we equipped murine T cells both with gp100-specific TCR α/β genes and an inducible cytokine construct. T cells were antigen-responsive and, besides being producers of IL-12 or IL-18, produced enhanced levels of IFN γ . The production of IFN γ was prominent in case of TCR+iIL-12 T cells and highest in TCR+iIL-12+iIL-18 T cells, which is in line with previous reports where T cells were exposed to a combination of recombinant IL-12 and IL-18 (33). TCR T cells harboring iIL-18, when transferred to mice bearing melanoma positive for the gp100 target antigen, resulted in enhanced and more durable anti-tumor responses when compared to T cells engineered with a TCR only or TCR and iIL-12. Furthermore, treatment with TCR+iIL-18 T cells, but not TCR+iIL-12 T cells, prolonged survival when compared to treatment with TCR T cells.

The low survival in the TCR+iIL-12 T cell treatment group is most likely a result of IL-12-mediated toxicities. Mice receiving TCR+iIL-12 or TCR+ iIL-12+iIL-18 T cells demonstrated elevated plasma levels of IFN γ and TNF α , clear weight loss, edema-like symptoms and in some cases death. The detection of IFN γ and TNF α in plasma is considered a consequence of IL-12-mediated productions of these cytokines by tumor-infiltrating immune cells (34). IFN γ is most likely derived from administered T cells following infiltration and activation in the tumor tissue. This notion is strengthened by our observation that CD335-positive NK cells, as an alternative source of IFN γ , were only present at low frequency within the tumors of treated mice. TNF α is most likely derived from macrophages since we detected an enhanced frequency of CD68 as well as F4/80-positive macrophages in tumors isolated from mice treated with TCR+iIL-12 T cells, and *in vitro* assays revealed no enhanced TNF α production by these T cells. Collectively, our findings extend previous clinical studies, where systemic administration of recombinant protein or cDNA encoding IL-12 was reported to result in enhanced production of IFN γ and TNF α , changes in blood vessel wall permeability, edema and even sepsis-like symptoms (34,35). In fact, a recent study demonstrated that treatment of melanoma patients with iIL-12-transduced TILs was accompanied by liver dysfunction, high fever, and hemodynamic instability (36). To further assess the observed toxicities, we also treated mice without tumor-implant with TCR+iIL-12 T cells and still observed enhanced weight loss when compared to mice treated with TCR T cells (see supplementary figure 3). These findings extend our *in vitro* observations showing target-independent release of IL-12 by these T cells (Figure 2A) as well as those by Zhang and colleagues, who also reported non-specific release of IL-12 and reduced fitness when culturing iIL-12-expressing TILs (36). It is noteworthy that none of the mice without a tumor implant developed edema or showed therapy-related mortality, arguing that although toxicities are related to antigen-independent release of IL-12, they may exacerbate in the presence of antigen-positive tumor cells. Non-specific release of IL-12, in particular in the setting of TCR-engineered T cells, may be explained by the size of the inserted construct. IL-12 is a relatively large cytokine with 550 aa, while IL-18 encompasses 192 aa and, like other smaller constructs we tested (data not shown), shows no antigen non-specific release.

Finally, we cannot exclude the contribution of host, co-treatment and tumor to the observed toxicities. In one of our earlier studies, where we tested iIL-12 in mouse models utilizing CAR-transduced T cells (18), we did not observe the toxicities as reported in this study, which may suggest that mouse strain (C57BL/6 vs NIH-III), pre-conditioning protocol (Busulfan/Cyclophosphamide vs no pre-treatment), and type of tumor (melanoma cells vs colon carcinoma cells) affect the extent of IL-12-mediated toxicities.

Treatment with TCR+iIL-18 T cells, not revealing any toxicity, resulted in clearly enhanced anti-tumor responses. To better understand the immune-enhancing effect of this treatment, we have assessed numbers and phenotype of intra-tumoral T cells as well as the presence of other immune cell types within tumors. Notably, we observed an enhanced density of TCR-transduced TILs, in particular of the CD8 subset, when mice were treated with TCR+iIL-18 but not TCR+iIL-12 T cells. Enhanced numbers of CD8⁺ T cells within tumors are generally considered a beneficial factor with respect to tumor evolution and therapy response (7,37,38). The differential treatment effect with respect to numbers of TILs can be explained by two lines of arguments. First, our *in vivo* analysis of tumors showed that treatment with TCR+iIL-12 when compared to TCR+iIL-18 T cells results in an enhanced presence of macrophages (see figure 7 and supplementary figure 4). These results, together with enhanced TNF α levels in sera, argue that macrophages and their product TNF α may limit the infiltration of CD8⁺ T cells as was observed previously using various mouse models (39,40). Second, *in vitro* analysis of T cells demonstrated that iIL-18 when compared to iIL-12 provides T cells with high expression of co-stimulatory receptors and a low expression of co-inhibitory receptors (see supplementary figure 5). These traits may be representative of a preferred T cell phenotype at the time of adoptive transfer for an effective tumor response. Interestingly, when studying the phenotype of TILs, we observed no significant differences in the expression of co-stimulatory or inhibitory receptors by CD8⁺ TILs. When zooming in on TCR transgene-expressing TILs, however, we did observe that treatment with TCR+iIL-18 T cells resulted in enhanced expression of the co-stimulatory receptor ICOS and the co-inhibitory receptors PD-1 and CTLA-4, whereas treatment with TCR+iIL-12 T cells resulted in enhanced expression of the co-stimulatory receptors 4-1BB and CD40L and the co-inhibitory receptors LAG3 and CTLA-4 (see supplementary figure 6). These latter data are difficult to interpret given the fact that numbers of TCR⁺ TILs are extremely low in case of treatment with TCR+iIL-12 T cells. With respect to treatment with TCR+iIL-18 T cells, ICOS may contribute to enhanced tumor infiltration and prolonged T cell persistence as was demonstrated in adoptive T cell therapy studies ((41); Kunert, manuscript in preparation). PD-1, on the other hand, may mark successful antigen-specific TCR triggering and T cell activation (42). Notably, the fact that TCR+iIL-12 T cell treatment, when compared to TCR+iIL-18 T cells, results in the highest CTLA-4 and LAG3 expressions may provide an additional explanation for their lowered ability to mediate tumor clearance.

The above findings argue that IL-12 has potent immune-stimulatory effects, which may rapidly initiate the expression of immune checkpoints. In an effort to downscale negative feedback on T

cell activation, we titrated down TCR+iIL-12 T cells and observed that lowering the numbers of these cells did not change the effects this cytokine has on the production of IFN γ nor the expression of PD-1 (supplementary figure 7; same results were observed for production of IL-10 and expression of FOXP3, data not shown), arguing that it is difficult to maintain beneficial effects of IL-12 towards anti-tumor T cell activity while preventing the induction of a negative feedback. These data, together with the toxic effects we noted when using TCR+iIL-12 T cells, do not favor further therapeutic studies with T cells carrying iIL-12.

In conclusion, our findings demonstrate that the therapeutic use of TCR-engineered T cells equipped with inducible IL-12 leads to limited anti-tumor effects and severe toxicities *in vivo*, in part due to antigen non-specific release, whereas equipment of TCR T cells with inducible IL-18 results in enhanced anti-tumor responses without toxicities, most clearly related to increased accumulation of CD8 and TCR-transgene-positive T cells within tumors. Taken together, these findings advocate further studies towards the use of iIL-18 T cells to address therapy resistance in the setting of adoptive T cell therapy.

ACKNOWLEDGEMENTS

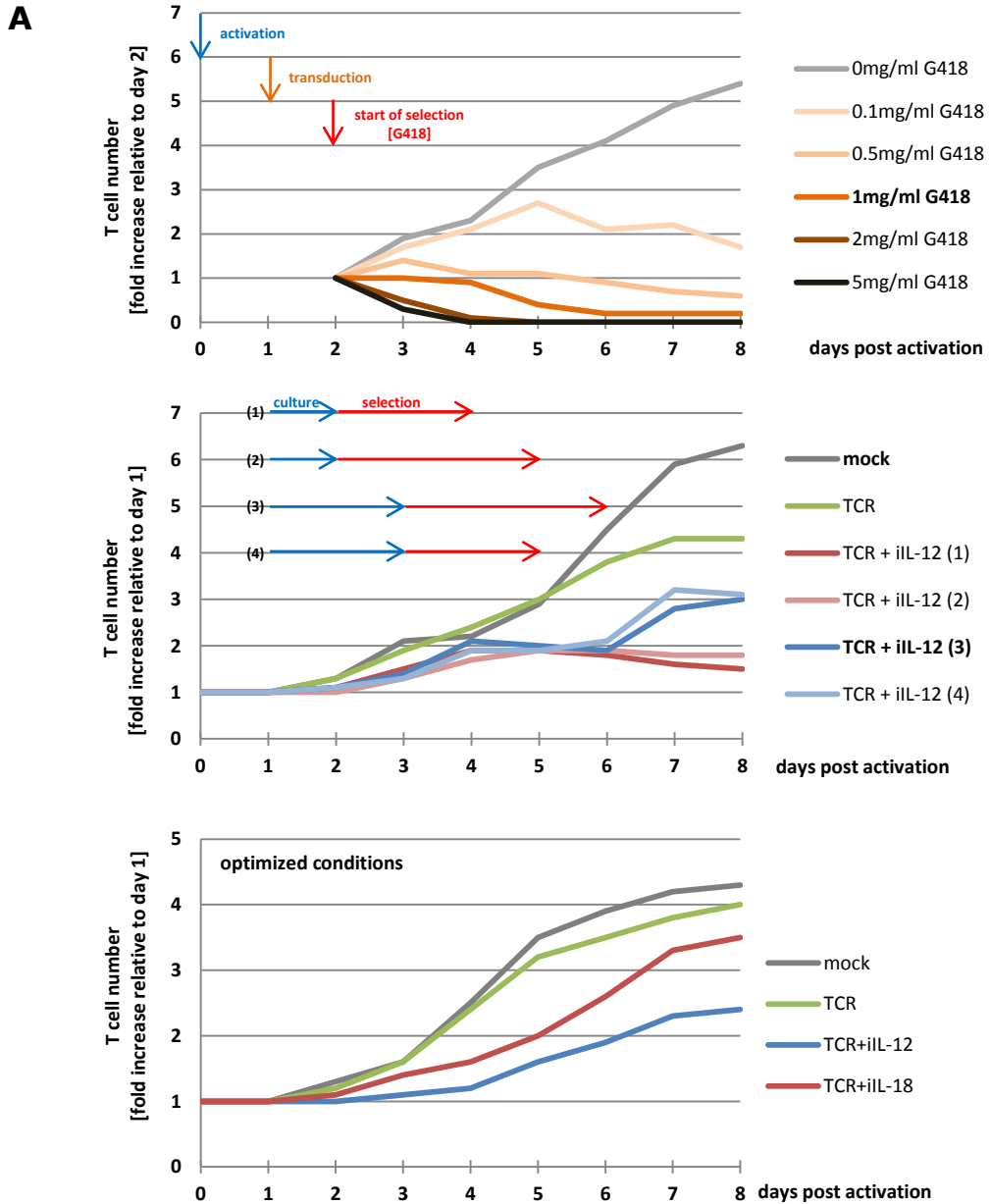
The authors would like to thank the team of the Erasmus Optical Imaging Center, in particular Adriaan Houtsmuller, Gert van Capellen and Gert-Jan Kremers, for their support in the quantification of *in situ* stainings. Part of research was funded by the EU 7th framework ITN Grant ATTRACT.

