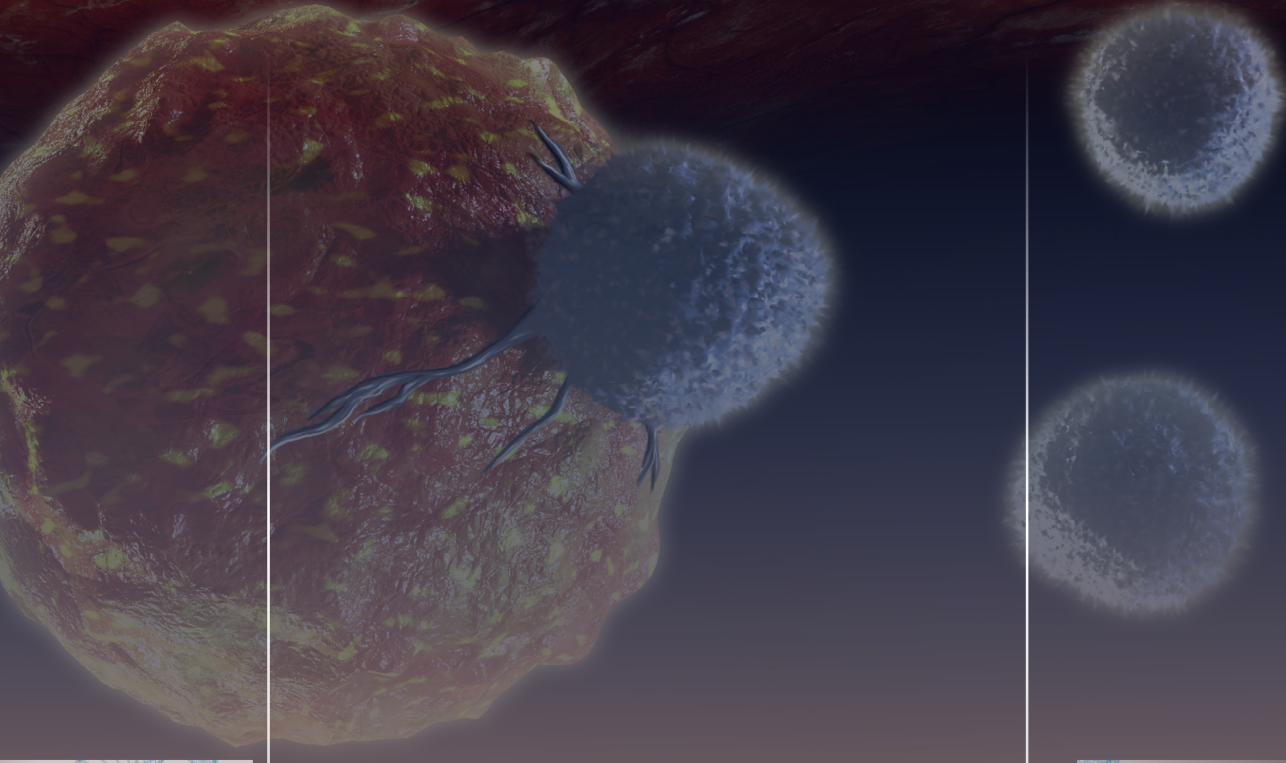

Engineering of T cell receptor genes to advance T cell therapy:
studies into TCR pairing, signaling and binding strength

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ENGINEERING OF T CELL RECEPTOR GENES TO ADVANCE T CELL THERAPY: STUDIES INTO TCR PAIRING, SIGNALING AND BINDING STRENGTH

Engineering van T-cel receptor genen ter verbetering van T-cel therapie: Studies naar TCR dimerizatie, signalering en bindingssterkte

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Prof.dr. R.W. Hendriks

Copromotor: Dr. R. Debets

> “In science it often happens that scientists say, ‘You know that’s a really good argument; my position is mistaken,’ and then they would actually change their minds and you never hear that old view from them again. They really do it. It doesn’t happen as often as it should, because scientists are human and change is sometimes painful. But it happens every day. I cannot recall the last time something like that happened in politics or religion.”

Carl Sagan

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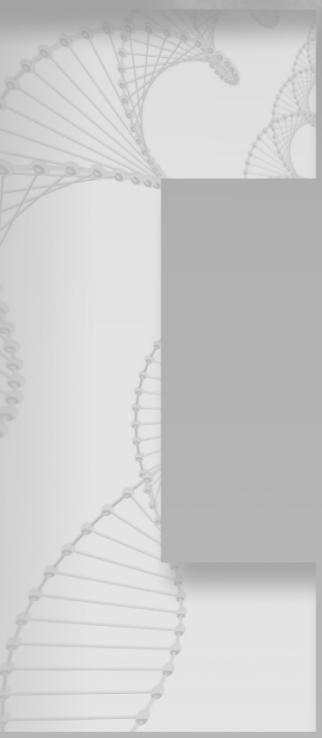


1

General introduction

1.1 T CELL THERAPY TO TREAT MELANOMA: IMPRESSIVE FIRST CLINICAL SUCCESSES

The incidence of cutaneous melanoma has increased dramatically over the past 40 years. The yearly increase in incidence rates in the Netherlands is on average 4.1%. Although the 10-year survival rates improved over the last fifteen years, the yearly mortality rates are further increasing with 2.3%, mainly in elderly patients (1). In case cutaneous melanoma metastasizes, the 10-year survival rate drops dramatically to less than 10%. Table 1.1 lists the U.S. Food and Drug Administration (FDA)-approved treatments for melanoma. The current standard cares of treatment for melanoma are either administration of the alkylating agent Dacarbazine (2), which induces DNA damage, or administration of high-dose IL-2 (3), which serves as a T cell growth factor. However, both treatments demonstrate fairly low response rates and significant adverse effects. More recent FDA-approved treatments for melanoma include: Ipilimumab, an antibody that blocks the T cell inhibitory molecule CTLA-4 to lower the threshold of T cell activation (4, 5); Vemurafenib, a drug that inhibits the serine-threonine protein kinase B-RAF (BRAF), a kinase that is constitutively active in 36 to 54% of melanoma patients due to a V600E mutation (6); and pegylated interferon α2b, used as an adjuvant that demonstrates anti-proliferative effects on melanoma cells and modulates immune responses (7). In

Table 1.1. FDA-approved therapeutic drugs to treat metastatic melanoma

Treatment	Year of FDA approval	Mechanism of action	OR in % (#pts)	CR in %	Toxicities in %[a]	Reference
Dacarbazine	1975	Alkylates DNA	12 (149)	3	36	(2)
High-dose IL-2	1992	Induces T cell growth	16 (270)	6	45	(3)
Ipilimumab	2011	Blocks T cell CTLA-4	28 (137)	2[b]	23	(4)
Vemurafenib	2011	Inhibits BRAF kinase activity	48 (219)	1	38[c]	(6)
PEG-IFN	2011	Reduces tumor cell proliferation	6.2[d] (627)	N.A.	33	(7)
Nivolumab	Applied	Blocks T cell PD-1	28 (94)	14	14	(8)
Ipilimumab and Nivolumab	N.A.	Blocks T cell CTLA-4 and PD-1	40 (53)	10	53	(9)
Lambrolizumab	2013	Blocks T cell PD-1	38 (135)	4	0	(10)

[a] Grade 3 and 4 according to National Cancer Institute's (NCI) Common Toxicity Criteria (CTC)

[b] Long-term follow up revealed an unexpected CR of 17%, which is likely explained by continued tumor eradication months or years after treatment (5)

[c] Adverse events, including grade 2, leading to modification of dosage or interruption of treatment

[d] Percentage of patients with treatment-mediated relapse-free survival following surgical resection

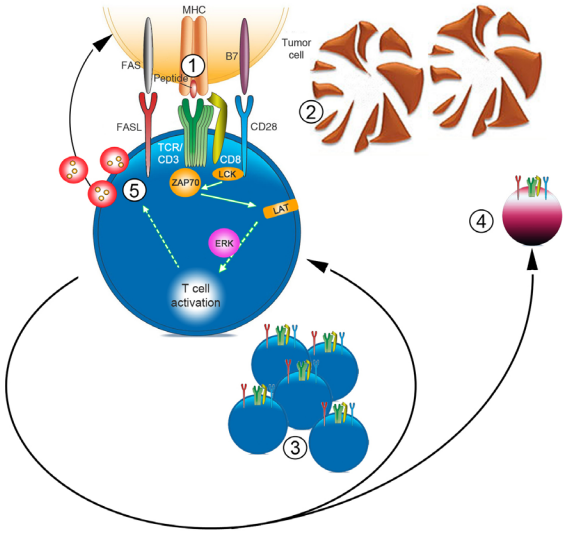
Abbreviations used in this table: CTLA-4, Cytotoxic T-lymphocyte Antigen-4; CR, complete responses; N.A., not applicable; OR, objective responses; PD-1, Programmed Death-1; Pegylated Interferon α2b (PEG-IFN); and pts, patients.

addition, Nivolumab, an antibody that blocks the T cell inhibiting PD1 receptor (CD279), is currently under consideration for FDA approval (8). Interestingly, the combined use of Ipilimumab and Nivolumab, with the intent to lower the T cell activation threshold and prevent T cell exhaustion, demonstrated an objective response rate of 40% and a complete response rate of 10% (9). The response rate of the combined antibodies was higher when compared to single use of either Ipilimumab or Nivolumab, underscoring the potency of combining immune-therapies when considering the mechanistic background. Another PD-1 blocking antibody (Lambrolizumab) has recently been developed, which in a first clinical study demonstrated a competitive objective response rate (38%) and no grade 3/4 toxicities when compared to Nivolumab (10).

In addition to the above-mentioned drugs, there is also a large array of new treatments under development and/or tested in clinical trials with the intent to provide anti-tumor T cell immunity (11, 12). Treatments include the use of vaccines, such as peptides (13) or antigen-loaded autologous dendritic cells (14-16) to actively induce a T cell mediated anti-tumor response. Also, the adoptive transfer of tumor infiltrating lymphocytes (TILs) has been tested clinically and has demonstrated impressive results in patients suffering from metastatic melanoma (17). Advantages of T cells when compared to more standard drugs in the treatment of melanoma and tumors in general are listed in figure 1.1.

The T cell population can broadly be divided into CD4 and CD8 T cells. CD4 T cells are commonly described as 'helper' T cells, whereas CD8 T cells are described as 'cytotoxic' T cells. Helper T cells facilitate immune responses and can be subdivided in various subsets, depending on production of signature cytokines, of which Th1 and Th17 may have the most pronounced anti-tumor activities (18, 19). Cytotoxic T cells are optimally equipped to kill tumor cells and constitute the T cell subset of interest for this thesis. CD8 T cells are able to survey individual cells for expression of intracellular proteins, as explained in box 1.1. Besides tumor reactive CD8 T cells, other cells have a part in the net anti-tumor response such as T cell activating B-cells, dendritic cells or CD4-helper T cells but also inhibiting regulatory T cells, tumor associated macrophages, myeloid-derived suppressor cells or other T cell inhibiting factors as more elaborately described in chapter 7 and reviewed in Malyguine *et. al.* (20).

Adoptive T cell therapy is based on the isolation of tumor-specific T cells from tumor tissue or peripheral blood, expansion to clinical numbers (generally 10^8 or more per injection) and re-infusion in patients (21). This therapy extends the early successes of bone marrow transplantation after it became evident that T cells present in transplants were of critical importance in the treatment of leukemia (22, 23). Adoptive T cell therapy using autologous TILs, often supported by stringent patient pre-conditioning and IL-2 injections to enhance the clinical effect of T cell administration (24), has shown significant objective clinical response rates that ranged from 49 to 72% (based on Response Evaluation Criteria In Solid Tumors (RECIST)). Notably, ongoing complete responses are observed in



T cells can

1. specifically recognize tumor cells
2. serially kill tumor cells
3. self-replicate
4. form immunological memory to provide future protection
5. switch mechanisms of killing

- in example, exocytosis of lytic molecules, such as perforins and granzymes, and activation of the FAS pathway

Figure 1.1. Advantages of T cells when compared to drugs in the treatment of tumors. A schematic representation of primary characteristics of T cells that are considered advantages when comparing T cell therapy with standard drug treatment of metastatic melanoma. Abbreviations used in this figure: CD.., cluster of differentiation ..; ERK, extracellular-signal-regulated kinases; FAS, tumor necrosis factor superfamily, member 6; FASL, FAS ligand; LAT, linker for activation of T cells; LCK, lymphocyte-specific protein tyrosine kinase; MHC, major histocompatibility complex; TCR, T cell receptor; and ZAP70, zeta-chain associated protein kinase 70; (see also box 1.1 for details). Figure was adapted from: Appelbaum, Nature, 17; 411:385.

20% of the patients (25) (see also chapter 2). More recently, a modified *ex vivo* culture protocol was initiated (so-called 'young TIL protocol') (26), which shortened culture time and omitted selection for recognition of antigen-positive cells. Using this protocol, the success rate of obtaining TILs increased, TILs were younger, and functionally more active than non-young TILs, and clinical activities of young TILs resulted in objective responses of 48% and 40% and complete responses of 9% and 10%, respectively (27, 28). So far, tumor-reactive TILs were only successfully isolated from melanoma. Other tumor types have demonstrated T cell infiltration, but appear to lack tumor-specific T cells (17).

Box 1.1. T cell receptor expression and target cell recognition

The T cell's specificity is governed by its T cell receptor (TCR). A TCR consists of an α- and β-chain (leaving γ and δ-chains out of the scope of this thesis) which are the products of recombining of various segments encoded by TCR-Variable (α: ~70, and β: 52 genes), Diversity (β: 2 genes) and Joining genes (α: 61, and β: 13 genes). Recombination and other processes that tweak the genetic sequence result in ~10^{18} potentially different TCR specificities. A TCR binds to major histocompatibility complexes (MHCs) class I or II, which present peptide fragments of intra- and extracellular proteins, respectively, and are termed T cell antigens. Antigen binding by T cells induces the formation of immune-synapses and recruitment of various co-receptors (29). CD8αα or CD8αβ and CD4 co-receptors also bind MHC class I or II molecules, respectively, but at sites distant from the sites bound by TCR and independently of presented peptides. These co-receptors increase the stability of the TCR:peptide-MHC complex and are of critical importance during T cell selection in the thymus and T cell homeostasis in the periphery. T cell selection in the thymus will remove T cells with TCRs that have a too low or too high affinity for self-peptide-MHC (these T cells will die by neglect or negative selection, respectively) and yield a functional T cell repertoire of ~$2.5x10^{8}$ different TCRs.

TCR are expressed on the cell surface in a complex with invariant CD3 proteins (see also chapter 2 for details on assembly and expression of TCR/CD3 complex). CD3 proteins contain immune receptor tyrosine-based activation motifs (ITAMs) that provide means for intracellular signaling upon antigen binding. Antigen presenting cells (APCs) can efficiently present pathogen-derived peptide-fragments in MHC molecules and activate T cells through engaging the TCR/CD3 complex and, by exposing co-stimulatory ligands, engaging co-stimulatory receptors expressed by T cells. Co-stimulatory receptors expressed by T cells generally belong to the CD28 co-stimulatory family of molecules, such as CD28, CD134, and CD137 (30). T cell co-stimulation is required to optimally induce T cell proliferation and differentiation into effector T cells. In addition, T cell co-stimulatory signals prevent T cell anergy and apoptosis, rearrange the cytoskeleton, activate transcription factors and boost the secretion of cytokines or chemokines. Lack of T cell co-stimulation, which often occurs in tumors that present antigen but have down-regulated the expression of co-stimulatory ligands, may lead to impaired T cell persistence and T cell exhaustion.

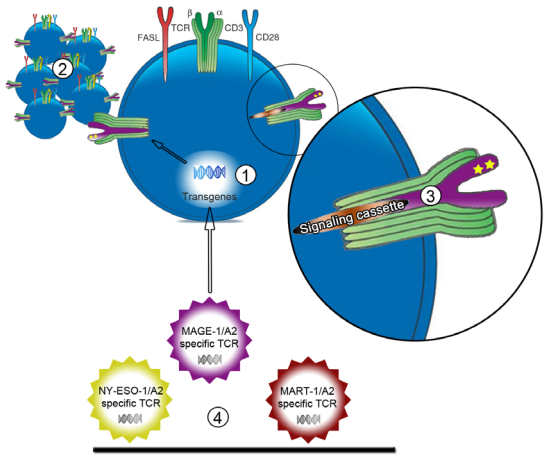
TCR-mediated antigen binding is reported to induce conformational changes in CD3-proteins which may initiate the first steps in downstream signaling (31). TCR signaling involves activation of LCK, often recruited to the TCR/CD3 complex by CD8/CD4 and CD28 molecules, ZAP70, recruited by phosphorylated CD3ζ, and subsequent activation of LAT and the RAS/MAPK/ERK pathway. T cell signaling results in cytoskeletal changes and release of cytotoxic effector molecules, such as perforins and granzymes, enhanced gene transcription of cytokines, such as IFNγ, IL-2, and TNFα, and enhanced expression of apoptosis-inducing ligands, such as FasL. See figure 1.1 for a schematic illustration of T cell antigen binding and the first steps in downstream signaling and tumor cell killing.

1.2. GENE-ENGINEERING OF T CELLS: THERAPEUTIC POTENTIAL AND NEW CHALLENGES

T cells can be re-directed towards tumor antigens through genetic introduction of tumor-specific TCRα and TCRβ genes, after which TCR-engineered T cells can be administered to patients (TCR gene therapy). The therapeutic use of TCR transgenes is explained in figure 1.2.

To obtain TCR genes, one generally acquires tumor-reactive T cells as a source for TCR genes. To this end, several methods can be employed. (I) Tumor-reactive T cells can

be isolated from patients' TILs (32), tumor draining lymph nodes or peripheral blood (33). In some cases, where frequencies of patient-derived TILs are low, vaccination with tumor-specific peptides or tumor antigen-loaded DCs prior to isolation of T cells may boost the numbers of specific T cells (34-36). Combinatorial coding with peptide-MHC has recently been developed and may facilitate the detection and isolation of multiple T cells specific for various antigens, significantly shortening the process to acquire pure T cell populations of desired specificities (37). (II) Tumor-reactive T cells can be isolated from allo-reactive settings that circumvent self-tolerance and generally yield T cells with a higher avidity than those derived from patients. Examples of such settings include peptide-vaccination of MHC-mismatched healthy donors (38), immunization of mice transgenic for human-MHC or human TCR loci (39, 40), *in vitro* T cell stimulation using T2 cells pulsed with peptide, B cells loaded with allogeneic peptide-MHC monomers (41) or



TCR gene-engineering of T cells allows

1. targeting tumor antigens of interest
2. timely and cost-effective production of therapeutic T cells at clinical scale
3. genetic engineering to address TCR mis-pairing and enhance TCR affinity and/or functional expression
4. 'off-the-shelf' treatment

Figure 1.2. The therapeutic use of TCR transgenes. A schematic representation of reasons to therapeutically use TCR transgenes. Stars indicate amino acid substitutions in complementarity determining regions (CDR) to enhance TCR affinity (see box 1.2 for details). Signaling cassettes are described below and consist of transmembrane and/or intracellular accessory and/or co-stimulatory domains genetically fused to TCRαβ. Abbreviations used in this figure (in addition to the ones explained in the legend of figure 1.1): A2, human leukocyte antigen A*0201; MAGE, melanoma antigen gene; MART-1, melanoma-associated antigen recognized by T cells 1; and NY-ESO-1, New York esophageal squamous cell carcinoma 1.

autologous dendritic cells gene-transferred with RNA encoding peptide and MHC (42). Once the desired tumor-reactive T cells have been obtained, then the corresponding TCR genes can be aquired by molecular methods such as rapid amplification of cDNA ends (RACE) (43). To introduce TCR genes into patient T cells, one of the most commonly utilized gene delivering systems, also for the generation of clinical batches of T cells, makes use of retro- or lentiviruses. In addition, several other vehicles and methods can be used for TCR gene introduction, such as DNA plasmid transfer, transposons-mediated transfer, and RNA electroporation, techniques that do show differences with respect to gene-transfer efficiency and stability of transgene expression (briefly reviewed in Gilham *et. al.* (44)).

The first clinical trials have clearly demonstrated feasibility and anti-tumor activity of TCR gene therapy, but at the same time revealed treatment-related toxicity. A summary of these findings is presented in table 1.2. Objective clinical responses, although variable and tested in a limited number of patients, ranged from 12 to 67%. Treatment-related toxicities varied from severe, but transient and/or treatable inflammation of organs, to neuro- and cardiological which resulted, in some cases, in death of patients. Current challenges of TCR gene therapy aim at preventing toxicity and further increasing therapeutic efficacy. These challenges are discussed in more detail below, including strategies to address these challenges with an emphasis on those strategies that use genetically modified TCR transgenes (main topic of this thesis).

1.2.1 Therapy-related toxicities

As mentioned above, TCR gene therapy in patients bears the risk to induce on- and off-target toxicities. On-target toxicities are toxicities that occur in healthy organs that express the T cell target antigen, whereas off-target toxicities occur in healthy organs that do not express the target antigen. Both types of toxicities are likely to be halted by the use of so-called 'suicide genes' which are not the topic of the current chapter, but are described in more detail in chapters 2 and 7.

1.2.1.1 How to prevent or limit on-target toxicities

Several TCR gene therapy studies reported the onset of on-target toxicities as described in table 1.2. Targeting melanoma-associated antigen recognized by T cells 1 (MART-1) (AAG epitope), glycoprotein 100 (gp100) (KTW epitope), or carcinoma embryonic antigen (IMI epitope) with affinity-enhanced TCRs resulted in severe, although treatable, toxicities (40, 45). More recently, a study using a TCR specific for an alternative MART-1 epitope (ELA) demonstrated lethal cardiotoxicity. On-target toxicities can be addressed by targeting antigens that show a highly specific or even exclusive expression on tumor cells when compared to healthy cells. Indeed, when targeting an alternative antigen,

Table 1.2. Current overview of clinical TCR gene therapy trials

TCR specificity (epitope)	Tumor type	Toxicities	OR in % (#pts)	Reference
MART-1 / HLA-A2 (AAG)	Metastatic melanoma	None reported	12 (15)	(32)
MART-1 / HLA-A2 (AAG) (high affinity TCR)	Metastatic melanoma	Severe inflammation of skin, eye or ear in 8 out of 20 pts	30 (20)	(45)
gp100 / HLA-A2 (KTW) (murine TCR)	Metastatic melanoma	Severe inflammation of skin, eye or ear in 1 out of 16 pts	19 (16)	(45)
CEA / HLA-A2 (IMI) (murine TCR)	Metastatic colorectal carcinoma	Severe inflammation of colon in 3 pts	33 (3)	(40)
NY-ESO-1 / HLA-A2 (SLL) (affinity enhanced TCR)	Metastatic synovial carcinoma	None reported	67 (6)	(46)
NY-ESO-1 / HLA-A2 (SLL) (affinity enhanced TCR)	Metastatic melanoma	None reported	45 (11)	(46)
MAGE-A3 / HLA-A2 (KVA) (affinity enhanced and murine TCR)	Metastatic melanoma	Mental changes in 3 pts and comas and death in 2 pts	57 (9)	(62)
MART-1 / HLA-A2 (ELA) (affinity enhanced cysteine-modified and murine TCR)	Metastatic melanoma	Lethal cardiotoxicity in 1 pt	0 (1)	note a
MAGE-A3 / HLA-A1 (EVD) (affinity enhanced TCR)	Metastatic melanoma	Lethal cardiotoxicity in 2 pt	0 (2)	note b

[a] Prof.dr. John Haanen and [b] Dr. Bent Jakobsen, Cellular Therapy against Cancer Symposium, London, Febr. 27th - March 2nd, 2013.
Abbreviations used in this table (in addition to the ones explained in the legends of table 1.1 and figure 1.1 and 1.2): CEA, carcinoembryonic antigen; and gp100, glycoprotein 100.

NY-ESO-1 (SLL), no toxicities and a strong anti-tumor response were observed (46). Metastatic melanoma is an immunogenic tumor and expresses several tumor-associated antigens (TAA). Such TAA can generally be divided into four groups (47, 48):

- Differentiation antigens: cell surface proteins that are selectively expressed at different stages of development or cell activation. Expression of these antigens may discriminate tumor cells from surrounding healthy cells, but expression is not absent on healthy cells. Examples include MART-1, gp100, tyrosinase related protein 1 (TRP-1) and TRP-2.
- Over-expressed antigens: cell surface proteins that are highly, but not selectively, expressed on tumor cells when compared to healthy cells. Examples include CEA and ERBB2.
- Cancer Testis Antigen (CTA): a large group of immunogenic proteins that are expressed by tumors of different histology and, depending on the specific CTA, not or

only a limited number of healthy and adult cell types. Examples include melanoma antigen gene A1 (MAGE-A1), MAGE-C2 and NY-ESO-1.

- Neo-antigens: new proteins as a result from gene mutations or aberrations that are present in tumor cells. These proteins are uniquely expressed by tumor and not by healthy cells. Examples include protein 53 (p53), Ras and cyclin-dependent kinase 4 (CDK4).

With respect to tumor-restricted expression, neo-antigens and CTA may represent the preferred groups of target antigens. In an elaborate study that ranked known tumor antigens according to multiple characteristics that are considered important for T cell targeting, several neo-antigens and CTA were identified as high-priority antigens (49). Neo-antigens are unique to tumor tissues, not present in healthy tissues, but their expression may be restricted to a single or a limited number of patient(s). Current developments of second-generation sequencing techniques may make it feasible to identify tumor-specific mutations (50, 51). Although up to 100,000 mutations can be encountered in tumor genomes (52), 85% of these are considered 'passenger' mutations, whereas the remaining 15% may be 'driver' mutations (53). Passenger mutations are not related to oncogenic development, making them less suitable as T cell target antigens. Driver mutations are related to oncogenesis, may be linked to known genes (~400), and may provide tumors with a selective growth advantage (52, 54). However, not all driver mutations result in new immunogenic antigens. Thus, although new techniques allow fast analysis of mutations, additional techniques or screening methods may be necessary to determine whether a tumor expresses an immunogenic epitope that is suitable as a target for T cell therapy (55).

CTAs represent an expanding class of tumor-associated proteins. Currently, over 250 CTA genes have been identified of which more than 50% are located on the X-chromosome, including CTA families such as MAGE, GAGE, BAGE, SSX and NY-ESO-1 (see for a full description of CTAs: http://www.cta.lncc.br). CTA can serve as potential T cell target antigens for several reasons. First, using extensive RT-PCR of various tissues, 39 CTA genes, of which 35 CTA genes are located on the X-chromosome, demonstrated expression that was limited to testes and placentas, which do not express MHC-molecules and are therefore not targeted by T cells (56). In tumor tissues, aberrant epigenetic regulation, such as promotor demethylation, appears to drive the expression of CTAs (57). Second, CTAs are expressed in tumor tissues of various histological origins. In fact, expression in tumor tissues has been associated with advanced stages of disease and unfavorable patient prognosis (58). Third, both humoral and cell-mediated immune responses to CTA proteins have occurred in tumor-bearing patients and pointed to the immunogenic nature of these antigens (35, 36, 59). Fourth, X-chromosome linked CTAs are coordinately expressed in tumor tissues, which allows the simultaneous targeting of multiple CTAs thereby potentially decreasing the risk of tumor recurrence following T cell therapy (60).

Fifth, expression of some CTAs may be restricted to tumor stem cells and these CTAs may provide unique targets to treat tumor recurrence and metastasis (61). Unexpectedly, in a recent clinical study using TCR-engineered T cells directed against MAGE-A3/HLA-A2 (KVA epitope; see table 1.2), 2 patients with metastatic melanoma lapsed into comas and died, most likely because of T cell recognition of rare neurons positive for MAGE-A12 and possibly MAGE-A9 and expressing the shared KVA epitope (62).

Taken this observation into account, we consider neo-antigens and those CTA epitopes that are not expressed by healthy adult tissues and not shared among multiple CTAs as potentially safe and effective T cell target antigens. However, whatever the choice of antigen, it should be recommended to perform stringent preclinical testing to confirm that healthy cells do not express the target antigen prior to proceeding with the clinical testing of TCR-engineered T cells.

1.2.1.2 How to prevent or limit off-target toxicities

TCR mis-pairing, a phenomenon that is inherent to the generation of TCR-engineered T cells, is considered a potential cause of off-target toxicities. Introduced TCRα and TCRβ chains can form TCR heterodimers with endogenously expressed TCRβ and TCRα chains, respectively (63, 64), and these newly formed heterodimers are of unknown specificity and can yield self-reactive T cells (65, 66). Although in clinical TCR gene therapy 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 can induce new and harmful recognition of self-antigens under conditions that significantly enhance *in vivo* T cell proliferation (65). TCR mis-pairing can be prevented or limited by the genetic modification of TCR transgenes or by the silencing of endogenous TCRs. Strategies that prevent or reduce TCR mis-pairing through genetically modifying the TCR transgene are reviewed in chapter 2. These strategies include the murinization of the TCR, addition of cysteine amino acids to the TCR, and equipping the TCR with a signaling cassette that consists of the CD3ζ accessory molecule (i.e., TCR:ζ; see figure. 1.2). A signaling cassette introduced into TCRs, designed in analogy to signaling cassettes used in Chimeric Antigen Receptors (CARs) (44), typically replaces TCR transmembrane and intracellular domains with accessory and/or co-stimulatory molecules to enhance the functional expression of the TCR transgene. In addition, RNA interference technologies have been shown to specifically interrupt the expression of endogenous but not transgenic TCR chains (67, 68). Another method to abolish the expression of endogenous TCR chains encompasses the use of zinc finger nucleases and a sequential knock-out of endogenous TCRα and β chains and introduction of TCRα and β transgenes, combined with cell sorting for CD3-negative and CD3-positive T cells (69). The latter two techniques are relatively new and not yet widely or clinically applied, but without doubt appear promising to address TCR mis-pairing-related toxicities.

A second cause of off-target toxicities became evident in a recent study in which MAGE-A3/HLA-A1 (EVD epitope; see table 1.2) was targeted in metastatic melanoma and multiple myeloma patients. In this study, 2 patients suffered from cardiotoxicity and died, most likely because of T cell recognition of a similar but not identical peptide from the muscle protein Titin (Dr. Bent Jakobsen, Cellular Therapy against Cancer Symposium, London, Febr. 27th - March 2nd, 2013).

Collectively, the studies referred to in the above paragraphs on toxicities clearly warrant for the inclusion of a strategy to address TCR mis-pairing as well as stringent safety screening to exclude recognition of healthy cells by TCR-engineered T cells to reduce the risk of toxicities in future trials.

1.2.2 Efficacy of TCR gene therapy

Here, I introduce how to potentially further improve the efficacy of TCR gene therapy by enhancing T cell co-stimulation, TCR affinity and level of TCR surface expression.

1.2.2.1 T cell co-stimulation

T cells are routinely stimulated with anti-CD3 antibodies and high concentrations of IL-2 to allow for extensive *ex vivo* proliferation of T cells and significant efficiency of retroviral transduction to acquire clinical-scale numbers of therapeutic T cells (70, 71). This procedure, however, induces insufficient T cell co-stimulation and accelerates T cell differentiation, which may result in hampered T cell persistence and anti-tumor T cell responses *in vivo* (72) (see also box 1.1). One way to enhance T cell co-stimulation is to equip the TCR with a signaling cassette that consists of a co-stimulatory molecule, such as CD28. Preclinical studies have shown that the addition of CD28 to the signaling cassette of a CAR potentiates T cell cytokine secretion, including IL-2, prevents apoptosis and sustains CD8 T cell proliferation (73, 74). In addition, clinical testing of a CD19-specific CAR bearing a CD28-containing signaling cassette, in a manner that allowed intra-patient and side-by-side comparison with the same CAR containing a non-CD28 signaling cassette, revealed enhanced T cell persistence (75). In fact, several clinical trials have reported prolonged *in vivo* persistence as well as enhanced anti-tumor reactivity of CARs that contained co-stimulatory molecules such as CD28 and CD137 (between 6 and 26 weeks (76-78)) when compared to trials with CARs that did not contain a co-stimulatory molecule (between 3 and 9 weeks (79-83)). The observed enhanced persistence of T cells is most likely explained by a substantial presence of less-differentiated central memory T cells, and may allow for a clinical protocol that depends less on supportive treatment to aid proliferation of administered T cells. A study by Kalos and colleagues confirms this notion, as in this study CAR-expressing T cells with a central memory phenotype persisted, potentially at the expense of more differentiated effector memory T cells, using a protocol without IL-2 administrations (77).

Another way to enhance T cell co-stimulation is to stimulate T cells *ex vivo* with human artificial antigen presenting cells (aAPCs) that express both HLA-A*0201 loaded with the peptide of interest and co-stimulatory ligands, with stimulations often taking place in the presence of common-γ cytokines other than IL-2 (84, 85). This approach educates T cells with a certain HLA-A2-restricted specificity, a young phenotype ($CD45RA^+$ $CD62L^+$) and superior T cell functions (84). In a clinical study, T cells educated with aAPC presenting MART-1 peptide and the co-stimulatory receptor ligands CD80 and CD83, and stimulated in the presence of IL-2 and IL-15, resulted in objective responses and in peripheral persistence of therapeutic T cells of up to 12 months (85). Notably, in the latter study, the clinical protocol did neither include patient preconditioning with chemotherapy nor administration of IL-2.

1.2.2.2 TCR affinity

Functional T cell avidity can be enhanced via increasing the TCR's affinity for peptide-MHC (this paragraph) or via increasing its level of TCR surface expression (the next paragraph) (see box 1.2 for a description of TCR affinity and T cell avidity). In case of T cells specific for pathogens, K_D values are generally between 0.18 and 25 μM (mean = 8.2 μM). In contrast, T cells specific for tumor antigens, most often self-antigens, revealed K_D values between 11 and 387 μM (mean = 96.6 μM), the likely consequence of thymic selection of TCRs with a low affinity for self-peptide MHC ligands (86). Since TCR affinity is a strong determinant for functional T cell avidity (87), one could argue that there is a window for affinity-enhancement of tumor specific TCRs to enhance anti-tumor responses. In support of this argument, clinical TCR gene therapy studies have revealed improved objective response rates when using affinity-enhanced TCRs (see table 1.2). One example is the high-affinity MART-1/HLA-A2 specific TCR that mediated an objective response rate of 30%, whereas the lower affinity variant resulted in an objective response rate of 12% (32, 45). Other examples include the affinity-enhanced NY-ESO-1/HLA-A2 specific TCR and affinity-enhanced MAGE-A3/HLA-A2 specific TCR that demonstrate significant objective response rates (46, 62). Affinity-enhanced TCRs for use in TCR gene therapy can be obtained from allo-reactive settings to circumvent self-tolerance, as described above, and phage, yeast, or T cell display selections to acquire TCR variants harboring amino acid substitutions in their complementarity-determining regions (CDRs).

1.2.2.3 TCR expression levels

To enhance the level of TCR surface expression one can use strategies to enhance the expression in individual T cells or in the T cell population as a whole. The expression of TCR transgenes at a cellular level can be enhanced by optimizing various aspects of the TCR gene transfer methodology, such as: choice of gene delivery method (shortly described

Box 1.2. TCR affinity and T cell avidity

A measure of TCR affinity for peptide-MHC is the dissociation constant (K_D), the rate by which a TCR:peptide-MHC complex dissociates into single TCR and peptide-MHC molecules. The K_D itself is defined as the ratio between the off-rate (K_{off}) and on-rate constant (K_{on}). Typically, as a result of negative selection in the thymus, T cells bear TCR with K_D values in the range of 1-100 μM (88-90). TCRs interact with peptide-MHC via loops of the three complementarity determining regions (CDRs) in each of the TCRα and TCRβ chains. CDR1α/β and CDR2α/β, which are germline encoded genes, bind the MHC molecule and position the highly variable CDR3α/β to interact with the peptide present in the MHC groove (88, 89). TCR affinity can be increased by amino acid substitutions in CDRs to reduce K_{off} or increase K_{on} rates and hence lower the K_D.

T cell avidity is the T cell's ability to interact with a target cell, and is defined as the sum of affinities of all cellular interactions, such as TCR:peptide-MHC, CD28:B7, CD4/8:MHC, PD-1:PD-L1 and so on. Functional T cell avidity is the T cell's ability to respond to antigen-positive tumor cells and can be enhanced via increasing the TCR's affinity for peptide-MHC or via increasing its level of cell surface expression. Notably, functional T cell avidity does not fully correlate with T cell affinity (chapter 5 and (91, 92)).

above); use of optimal vector elements (i.e., splice signals, constitutive RNA transport elements, posttranscriptional regulatory elements (93, 94); use of multi-cystronic vectors (95); and codon optimization of TCR transgene (96) (for an overview see Coccoris *et. al.* (97)). As referred to earlier, genetically introduced TCRs need to compete with endogenous TCRs for the limited availability of CD3ζ (98) (see also box 1.1 and chapter 2). The following genetic strategies have been reported to provide transgenic TCRs with a competitive advantage (see chapter 2 for a more complete overview of these strategies): (I) replacement of positively charged residues in the TCRα transmembrane region with hydrophobic residues to enhance the stability of surface expressed TCRs (99); (II) removal of intracellular TCRαβ glycosylation sites to prevent degradation and enhance the TCR's ability to move within the plasma membrane (100); (III) co-transduction of the TCR transgene with CD3γ, δ, ε, and ζ genes to decrease the competitive pressure for endogenous CD3 chains (101); (IV) RNA interference or zinc finger nuclease technologies to ablate the expression of endogenous TCR chains (67-69); and (V) genetic incorporation of a CD3ζ signaling cassette into TCR (i.e., TCR:ζ) to create a TCR that expresses independently of CD3 and demonstrates a level of surface expression that goes beyond those of endogenous TCR (63, 64).

The expression of TCR transgenes at a T cell population level, i.e., the frequency of TCR-transduced T cells, can be increased by selectively enriching T cells that highly express the TCR transgene. Patients treated with TCR gene therapy have received T cell populations with fractions of TCR-transduced T cells being as low as 3% (32). There are several methods to improve percentages of TCR-engineered T cells in a T cell population, including fluorescent- and magnetic-activated cell sorting (FACS and MACS, respectively). MACS is well suited for rapid isolation of single cells and has already been ap-

plied under General Medical Practice conditions and tested clinically. In example, MACS allowed for the isolation and increase in frequencies of CMV-specific CD8 T cells (>95%), which resulted in the control of CMV re-activation in 9 patients who received a stem cell transplantation and the MACS-prepared CMV-specific T cells (102).

1.3. SCOPE OF THIS THESIS

In this thesis, I have worked along the following lines of research (see figure 1.3):

1. Equipment of TCR transgenes with signaling cassettes
2. Enhancement of TCR affinity
3. Enrichment of TCR-engineered T cells using MACS technology

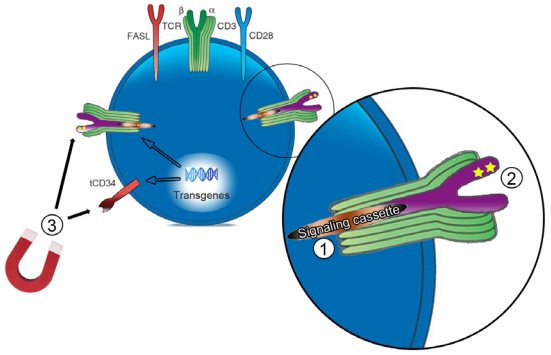
First, in **chapter 2** an overview is provided of strategies to prevent or limit TCR mis-pairing by genetically modifying TCR transgenes.

In **chapter 3** we compared various TCRs for their ability to prevent TCR mis-pairing, become functionally expressed and mediate antigen-specific functions. We focused on TCR:ζ variants with minimized CD3ζ content, which we generated by applying two different strategies, i.e., domain exchange and 3D modeling strategies. In our studies, we also included a TCR that was modified via murinization and cysteine-modification and compared all TCRs with a previously characterized TCR:ζ., which has demonstrated strong functional properties.

In **chapter 4** we designed and tested multiple signaling cassettes consisting of various accessory molecules with or without the co-stimulatory CD28 molecule. These were compared and selected for optimal functional expression by T cells. Next, we evaluated the most optimal cassette, i.e., a CD28-CD3ε signaling cassette, for its ability to prevent TCR mis-pairing, provide functional T cell avidity, and form antigen-specific immune synapses.

In **chapter 5** we investigated whether the extent of TCR affinity enhancement that leads to improved T cell function differs from the extent of TCR affinity enhancement that leads to self-reactivity. Using phage-display, we generated a set of TCRs that covered an almost 3-log range of affinities and analyzed whether these TCRs result in gain of antigen sensitivity and/or loss of antigen specificity when compared to wt TCR.

In **chapter 6** we compared different types of peptide-MHC multimers and CD34 mAb to MACSort TCR and/or CD34 engineered T cells. T cell sorts were compared with respect to



1. Enhance correct pairing and functional TCR expression via the incorporation of signaling cassettes
  - chapters 3 and 4

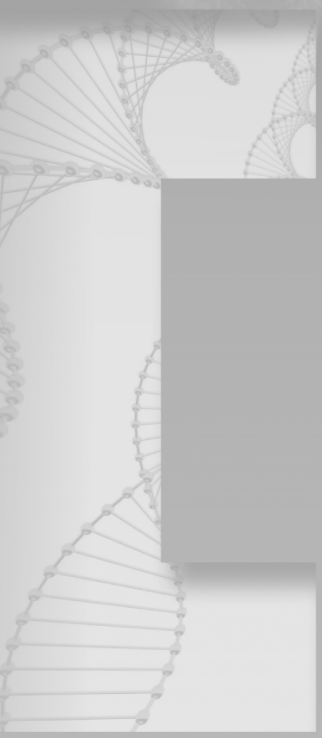
2. TCR affinity-enhancement via amino acid substitutions in CDR
  - chapter 5

3. MACS enrichment of TCR-engineered T cells
  - chapter 6

Figure 1.3: Strategies designed and tested in current thesis to advance TCR gene therapy. A schematic representation of a T cell expressing a therapeutic TCR, containing a signaling cassette and amino acid substitutions in CDR, and a truncated CD34 surrogate marker that can both be targeted for magnetic activated cell sorting (MACS). Abbreviations used in this figure (in addition to the ones explained in the legends of table 1.1 and 1.2 and figure 1.1 and 1.2): tCD34, truncated CD34.

T cell yield, enrichment for engineered T cells, stability of TCR transgene expression and functional avidity.

Finally, in **chapter 7**, I have discussed experimental results in the context of recent developments in the field of T cell therapy, and I have proposed a combination of the presented strategies to advance TCR gene therapy.



2

T cell receptor gene therapy: strategies for optimizing transgenic TCR pairing

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ABSTRACT

T cell receptor (TCR) gene therapy provides patients with autologous T cells that are genetically engineered with TCRαβ chains, and constitutes a promising approach for the treatment of tumors and virus infections. Among the current challenges of TCR gene therapy is the optimization of TCRα and β transgene pairing to enhance the functional avidity of therapeutic T cells. Recently, various genetically modified TCRs have been developed that enhance TCR pairing and minimize mis-pairing – i.e., pairing between transgenic and endogenous TCR chains. Here, we classify such receptors according to their CD3-dependence for surface expression and review their abilities to address functional T cell avidity. In addition, we discuss the anticipated clinical value of these and other strategies to generate high-avidity T cells.

INTRODUCTION

Adoptive transfer of autologous tumor-infiltrating or peripheral blood T cells results in clinical responses when treating melanoma (103, 104) as well as virus infections and virus-associated tumors (105-110). Notably, objective response rates were as high as 51% when melanoma patients were treated with non-myeloablative chemotherapy prior to transfer of autologous tumor-infiltrating lymphocytes (TIL) (111). Recently, myeloablative irradiation as a more stringent patient preconditioning regimen resulted in response rates of up to 72% (112). In comparison, patients with metastatic melanoma, of which the incidence has increased dramatically over the last few decades, face a 10 year survival rate of less than 10% after standard systemic treatment with either chemotherapy, such as dacarbazine, or high-dose interleukin-2 (IL-2) (113).

T cell receptor (TCR) gene therapy has extended the principle of therapy with T cells that are non-modified to T cells that have been retrovirally transduced with genes encoding TCRαβ. TCR gene therapy does not depend on the isolation and expansion of TILs for patient treatment, often laborious and having a limited success rate, and constitutes a more universal variant of T cell therapy. The genetic introduction of a single set of TCRα and β genes enables the generation of large numbers of T cells with a defined specificity that is generally not present in the endogenous T cell repertoire due to tolerance towards self-antigens, such as many tumor-antigens. Clinical TCR gene therapy, using TCRs directed at the HLA-A2-restricted MART-1_{27-35} antigen (epitope: amino acids 27-35) (termed MART-1/A2), resulted in an objective response rate of 12% in patients with metastatic melanoma (32). Recently, the clinical use of a highly reactive murine TCR specific for human $gp100_{154-162}$/HLA-A2 (gp100/A2) and a high-affinity TCR specific for MART-1/A2 revealed objective response rates of 19% and 30%, respectively, which coincided with toxicities such as melanocyte destruction and severe inflammation of target tissues in ears and eyes (45). Reasons that might explain the observed drop in therapeutic effectiveness of TCR-transduced T cells compared to TIL include suboptimal surface expression and/or function of TCR transgenes when compared to natural TCRs, resulting in therapeutic T cells with suboptimal avidity.

Recently, various genetic strategies have been developed to optimize the functional avidity of TCR-engineered T cells, which are presented in table 2.1. Here we focus on genetic modifications of receptor transgenes that are designed to enhance the ability of TCRα and β transgenic chains to correctly pair and, consequently, enhance the functional expression of transgenic TCRαβ. This review first describes the TCRαβ/CD3 complex, after which it describes and discusses genetically modified receptors, with special emphasis on TCR pairing and mis-pairing. Lastly, we discuss the value of these receptors and other strategies to generate high-avidity T cells for clinical TCR gene therapy.

Table 2.1. Genetic strategies to improve the functional avidity of TCR-engineered T cells

Receptor modifications that address TCR pairing	
CD3 dependent receptors	• Murinized TCR • Cysteine-modified TCR • Exclusive TCR heterodimer
CD3- independent receptors	• TCR:ζ • scTCR • TCR-like antibody
Other strategies to enhance the functional avidity of gene-engineered T cells	
Vectors and transgene cassette to increase transduction efficiency	• Optimized gammaretroviral vectors combined with 2A transgene cassette
TCR modifications aimed at improving TCR surface expression and function	• Codon optimization • Affinity maturation • Incorporation of stimulatory T cell molecules
Defined T cell populations to enhance T cell responsiveness	• TCRαβ gene transfer in monoclonal cytotoxic T cells

Box 2.1. Glossary

Autoreactive T cells: T cells that recognize self-antigens and are capable of destroying patient's healthy cells and tissues.

Clinical adoptive T cell transfer: therapy that is based on re-infusion of mostly autologous tumor infiltrating lymphocytes or peripheral lymphocytes that have been activated, selected *in vitro* for tumor-reactivity, and expanded to clinically relevant numbers.

Functional T cell avidity: in general, the combined strength of multiple protein-protein interactions between a T cell and a pMHC ligand or an antigen-positive target cell (strength of single protein-protein interactions determine affinity). In particular, T cell avidity is determined by the strength of T cell-pMHC interactions. Functional T cell avidity defines the sensitivity of T cells to respond to pMHC ligands or antigen-positive target cells.

Objective clinical response rate: according to the 'RECIST' criteria all responses that fall in the following categories are considered an objective response: complete response, partial response, and stable disease (http://imaging.cancer.gov/clinicaltrials/imaging/).

Peptide-MHC: major histocompatibility complex (MHC) glycoproteins are divided into 2 classes (i.e. MHC I and II) and expressed on the cell surface displaying short peptides. Complexes of peptide and MHC (pMHC) are considered antigens to T cells and constitute ligands for TCRαβ.

TCR heterodimerization: pairing of a TCR α-chain and a corresponding TCR β-chain that are both required to recognize an antigen.

T cell cytokines: proteins that are generally produced by antigen-activated T cells to aid an immune response. Cytokines may have different effects on recipient cells, such as cellular activation or migration. Some cytokines, such as IFNγ, have clear anti-tumor effects.

T cell cytotoxicity: killing of target cells (i.e. tumor cells) by T cells upon antigen recognition often through the release of lytic granules containing perforin (forms pores in target cell) and granzymes (activates caspase cascade that leads to apoptosis).

TCR gene transfer: transfer of genes encoding TCR into target cells. In general, retrovirusses are used that allow stable integration of TCR transgenes in the genome of T cells (i.e. T cell transduction).

TCR/CD3: STRUCTURE AND INITIAL SIGNALING

TCRαβ is a heterodimer that consists of a TCRα and TCRβ chain, which both contain a variable (V) and a constant (C) domain. TCR V gene segments encode two out of three complementarity determining regions (CDR) that comprise the peptide/Major Histocompatibility Complex (pMHC) binding site. The V, Diversity (D, only in case of TCRβ) and Joining (J) domains of both TCR chains form the third CDR. TCR chains each have a single membrane-spanning domain, a very short cytoplasmic tail, and are covalently linked through disulphide bonds. Surface expression and proper function of the variable TCRαβ requires intracellular assembly with invariant CD3 components (see also box 1.1). There are 4 different CD3 proteins that form two heterodimers (CD3δε and CD3γε) and one homodimer (CD3ζζ) before their association and surface expression with TCRαβ subunits (the structure of a surface expressed TCR/CD3 complex is schematically illustrated in figure 2.1A).

The assembly of CD3 dimers with TCRαβ occurs in a strict temporal and spatial fashion. First, three transmembranal tri-polar interactions are formed between CD3δε and TCRα, CD3γε and TCRβ, and CD3ζζ and TCRα (CD3 molecules provide an acidic amino acid and TCRα or β chains provide a basic amino acid to form polar interactions (114)). Notably, CD3ζ is considered a limiting factor for the total number of TCR/CD3 complexes expressed on a T cell's surface (98). Second, tetracysteine motifs in the membrane-proximal stalk regions of CD3γε and CD3δε dimers interact with TCR connecting peptide motifs (115, 116). Third, extracellular interactions between CD3δε and a loop in TCR-Cα help to stabilize CD3γε within the complex (which itself interacts with a TCR-Cβ loop) (117).

CD3 components contain immune receptor tyrosine-based activation motifs (ITAMs) that endow the TCR/CD3 complex with means for intracellular signaling. CD3δ, CD3ε, and CD3γ each contain 1 ITAM, whereas CD3ζ contains 3 ITAMs, supplying each TCR/CD3 complex with a total of 10 ITAMs. Following pMHC binding, the TCR/CD3 complex initiates synapse formation and T cell activation, possibly through conformational changes of TCRαβ and CD3ε (117, 118). These conformational changes might establish a molecular platform that allows recruitment of NCK, phosphorylation of CD3 ITAMs and subsequent docking and activation of a large number of signaling components (119-121).

TCR GENE THERAPY: TRANSGENE MODIFICATIONS THAT ADDRESS TCR PAIRING

TCRα and β chains that are genetically modified to enhance their ability to correctly pair are expected to yield improved surface expression levels of the transgenic TCRαβ

heterodimer. An expected consequence of enhanced TCR pairing is a concomitantly decreased TCR mis-pairing. TCR mis-pairing is a recognized phenomenon in the field of TCR gene therapy, which defines the incorrect pairing between a TCRα or β transgene and an endogenous TCRβ or α chain, respectively, and results in diluted surface expression of the therapeutic TCRαβ. Although there is currently no clinical evidence for TCR mis-pairing induced autoreactivity, the generation of autoreactive TCRs upon TCR mis-pairing cannot be excluded. In fact, T cells expressing mis-paired TCRs and expanded under high IL-2 conditions (similar to the current clinical setting) were demonstrated to induce Graft-versus-Host Disease (GvHD) in a preclinical model (122). Strategies that increase preferential TCR pairing and counteract TCR mis-pairing are anticipated to yield T cells with higher avidity for the antigen of interest and at the same time avoid the generation of unknown TCR specificities and the development of potential off-target autoimmune reactivity. Here we divide genetically modified receptors into two classes, termed CD3-dependent and independent receptors, with examples of both classes listed in table 2.1. Receptor modifications, described in more detail below, affect TCR pairing and mis-pairing as well as surface expression and redirected T cell function to different extents. An inventory of observed and anticipated effects of modifications on various receptor characteristics is given in table 2.2.

CD3-DEPENDENT RECEPTORS

CD3-dependent receptors are designed to have a competitive advantage towards endogenous TCR chains for surface expression and to improve the avidity of T cells by functional allelic exclusion of endogenous TCR chains. Examples of CD3-dependent receptors are depicted in figure 2.1.

Murinized TCR

Murinization of human TCRs is defined by replacement of human TCR-Cα and Cβ by the corresponding murine TCR-Cα and Cβ domains (fig. 2.1B). Although human and murine TCR-C domains show a high degree of homology, it is anticipated that small differences affect the stability of TCR/CD3 interactions and hence TCR surface expression levels.

Evidence for a competitive advantage of murinized TCR for interacting with human CD3 molecules comes from the observation that murine, and not human, TCR-C domains bind more strongly to human CD3ζ (123). TCR murinization as a mean to enhance preferential TCR pairing was investigated using the $MDM2_{81-88}$/HLA-A2 (MDM2/A2) and Wilms Tumor antigen $1_{126-134}$/HLA-A2 (WT1/A2) specificities (124, 125) and more extensively using the $p53_{264-272}$ HLA-A2 (p53/A2) and MART-1/A2 specificities (123). Gene

transfer of murinized TCRs in human T cells resulted in enhanced and more sustained levels of surface expression when compared to fully human TCRs as determined by flow cytometry (123-125). With regard to TCR function, murinized MART-1/A2, p53/A2 and WT1/A2 TCR demonstrated increased T cell activity relative regarding cytotoxicity as well as interferon-γ (IFNγ) and granulocyte macrophage-colony stimulating factor (GM-CSF) production in response to antigen-positive tumor cell lines (123). Interestingly, this increase in T cell activity was lost in T cell populations following various rounds of antigen-specific stimulation and having high and equal frequencies of T cells transduced with either modified or non-modified TCRs, which suggested that enhanced T cell reactivity is related to an increased frequency of TCR-positive T cells rather than an increased potency of murinized (or cysteine-modified, see below) TCRs.

In another study, however, murinized MDM2/A2 TCR did not mediate a change in cytotoxicity and cytokine production upon antigenic stimulation (124, 125). Notably, MART-1/A2 binding by human or murinized TCR in Jurkat T cells was reduced by 80 and 20%, respectively, upon introduction of a second non-related human TCR. The reduced expression of a murinized MART-1/A2 TCR suggests that TCR murinization addresses TCR mis-pairing, yet not to a full extent. In addition, murinized WT1/A2 TCR did not reveal reduced TCR mis-pairing compared to non-modified TCR when tested in TCR-engineered Jurkat as well as primary human T cells. Instead the level of TCR mis-pairing appeared to be determined by sequences of the TCR-V domains (125), in part providing a possible explanation for the discrepancy between the above-mentioned findings with different TCRs.

Cysteine-modified TCR

Introduction of cysteine amino acids at a structurally favourable position allows formation of an additional disulphide bridge and promotes correct pairing between the two TCR chains (126) (fig. 2.1C). Site-directed mutations of TCR-Cα Threonine-48-Cysteine and TCR-Cβ Serine-57-Cysteine resulted in a TCRαβ heterodimer linked by 2 interchain bonds (i.e., an introduced plus an endogenous transmembrane disulphide bridge (Cα-95 and Cβ-131) (127, 128)). It is noteworthy that the endogenous cysteines are not dispensable for surface expression of this modified TCR (125). Introduction of cysteine-modified WT1/A2 TCR in human T cells resulted in increased TCR expression and pMHC multimer binding compared to non-modified TCR, which corresponded to enhanced peptide-specific T cell cytotoxicity and IFNγ production (127).

Interestingly, increased pMHC binding was also noted when T cell populations were standardized for equal TCR expression levels, which suggested enhanced preferential pairing of cysteine-modified TCRs. Indeed, experiments measuring TCRβ that remains surface expressed following antigen-specific TCR down-regulation demonstrated that

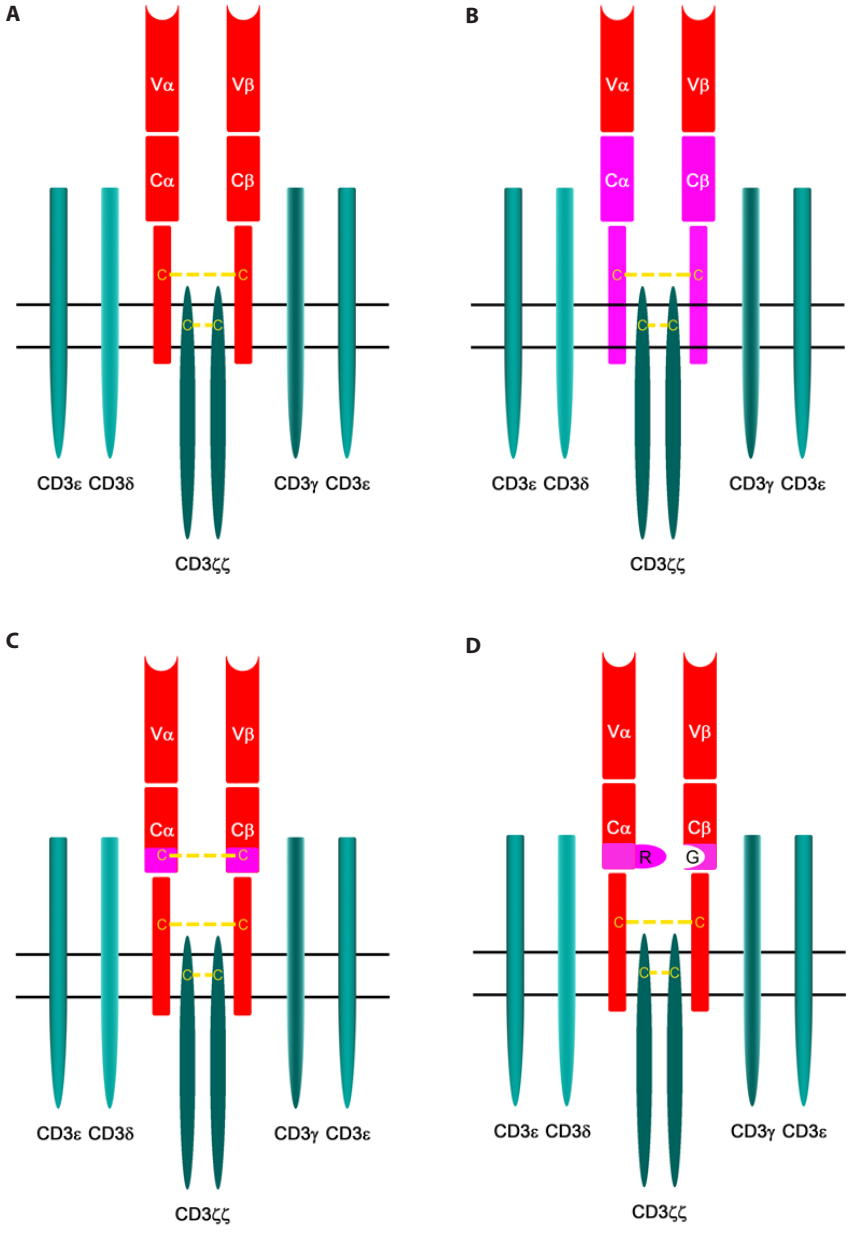

Figure 2.1. Genetic strategies that address TCR pairing (1): receptor transgenes that are dependent on endogenous CD3 for expression and function. Genetic modifications that result in CD3-dependent receptors, using the non-modified TCR as a reference (depicted in **A**), are the following: murinized TCR in which human constant domains are exchanged for their murine counterparts (**B**); cysteine-modified TCR in which additional cysteine residues provide a second interchain disulfide bond (i.e., TCR-Cα T48C and TCR-Cβ S57C) (**C**); and exclusive TCR heterodimers (based on the mutations TCR-Cα S85R and TCR-Cβ A88G) (**D**). TCRα and TCRβ variable and constant domains are indicated by Vα, Vβ, Cα and Cβ, respectively. The amino acids cysteine, arginine and glycine are abbreviated as 'C', 'R', and 'G'.

cysteine-modified, but not non-modified TCRβ, correctly pairs with corresponding TCRα chains (127). In contrast, the ability of cysteine-modified TCR to mis-pair was at best modestly decreased when compared to non-modified TCRs (125, 127). Surface expression levels of cysteine-modified TCRs were clearly detectable following gene transfer of single TCRα or β chains, or corresponding combinations of cysteine-modified and non-modified TCRα and β chains (125, 127). As might be expected, giving the single amino acid change, cysteine-modified and non-modified TCRs associate to CD3ε and CD3ζ to similar extents as determined by flow cytometry and immune precipitation (125).

When combining TCR murinization and cysteine-modification, human primary T cells revealed enhanced levels of p53/A2 TCR surface expression as well as p53-specific IFNγ release when compared to TCR that were either murinized or cysteine-modified (128). In these comparative analyses, TCR murinization revealed higher levels of surface expression and IFNγ release than cysteine-modification. Although expression and functional data are promising, no characterization regarding TCR pairing or CD3 complex formation of this dual-modified TCR has yet been performed.

Exclusive TCR heterodimer

To generate an exclusive TCR heterodimer, sterical and electrostatic forces were exploited to facilitate correct pairing between TCRα and β transgenes and at the same time inhibit pairing between exogenous and endogenous TCRα and β chains. The crystal structures of murine 2C/H-2K^{b} and human Tax$_{11\text{-}19}$/HLA-A2 specific TCRs identified Serine-85-Arginine for TCR-Cα and Arginine-88-Glycine for TCR-Cβ as mutations that would yield the required changes in electrostatic charges. These mutations were expected to generate a reciprocal 'knob-into-hole' configuration, and to minimally distort secondary and tertiary structures (schematically illustrated in figure 2.1D) (129). Initial tests were performed using primary human T cells transduced with a single non-modified or mutated MDM2/A2 TCRβ chain. Voss and colleagues observed that the expression of mutated TCRβ was reduced two-fold compared to non-mutated TCRβ, implying that mutated TCRβ chains have a reduced ability to mis-pair with endogenous TCRα chains. Further experiments using a TCR-negative thymoma cell line demonstrated that mutated TCRα has an even ten-fold lower ability to mis-pair than mutated TCRβ. Authors suggested that the large Arginine side chain in TCRα-S85R may result in enhanced steric interference when compared to the small Glycine side chain in TCRβ-R88G upon pairing with corresponding non-mutated TCR chains. In contrast to their ability to decrease TCR mis-pairing, mutated TCR chains also showed decreased expression of correctly paired TCRαβ resulting in compromised tetramer binding and antigen-specific cytolysis, but not IFNγ secretion, when transduced in primary human T cells.

CD3-INDEPENDENT RECEPTORS

CD3-independent receptors are comprised of TCR or TCR-like receptors that, in contrast to wild-type TCR, are expressed independently of endogenous CD3 molecules, and as such do not compete with endogenous TCR for surface expression. Examples of CD3-independent receptors are depicted in figure 2.2.

TCR:ζ

TCRα and β chains can be endowed with CD3ζ-driven dimerization by fusing each chain to a complete human CD3ζ molecule (abbreviated as TCR:ζ, see figure 2.2A). Already in 2000 it was reported that transduction of primary human T cells with either TCRα:ζ or TCRβ:ζ does not result in cell surface expression, pointing out that these TCR chains lack the ability to mis-pair with endogenous TCR chains (64). TCR pairing properties of TCR:ζ were more extensively studied in dual TCR Jurkat T cells expressing MelanA$_{26-35}$/HLA-A2 (MelanA/A2) specific TCR and either MAGE-A1$_{161-169}$/A1 (M1/A1) specific TCRαβ or TCR:ζ. In this study flow cytometric measurement of Fluorescent Resonance Energy Transfer (FRET) indicated high preferential pairing for TCRα:ζ and β:ζ and absence of TCR mis-pairing (63). TCR:ζ's surface expression is characterized by high mean fluorescence intensity levels and by being independent of the expression levels of endogenous TCR. Immune precipitation revealed that TCR:ζ did not associate with CD3ε, CD3γ, or CD3δ, and only marginally with CD3ζ. Studies into pMHC binding and T cell signaling and function, such as activation of nuclear factor of activated T-cells (NFAT), cytotoxicity and cytokine production, demonstrated that TCR:ζ performs equally well as non-modified TCR (63, 64, 130). So far, the TCR:ζ format has been applied to TCRs specific for several tumor and virus antigens, including M1/A1, gp100$_{154-162}$/HLA-A2 (gp100/A2), BMFL-1$_{280-288}$/HLA-A2 (BMFL-1/A2), JC$_{100-108}$/HLA-A2 (JC/A2) and EBNA-4$_{416-424}$/HLA-A11 (EBNA4/A11) by various laboratories (64, 131-133).

Single-chain TCR

Single-chain TCRs combine the V domains of TCRα and TCRβ into one chain. Generally, a TCR-Vα domain is attached to a TCR-Vβ domain, interspersed by a linker sequence, and followed by a TCR-Cβ domain which is fused to the complete human CD3ζ to provide downstream signaling and T cell activation (64, 134) (abbreviated as scTCR:ζ, see figure 2.2B). Reformatting a parental M1/A1 specific TCR, derived from the cytotoxic T lymphocyte (CTL) 82/30 (135), to generate a scTCR:ζ led to increased surface expression but reduced pMHC binding when compared to two-chain TCR:ζ (64). Lowered pMHC binding by scTCR:ζ was reflected by compromised T cell cytotoxicity and production of IFNγ

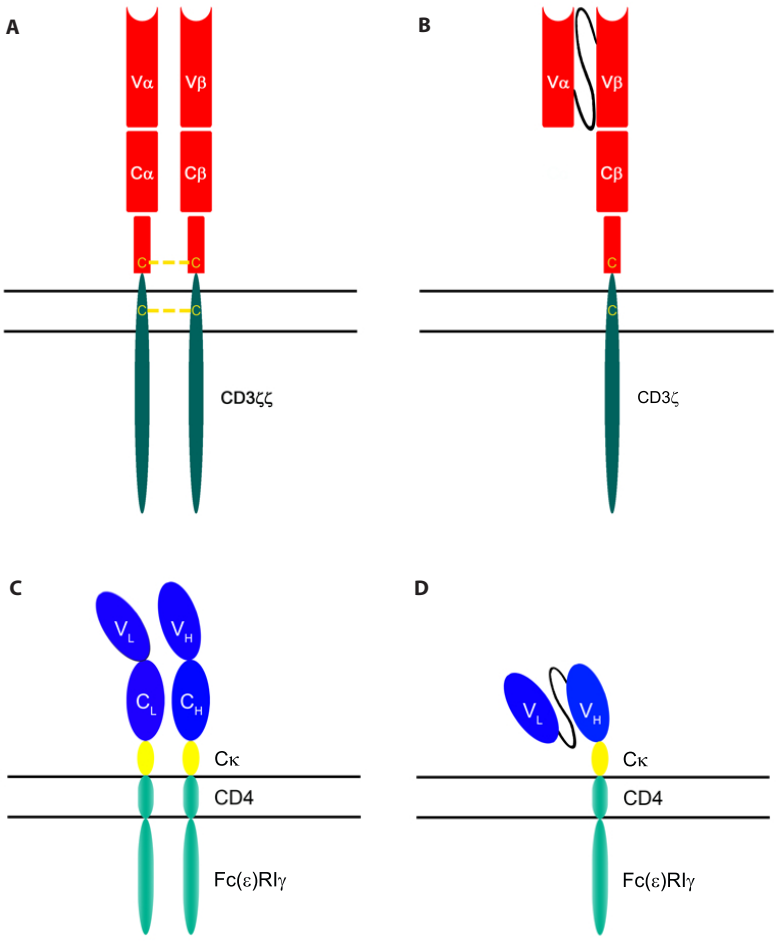

Figure 2.2. Genetic strategies that address TCR pairing (2): receptor transgenes that are independent of endogenous CD3 for expression and function. Genetic modifications that result in CD3-independent receptors are the following: TCR coupled to complete human CD3ζ (i.e., TCR:ζ) (**A**); single chain (sc)TCR, comprising TCR-Vα and Vβ (interspersed by a flexible linker) followed by TCR-Cβ and coupled to complete human CD3ζ (i.e., scTCR:ζ) (**B**); pMHC-specific Fab or scFv modified to allow surface expression and antigen-specific signaling (i.e., pMHC Fab:γ or scFv:γ) (**C** and **D**). TCRα and TCRβ variable and constant domains are indicated by Vα, Vβ, Cα and Cβ, respectively. Antibody heavy and light chains and their variable and constant domains are indicated by V_H, C_H, V_L and C_L, respectively. The Cκ linker-, CD4 transmembrane-, and Fc(ε)RIγ intracellular domain are abbreviated as Cκ, CD4 and Fc(ε)RIγ, respectively.

and TNFα, but not GM-CSF, in response to antigen-positive melanoma cells. Single-chain TCRs can express independently of CD3 (136), but in analogy to scFv (described below), these receptors are expected to weakly heterodimerize with endogenous CD3ζ/η, which may negatively affect antigen-specific T cell activation (137, 138).

TCR-like antibodies

Antibodies that recognize pMHC ligands represent a novel generation of receptors for immunogene therapy. Since antibody-based receptors, whether be it non-MHC or MHC-restricted antibodies, lack TCR-V and C domains, they are not expected to mis-pair with endogenous TCRα and β chains. General reviews on conventional non-MHC-restricted antibody-based receptors and their use to redirect T cell responses are provided by Gross and colleagues (139), Abken and colleagues (140) and Sadelain and colleagues (141).

TCR-like antibodies are derived *in vitro* from phage-displayed antibody fragments following selection with pMHC multimers. They are subsequently engineered for gene transfer after which T cells are able to bind and functionally respond to MHC-restricted tumor antigens (reviewed in (142, 143)). For example, selections from a large non-immune phage antibody repertoire yielded a fully human Fab fragment, termed G8 Fab, which specifically recognized M1/A1 complexes (144). The G8 Fab heavy (V_HC_H) and light chains (V_LC_L) were fused to the human CD4 transmembrane region and the Fc(ε)RIγ intracellular domain (general abbreviation: pMHC Fab:γ, see figure 2.2C) and upon gene transfer enabled T cells to bind tetramers (145, 146). Stimulating T cells engineered with G8 Fab:γ or its single chain variant, in which V_H and V_L were combined into one chain (general abbreviation: pMHC scFv:γ, see figure 2.2D), with M1/A1 antigen-positive melanoma cells leads to T cell cytotoxicity and production of cytokines (145, 146).

Interestingly, when compared to natural TCRs, TCR-like antibodies may use different contact residues but bind pMHC with a diagonal docking mode that is similar to that of natural TCRs (147, 148).

Thus far, phage-display and pMHC multimer selections yielded more than a dozen MHC-restricted Fab molecules, particularly true for HLA-A2-restricted antigens, and constitute a formally validated method to obtain TCR-like molecules (143). In contrast to full-length or modified TCR, the generation of these reagents is an entirely *in vitro* procedure and does not depend on the availability of T cells making these receptors promising candidates for gene therapy.

OTHER STRATEGIES TO ENHANCE THE FUNCTIONAL AVIDITY OF GENE-ENGINEERED T CELLS

The functional avidity of TCR-engineered T cells can also be improved via strategies other than those modifying receptors to address TCR pairing, such as the use of (I) optimal vectors and transgene cassettes to increase transduction efficiency, (II) genetically modified TCR formats aimed at improving TCR surface expression and function, or (III) defined

T cell populations to enhance T cell responsiveness. An overview of these strategies is included in table 2.1.

Vectors and transgene cassette to increase transduction efficiency

Generally, TCR gene transfer studies have made use of gammaretroviral vectors for transgene delivery, and clinically there is experience with gammaretroviral MFG

Table 2.2 – Comparison of modified receptors that address TCR pairing[a]

Receptor format	Assembly with endogenous CD3ε[b]	Reduction of TCR mis-pairing[c]	Preferential TCR pairing[d]	Cell surface expression[e]	T cell function[f]	Reference(s)
Non-modified TCR	0	0	0	0	0	
Murinized TCR	+	++/0[g]	++	+	++/0[g]	(123, 124)
Cysteine-modified TCR	0	+	+	0	+	(127)
Murinized and cysteine-modified TCR	N.D.	N.D.	N.D.	++	++	(128)
Exclusive TCR heterodimer	N.D.	++	-	--	-	(129)
TCR:ζ	--	++	++	++	0	(63, 64)
scTCR:ζ	N.D.	N.A.	N.A.	++[h]	--[h]	(64, 136)
Fab:γ / scFv:γ	--	N.A.	N.A.	-[h]	--[h]	(137, 145, 146)

[a] Modified receptors (schematically depicted in figures 2.1 and 2.2) were scored for several characteristics. Performances of the modified receptors were monitored in receptor-transduced human T cells and are scored '0' for equal performance; '+' or '-' for ≥ 1,5-fold and '++' or '--' for ≥ 2-fold increased or decreased performance, respectively, when compared to non-modified TCR. N.D. is not determined. N.A. is not applicable. Results reflect intra-study comparisons between modified and non-modified receptors.

[b] Assembly with endogenous CD3ε has been measured by flow cytometry, immunoprecipitation or FRET.

[c] Reduction of TCR mis-pairing has been measured by FRET between non-corresponding TCR chains following transduction with two TCR chains (i.e., TCRα + β) and/or flow cytometry of surface expressed TCR transgenes following transduction with single TCR chains (i.e., TCRα or β).

[d] Preferential TCR pairing has been measured indirectly using flow cytometry to determine the fraction of TCRβ positive T cells that bind pMHC or directly using FRET between corresponding TCR chains (i.e., TCR:ζ).

[e] Cell surface expression has been measured by flow cytometry following staining with pMHC ligands and using MFI as a readout.

[f] T cell function has been measured by cytolytic activity or IFNγ production.

[g] Conflicting findings between studies: See main text for details.

[h] Head-to-head comparisons to non-modified TCR are lacking, but performances of receptors are compared to either TCR:ζ (for scTCR:ζ) or CTL clone with identical specificity (for TCR-like antibodies).

vectors or MFG-derived vectors. Notably, mature T cells in contrast to hematopoietic progenitor cells are resistant to oncogenic transformation (149), and have shown no evidence of adverse effects of retroviral gene transfer when transferred in high numbers to patients.

There is accumulated evidence that transduction efficiencies differ substantially between different vectors, with a major role for the viral origin of the Long Terminal Repeats (LTRs) and splice and start sequences. In this context, it of interest to mention that the pMP71 gammaretroviral vector, which has a Myeloproliferative Sarcoma Virus (MPSV) LTR and optimal 5' sequences, demonstrated highly improved TCR transduction efficiencies (93, 94). An additional optimization of TCR expression is accomplished by introducing TCRα and TCRβ genes into one vector downstream of a single promotor and separated by an internal ribosomal entry site (IRES) or a 2A peptide sequence. In clinical studies, both IRES and 2A sequences have been used to separate TCRα and β genes (45). Recent work suggests that placing the TCRβ chain in front of the TCRα chain, especially when separated by a 2A sequence, renders optimal functional TCR expression levels for most TCRs tested (95).

TCR modifications aimed at improving TCR surface expression and function

Examples of genetic strategies that augment the expression and function of TCR transgenes include: (I) codon optimization; (II) affinity maturation; and (III) incorporation of stimulatory T cell molecules.

First, the functional expression of TCR transgenes can be enhanced by the use of codon optimized synthetic genes. The genetic code is redundant in that many of the 20 amino acids are encoded by multiple codons. Highly expressed mammalian genes share a similar codon usage, suggesting that codon usage can affect protein production. Recent data show that codon optimization of TCR genes, which also takes into account structural factors (150), has a beneficial effect on surface expression and *in vitro* and *in vivo* function of TCR-engineered T cells (96, 151, 152).