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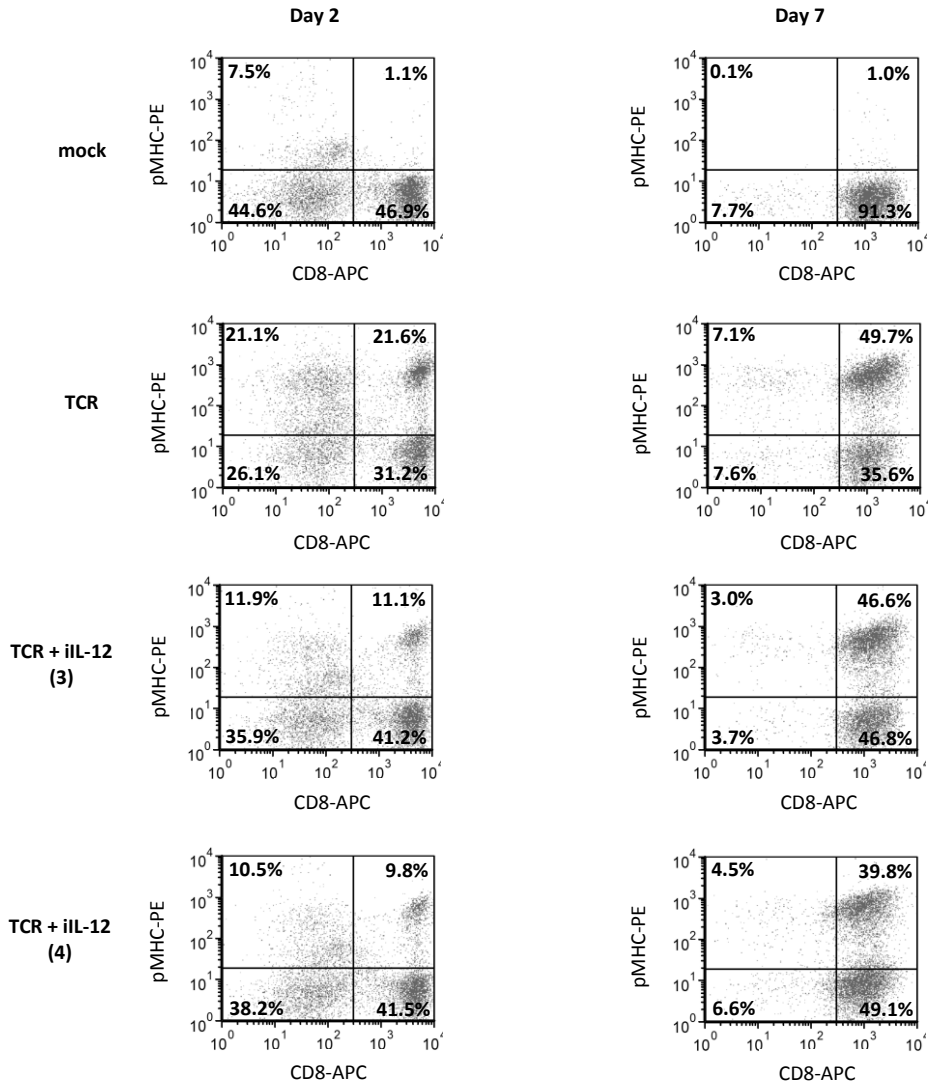
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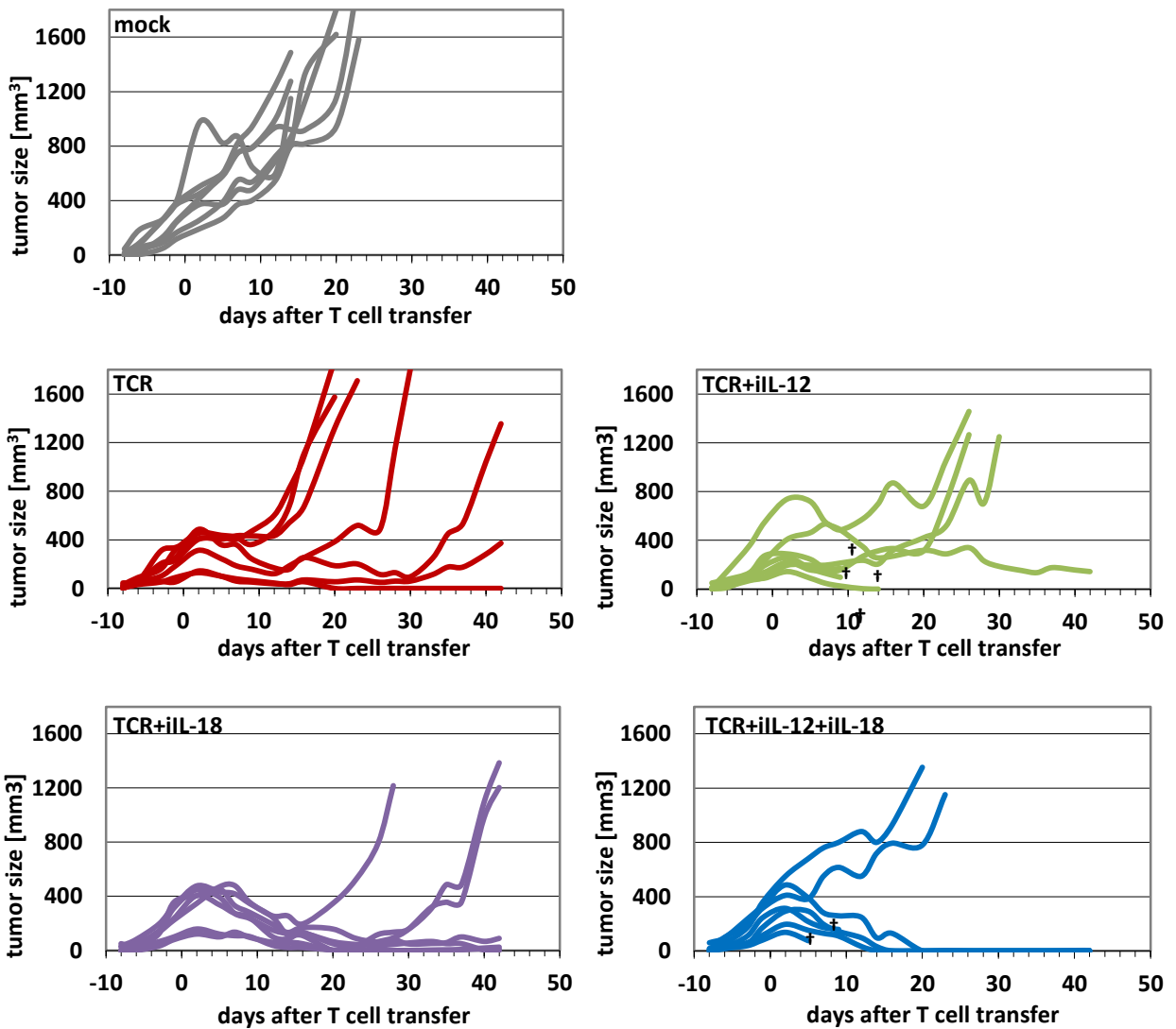
Supplementary figure 1. Generation of T cells expressing TCR and inducible IL-12 or IL-18

Optimal transduction and selection procedure with respect to numbers of T cells expressing both gp100/HLA-A2-specific TCR and inducible (i)IL-12 or IL-18. **(A)** depicts the effect of titrated amounts of neomycin (upper panel) on the number of untransduced (mock) T cells and the effect of start (relative to transduction) and duration of neomycin treatment (middle panel) on the number of transduced T cells from healthy donors. Mock and transduced T cells were cultured according to (Pouw et al., 2007) and used as controls; T cell numbers are indicated as fold increase compared to indicated days; and the test condition yielding highest fold-increase is indicated in bold. Upon identifying the optimal procedure, T cells were transduced with either empty retroviral vector (mock); gp100 TCR (TCR); gp100 TCR and iIL-12 (TCR+iIL-12); and gp100 TCR and iIL-18 (TCR+iIL-18). These T cell populations were seeded at 1×10^6 cells/ml in T75 flasks, and cultured in mouse T cell medium →

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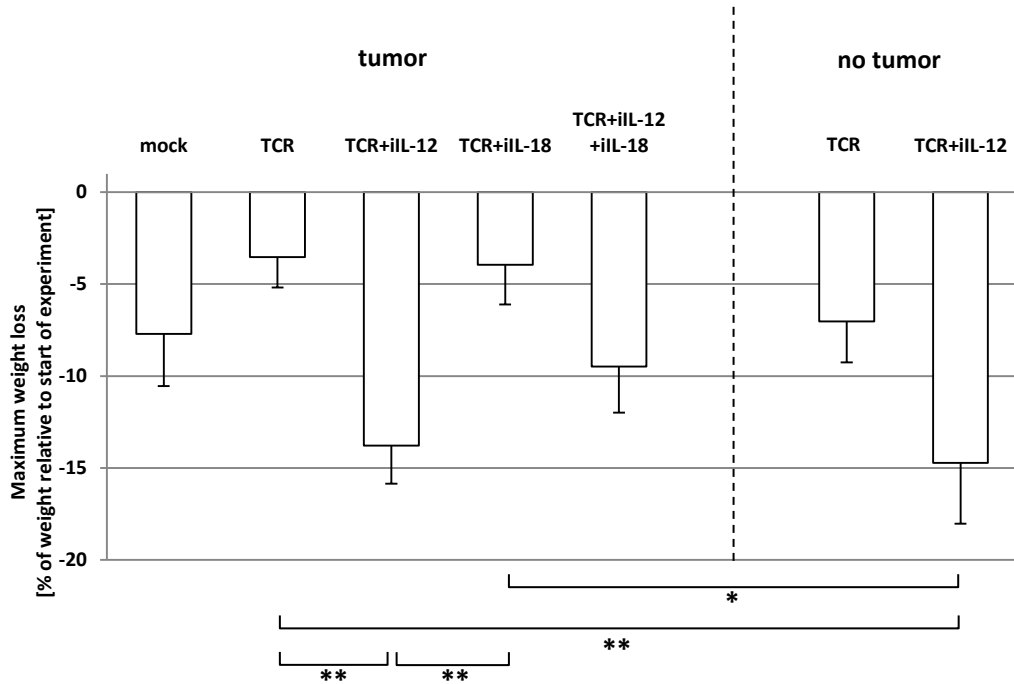


including 50 IU/ml human rIL-2 up to day 8 following activation of freshly isolated splenocytes. T cell yield was monitored microscopically using Trypan Blue exclusion (lower panel). (**B**) T cells transduced with both TCR and inducible mediator were labeled with CD8-APC antibody and gp100/HLA-A2-PE tetramer before start of selection (day 2 after T cell activation) and at the end of selection (day 7 after T cell activation). T cells were gated for live cells and dotplots are representative of three different experiments. Mock and TCR-transduced T cells were stained as controls. Percentages in upper right quadrants represent fractions of CD8⁺ T cells binding to pMHC complex; test procedure 3 or 4 as in (A) that yielded highest TCR expression is indicated in bold in (A, middle panel).



Supplementary figure 2. Treatment of mice with TCR+iIL-18 T cells results in prolonged anti-tumor response

HLA-A2 transgenic mice bearing established B16:A2-YLEP tumors were conditioned and treated with T cells as described in legend to figure 3. Tumor growth was measured by caliper 3 times a week and tumor volumes were estimated with the formula $0.4 \times (A \times B)$ where A represents the largest diameter and B the diameter perpendicular to A. Depicted are the individual tumor growth curves of mice for each treatment group. Mice exceeding a tumor volume of 1200 mm³ were sacrificed while growth lines marked with '+' indicate mice dying of causes unrelated to the outgrowth of tumors.



Supplementary figure 3. Treatment with TCR+iIL-12 T cells is accompanied by excessive weight loss

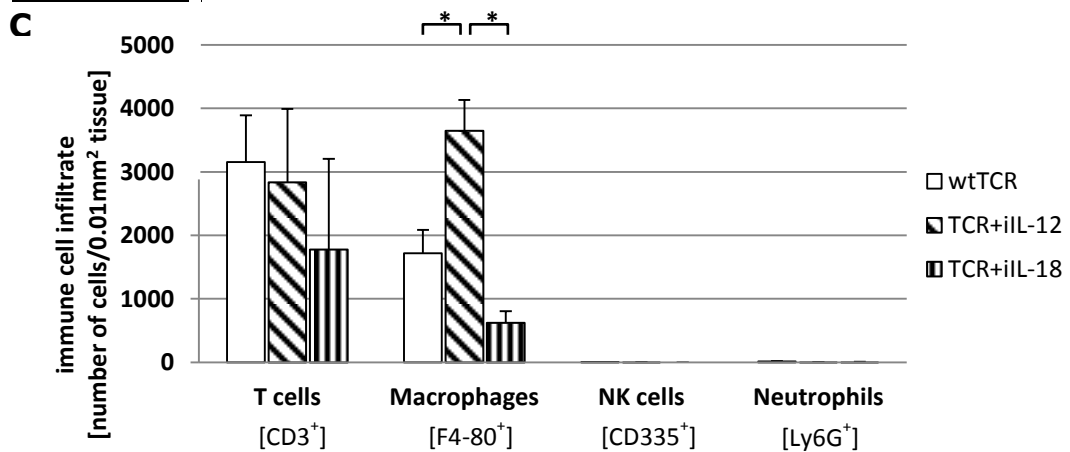
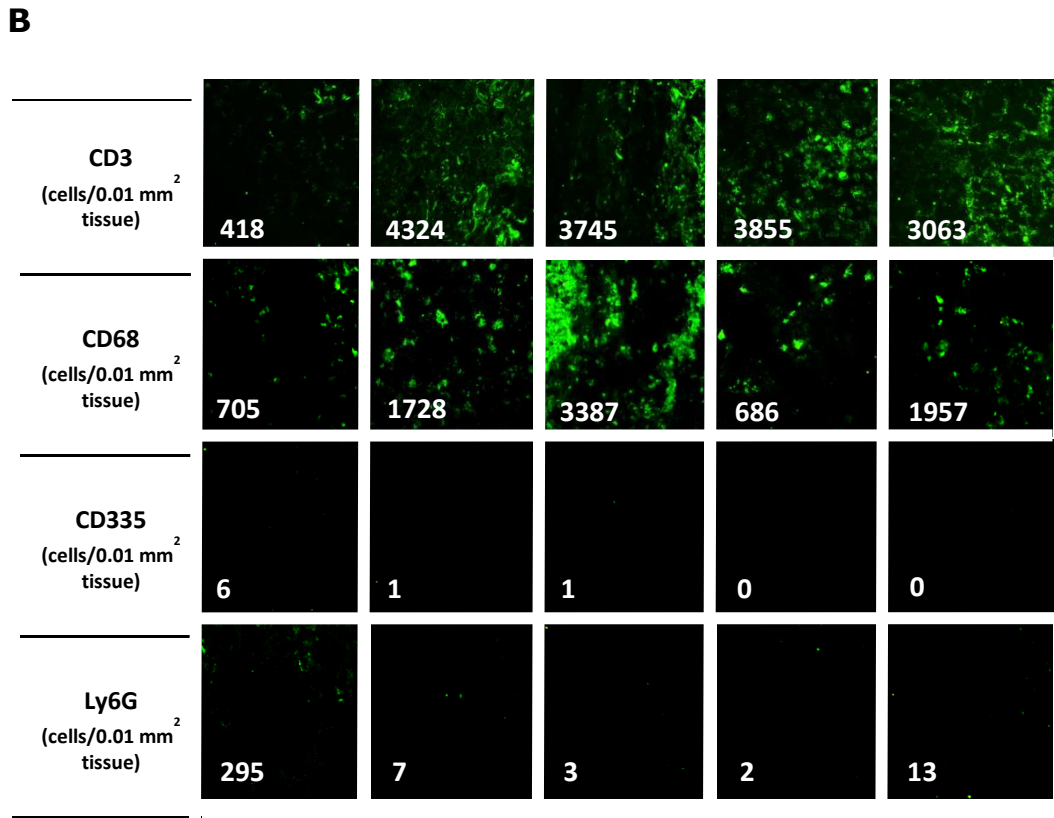
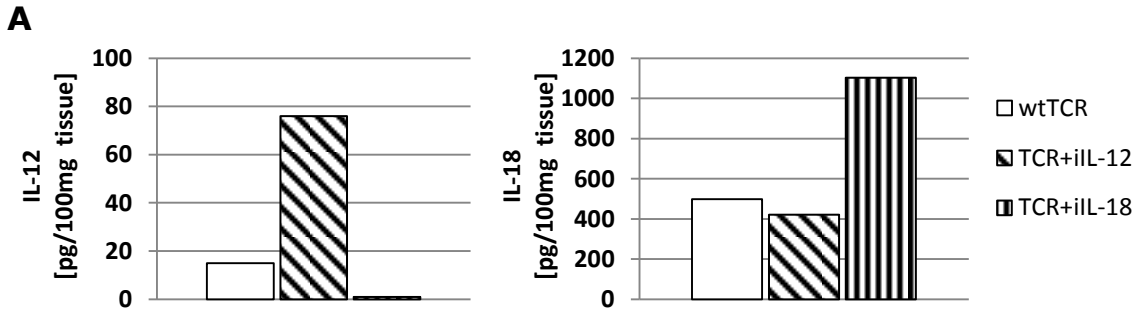
HLA-A2 transgenic mice bearing either established B16:A2-YLEP tumors or no tumor were conditioned and treated with T cells as described in legend to figure 3. Body weight of treated mice was recorded starting at either the day of tumor inoculation or in case of tumor-free mice at day of conditioning and every third day thereafter. Shown is the maximum weight loss during treatment \pm SEM (n=6-7) compared to initial weight measurements (weight range: 14.6-26.6 grams). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05; **p<0.01; ***p<0.005.