Second, the functional expression of TCR transgenes can be increased through *in vitro* affinity evolution of CDR-mutated TCRs via phage-display methodology. For example, HIV-1-specific TCRs have recently been selected with ligand-binding affinities that improved from the nM range to a high affinity pM interaction and provided T cells with HIV-1-specific and highly-avid responsiveness (153). In addition, the phage-display methodology has also been applied to TCR-like antibodies. The M1/A1 specific Hyb3 Fab:γ, the affinity-matured variant of G8 Fab:γ, was obtained by combining light-chain shuffling, heavy-chain targeted mutagenesis and more stringent *in vitro* selections, and displayed an increased ligand-binding affinity (K_D of 14 nM) yet an identical peptide-specificity (154). Following expression by human T cell transductants, Hyb3 Fab resulted

in improved ligand-specific T cell responses (154). Interestingly, amino acid changes to the TCR–C domains, resulting in a decreased N-glycosylation, have also been reported to enhance TCR-pMHC interactions and to result in enhanced functional T cell avidity as measured by cytokine release and lytic activity (100).

Third, the TCR's functional expression can be improved by genetic incorporation of transmembrane and/or intracellular domains of accessory, co-stimulatory and kinase molecules. In extension to the genetic introduction of CD3ζ or Fc(ε)RIγ into receptors, as discussed for TCR:ζ, scTCR:ζ and TCR-like antibodies, other 'building blocks' have been analyzed for their effect on surface expression and function of various receptors. For example, the Ovalbumin$_{257\text{-}264}$/H-2K^{b} specific scTCRs coupled to the transmembrane domain of CD3ζ followed by the intracellular domain of CD28 and the co-receptor molecule LCK (i.e., TCR-ζ-28-LCK) constituted a promising format with respect to T cell activation and peptide-specific T cell functions (136), which confirmed an earlier study performed by Geiger and colleagues (155). Despite constructive efforts to optimize scTCRs, it has generally been observed that these receptors are less responsive than non-modified TCR to low concentrations of antigen (64, 136). The coupling of the co-stimulatory molecule CD28 to EBNA-4/A11 specific TCR:ζ (i.e., TCR:28ζ) (131, 156) and MA1/A1 specific G8 Fab:γ (157) resulted in increased antigen-specific T cell responses. In addition, the incorporation of other TNF receptor super family members, such as CD134 and CD137, into TCR molecules may further optimize T cell functions as suggested by studies with scFv receptors that incorporate co-stimulatory molecules (158).

Defined T cell populations to enhance T cell responsiveness

A strategy that makes use of monoclonal T cells as recipient cells for gene transfer results in enhanced peripheral T cell persistence and sustained immune responses against tumors (159). The generation of dual-specific T cells is expected to result in ongoing T cell stimulation via the endogenous TCR and prevention of tumor-induced T cell anergy (82, 159). Treatment of neuroblastoma patients with EBV-specific CTL expressing a receptor directed against diasialoganglioside GD2 showed enhanced T cell survival and tumor regression in half of the patients treated (82, 160) (clinical findings are also presented in table 2.3). TCR gene transfer into restricted T cell populations is anticipated not only to enhance T cell responsiveness but also to reduce the risks of auto-immune reactivities for two reasons: recipient T cells are non-self reactive and chances that new TCR reactivities are generated as a consequence of TCR mis-pairing is reduced or even absent (161). Defined T cell populations may be transduced with genetically modified receptors that address TCR pairing to further reduce the chances of generating self-reactive T cells. A note of concern, the choice of defined T cell populations might affect the anti-tumor efficacy of TCR-engineered T cells. For example, TCRαβ-engineered γδT cells showed no

evidence of TCR mis-pairing, yet demonstrated compromised anti-tumor efficacy in an OVA mouse model when compared to TCRαβ-engineered αβT cells (162).

THERAPEUTIC IMPLICATIONS

Clinical application of receptor gene therapy with either TCRs to treat melanoma, or antibody-based receptors (so-called Chimeric Antigen Receptors, CARs) to treat renal cell cancer, ovarian cancer, neuroblastoma or lymphoma have shown some successes,

Table 2.3. Clinical TCR and CAR gene therapy trials[a]

Antigen-specific TCR or CAR	Disease	Treatment response	On-target toxicity	Institute/ company	Reference
MART-1/A2 (DMF4) TCR	Metastatic melanoma	17 pts. treated: 2PR, 1MR	None	National Cancer institute, Bethesda, USA	(32)
MART-1/A2 (DMF5) TCR	Metastatic melanoma	20 pts. treated: 6PR	Melanocyte destruction and inflammation in ears and eyes	National Cancer institute, Bethesda, USA	(45)
gp100/A2 TCR	Metastatic melanoma	16 pts. treated: 1CR, 2PR	Melanocyte destruction and inflammation in ears and eyes	National Cancer institute, Bethesda, USA	(45)
CAIX CAR	Renal cell carcinoma	No response	Liver toxicity	Erasmus University Medical Center, Rotterdam, Netherlands	(163)
FR CAR	Ovarian carcinoma	No response	None	National Cancer institute, Bethesda, USA	(79)
GD2 CAR[b]	Neuroblastoma	8 pts. treated: 1CR, 1SD, 2 tumor necrosis	None	Baylor College of Medicine, Houston, USA	(82)
CD20 CAR[c]	Indolent B-cell lymphoma or mantle cell lymphoma	7 pts. treated: 2CR, 1PR, 4SD	None	University of Washington, Seattle, USA	(83)

[a] Evaluation of clinical trials performed with T cells retrovirally transduced with either TCR or CAR with respect to treatment response and on-target toxicity.

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

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

Abbreviations: TCR is T cell receptor; CAR is chimeric antigen receptor; CAIX is carbonic anhydrase IX; FR is folate receptor; GD2 is diasialoganglioside; CR is complete response; PR is partial response; MR is minor response; and SD is stable disease.

summarized in table 2.3, but generally not in a substantial number of patients (32, 45, 79, 82, 83, 163). Strategies that enhance the functional avidity of TCR-engineered T cells, such as the ones described in the present review, are expected to improve anti-tumor responses in clinical studies.

A TCR transgene can be genetically modified in various ways to enhance its functional expression, including optimization of preferential TCR pairing, codon usage, ligand-binding affinity and/or signaling potency. We consider it of critical importance that the TCR format of choice is modified to improve functional surface expression, and at the same time minimizes the risk of mis-pairing with endogenous TCR chains. Although based on *in vitro* data the murinized TCRαβ and TCR:ζ might represent good candidate TCR formats (see table 2.2), there is a need for *in vivo* data with respect to anti-tumor efficacy as well as off-target toxicity to justify their use in clinical studies.

Additional challenges of the clinical use of TCR-engineered T cells include: (I) toxicities (see table 2.3), (II) limited antigen specificity, (III) decreased peripheral persistence and (IV) potential immunogenicity of receptor-engineered T cells. First, and with respect to toxicities, two clinical studies, one with CAR-engineered T cells to treat renal cell carcinoma (RCC) patients and one with TCR-engineered T cells to treat metastatic melanoma, show 'on-target' toxicities that are related to the expression of the target antigen on healthy tissue (45, 163). These results argue for the use of TCR recognizing non-self antigens or cancer testis antigens. This latter group of antigens represents a small number of gene families that are expressed in many tumors, but not in healthy tissues except immune-privileged male germline and thymic medullary epithelial cells (164, 165), and may encode candidate target antigens for T cell therapy. One should note that in an event toxicities do occur, auto-reactive T cells might be removed via drug-induced T cell suicide (166, 167) or tag-mediated T cell killing (168, 169).

Second, TCR gene transfer results in T cells that are specific for a single (therapeutic) antigen, which is in contrast to TILs that may cover a few antigen specificities (111). To enhance antigen-reactivity and consequently reduce the risk of immune escape, one could opt to use two instead of one set of TCRαβ genes to engineer T cells. Third, peripheral persistence of TCR-engineered T cells is decreased when compared to TILs following adoptive transfer (32, 45, 111). T cell persistence is reported to be inversely associated with differentiation state and replicative history of transferred T cells (170). One way to obtain less differentiated T cells might be to expose T cells to common-γ cytokines other than IL-2 prior to adoptive T cell transfer. For example, treatments with either IL-15+IL-21 or IL-7+IL-15 have been shown to generate gene-engineered T cells with a less differentiated CD8 T cell phenotype (i.e., central memory phenotype) and potent antigen reactivity and prolonged peripheral persistence (171, 172). Alternatively, one can also use less differentiated T cell populations as recipient cells for gene transfer (173, 174).

Finally, TCR-engineered T cells may evoke an immune response in patients. Studies with CAR-engineered T cells to treat RCC patients revealed humoral and cellular responses directed at the CAR's murine CDR domains (163, 284). With respect to genetically modified TCRs, the TCR-V domains remain unchanged, but the extent of modifications to the TCR-C domains varies between the different receptor formats. Although severity of potential immunogenicity might be estimated based on the extent of genetic modifications, for all described receptors this needs to be verified in *in vivo* studies. Another way to address the potential immunogenicity of receptor-transduced T cells might be by immune suppressive preconditioning of patients in order to limit or delay the patient's immune response towards transduced T cells. This latter option, combining chemotherapy with T cell therapy, potentiates the responses of the adoptively transferred T cells (175). Combining T cell therapy with other therapies, such as those stimulating the innate immune system, also appear promising and await clinical testing (176).

For future clinical trials, we propose to combine strategies that on the one hand genetically modify TCR transgenes to enhance preferential TCR pairing and minimize TCR mis-pairing and on the other hand further enhance the efficacy and safety of TCR-engineered T cells, such as the use of T cells with an optimal T cell differentiation state and a TCR specific for a tumor-specific antigen.

CONCLUDING REMARKS

There are numerous ways to increase the functional avidity of TCR-engineered T cells (presented in table 2.1), but those that address TCR pairing recently got special attention in the field of TCR gene therapy since they are expected to enhance the functional avidity as well as the potential safety of T cells (see table 2.2).

Genetically modified receptors that address TCR pairing can be classified according to the CD3-dependence for surface expression. Classification into CD3-dependent and CD3-independent receptors represents not only a convenient way of listing genetic modifications, but points to fundamental differences in cell surface expression and downstream signaling. The abilities of CD3-dependent receptors to address TCR mis-pairing are not absolute. On the one hand, the murinized and cysteine-modified TCRs, and especially the combination of these formats, do show enhanced preferential pairing, but are less potent at addressing TCR mis-pairing. On the other hand, the exclusive TCR heterodimer addresses TCR mis-pairing to a fair extent, but unfortunately shows limited preferential TCR pairing, pMHC binding and redirected T cell function. In contrast, CD3-independent receptors (with most data available for TCR:ζ) combine high preferential pairing with none or clearly less TCR mis-pairing. Murinized TCR, cysteine-modified TCR and TCR:ζ,

Box 2.2. Outstanding questions

- How do the various modified receptors perform *in vivo* with respect to anti-tumor efficacy as well as safety? Is there a winner?
- What combination of strategies performs best in vivo without showing toxicities: modified receptors, T cell phenotype, antigen choice?
- Would TCR mis-pairing lead to off-target toxicities in clinical TCR gene therapy when conditions would promote expansion of adoptively transferred T cells more strongly? And if so, would the modified receptors prevent or decrease the extent of such toxicities?
- Would it be feasible to identify and remove T cells with mis-paired TCRs prior to adoptive transfer?

but not scTCR and MHC-restricted scFv, show good pMHC binding and TCR-mediated T cell functions.

Receptor modifications to enhance functional T cell avidity other than those that specifically address TCR pairing include codon optimization, affinity maturation, and incorporation of stimulatory T cell molecules. A combined effort of such strategies towards improved T cell avidity is expected to potently enhance the anti-tumor efficacy of clinical TCR gene therapy. Importantly, various receptor modifications, especially those that address TCR pairing, as well as combinations of receptor modifications need validation in *in vivo* studies prior to their translation to clinical trials (see box 2.2. Outstanding questions). In addition to the design of an optimal TCR transgene, key parameters that may affect the clinical impact of TCR gene therapy include the choice of vector and transgene cassette, definition of T cell population, and combination therapies. In our view of critical importance to the safety of TCR gene therapy is the choice of T cell target antigen.

Taken together, we consider CD3-independent receptors valuable transgenes to optimally address TCR pairing and functional T cell avidity. Combining such receptors with strategies to further enhance anti-tumor activity and safety of TCR-engineered T cells holds great promise to the development of clinical TCR gene therapy.

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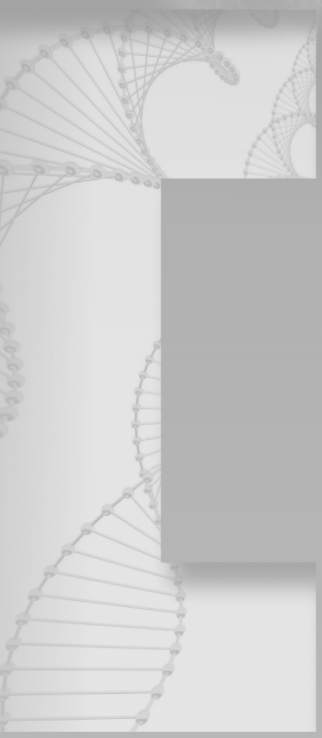

3

T cell receptor fused to CD3ζ: transmembrane domain of CD3ζ prevents TCR mis-pairing, whereas complete CD3ζ directs functional TCR expression

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ABSTRACT

TCR gene therapy represents a feasible and promising treatment for patients with cancer and virus infections. Currently, this treatment rationale is hampered by diluted surface expression of the TCR transgene and generation of potentially self reactive T-cells, both a direct consequence of mis-pairing with endogenous TCR chains. As we reported previously (63, 64), TCR mis-pairing can be successfully addressed by a TCR:CD3ζ fusion protein (i.e., TCR:ζ). Here, we set out to minimize the content of CD3ζ in TCR:ζ, specific for MAGE-A1/HLA-A1, without compromising TCR pairing and function. Domain-exchange and 3D-modeling strategies defined a set of minimal TCR:ζ variants, which, together with a murinized and cysteine-modified TCR (TCR:mu+cys), were tested for functional TCR expression and TCR pairing. Our data with Jurkat T cells show that the CD3ζ transmembrane domain is important for cell-surface expression, whereas the CD3ζ intracellular domain is crucial for T-cell activation. Notably, the inability of TCR:ζ to mis-pair was not observed for TCR:mu+cys, depended exclusively on the transmembrane domain of CD3ζ and could not be recapitulated by a limited number of structurally defined CD3ζ transmembrane amino acids. The extracellular CD3ζ domain was dispensable for TCR:ζ's ability to prevent TCR mis-pairing, bind pMHC and mediate NFAT activation. In primary human T cells, however, minimal TCR:ζ without CD3ζ's extracellular domain but not TCR:ζ nor TCR:mu+cys revealed compromised cell surface expression and T cell function. Taken together, our study demonstrates that CD3ζ's transmembrane domain dictates TCR:ζ's inability to TCR mis-pair, but only TCR coupled to complete CD3ζ and not its minimal variants were functionally expressed in primary T cells.

INTRODUCTION

T cell receptor (TCR) gene therapy is based on retroviral transduction of T cells with tumor or virus-specific TCRαβ transgenes. Clinical TCR gene therapy trials proved feasible and applicable towards multiple tumor types, such as metastatic melanoma, colorectal carcinoma and synovial sarcoma (32, 40, 45, 46). This first series of studies with TCR-engineered T cells, although showing variable clinical responses in limited numbers of patients, demonstrate that responses generally lag behind those observed with non gene-engineered T cells (27, 28, 103, 104, 111). In addition, in case of TCRs directed against MART-1, gp100 or CEA, but not the cancer testis antigen NY-ESO-1, treatment resulted in on-target toxicity, i.e., severe inflammation of healthy tissue expressing the target antigen (40, 45, 46).

The efficacy and safety of clinical TCR gene transfer may be further enhanced by strategies that address TCR mis-pairing. TCR mis-pairing is a recognized phenomenon of TCR gene transfer, and defined by the incorrect pairing of TCRα or TCRβ transgenes with endogenous TCRβ or TCRα chains, respectively. TCR mis-pairing leads to the formation of unknown TCR specificities, which dilute the surface-expression of the therapeutic TCRαβ heterodimer and can potentially result in off-target toxicity. Although, there is no clinical evidence for TCR mis-pairing-induced autoreactivity, preclinical data derived from a mouse model, demonstrate that TCR mis-pairing can lead to graft-versus-host disease (65). Strategies that prevent TCR mis-pairing are therefore expected to improve T cell avidity by increasing the level of cell surface expression of therapeutic TCRαβ heterodimer and at the same time reduce potential off-target toxicity, as described in chapter 2. These strategies include the murinization of TCRα and –β constant domains (123), introduction of additional cysteine residues in TCRα and –β to form an extra disulfide bridge (127), the exchange of structurally important amino acids between TCRα and –β (129), or the replacement of parts of TCRα and –β constant domains by complete human CD3ζ (TCR:ζ) (64).

In previous studies, TCR:ζ has been extensively characterized regarding its ability to address TCR mis-pairing and functionally retarget T cells towards several tumor and virus antigens, such as MAGE-1/HLA-A1, gp100/HLA-A2, BMFL-1/HLA-A2, JC/HLA-A2 and EBNA-4/HLA-A11 (63, 64, 131-133, 177). Notably, TCR:ζ's surface expression, which is enhanced when compared to wt TCR, and its ability to form immune synapses are independent of endogenous CD3 proteins. This receptor, possibly as a consequence of conformational changes, results in enlarged synapse sizes in TCR-transduced T cells (63, 177). Despite these unique properties, TCR:ζ and wt TCR do not differ with respect to the molecular 'make-up' of immune synapses and their ability to mediate antigen-specific T cell functions.

In the current study, we set out to minimize the content of CD3ζ present in TCR:ζ, specific for MAGE-A1/HLA-A1, without compromising the pairing and functional properties of TCR:ζ, and with the intent to potentially decrease the immunogenicity of this receptor. In analogy to a study by Sommermeyer and colleagues, who defined a limited number of amino acids that preserved the benefits of murinized TCRs (178), the present effort would define those domains or amino acids of CD3ζ responsible for improved TCR pairing and function. To generate minimal TCR:ζ variants we applied a domain-exchange as well as a 3-dimensional (3D) modeling strategy, and tested variants for TCR pairing and functional expression. We observed that the CD3ζ transmembrane domain, but not a limited number of structurally defined amino acids, is critical for TCR:ζ's surface expression and its inability to mis-pair with endogenous TCR chains, whereas the intracellular CD3ζ domain is critical for T cell activation. A minimal TCR:ζ variant that lacked the extracellular CD3ζ domain was best at preserving both TCR pairing and function in Jurkat T cells, but was not functionally expressed in primary T cells.

MATERIALS AND METHODS

Cells and reagents

Jurkat T cell clone 19 (63), which expresses MelA/HLA-A2 (MelA) TCR, and EBV-transformed B cell blasts (APD) were cultured in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) containing 10% Fetal Bovine Serum (FBS, Stonehouse, Gloucestershire, UK), streptomycin (100 µg/ml) and penicillin (100 U/ml). The human embryonic kidney cell line 293T, the packaging cell line Phoenix-A, and melanoma cell line Mel2A were cultured in DMEM medium (BioWhittaker) supplemented with 10% FBS, 2 mM L-glutamine, non-essential amino acids and antibiotics. T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere (179) and cultured in HEPES buffered RPMI 1640 medium supplemented with 10% Human Serum, 2 mM L-glutamine, streptomycin and penicillin. Monoclonal Abs included: FITC- and non-conjugated anti-TCR-Vα19 mAb (Pierce Biotechnology, Inc., Rockford, IL); PE- and non-conjugated anti-TCR Vβ27 mAb (BD Biosciences, San Jose, USA); PE- and non-conjugated anti-TCR-Vβ9 mAb (BD Biosciences and Coulter-Immunotech, Marseille, France, respectively); PE-conjugated anti-CD107a mAb (BD biosciences); APC-and non-conjugated anti-CD3ε mAb (OKT3) (BD Biosciences and Coulter-Immunotech, respectively); Cy5- and non-conjugated Rabbit-anti-Mouse (RαM) IgG Fab (Jackson ImmunoResearch, Suffolk, UK). Peptide/MHC (pMHC) monomers included: MAGE-1 (M1: EADPTGHSY)/HLA-A*0101; and Melan-A (MelA: ELAGIGILTV)/HLA-A*0201 biotinylated peptide/MHC monomers (Sanquin, Amsterdam, Netherlands), and these pMHC monomers were tetramerized as described previously (180). Other

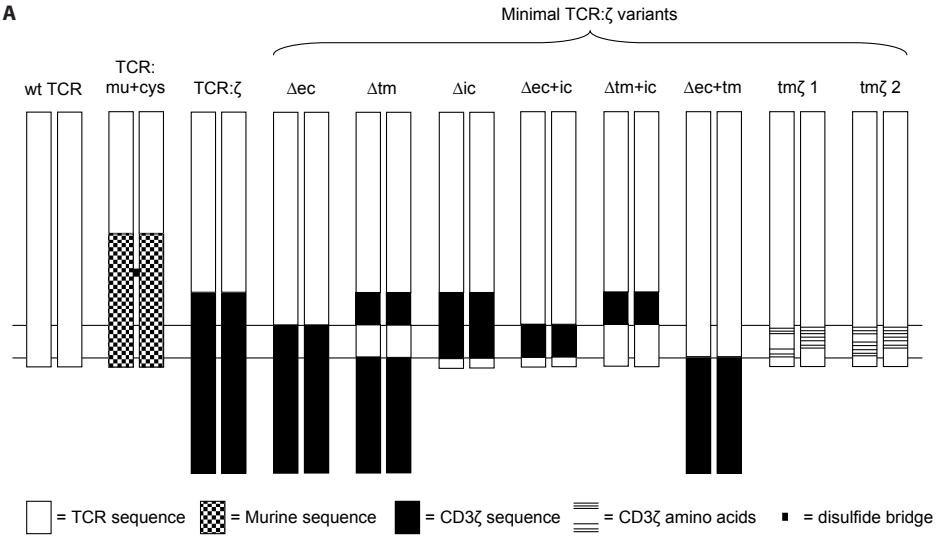
reagents included: Retronectin (human fibronectin fragments CH-296, Takara Shuzo Co. Ltd., Otsu, Japan); PMA (Sigma-Aldrich, St. Louis, USA); PHA (Remel Europe, Dartford, England); golgistop (BD biosciences) and streptavidin-PE (BD biosciences).

Cloning of minimal TCR:ζ variants

We have generated a panel of 11 TCRs, as schematically represented in figure 3.1, that are specific for MAGE-A1/HLA-A1 (M1/A1) and have the TCR-V gene usage TRAV19/J39/C and TRBV9/D2/J2-3/C2 (with TCR-V(D)J gene nomenclature according to http://imgt.cines.fr), originally derived from CTL clone MZ2-82/30 as described previously (64). Control TCRs (n=3) included wt TCR (63), TCR:ζ (63, 64) and a murinized plus cysteine-modified TCR (i.e., TCR:mu+cys), the latter designed according to Cohen and colleagues (128). TCR:mu+cys was generated via overlap PCR to fuse together M1/A1 TCR-V and murine TCR-C domains (Vα-mCα and Vβ-mCβ2), which were ligated in pBullet vectors (64) via *Sal*I-*Xho*I (TCRα) and *Nco*I-*Xho*I (TCRβ). Cysteine mutations (TCRα T189, and TCRα S191) were generated using QuickChange Site-Directed Mutagenesis Kit (Fynnzymes, Espoo, Finland) according to the manufacturer's instructions. The minimal TCR:ζ variants (n=8) were generated via either one of two approaches. First, six variants were made using a domain-exchange strategy in which extracellular (ec), transmembrane (tm) or intracellular (ic) domains of CD3ζ, and combinations of these domains, were exchanged for corresponding TCR domains. These six variants were named as follows: TCR:ζ Δec; TCR:ζ Δtm; TCR:ζ Δic; TCR:ζ Δec+ic; TCR:ζ Δtm+ic; and TCR:ζ Δec+tm (Δ indicates lack of CD3ζ domain(s), and replacement by corresponding TCR domain(s)). The exact boundaries of ec, tm and ic domains of CD3ζ, TCRα and TCRβ are provided in the legend to figure 3.1. These variants were generally constructed by overlap PCR and gene synthesis, as described in detail in *supplementary methods*. Second, two additional TCR:ζ variants were made using a 3D-modelling strategy in which a limited number of amino acids of TCR tm were exchanged for CD3ζ tm amino acids at structurally favorable positions. These two variants were named as follows: TCR tmζ1 and 2, with the exact tm sequences provided by figure 3.1. The modeling software to design these variants, and their construction, generally by gene synthesis, is described in detail in *supplementary methods*. All TCR constructs made (n=11) were sequence verified (Service XS, Leiden, Netherlands).

Retroviral TCR gene transfer into T cells

TCR cDNAs were used to transduce Jurkat T cell clone 19 as well as human PBMC. To this end, Moloney Murine Leukemia retroviruses were produced by a co-culture of the packaging cells 293T and Phoenix-A following calcium-phosphate transfections (63, 131). Packaging cells were transfected with TCR cDNAs, pHIT60 MLV GAG/POL, and VSV-G



B

CD3ζ tm	P K L C Y L L D G I L F I Y G V I L T A L F L
TCRα:ζ tmζ 1	N L S C I G F D I L L L K Y A G F L T L M F L
TCRα:ζ tmζ 2	N L C Y I G D R I L L L K G A G L T L L F L L
TCRβ:ζ tmζ 1+2	N L C I L L D K A L L Y Y V L V L T L V F M A M

Figure 3.1. Scheme of minimal TCR:ζ variants. (**A**) Minimal TCR:ζ constructs were modified via replacement of either extracellular (Δec), transmembrane (Δtm), intracellular (Δic), extra and intracellular (Δec+ic), transmembrane and intracellular (Δtm+ic) or extracellular and transmembrane CD3ζ domains (Δec+tm) (black) by corresponding TCRα and TCRβ domains (white). Boundaries for the CD3ζ/TCRαβ ec, tm and ic domains were defined as follows. CD3ζ (genbank accession number: CAI21380.1) ec, tm and ic: nucleotide (nt) 73-90 (aa 25-30); nt 91-153 (aa 31-51); and nt 154-489 (aa 52-163). TCRα ec, tm and ic: nt 703-765 (aa 235-255); nt 766-825 (aa 256-275); and nt 826-840 (aa 276-280). TCRβ ec, tm and ic: nt 790-846 (aa 264-282); nt 847-912 (aa 283-304); and nt 913-933 (aa 305-311). In addition, two minimal TCR:ζ constructs were designed in which defined amino acids of tm CD3ζ were transplanted onto structurally favorable positions in TCRα and TCRβ tm domains, and were termed minimal TCR:tmζ1 and 2 (see figure 3.1B). Control TCRs include: wt TCR, TCR:ζ and a TCR containing murine constant domains and additional cysteines (TCR:mu+cys, as described in (128). (**B**) Transmembrane amino acids of CD3ζ and minimal TCR:ζ variants TCRα:tmζ1, TCRα:tmζ2, and TCRβ:tmζ (CD3ζ amino acids underlined).

envelope plasmids for Jurkat T cell transductions, or with TCR cDNAs, pHIT60 MLV GAG/POL, and pCOLT-GALV-envelope encoding plasmids for human PBMC transductions. The transduction procedure used was optimized for human T cells and described previously (71).

Flow cytometry and FACSort

TCR-transduced T cells ($1x10^4$ cells) were monitored by flow cytometry for surface expression of transgenic TCR using FITC-conjugated anti-TCR-Vα19, PE-conjugated anti-TCR-Vβ9 mAbs and/or R-PE-conjugated M1/A1 tetramer; endogenous TCR (in case of Jurkat T cells) using PE-conjugated anti-TCR-Vβ27 mAb and/or R-PE-conjugated MelanA/A2 tetramer; and endogenous CD3 using APC-conjugated anti-CD3ε mAb. After T cells were washed, they were incubated with mAbs for 30 min on ice, or 15 min at RT for pMHC tetramers. Next, T cells were washed and fixed with 1% PFA (Brunschwig, Amsterdam, the Netherlands) before measurements on a FACSCalibur dual-laser flow cytometer (Beckton Dickinson, Alphen a/d Rijn, the Netherlands). Samples were analyzed using BD Cellquest software and displayed as dotplots or histograms. Enrichment of M1/A1 TCR-expressing Jurkat Clone 19 T cells was performed by two-color Fluorescent Activated Cell Sorting (FACS) following staining with FITC conjugated anti-TCR-Vα19 and PE conjugated anti-TCR-Vβ9 mAbs.

Flow cytometry-based FRET

PE-labeled mAbs were used as donor and Cy5-labeled RαM antibody as acceptor and used in the following donor/acceptor combinations (as described in (63)): anti-TCR-$Vβ27^{PE}$ + anti-TCR-Vα19/ $GαM^{Cy5}$ and anti-TCR-$Vβ9^{PE}$ + anti-CD3ε (OKT3)/$GαM^{Cy5}$. Staining was performed sequentially, with extensive washing steps in between, using the following order of staining steps: first non-conjugated mAbs, second Cy5-labeled Abs, and last PE-labeled donor Abs. Fluorescence intensities of emissions at 570 nm (donor channel, excitation at 488nm), 670 nm (acceptor channel, excitation at 635), and over 670 (FRET channel, excitation at 488 nm) were measured and collected on a FACSCalibur. Data were analyzed with the FLEX software on a per-cell basis (181).

NFAT reporter gene assay

Gaussia-Luciferase reporter gene under the control of 6 NFAT response elements, a minimal IL-2 promotor, and a TATA box (in short: GLuc-NFAT(RE)6) was used to quantify TCR-mediated stimulation. Gluc-NFAT(RE)6 was generated by digesting a plasmid containing NFAT-6-luc (182) with *Nco*I-*Hin*DIII and ligating this fragment (containing the 6 NFAT response elements, minimal IL-2 promotor and TATA box), together with a *Eco*RI-*Nco*I linker, in *Eco*RI-*Hin*DIII digested pGluc-basic vector (New England Biolabs, Ipswich, USA). $5x10^6$ Jurkat TCR-transduced T cells were transiently nucleofected with an Amaxa nucleofector (AmaxaBiosystems, Cologne, Germany) according to previous optimizations (130, 183). Briefly, T cells were resuspended in 100 µl supplemented buf-

fer V to which 5 µg GLuc-NFAT(RE)6 was added and pulsed with the Nucleofector set at program C-16. Next, T cells were immediately transferred to 2.5 ml warm Jurkat T-cell medium in T25 flasks for O/N recovery at 37°C and 5% CO_2. Non-tissue culture-treated 96-well plates were coated with non-conjugated mIg Ab, anti-TCR-Vβ9 mAb (100 ng) or biotinylated M1/A1 or JC/A2 monomers (titrated from 126 nM down to 4 nM). The latter added to streptavidin-coated plates O/N at 4°C. Twenty hours post-transfection, $0.2x10^6$ T cells at a concentration of $1x10^6$/ml were transferred to each well in the 96-well plates and stimulated for 6 hours at 37°C and 5% CO_2. Subsequently, 25 µl supernatant was transferred to 96 wellplate read-out plates (Corning incorporated, Costar assay plate, Lowell, USA) and placed in a luminometer. Next, 50 µl assay buffer was added (Gluc substrate; New England Biolabs, Ipswich, USA). Light units indicative of the Gaussia Luciferase-mediated enzymatic transition of coelenterazine into coelenteramide were measured according to the manufacturer's instructions (Mediators, Vienna, Austria) and expressed (in RLU) relative to wt TCR luciferase activities (100% for each concentration).

CD107a mobilization assay

TCR-transduced human PBMC ($1x10^6$) were resuspended in 0,15 ml T cell medium, 0,5 µl Golgistop (BD biosciences), and 100 µl anti-CD107a-PE (BD biosciences). Mel2A and APD target cells ($1x10^6$) were resuspended in 0,25 ml T cell medium (± 10^{-5} M M1 peptide). Next, 50 µl target cells and 50 µl T cells were mixed in a tissue culture treated 96-well plate (Greiner bio-one) and incubated for 2 h at 37°C and 5% CO_2. After the cells were washed, they were stained with anti-Vα19-FITC and anti-CD3ε-APC for 30 minutes at 4°C in the dark. Next, cells were washed and fixed with 1% PFA and measured on a FACS Calibur dual-laser flow cytometer. T cells gated for viability (FSC and SSC) and positive for CD3ε and TCR-Vα19, were assessed for surface expression of CD107a.

Statistical analyses

Differences among TCRs in various assays were tested with Student's *t*-tests (unpaired; two-tailed) using Graphpad Prism 4 software. Differences with p-values <0.05 were considered significant.

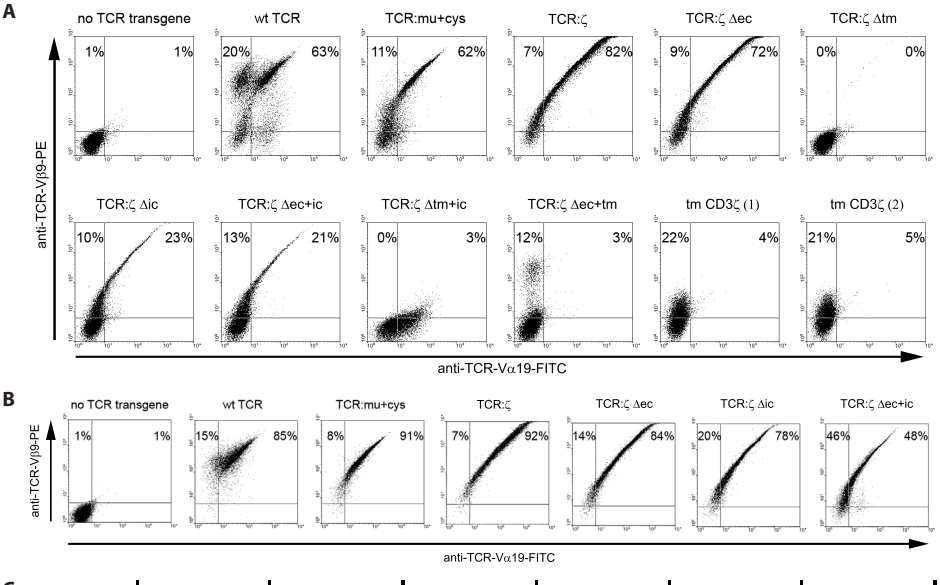
RESULTS

CD3ζ transmembrane domain is required for surface expression of TCR:ζ

Jurkat T cells containing MelA/A2 TCR were used as recipient cells for a panel of 6 M1/A1 minimal TCR:ζ's, each incorporating a different but minimal of ec, tm and/or ic CD3ζ domains (see figure 3.1 for details). These dual-TCR Jurkat T cells were assessed for surface expression of transgenic TCR by flow-cytometry. Minimal TCR:ζ Δec, Δic, Δec+ic, all containing the CD3ζ tm domain, revealed a surface expression pattern like that of parental TCR:ζ with a typical diagonal, high mean fluorescence intensity (MFI) and absence of single TCR-chain positive cells (see fig. 3.2A; (63)). TCR:ζ Δic and Δec+ic revealed lower levels of surface expression than TCR:ζ Δec or TCR:ζ. In contrast, TCR:ζ Δtm, Δtm+ic and Δec+tm transduced Jurkat T cells showed no surface expression of either or both TCRα:ζ and TCRβ:ζ chains (fig. 3.2A). Further analysis of these latter TCRs revealed that mRNA, but not intracellular protein were consistently present, suggesting that not gene transcription but more likely protein translation and/or transportation to the cell surface were hampered (*supplementary fig. 3.1*). Notably, the TCR:mu+cys, a murinized and cysteine-modified TCR used as a control TCR, shows an extended diagonal surface expression pattern and no or less single TCR-chain positive T cells than wt TCR, similar to TCR:ζ or its CD3ζ tm-containing variants (fig. 3.2A) and suggestive for high preferential TCR pairing.

CD3ζ transmembrane domain critically determines the inability of TCR:ζ to associate with endogenous CD3 and TCR chains

The minimal TCR:ζ variants were assessed for their inability to associate with endogenous CD3, considered a unique characteristic of TCR:ζ, using conventional flow cytometry and flow cytometric Fluorescence Resonance Energy Transfer (FRET). Flow cytometry analyses after double-staining for TCRβ and CD3ε revealed diagonal dotplots in Jurkat T cells expressing wt TCR and TCR:mu+cys, but not TCR:ζ or any of its minimal TCR variants (fig. 3.3A). These data suggest on the one hand a CD3-independence of minimal TCR:ζ variants that contain CD3ζ tm, similar to the reported CD3-independence of TCR:ζ, and confirm on the other hand a CD3-dependence of wt TCR and TCR:mu+cys (see chapter 2) . The lack of competition for CD3-proteins by TCR:ζ Δec, Δic, Δec+ic was also reflected in the expression of endogenous TCR. TCR:ζ or these variants did not alter the MFI of endogenous TCRβ, whereas wt TCR or TCR:mu+cys approximately halved the MFI of endogenous TCRβ (data not shown). Subsequent studies focused only on the three minimal TCR:ζ variants that were expressed on the cell surface. Flow cytometric FRET confirmed lack of association between TCR:ζ or its minimal variants, but not wt TCR or TCR:mu+cys, and CD3ε (fig. 3.3B). Flow cytometric FRET was also applied to address the



C

	wt TCR	TCR:mu+cys	TCR:ζ	TCR:ζ Δec	TCR:ζ Δic	TCR:ζ Δec+ic
MFI TCR-Vα19	64 ± 10	66 ± 11	242 ± 46 P< 0.005	163 ± 37 P< 0.05	133 ± 38	76 ± 26
MFI TCR-Vβ9	633 ± 124	540 ± 108	1646 ± 275 P< 0.005	1145 ± 248	998 ± 265	578 ± 194
% TCRαβ heterodimer	89 ± 2.1	92 ± 1.1	88 ± 2.2	79 ± 2.5 P< 0.05	74 ± 3 P< 0.005	48 ± 4.1 P< 0.0001

Figure 3.2. Minimal TCR:ζ Δec, Δic and Δec+ic express at T cell surface. Jurkat T cells expressing MelA/A2 TCR (Jurkat cl. 19) were transduced with one of the following M1/A1 TCRs: minimal TCR:ζ Δec, Δtm, Δic, Δec+ic, Δtm+ic, Δec+tm, TCR tmζ 1, tmζ 2, TCR:ζ, wt TCR, TCR:mu+cys, or no TCR transgene. (**A**) Surface expression levels of transgenic MA1/A1 TCRs were measured via flow cytometry using anti-TCR-Vα19^{FITC} and anti-TCR-Vβ9^{PE} mAbs. Representative dotplots out of 5 individual measurements are displayed. See *supplementary figure 3.1* for extended analysis of intracellular protein and mRNA expression of those minimal TCR:ζ variants that did not show surface expression of both TCR-Vα19 and TCR-Vβ9, i.e., minimal TCR:ζ Δtm, Δec+tm, Δtm+ic, TCR:tmζ 1 and tmζ 2. (**B**) TCR surface expression of Jurkat T cell lines transduced with minimal TCR:ζ Δec, Δic and Δec+ic or controls wt TCR, TCR:ζ and TCR:mu+cys after FACSort with anti-TCR-Vα19^{FITC} and anti-TCR-Vβ9^{PE} mAbs. Representative dotplots out of 5 individual measurements are displayed and percentages of stained T cells in upper left and upper right quadrants are indicated. (**C**) Mean Fluorescence Intensities or percentages (both + SEM) of TCRαβ in upper right quadrants in (**B**), n=6-11 independent measurements (statistically significant differences in comparison to wt TCR are calculated with Student's *t*-tests; p-values indicated in figure).

extent TCRs were prone to TCR mis-pairing. Using antibodies specific for endogenous TCR-Vβ27 (PE-fluorochrome, donor) and the TCR-Vα19-transgene (Cy5-fluorochrome, acceptor), we observed no FRET signals above background for TCR:ζ Δec, Δic, or Δec+ic variants, similar to parental TCR:ζ, indicating that these variants preserve TCR:ζ's ability to successfully address TCR mis-pairing (fig. 3.3C). Notably, when using a sensitive methodology such as FRET, TCR:mu+cys TCRα mis-paired with endogenous TCRβ to the

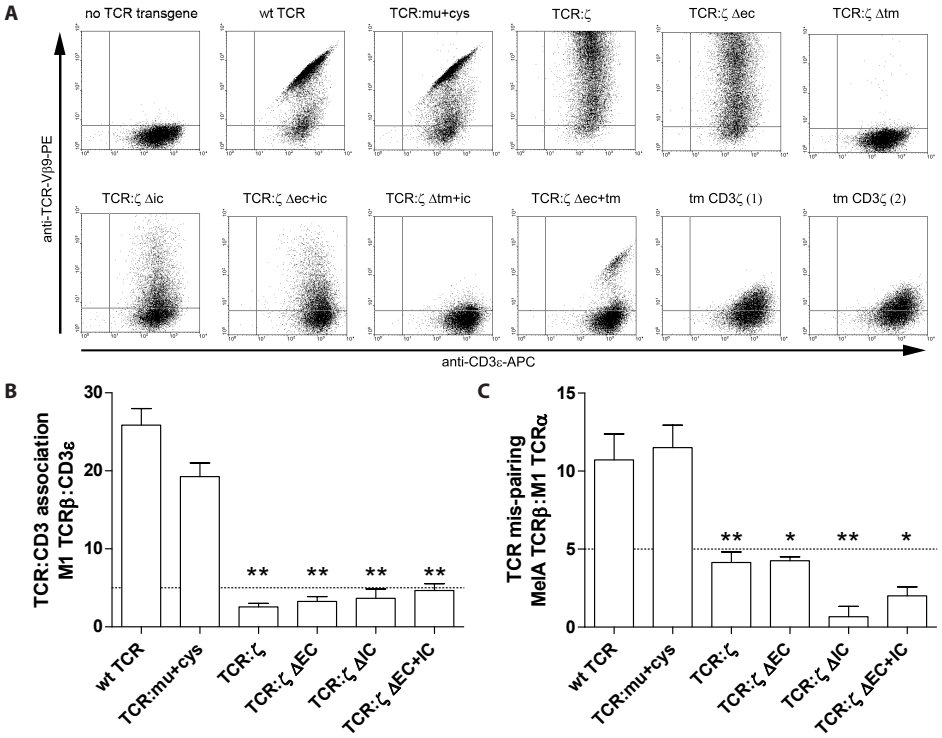


Figure 3.3. Minimal TCR:ζ Δec, Δic and Δec+ic show neither CD3 association nor TCR mis-pairing. (A) Jurkat T cells expressing minimal TCR:ζ Δec, Δic and Δec+ic, TCR:ζ, wt TCR, TCR:mu+cys transgenes were tested for surface expression of transgenic TCR and CD3ε via flow cytometry using anti-TCR-Vβ1^{PE} and anti-CD3ε^{APC} antibodies. Representative examples out of 5 individual measurements are displayed. **(B)** CD3ε association and **(C)** TCR mis-pairing were determined of minimal TCR:ζ Δec, Δic and Δec+ic and control TCRs using Fluorescence Resonance Energy Transfer (FRET) between anti-Vβ9^{PE}->anti-CD3ε-RαM^{Cy5} and anti-Vβ27^{PE}->anti-Vα19-RαM^{Cy5} mAbs, respectively. Please note that FRET was measured using TCR-transduced but non-sorted Jurkat T cells after gating on M1/A1-specific TCR expressing cells. Dotted line represents the level of background signal (5%) and bars represent mean FRET values + SEM, n=4 independent measurements (statistically significant differences in comparison to wt TCR are calculated with Student's *t*-tests: * = $p<0.05$; ** = $p<0.005$).

same extent as wt TCRα. Additional proof for the absence or presence of TCR mis-pairing came from single TCR chain transductions of Jurkat T cells. *Supplementary figure 3.2* demonstrates significant cell surface expression of a TCRαβ heterodimer (indicative of TCR mis-pairing) upon transduction with single chains (either TCRα or -β) of wt TCR or TCR:mu+cys, but not minimal TCR:ζ variants or TCR:ζ.

Next, we attempted to identify a limited number of CD3ζ tm amino acids important for TCR:ζ's surface expression and inability to mis-pair. A 3D-modeling strategy was applied to define a set of CD3ζ transmembranal amino acids that was subsequently transplanted onto favorable positions in the transmembrane domain of TCR chains. This resulted in TCR:ζ variants TCR:tmζ1 and -2 which were retrovirally introduced in Jurkat T

cells. Flow cytometry revealed no cell surface expression of minimal TCR:tmζ1 or -2 heterodimers on these dual-TCR Jurkat T cells (fig. 3.2A). TCRα:tmζ1 and -2 chains were not able to complex with TCRβ:tmζ or endogenous TCRβ. Also, TCRβ:tmζ showed moderate levels of surface expression through mis-pairing with the endogenous TCRα. Analysis of mRNA and intracellular protein suggested difficulties in protein transportation to the cell surface of particularly both TCRα-chains (*supplementary fig. 3.1*). Thus, we were unable to attribute strong CD3-independent cell surface expression and prevention of TCR mis-pairing to a subset of CD3ζ transmembranal amino acids.

Minimal TCR:ζ Δec performs best at preserving the ability of TCR:ζ to bind pMHC and activate NFAT

To study TCR-mediated functions, we have FACSorted Jurkat TCR-transductants with TCRα and -β mAbs to enhance and equalize surface expression levels of the different TCR formats (fig. 3.2B). Enrichment resulted in similar surface expression levels of TCRs (range between 74 - 92%), except for TCR:ζ Δec+ic which fell behind (48%) (fig. 3.2C). MFIs were within the same range for TCR:ζ Δec, Δic and parental TCR:ζ (TCR:ζ Δec: 163 and 1145; TCR:ζ Δic: 133 and 998; and TCR:ζ: 242 and 1646 for TCRα and -β chains, respectively), and were lowered for TCR:ζ Δec+ic (76 and 578) (fig. 3.2C). In fact, MFIs of TCR:ζ Δec+ic were within the same range as those for wt TCR and TCR:mu+cys (wt TCR: 64 and 633; TCR:mu+cys: 66 and 540). Please note that levels of surface expression of TCR:ζ and its variants do not take into account TCR stainings in upper left quadrants of flow cytometry dot plots. Since these stainings extend the diagonal of flow cytometry dotplots and are *not* due to TCR mis-pairing (fig. 3.3C), levels of surface expression, as put in figure 3.2C, may underestimate total surface expression levels of TCR:ζ and its variants.

Subsequently, transgenic TCRs were standardized for average cell surface expression levels (with % surface expression of wt TCR, see figure 3.2C, set to 1.0) and compared for their ability to recognize and bind pMHC. The TCR:ζ Δec transgene revealed a slightly lower percentage of pMHC binding compared to the TCR:ζ transgene, detected at all measured concentrations, although differences reached no statistical significance (fig. 3.4A). Wild-type TCR, TCR:mu+cys, and TCR:ζ transduced Jurkat T cells showed the highest percentages of pMHC positive populations (fig. 3.4A). Again, when analyzing the MFIs of pMHC binding, we noted that Jurkat T cells expressing TCR:ζ Δec or parental TCR:ζ were the two T cell lines with the highest TCR expression levels (figs. 3.4B and 3.2C). Although TCR:ζ Δec shows a pMHC binding approximately half of that of parental TCR:ζ, both show a significantly increased MFI compared to wt TCR (TCR:ζ vs wt TCR: $p<0.05$ at all concentrations; and TCR:ζ Δec vs wt TCR: $p<0.05$ at 4 out of 6 concentrations). TCR:ζ Δic and Δec+ic variants showed a negligible pMHC binding, whereas TCR:mu+cys showed a pMHC binding that was slightly lower than wt TCR.

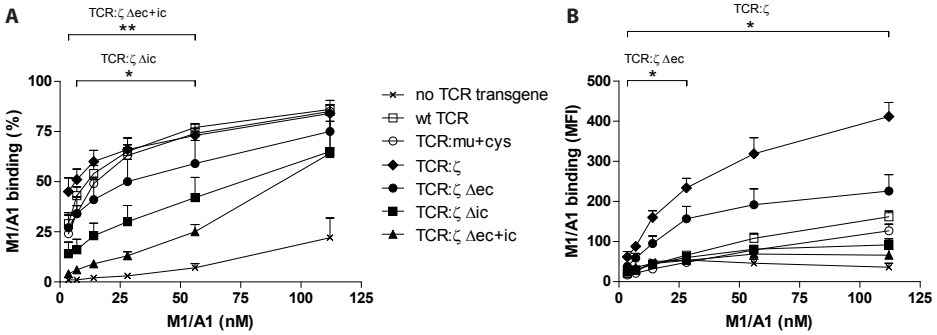


Figure 3.4. T cells transduced with minimal TCR:ζ Δec, Δic or Δec+ic show decreased ability to bind pMHC, which is least compromised for TCR:ζ Δec. Jurkat T cells expressing minimal TCR:ζ Δec, Δic and Δec+ic, TCR:ζ, wt TCR, TCR:mu+cys transgenes or no TCR transgene were tested for their ability to bind M1/A1-tetramer-PE complexes. The amount of M1/A1-tetramer used to stain 0.5 x 10^6 cells (30 min, RT) was titrated from 112 nM down to 3.5 nM. Percentages **(A)** and MFIs **(B)** of pMHC binding by T cells were measured via flow cytometry. Percentages of pMHC binding were standardized for average cell surface expression levels (with % surface expression of wt TCR, see figure 3.2C, set to 1.0). Curves represent mean percentages of MFI or pMHC binding + SEM, n=4 independent measurements (statistically significant differences in comparison to wt TCR are calculated with Student's *t*-tests: * = $p<0.05$; ** = $p<0.005$).

Next, we measured antigen-specific T cell activation by employing a Gaussia Luciferase reporter assay based on six response elements of Nuclear Factor of Activated T cells (NFAT), a transcription factor that is a key step in T cell activation. With transgenic TCRs being standardized for average cell surface expression levels (fig. 3.2C), we observed that TCR:ζ Δec and parental TCR:ζ proved to be most potent in activating Jurkat T cells by inducing the highest level of luminescence, which was significantly higher when compared to wt TCR (fig. 3.5). TCR:ζ Δic and Δec+ic variants, however, did not mediate T cell activation, even though these TCRs were able to bind pMHC. To further investigate the potency of TCR:ζ Δic and Δec+ic to induce intracellular T cell signaling, we stimulated Jurkat T cells with anti-TCR-Vβ9 mAbs and demonstrated that these two TCRs, both lacking the intracellular CD3ζ immunoreceptor tyrosine-based activation motifs (ITAMs), were not able to mediate T cell activation (*supplementary fig. 3.3*). Stimulations of the endogenous TCRβ with anti-TCR-Vβ27 mAb did not show reduced NFAT activation upon transduction with TCR:ζ Δec, Δic or Δec+ic, in line with parental TCR:ζ ((63) and data not shown). In contrast, stimulation with anti-TCR-Vβ27 mAb did show significantly reduced NFAT activation upon transduction with wt TCR or TCR:mu+cys, a result of down-regulated surface expression of CD3-dependent TCRs in dual TCR T cells.

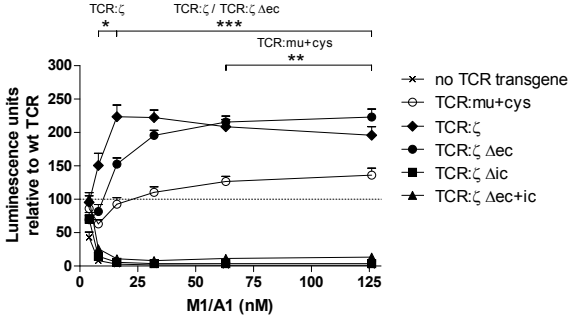


Figure 3.5. T cells transduced with minimal TCR:ζ Δec show a potent antigen-specific NFAT response. Jurkat T cells expressing minimal TCR:ζ Δec, Δic and Δec+ic, TCR:ζ, wt TCR, TCR:mu+cys transgenes or no TCR transgene were tested for their ability to mediate activation of Nuclear Factor of Activated T cells (NFAT). TCR-transduced Jurkat T cells were nucleofected with a Gaussia Luciferase reporter construct under control of 6 NFAT response elements, and stimulated for 6h with pMHC monomers. The concentration of monomers was titrated from 126 nM down to 4 nM. Luciferase activities of wt TCR T cells for 4, 8, 16, 32, 63, and 126 nM pMHC were: 588926, 670147, 656821, 444174, 181790, and 37084 relative luminescence units (RLU), respectively, and were all set at 100% (dotted line). Luciferase activities of TCR-transduced T cells were standardized for average cell surface expression levels (as explained in legend to figure 3.4A). Curves represent mean luminescence units + SEM, n=6-14 independent measurements (statistically significant differences in comparison to wt TCR are calculated with Student's *t*-tests: * = $p<0.05$, *** = $p<0.0005$).

Minimal TCR:ζ Δec shows a compromised surface expression and function in primary human T cells

The next step was to test the minimal TCR:ζ variant that performed best in Jurkat T cells with respect to surface expression, absence of TCR mis-pairing, pMHC binding and activation of NFAT, i.e., TCR:ζ Δec, in primary human T cells. Anti-CD3 mAb-activated human PBMC were retrovirally transduced with TCR:ζ Δec, TCR:ζ, wt TCR and TCR:mu+cys. To enhance the functional expression of TCR:ζ Δec, we codon optimized CD3ζ Δec and aligned the two chains in a TCRβ-2A-TCRα configuration in a pMP71 retroviral vector (93, 95, 96, 151). Surface expression of TCR transgenes and pMHC binding was assessed by flow-cytometry in cultures of TCR-engineered T cells (> 90 % CD8-positive T cells). Please note that, for reasons explained above, percentages of surface expression displayed in the upper right quadrants of flow cytometry dotplots may provide an underestimation of the actual levels of surface expression of TCR:ζ and TCR:ζ Δec (with and without codon optimization) (fig. 3.6A). TCR:ζ Δec revealed a weak surface expression and pMHC binding, which was, unexpectedly, not improved when using TCR:opt.ζ Δec in a β-2A-α configuration in pMP71 (figs 3.6A and B). Notably, the surface expression levels of TCR:ζ and TCR:mu+cys were higher when compared to wt TCR (fig. 3.6A). However, when looking at the pMHC binding, only TCR:ζ, but not TCR:mu+cys, demonstrated enhanced performance when compared to wt TCR (fig. 3.6B).

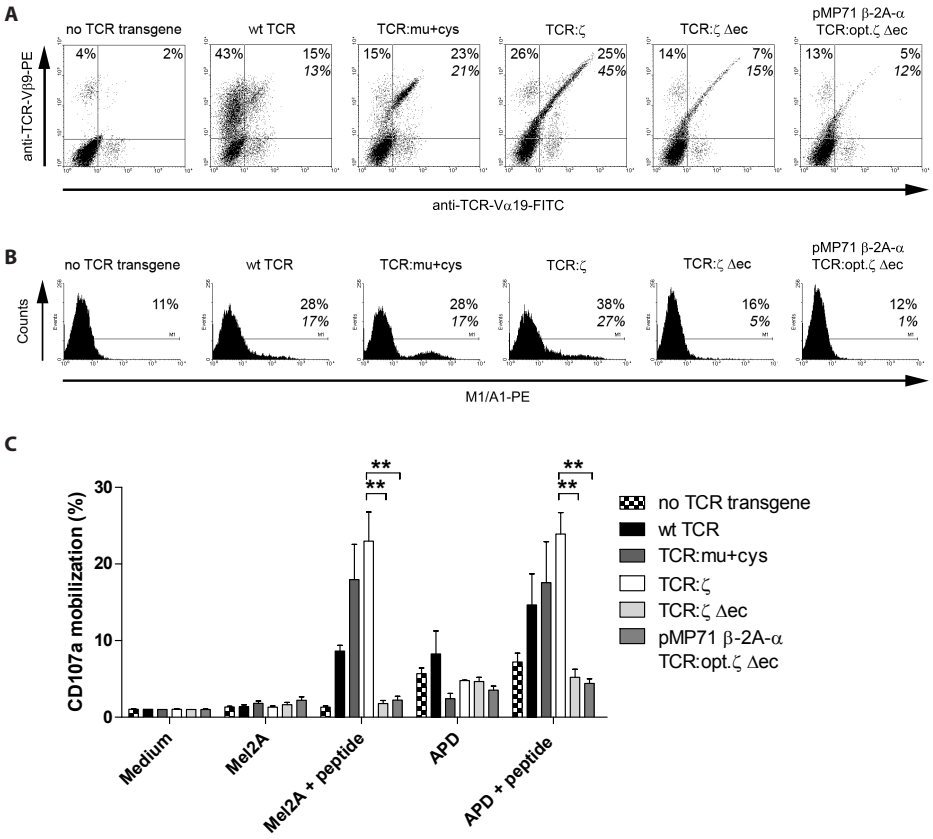


Figure 3.6. Minimal TCR:ζ Δec demonstrates compromised surface expression and antigen-specific function in primary human T cells. Primary human T cells were transduced with one of the following M1/A1 TCRs: TCR:ζ Δec, pMP71 β-2A-α TCR:opt.ζ Δec, TCR:ζ, wt TCR, TCR:mu+cys, or no TCR. **(A)** Surface expression of transgenic TCR was measured via flow cytometry. Anti-TCR-Vα19^{FITC} and anti-TCR-Vβ9^{PE} antibodies were used to stain TCR-transduced T cells. Representative examples out of 5 individual measurements of two healthy donors are displayed and percentages of stained T cells in upper left and upper right quadrants are indicated. Percentages indicated in italics are either percentages of upper right quadrants (wt TCR, TCR:mu+cys) or the sum of percentages of upper left and upper right quadrants (TCR:ζ, TCR:ζ Δec, TCR:opt.ζ Δec), corrected for the corresponding percentage(s) of Mock T cells. These percentages (in italics) may better represent the actual levels of surface expression of TCR:ζ and its variants (see Result section for details). **(B)** pMHC binding by transgenic TCR was measured via flow cytometry. M1/A1-tetramer-PE complexes (15 nM) were used to stain TCR-transduced T cells, and percentages of positive cells in histograms are indicated. Representative examples out of 5 individual measurements of two healthy donors are displayed and percentages of stained T cells in selected histogram region are indicated (in italics: corrected for pMHC binding observed by Mock T cells). **(C)** CD107a-mobilization to cell surface of TCR-transduced T cells. TCR-transduced T cells were stimulation for 2h with medium, Mel2A or APD (both M1 negative, HLA-A1 positive), or Mel2A and APD loaded with 10^{-5} M M1 peptide, after which T cells were analyzed for CD107a expression by flow cytometry (see materials and methods section for details). Bars represent mean CD107a values + SEM, 2 independent measurements of two healthy donors (statistically significant differences are calculated with Student's *t*-tests: ** = $p<0.005$). Please note that at the time of flow cytometry analyses, TCR-engineered primary human T cells contained > 90 % CD8-positive T cells.

Finally, primary human T cells expressing TCR transgenes were stimulated with antigen-positive and negative target cells, after which CD107a mobilization on the cell surface was measured within the TCR-Vα19 positive T cell population as a measure for cytotoxicity. TCR:ζ Δec, whether or not with codon optimized CD3ζ and in an optimal vector cassette, mediated negligible antigen-specific responses in contrast to TCR:ζ ($p<0.005$; fig. 3.6C). Both TCR:ζ and TCR:mu+cys show a higher percentage of CD107a positive cells than T cells with wt TCR transgenes.

DISCUSSION

In this study, we generated novel MAGE-A1-specific TCRs fused to partial rather than complete human CD3ζ in an effort to identify a minimal TCR:ζ that preserved the TCR pairing and functional properties of TCR:ζ. To this end, a panel of 8 minimal TCR:ζ's (see figure 3.1 for details) were tested for surface expression, association with endogenous CD3 and TCR chains, pMHC binding and TCR transgene-mediated functions in Jurkat T cells and primary human T cells. Our observations revealed that for TCR:ζ and its variants: (I) intact CD3ζ transmembrane (tm) domain critically determines surface expression and inability to associate with endogenous CD3 and TCR chains in Jurkat T cells; (II) both CD3ζ extracellular (ec) and intracellular (ic) domains are dispensable for pMHC binding, whereas only CD3ζ ec domain is dispensable for T cell mediated signaling in Jurkat T cells; and (III) the combination of CD3ζ ec, tm and ic domains is required for surface expression and T cell function in primary human T cells.

In a first series of experiments, we have analyzed 6 minimal TCR:ζ variants, in which the ec, tm and/or ic domain(s) of CD3ζ were omitted (and replaced by corresponding TCR domain(s)). Cell surface expression analysis revealed that not all minimal TCR:ζ variants resulted in detection of a M1/A1 TCR heterodimer. In case the CD3ζ tm domain was absent (i.e., TCR:ζ Δtm, Δtm+ic, Δec+tm), TCRα:ζ and -β:ζ transgenes were not properly expressed as a TCR heterodimer (fig. 3.2A). In fact, TCR chains of these minimal TCR:ζ variants showed aberrant protein expression or transport to the cell surface (*supplementary figs. 3.1B* and *C*). TCR:ζ and its variants that were expressed (i.e., TCR:ζ Δec, Δic, and Δec+ic,), show similarly strong levels of cell surface expression (TCR:ζ Δec+ic to a lesser extent; fig. 3.2) and an inability to associate with CD3ε (figs. 3.3A and B). Functional assays, however, identified two groups of TCRs: (I) TCR:ζ and TCR:ζ Δec; and (II) TCR:ζ Δic and TCR:ζ Δec+ic. The latter group of TCRs, in contrast to the first group, revealed lowered to negligible binding of pMHC (figs. 3.4A and B) and an inability to induce NFAT activity upon stimulation with either pMHC (fig. 3.5) or anti-TCR-Vβ9 mAb (*supplementary fig. 3.3*). Our finding that TCR:ζ variants that lack the CD3ζ ic domain showed compromised pMHC binding is unexpected. Studies with other antigen-spe-

cific receptors argue that lack of, or not having access to, ITAM-bearing CD3ζ ic domain results in enhanced surface expression and pMHC binding. In example, inhibition of protein tyrosine kinases demonstrated that early TCR-mediated signaling normally provides a negative feedback loop that facilitates pMHC-induced TCR down-regulation and T cell apoptosis (184, 185). Moreover, studies with Chimeric Antibody Receptors coupled to CD3ζ (i.e., CAR:ζ) showed that CD3ζ ITAMs directly contribute to loss of transgene expression and enhanced sensitivity to apoptosis (186). In contrast, TCR:ζ is different from other receptors since it does not dimerize with endogenous TCR/CD3 complexes (63) and may signal differently (187). Moreover, recent studies into immune synapses suggested that TCR:ζ has a distinct conformation (177), and we propose that conformational changes due to extensive deletions, such as Δic and Δec+ic, cause the observed decrease in pMHC binding. The observation that TCR:ζ Δic and Δec+ic cannot mediate activation of NFAT, even upon stimulation with anti-TCR mAb, points out that TCR-mediated signaling either requires a modified TCR that contains ITAMs (i.e., TCR:ζ or minimal TCR:ζ Δec) or a (modified or wt) TCR that is able to recruit endogenous and ITAM-containing CD3 molecules.

In addition to the shared inability to associate with CD3ε, minimal TCR:ζ Δec, Δic, and Δec+ic and the parental TCR:ζ, all containing an intact CD3ζ tm domain, showed no association with endogenous TCR chains (fig. 3.3C and *supplementary fig. 3.2*). Notably, flow cytometry of TCR:ζ Δtm+ic and TCR:ζ Δec+tm showed cells that were single positive for either TCR-Vα19 or -Vβ9, respectively (fig. 3.2A), indicating that CD3ζ ec or ic domains do not completely prevent these TCRs from mis-pairing. Our finding that prevention of TCR mis-pairing, and consequently enhanced pairing between TCRα:ζ and -β:ζ, is governed by the CD3ζ tm domain is consistent with a report by Rutledge and colleagues (188), in which the CD3ζ tm domain was used to induce dimerization of monomeric proteins, such as the IL-2Rα chain.

In a next series of experiments, we have transplanted a limited set of CD3ζ tm amino acids onto TCRα and TCRβ with the intent to preserve surface expression and inability to TCR mis-pair and, at the same time, retain structural and spatial requirements to associate with endogenous CD3 chains (i.e., TCR:tmζ1 and -2; see figure 3.1). This 'transplantation set' of amino acids were identified through 3D modeling and included the ones defined by Call and colleagues to be critically involved in CD3ζ homodimerization (i.e., C2, D6, L9, Y12, T17 and F20) (189). We observed that TCR:tmζ heterodimers did not express at the cell surface (fig. 3.2A). In fact, TCRα:tmζ1 and -2 chains were not able to complex with TCRβ:tmζ nor endogenous TCRβ, and TCRβ:tmζ showed moderate levels of surface expression through mis-pairing with the endogenous TCRα. These data suggest that TCR:ζ properties related to the presence of an intact CD3ζ tm domain, such as enhanced cell surface expression and inability to associate with endogenous CD3 and TCR chains, cannot be attributed to a limited number of individual CD3ζ tm amino acids.

TCR:ζ's pairing and functional properties were best preserved in minimal TCR:ζ Δec. This TCR variant retains high surface expression levels (fig. 3.2), prevents pairing with endogenous CD3 and TCR chains (fig. 3.3), binds pMHC (although binding is somewhat reduced when compared to TCR:ζ; fig. 3.4), and potently activates NFAT (fig. 3.5). Interestingly, recent confocal microscopy studies demonstrated that minimal TCR:ζ Δec is able to form immunological synapses with similar sizes as those formed by parental TCR:ζ (177). In minimal TCR:ζ Δec there is only a single artificial boundary, i.e., the one between TCR ec and CD3ζ tm sequences, potentially preventing or diminishing humoral and/or cellular immunogenicity. Collectively, our observations warranted testing of minimal TCR:ζ Δec in primary human T cells. In bulk populations of human PBMC (non-sorted for either TCR or CD8 expression), we observed that the difference in surface expression between minimal TCR:ζ Δec and parental TCR:ζ was more pronounced when compared to Jurkat T cells (fig. 3.6A). Also, minimal TCR:ζ Δec showed lower pMHC binding and induced negligible CD107a mobilization to the cell surface when compared to TCR:ζ (figs. 3.6B and C), which most likely was accounted for by the low level of surface expression. These observations, perhaps unexpectedly, were not different when using minimal TCR:ζ Δec in an optimal vector, an optimal TCR cassette and with codon optimized CD3ζ tm+ic domains. Apparently, the absence of the membrane-proximal CD3ζ ec domain in minimal TCR:ζ Δec results in a stringent decrease in functional TCR expression. Interestingly, single chain (sc) Fv (190) and scTCR ((64) and data not shown) that do contain CD3ζ ec coupled to either CD3ζ tm+ic, CD4 tm + Fc(ε)RIγ ic, or Fc(ε)RIγ tm+ic, have indeed demonstrated significant surface expression in human T cells. In minimal TCR:ζ Δec, six CD3ζ ec amino acids (GDLDPK) were replaced by either TCRα (DVKLVEKSFETDTNLNFQNLS) or TCRβ (GFTSESYQQGVLSATILYE) ec amino acids (that cover the connecting-peptide motifs CPMα and CPMβ, respectively). The CPMα and CPMβ are reported to interact with tetracysteine motifs in the membrane-proximal stalk regions of CD3δε and CD3γε dimers, and as such contribute to TCR/CD3 complex formation and T cell activation (115, 116). In addition, a defective or mutated CPMα is compromised with respect to its association with CD8, translation of antigen-specific stimulation into phosphorylation of LCK, FYN, and ZAP70, and production of IL-2 (115, 191, 192). We observed that the presence of CPMα and -β in minimal TCR:ζ Δec appears not sufficient to induce TCR/CD3 complex formation (figs. 3.3A and B), suggesting a more critical role for intact TCR tm for association with CD3 chains (114). The extent to which the presence of CPMα and -β in minimal TCR:ζ Δec improved pMHC binding in T cells is difficult to assess due to poor expression levels in primary human T cells (fig. 3.6A) and non-specificity of pMHC binding in CD8α-transduced Jurkat T cells (data not shown). Nevertheless, earlier findings proved that M1/A1-specific TCR:ζ, not containing CPMα or –β, were able to associate with and depend on CD8α for ligand binding (177, 193), and may suggest a non-dominant role of CPM with respect to TCR:CD8α association.

An alternative TCR format that is designed to address TCR mis-pairing, in addition to TCR:ζ, is TCR:mu+cys (see also chapter 2). In TCR:mu+cys two separate strategies have been combined: murinization of the TCR-C domain and introduction of cysteine amino acids at structurally favorable positions to allow formation of an additional disulfide bridge, which together result in enhanced functional expression (125, 128). Our studies with M1/A1-specific TCR:mu+cys, taken along as a control TCR in the present paper, confirmed this notion to some extent. In Jurkat T cells, TCR:mu+cys, when compared to wt TCR, showed similar levels of surface expression and pMHC binding, and somewhat enhanced levels of antigen-specific NFAT activation (figs. 3.2A, 3.4 and 3.5), whereas in primary human T cells TCR:mu+cys showed enhanced levels of surface expression and similar levels of pMHC binding and antigen-specific CD107a mobilization to the cell surface (fig. 3.6). However, we did observe that TCR:mu+cys mediates a significantly enhanced antigen-specific IFNγ response (data not shown). These findings, although not fully in accordance with previous reports and potentially unique to the TCR-V regions of the M1/A1 TCR (125, 128), generally argue that TCR:mu+cys improved functional TCR expression. Expectedly, enhanced functional TCR expression was related to enhanced preferential pairing between the two modified TCR:mu+cys chains, which was suggested by the flow cytometric absence of single TCRα or TCRβ positive cells (fig. 3.2A). Unexpectedly, however, flow cytometric FRET (fig. 3.3B) and single TCR chain transductions (*supplementary fig. 3.2*) clearly demonstrated that TCR:mu+cys mis-paired with endogenous TCR chains to the same extent as wt TCR. Also *in vivo*, murine TCRs with a cysteine modification did not fully prevent the pathology related to TCR mis-pairing, i.e., TCR transfer-induced Graft versus Host disease (65). We therefore propose that from a safety point of view, TCR:ζ but not TCR:mu+cys provides a better alternative to wt TCR.

In conclusion, our studies showed that CD3ζ domains separate various properties of TCR:ζ, i.e., the CD3ζ tm domain determines surface expression and lack of association with endogenous CD3 and TCR chains, whereas CD3ζ ic domain contributes to T cell signaling. Functional expression of TCR:ζ in primary human T cells, however, required the complete rather than minimized content of CD3ζ.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY METHODS

Cloning of minimal TCR:ζ variants

TCR:ζ variants generated by a domain exchange strategy or 3D-modeling strategy were acquired through cloning or gene synthesis. Exact amino acid constitutions of PCR, digestion, or gene synthesis products are indicated below. Primer sequences will be provided upon request.

TCR:ζ Δec: TCR chains (TCRα: nucleotide (nt) 1-702; TCRβ: nt 1-789) were fused together with CD3ζ tm+ic domains (CD3ζ: nt 91-489) (started from 1st methionine). PCR products were digested with *Nco*I-*Xho*I (TCRα:ζ) and *Xho*I- *Xho*I (TCRβ:ζ) and ligated in digested wt TCR pBullet vectors. For experiments in primary human T cells, the TCR:ζ Δec chains were put in a pMP71 vector in a TCRβ-2A-TCRα configuration. Overlap PCR fused together both TCR ec domains to codon optimized CD3ζ tm+ic domains (the latter domains from Geneart, Regensburg, Germany), after which PCR products were digested with *Not*I-*Mlu*I (TCRβ) and *Mfe*I-*Eco*RI (TCRα) and sequentially ligated in digested pMP71 vector.

TCR:ζ Δtm: TCR chains were partially constructed by hybridized oligomers (oligomers ordered via Eurogentec, Maastricht, Netherlands), digested with *Bam*HI-*Sac*I, that covered nt: CD3ζ: 76-90; TCRα: 766-825; CD3ζ: 154-220 or CD3ζ: 76-90; TCRβ: 847-912; CD3ζ: 154-220. The additional CD3ζ sequence (nt 221-489) was acquired through a *Sac*I-*Xho*I digestion of TCRα:ζ, after which both fragments were ligated in *Bam*HI-*Xho*I digested TCRα:ζ pBullet vector. To generate TCRβ:ζ Δtm, an additional *Nco*I-*Bam*HI fragment of TCRβ:ζ was required (to circumvent digestion of an internal *Xho*I site) and all fragments were ligated into a *Nco*I-*Xho*I digested pBullet vector.

TCR:ζ Δic: TCR chains were obtained via *Nco*I-*Bam*HI digests of parental TCR:ζ (covering nt TCRα: 1-702; CD3ζ: 73-75 or TCRβ: 1-789; CD3ζ: 73-75) and hybridized oligomers (Eurogentec), the latter digested with *Bam*HI-*Xho*I (CD3ζ: 76-153; TCRα: 826-840 or CD3ζ: 76-153; TCRβ: 913-933). Variants were finalized by ligation in *Nco*I-*Xho*I digested pBullet vectors.

TCR:ζ Δec+ic: TCR chains were obtained via hybridized primers (Eurogentec) (covering nt TCRα: 724-765; CD3ζ: 91-153; TCRα: 276-280 or CD3ζ: 108-153; TCRβ: 913-933), digested with *Hin*DIII-*Xho*I (TCRα) or *Bfu*A1-*Xho*I (TCRβ). For TCRβ a *Nco*I-*Bfu*A1 digestion fragment from TCRβ:ζ was acquired to circumvent an internal *Xho*I site, and fragments were ligated in digested wt TCR pBullet vectors.

TCR:ζ Δtm+ic: TCRα was generated via gene synthesis (Geneart) (nt TCRα: 1-702; CD3ζ: 73-90; TCRα: 826-840) and ligated via *Nco*I-*Xho*I in a pBullet vector. TCRβ was obtained via a *Nco*I-*Bam*HI digestion of TCRβ:ζ Δic (nt TCRβ: 1-789; CD3ζ: 73-75), a *Bam*HI-*Xho*I fragment from hybridized primers (Eurogentec) (CD3ζ: 76-90; TCRβ: 847-933), and ligation of these fragments in a pBullet vector.

TCR:ζ Δec+tm: TCRα was obtained via *Bsp*I-*Xho*I digestion of TCRα:ζ Δtm and ligation of this fragment in a pBullet vector already containing wt TCRα. TCRβ was generated by *Nco*I digestion of wt TCRβ and ligation of this fragment in a pBullet containing TCRβ:ζ Δtm.

TCR:tmζ1 and -2: YASARA (www.yasara.org (194)) was used to build a model of the interacting transmembranal amino acids in the TCR/CD3 complex. The structure of the CD3ζζ transmembrane domain has been resolved by NMR and was introduced as such in our model (PDB file 2hac (189)). The other CD3 transmembrane helices were modeled based on the large CD3ζ helix. Predicted CD3 helices were used to identify transmembrane amino acids of CD3ζ important for interaction with the other helices in the complex. These amino acids were transplanted onto TCRα and TCRβ chains, preferentially replacing amino acids defined to mediate TCRα-TCRβ but not TCRα-CD3δε or TCRβ-CD3γε associations. This 3D-modelling exercise revealed two modified TCRα and one TCRβ tm domain(s) (see figure 3.1B). For both TCRα chains the tm domain with small flanking regions was generated through gene synthesis (Geneart), digested with *Hin*DIII and *Xho*I, and ligated in pBullet vector with wt TCR. CD3ζ amino acids substitute TCRα amino acids at positions 256, 260, 266, 270, 271, and 274 for variant tmζ1, and positions 255, 256, 259, 266, 269, 270, 273, and 274 for variant tmζ2. The TCRβ chain (combined with either TCRα chain) was generated through gene synthesis (Geneart) and ligated in a pBullet vector (64) using *Sal*I and *Not*I. CD3ζ amino acids substitute TCRβ amino acids at positions 282, 256, 289, 292, 296, 297, and 300.