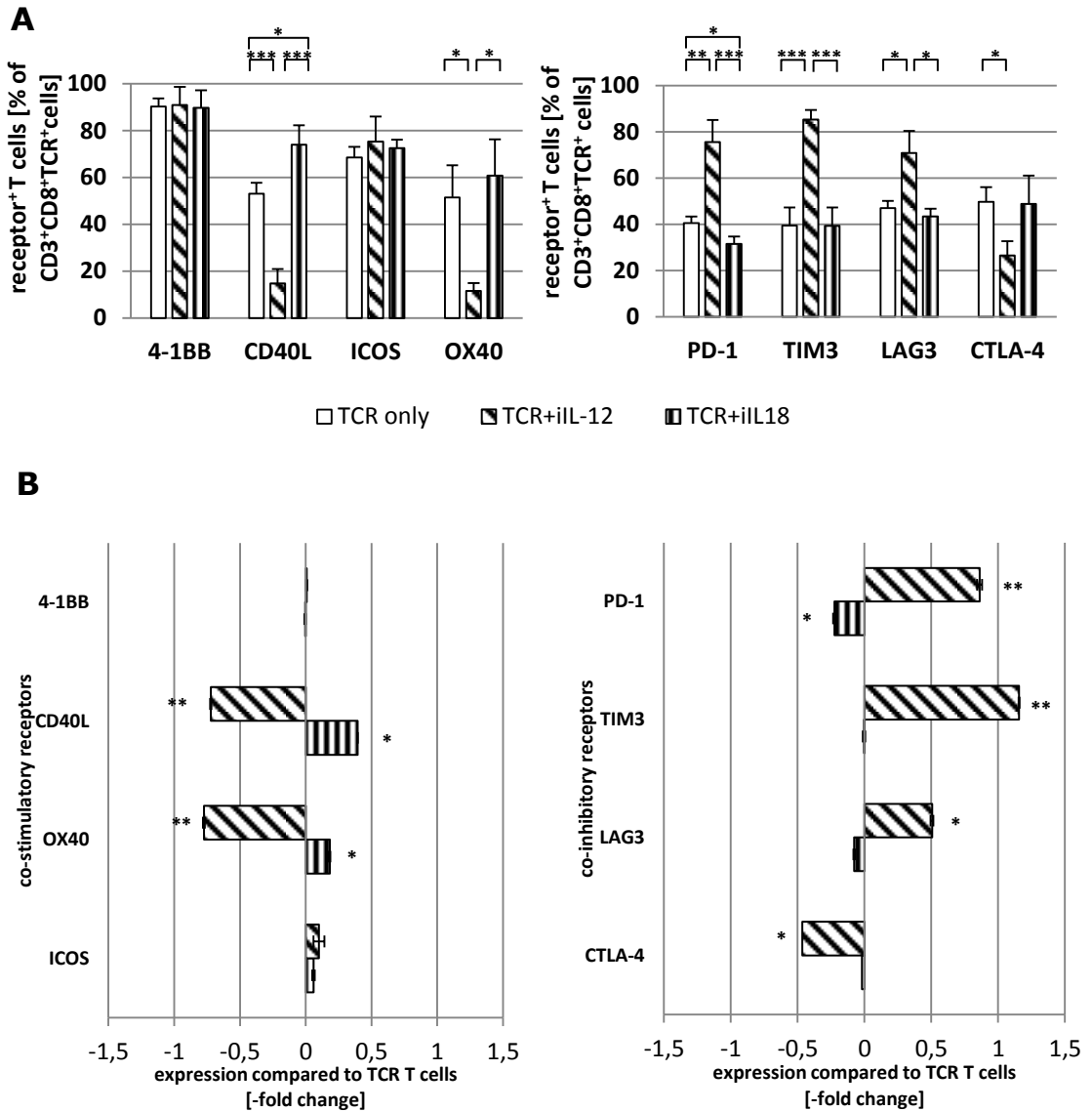
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Supplementary Figure 4. Mice treated with TCR+iIL-12 show an enhanced number of tumor-infiltrating macrophages

Following T cell treatment, mice with regressing were sacrificed (n=4 per group) on day 5 after T cell transfer. Part of tumor was lysed to measure levels of intra-tumoral IL-12 or IL-18; other part of tumor was used for in situ staining with CD3 (T cells), F4/80 (macrophages), CD335 (NK cells) and Ly6G (neutrophils) antibodies (all rat; secondary antibody: donkey anti-rat IgG Alexa Fluor 488). DAPI stainings were performed to quantify tissue areas containing nucleated tumor cells per picture (not depicted). Cell numbers in tissue stainings were quantified using FIJI software. (A) lists levels of IL-12 or IL-18 in tumors (n=2). (B) depicts exemplary images of the in situ stainings indicating the number of cells for that particular picture (200 \times magnified, Leica DM IL microscope and Leica DFC 3000G camera), while (C) shows the mean number of cells quantified from these stainings for each treatment group \pm SEM (n=4). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05

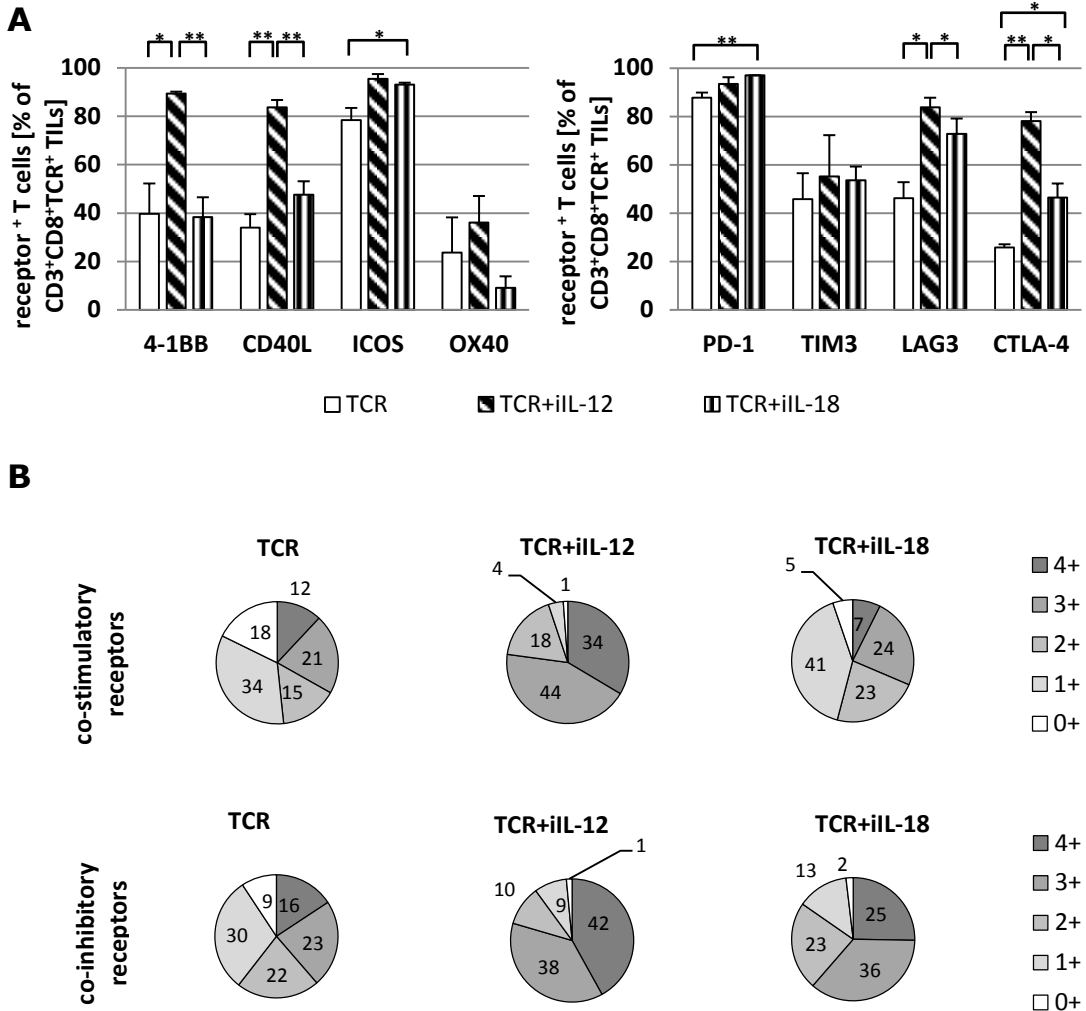






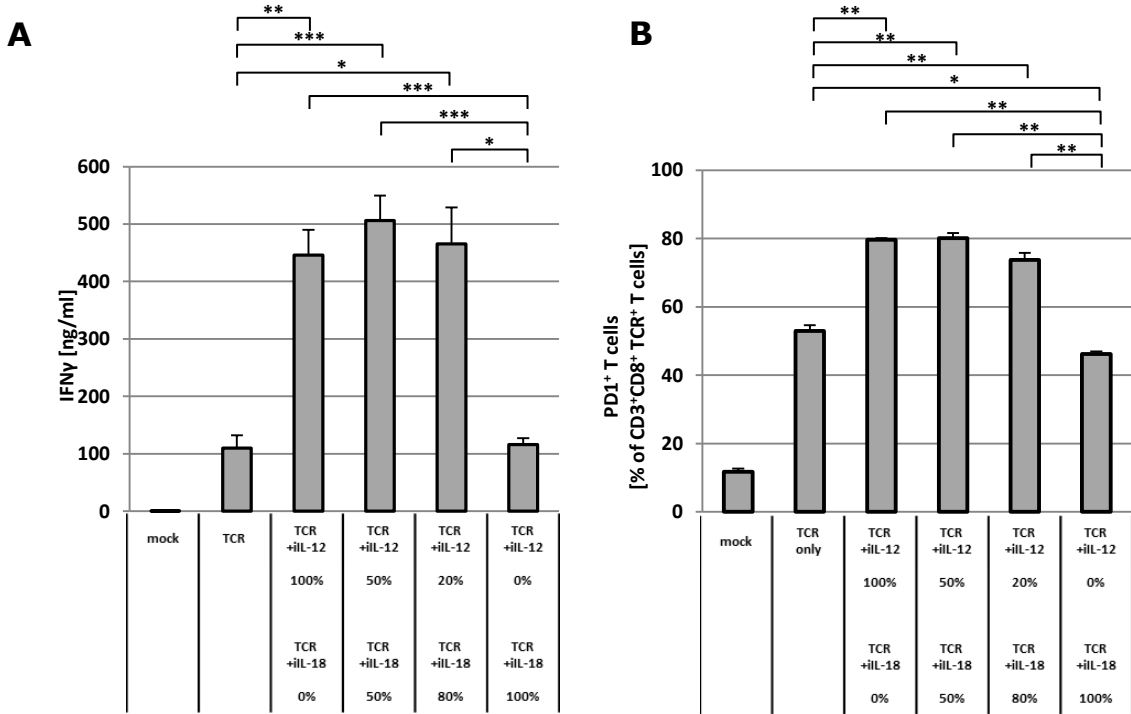
Supplementary figure 5. CD8 T cells engineered with TCR and iIL-18, when stimulated with tumor antigen in vitro, demonstrate no enhanced expression of ICOS but a decreased expression of PD1 in vitro.

T cells were transduced as described in legend to figure 2 and co-cultured with B16 melanoma cells that were positive or negative for the gp100 target antigen at an E:T ratio of 3:1. After 24h, T cells were harvested and stained for CD3, CD8, TCR and either the co-stimulatory receptors 4-1BB, CD40L, OX40, CD28, and ICOS, or the co-inhibitory receptors PD-1, TIM3, LAG3, CTLA4, and BTLA. (A) Expression levels of individual receptors on T cells positive for CD3, CD8 and TCR upon antigen-stimulation (%), mean±SEM, n=4 for all treatment groups). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05; **p<0.01; ***p<0.005. (B) Fold-changes in expression levels of individual receptors on TCR+iIL-12, TCR+iIL-18 and TCR+iIL-12+iIL-18 T cells compared to TCR T cells (mean±SEM; n=4).



Supplementary figure 6. Upon treatment with TCR+iIL-12 T cells, CD8 and TCR-transgene-positive TILs show enhanced expression of both co-stimulatory and co-inhibitory receptors

Following T cell treatment, mice with regressing tumors were sacrificed (n=4 per group). TILs were isolated as described in Materials and Methods and analyzed via flow cytometry for the expression of CD3, CD8, TCR, 4-1BB, CD40L, ICOS, OX40, PD-1, TIM3, LAG3 and CTLA-4. Bars in (A) show the mean expression levels of individual co-stimulatory (left panel) and co-inhibitory (right panel) receptors within the CD3⁺CD8⁺TCR⁺ TIL population. (B) provides an overview of the degree of co-expression of co-stimulatory receptors (upper charts) as well as co-inhibitory receptors (lower charts). Statistically significant differences between treatment groups were calculated with Student's t-test: *p<0.05; **p<0.01.



Supplementary figure 7. Production of T cell IFN γ as well as T cell expression of PD1 are governed by low numbers of TCR+iIL-12 T cells

T cells were transduced and co-cultured with B16 melanoma cells as described in legend to figure 2. In this series of experiments, T cell populations either comprised a single population of T cells (mock, TCR, TCR+iIL-12, TCR+iIL-18) or a combination of TCR+iIL-12 and TCR+iIL-18 T cells at the indicated ratios. After 24h, supernatants were collected and T cells were harvested. **(A)** Levels of IFN γ in culture supernatants are measured by ELISA and displayed as mean \pm SEM (n=4). **(B)** Percentages of PD-1 staining within CD3⁺ CD8⁺ TCR⁺ T cells are determined by flow cytometry and displayed as mean \pm SEM (n=3). Statistically significant differences between T cell populations were calculated with Student's t-test: *p<0.05; **p<0.01; ***p<0.005.



GENERAL DISCUSSION

In the preceding four chapters, I aimed to address major challenges that may currently impede TCR gene therapy of solid tumors through the following means:

1. Selection and validation of tumor specific target antigens and corresponding TCRs
2. T cell engineering to counteract local immune suppression

In this chapter, I aim to summarize my findings and put them into perspective with respect to recent developments in the field of immune-oncology. Finally, I aim to propose future designs for clinical trials with TCR-engineered T cells, incorporating beneficial aspects outlined in previous chapters.

7.1. Selecting suitable antigens and TCRs

Evidently, the search for an ideal T cell target antigen and a therapeutic TCR cannot be conducted independently from each other. This is due to the molecularly complex relationship between antigen expression/processing and TCR expression/affinity that within a window of set criteria results in full activation of TCR-transduced T cells (explained below). The following three subheadings detail and discuss our own and others' attempts to select antigens and obtain TCRs that provide optimal antigen:TCR efficacy.

7.1.1. Target antigens

Ideally, T cell target antigens are selectively expressed by tumor tissue and not by healthy tissue. Hence, they are not expected to evoke a response against self. At the same time, target antigens should have proficient immunogenicity to initiate an effective anti-tumor response. Since reported differences between cancer cells and healthy cells are generally based on self-peptides, these requirements have been one of the major challenges of TCR gene therapy. In most cases, disturbances of the cell-cycle or other regulatory mechanisms lead to altered expression levels of antigens that were already present in cancer cells, rather than presentation of entirely novel antigens (1,2). Initial clinical trials utilized TCRs directed against differentiation or over-expressed self- antigens in tumor tissues such as MART-1, CEA or gp100 (3-6). The on-target toxicity observed in these trials however, led to a shift in preferred target targets. Recent trials have focused more on cancer germline antigens (CGAs) such as NY-ESO1 (5). One of the favorable characteristics of these antigens is, at least for some CGAs, that expression is limited to cancer as well as germline cells (i.e. gonads, where lack of MHC expression renders these antigens invisible to TCRs). While expression of CGAs is thus more restricted to immune-privileged organs, the average expression levels of these antigens are lower compared to that of differentiation or over-expressed antigens and more heterogeneous between different tumor types and even within

individual tumors (7). In **chapter 3** of this thesis we have established that MAGE-C2 (MC2), a member of the CGA family, represents an antigen that can be targeted in tumor cell lines derived from different histologies such as melanoma, head and neck cancer, triple negative breast cancer as well as bladder cancer. Furthermore, we have established that the level of T cell response directly correlates to the level of MC2 expression, but not to the expression level of co-signaling molecules that are involved in T cell activation. In **chapter 4** we provided additional evidence that MC2 expression is virtually absent in most healthy tissues, with the exception of the testis. Summarizing our own findings, MC2 fulfilled criteria that we set for an ideal target antigen to be targeted in a clinical trial, namely a safe expression profile and initiating T cell responsiveness. In addition, MC2 is expressed in various tumor types, and MC2 expression levels have been reported to correlate to poor patient prognosis as well as mesenchymal to epithelial transition in breast cancer (8,9), highlighting its role in driving cancer development. It is noteworthy that the strongest arguments for MC2 as a safe, yet effective target antigen come from observations of a clinical trial, where clearance of solid melanoma tumors occurred due to high numbers of endogenous MC2-specific T cells. At the same time, no signs of T cell mediated toxicity were observed. Validation of the MC2-specific TCRs, which we identified from these patient-derived T cell clones, is discussed in more detail in the next subheading.

When discussing selection of target antigens, one particular group of antigens has been receiving increased interest recently: neoantigens. These are antigens derived from somatic mutations occurring throughout carcinogenesis. Studies have shown that tumor-infiltrating lymphocytes that specifically recognize mutated antigens do occur in patients (10-13). In addition, boosting endogenous T cell responses using checkpoint inhibitors seems to have a higher impact on patients with a higher mutational burden (14,15). Moreover, targeting neo-epitopes either via vaccination (16,17) or transfer of peripheral blood mononuclear cell (PBMC) or tumor-infiltrating lymphocyte (TIL) populations (18-20) resulted in measurable tumor regression in melanoma and colorectal cancer patients. While targeting neoantigens has proven its feasibility as indicated by the above studies, detection and prediction of these mutations remains challenging. The diverse genetic landscapes in tumors, due to different histological origins or the fundamental genetic variation between patients, makes this process a highly personalized approach. In fact, overlap in specific mutations remains rather rare and just like CGAs and overexpressed antigens, immunogenic mutations remain subject to dynamic changes induced by T cell pressure (18,21,22). The isolation or selective expansion of neoantigen-specific T cells from autologous patient material is a critical element towards development of such personalized adoptive T cell therapy. Novel approaches to facilitate these processes encompass expansion from TILs or peripheral blood through co-culture with autologous APCs expressing neoantigen minigenes (12,23) or cell sorting with the use of peptide MHC multimer libraries (24,25). Notably, both TIL and vaccination approaches, rely on the condition that a patient's endogenous T cell repertoire contains T cell clones with sufficient avidity towards their target.

7.1.2. T cell receptors

Once a target antigen is chosen or selected, one can start isolating and selecting corresponding TCRs for therapeutic use. Also the latter selection has to adhere to several criteria. In order to trigger an adequate immune response, TCRs require sufficient affinity for their respective target antigen to ensure optimal T cell activation and subsequent display of effector functions, while at the same time the TCR requires sufficient specificity to prevent or limit T cell activation when encountering peptide-MHC complexes that are similar yet not identical to the cognate peptide-MHC complex. The range of a TCR's affinity, contributing to a T cell's avidity, may differ depending on the source of T cells. On the one hand, TCRs derived from patient material (TILs, PBMC after peptide vaccination, etc.) have passed thymic selection, resulting in TCRs of only low to intermediate affinity for self-antigens (26,27). On the other hand, TCRs derived from a non-tolerant repertoire of T cells that were co-cultured with artificial antigen-presenting cells pulsed with peptides of interest (28) or from transgenic mouse models equipped with human TCR and HLA repertoires (29) can reach high affinities for the chosen target antigen. The affinity requirements for a TCR intended for therapeutic use depend on properties of the cognate antigen, such as extent and homogeneity (i.e., fraction of cells within a tissue) of expression, and epitope binding to MHC. The MC2 specific TCRs we evaluated in **chapter 3** of this thesis were derived from patient T cell clones. As these TCRs are derived from a tolerant repertoire, our expectations were that their affinities would be low to intermediate, and their specificities high. Indeed, four TCRs recognizing MC2 epitopes in the context of HLA-A2 (TCRs 4, 6, 11 and 16) displayed a low to intermediate range of affinities for their cognate epitopes. Responses of TCR-engineered T cells towards tumor cell lines that demonstrated low expression of MC2 were negligible, whereas enhancement of MC2 expression (see 7.1.3) enhanced T cell responses. No T cell responses to healthy cell lines were observed. TCRs 6 and 16 showed strongest MC2-specific activation of T cells and displayed a clear breadth of responsiveness across tumor cell lines of different histological origins (see chapter 4, table I for an overview). Safety assessment of both TCRs was conducted in **chapter 4**. Although both TCR 6 and TCR 16 were derived from an autologous repertoire and have a low-risk of cross-reactivity versus self-antigens, negative thymic selection of T cells may not have been 100% waterproof. The reporting of autoimmune side effects following adoptive transfer of T cells using patient-derived TCRs, or checkpoint blockade-mediated reactivation of endogenous T cells substantiate this notion. The thymic escape of self-reactive T cells is most likely a trade-off between producing a self-tolerant yet sufficiently diverse and responsive TCR repertoire (30). In order to assess the risk of cross-reactivity, we carried out initial, unbiased testing of TCR-transduced T cells by exposing them to a library of 114 HLA-A2-eluted peptides. In these assays, none of the TCRs showed signs of activation aside from responses towards their respective cognate peptides. For a more individual, TCR-specific approach we determined recognition motifs, meaning the amino acids and their positions within the target epitope that are critical for recognition by the TCR. To this end we used a set of altered peptide ligands (APLs). Subsequently, we determined the frequency of these recognition motifs within the human antigenome (31) and tested whether T

cells transduced with TCR 6 or TCR 16 were able to respond to these matched peptides in an artificial (peptide loaded cells) or endogenously expressed and presented (cell lines) setting. It is noteworthy that TCR 16 showed a highly restrictive recognition motif, with 7 out of 9 consecutive amino acids that could not be altered without losing TCR recognition (xLKDVEERx). Antigenome screens revealed only a single additional antigen that harbors this recognition motif: MAGE-C1 (MC1). Subsequent co-culture of TCR-engineered T cells with target cells loaded with the MC1 peptide revealed a 5-fold higher EC_{50} value of TCR 16 when compared to the cognate peptide. Co-culture with tumor cells expressing native MC1, however, failed to initiate T cell activation. Only one other TCR with such a restricted recognition motif and lack of cross-reactivities has been described to date in vitro: a TCR specific for the over-expressed survivin antigen (32). TCR 6, possessing a less restricted recognition motif, was predicted to recognize 27 additional epitopes, yet the peptide of only a single antigen was able to trigger T cell activation at a 2.5-fold higher EC_{50} value when compared to the MC2 peptide. Also this antigen turned out to be a CGA, namely MAGE-B4 (MB4). Similar to TCR 16, TCR 6 failed to initiate T cell activation when co-cultured with tumor cells expressing MB4.

It is important to note that the use of APLs, in which an alanine or glycine is generally used to substitute a single amino acid, does not cover all possible amino acid changes at any given position and may thus under-estimate the cross-reactivity of a TCR. To improve the assessment of a TCR's recognition motif, it would be possible to use extensive peptide libraries that contain random amino acid substitutions at multiple positions (30,33). Notwithstanding that testing such extended libraries provides a better coverage of non-cognate peptides (including foreign peptides), we would like to emphasize that cross-reactivities against self-peptides as observed in patient studies using TCR-engineered T cells so far (34,35) have been identified by the proposed assays in **chapter 4**. In fact, we would like to argue that the proposed identification of recognition motifs, together with other assays mentioned in **chapter 4**, should be considered a useful tool to significantly narrow down the list of cross-reactive self-peptides when assessing clinical TCRs. Taken together, in **chapters 3 and 4**, we have established that the patient-derived TCRs we obtained, once introduced into T cells, provide functional avidity towards their cognate peptide as well as negligible cross-reactivity against self-peptides.

7.1.3. Enhancing T cell efficacy through epigenetic drugs

The optimal window of TCR:antigen interactions, as pointed out earlier, is governed by a molecularly complex relationship between antigen expression (including affinity of peptide for MHC) and TCR affinity for pMHC. Despite the fact that this window may be narrow, it can be shifted towards enhanced TCR:antigen interactions and hence enhanced T cell responses. For this purpose, different TCR-modifications or patient pre-treatments can be employed to enhance the efficacy of T cell treatments (see figure 1).