Intracellular flow cytometry

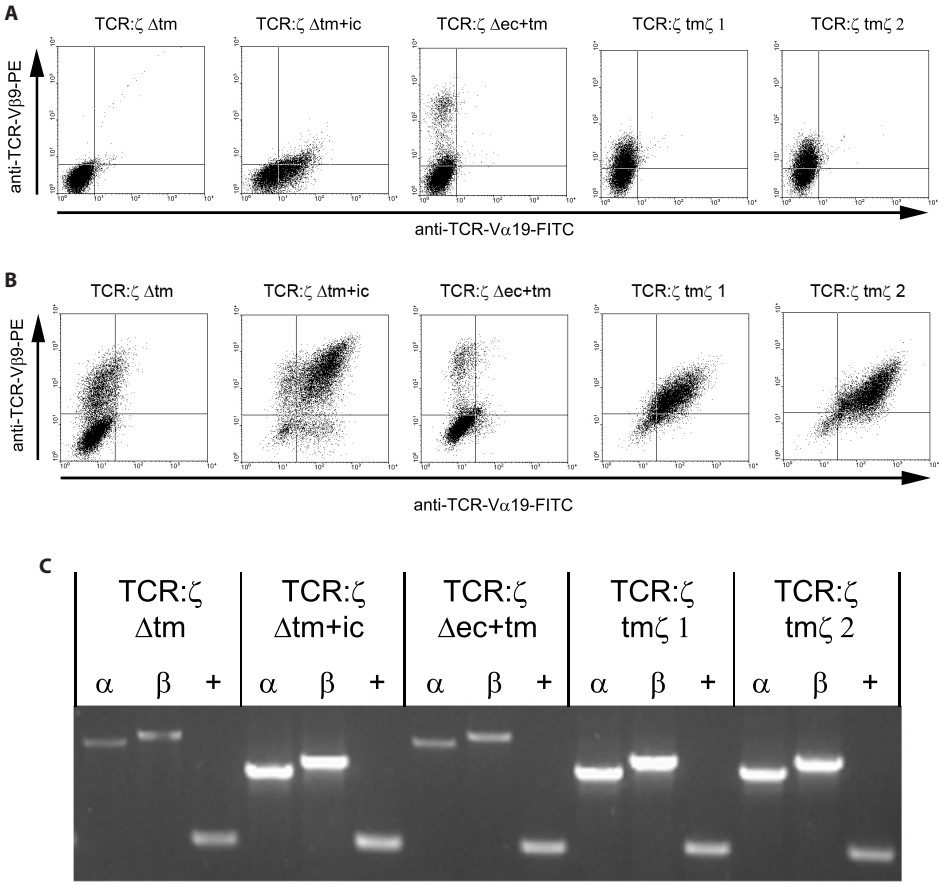
TCR-transduced T cells ($1x10^4$ cells) were monitored by flow cytometry for intracellular expression of transgenic TCR using FITC-conjugated anti-TCR-Vα19 and PE-conjugated anti-TCR-Vβ9 mAbs. After T cells were washed, they were permeabilized with the permeabilization kit (BD Biosciences) for 10 minutes at room temperature (RT) and washed again. T cells were stained with TCR-Vα19 and TCR-Vβ9 mAbs for 20 minutes on ice. Next, T cells were washed and measured on a FACSCalibur dual-laser flow cytometer.

mRNA isolation and RT-PCR

mRNA was isolated by means of TRIzol (Invitrogen). $1x10^6$ cells were lyzed in 1 ml TRIzol and incubated for 5 minutes at RT. The lysate was mixed with 0,2 ml Chloroform (Sigma-Aldrich), incubated for 2 minutes at RT, and subsequently centrifuged for 15 minutes at 12,000g and 4°C. The aqueous phase was acquired, supplemented with 0,5 ml isopropanol (Sigma-Aldrich) and 1 µl glycogen (Roche, Woerden, the Netherlands), and centrifuged for 10 minutes at 12,000g and 4°C. The pellet was resuspended in 1 ml ethanol (Merck, New Jersey, USA), mixed, and centrifuged for 5 minutes at 7,500g and 4°C. The pellet was

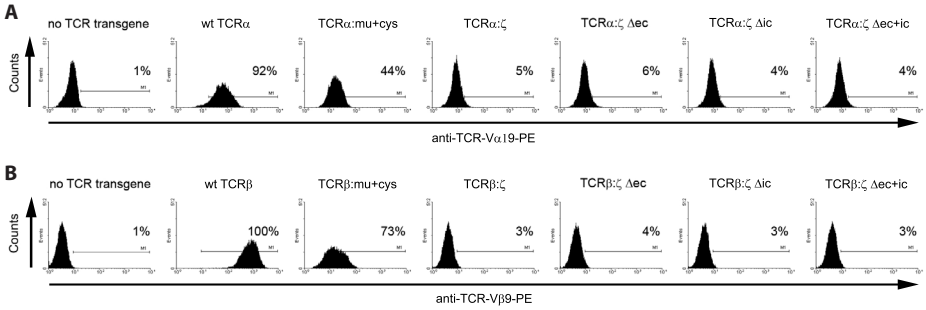
reconstituted in RNase-free water. For cDNA synthesis 5 µg mRNA was mixed with 0.5 ng oligo-dT primers (Invitrogen), 0.05 ng random hexamer primers (Promega) and water to 12 µl. The mixture was heated to 70°C for 10 minutes and quickly cooled on ice. 1st strand buffer (Invitrogen), 0.5 mM dNTPs (Promega), 0.005 M DTT (Invitrogen), and RNasin (Promega) was added to the mixture before incubation at 42°C for 2 minutes. Superscript III RTase (Invitrogen) was added to the mixture and placed at 42°C for 50 minutes, followed by 10 minutes at 70°C. The subsequent PCR was performed with transgene and GAPDH specific primers, sequences of which will be provided upon request.

SUPLEMENTARY FIGURES

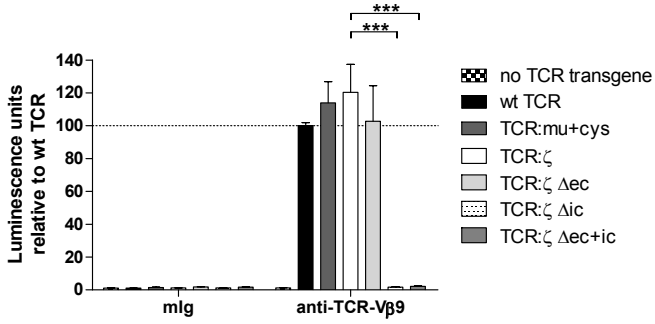


Supplementary figure 3.1. Minimal TCR:ζ variants without a complete CD3ζ transmembrane domain show aberrant protein production or transportation to cell surface. Jurkat T cells expressing MelA/A2 TCR were transduced with one of the following M1/A1 TCRs (those that were not surface-expressed, see figure 3.2A): minimal TCR:ζ Δtm, Δtm+ic, Δec+tm, TCR tmζ 1 or tmζ 2. Surface **(A)** and intracellular **(B)** expressions of transgenic TCR were measured via flow cytometry using anti-TCR-Vα19^{FITC} and anti-TCR-Vβ9^{PE}

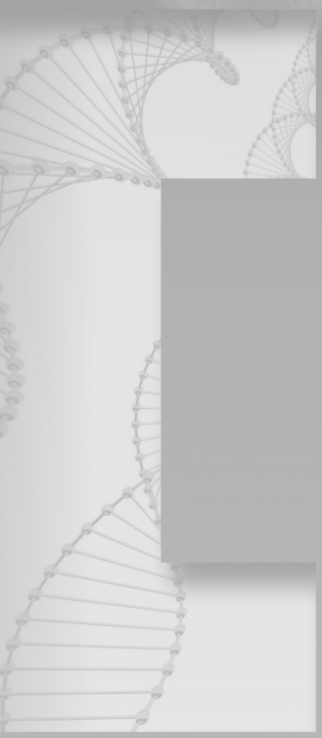
mAbs. Representative dotplots out of 5 individual measurements are displayed. T cells were permeabilized with the permeabilization 2 kit before mAb staining for intracellular transgenic TCR expression measurements. **(C)** mRNA expression of TCR transgenes (TCRα = α; TCRβ = β) and GAPDH (+; positive control). mRNA was isolated by means of TRIzol after which cDNA synthesis was performed with superscript III reverse transcriptase. PCR was performed with TCR transgene and GAPDH specific primers, and products were loaded on an agarose gel. TCRα and TCRβ transgenes vary in size between 795 nt – 1164 nt and 891 nt – 1248 nt, respectively, due to altering TCR and CD3 domain compositions. GAPDH primers amplify a sequence of 319 nt. Data of one out of two independent experiments with similar results are shown. See *supplementary methods* for details.



Supplementary figure 3.2. Single TCRα or TCRβ chains of wt TCR and TCR:mu+cys, but not TCR:ζ Δec, Δic, Δec+ic or parental TCR:ζ, express at the surface of T cells. Jurkat T cells expressing MelA/A2 TCR were transduced with either the TCRα-chain **(A)** or the TCRβ-chain **(B)** of minimal TCR:ζ Δec, Δic, Δec+ic, parental TCR:ζ, wt TCR , TCR:mu+cys or no TCR transgene. Surface expression of transgenic TCR was detected via flow cytometry using anti-TCR-Vα19^{FITC} or anti-TCR-Vβ9^{PE} mAbs and presented as histograms. Percentages of TCRα or TCRβ expression are indicated. Representative examples out of 2 individual measurements are displayed.



Supplementary figure 3.3. TCR:ζ Δic and Δec+ic show no NFAT activation upon stimulation with anti-TCR mAb. Jurkat T cells expressing minimal TCR:ζ Δec, Δic, Δec+ic, parental TCR:ζ, wt TCR, TCR:mu+cys or no TCR transgene were tested for their ability to mediate activation of Nuclear Factor of Activated T cells (NFAT). TCR-transduced Jurkat T cells were nucleofected with a Gaussia Luciferase reporter construct under control of 6 NFAT response elements, and stimulated for 6 hours by anti-TCR-Vβ9 mAb or control mIg. Luciferase activities of wt TCR for TCR-Vβ9 mAb stimulation was 1069540 relative luminescence units, which was set to 100% (dotted line). Bars represent mean luminescence units + SEM of 15 to 27 independent measurements. Statistical significance is based on Student's *t*-tests: *** = p<0.0005.



4

T cell receptors genetically linked to CD28 and CD3ε do not mis-pair with endogenous TCR chains and mediate enhanced and antigen-specific T cell activation

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Manuscript submitted

ABSTRACT

Adoptive transfer of T cells that are gene-engineered to express a defined T cell receptor (TCR gene therapy) represents a feasible and promising therapy for patients with tumors. TCR gene therapy, however, is challenged by a transient presence and effectiveness of transferred T cells, which is anticipated to be improved by adequate T cell co-stimulation. Here, we report the identification and characterization of novel TCR transgenes that genetically incorporate signaling cassettes which consist of the accessory molecules Fc(ε)Rlγ, CD3ε or CD3ζ with or without the co-stimulatory molecule CD28. Two-chain TCR transgenes linked to CD28 and CD3ε, i.e., TCR:28ε, demonstrated enhanced binding of peptide-MHC and mediated enhanced CD107a mobilization and cytokine production following stimulation with antigen when compared to wild type (wt) TCR. TCR:28ε does neither mis-pair with endogenous TCR nor associate with endogenous CD3. In addition, this co-stimulatory TCR does not show off-target recognition as evidenced by preserved specificity towards a panel of altered peptide ligands as well as antigen-positive and negative melanoma cells. Notably, TCR:28ε induces the formation of immune synapses with significantly enhanced accumulation of TCR transgenes and markers of early TCR signaling. Taken together, our data show that TCR transgenes that harbor a signaling cassette consisting of CD28 and CD3ε confer T cells with correctly paired TCR dimers and enhanced and antigen-specific T cell activation.

INTRODUCTION

Metastatic melanoma is a highly lethal disease with an incidence that continues to rise and for which currently no standard curative treatments are available. Adoptive transfer of tumor infiltrating lymphocytes (TILs) as well as T cell receptor (TCR)-engineered T cells has shown clinical successes in the treatment of metastatic melanoma (97, 195). In example, T cells expressing TCR transgenes that were directed against the human leukocyte antigen (HLA)-A2-restricted antigens MART-1, gp100 or NY-ESO-1 mediated objective clinical responses in patients with metastatic melanoma that ranged between 12 and 45% (32, 46). Despite these potent anti-tumor responses, clinical responses are currently challenged by both toxicities and a transient nature of tumor regression in the majority of patients.

T cell therapy-mediated toxicities became evident from studies with TCRs, in particular those of high-affinity, directed against antigens that are over-expressed on melanoma but also expressed on healthy cells, albeit a minute number. Toxicities included severe inflammation of skin, eyes, and ears (for MART-1, gp100) and neurological toxicities, with the latter resulting in comas and death in two patients (a MAGE-A3 derived-epitope that is shared with MAGE-12) (45, 62). The observed toxicities can be addressed by using TCRs directed against antigens that are highly selectively expressed by tumor but not healthy tissues, such as neo-antigens and potentially some defined and non-shared Cancer Testis Antigens (CTA). Besides the above-mentioned on-target toxicities, TCR-gene engineering may result in recognition of off-target (i.e., unknown) self-peptides as a consequence of new TCR dimers that are formed between introduced and endogenous TCR chains (i.e., TCR mis-pairing). Although there has been no formal proof of TCR mis-pairing-mediated toxicity in patient studies, preclinical studies clearly demonstrated the destructive ability of T cells that express mixed TCR dimers towards healthy cells (65, 66). These findings warrant for measures to prevent or limit TCR mis-pairing, such as genetic modification of TCR transgenes (see chapter 2) or disruption of endogenous TCR chains via zinc-finger nucleases (69).

The transient nature of tumor regression following T cell therapy became clear from observations that anti-tumor responses are most often incomplete in 80 to 90% of patients (27, 111). The compromised anti-tumor responses coincided with a limited persistence of transferred T cells (25). T cell persistence and anti-tumor activity appear sensitive to T cell co-stimulation as evident from a recent clinical study in which T cells engineered with a CD19-specific CAR that incorporated CD137 and CD3ζ were used to treat patients with B cell lymphoma. In this study, T cell persistence was significant (detectable up to 6 months) and complete clinical responses were observed in two out of three patients (77). Similarly, clinical studies using CD19-targeted T cells with CARs that incorporated CD28 and CD3ζ showed beneficial effects on long-term T cell persistence

and clinical responses (76, 78). In addition to genetic engineering, MART-1-specific T cells stimulated with artificial antigen presenting cells that expressed the CD28 ligands and used to treat patients with metastatic melanoma also revealed enhanced T cell persistence and clinical responses (85). Importantly, inclusion of T cell co-stimulation in these clinical protocols relieved the requirement for patient preconditioning with chemotherapy and/or *in vivo* IL-2 administration (77, 85).

Here, we designed and generated TCRs directed against MAGE-1/HLA-A1 and gp100/HLA-A2 that incorporated signaling cassettes to simultaneously address TCR mis-pairing and enhance T cell co-stimulation. The modular design of signaling cassettes allowed us to compare and test combinations of the accessory molecules Fc(ε)RIγ, CD3ε or CD3ζ with or without the co-stimulatory molecule CD28. Our results showed that TCRs bearing a cassette consisting of CD28 and CD3ε (i.e., TCR:28ε) resulted in maximum ability to bind peptide-MHC, no TCR mis-pairing, and no loss of antigen and peptide-fine specificity. Moreover, T cells expressing TCR:28ε mediated enhanced T cell responses, and highly active immune synapses and early T cell signaling.

MATERIALS AND METHODS

Cells and reagents

T lymphocytes derived from healthy donors were isolated and expanded using a feeder system as described elsewhere (179) and cultured in HEPES-buffered RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% human serum (Sanquin, Amsterdam, The Netherlands), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Jurkat T cells expressing a single MelanA/HLA-A2 specific TCR (clone J.19 (63)), J.19 transduced with CD8α (J.19-CD8 (177)), the B cell lines APD and BSM, and the TAP-deficient TxB hybrid T2 cells were cultured in RPMI 1640 medium (BioWhittaker) containing 10% Fetal Bovine Serum (FBS, Stonehouse, Gloucestershire, UK) and antibiotics. The packaging cell lines 293T and Phoenix-A, the C1R B cell lines expressing either wild type HLA-A2 or the D227K/T228A mutant of HLA-A2 (196), and the melanoma cell lines G43, MZ2-MEL43, GE-F-, FM3 and MEL2A were cultured in DMEM medium (BioWhittaker) containing 10% FBS, 2 mM L-glutamine, non-essential amino acids and antibiotics. Antibodies used in this study were: PE-conjugated TCR-Vβ9 (clone BL37.2 for flow cytometry and clone IM2355 for microscopy, Beckman-Coulter, Marseille, France); non and FITC-conjugated TCR-Vα19 (clone 6D6.6; Pierce Biotechnology, Inc., Rockford, IL); PE-conjugated TCR-Vβ27 (clone CAS1.1.3; Beckman-Coulter); non-conjugated CD3ε (clone OKT3; Beckman-Coulter); PerCP-conjugated CD3ε (clone SP34-2; BD Biosciences, San Jose, CA); non-conjugated CD8α (clone UCHT4; Adipogen, San Diego, CA); PE-con-

jugated CD107a (clone H4A3; BD Biosciences); AlexaFluor647-conjugated pCD3ζ (SC-9975; Santa Cruz Biotechnology, Heidelberg, Germany); pLCK (clone SC-28445-R; Santa Cruz Biotechnology); pERK (clone SC-7976; Santa Cruz Biotechnology); pZAP70 (clone SC-33526; Santa Cruz Biotechnology); Cy5-conjugated RαM (Jackson immunoresearch, West Grove, PA); and AlexaFluor647-conjugated GαM (Life technologies-Invitrogen, Paisley, UK). AlexaFluor647-conjugated CD3ε, CD8α, and CD45 antibodies were acquired and conjugated as described previously (177). MAGE-A1$_{161-169}$/HLA-A*0101 (M1/A1) and gp100$_{280-288}$/HLA-A*0201 (gp100/A2) tetramers were generated from biotinylated monomers (Sanquin, Amsterdam, Netherlands) and Streptavidin-PE (BD Biosciences) as described in chapter 6. Other reagents used in this study include: Retronectin (human fibronectin fragments CH-296, Takara Shuzo Co. Ltd., Otsu, Japan); PMA (Sigma-Aldrich, St. Louis, MO); PHA (Remel Europe, Dartford, England); golgistop (BD Biosciences); Influenza (INF) peptide (CTELKLSDY), M1 peptide (EADPTGHSY) and gp100 peptide (YLEPGPVTA) (Proimmune). Altered peptide ligands of gp100 peptide (A1 to A8, and G9) were synthesized as described previously (132).

TCR gene constructs

The M1/A1 TCR has been derived from CTL clone MZ2-82/30 (64, 135) and is composed of TRAV19/J39/C and TRBV9/D2/J2-3/C2, and the gp100/A2 TCR has been derived from CTL clone 296 and is composed of TRAV13-1/J52/C and TRBV27/J2-7/D2/C2 ((132) with TCR-V(D)J gene nomenclature according to http://imgt.org). A single-chain M1/A1 TCR platform (64) was used to incorporate various human signaling cassettes that consisted of Fc(ε)RIγ (γ), CD3ε (ε) or CD3ζ (ζ) without or with CD28 (28γ, 28ε, 28ζ). See figure 4.1A for a schematic overview of all 8 scTCR constructs and table 4.1 for the amino acid boundaries of fused molecules. The γ cassette comprised of extracellular amino acids of the immunoglobulin κ light chain (Cκ) constant domain, the transmembrane domain of CD4 and the intracellular domain of Fc(ε)RIγ (190), whereas the ε and ζ cassettes (the latter described in (64)) comprised the complete CD3ε or CD3ζ, which were amplified from human PBMC cDNA. In CD28-containing cassettes, the transmembrane and intracellular domains of CD28 preceded the intracellular domains of γ, ε or ζ. The cassettes comprising Fc(ε)RIγ or CD3ζ coupled to CD28 were derived from cassettes incorporated into Chimeric Antigen Receptors (CARs) (10) and TCRs (131), respectively. The 28ε cassette was derived via overlap PCR using template DNAs containing either molecule. In case of ε and 28ε an additional extracellular cysteine residue was introduced at the 5' end of the signaling cassette yielding cys-ε and cys-28ε. The resulting scTCRs were introduced in the retroviral vector pBullet via *Not*I or *Bam*HI cloning sites. In addition, the 28ε cassettes were cloned into both pBullet-TCRα (amino acids ending at SPESS) and TCRβ (amino acids ending at WGRAD) via *Not*I and *Bam*HI. See figure 4.2A for a schematic representation

Table 4.1. Composition and functional responses of signaling cassettes in a scTCR format[a]

Signaling cassette	Linker with cloning site[b]	Boundaries within signaling cassette[c]	TCR surface expression[d]	pMHC binding[e]	Cyto-toxicity[f]	IFNγ release[g]
γ	GGR (*Not*I)	RADAA…LLDRS (ec Cκ) QPMAL...LGIFF (tm CD4) RLKIQ...EKPPQ (ic Fc(ε)RIγ)	+	+	-	-
28γ	AAAK (*Not*I)	FWVLV…AAYRS (tm+ic CD28) RLKIQ…EKPPQ (ic Fc(ε)RIγ)	++	+	-	-
ε	GS (*Bam*HI)	DGNEE...NQRRI (ec+tm+ic CD3ε)	++	+	-	-
28ε	GSPK (*Bam*HI)	FWVLV…AAYRS (tm+ic CD28) KNRKA…NQRRI (ic CD3ε)	++	+	+++	+++
cys-ε	CGDGS (*Bam*HI)	DGNEE...NQRRI (ec+tm+ic CD3ε)	++	+	+	-
cys-28ε	CGDGSPK (*Bam*HI)	FWVLV…AAYRS (tm+ic CD28) KNRKA…NQRRI (ic CD3ε)	++	++	++	+++
ζ	CGDLDPK (*Bam*HI+ec CD3ζ)	LCYLL...ALPPR (tm+ic CD3ζ)	+	-	+	+
28ζ	CGDLDPK (*Bam*HI)	FWVLV…AAYRS (tm+ic CD28) LRVKF…ALPPR (ic CD3ζ)	++	+	+	-

[a] Signaling cassettes consisting of extracellular (ec) Cκ + transmembrane (tm) CD4 + intracellular (ic) Fc(ε) RIγ (γ); tm+ic CD28 + ic Fc(ε)RIγ (28γ); ec+tm+ic CD3ε (ε); tm+ic CD28 + ic CD3ε (28ε); ec+tm+ic CD3ε with a N-terminal cysteine (cys-ε); tm+ic CD28 + ic CD3ε with a N-terminal cysteine (cys-28ε); ec+tm+ic CD3ζ (ζ); and tm+ic CD28 + ic CD3ζ (28ζ) were fused to single-chain (sc)TCR using different cloning sites and protein domains as described in the legend to figure 4.1. Expression and antigen-specific functions of these scTCR were tested, following expression on primary human T cells, and summarised findings, that correspond to data shown in *supplementary figure 4.1*, are shown.

[b] The cloning sites are underlined in the linker sequences

[c] The first and last five amino acids of the boundaries between the various components of the signaling cassettes are provided

[d] TCR surface expression (measured by flow cytometry with M1/A1-tetramer): 0-50% = -; 51-75% = +; 76-90% = ++; >90% = +++

[e] Peptide-MHC binding: 0-10% = -; 11-25% = +; 26-50% = ++; >50% = +++

[f] Antigen-specific cytotoxicity at E:T ratio 40:1: 0-10% = -; 11-25% = +; 26-50% = ++; >50% = +++

[g] antigen-specific IFNγ release: 0-100 pg/ml = -; 101-250 pg/ml = +; 251-500 pg/ml = ++; >500 pg/ml = +++.

Abbreviations used: Cβ, TCRβ constant domain; cys, cysteine; ec, extracellular; ic, intracellular; sc, single-chain; tm, transmembrane; Vα, TCRα variable domain; Vβ, TCRβ variable domain.

of the two-chain TCR:28ε. Primer sequences used for cloning TCR and signaling cassettes can be provided upon request. All TCR constructs were sequence verified (Service XS, Leiden, Netherlands).

Retroviral gene transfer into T cells

Moloney Murine Leukemia retroviruses were produced by co-cultures of 293T and Phoenix-A cells. Cells were calcium phosphate-transfected with TCR transgenes, the

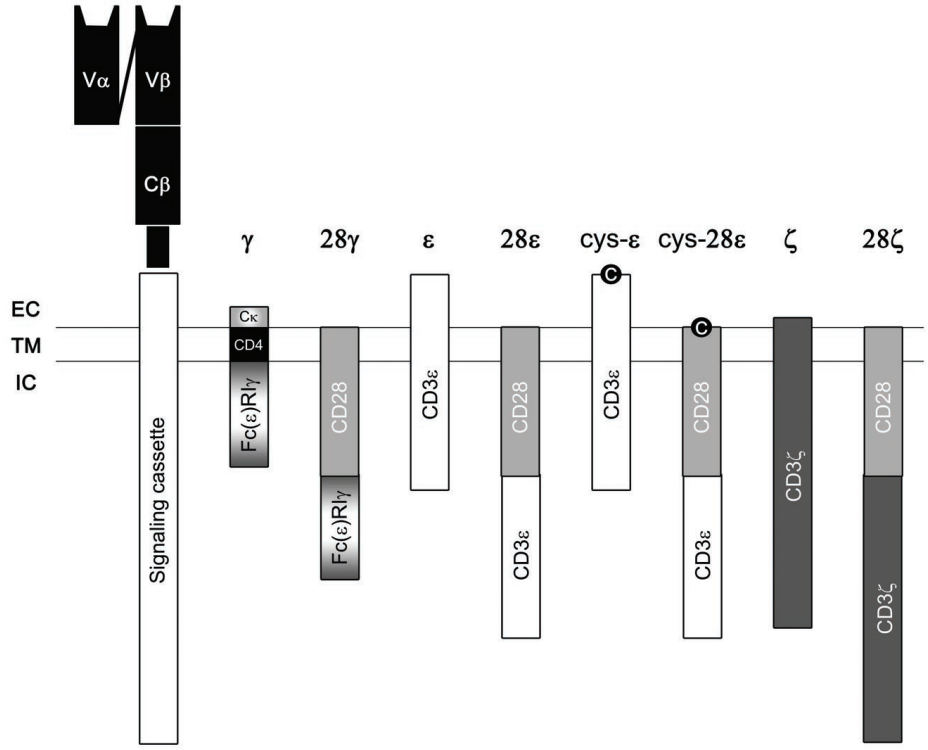

Figure 4.1. A schematic representation of signaling cassettes tested for functional expression on primary human T cells. Single-chain (sc)TCRs were generated bearing one of the following eight signaling cassettes which are schematically represented: ec Cκ + tm CD4 + ic Fc(ε)RIγ (γ); tm+ic CD28 + ic Fc(ε)RIγ (28γ); ec+tm+ic CD3ε (ε); tm+ic CD28 + ic CD3ε (28ε); ec+tm+ic CD3ε with an N-terminal cysteine (cys-ε); tm+ic CD28 + ic CD3ε with an N-terminal cysteine (cys-28ε); ec+tm+ic CD3ζ (ζ); and tm+ic CD28 + ic CD3ζ (28ζ). These protein domains correspond to the following sequences (numbers relative to 1st Methionine): ec Cκ, RADAAPTVSQSFGLLDRS (GI:52119); tm $CD4_{395-418}$ (GI:54696483); ic $Fc(\varepsilon)RI\gamma_{45-86}$ (GI:4758343); ec+tm+ic $CD3\varepsilon_{23-207}$ (GI:4502670); ic $CD3\varepsilon_{153-207}$; ec+tm+ic $CD3\zeta_{27-163}$ (GI:4557430); ic $CD3\zeta_{51-163}$; and tm+ic $CD28_{151-218}$ (GI:338444). Abbreviations used: Cβ, TCRβ constant domain; cys, cysteine; ec, extracellular; ic, intracellular; sc, single-chain; tm, transmembrane; Vα, TCRα variable domain; Vβ, TCRβ variable domain.

helper vectors pHIT60 MLV GAG/POL and either pVSV-G ENV or pCOLT-GALV ENV in case of Jurkat T cells or primary human T cells, respectively. Transduction of human T cells was optimized and described previously (71).

NFAT reporter gene assay

The NFAT reporter gene assay was performed as described in chapter 3. Briefly, Jurkat clone J.19 ($5x10^6$ cells) were nucleofected with 5 µg Firefly luciferase reporter gene encompassing 6 response elements of the Nuclear Factor of Activated T cells (NFAT-6-Luc) using Amaxa's nucleofector and program C-16 (according to the manufacturer's protocol, Amaxa Biosystems, Cologne, Germany). Next, T cells were transferred to pre-warmed medium and incubated O/N at 37°C and 5% CO_2. Non-tissue culture-treated 96-well plates were coated O/N at 4°C with anti-TCR-Vβ9, anti-TCR-Vβ27 or non-conjugated control mAb. T cells ($0.2x10^6$) were incubated in pre-coated 96-well plates for 6h at 37°C and 5% CO_2, and lysates (Cell Lysis Buffer, Promega, Madison, WI) were collected and used to measure photon emissions in a luminometer (Mediators, Vienna, Austria) using luminescent substrates according to the manufacturer's instructions. Results were expressed as relative luminescence units (RLU) relative to non-stimulated controls (medium only: set at 1.0).

IFNγ release

T cell IFNγ release was determined as described previously (131). T cells were stimulated with B cells loaded without or with peptides, or melanoma cell lines.

Cytotoxicity assay

T cell cytolytic activity was determined with a ^{51}Cr-release assay after 4h incubation with target cells (190). The percentage of specific ^{51}Cr-release was determined as follows: ((test counts – spontaneous counts) / (maximum counts – spontaneous counts)) x 100%.

Flow cytometry and FACSort

TCR-transduced T cells were washed and incubated with TCR mAbs or peptide-MHC for 30 min on ice or 15 min at RT, respectively, after which T cells were washed again and fixed with 1% PFA (Brunschwig, Amsterdam, the Netherlands). To detect CD107a expression, T cells were stimulated with target cells and analyzed as described in chapter 3. T cells were gated according to forward and sideward scatter properties using a FACSCalibur (Beckton Dickinson, Alphen a/d Rijn, the Netherlands) equipped with CellQuest software (BD Biosciences). Enrichments of T cells were performed by two-color Fluorescent Activated Cell Sorting (FACS) following staining with TCRα and TCRβ mAbs (in case of M1/A1) or peptide-MHC (in case of gp100/A2).

Flow cytometric FRET

TCR-transduced clone J.19 T cells were stained with TCR-Vβ9-PE or TCR-Vβ27-PE mAbs to provide donor fluorochromes and either with non-conjugated TCR-Vα19, CD3ε or CD8α mAbs followed by RαM-Cy5 mAb to provide acceptor fluorochromes as described previously (63). Immune stainings were performed sequentially, with extensive washing steps in between, and using a fixed order of antibodies. First, non-conjugated mAbs followed by Cy5-labeled mAbs, and second, PE-labeled mAbs. Fluorescence intensities of donor fluorochromes (excitation at 488 nm, emission at 570 nm), acceptor fluorochromes (excitation at 635, emission at 670 nm) and FRET signals (excitation 488 nm, emission at 670 nm) were measured and collected on a FACSCalibur. Data of viable T cells were analyzed with ReFLEX software on a per-cell basis (181).

Acceptor photobleaching for microscopic FRET

TCR-transduced clone J.19 T cells and primary human T cells were prepared for confocal microscopy as described previously (177). Briefly, target APD B cells were loaded with 10^{-5} M of M1 or INF peptide and subsequently labeled with 5 µM CFSE (Life technologies-Invitrogen). T cells and target cells were mixed and fixed with 4% formaldehyde. T cells were stained with TCR-Vβ9-PE mAb (donor) and either with CD3ε-AlexaFluor647, CD8α-AlexaFluor647 or CD45-AlexaFluor647 mAbs (acceptor), covered with 50:50 glycerol:PBS, and analysed with a confocal microscope (Zeiss LSM 510; Carl Zeiss AG, Jena, Germany). Data was collected using ImageJ software (NIH, Bethesda, MD). Immune synapses were defined as contact regions between conjugated T and APD B cells that show an accumulated number of TCR molecules. Confocal laser scanning images were taken of 4 µm thick optical sections. The FRET donor fluorochrome was excited with a 543-nm HeNe laser, whereas the FRET acceptor fluorochrome was excited and photobleached with a 633-nm HeNe laser. Confocal images were taken in the donor, acceptor and CFSE channels (the latter channel for target cells) prior to and post-photobleaching. FRET efficiencies per pixel were calculated using the AccPbFRET program (197).

Molecular densities in immune synapses

T cells and target cells were prepared and co-cultured as described for acceptor photobleaching. T cells were stained with TCR-Vβ9-PE mAb and one of the following mAbs: CD3ε-AlexaFluor647, CD8α-AlexaFluor647, CD45-AlexaFluor647, pCD3ζ-AlexaFluor647, pLCK, pERK or pZAP70. The latter three mAbs were used in combination with AlexaFluor647 GαM. Next, T cells were covered with 50:50 glycerol:PBS and imaged with a confocal microscope. The relative density of synaptic molecules was calculated by the

ratio of Mean Fluorescence Intensities (MFI) per pixel inside versus outside the synapse. Images were background-corrected before calculation of molecular densities as described previously (177).

Synapse sizes

Synapses were defined as contact regions between T cells and antigen presenting cells where the number of introduced TCRβ molecules accumulated. Areas of single synapse were calculated by ImageJ software (177).

T cell counting

T cell viability was determined by tryphan blue exclusion and viable T cells were counted microscopically using Bürker chambers and a Leitz Laburlux 12 microscope (Leica Geosystems BV, Rijswijk, The Netherlands). T cell expansions are represented as fold increases in absolute cell numbers during a 6-day culture on feeder cells.

Statistical analysis

Statistical significant differences between TCR:28ε and wt TCR were tested by non-paired and two-tailed Student's *t*-tests using Graphpad Prism4 software. Differences with p-values <0.05 were considered significant.

RESULTS

Single-chain TCRs that contain CD28, in particular in combination with CD3ε, demonstrate enhanced functional expression in human T cells

We used single-chain (sc) TCRs to test signaling cassettes consisting of Fc(ε)RIγ (γ), CD3ε (ε) and CD3ζ (ζ) accessory molecules without or with the CD28 co-stimulatory molecule for their effect on the functional expression of TCR transgenes (fig. 4.1 and table 4.1). The scTCRs were specific for MAGE-A1/HLA-A1 (M1/A1) and gene-transferred into primary human T cells, sorted with TCR antibodies, and analyzed for surface expression, binding of peptide-MHC, cytotoxicity and IFNγ release. Findings are presented in *supplementary figures 4.1A to D*, and are summarized in table 4.1. The various scTCRs showed comparable levels of surface expression, but differed with respect to binding of peptide-MHC. Single-chain TCR:γ and in particular scTCR:ζ, bound less peptide-MHC when compared to scTCR:ε. Notably, the ability of scTCRs to bind peptide-MHC increased in case acces-

sory molecules were combined with a CD28 co-stimulatory molecule. When analysing T cell cytotoxicity and IFNγ release of scTCR T cells in response to M1 peptide-loaded target cells, the CD28-CD3ε (28ε) signaling cassette consistently provided T cells with the best functional properties. Addition of a membrane proximal cysteine residue, that was reported to enhance functional expression of TCRαβ (198), did not enhance the general properties of the 28ε signaling cassette.

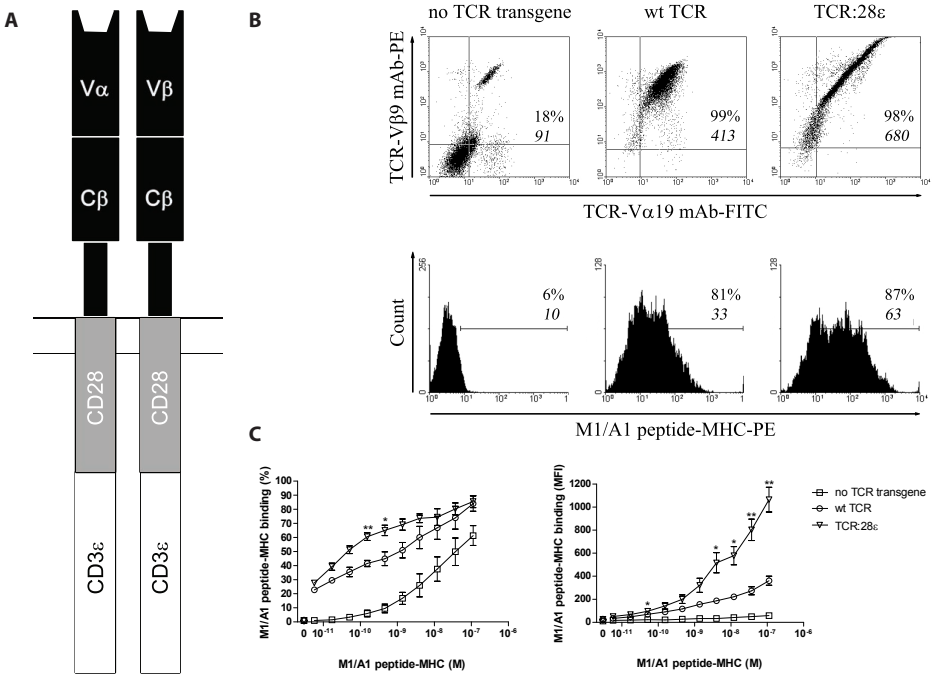

Figure 4.2. TCR:28ε shows enhanced peptide-MHC binding. Primary human T cells were transduced with empty virus particles (no TCR transgene) or M1/A1-specific two-chain TCR (wt TCR) or TCR:28ε. A schematic representation of two-chain TCR:28ε is given in (**A**). See materials and methods and table 4.1 for details on the 28ε signaling cassette. T cells were FACSorted with TCR-Vα19 and TCR-Vβ9 mAbs and (**B**) analysed by flow cytometry following staining with mAbs directed against TCRα and TCRβ (first row) or M1/A1 peptide-MHC (second row). Dot plots and histograms represent measurements of PBMC, n=3, from two healthy donors with similar results. In case of dot plots, percentages (%) correspond to the upper quadrants and Mean Fluorescence Intensities (MFI) (in italics) correspond to the x-axes of the upper right quadrants. In case of histograms, % and MFI correspond to marker-positive cells. Non-stained T cells were used to gate viable cells and set ordinates and markers at < 1% T cells. In (**C**) FACSorted T cells were stained with titrated amounts of M1/A1 peptide-MHC (range: 10^{-11} to 10^{-7} M) and results were expressed as % or MFI, n=3 independent measurements. Statistically significant differences between TCR:28ε and wt TCR were calculated with Student's *t*-test: *: p<0.05; ** : p<0.005.