The first modification is TCR affinity enhancement, which has been applied for clinical TCRs derived from tolerant patient repertoires or vaccinated mice ((6,22,36), see also Table 2 of **Chapter 2**). In order to increase the affinity of a TCR for its cognate peptide, amino acid in one or several of the complementarity determining regions (CDRs) of the TCR are substituted ((37); Govers, manuscript submitted). Although TCR affinity clearly governs T cell avidity, it is important to realize that there may be a strict window of TCR affinities within which antigen-specificity is preserved. TCR affinities outside such a window may result in loss of specificity and/or T cell function, i.e., minor affinity enhancements resulting in a $K_D \geq 4\mu\text{M}$ improved antigen-specific T cell function, while TCRs enhanced to a $K_D < 1\mu\text{M}$ drastically lost peptide-fine specificity ((38); Govers manuscript submitted). Thus, T cells transduced with an affinity enhanced TCR may result in enhanced clinical response rates, such a modification may be accompanied by an increased degeneracy of the TCR recognition motif (Govers, manuscript submitted; **chapter 4** of this thesis). This increases the general risk of off-target toxicity as seen in adverse effects described in recent clinical trials (35,39) and highlights the need for thorough safety assessments as discussed in **chapter 4**.

The second modification is upregulation of target antigens, which we applied through the use of epigenetic drugs in **chapter 3**. In particular two types of epigenetic drugs are currently investigated in combination with immune therapy: histone deacetylase (HDAC) inhibitors and DNA-methylation (DNMT) inhibitors. HDACs aim at reducing DNA packaging around histones, thus allowing for the (over-)expression of genes usually repressed in cancer cells. DNMTs prevent methylation of genes which is also a means to reduce expression. While both these types of agents were initially thought to contribute towards an anti-tumor response by primarily de-repressing cancer suppressor genes or cell cycle regulators in hematological diseases (reviewed in (40,41)), in recent years the understanding emerged that their epigenetic effect impacts anti-tumor immune responses as well. One recently appreciated mechanism is enhancement of tumor cell immunogenicity through expression of usually silenced genes. These genes may be derived from benign retroviruses (42-44) or from for instance CGAs such as MC2 or NY-ESO1. We were able to show that treating tumor cell lines of various origins with the DNMT inhibitor Azacytidine and the HDAC inhibitor Valproate upregulated existing or enabled de novo gene expression of MC2. Treatment of cell lines of healthy origin did not affect CGA expression. De-repression of CGAs is usually the combined result of DNA de-methylation, dissociation of polycomb proteins and shifting of euchromatin markers such as for example BORIS (a CCCTC-binding factor (CTCF)-paralog) at the cancer-germline promoter site (45). Healthy cells demonstrate strict epigenetic silencing of CGAs, and are usually devoid of for instance BORIS, which prevents transcriptional activity of certain chromatin areas (46,47). In contrast, tumor cells are generally intrinsically disrupted in their gene silencing, in which case treatment with epigenetic drugs is thought to further counteract gene silencing while healthy cells remain unaffected (48,49). Next to making tumor cells more 'visible' to the immune system, another mechanism by which epigenetic drugs enhance tumor immunogenicity is enhancement of the production of chemokines involved in immune cell migra-

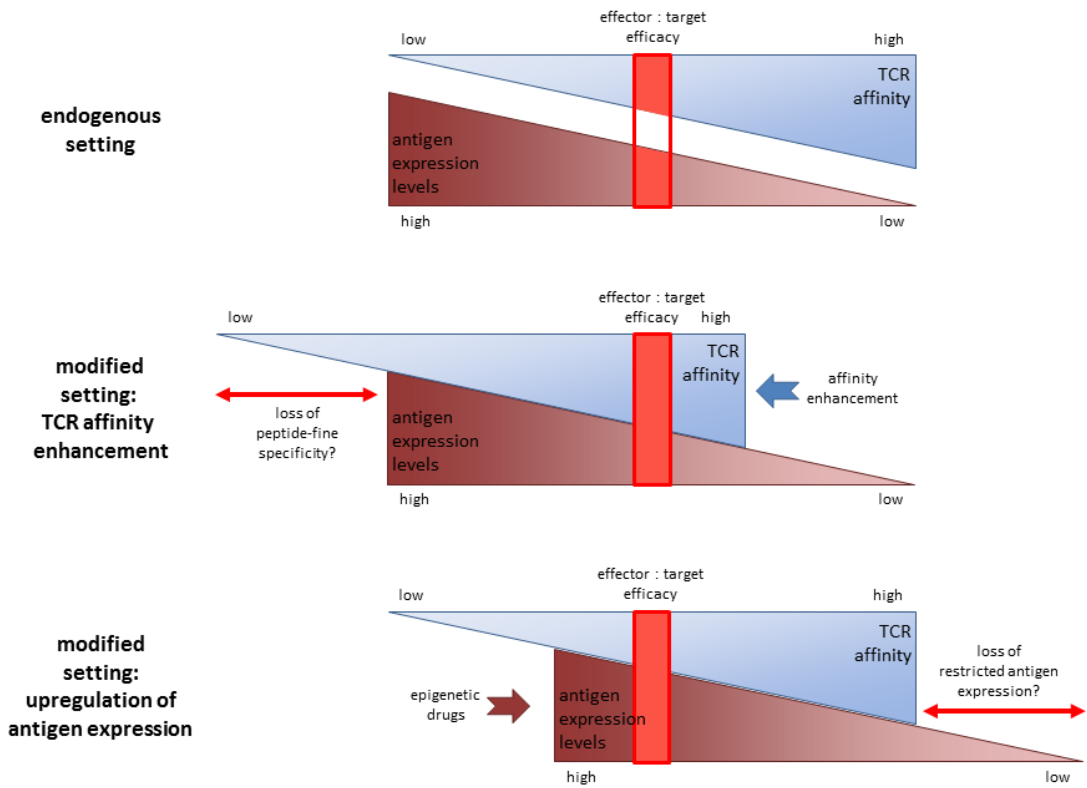


Figure 1. Manipulating TCR:antigen interactions to enhance TCR gene therapy

TCR:antigen interaction dictates T cell responsiveness, which occurs within a window (schematically illustrated by red rectangle) that is set by antigen expression (bottom triangle) and TCR affinity (upper triangle). In the endogenous setting (top panel) low affinity TCRs (as a consequence of thymic selection against self-antigens) or low or no expression of CGAs may prevent effective anti-tumor responses. One can shift the TCR:antigen interactions towards improved T cell responses by enhancing a TCR’s affinity (middle panel); this shift potentially introduces the risk of reducing TCR specificity. One can also favorably shift the TCR:antigen interactions by enhancing antigen expression through epigenetic drugs (lower panel); this shift requires to check for maintenance of tumor-specific antigen expression.

tion as well as production of IFN γ and TNF α by effector cells (50). These epigenetic drugs may trigger the viral defense pathway through enhanced levels of retroviral RNAs (42), resulting in up-regulation of the type I and type II interferon families, such as interferon-gamma receptor 1 and STAT, as well as antigen processing and subsequent presentation (51). Interestingly, the up-regulation of type I interferons such as IFN α may further potentiate the anti-tumor effects of Azacytidine (52).

When it comes to the utilization of epigenetic drugs in combination with immune therapy, there are already several exemplary clinical trials ongoing. The majority of these trials focusses on combination treatments of PD-1 blockade (Nivolumab, Pembrolizumab) with DNMTis (Azacytidine, Vorinostat) (53). Previous trials revealed how crucial timing and dosage of these agents are in a clinical setting. For instance, epigenetic drugs are intrinsically toxic to hematopoietic cells (hence

their initial use in treating hematological malignancies). Recent preclinical studies demonstrated that gene expression effects of epigenetic drugs on tumor cells may already occur at low doses, thus reducing toxicity risks (54,55).

In line with these findings and our own observations, we plan to include epigenetic pre-treatment of patients as part of a Phase I clinical trial that utilizes TCR 16 transduced T cells for patients suffering from MC2/HLA-A2-positive melanoma as well as head-and-neck cancer.

Box 1. Major Findings of chapters 3 and 4

Selecting and testing antigen and TCRs

- Patient blood with enhanced frequencies of tumor-specific T cell clones, potentially following treatment, provide source a good starting point to obtain effective and safe TCRs.
- Expression of MAGE-C2 (MC2) may be upregulated through the use of epigenetic drugs such as Azacytidine and Valproate in cancer cell lines of various histological origins.
- Epigenetic drugs do not upregulate MC2 expression in healthy cell lines.
- Responsiveness of MC2-specific TCRs is directly correlated to expression levels of MC2, but independent of expression levels of CD80, CD86, programmed death-ligand 1 (PD-L1) and PD-L2.
- Epigenetic drug-pretreatment of tumors in combination with MC2-specific TCRs provides an effective therapy when testing cell lines from melanoma, head-and-neck, bladder and triple-negative breast cancers *in vitro*.

In vitro safety assessment of TCRs

- TCR gene therapy trials of recent years highlight the need to thoroughly assess the risk of on-target and off-target toxicity.
- Transcriptomic analyses and RT-PCRs, and in some cases immune histochemistry, of healthy tissues are suitable tools to assess the expression of candidate target antigens and the risk of on-target toxicity.
- Initial safety assessment of TCRs can be conducted by testing responsiveness of transduced T cells to HLA-restricted peptide libraries and cell lines transduced with allogenic HLA molecules.
- For TCRs with a therapeutic intent, it is recommended to identify individual recognition motifs to properly assess risk of off-target toxicity.

7.2. T cell engineering to counter the immune suppressive tumor microenvironment

Solid tumors possess numerous ways of avoiding detection or inhibiting a response mounted by the immune system (in part reviewed in **chapter 2**; also see **Chapter 1**, figure 2 for an overview of evasive mechanisms). Next to up-regulation of checkpoint inhibitors or down-regulation or loss of HLA (or other antigen-presenting) molecules, tumors may prevent T cells from entering the tumor site. Furthermore, enhanced recruitment of immune inhibitory cell types and changes in the cytokine milieu may interfere with the activity of T cells. The following two subheadings propose approaches to modify therapeutic T cells in an attempt to counter immune-suppressive mechanisms in the tumor micro-environment.

7.2.1 Co-stimulatory TCRs and enhanced T cell fitness

Full activation of therapeutic T cells requires multiple interactions, primarily the binding of TCR to its cognate antigen and secondarily the binding of a co-stimulatory receptor on the T cell to a corresponding ligand on an antigen presenting or tumor cell (i.e. CD28 and CD80/86) (56). Tumors are known to down-regulate or completely abrogate the expression of co-stimulatory ligands (see **Chapter 1**, figure 2 for an overview of evasive mechanisms). Due to the lack of co-signaling, T cells can become anergic despite continuous TCR stimulation, reducing their proliferation and anti-tumor effector functions (57). In **chapter 5** we propose to address early T cell anergy by integrating co-stimulatory signaling elements into TCRs. This approach has proven feasible in CAR-mediated T cell therapy (58-60) and has also been shown by our laboratory to enhance treatment responses in TCR-mediated T cell therapy (61,62). The latter observations were obtained using an expression cassette, which integrated CD28 and CD3 ϵ signaling elements into a gp100-specific TCR, and which was defined by re-iterative optimization experiments. We made further attempts, using this expression cassette, to enhance TCRs by replacing the intracellular domain of CD28 with those of OX40, ICOS, 4-1BB and CD40L. Initial in vitro assays revealed that the TCR:28-4-1BB ϵ and the TCR:28-CD40L ϵ variant were not expressed by T cells and that TCR:28 ϵ , TCR:28-OX40 ϵ and TCR:28-ICOS ϵ , although mediating T cell function, showed reduced affinity towards the cognate peptide when compared to the wtTCR (see **chapter 5** for details). However, when evaluating the impact of TCR:28 ϵ , TCR:28-ICOS ϵ and TCR:28-OX40 ϵ TCRs in our gp100 melanoma mouse model, we observed that all three co-stimulatory TCRs improved therapy significantly when compared to wtTCR. TCR:28-ICOS ϵ T cells in particular led to complete cures in 50% of the mice and more than doubled the time till tumor recurrence up to 40 days after T cell injection. We found that one of the most outstanding features of these co-stimulatory TCR T cells was their enhanced persistence in blood and increased numbers in regressing tumors, which in general is considered a beneficial factor with respect to therapy response (63,64). Notably, the number of therapeutic T cells within the blood one week after transfer correlated with the day of relapse, similar to

observations made in clinical trials (65). To better understand enhanced T cell persistence, we are currently investigating the impact of co-stimulatory signaling on T cell phenotype and metabolism. With regard to the former, the enhanced expression of CD40L in TCR:28-ICOS ϵ T cells is of particular interest. The interaction of CD40L on T cells with CD40-bearing dendritic cells (DCs) within the tumor was determined essential in successful tumor clearance through adoptive T cell therapy (66,67). With regard to the latter, skewing the energetics of T cells towards glycolysis has been described as a result of triggering both ICOS and OX40 pathways (68-70), possibly providing them with a proliferative advantage within the tumor microenvironment.

With the aim to translate our findings from this gp100 model into a clinical trial, we will test the use of TCR:ICOS specific for an antigen with a more restricted, safer expression (i.e. MC2) and evaluate such TCRs further.