Two-chain TCRs that harbor CD28 and CD3ε mediate improved binding of peptide-MHC and antigen-specific T cell functions

Following the above-mentioned screen of different scTCR formats, we have translated the 28ε cassette to a two-chain TCR format (i.e., TCR:28ε; fig. 4.2A). Primary human T cells were gene-transduced with M1/A1 wt TCR or two-chain TCR:28ε, and FACSorted to obtain T cell populations with high and equal levels of TCR expression (> 95%) (fig. 4.2B). Following staining with TCRα and β mAbs, flow cytometry dot plots of TCR:28ε-positive T cells revealed a typical diagonal with enhanced MFI values and equal staining intensities for both TCRα and TCRβ chains. This staining pattern suggests correct pairing of TCRα and TCRβ transgenes and was similar to the pattern that has previously been reported for TCR:ζ (see chapter 3). Following staining with peptide-MHC, TCR:28ε T cells demonstrated a significantly enhanced percentage and MFI of peptide-MHC binding over a range of peptide-MHC concentrations when compared to wt TCR (fig. 4.2C).

To investigate T cell function, we have stimulated TCR-engineered T cells with anti-TCR antibodies, peptide-loaded cells and native melanoma cells and analyzed activation of NFAT, mobilization of CD107a and production of cytokines. Using a luciferase reporter assay with Jurkat T cells we observed a significantly higher activation of NFAT upon stimulation of TCR:28ε T cells with TCR-Vβ9 antibody (against the exogenous M1/A1 TCR) when compared to wt TCR T cells (fig. 4.3A). T cells transduced with wt TCR, but not TCR:28ε, demonstrated decreased NFAT activation upon stimulation with TCR-Vβ27 antibody (against the endogenous MelanA/A2 TCR). These findings proof enhanced functional expression of TCR:28ε, not being at the expense of the functional expression of endogenous TCR. When assessing CD107a mobilization in primary human T cells, we observed that TCR:28ε outperformed wt TCR upon stimulation with M1 peptide, and that both TCR:28ε and wt TCR performed equally well upon stimulation with native, M1/A1-positive, melanoma cells (fig. 4.3B). Using TCRs directed against a second antigen, i.e., gp100/A2, we confirmed enhanced peptide-MHC binding (s*upplementary fig. 4.2A*) and peptide-induced mobilization of CD107a by TCR:28ε (fig. 4.3C). Enhanced responsiveness towards peptide-loaded targets cells of both M1/A1 and gp100/A2 specific TCRs was also observed upon assessing TCR:28ε T cells for their production of IFNγ and IL-2 (fig. 4.5 and s*upplementary figs. 4.2B and C and 4.3*).

TCR:28ε does not mis-pair with endogenous TCR chains

To assess whether TCR:28ε is prone to mis-pair with endogenous TCR chains, we generated dual TCR T cells with defined sets of TCR transgenes and performed flow cytometric Fluorescence Resonance Energy Transfer (FRET) to determine the molecular association between different TCR chains. To this end, we transduced clone J.19-CD8 T cells, already

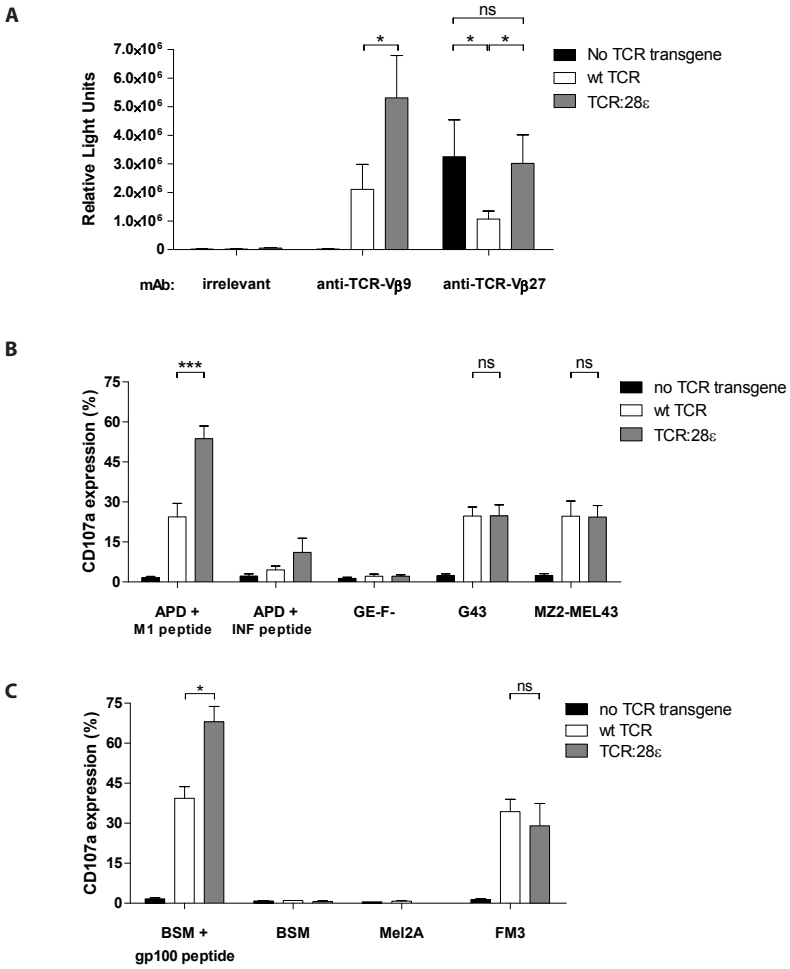

Figure 4.3. TCR:28ε T cells demonstrate enhanced activation of Nuclear Factor of Activated T cells and antigen-specific mobilization of CD107a. Jurkat clone 19 T cells (**A**) and primary human T cells (**B**, **C**) were transduced with empty virus particles, M1/A1 or gp100/A2 wt TCR or TCR:28ε, FACSorted with TCRα and TCRβ mAbs (in case of M1/A1) or peptide-MHC (in case of gp100/A2), and tested for their ability to (**A**) mediate activation of Nuclear Factor of Activated T cells (NFAT) or (**B**, **C**) mobilize CD107a to the cell surface upon stimulation with antigen. In (A) M1/A1 TCR Jurkat T cells were nucleofected with a Firefly luciferase reporter construct under control of NFAT response elements, and stimulated for 6h with irrelevant, anti-TCR-Vβ9 or anti-TCR-Vβ27 mAb coated to well plates. Bars represent mean activation of NFAT expressed in % RLU + SEM, n=5 independent measurements. Mock-transduced T cells showed negligible levels of NFAT activation (data not shown). In (B) M1/A1 TCR T cells were stimulated with the APD B cell line ($A1^+$) loaded with M1 or INF (influenza) peptide (10^{-5} M final), and the melanoma cell lines GE-F- ($M1^-/A1^+$), G43 and MZ2-Mel43 (both $M1^+/A1^+$). In (C) gp100/A2 TCR T cells were stimulated with the BSM B cell line ($A2^+$) loaded with or without gp100 peptide (10^{-5} M final), and the melanoma cell lines FM3 ($gp100^+/A2^+$) and Mel2A ($gp100^+/A2^-$). CD107a mobilization was measured by flow cytometry and expressed in % of CD107a-positive cells within the population of CD3-positive cells. Bars represent mean + SEM, n=2-10 independent measurements. Statistically significant differences between TCR:28ε and wt TCR were calculated with Student's *t*-test: ns: non-significant; *: $p<0.05$; ***: $p<0.0005$.

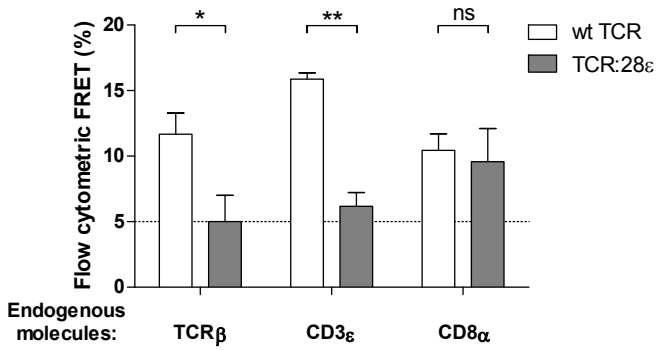

Figure 4.4. TCR:28ε does not mis-pair with TCR nor associate with CD3. Jurkat clone 19 T cells expressing MelanA/A2 TCR and CD8α were transduced with M1/A1 wt TCR or TCR:28ε and FACSorted with TCRα and TCRβ mAbs. T cells were stained with the following three sets of antibodies: (1) TCR-Vα19 + Rabbit-anti-Mouse (RαM)-Cy5 (acceptor) + TCR-Vβ27-PE (donor); (2) CD3ε + RαM-Cy5 (acceptor) + TCR-Vβ9-PE (donor); and (3) CD8α + RαM-Cy5 (acceptor) + TCR-Vβ9-PE (donor). Flow cytometry was used to measure Fluorescence Resonance Energy Transfer (FRET) and bars represent mean % + SEM, n=3-6 independent measurements. Notably, FRET efficiencies <5%, with the dotted line representing a 5% background signal, do not imply that there is no pairing of molecules but do not allow distinction between significant and non-significant interactions. Mock-transduced T cells (no TCR transgene) showed negligible levels of FRET (data not shown). Statistically significant differences between TCR:28ε and wt TCR were calculated with Student's *t*-test: ns: non-significant; *: $p<0.05$; ** : $p<0.005$.

expressing the MelanA/A2 wt TCR and the CD8α co-receptor, with the M1/A1 wt TCR or TCR:28ε genes. Measurements of flow cytometric FRET using the MelanA/A2 TCR-Vβ27 and the M1/A1 TCR-Vα19 specific mAbs revealed significant energy transfer in case of wt TCR, but not TCR:28ε where values were below the 5% background signal (fig. 4.4). Measurements of FRET using the M1/A1 TCR-Vβ9 and either CD3ε or CD8α mAbs showed that TCR:CD3 associations were only observed for wt TCR but not TCR:28ε and TCR:CD8α associations were present for both TCR formats (fig. 4.4). These experiments show that TCR:28ε does not mis-pair with endogenous TCR chains nor does it associate with endogenous CD3 molecules, similar to observations reported for TCR:ζ in chapter 3 and Sebestyén *et. al.* (63).

TCR:28ε displays a similar peptide-fine specificity as wt TCR

Next, we studied whether the 28ε cassette alters the TCRs' peptide-fine specificity. To this end, we loaded T2 cells with altered gp100 peptide ligands (APL), as described by Schaft and colleagues (132), and measured IFNγ release following stimulations of TCR-transduced primary human T cells. These APLs deviate from the wt gp100 peptide (YLEPGPVTA) by a single amino acid mutation to alanine or, in case of the last amino acid, glutamic acid, indicated by an 'A' or 'G', respectively, with the number corresponding to the amino acid position within the peptide. We observed that both TCR:28ε and

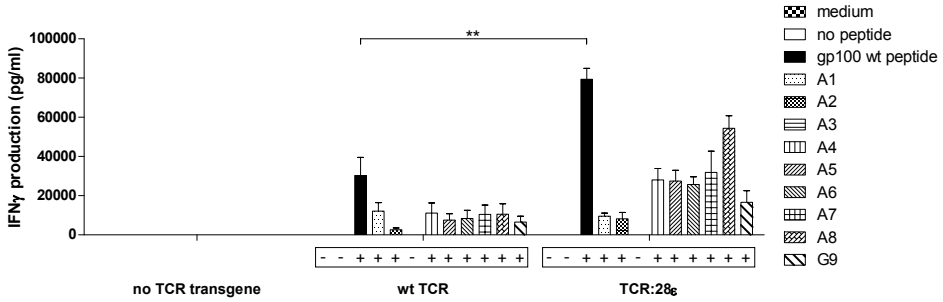

Figure 4.5. TCR:28ε shows a peptide-fine specificity that is similar to that of wt TCR. Primary human T cells were transduced with empty virus particles, gp100/A2 wt TCRs or TCR:28ε and FACSorted with peptide-MHC. T cells were O/N stimulated with T2 cells that were not loaded or loaded with gp100 wt peptide or altered peptide ligands (APL) (10^{-5} M final). APLs are coded by the letters A (Alanine) or G (Glycine) followed by a number that corresponds to the position of the amino acid that is altered into A or G in the gp100 wt peptide sequence (YLEPGPVTA). IFNγ release was measured by ELISA and expressed in pg/ml, n=3 independent measurements. Notably, both wt TCR and TCR:28ε did not mediate a response to the A3 APL as indicated by '-', in contrast to other APLs as indicated by '+' (enhanced response compared to medium). Statistically significant differences (with respect to gp100 wt peptide responses) were calculated with Student's *t*-test: ns: non-significant; **: p<0.005.

wt TCR mediated IFNγ release to all APLs, except when an Alanine replaced a Glutamic acid at position 3 (fig. 4.5). This peptide response pattern was identical to that of the parental CTL-296 clone (132) and showed that incorporation of the 28ε cassette did not alter the peptide-fine specificity of the parental TCR.

TCR:28ε induced immune synapses with enhanced accumulation of TCR, CD3ε, CD8α co-receptor and phosphorylated LCK and ERK

The induction of immune synapses and their molecular profile is considered a critical measure of T cell activation. To study the ability to induce and the composition of immune synapses, TCR-transduced Jurkat T cells were stimulated with M1 peptide-loaded target cells and studied by confocal microscopic FRET. We observed that TCR:28ε is able to induce immune synapses and, in extension to data obtained with flow cytometric FRET, TCR:28ε present in synapses does not associate with CD3ε nor CD45, but does associate with CD8α (figs. 4.6A and B). In addition, the immune synapses induced by either TCR:28ε or wt TCR are of similar size (fig. 6C). Notably, when repeating analyses with TCR-engineered primary human T cells, TCR:28ε, but not wt TCR, demonstrated clear antigen-induced synapse formation (fig. 4.7A and B). To investigate synaptic accumulation of T cell membrane molecules in more detail, we used quantitative analysis of confocal microscopy data and observed that TCR:28ε mediated a 5-fold increase in the accumulations of TCRβ transgene, endogenous CD3ε and CD8α molecules when compared to wt TCR (fig. 4.8A). When measuring markers of early T cell activation, we noted

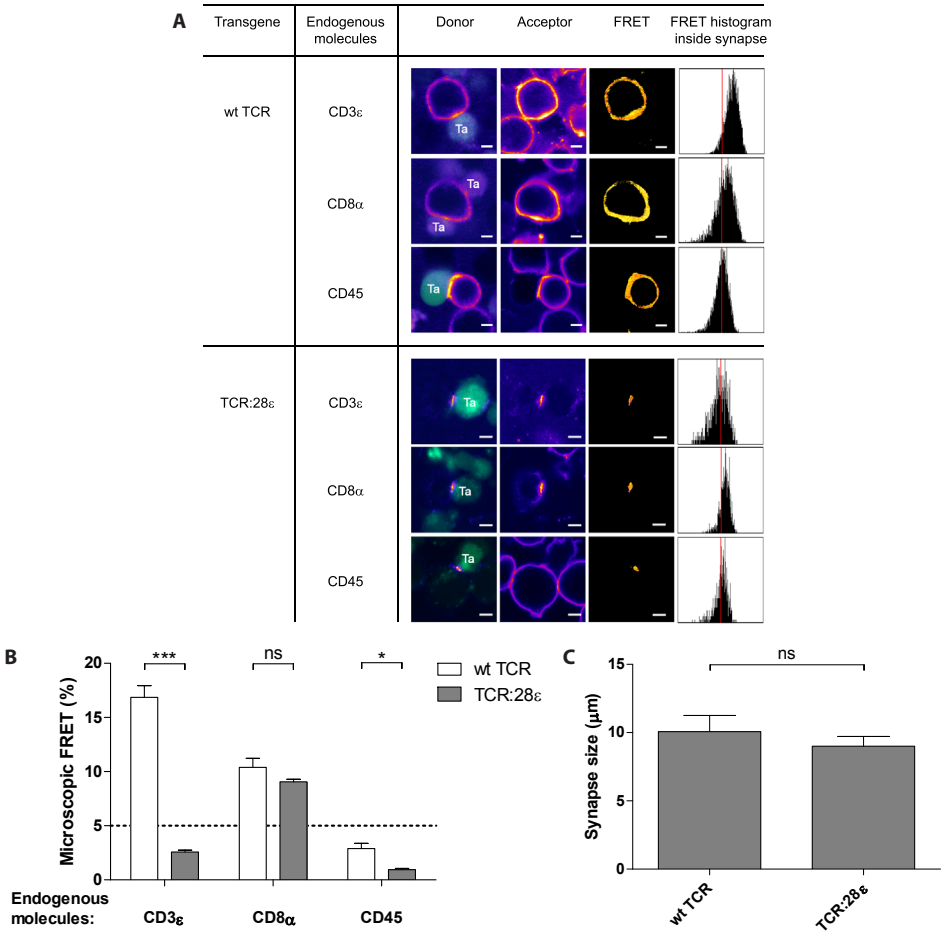

Figure 4.6. TCR:28ε induces formation of antigen-dependent immune-synapses in T cell line. Jurkat T cells were transduced with M1/A1 wt TCR or TCR:28ε, as described in the legend to figure 4.4, and analysed for molecular associations between the TCRβ transgene and endogenous CD3ε, CD8α, or CD45 molecules in immune synapses. APD B cells (marked as target cells; Ta) were stained with CFSE, loaded with M1 peptide and used to stimulate TCR T cells. T cells were labeled with the donor mAb TCR-Vβ9-PE and one of the following acceptor mAbs: CD3ε-AlexaFluor647, CD8α-AlexaFluor647 or CD45-AlexaFluor647. Confocal microscopy was used to measure Fluorescence Resonance Energy Transfer (FRET). In (**A**) representative pictures are shown of immune synapses of Jurkat T cells (magnification: 200x and with a white scale bar of 5 µm), including FRET histograms of corresponding synapses. Vertical lines in the center of histograms denote zero FRET efficiency per pixel in the synapse region. TCR T cells stimulated with APD B cells loaded with irrelevant peptide showed low background levels of FRET (except for FRET between wt TCR and CD3ε) (data not shown). Mean % + SEM of microscopic FRET measured with Jurkat T cells are shown in (**B**), n=3 independent measurements of 15 cells per measurement. Data of wt TCR presented in (A) were modified from Roszik and colleagues (177). In (**C**) mean µm^2 + SEM of synapse sizes are shown, n=4 independent measurements. Synapses were defined as regions of contact between T cells and peptide-loaded target cells with an accumulation of TCR-Vβ9 molecules and their areas were calculated as described in materials and methods. Mock-transduced T cells did not induce the formation of immune synapses (data not shown). Statistically significant differences between TCR:28ε and wt TCR were calculated with Student's *t*-test: ns: non-significant; *: $p<0.05$; ***: $p<0.0005$.

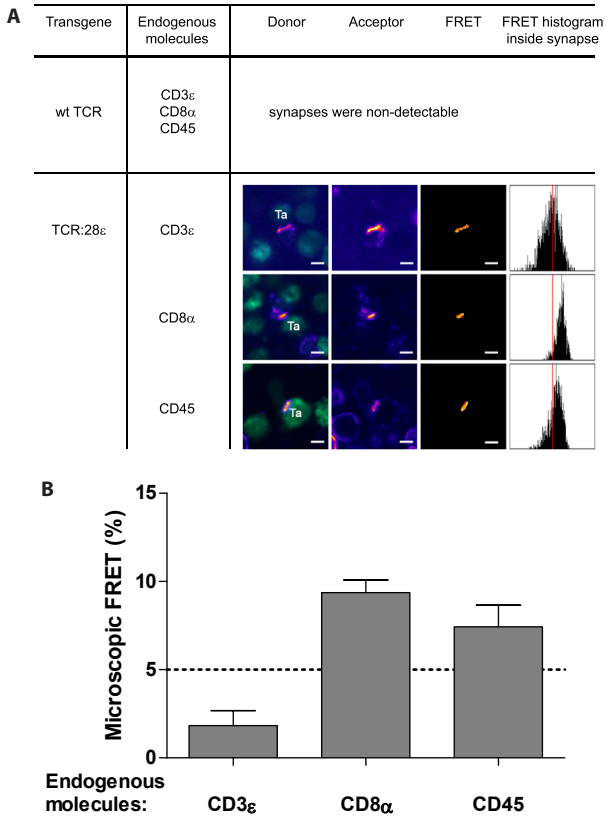

Figure 4.7. TCR:28ε, but not wt TCR, induces formation of antigen-dependent immune-synapses in primary human T cells. Primary human T cells were transduced with M1/A1 wt TCR or TCR:28ε, as described in the legend to figure 4.2, and analysed for molecular associations between the TCRβ transgene and endogenous CD3ε, CD8α, or CD45 molecules in immune synapses as described in the legend to figure 4.6. In (**A**) representative pictures are shown of immune synapses of primary human T cells (magnification: 200x and with a white scale bar of 5 μm), including FRET histograms of corresponding synapses. Mean % + SEM of microscopic FRET measured with primary human T cells are shown in (**B**), n=3 independent measurements of 15 cells per measurement.

that TCR:28ε mediated accumulations of phosphorylated-LCK (pLCK) (7-fold increase), and to a lesser extent pERK (1.6-fold increase) (fig. 4.8B). However, TCR:28ε mediated a significantly decreased accumulation of pCD3ζ (2.3-fold) and a non-changed accumulation of pZAP70 (fig. 4.8B).

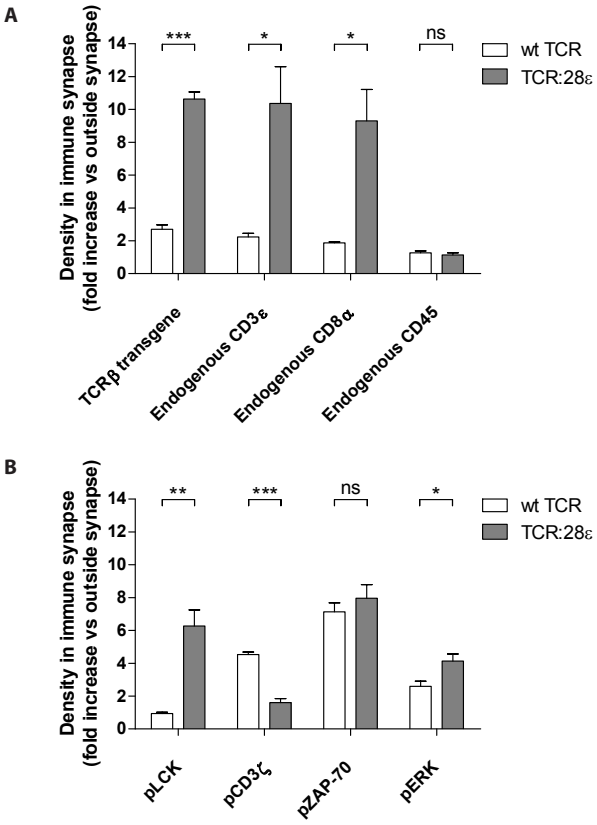

Figure 4.8. TCR:28ε T cells demonstrate enhanced synaptic accumulation of TCR, CD3ε, CD8α, pLCK and pERK. Jurkat T cells were transduced with M1/A1 TCRs, as described in legend to figure 4.4, stimulated with APD B cells loaded with M1 peptide, and measured for densities of (**A**) membrane and (**B**) intracellular proteins present in synapses. T cells were stained with TCR-Vβ9-PE mAb and one of the following mAbs: CD3ε-AlexaFluor647, CD8α-AlexaFluor647, CD45-AlexaFluor647, pCD3ζ-AlexaFluor647, pLCK, pERK and pZAP70. In case of the latter 3 non-conjugated mAbs, staining was followed by a secondary isotype-specific AlexaFluor647-conjugated mAb. T cells were analysed by confocal microscopy and bars represent densities of molecules (fold increase of signals inside versus outside synapses) + SEM, n=3 independent measurements of 15 cells per measurement. TCR T cells stimulated with irrelevant peptide showed low background signals (data not shown). Data of wt TCR presented in (A) were modified from Roszik and colleagues (177). Statistically significant differences between TCR:28ε and wt TCR were calculated with Student's *t*-test: ns: non-significant; *: $p<0.05$; **: $p<0.005$; ***: $p<0.0005$.

DISCUSSION

In this study, using a single-chain TCR platform, we have tested signaling cassettes that consisted of the accessory molecules Fc(ε)RIγ, CD3ε or CD3ζ, without or with the co-stimulatory molecule CD28, for their effects on the functional expression of TCR chains. The combined use of CD28 and CD3ε proved most optimal with respect to various pa-

rameters of T cell activation. When incorporating this cassette into both TCRα and TCRβ, we observed that a two-chain TCR:28ε provides T cells with the following beneficial properties for T cell therapy: (I) enhanced peptide-MHC binding; (II) more potent T cell responses upon stimulation with peptide and equal responses upon stimulation with antigen-positive melanoma cells; (III) no TCR mis-pairing; (IV) no change in peptide-fine specificity; and (V) antigen-dependent formation of immune synapses that are enriched in TCR/CD3, CD8 and markers of early T cell activation.

The enhanced potency of T cells transduced with TCR:28ε to bind peptide-MHC (figs. 4.2B and C, and s*upplementary fig. 4.2A*) and respond to peptide (figs. 4.3, 4.5 and s*upplementary figs. 4.2B and C*) is not related to a changed dependency for CD8α (*supplementary fig 4.3*) but most likely a direct consequence of enhanced surface expression per cell (see MFI values in figure 4.2B and C, and s*upplementary figure 4.2A*). In contrast to wt TCR (98), TCR:28ε does not compete for endogenous CD3 proteins to become surface-expressed as evidenced by the lack of association between TCR:28ε and CD3ε (fig. 4.4) and enhanced activation of NFAT when stimulating either TCR:28ε or the endogenous TCR (fig. 4.3A). Unexpectedly, T cell responses towards antigen-positive tumor cells were equal when compared to wt TCR-transduced T cells. When testing TCR:28ε T cells in more detail, we observed a lowered effective dose of peptide yielding half-maximum response (data not shown), and a compromised response in the absence of CD8α binding (s*upplementary fig. 4.3*). In our opinions these finding do not argue against further clinical development of TCR:28ε, which compared to wt TCR provides co-stimulation and no TCR mis-pairing. Nevertheless, we speculate that TCR:28ε enhances the potency of T cell responses but does so with a reduced affinity. Along this observation, earlier studies have reported that binding of peptide-MHC multimers may not always correlate with antigen sensitivity (91) and that inclusion of CD28 into a CAR or a TCR predominantly enhances the potency of T cell cytokine production (199).

TCR:28ε does not mis-pair with endogenous TCR as evidenced by flow cytometric FRET (fig. 4.4) nor does it have a changed peptide-fine specificity when compared to wt TCR (fig. 4.5) and is therefore most likely less prone to induce off-target toxicities (65, 66). In addition, our studies provide the following lines of evidence that TCR:28ε does not hamper safety by constitutively activating T cells. First, we did not observe activation of NFAT following non-specific TCR stimulation (fig. 4.3A). Second, we did neither observe CD107a mobilization nor release of IFNγ and IL-2 in the absence of antigen (figs. 4.3B and C, 4.5 and s*upplementary figs 4.2B and C*). Thirdly, TCR:28ε T cells revealed no enhanced proliferation during routine culture conditions (s*upplementary fig. 4.4*).

To study T cell activation in more depth, we analysed the formation of immune synapses. TCR:28ε is able to mediate the formation of immune synapses between Jurkat T cells and target cells similar to wt TCR (figs. 4.6A and B). Also, the composition of immune synapses is similar, except for a lack of association between TCR:28ε and CD3ε. Notably,

TCR:28ε but not wt TCR mediated detectable synapse formation when using primary human T cells (figs. 4.7A and B), implying an enhanced potency of the former TCR to mediate the formation of immune synapses. Alternatively, we cannot exclude that TCR:28ε may differ from wt TCR with respect to its temporal organization of synapses. In fact, a reduced association with CD45 may provide TCR:28ε with a kinetic advantage (fig. 6B). In this respect, it is noteworthy that primary human T cells may demonstrate prolonged time requirements to develop molecular clusters when compared to Jurkat T cells and may proof better suited to perform further experiments into the kinetics and stability of immune synapses (200, 201). When quantifying the presence of synaptic molecules, we showed significant accumulation of TCR transgene, CD3ε and CD8α when comparing immune synapses of TCR:28ε to wt TCR T cells (fig. 4.7A). Synapses induced by wt TCR and TCR:28ε have identical sizes (fig. 4.6C), arguing that TCR:28ε forms highly organized complexes with endogenous TCR/CD3 and CD8 molecules inside synapses. Such highly organized TCR microclusters potentially facilitate antigen-dependent T cell activation (202-204). When investigating early T cell activation markers, we observed significantly enhanced densities of phosphorylated LCK and ERK in TCR:28ε-induced immune synapses (fig. 4.8B). Enhanced densities of pLCK may be a direct consequence of enhanced densities of TCR:28ε and CD8 since both CD28 (contained in TCR:28ε) and CD8 harbor a motif for LCK recruitment. Currently, we are testing TCR:28ε and CD8α with a mutated LCK-binding domain (205, 206) to identify the molecular source of pLCK. Notably, pERK can phosphorylate LCK at Serine-59 preventing inactivation of pLCK by the phosphatase SHP-1 (207), thereby providing an alternative reason and possibly a positive feedback loop for the enhanced activation of LCK. Interestingly, the motif of CD28 that recruits LCK also acts as a docking site for the adaptors GRB2 and FLN3 (208), whereas a second membrane-proximal proline-rich motif acts as a docking site for the kinase ITK (209). In TCR:28ε T cells, despite synaptic accumulation of pLCK, levels of pCD3ζ were significantly lower when compared to wt TCR (fig. 4.8B). The lack of CD3ζ phosphorylation observed in TCR:28ε T cells may be due to an inability of this receptor to associate with endogenous CD3ζ and position this molecule sufficiently close to pLCK to become phosphorylated. Despite the presence of fewer pCD3ζ scaffolds, the accumulated levels of pZAP70 in TCR:28ε T cells were similar to those in wt TCR T cells (fig. 4.8B), which may argue that ZAP70, even when docking to pCD3ζ is hampered, becomes phosphorylated by the enhanced presence of pLCK. Interestingly, TCR:28ε resulted in enhanced activation of two early TCR signaling pathways, namely activation of NFAT (fig. 4.3A) as well as pERK (fig. 4.8B). In this respect, the CD3ε molecule present in the CD28-CD3ε signaling cassette may serve as docking site for kinases and adaptors such as GRK2, CAST and NCK (210). CD3ε-bound GRK2 and CAST, together with calcium, are involved in the activation of NFAT and production of IL-2 (210, 211), whereas CD3ε-recruited NCK, in conjunction with SLP-76, leads to activation of the RAS/RAF/ERK pathway, cytoskeletal reorganization

and formation of immune synapses (212, 213). Taken together, the CD28-CD3ε signaling cassette yields a TCR that takes advantage of two critical components of T cell activation: CD3ε, linked to the initiation of TCR signaling, and CD28, linked to T cell co-stimulation.

So far, most studies analysing signaling cassettes were performed with CARs. Inclusion of CD28 into CARs, generally in combination with a CD3ζ domain, resulted in enhanced T cell activation and proliferation, *in vivo* T cell persistence and anti-tumor effects. Signaling cassettes were further tested with other members of the CD28-family (186, 214-217), such as CD137 (4-1BB) and CD134 (OX40), which showed promising results with respect to cytolysis, initiation or sustainment of an effective T cell response and prevention of CAR-mediated proliferation of regulatory T cells (44). Importantly, clinical trials using CAR:CD28-CD3ζ or CAR:CD137-CD3ζ demonstrated significant objective responses in patients with B cell leukemia (76-78). In this manuscript, we showed that a signaling cassette consisting of CD28 and CD3ε in the setting of TCRs improves the potency of T cell responses without compromising antigen specificity and warrants further development of co-stimulatory TCRs for clinical testing.

ACKNOWLEDGEMENTS

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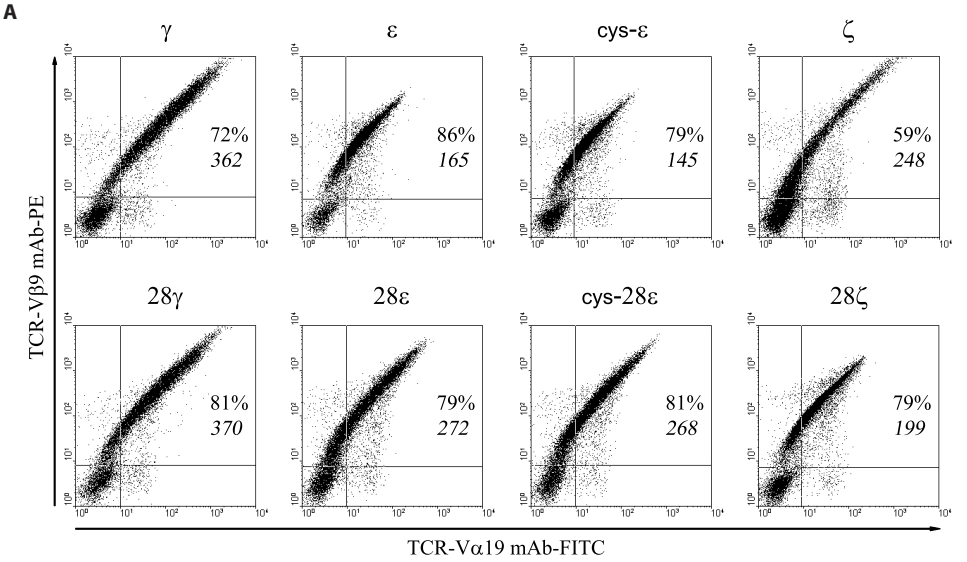
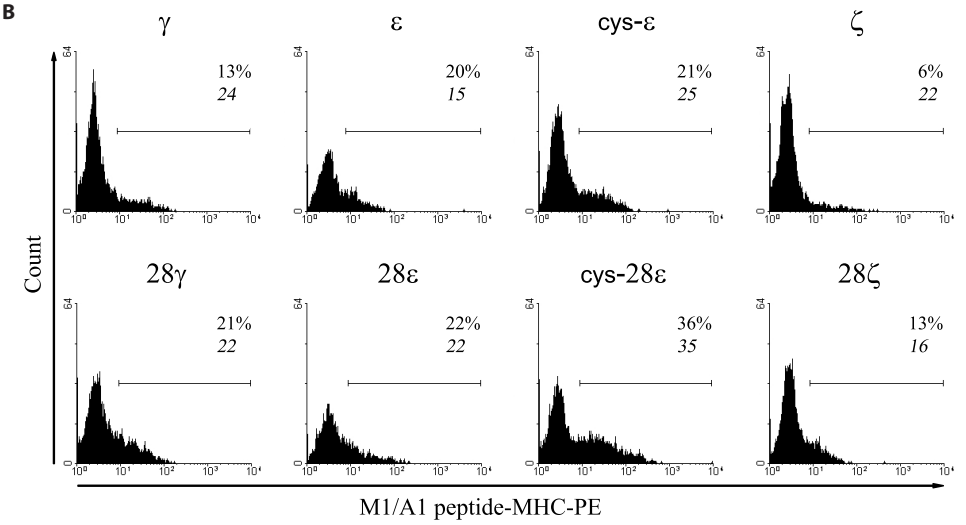
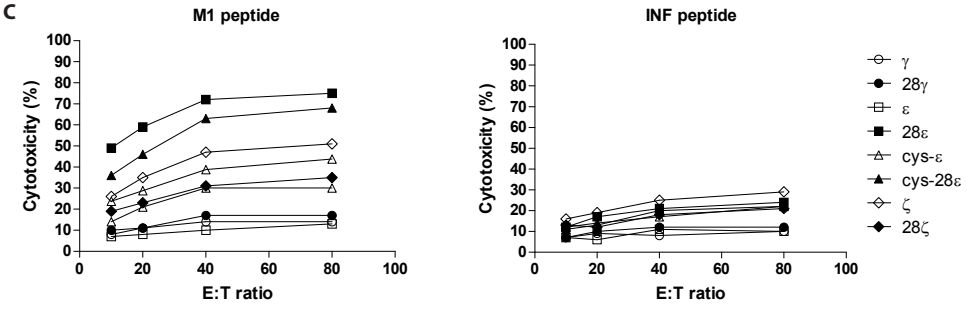
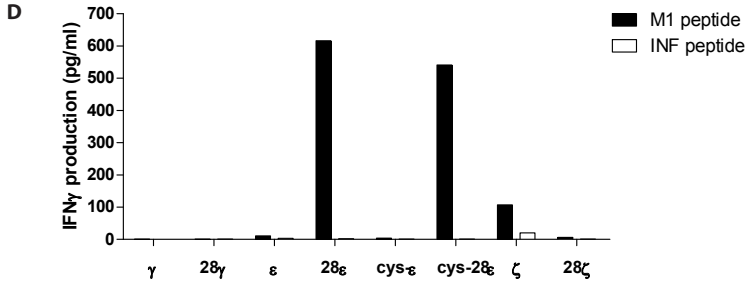
SUPLEMENTARY FIGURES

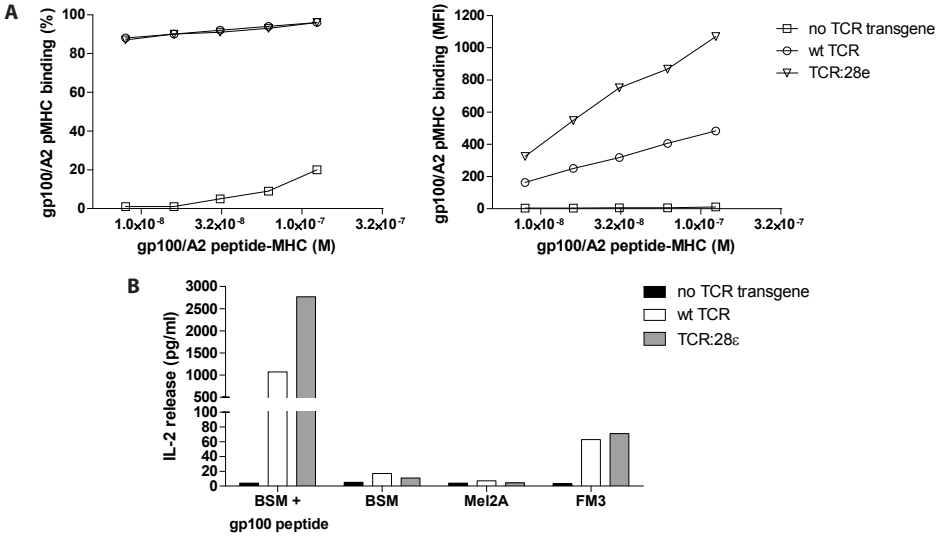


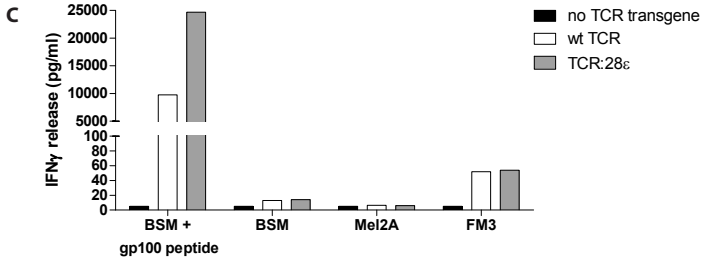




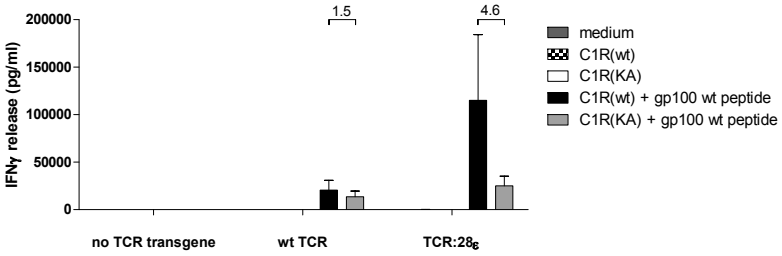


Supplementary figure 4.1. Single-chain TCRs equipped with a CD28-CD3ε signaling cassette show enhanced functional expression. Primary human T cells were transduced with single chain (sc)TCRs specific for M1/A1, FACSorted with TCRα and TCRβ mAbs and analyzed for TCR surface expression (**A**), peptide-MHC binding (**B**), cytotoxicity (**C**), and IFNγ release (**D**). In (A) and (B), flow cytometry was used either with TCRα and TCRβ mAbs or M1/A1 peptide-MHC. In case of dot plots, percentages (%) correspond to the upper quadrants, and Mean Fluorescence Intensities (MFI) (in italics) correspond to the x-axes of the upper right quadrants. In case of histograms, % and MFI correspond to marker-positive cells. Non-stained T cells were used to gate viable cells and set ordinates and markers at <1% T cells. In (C) and (D), T cells were co-cultured with either M1 or INF peptide-loaded APD B cells (10^{-5} M final) and assessed for their cytotoxic capacity and ability to produce IFNγ. T cell-mediated cytotoxicity was measured by ^{51}Cr-release at E:T ratios of 1:10, 1:20, 1:40 and 1:80 at 4h and expressed in % specific ^{51}Cr release, whereas IFNγ release was measured by ELISA in supernatants collected at 24h and expressed in pg/ml. Antigen-negative target cells or Mock-transduced T cells (no TCR transgene) showed negligible responses (data not shown). Data presented in this figure are representative for three independent experiments with similar results, and are summarized in table 4.1.