7.2.2 Smart T cells and production of immune-stimulating cytokines

Cytokines impact T cell differentiation and effector function (reviewed in (71)) and tumors have been shown to drive differentiation of effector T cells towards a more exhausted phenotype. While it is possible to drive therapeutic T cells into a preferential differentiation and/or effector state using cytokines ex vivo prior to transfer into the patient (72,73), other can be used enhance T cell effector functions. Two examples of cytokines we would like to administer locally in order to enhance T cell function are the pro-inflammatory cytokine IL-12 which is known to enhance cytolytic activity of T cells (74) and IL-18, a pro-inflammatory cytokine able to drive T and NK cell maturation (75). Systemic application of IL-12 in patients was is not considered feasible due to toxic side effects (76). Systemic application of IL-18 on the other hand, while non-toxic yielded no clinical responses (77,78). In **chapter 6** of this thesis we have set up a system that allows for inducible, local production of either IL-12 or IL-18 at the tumor site. We optimized transduction conditions in order to sequentially equip primary T cells with a gp100-specific TCR, and either murine IL-12 and/or IL-18 under the control of a nuclear-factor of activated T-cell (NFAT)-sensitive promoter (iIL-12 and iIL-18, respectively). Initial assessments of these double transduced T cells in vitro indicated that antigen-specific activation was required to induce cytokine production. However, when assessing the value of TCR+(i)cytokine in our in vivo model, we observed that use of TCR+iIL-12 T cells in tumor bearing mice triggered severe side effects such as severe weight loss as well as edema and reduced overall survival compared to treatment with TCR T cells without iIL-12. Alongside these toxicities we observed enhanced infiltration of the tumor by macrophages and enhanced serum levels of IFN γ and TNF α . The later finding mirrors observations from studies that tested systemic administration of IL-12 (76,79). Notably, treatment of melanoma patients with iIL-12 TILs resulted in similar side effects (80). In contrast, the use of TCR+iIL-18 T cells enhanced anti-tumor responses and prolonged survival without any kind of overt side effects. This can be in part accredited to an enhanced frequency of therapeutic CD8 $^+$ T cells within tumors

(63,64) compared to the mice treated with TCR+iIL-12 T cell . Our findings highlight that while the inducible release of cytokines at the tumor site seems a promising approach to enhance T cell therapy, the choice of cytokine (or other mediator) and confirmation of restricted release are crucial. Our in vitro studies showed that the T cell activation leads to production of massive amounts of IL-12 under antigen-positive conditions as well as unspecific release of small amounts of IL-12 under antigen-negative conditions. In vivo we observed an enhanced infiltration of macrophages into the tumors of TCR+iIL-12 T cell-treated mice, which may argue that the effect is localized, but it cannot be excluded that part of the produced IL-12 is leaking from the tumor microenvironment into the periphery, resulting in the systemic effects we observed. The type of tumor and its antigen targeted (location, vascularity, etc.) or the targeting receptor may further affect the risk for toxicities. Indeed, Chmielewski and colleagues did not observe toxic side effects in a colon-carcinoma model using CAR+iIL-12 T cells (81), while we in a melanoma model and Zhang and colleagues in melanoma patients did observe side effects when using TCR+iIL-12 T cells (80). As our findings with iIL-18 indicated, inducible production of cytokines without leakage and no side effects can be achieved. In extension to IL18, we are currently also assessing the value of inducible chemokine constructs which have both proven their importance and value in clinical trials (82-86).

7.3. Suggested improvements of TCR gene therapy

Cancer is a highly diverse disease that is challenging to target specifically and that actively counteracts the immune system. The latter probably being one of the major factors that allows its growth and treatment resistance to begin with (2,87). In order to provide a most optimal therapy approach using TCR engineered T cells, these challenges can be addressed at multiple levels as exemplified in this thesis: i.e., selection of target antigen and corresponding TCRs, costimulatory TCRs, inducible cytokine production and epigenetic pre-treatment of patients (see figure 2).

A proposed future TCR T cell trial should center around the proper selection of a tumor-restricted, sufficiently expressed target antigen (i.e., MC2) as well as the use of a TCR with sufficient affinity for its target (i.e., TCR 16) as described in 7.1. Both, antigen and TCR should undergo stringent safety assessment to limit the risk of on- and off-target toxicities. To further enhance anti-tumor T cell responsiveness in particular against CGAs, epigenetic drugs (i.e. Azacytidine and Valproate) should be used to further boost antigen expression and sensitize tumors to T cell treatment. In order to counteract the immune suppressive tumor micro-environment, multiple approaches are available and may be individually selected to match dominant suppressive mechanisms: therapeutic T cells could be equipped with co-stimulatory TCRs (i.e., TCR:28-ICOS ϵ), making their activation independent of co-signaling ligand expression by tumor cells, or inducible and local production of cytokines (i.e., iIL-18) may be employed to enhance effective T cell responses.

Box 2. Major Findings of chapters 5 and 6**Co-stimulatory T cells**

- An optimal expression cassette (harboring tmCD28 and icCD3 ϵ) enables T cell surface expression of TCRs equipped with ic domains of the co-stimulatory receptors CD28, ICOS or OX40
- TCRs equipped with CD28, ICOS or OX40 delay tumor relapse in a gp100 melanoma mouse model.
- TCR:28-ICOS ϵ T cells mediate enhanced tumor-free survival.
- TCR:28-ICOS ϵ and TCR:28-OX40 ϵ T cells persist longer in peripheral blood than wt TCRs and show enhanced numbers within the tumor.

Inducible cytokine production

- Therapeutic T cells transduced with a TCR and an inducible cytokine construct produce such a cytokine only upon antigen-specific activation.
- Adoptive transfer of TCR T cells with inducible (i)IL-12 to melanoma-bearing mice resulted in severe, edema-like toxicity, enhanced levels of inflammatory cytokines in blood, enhanced infiltration of macrophages into the tumor and reduced overall survival.
- Adoptive transfer of TCR T cells with iIL-18 to melanoma-bearing mice resulted in significantly reduced tumor burden and prolonged overall survival, without side effects.
- iIL-12 T cells show enhanced expression of co-inhibitory receptors, while iIL-18 T cells exhibited a favorable profile of T cell co-stimulatory and inhibitory receptors.

While the above-mentioned approaches carry the potential to improve therapy for patients, resulting in tumor clearance and prolonged survival, it is important to realize that cancer is a dynamic disease. The mechanisms underlying immune evasions may be distinctive for certain tumor types or change depending on the choice of therapy (88). Clinical trials with checkpoint inhibitors in advanced melanoma show long lasting effects in a fraction of patients, however ~25% of these patients relapse after a median follow-up of 21 months (89). While initial blockade of the PD-1/PD-L1 axis is sufficient to treat the disease, other immune suppressive mechanisms such as defects in interferon-receptor signaling pathway or antigen presentation may arise as the dominant drivers of immune evasion (90). As stated in **chapter 1** of this thesis, many patients suffer from such relapses after initial response to T cell therapy due to acquisition of or change within an immune suppressive tumor micro-environment. It is noteworthy that the proposed approaches in

this thesis are designed to counter selected mechanisms such as lack of co-stimulation or decreased T cell activity, and may be included (or not) dependent on the tumor type and (other) treatment components of choice.

Therefore, in extension to research conducted with the intention to improve T cell therapy, it is necessary to gain a better, more basic understanding of how this myriad of evasive mechanisms are connected with each other, how to detect their emergence throughout therapy and how to counter them successfully. To this end, research is extending its efforts in regard to immune monitoring of patients undergoing immune therapy as well as its attempts to understand the processes underlying immune evasion in preclinical models.

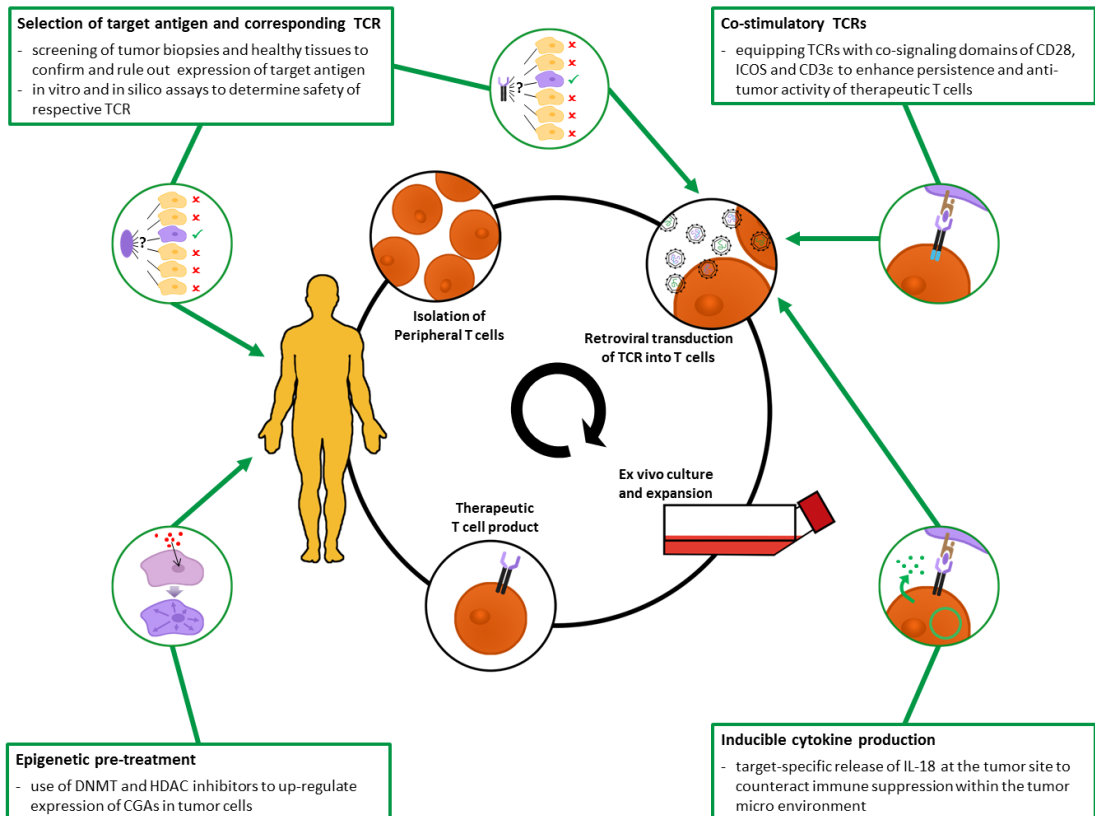


Figure 2. Improving TCR gene therapy requires multiple angles

Depicted is the standard procedure of TCR gene therapy (within circle), amended with individual approaches to enhance therapy efficacy and safety as indicated in this thesis (within green boxes).

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**SUMMARY –
ENGLISH/NEDERLANDS**

Summary

Immune therapy has proven its feasibility in cancer treatment and in some cases even its preeminence over other treatment modalities such as chemotherapy. Despite promising results observed in clinical trials utilizing or targeting various components of the patient's own immune system, new challenges emerged as a significant fraction of patients demonstrate inherent or acquired non-responsiveness to therapy or therapy-related toxicity. In this thesis, I provide an overview over pre-clinical approaches we have undertaken in order to optimize T cell receptor (TCR) gene therapy, a treatment that involves genetic introduction of a TCR specific for a selected tumor antigen into patient derived T cells.

Chapter 1 starts with a short overview of some of the currently employed immune therapies: checkpoint inhibitor treatment, vaccination therapy and adoptive T cell therapy. Given the focus of this thesis, this chapter continues to outline the current challenges facing TCR gene therapy, namely the search for safe, effective target antigens as well as overcoming tumor-mediated T cell evasion. Following these challenges, we designed approaches to improve TCR gene therapy along two lines:

- (1) *Selection and validation of tumor specific target antigens and corresponding TCRs; and*
- (2) *T cell engineering to counteract local immune suppression.*

These research lines are explained and introduce the work presented and discussed in subsequent chapters. Lastly, this chapter provides the aims of this thesis and how individual chapter contribute to addressing the main challenges described above.

Chapter 2 provides a literature review covering the challenges mentioned in chapter 1, which is evident from its subheadings: 'Choice of Target Antigen', 'T cell Fitness' and 'Sensitization of Micro Milieu for T cell Therapy'. This review deals with translational and clinical approaches, some of which find application within this thesis.

In **chapter 3** we have used patient blood with enhanced frequencies of tumor-specific T cell clones as a source for TCRs. The utilized T cell clones demonstrated specificity for the cancer germline antigen (CGA) MAGE-C2 (MC2) and were obtained from patients that showed clinical responses following vaccination without apparent side effects. We have isolated ten sets of TCR α/β chains from eight clones and introduced these into retroviral vectors for *in vitro* assessment. Four of these TCRs revealed functional expression in T cells as verified by pMHC stainings as well as co-culture with peptide loaded cells. T cell performances and tumor-specific recognition were further assessed through co-culture with cell lines of melanoma, head-and-neck, bladder and triple-negative breast cancer origin. Another observation from this chapter was that expression of MC2 becomes

pregulated through use of the DNA methyl transferase inhibitor Azacytidine and the histone deacetylase inhibitor Valproate in various cancer cell lines. Notably, the use of epigenetic drugs neither induced MC2 expression nor did it evoke MC2-specific T cell activation by healthy cell lines. With tumor cell lines however, epigenetic drug treatment led to an enhanced responsiveness of MC2-specific TCRs, which was dependent on expression of antigen, but not CD80, CD86, PD-L1 and 2.

Chapter 4 revolves around the safety assessment of antigens and TCRs. Due to the occurrence of adverse events in clinical TCR gene therapy trials, we advocate that target antigens as well as TCRs intended for clinical use need to undergo stringent testing in a series of *in vitro* and *in silico* assays. Using the MC2 antigen and two of the MC2-specific TCRs from chapter 3 as examples, we proposed a pipeline consisting of two major elements. First, antigen safety is defined by sufficient expression and presentation by tumor cells, and absent expression by healthy cells. Using RNA- and tissue libraries of healthy samples as well as tumor cell lines, together with qPCR analysis and histochemistry, we were able to demonstrate that the MC2 expression profile is safe and does justify its targeting in human cancers. Secondly, TCR safety is defined by a low potential for cross-reactivity. For initial evaluation, TCR transduced T cells were exposed to HLA-A2-restricted peptide libraries. For a subsequent, more TCR-specific approach, we assessed the recognition motif using altered peptide ligands, after which peptides with matching recognition motifs were evaluated for their ability to induce T cell activation. TCRs assessed in this manner revealed a single other, non-cognate peptide derived from the CGAs MAGE-C1 or MAGE-B4. T cell avidities for these non-cognate peptides were decreased compared to the cognate peptide, and T cells showed no response to target cells natively expressing these antigens. In both cases the cross-reactive epitopes were part of another CGA (MAGE-C1 or MAGE-B4).

Chapter 5 further builds on an approach to engineer T cells in order to counter immune suppression mediated by reduced expression of co-stimulatory ligands. Following inclusion of intracellular (ic) domains of the co-signaling receptors CD28, OX40, ICOS, 4-1BB and CD40L into a TCR, we generated a panel of co-stimulatory TCRs that were expected to signal via co-stimulatory pathways upon antigen binding but independent of co-stimulatory ligand binding. Transduction of these constructs into primary T cells revealed that both the 4-1BB- as well as the CD40L-TCR variant were not functionally expressed and the three other TCR constructs yielded T cells with reduced functional avidity when compared to the wt TCR. When assessing their capacity in immune competent mice however, it became apparent that all co-stimulatory TCRs delayed tumor recurrence and in particular, TCR:28-ICOS ϵ T cells mediate enhanced tumor-free survival. Both TCR:28-ICOS ϵ and TCR:28-OX40 ϵ T cells persisted longer in peripheral blood than wt TCR T cells and showed enhanced numbers within regressing tumors.

In **chapter 6** we propose the generation of therapeutic T cells possessing the capacity to reverse local immune suppression through the release of cytokines at the tumor site. For this purpose, we have established a protocol to generate T cells equipped with both a TCR as well as an inducible (i)IL-12 or iIL-18 construct under the control of a nuclear-factor of activated T-cell (NFAT)-sensitive promoter. Both T cell variants demonstrated antigen-specific release in vitro. While adoptive transfer of TCR+iIL-18 T cells to melanoma-bearing mice resulted in significantly reduced tumor burden and prolonged overall survival, transfer of TCR+iIL-12 T cells led to severe, edema-like toxicity and reduced overall survival, accompanied by enhanced levels of IFN γ and TNF α in blood as well as enhanced infiltration of macrophages into the tumor.

In **chapter 7** the two main challenges of TCR gene therapy as mentioned in chapter 1 are discussed according to the results of chapters 3 to 6. I have placed the approaches put forward by this thesis into context of recent developments within the TCR gene therapy field, have made an effort to explain findings using illustrations, and have drafted an 'ideal' treatment design. Furthermore, I have discussed which additional investigations are necessary in order to further improve and utilize these approaches.

Samenvatting

In de behandeling van kanker heeft immunotherapie zich de laatste jaren gevestigd als een standaard behandelingsoptie voor bepaalde kankersoorten, en een plaats afgedwongen naast de reeds bestaande behandelingsopties zoals chemotherapie. In sommige gevallen blijkt immunotherapie zelfs een betere behandelingsmethode dan deze "klassieke" behandelingsstrategieën.

Ondanks veelbelovende resultaten van immunotherapie tijdens (pre-)klinische onderzoek is er vooralsnog een significant deel van de patiënten dat niet reageert op de therapie of last heeft van bijwerkingen. In dit proefschrift geef ik een overzicht van de preklinisch benadering die wij in het laboratorium hebben gehanteerd om T cel receptor (TCR) gentherapie te verbeteren, zowel qua therapeutische veiligheid als effectiviteit. TCR T cel therapie is een behandeling waarbij witte bloedlichaampjes van een patiënt, zogenaamde T cellen, worden afgenomen en genetisch worden gemoduleerd om een TCR tot expressie brengen. Vervolgens worden deze cellen gekweekt waardoor het cel aantal fors toeneemt waarna ze worden teruggeplaatst in de patiënt.

Hoofdstuk 1 start met een korte opsomming van verschillende immunotherapieën zoals checkpoint therapie, vaccinatie therapie en T cel therapie. Verder beschrijft dit hoofdstuk waar de uitdagingen liggen in het verbeteren van TCR gentherapie, namelijk de selectie van veilige, effectieve antigenen en het overwinnen van tumor gemedieerde immuun ontwijking. Hiertoe wordt een tweetal manieren gepresenteerd:

- (1) Selectie en validatie van tumor specifieke antigenen en de daarbij horende TCRs.
- (2) Ontwikkeling van T cellen welk de lokale immuunsuppressie tegen gaan.

Deze twee manieren worden in de latere hoofdstukken meer in detail besproken. Tenslotte wordt in hoofdstuk 1 het doel van dit proefschrift uiteen gezet en wordt beschreven hoe de individuele hoofdstukken specifiek bijdragen om dit doel te verwezenlijken.

Hoofdstuk 2 geeft een overzicht van de literatuur van de in hoofdstuk 1 genoemde uitdagingen aan de hand van de volgende drie hoofdonderdelen: keuze van het antigeen herkend door T cellen; fitheid van T cellen; en het gevoelig maken van tumoren voor T cellen.

In **Hoofdstuk 3** beschrijven we hoe we uit het bloed van patiënten T cellen specifiek voor het zogenaamde 'Cancer Germline Antigen (CGA)' MAGE-C2 (MC2) hebben geïsoleerd. Uit deze T cellen hebben wij de TCRs in handen gekregen. Tien TCR α/β ketens van verschillende T cel klonen zijn in retrovirale vectoren gezet, die op hun beurt gebruikt zijn voor genetische introductie in T cellen. Vier van deze 10 TCRs werden door T cellen tot expressie gebracht, aangetoond door

middel van flow cytometrie, en resulteerden in de verwachte T cel functie, dat laatste aangetoond middels *in vitro* blootstelling aan antigeen (dwz buiten proefdieren om). Herkenning van tumoren is verder geanalyseerd door T cellen te co-cultiveren met cellijnen afkomstig van huid, hoofd-hals, blaas en borstkanker. In dit hoofdstuk worden ook experimenten beschreven die het belang van de mate van expressie van MC2 onderstrepen en dat deze expressie positief wordt beïnvloed door medicatie die de toegankelijkheid van DNA voor gentranscriptie bevordert. Belangrijk te melden is dat het gebruik van deze zogenaamde epigenetische middelen MC2 expressie in noch een T cel reactie tegen cellijnen van gezond weefsel veroorzaakt, hetgeen een basis vormt voor mogelijke combinatietherapie.

Hoofdstuk 4 beschrijft een voorstel hoe veilige antigenen en TCRs voor T cel therapie aan de hand van *in vitro* en *in silico* (dwz computer-gerelateerde) technieken geselecteerd kunnen worden. Uit patiënten studies met TCR T cellen werden onder sommige condities ernstige bijwerkingen geconstateerd. Wij bepleiten dat zowel gekozen antigenen als TCRs die geselecteerd worden voor klinisch gebruik aan grondig onderzoek moeten worden blootgesteld voordat deze TCRs aan patiënten gegeven kunnen worden. Hiertoe raden we het volgende stappenplan aan, bestaande uit 2 belangrijke componenten. Ten eerste wordt de toepasbaarheid en veiligheid van een antigeen gedefinieerd door voldoende expressie van antigeen door tumorweefsel en afwezigheid van deze expressie door gezond weefsel. Hiervoor gebruikte technieken analyseren zowel antigeen expressie op RNA als eiwitniveau. Met deze technieken laten we in hoofdstuk 4 zien dat MC2 een geschikt antigeen is voor T celtherapie. Ten tweede wordt de toepasbaarheid en veiligheid van een TCR gedefinieerd door voldoende reactiviteit van deze TCR voor het bedoelde antigeen ('cognate antigen'), en afwezigheid van of minimale kruis-activiteit voor andere antigenen. Hiervoor gebruikte technieken analyseren TCR-gemedieerde T cel reactiviteit tegen. Met deze technieken laten we in hoofdstuk 4 zien dat MC2 TCR6 een geschikte TCR is voor T celtherapie, en deze TCR is ook uitgangspunt van een geplande klinische test bij patiënten met huid en hoofd-hals-kanker in 2018.

Hoofdstuk 5 gaat verder in op het verbeteren van T cel fitheid. Door het toevoegen van co-stimulatoire domeinen (afkomstig van bijvoorbeeld CD28, OX40, of ICOS) in TCR ketens, en deze te introduceren in T cellen, wordt verondersteld dat deze T cellen beter bestand zullen zijn tegen het immuun-suppressieve milieu van tumorweefsel. Dergelijke co-stimulatoire TCRs resulteren in enige afname van T cel reactiviteit *in vitro*, echter in *in vivo* proeven met immuun competente muizen laten deze nieuwe TCRs een verbetering zien in anti-tumor response en overleving. Deze effecten gaan gepaard met hoge aantallen T cellen in bloed en tumor. In het bijzonder is de TCR met ingebouwd ICOS domain effectief welke leidt tot zelfs 50% genezing van muizen met huidtumoren (waar dat normaal 10% is).

In **Hoofdstuk 6** beschrijven we een andere methode om de therapeutische potentie van T cellen te verbeteren, met de nadruk op afgifte van moleculen in het tumorweefsel om de anti-tumor T cel response verder te ondersteunen. T cellen krijgen naast een TCR ook een induceerbaar (i)IL-12 of iIL-18 construct, waarin de expressie en afgifte het genoemde molecuul gecontroleerd wordt door T cel activatie. Zowel iIL-12 als ook iIL-18 T cellen laten *in vitro* zien dat IL-12 of IL-18 productie inderdaad bepaald wordt door T cel activatie met antigeen. *In vivo* veroorzaken deze T cellen een significante afname van de tumorgroei en een langere overleving. Het behaalde voordeel was vooral het geval voor behandeling met iIL-18 T cellen, en blijkt gerelateerd aan hoge aantallen CD8 T cellen in bloed en tumor. Behandeling met iIL-12 T cellen resulteert helaas in ernstige bijwerkingen, waaronder oedeem en dood.

In **Hoofdstuk 7** worden uitdagingen van de TCR T cel therapie (zoals eerder omschreven in hoofdstuk 1) besproken aan de hand van de resultaten in hoofdstuk 3 tot en met 6. Ik heb de verschillende uitkomsten die in dit proefschrift worden beschreven geplaatst in de context van recente ontwikkelingen binnen de T cel therapie. Daarnaast worden bevindingen samengevat door middel van illustraties. Ook worden er voorstellen gedaan om de patiëntbehandeling met TCR T cellen te verbeteren door gebruik te maken van benaderingen die in dit proefschrift worden besproken, en welke aanvullende onderzoeken er nodig zijn om combinatie behandelingen met T cellen te testen.



ACKNOWLEDGEMENTS

While a PhD thesis carries the name of only a single person on the cover, it is clear that in order to put it together the help and support of many others is needed. Here I would like to acknowledge the people that helped me pursue and reach this point, be it through their direct involvement in my research or their support along the way.

To **Dr. Reno Debets**, my co-promotor: Reno, I have to start by thanking you for giving me the opportunity to work in your group. Over these past few years I have grown as a researcher and as a person, not only thanks to the support and guidance you provided, but also thanks to the challenges you entrusted or confronted me with. Thank you for the countless times I could drop by your office with “just a quick question”. We had many discussions: often calm, sometimes heated, often agreeing, sometimes agreeing to disagree, but we always emerged from these discussions with a clear path forward. I truly admire your deep knowledge of immunology as a subject, your inquisitive nature and your dedication to research itself, but also to the members of your group.

To **Prof. Dr. Stefan Sleijfer**, my promotor: Stefan, thank you for seeing the potential in my work and giving me the opportunity to pursue my PhD project at the Department of Medical Oncology after the initial ATTRACT project had ended. I very much enjoyed our meetings and discussions, especially since your clinical expertise never failed to provide a fresh view on the data at hand.

To **Prof. Dr. Joachim Aerts**: Joachim my thanks also go to you for allowing me to join your group and collaborate on the Multomab project. I admire the research you promote in DC-vaccination, the efforts you extend in implementing it and the fact that your questions to research are always driven by the goal to improve therapy and well-being of your patients.

To the remaining members of my ‘kleine commissie’: **Prof. Dr. Peter Sillevis-Smitt** and **Prof. Dr. Thomas Blankenstein**, thank you both for thoroughly reading and assessing my thesis. To the remaining members of my PhD commissie, **Prof. Dr. Ton Schumacher**, **Prof. Dr. Peter Katsikis**, **Prof. Dr. Clemens Löwik**, **Dr. Sonja Buschow**, and **Dr. Martijn Lolkema**, thank you very much for taking part in my committee.

Getting along with your colleagues might not be a prerequisite for great research, but it does make the task so much more easy and fun. I consider myself very lucky to have had the opportunity to work alongside so many skilled and amicable individuals.