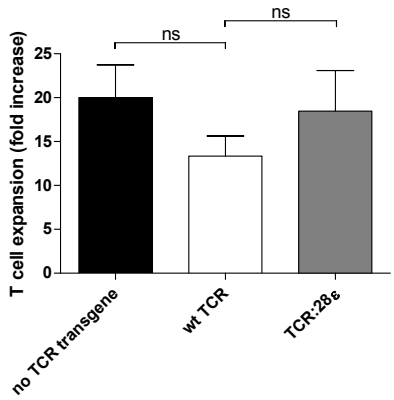




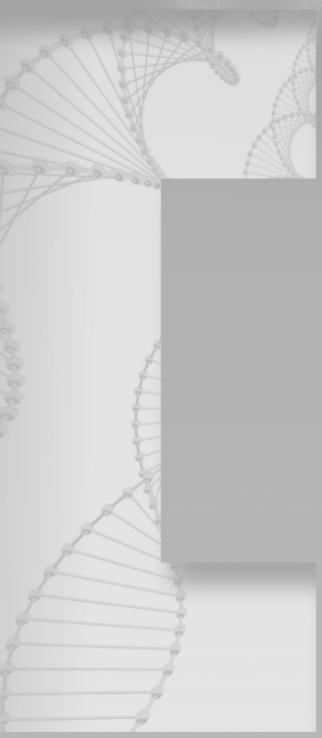
Supplementary figure 4.2. TCR:28ε T cells specific for gp100/HLA-A2 demonstrate enhanced binding of peptide-MHC and peptide-induced release of IFNγ and IL-2. Primary human T cells were transduced with empty virus particles, gp100/A2 wt TCR or TCR:28ε and FACSorted with peptide-MHC. In (**A**), T cells were stained with titrated amounts of peptide-MHC (range: $5x10^{-6}$ to 10^{-8} M). In (**B**) and (**C**), T cells were stimulated for 24h with BSM B cells that were loaded or not loaded with gp100 wt peptide, or the melanoma cell lines MEL2A ($gp100^{-}/A2^{+}$) and FM3 ($gp100^{+}/A2^{+}$), and were subsequently analyzed for their release of IFNγ or IL-2. Representative data of flow cytometry stainings and IFNγ and IL-2 measurements are shown (n=2).



Supplementary figure 4.3. TCR:28ε shows an enhanced CD8 dependency. Primary human T cells were transduced with empty virus particles or gp100/A2 wt TCR or TCR:28ε and FACSorted with peptide-MHC. T cells were O/N stimulated with C1R cells expressing wt HLA-A2 or the non-CD8α binding KA mutant of HLA-A2 that were not loaded or loaded with gp100 wt peptide (10 M^{-5} final). IFNγ release was measured by ELISA and expressed in pg/ml, n=4 independent measurements. Fold difference (non-significant according to Student's *t*-test) between TCR:28ε and wt TCR T cells in response to peptide-loaded C1R(wt) and C1R(KA) target cells are indicated.



Supplementary figure 4.4. TCR:28ε T cells show no enhanced proliferation. Proliferation of primary human T cells transduced with empty virus particles or M1/A1 wt TCR or TCR:28ε was determined by counting T cells following harvest from feeder cells, as described in materials and methods. Bars represent mean values + SEM, n=13-18 independent measurements. No significant (ns) differences were found between wt TCR and TCR:28ε according to Student's *t*-test.



5

CDR3α mutations in gp100/HLA-A2 TCR causing minor affinity-enhancement demonstrate improved T cell function without gain in self-reactivity

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Manuscript under revision

ABSTRACT

Adoptive therapy with T cells is likely to benefit from affinity-enhancement of T cell receptor (TCR) transgenes. However, it is questioned whether affinity-enhanced TCRs selectively lead to improved T cell function without increase of self-recognition. Here, we used phage display to obtain affinity-enhanced TCRs specific for $gp100_{280-288}$/HLA-A*0201 (K_D range: 18.5 μM to 26 nM) that harbor amino acid substitutions restricted to individual complementarity determining regions (CDR). Following gene transfer into human T cells, we demonstrated that TCRs with the highest affinity and mutations in CDR2β, but not CDR3β, improved peptide-HLA binding most significantly. In contrast, improvement of antigen-specific T cell functions was restricted to TCRs with relatively minor affinity enhancements (K_D ≥4 μM) and mutations in CDR3α. Importantly, high-affinity TCRs (K_D <1 μM), which exclusively harbored mutations in CDR2β, lost peptide fine-specificity and gained reactivity towards HLA-A2. Notably, TCRs with nM affinities mediated target cell-independent T cell activation and differentiation when introduced in HLA-A2-positive donor T cells. In conclusion, perimeters of TCR affinities that determine gain of function are different from those that determine gain of self-reactivity and, in this study, are best distinguished by TCR-CDR3α mutations. These findings may facilitate the generation of functionally enhanced and therapeutically safe T cells.

INTRODUCTION

Adoptive T cell therapy to treat viral infections or tumors is based on the ability of T cell receptors (TCRs) to selectively recognize antigens, i.e., peptides that are presented by Major Histocompatibility Complex (MHC) molecules. The contact between TCR and peptide-MHC is governed by loops of the three complementarity determining regions (CDRs) of both TCRα and TCRβ chains. CDR1 and CDR2 represent germ line-encoded regions that interact with MHC heavy chain alpha helices and position the highly variable CDR3 region for direct interactions with the peptide (88, 89). The combined binding strength of CDRs determines the affinity of the TCR for peptide-MHC (see also box 1.2). T cell selection in the thymus and peripheral maintenance of low affinity interactions with self-peptide-MHC yields a functional repertoire of ~2.5 x 10^8 different TCRs with dissociation constant (K_D) values for their cognate peptide-MHC typically in the range of 1-100 μM (88-90, 218). T cell responsiveness towards target cells, i.e. functional T cell avidity, is determined in large part by TCR:peptide-MHC interactions (219, 220). Studies with viral and tumor antigens demonstrate that high avidity T cells are able to mediate maximum antigen reactivity and protection from disease (219-221).

Procedures have been developed that yield high-affinity TCR variants with the intention of obtaining higher avidity, and hence therapeutically more effective T cells. One approach is that TCRs with moderately high affinities can be obtained from a pool of non-tolerant TCRs, such as *in vitro* systems of MHC-mismatched cytotoxic T cells (38) or mice that are transgenic for human MHC (Human Leukocyte Antigen, HLA) following immunization with human antigens (222). Gp100 and CEA-specific TCRs have been derived from HLA-A*0201 transgenic mice and used in clinical trials (40, 45), but patients developed a humoral response against these non-human TCRs (223). Mice that are transgenic for human TCR loci, with murine TCR loci being inactivated, provide a non-tolerant repertoire of completely human TCRs (39). Alternatively, TCR affinities can be enhanced by rationally designed mutagenesis of CDR loops (224, 225). Lastly, high-affinity TCR variants can be selected from a library of CDR site-directed mutants by phage, yeast or T cell display (226-230). Generally, T cells expressing higher than average affinity TCRs show enhanced functional avidity and less dependence on CD8 co-receptor (92, 153, 224, 228, 231). Notably, the clinical use of improved-affinity TCRs in adoptive transfer of TCR-engineered T cells to treat melanoma patients enhanced response rates (45). Although high affinity TCRs, when directed against differentiation antigens that are over-expressed on tumors, can result in on-target toxicities on normal tissues (40, 45), an improved-affinity TCR directed against a shared peptide epitope from the Cancer Testis Antigen (CTA) NY-ESO-1/HLA-A*0201 and LAGE-1/HLA-A*0201 has been successfully used in clinical TCR gene therapy without observations of either on-target or off-target toxicities (46). However, apart from the nature of the target antigen, studies

have shown that affinity-enhanced TCR bear the risk of reduced antigen-specificity and increased affinities for self-peptide-MHC (92, 228). One of the current challenges in the development of affinity-enhanced TCRs for adoptive T cell therapy is to control the risk of generating self-reactive TCRs.

In the present study, we questioned whether selected CDR amino acid substitutions are able to improve functional T cell avidity without loss of specificity. Starting from a TCR specific for $gp100_{280-288}$/HLA-A*0201 (gp100/A2), we generated phage libraries that harbored amino acid substitutions either in CDR2β, CDR3α or CDR3β and selected TCR variants with K_D values ranging from 18.5 μM to 26 nM. Following gene transfer into human T cells we demonstrated that improved peptide-HLA binding was most significant for TCRs with the highest affinity enhancement. Gain in TCR function, however, appeared restricted to a lower K_D limit of ~4 μM and was only observed for TCR variants with amino acid substitutions in CDR3α. These CDR3α TCR variants (but not the CDR2β variants with K_D values less than 1 μM) preserved the antigen and peptide-fine specificity of wild type TCR. Extending our findings to a therapeutic setting, we note that, when introduced in HLA-A2-positive donor T cells, TCRs with nM affinities conveyed significant T cell activation and differentiation in the absence of tumor target cells. In conclusion, affinities with K_D values down to 4 μM, in this study best captured by amino acid substitutions in CDR3α, with a potential safe guard set at 1 μM, may facilitate the generation of functionally optimal therapeutic T cells.

MATERIALS AND METHODS

Cells and reagents

T lymphocytes were derived from healthy donors, isolated and expanded in HEPES-buffered RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, 10% human serum, streptomycin, penicillin and 360 IU/ml rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands) were stimulated every other week with a mixture of irradiated allogeneic feeder cells as described elsewhere (232). Both the retroviral packaging cell lines, 293T and Phoenix-A, and melanoma cell lines were cultured in DMEM medium (BioWhittaker) supplemented with 2 mM L-glutamine, non-essential amino acids, 10% FBS and antibiotics. Melanoma cell lines included C1R wild-type (wt) and C1R-227/228KA cell lines, expressing wt HLA-A2 and a mutant non-CD8α binding HLA-A2, respectively ((196); a kind gift of Prof.dr. Andrew Sewell, Cardiff University, UK) and melanoma cell lines positive for gp100 and HLA-A2: FM3, MEL624, DAJU, BLMgp100, 453AO; positive for gp100 but negative for HLA-A2: MEL2A, MZ2-MEL43, G43; and negative for gp100 but positive for HLA-A2: BLM, 607B, 518A2 and Brown. Monoclonal

antibodies used in this study were: PE-conjugated anti-TCR-Vβ19 (TCR nomenclature according to http://imgt.org; clone E17.5F3.15.13; Beckman-Coulter, Marseille, France); FITC-conjugated anti-CD3ε (clone SK7; BD Biosciences, San Jose, CA); PerCP-conjugated anti-CD3ε (clone SP34-2; BD biosciences); non-conjugated anti-CD3ε (clone OKT3; Beckman-Coulter); PerCP-conjugated anti-CD8α (clone SK1; BD biosciences); APC-Cy7-conjugated anti-CD27 (clone M-T271; BD biosciences); APC-conjugated anti-CD28 (clone CD28.2; BD biosciences); PE-Cy7-conjugated anti-CD45RA (clone L48; BD biosciences); PE-conjugated anti-CD107a (clone H4A3; BD Biosciences); APC-conjugated anti-CD137 (clone 4B4-1; BD biosciences); and PE-conjugated anti-CCR7 (clone 150503; R&D systems, Abingdon, UK). $Gp100_{280-288}$/HLA-A*0201 (gp100/A2) peptide-HLA-tetramers were generated from biotinylated monomers (Sanquin, Amsterdam, Netherlands) and PE-conjugated streptavidin (BD Biosciences) as described in chapter 6. Other reagents used in this study include: Retronectin (human fibronectin fragments CH-296, Takara Shuzo Co. Ltd., Otsu, Japan); PMA (Sigma-Aldrich, St. Louis, USA); PHA (Remel Europe, Dartford, UK); Golgistop (BD Biosciences); $gp100_{280-288}$ wild type (wt) peptide (YLEPG-PVTA) (Proimmune, Oxford, UK) or Altered Peptide Ligands (APL) in which single amino acids were substituted (A1 to A8 and G9).

Phage libraries and selections

A wildtype (wt) TCR specific for gp100/A2 with the genes: TRAV17*01/J29*01/TRAC and TRBV19*01/D1/J2*07/TRBC1 was used as a starting TCR to obtain affinity-enhanced TCR variants (a kind gift of Prof.dr. Cassian Yee, Fred Hutchinson Cancer Research Centre, Seattle, WA, USA). Phage libraries of TCRs with CDR amino acid substitutions were generated and TCR variants were selected from these libraries with peptide-HLA as described previously (153). In short, CDR2β, CDR3α and CDR3β libraries were constructed by splicing overlap extension PCR (SOE-PCR) using a panel of 5-NNK mutagenic oligonucleotides that spanned the codons encoding the CDR loop regions. M13KO7 helper phage was used to rescue TCR-displaying phage particles, which were subsequently subjected to three cycles of panning against gp100 peptide complexed to biotinylated-A2. TCR-phage:gp100/biotinylated-A2 complexes were captured with streptavidin-labeled magnetic beads. Binding of selected phages to gp100/A2 was validated using ELISA and corresponding CDR amino acid substitutions were analyzed by DNA sequencing. A panel of 8 TCRs each containing 2 or 3 amino acid substitutions in either CDR3α, CDR2β or CDR3β regions was used for the present study (see table 5.1).

Table 5.1. CDR amino acid substitutions and peptide-HLA binding properties of affinity-enhanced gp100/A2 specific TCR[a]

TCR[b]	Clone no.	CDR3α (110-129)	CDR2β (68-73)	CDR3β (111-131)	K_{on} ($M/s^{-1}x1000$)	K_{off} (s^{-1})	K_D (μM)	$t_{1/2}$ (s)
wt		ATDGDTPLVFGKGTRLSVIA	SQIVND	ASSIGGPYEQYFGPGTRLTVT	34	0.635	18.5	1.10
1	**ADB97**	S M			125	0.99	7.9	0.70
2	**ADB98**	S MQ			197	0.79	4.0	0.88
3	**ADB217**			F A	75	0.194	2.6	3.60
4	**ADB243**			Y A	65	0.122	1.9	5.70
5	**ADB242**			W A	74	0.082	1.1	8.50
6	**ADB212**		W TG		275	0.0683	0.402	10.2
7	**ADB241**		WGT		325	0.0115	0.035	60.3
8	**ADB213**		WG G		304	0.0078	0.026	89.2

[a] The wt TCR consists ofTRAV17*01/J29*01/TRAC and TRBV19*01/D1/J2*07/TRBC genes. Affinity-enhanced TCR protein variants were obtained from phage libraries of expressed TCR genes with CDR amino acid substitutions following selections with $gp100_{280-288}$ peptide-HLA-A2. TCR genes were cloned and produced as soluble, disulfide-linked heterodimeric TCR proteins. Binding kinetics of soluble TCR proteins were measured using biotinylated peptide-HLA-A2 antigen immobilized to a streptavidin-coated flow cell in a BIAcore 3000 (see materials & methods section for details).

[b] Affinity enhanced TCRs were numbered 1 to 8 according to decreasing K_D values.

Soluble TCRs and Surface Plasmon Resonance measurements

Soluble proteins of wt and affinity-enhanced TCR variants were produced as disulfide-linked heterodimeric TCRs (126). Surface Plasmon Resonance (SPR) analysis of TCRs was performed essentially as described elsewhere (153). Briefly, binding kinetics of soluble TCRs were measured using biotinylated $gp100_{280-288}$/HLA-A2 (233) immobilized to a streptavidin-coated flow cell in a BIAcore 3000 and performing 60s injections (20 μl/min). For TCRs with half-lives ($t_{1/2}$) of <9s, binding at equilibrium was recorded against gp100/A2 concentrations in μM. K_D values were determined by non-linear curve fitting using Graphpad Prism, whereas K_{on} values were calculated using $K_{on}=K_{off}/K_D$. For TCRs with $t_{1/2}$>9s, K_{off} values were determined by dissociation fitting using BIAevaluation, and K_{on} values were fitted to the 1:1 Langmuir binding model using BIAevaluation, and K_D values were calculated using $K_D=K_{off}/K_{on}$. For TCRs with $t_{1/2}$>50s, binding of gp100/A2 was fitted to a single cycle kinetics model again using BIAevaluation. Affinity enhanced TCRs were numbered 1 to 8 according to decreasing K_D values (table 5.1).

TCR constructs

TCRs were selected from phage libraries and, in soluble format, characterized using surface plasmon resonance. Genes for both the α and β chains of wt TCR and affinity-

enhanced TCR variants were codon-optimized for expression in human cells (Geneart, Regensburg, Germany) and sub-cloned into the pMP71 retroviral vector with the TCRα and TCRβ joined together as a single open reading frame intervened by a 2A skipping sequence preceded by codons encoding a furin protease cleavage site (i.e. pMP71:TCRα-2A-TCRβ; (234)). All constructs were confirmed by DNA sequencing.

Retroviral gene transfer into T cells

Co-cultures of the packaging cells 293T and Phoenix-A were used to produce Moloney Murine Leukemia retroviruses. Packaging cells were calcium phosphate-transfected with pMP71:TCRα-2A-TCRβ and the helper vectors: pHIT60 and pCOLT-GALV. Transduction of human T cells was performed as described previously (43).

Flow cytometry

TCR-engineered primary human T cells were washed with PBS and incubated with gp100/A2 peptide-HLA-tetramers for 15 min at RT or incubated with CD3ε-FITC, CD8α-PerCP, CD45RA-PE-Cy7, CD27-APC-Cy7, CD28-APC, CCR7-PE, CD107a-PE, and/or CD137-APC mAbs (up to 6 colors simultaneously) for 30 min on ice. Next, T cells were washed and fixed with 1% PFA (Brunschwig, Amsterdam, the Netherlands). CD107a expression was detected as described in chapter 3. Briefly, $2x10^5$ T cells were re-suspended in a mixture of T cell medium, Golgistop and anti-CD107a mAb. Next, tumor target cells and T cells were mixed in a 1:1 ratio and incubated for 2h at 37 °C and 5% CO_2. T cells were analyzed by flow cytometry using a FACSCalibur or a FACSCanto cytometer (BD Biosciences), equipped with Cell Quest or FACS DIVA software (BD Biosciences), respectively. Viable lymphocytes were gated according to forward and side scatter properties and data were analyzed within CD3+ or CD3+/CD8+ dual-positive T cells. Sets of T cell differentiation markers to define naïve, central memory, effector memory and end-stage T cells are provided in figure 5.7.

Measurement of cytokines

T cell release of cytokines was determined as described previously (131). BLM cells loaded without or with titrated amounts of gp100 peptide and a panel of melanoma cell lines were used as target cells. T cells ($6x10^4$) were incubated overnight with target cells in a 3:1 ratio and tested in triplicate. IFNγ, IL-2 or TNFα levels present in supernatants were determined using a human IFNγ (Pelikine compact, Sanquin), IL-2 (U-cytech biosciences, Utrecht, The Netherlands) or TNFα (Pelikine compact, Sanquin) ELISAs, respectively, and a Titertek plus ELISA reader (Merlin, Breda, Netherlands) according to the manufacturer's instructions.

T cell counts

T cells were counted when harvested from feeder cultures and after 4 days of culture in IL-2 supplemented medium. Trypan blue exclusion was applied to determine T cell viability and viable T cells were counted microscopically using Bürker chambers and a LeitzLaburlux 12 microscope (Leica Geosystems BV, Rijswijk, The Netherlands).

Statistical analyses

Differences between affinity-enhanced and wt TCR or between APL and gp100 wt peptide were assessed for their significance with Student's *t*-tests (unpaired; two-tailed, unless stated otherwise) using Graphpad Prism4 software. Differences with p-values <0.05 were considered significant.

RESULTS

Amino acid substitutions in CDR2β and CDR3α, but not CDR3β, resulted in enhanced binding of peptide-HLA by TCR-engineered T cells

Affinity-enhanced TCR variants were selected for gp100 peptide:HLA-A2 complex binding from phage libraries displaying TCRs with amino acid substitutions either in CDR2β, 3α or 3β. We obtained and characterized 8 TCR variants with affinities that were 2 to 712-fold higher when compared to the parental wt TCR. TCRs were numbered 1 to 8 according to their K_D values (range: 7.9 to 0.026 μM), which grouped affinity-enhanced TCR variants according to the CDR that contained amino acid substitutions (table 5.1). TCR gene transfer in primary human T cells and flow cytometric analysis of TCRβ transgene expression revealed similar cell surface expression levels for all introduced TCRs, except for TCR 6 and 8 (*supplementary fig. 5.1*). The latter two TCRs lost the epitope recognized by anti-TCR-Vβ19 mAb due to CDR2β amino acid substitution(s) as also observed in reports of affinity-enhanced NY-ESO-1/HLA-A2 TCRs (92), and is probably related to an asparagine to glycine substitution (i.e., N72G). Analyses of binding of titrated amounts of gp100/A2 peptide-HLA tetramers revealed significantly higher percentages and Mean Fluorescence Intensities (MFI) with T cells transduced with TCRs 6 to 8 when compared to T cells transduced with wt TCR (fold decrease in K_D: 46 to 712). It is noteworthy that the observed ability of TCR6 and 8 to bind gp100/A2 peptide-HLA tetramers provides phenotypical evidence for the surface expression of these two TCRs (figs. 5.1A and B). Interestingly, TCRs 1 and 2 (fold decrease in K_D: 2 to 5) result in significantly increased MFI values, whereas TCRs 3 to 5 (fold decrease in K_D: 7 to 17) did not result in improved binding of gp100/A2 peptide-HLA complexes.

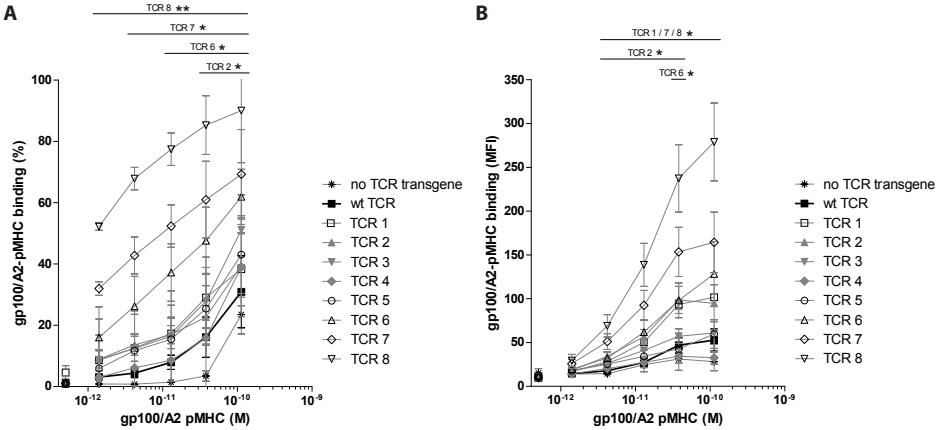


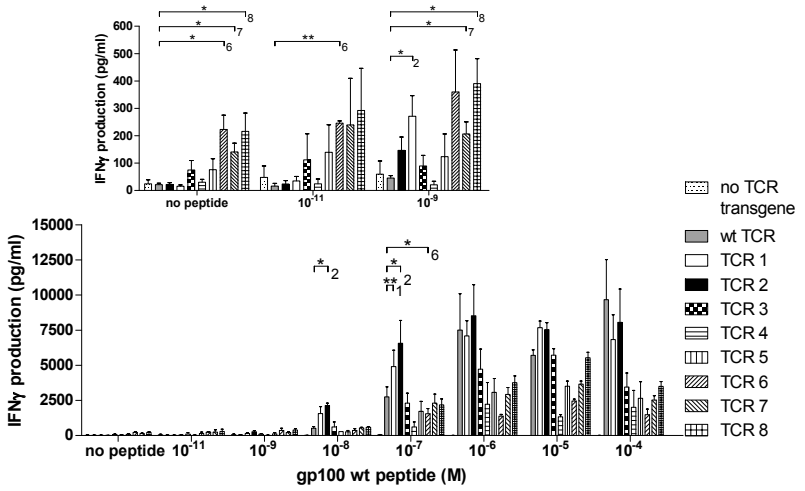
Figure 5.1. Primary human T cells showed increased binding of peptide-HLA when expressing TCRs with enhanced affinity. Primary human T cells were transduced with empty virus particles (no TCR transgene), a gp100/A2 specific wt TCR or affinity-enhanced TCR variants (TCRs 1 to 8; see table 5.1). Gene-engineered T cells were stained with titrated amounts of gp100/A2 peptide-HLA tetramer (range: 10^{-12} to 10^{-10} M) and analyzed by flow cytometry. **(A)** Percentages (%) and **(B)** Mean Fluorescence Intensities (MFI) of peptide-HLA tetramer binding is presented as curves connecting mean values +/- SEM for each TCR, n=4 independent measurements. In (A) and (B) data of wt TCR are presented as a bold line. Similar results were obtained with a second healthy donor (data not shown). Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: * = p<0.05; ** = p<0.005.

5

TCRs with a lower K_D limit of 4 μM and amino acid substitutions in CDR3α demonstrated improved antigen-specific T cell functions

To investigate how TCR affinity relates to T cell sensitivity, we tested T cell IFNγ release following stimulations with titrated amounts of gp100 wt peptide. Findings are presented in figure 5.2 and demonstrated that TCRs 1, 2, 6, 7 and 8 mediate enhanced IFNγ release when compared to wt TCR at the lower end of peptide concentrations used (10^{-9}, 10^{-8} and 10^{-7} M of wt peptide). Notably, TCRs 1 and 2 were the only TCRs mediating antigen-specific IFNγ release, i.e., showing no IFNγ release in the absence of peptide. TCRs 3 to 5 showed no benefit when compared to wt TCR. In fact, these TCRs, as well as TCRs 6 to 8, mediated a decreased IFNγ release when compared to wt TCR at the higher end of peptide concentrations used (10^{-6}, 10^{-5}, and 10^{-4} M of wt peptide) (fig. 5.2A). The half-maximal effective concentrations (EC_{50}), used as measures for T cell sensitivity, revealed 2.3 and 4.4-fold increases in sensitivity for TCRs 1 and 2, respectively, when compared to wt TCR (fig. 5.2B). Although not entirely antigen-specific, TCRs 6 and 7 revealed 2.2-fold increases in EC_{50}, and, most notably, TCR 8 mediated a 1.4-fold increase. Analysis of CD107a surface expression in response to stimulation with titrated amounts of gp100 wt peptide revealed similar differences among the different TCRs, although CD107a sur-

A



B

TCR variant	wt TCR	TCR 1	TCR 2	TCR 3	TCR 4	TCR 5	TCR 6	TCR 7	TCR 8
EC_{50} (M)	$1.1x10^{-7}$	$0.47x10^{-7}$	$0.25x10^{-7}$	$1.16x10^{-7}$	$1.08x10^{-7}$	$0.98x10^{-7}$	$0.50x10^{-7}$	$0.51x10^{-7}$	$1.5x10^{-7}$

Figure 5.2. TCRs 1 and 2 with a lower K_D limit of 4 µM mediate enhanced sensitivity of antigen-specific IFNγ release. Primary human T cells transduced with wt or affinity-enhanced TCRs 1 to 8, as described in the legend to figure 5.1, were tested for their ability to produce IFNγ following stimulation with BLM melanoma cells that were either unloaded (no peptide) or loaded with titrated amounts of gp100 wt peptide (range: 10^{-11} to 10^{-4} M). **(A)** IFNγ was measured in supernatants from overnight cultures by ELISA and expressed as pg/ml and **(B)** half maximal effective concentrations of gp100 wt peptide (EC_{50}) were calculated with 4-parametric logistics using Masterplex ReaderFit software. Bars represent mean values + SEM, n=2 to 6 independent measurements. Similar results were obtained with a second healthy donor (data not shown). Statistically significant differences relative to wt TCR were calculated with paired Student's *t*-tests: * = p<0.05, ** = p<0.005.

face expression appeared less sensitive and hence less discriminative than IFNγ release (*supplementary fig. 5.2*).

We next questioned whether the observed changes for TCR variants in T cell sensitivity were related to loss of CD8αβ dependency. To address this issue, we first analyzed the ability of TCR variants to bind gp100/A2 peptide-HLA in the absence of CD8αβ (using $CD4^+$ T cells). Experiments demonstrated that enhanced TCR affinity did not lead to a proportional increase of CD4 T cells that bind peptide-HLA tetramers (fig. 5.3A). In fact, the proportion of CD4 T cells binding peptide-HLA is fairly constant and similar to the proportion of total CD4 T cells following transduction with individual TCR variants. Second, we analyzed the ability of the TCR variants to mediate IFNγ release in the absence of HLA-A2:CD8αβ binding. To this end, C1R-227/228KA cells that harbor mutated HLA-A2 and are unable to bind CD8α (196) and wt C1R cells were loaded with gp100 wt peptide and used in T cell stimulation assays. All TCRs, including the wt TCR, triggered IFNγ releases that were independent of the presence of CD8αβ binding (fig. 5.3B). Collectively,

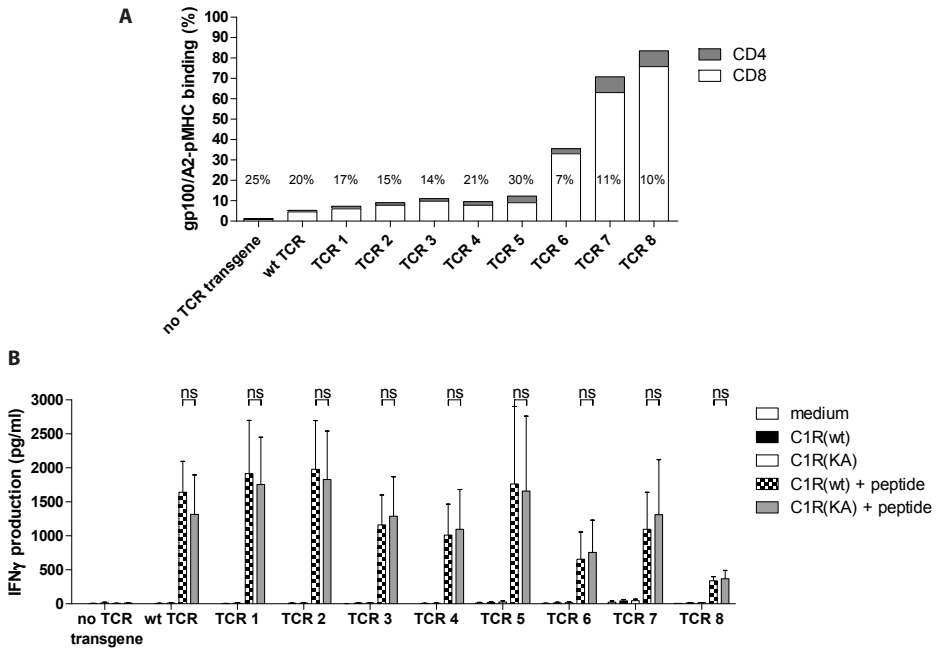


Figure 5.3. Affinity-enhanced TCRs bind peptide-HLA and mediate IFNγ production independently of CD8. Primary human T cells transduced with wt or affinity-enhanced TCRs were **(A)** stained with both gp100 peptide-HLA-A2 and CD4 mAb and **(B)** tested for IFNγ production following stimulation with gp100 wt peptide-loaded C1R and C1R-KA cells (final concentration of peptide: 10^{-5} M) (IFNγ levels expressed in pg/ml). In (A) stacked and grey bars show mean % of total T cells and CD4 T cells that bind gp100/A2 peptide-HLA, respectively, n=4 independent measurements. For reasons of simplicity, SEM values (in all cases less than 10% of mean values) have been left out of figure. The % of CD4 T cells within total population of T cells that bind gp100/A2 peptide-HLA has been indicated above the stacked bars. In (B) bars represent mean values + SEM, n=3 independent measurements. Similar results as those presented in (A) and (B) were obtained with a second healthy donor. Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: ns = non-significant.

these data suggest that the wt TCR, and consequently also the affinity-enhanced TCR variants, are able to bind peptide-HLA and activate T cells independently of CD8αβ.

TCRs with a lower K_D limit of 4 μM and amino acid substitutions in CDR3α retained peptide-fine specificity

Next, we studied how TCR affinity relates to T cell specificity. To this end, we tested T cell IFNγ release following stimulations with gp100 altered peptide ligands (APL) containing alanine or glycine substitutions, as described by Schaft and colleagues (132). Wild type TCR recognized, in addition to wt gp100 peptide, APLs A1, A2, A4, A7, A8 and G9 (fig. 5.4). Although APL A2 resulted in a significant increase in IFNγ release compared to medium stimulation, at the same time it also resulted in a clear and significantly reduced

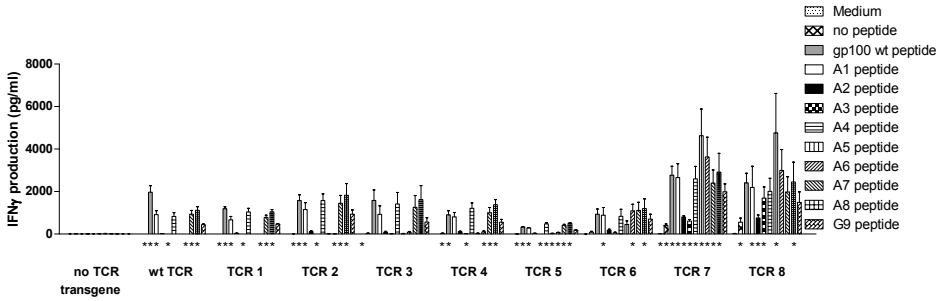


Figure 5.4. TCRs 1 and 2 with a lower K_D limit of 4 µM preserve peptide-fine specificity of wt TCR. Primary human T cells transduced with wt or affinity-enhanced TCRs were tested for IFNγ release following stimulation with BLM melanoma cells that were loaded with altered peptide ligands (APL, final concentration: 10^{-5}M). APLs are coded by the letters A (alanine) or G (glycine) followed by a number, which corresponds to the amino acid and position that is altered in the wt gp100 peptide (YLEPGPVTA). T cell stimulations with wt gp100 peptide or no peptide were included as controls. Bars represent mean values + SEM, n=3 to 5 independent measurements. Statistically significant differences relative to medium were calculated with paired Student's *t*-tests. For reasons of simplicity, significant responses towards APLs (p-values < 0.05) are indicated by asterisks and placed below the bars. Note that APL responses for wt TCR are all significant, except for A3, A5 and A6. This APL response is only preserved for TCRs 1 and 2, and non-significant responses have reached significant in case of TCRs 6 to 8. P-values < 0.005 were observed for responses of wt TCR to G9; TCR 1 to wt peptide, A7, A8, and G9; TCR 5 to wt peptide, A1, A2, A4, A7, A8, and G9; and TCR 7 to A2 and A3.