Cor, my roomie for the last two years. Your concern with detailed research and knowledge in setting up clinical trials are second to none. I often found myself very grateful for being able to ask you directly for advice, be it on scientific writing or your opinion on the latest Multomab data. Aside from scientific matters I think I can still learn a lot from you when it comes to singing - a skill of yours which is also second to none (at least within the TI group).

To the members of the PhD cave, the people that can best understand what a “heeeeeeeel zwaaaaaar leven” we sometimes lead:

Yarne, apart from the fact that your arrival in our group freed me from my PhD-solitude, I was happy to finally share the cave with someone that’s willing to look critically at my work and discuss research with, although that meant dealing with the occasional urge of yours to share some Justin Bieber or Helene Fischer classics with the entire cave. Thanks for all the support you provided and still provide with regard to the Multomab study and with scripting in R. I told you before that in some aspects we are polar opposites; however, the different ways we use to approach similar issues taught me a lot as well. You are driven and hard-working guy, so I know you’ll reach the goals you set yourself. I’m very grateful to have you as my paranimf today!

Dora, my savior when it comes to practicing my native language every now and then, despite a few subtle differences ('Januar'! Nicht 'Jänner'!). There's no other way to put it: you are a very smart cookie (or 'Mozartkugel' in your case). The quality of and dedication to your work and your enthusiasm about research are admirable. At the same time you always keep your calm, almost zen-like attitude, hardly ever stressing out. Please teach me your secret! Thanks for all your feedback, support and of course: the laughs.

Priscilla, always starting early, always leaving late, you're one of the most hard-working PhD's I've come across. Starting up a new project and research angle from scratch in a group is never easy, but I know your dedication will lead to great findings and great publications. As a Feyenoord fan, I'm sure you know that patience always pays off. ;)

Maud, not only are you a very talented researcher, but your positive attitude and smile were always highly contagious. Please make sure you carry both of these with you throughout your PhD and beyond! They certainly helped me to stay positive as well.

Albeit, not dwelling in the PhD cave, there were several other PhD candidates that I had the pleasure to work alongside: **Mesha**, thanks you for making the sometimes monotonous lab work more interesting with your dry sense of humor. **Bas**, thanks for all the laughs during breaks and for making sure all antibody vials are really, really, reeeeeaaaally tightly closed. **Pim**, seeing you balance your lab work with your clinical duties, doing all those assays even after a long day of clinical work was inspiring. **Zineb**, thanks for all the coffees and philosophizing over research and life and whether there's a life after research.

To our technicians, the MacGyvers (80's TV reference, please look it up. It's a compliment, trust me!) of our group we couldn't do without:

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Cor, you were the one who right after my interview took me to the Erasmus MC and guided me around my soon to be new workplace. Similarly during my first weeks you showed me the fun and the pain of the "straight-forward procedure" that is TCR-cloning. Thank you for all your help during the following years, be it through in vivo experiments or the cloning of new TCRs. I appreciate the fact that whenever there is a problem or a shortness of hands, you never hesitate to drop everything and go to any lengths to make sure we reach the goals we set.

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During my PhD I had the pleasure to supervise three very enthusiastic and skilled students: **Marvin, Bianca** and **Luc**. The projects you three worked on contributed and shaped several chapters of this thesis and will keep on shaping publications yet to come. I truly enjoyed sharing knowledge, techniques and laughs with you, be it in the lab or in project meetings. I hope that you enjoyed your time here as much as I did and that it equipped you with useful skills to pursue your future careers.

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And of course there is a long list of people that had nothing to do with the research presented here itself, but that hopefully after today's presentation and looking at this thesis will have a better understanding of what it is that I do (or at least nod politely and pretend that they do). I am very grateful for their support and the distractions they provided to me when I needed them.

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LIST OF PUBLICATIONS

Published Articles

Kunert A, Straetemans T, Govers C, Lamers CHJ, Mathijssen R, Sleijfer S, Debets R. "TCR-engineered T cells meet new challenges to treat solid tumors: choice of antigen, T cell fitness and sensitization of tumor milieu"; *Frontiers in Immunology*, 4:363, 2013

Klaver Y, **Kunert A**, Sleijfer S, Debets R, Lamers CHJ. "Adoptive T cell therapy: a need for standard immune monitoring"; *Immunotherapy*, 7(5):513-33, 2015

Kunert A, Van Steenberghe-Langeveld S, Van Brakel M, da Silva M, Coulie P, Lamers CHJ, Sleijfer S, Debets R. "Intermediate-affinity TCR for MAGE-C2, in combination with epigenetic drug treatment of target cells, yields tumor-selective therapeutic T cells"; *Journal of Immunology*, 197:2541-2552, 2016

Kunert A, Obenaus M, Lamers CHJ, Blankenstein T, Debets R. "T cell receptors for clinical therapy: in vitro assessment of toxicity risk"; *Clinical Cancer Research*, 23(20):6012-6020, 2017

Kunert A, Chmielewski M, Berrevoets C, Wijers R, Abken H, Debets R. "Intra-tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and counteracts immune evasion of solid tumors"; *Oncoimmunology*;7(1), ePub ahead of print, 2017

Aerts JGJV, de Goeje PL, Cornelissen R, Kaijen-Lambers MEH, Bezemer K, van der Leest CH, Mahaweni NM, **Kunert A**, Eskens FALM, Waasdorp C, Braakman E, van der Holt B, Vulto AG, Hendriks RW, Hegmans JPJJ, Hoogsteden HC. "Autologous Dendritic Cells Pulsed with Allogeneic Tumor Cell Lysate in Mesothelioma: From Mouse to Human"; *Clinical Cancer Research*, ePub ahead of print, 2017

Kunert A, Debets R. "Engineering T cells for adoptive therapy: outsmarting the tumor"; *Current Opinion in Immunology*, (in press) 2018

Unpublished Manuscripts

De Goeje PL, Klaver Y, Kaijen-Lambers MEH, Bezemer K, Langerak AW, **Kunert A**, Lamers CHJ, Hendriks RW, Debets R, Aerts JGJV. "Autologous dendritic cell therapy of mesothelioma patients enhances frequencies of peripheral CD4 T cells expressing HLA-DR, PD1 or ICOS"; (manuscript submitted)

De Goeje PL, Poncin M, Bezemer K, Kaijen-Lambers MEH, Groen HJ, Smit EF, Dingemans AMC, Hendriks RW, **Kunert A**, Aerts JGJV. "Induction of peripheral effector CD8 T cell proliferation by paclitaxel/carboplatin/bevacizumab"; (manuscript submitted)

Kunert A, Basak E, Hurkmans D, Klaver Y, van Brakel M, Oostvogels A, Lamers CHJ, Koolen SL, van der Veldt A, Mathijssen RH, Aerts JGJV, Debets R. "Identifying T cell profiles that associate with clinical response to anti PD-1 treatment in NSCLC patients"; (manuscript in preparation)

Kunert A, Berrevoets C, Wijers R, Peters M, Debets R. "TCRs equipped with ICOS enhance T cell persistence and mediate sustainable anti-tumor responses upon adoptive T cell therapy"; (manuscript in preparation)





PHD
PORTFOLIO



PhD portfolio

General information:

Name: Andre Kunert
 Research school: Molecular Medicine
 Period: 01-01-2012 until 30-06-2017
 Supervisor: Dr. Reno Debets
 Promotor: Prof. Dr. Stefan Sleijfer

1. PhD training	Year	ECTS
<i>1.1 General academic skills</i>		
• Dutch Language Course A1	2012	1.1
• Dutch Language Course A2	2013	1.1
<i>1.2 Research skills</i>		
<i>1.3 In-depth courses (e.g. Research School, Medical Training)</i>		
• Molecular Immunology Course	2013	3
• Basic Course on R	2017	1.8
<i>1.4.1 Poster Presentations</i>		
• Post Graduate School Molecular Medicine Day, Rotterdam	2013-2015	1.1
• Dutch Society for Immunology, Winter Meeting, Noordwijkerhout	2013-2015	1.1
• Cancer Immunotherapy (CIMT), Mainz, Germany	2015-2017	1.1
• Keystone Symposia C7, Whistler, Canada	2017	0.4
<i>1.4.2 Oral Presentations</i>		
• Josephine Nefkens Institute Scientific Research Meeting	2012-2017	4
• EU (FP7 ATTRACT) progress, Berlin	2012	1
• EU (FP7 ATTRACT) progress, London	2013	1
• EU (FP7 ATTRACT) progress, Milano	2013	0.5
• Annual departmental meeting on science, Rotterdam	2013	1
• 30th T Cell Consortium (TCC) Meeting	2014	1
• Daniel den Hoed Day, Rotterdam	2015-2017	2.5
• Post Graduate School Molecular Medicine Day, Rotterdam	2015	1
• Dutch Tumor Immunology meeting, Breukelen	2015-2016	2
• Medical Oncology Meeting	2015	0.5
• Dutch Society for Immunology, Noordwijkerhout	2015, 2017	2

1.5.1 National Conferences		
<ul style="list-style-type: none"> Dutch Society for Immunology, Winter Meeting, Noordwijkerhout 	2012-2015	2.3
<ul style="list-style-type: none"> Molecular Medicine Day, Rotterdam 	2012-2015	1.2
<ul style="list-style-type: none"> Dutch Tumor Immunology meeting, Breukelen 	2012-2016	2
1.5.2 International Conferences		
<ul style="list-style-type: none"> Symposium on Adoptive T cell therapy, Berlin 	2012	0.9
<ul style="list-style-type: none"> Cellular Therapy of Cancer Symposium, London, UK 	2013	1.1
<ul style="list-style-type: none"> Cancer Immunotherapy (CIMT), Mainz, Germany 	2015-2017	2.9
<ul style="list-style-type: none"> Keystone Symposia C7, Whistler, Canada 	2017	1.4
1.6 Seminars and Workshops		
<ul style="list-style-type: none"> EU (FP7 ATTRACT) Summer School 2 (adoptive T cell therapy), Berlin 	2012	0.3
<ul style="list-style-type: none"> EU (FP7 ATTRACT) Workshop (clinical trial design, GMP Cell processing and patient management), Rotterdam 	2012	0.6
<ul style="list-style-type: none"> EU (FP7 ATTRACT) progress, Summer School 3 (T cell fitness and gene engineering to advance T cell therapy), Milano 	2013	0.9
1.7 Didactic skills		
1.8 Other		
<ul style="list-style-type: none"> JNI oncology lectures (~6x /year), EMC 	2012-2017	0.6
<ul style="list-style-type: none"> T cell consortium (TCC) meetings (~6x /year), EMC 	2013-2017	0.5
<ul style="list-style-type: none"> JNI scientific research meetings (~36x /year), EMC 	2012-2017	6.6
<ul style="list-style-type: none"> Tumor Immunology Platform (TIP) meetings (~36x /year), EMC 	2012-2017	7.2
2. Teaching activities		
2.1 Lecturing		
<ul style="list-style-type: none"> Biomedical Research Techniques 	2014-2017	1.2
2.2 Supervising practicals and excursions		
2.3 Supervising theses		
<ul style="list-style-type: none"> HLO student 	2013/14	3
<ul style="list-style-type: none"> Master student 	2014/15	3
2.4 Other		
<ul style="list-style-type: none"> Laboratory Training, Cologne (Lab of Prof. dr. Hinrich Abken) 	2013	2
(28h workload = 1ECTS) Total ECTS		64.9



ABOUT THE AUTHOR

André Kunert was born in Laubach, Germany on the 23rd of September, 1984. He attended the Theo Koch Gymnasium where he graduated in 2004 top of his class and laureate of the Direktor Hühthwohl Trust. In 2005 André enrolled at the University of Hawaii in Hilo (UHH), USA where he pursued a Bachelor's degree in Biology. In 2006 he returned to Germany where he continued his study at the Justus-Liebig-University (JLU) in Giessen. He obtained his Bachelor of Science degree in 2009 after writing his thesis on the role of caspases 1 and 3 in the processing of the Interleukin-33 precursor at the Institute of Immunology (supervised by Prof. Dr. Michael Martin). Inspired by his internships and thesis work, André decided to focus on human biology and immunology during his Master's study at the JLU. In 2011 he received his Master's degree after completing his internship and thesis work on the role of decapping-protein-1-a (DCP1a) in stress- and cytokine-induced gene expression at the Rudolf-Buchheim-Institute of Pharmacology (supervised by Prof. Dr. Michael Kracht).

Although enjoying his studies on basic immunology, André aimed to conduct his PhD in a translational research setting. In line with this, he started his PhD project in the beginning of 2012 in the laboratory of Tumor Immunology, supervised by Dr. Reno Debets, at the Department of Medical Oncology, chaired by Prof. Dr. Stefan Sleijfer at the Erasmus Medical Center in Rotterdam. The first two years of his PhD study involved training within the ATTRACT (Advanced Teaching and TRaining for Adoptive Cell Therapy) consortium of the EU Framework Program (FP) 7. The workshops, meetings and exchanges included in this training led to close collaborations with other European Research groups focusing on cellular immune therapy, in example with the group of Prof. Dr. Hinrich Abken from Cologne. Throughout his PhD study, André's work revolved around the identification of safe and effective target antigens and T cell receptors (TCRs) for TCR gene therapy as well as the manipulation of therapeutic T cells and the tumor microenvironment to favor anti-tumor immune responses. The results of his work are described in this thesis.

From July 2016 onwards, André continued his research in immunotherapy as a post-doctoral fellow shared between the Tumor Immunology group of Dr. Reno Debets and the Pulmonary Medicine group of Prof. Dr. Joachim Aerts. Next to his ongoing research into TCR gene therapy and the tumor microenvironment, he is working on immune monitoring of patients undergoing checkpoint therapy.