IFNγ release compared to wt peptide. The wt TCR did not recognize APLs A3, A5 and A6 (fig. 5.4). TCRs 1 and 2 were the only TCR variants that, identical to the wt TCR, recognized all APLs except A3, A5 and A6, and also showed a significantly lowered A2 response. TCRs 6 to 8, and to a lesser extent TCRs 3 to 5, lost peptide-fine specificity and were able to recognize APL A3, A5 and A6, with highest IFNγ levels produced upon stimulations with A5 and A6.

TCRs with K_D values lower than 1 µM and amino acid substitutions in CDR2β resulted in peptide-independent but HLA-A2-dependent reactivity towards melanoma cells

To follow up on our findings with respect to sensitivity and specificity, we tested T cell IFNγ release following co-culture with a panel of melanoma cell lines. In line with our findings with gp100 wt peptide and APLs, TCRs 1 and 2 significantly enhanced IFNγ responses in co-culture with various gp100/A2-positive melanoma cells when compared to wt TCR, but did not respond to melanoma cells lacking either HLA-A2 or gp100 expression (fig. 5.5). TCRs 3 to 5 did not result in improved IFNγ responses, but rather showed a trend of reduced IFNγ release when stimulated with gp100/HLA-A2-positive melanoma cells. In addition, TCRs 3 and 5 mediated minor, but significant, reactivity towards gp100-negative and HLA-A2-positive melanoma targets. TCRs 6 to 8 produced

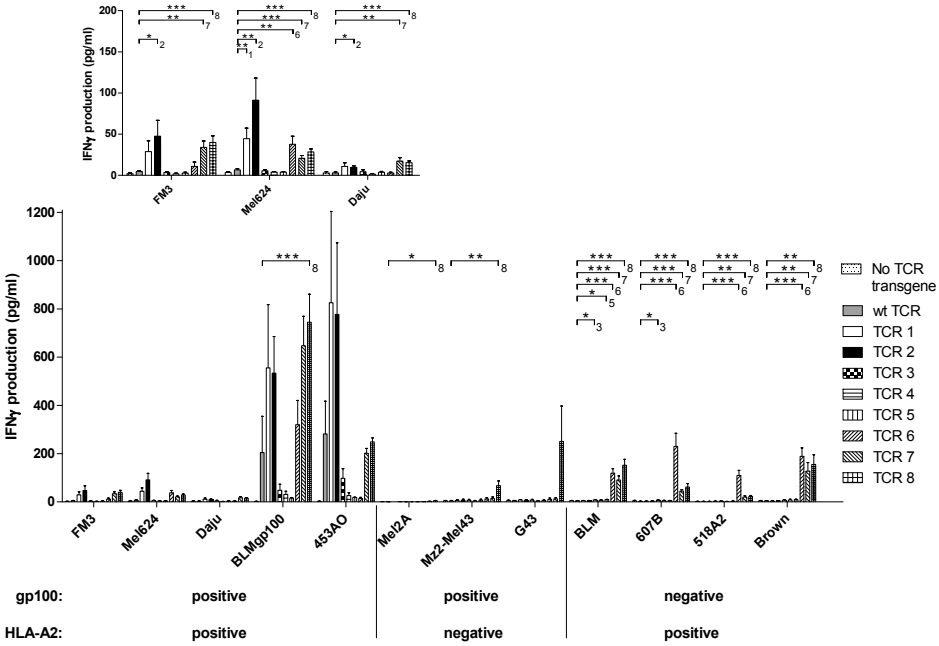


Figure 5.5. TCRs 1 and 2 with a lower K_D limit of 4 µM result in enhanced reactivity towards antigen-positive but not antigen-negative tumor cells. Primary human T cells gene-engineered with wt and affinity-enhanced TCRs were tested for IFNγ release following stimulation with melanoma cell lines that were either positive or negative for the gp100 antigen and/or HLA-A2. The presence or absence of gp100 and HLA-A2 is indicated below the x-axis. Bars represent mean values + SEM, n=4 to 11 independent measurements. Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: * = p<0.05, ** = p<0.005, *** = p<0.0005.

high and significantly enhanced IFNγ responses towards gp100/HLA-A2-positive melanoma cells, in some cases even more pronounced when compared to TCRs 1 and 2, but resulted in similarly high and significant responses to gp100-negative and HLA-A2-positive melanoma cells. Identical responses for the TCR variants were observed when measuring IL-2 and TNFα release (*supplementary figs. 5.3A and B*).

CDR2β-mutated TCRs with K_D values lower than 1 µM mediate constitutive activation when expressed in HLA-A2-positive T cells

The observed HLA-A2-driven reactivity of high affinity TCRs may prevent the use of these TCRs in a therapeutic setting. To address this issue, we have transduced TCRs 1-8 in both HLA-A2-negative as well as HLA-A2-positive and gp100-negative peripheral blood T cells. In a first series of experiments, we tested (constitutive) surface expression of the T cell activation markers CD107a and CD137 under standard culture conditions. We observed only marginal expression of both CD107a and CD137 in HLA-A2-negative pri-

mary human T cells, which appeared independent of the TCR variant used (figs. 5.6A and B). In contrast, we observed significantly increased expression of CD107a and CD137 for TCR 6 to 8 in HLA-A2-positive T cells, suggesting reactivity towards HLA-A2-positive neighboring T cells. Next, we analyzed a panel of T cell differentiation markers, including CD45RA, CD27, CD28 and CCR7 (77, 235), previously used by our group to assess the differentiation status of gp100/A2 TCR-engineered human T cells (236). CD8-positive T cell

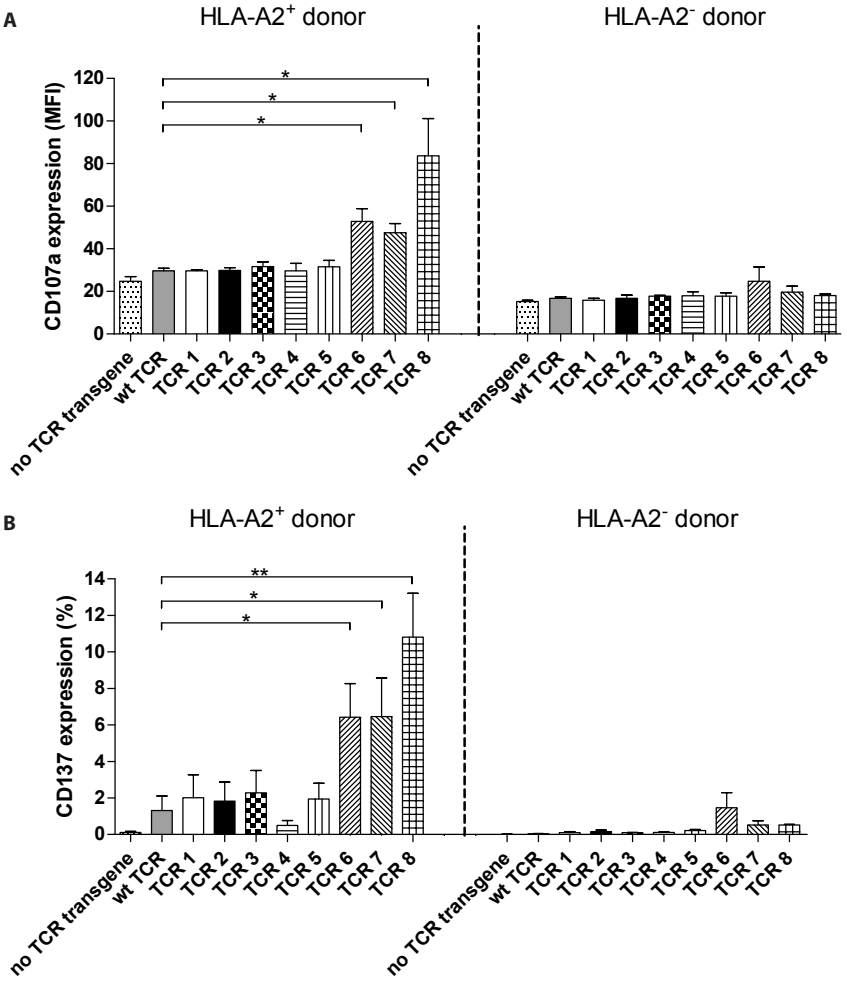


Figure 5.6. HLA-A2-positive T cells expressing TCRs with K_D values lower than 1 µM constitutively up-regulate expression of CD107 and CD137. HLA-A2-positive and negative primary human T cells were gene-engineered with wt and affinity-enhanced TCRs analyzed by flow cytometry for (**A**) CD107a expression (MFI) on CD3-positive T cells and (**B**) CD137 (%) on CD3-positive T cells that bound gp100/A2 peptide-HLA. Bars represent mean values + SEM, n=3 to 5 independent measurements. Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: * = $p<0.05$, ** = $p<0.005$.

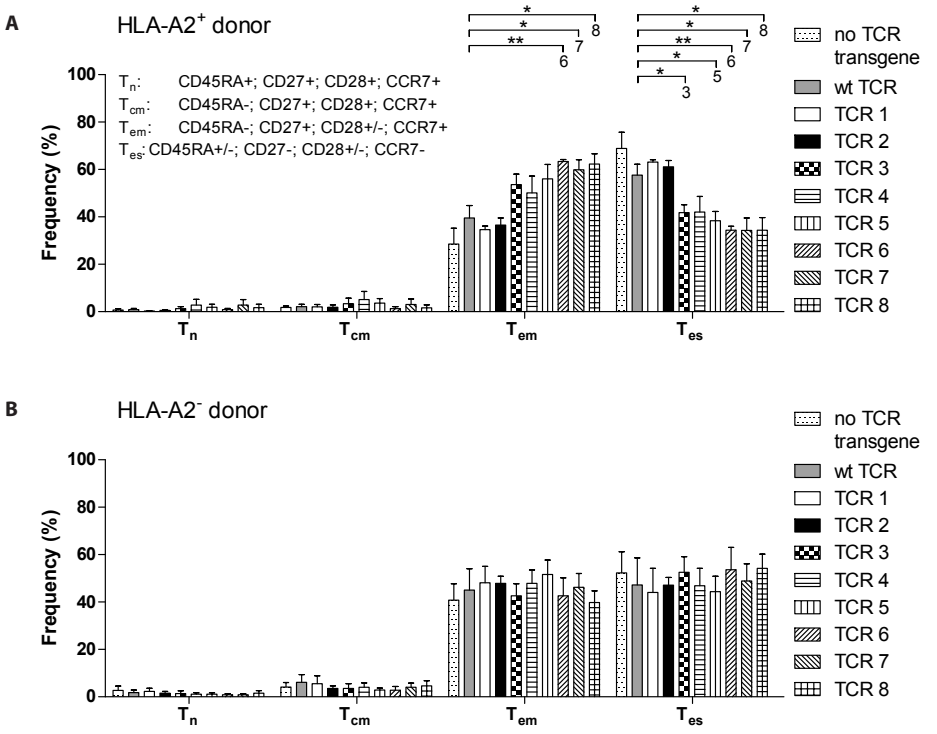


Figure 5.7. HLA-A2-positive T cells expressing TCRs with K_D values lower than 1 µM show enhanced numbers of effector memory CD8 T cells. Primary human T cells gene-engineered with wt and affinity-enhanced TCRs being (**A**) HLA-A2 positive or (**B**) HLA-A2 negative were stained for CD3, CD8, CD27, CD28, CD45RA, and CCR7 and expression levels were measured by flow cytometry. Naïve T cells (T_n), central memory T cells (T_{cm}), effector memory (T_{em}), and end-stage T cells (T_{es}) are defined by a combination of markers mentioned in inserts (see also (235, 236)). Sum of fractions of T cells defined by these markers (range: 71-94% of CD3, CD8-positive T cells) was set to 100%. Bars represent mean values + SEM, n=4 independent measurements. Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: * = $p<0.05$, ** = $p<0.005$.

populations showed, irrespective of HLA-A2 status, low fractions of naïve (T_n) and central memory T cells (T_{cm}) and high fractions of effector memory (T_{em}) and end-stage T cells (T_{es}) (figs. 5.7A and B, see inset for a definition of T cell subsets). T cells engineered with wt TCR, TCR 1 or 2 revealed similar fractions of T_{em} and T_{es} in both HLA-A2-positive and negative T cells. TCRs with nM affinities, however, mediated significant increases in the fractions of T_{em} and decreases in the fractions of T_{es} in HLA-A2-positive but not negative T cells. In addition to mediating an enhanced fraction of T_{em}, TCRs 6 to 8 also mediated decreased T cell growth in HLA-A2-positive T cells, which was only observed for TCR 6 but not for TCRs 7 and 8 in HLA-A2-negative T cells (*supplementary fig. 5.4*).

DISCUSSION

In this study, we have determined the antigen sensitivity and specificity of affinity-enhanced TCR variants, specific for gp100$_{280-288}$/HLA-A2, that harbor amino acid substitutions restricted either to CDR2β, 3α or 3β (table 5.1). With this panel of TCR variants, we were able to demonstrate that (I) amino acid substitutions in CDR2β and 3α, and K_D values between 18.5 and 4 μM and below 1 μM, enhanced binding of peptide-HLA by T cells; (II) amino acid substitutions in CDR3α, and K_D values between 18.5 and 4 μM, resulted in enhanced tumor-specific T cell functions without loss of antigen- and peptide-fine specificity; and (III) amino acid substitutions in CDR2β, and K_D values lower than 1 μM, resulted in antigen-independent activation and differentiation of T cells from an HLA-A2-positive donor.

Not all affinity-enhanced gp100/A2 reactive TCRs demonstrated an improved binding of peptide-HLA by T cells (fig. 5.1). In fact, TCRs with K_D values between 1-4 μM, in particular when containing a change of a proximal isoleucine to tyrosine in CDR3β (table 5.1; TCR 4: IGG into YGA), adversely affected peptide-HLA binding and T cell function (figs. 5.1B and 5.2). This observation argues that affinities of soluble TCRs do not simply translate to avidities of TCR-transduced T cells, which is governed not only by the TCR and its cellular surface density but also by accessory, co-receptor and adhesion molecules. Moreover, we observed that optimal functional T cell avidity towards gp100/A2-positive target cells, as measured by IFNγ release responses towards titrated amounts of peptide and native melanoma cell lines (figs. 5.2 and 5.5), was conferred by TCRs that harbored CDR3α amino acid substitutions with affinities no lower than 4 μM. In particular TCR 2 revealed decreased EC_{50} values of peptide responses and enhanced levels of IFNγ, IL-2 and TNFα release in response to antigen-positive melanoma cells when compared to wt TCR (figs. 5.2B and 5.5, and *supplementary figs. 5.3A and B*). Enhanced antigen-specific T cell responses were less apparent when looking at T cell cytotoxicity (*supplementary fig. 5.2*), which may be in agreement with the notion that T cell IFNγ release is a more sensitive readout than T cell cytotoxicity (237). Together, these data suggest that CDR amino acid substitutions that improve peptide-HLA binding as measured by BIAcore do not necessarily provide improved T cell function, and extend previous reports showing a lack of correlation between peptide-HLA binding of T cells and functional avidity of T cells (91, 92).

A pertinent question is whether TCR 2 with a K_D of 4 μM represents a functional threshold for affinity enhancement. It is hypothesized that affinities above a pre-set threshold, reflected by K_D values around 6 μM, induce thymocyte selection and activation at a same rate resulting in an 'all-or-nothing' quality of TCR/CD8 associations and TCR-mediated signaling (89, 238). As for thymocytes, activation of cytotoxic T cells may also require an optimal dwelling time between TCR and peptide-HLA (239). Studies

with primary human T cells transduced with affinity-enhanced TCRs directed against NY-ESO-1/HLA-A2 (240) or gp100/HLA-A2 (this report) point to the existence of a K_D threshold of around 4 to 5 μM. TCR 2 contained a triple amino acid substitution in its CDR3α that was centered around a leucine (table 5.1: DLV into SMQ). Notably, Sami and colleagues have demonstrated that leucine substitutions in CDR3α may favour interactions with CDR1α and 2α residues such that the overall stability of the TCR:peptide-HLA complex is enhanced (241), a scenario that may account for the properties found for TCR 2. Functional support for the existence of an affinity threshold comes from studies by Carreño and colleagues, who demonstrated that APLs responsible for low half-lives of TCR:peptide-HLA interactions, in analogy to self-peptide-HLA ligands, are able to induce T cell polarization but not assembly of functional synapses (242). Consequently, these low affinity interactions only result in partial proximal TCR signaling that is sufficient to deliver tonic signals essential for T cell persistence (89). Foreign peptide-HLA ligands, on the other hand, are of high affinity and are able to promote the assembly of functional synapses and result in complete proximal TCR signaling that is sufficient to evoke T cell effector functions (89, 242).

A second question is why T cell functions for TCRs with K_D values lower than 4 μM become compromised (as depicted in figure 5.2). This appears to be counterintuitive as high-affinity TCRs are generally expected to result in a decreased requirement for CD8α and enhanced T cell responsiveness (92, 153, 224, 228, 231). Using our panel of TCR variants, we were neither able to show different binding of gp100 peptide-HLA nor IFNγ release of T cells in the absence of an interaction between CD8αβ and HLA-A2 (fig. 5.3). We cannot exclude, however, that titrated levels of peptide-MHC-tetramers or gp100 wt peptide in these assays would potentially provide a window of CD8 dependency. An explanation for the compromised T cell functions may come from the off rate constant (K_{off}). The decrease in K_D values in our panel of TCRs, with K_D defined as the ratio between K_{off} and on rate constant (K_{on}), appears to be predominantly a consequence of decreased K_{off} values rather than increased K_{on} values (table 5.1). TCRs 6 to 8 show the lowest K_{off} values (on average: 0.0301/min) and may be less prone to dissociate from peptide-HLA, which may decrease the ability of the TCR to be serially triggered and consequently decrease the ability of the T cell to respond to antigen. Along this line it is noteworthy that lowered antigen-specific T cell functions, as mediated by TCRs 6 to 8, could not be compensated by higher peptide densities. In addition to our *in vitro* studies, *in vivo* studies have demonstrated that high-affinity TCR:peptide-HLA interactions are not a prerequisite for T cell activation, but rather required for sustained T cell expansion (241). Importantly, intermediate-affinity rather than high-affinity ligands provided optimal T cell responses to immunization, observed for both CD8-positive T cells (K_D values for intermediate affinity ligands around 3 μM) (244) and CD4-positive T cells (K_D values for intermediate affinity ligands around 44 μM) (245).

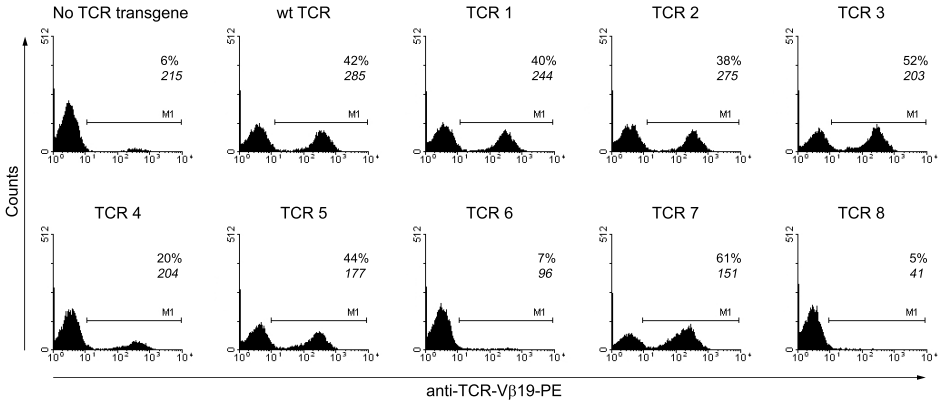
In addition to T cell sensitivity, we assessed the antigen and peptide-fine specificity of affinity-enhanced TCRs. Using APLs derived from the gp100 wt peptide, we demonstrated that the wt TCR was not able to trigger a response towards APL A3, A5 and A6, a recognition pattern that was preserved by TCRs 1 and 2. Since these TCRs contain CDR3α substitutions, and CDR3 amino acid substitutions have been shown to enhance TCR:peptide-HLA interactions specifically by improving peptide binding (241), we speculate that amino acid substitutions in CDR3 are more advantageous when compared to those in CDR2 with regard to retaining peptide-fine specificity. Higher affinity TCRs, however, revealed a gradual loss of peptide-fine specificity and, in particular, TCRs 6 to 8 displayed clear IFNγ responses upon stimulation with APLs A3, A5, and A6 (fig. 5.4). In addition, TCRs 6 to 8 revealed IFNγ, IL-2 and TNFα responses when stimulated with a panel of gp100-negative and HLA-A2-positive native melanoma cells (fig. 5.5 and *supplementary fig. 5.3*). Previous reports have demonstrated that amino acid substitutions in CDR2β may result in reactivity against self or allo-peptide-HLA (92, 224). Thus, TCRs 6 to 8 may show affinities against self-peptides that are in a more optimal affinity range explaining the responsiveness to HLA-A2-positive T cells. Our finding of an affinity perimeter related to self-reactivity, i.e., a K_D less than 1 μM, is in close accordance with previous studies that report upper affinity limits for antigen-specificity of around 0.5-3.0 μM (224, 231). To further assess how data on high affinity TCRs would extend to a therapeutic setting, we transferred our TCRs in PBMC derived from an HLA-A2-positive donor. TCRs 6 to 8 confer significant T cell activation and differentiation in the absence of target cells (figs. 5.6 and 5.7). Notably, TCRs 6 to 8 mediated minor cytotoxic T cell activity (about 20%) when using non-transduced HLA-A2-positive T cells as target cells (data not shown). Collectively, these results show that T cells that express TCRs 6 to 8 display an inherent reactivity towards HLA-A2 that is independent of gp100 peptide and clearly argue against the use of very high affinity TCRs for TCR gene therapy.

Taken together, our studies with affinity-enhanced gp100/HLA-A*0201 TCR demonstrate that perimeters of TCR affinities that determine gain of function (here K_D down to 4 μM) are different from those that determine gain of self-reactivity (here K_D lower than 1 μM), and are in this study best captured by CDR3α amino acid substitution(s), providing a window to generate functionally enhanced and safe therapeutic T cells.

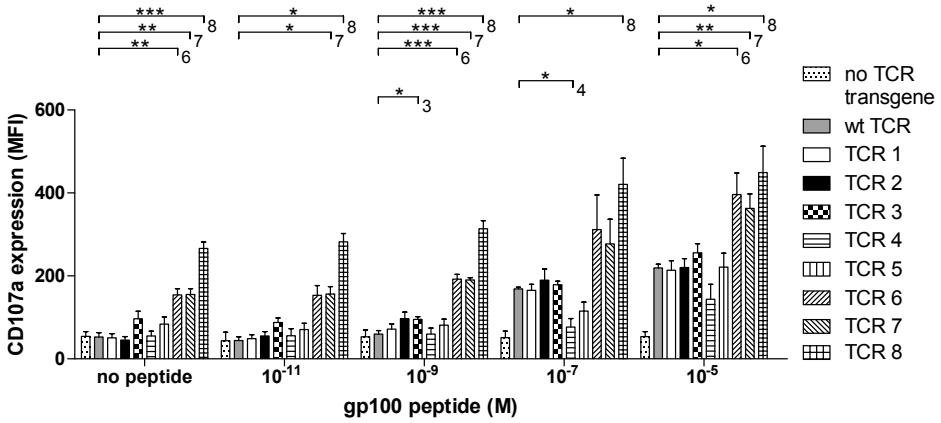
ACKNOWLEDGEMENTS

This work was supported by the European community 6th framework grant (018914) entitled: 'Adoptive engineered T cell targeting to activate cancer killing (ATTACK)'.

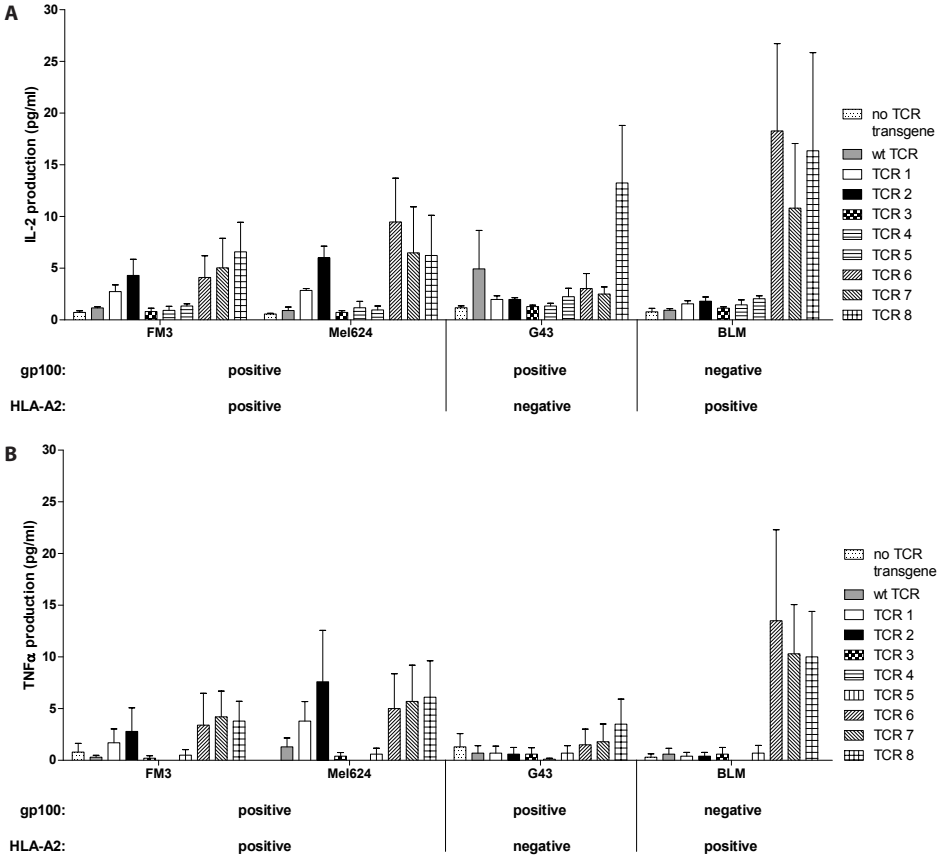
SUPLEMENTARY FIGURES



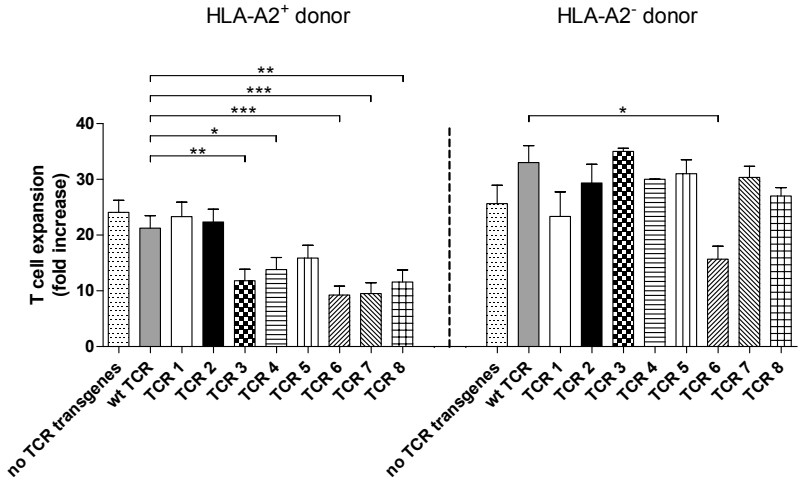
Supplementary figure 5.1. Surface expression of affinity-enhanced TCRs on primary human T cells. Primary human T cells transduced with wt or affinity-enhanced TCRs 1 to 8, as described in the legend to figure 5.1, were stained with anti-TCR-Vβ19-PE mAb and analyzed for surface expression of transgenic TCRβ via flow cytometry. Dot plots from a representative measurement (n=12) are shown including % and MFI values (italic) of TCR-positive T cells that were corrected for corresponding non-stained values (marker M1 set at ~1%). Similar results were obtained with a second healthy donor (data not shown).



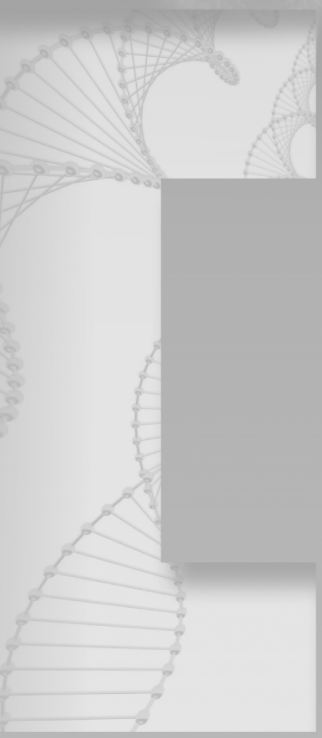
Supplementary figure 5.2. TCRs with K_D values lower than 1 µM mediated enhanced yet antigen non-specific CD107a mobilization. Primary human T cells transduced with wt or affinity-enhanced TCRs were tested for their ability to up-regulate surface expression of CD107a following stimulation with BLM melanoma cells that were either unloaded (no peptide) or loaded with titrated amounts of wt gp100 peptide (range: 10^{-5} to 10^{-11} M). CD107a mobilization was measured via flow cytometry and expressed as MFI of CD107a staining in CD3-positive cells. Bars represent mean values + SEM, n=3-4 independent measurements. Similar results were obtained with a second healthy donor. Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: * = p<0.05, ** = p<0.005, *** = p<0.0005.



Supplementary figure 5.3. TCRs with a lower K_D limit of 4 μM resulted in enhanced IL-2 and TNFα production towards antigen-positive but not antigen-negative tumor cells. Primary human T cells transduced with wt or affinity-enhanced TCRs were tested for **(A)** IL-2 and **(B)** TNFα production following stimulation with melanoma cell lines that were either positive or negative for the gp100 antigen and/or HLA-A2. The presence or absence of gp100 and HLA-A2 is indicated below the x-axes. Bars represent mean values + SEM, n=3 independent measurements.



Supplementary figure 5.4. TCRs with K_D values less than 1 µM mediated reduced T cell growth. HLA-A2-positive and negative primary human T cells were gene-engineered with wt and affinity-enhanced TCRs and assessed for T cell growth. T cells were harvested from feeder cultures and numbers of viable T cells were counted microscopically and expressed as fold increase. Bars represent mean values + SEM, n=3-19 independent measurements. Statistically significant differences relative to wt TCR were calculated with Student's *t*-tests: * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$.



6

Magnetic-Activated Cell Sorting of TCR-engineered T cells using tCD34 as a gene marker, but not peptide-MHC multimers, results in significant numbers of functional CD4 and CD8 T cells

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ABSTRACT

T cell sorting technologies with peptide-MHC multimers or antibodies against gene markers enable enrichment of antigen-specific T cells and are expected to enhance therapeutic efficacy of clinical T cell therapy. However, a direct comparison between sorting reagents for their ability to enrich T cells is lacking. Here, we compared the *in vitro* properties of primary human T cells gene-engineered with $gp100_{280-288}$/HLA-A2 specific TCRαβ upon Magnetic Activated Cell Sorting (MACS) with different peptide-MHC multimers or an antibody against truncated CD34 (tCD34). With respect to peptide-MHC multimers, we observed that streptamers, when compared to pentamers and tetramers, improved T cell yield as well as level and stability of enrichment of TCR-engineered T cells (> 65% of peptide-MHC-binding T cells, stable for at least 6 weeks). In agreement with these findings, streptamers, the only detachable reagent, revealed significant T cell expansion in the 1st week after MACS. Sorting TCR and tCD34 gene-engineered T cells with CD34 mAb resulted in the most significant T cell yield and enrichment of T cells (> 95% of tCD34 T cells, stable for at least 6 weeks). Notably, T cells sorted with CD34 mAb, when compared to streptamer, bind about two to three-fold less peptide-MHC but show superior antigen-specific up-regulated expression of CD107a and production of IFNγ. Multi-parameter flow cytometry revealed that CD4 T cells, uniquely present in CD34 mAb-sorted T cells, contributed to enhanced IFNγ production. Taken together, we postulate that CD34 mAb-based sorting of gene-marked T cells has benefits towards applications of T cell therapy, especially those that require CD4 T cells.

INTRODUCTION

Adoptive therapy with tumor-infiltrating T cells, preceded by lymphodepletion, shows significant clinical responses in melanoma patients (27, 111). In an effort to make T cell therapy a more universally applicable treatment, T cells have been gene-engineered to express virus and tumor-specific T cell receptors (TCR). T cells transduced with TCRs directed against the HLA-A2-restricted antigens MART-1, gp100, CEA or NY-ESO-1 have been tested in clinical trials and clinical responses have been observed in patients with metastatic melanoma, colorectal and synovial carcinoma (32, 40, 45, 46). Clinical responses, although variable and based on a relatively small number of patients, are promising but generally lag behind those observed with tumor-infiltrating T cells (27, 111). The clinical use of high-affinity TCRs enhances response rates and may provide a means to improve clinical responses (32, 45). However, high-affinity TCRs, when directed against differentiation antigens that are over-expressed on tumors but also present, albeit to a small extent, on normal tissues, result in on-target toxicities (40, 45). An alternative strategy to enhance functional responses of T cells is to enhance the frequency of TCR-transduced T cells. The threshold antigen concentration for T cell activation correlates inversely with the level of TCR expression in T cell populations (246, 247), providing a rationale for the enrichment of TCR-transduced T cells prior to their clinical use. T cell populations used in TCR gene therapy studies generally demonstrated a peptide-MHC binding of about 62%, being in some cases as low as 3% and in some cases as high as 97%, which presents a window for further improvement (32, 45, 46).

Magnetic Activated Cell Sorting (MACS) was developed from the late seventies onwards (248, 249) and now represents an established technology to enrich cells that is compatible to conditions of Good Medical Practice and can be applied in clinical cellular therapy trials (250-252). When MACS is used in combination with peptide-MHC multimers, this technology provides a versatile and potent platform to enrich T cells with a defined antigen-specificity. In example, T cells specific for multiple antigens, such as CMV, EBV1-3, Flu1 and MART-1 antigens, can be enriched using peptide-MHC tetramers from a single sample (253). Also, MART-1/HLA-A2-specific T cells have been enriched up to a 1000-fold from Peripheral Blood Mononuclear Cells (PBMCs) and tumor infiltrating lymphocytes from patients with metastatic melanoma without loss of *in vitro* reactivity (254). Currently, various forms of peptide-MHC multimers exist of which tetramers (180), pentamers (63), and streptamers (255, 256) represent the ones most extensively characterized. These peptide-MHC multimers differ in avidity and reversibility of binding to T cells (as detailed in table 6.1) and it is currently not known how these reagents compare to one another with respect to MACSort of T cells. In addition to peptide-MHC multimers, a surrogate gene marker such as truncated (t)CD34 (257) can be applied to enrich T cells with CD34 mAb-based MACS (258). Co-transduction of tCD34 and a Herpes Simplex

Table 6.1. Reagents used to MACSort TCR-engineered T cells[a]

Sort reagent	Label	2nd step sort reagent	TCR transgenes	Concentration[f] (ng/10^7 T cells)	pMHC valency	Detachable binding	Key reference(s)
gp100/A2 tetramer[b]	R-Phycoerythrin	Anti-PE microbeads	TCRαβ	700	4	No	(180)
gp100/A2 pentamer[c]	R-Phycoerythrin	Anti-PE microbeads	TCRαβ	500	5	No	(63)
gp100/A2 streptamer[d]	R-Phycoerythrin	Anti-PE microbeads	TCRαβ	200	8-12	Yes[g]	(255, 256)
CD34 mAb microbeads[e]	-	-	TCR-tCD34	N.D.	None	No	(258)

[a] Overview of specific characteristics of different sort reagents to enrich TCR-engineered T cells. N.D.: Not Determined.
[b] Tetramers contain 4 biotinylated peptide-MHC monomers, which are multimerized with streptavidin-PE to form a tetrahedral complex. A maximum of 3 peptide-MHC molecules are available per focal plane (260).
[c] Pentamers contain 5 peptide-MHC-PE monomers, which are multimerized through a coiled-coil structure. All 5 peptide-MHC molecules are available per focal plane (260).
[d] Streptamers contain between 8-12 streptagged peptide-MHC monomers, which are multimerized with streptactin-PE.
[e] CD34 mAb is directly coupled to magnetic microbeads.
[f] Concentrations of peptide-MHC multimers are based on molecular weight provided by manufacturers
[g] Release of peptide-MHC monomers is established by addition of an excess of D-biotin that has an high affinity for streptactin (K_d value of ~10^{-13} M; (256)).

Virus thymidine kinase (HSVtk) suicide gene in T cells, to allow ganciclovir-mediated elimination of alloreactive gene-modified T cells, and subsequent MACS resulted in T cell populations that were highly enriched for both tCD34 and HSVtk (259).

In this paper, we have compared tetramers, pentamers, streptamers and CD34 mAb for their ability to enrich TCR-engineered T cells (figure 6.1 provides a schematic representation of sort reagents used in our study). To this end, we gene-transduced human T cells with a $gp100_{280-288}$/HLA-A2 (gp100/A2) TCR and tested the different sort reagents for T cell output numbers, T cell yield at later time points, T cell expansion, enrichment for peptide-MHC binding and gp100/A2-specific functions. With respect to peptide-MHC multimers, streptamers significantly enhanced T cell yield, expansion and enrichment of peptide-MHC-binding T cells. MACS with CD34 mAb resulted in significant T cell yield, expansion and enrichment of tCD34-positive T cells, but not a significant enrichment of peptide-MHC-binding T cells. Notably, CD34 mAb-sorted T cells demonstrated enhanced antigen-specific T cell functions, which were related to CD4 T cells that were uniquely present in CD34 mAb-sorted T cells.

MATERIALS AND METHODS

Cells and reagents

T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere (179) and cultured in HEPES-buffered RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% human serum, 2 mM L-glutamine, and the antibiotics streptomycin and penicillin. The human embryonic kidney cell line 293T,

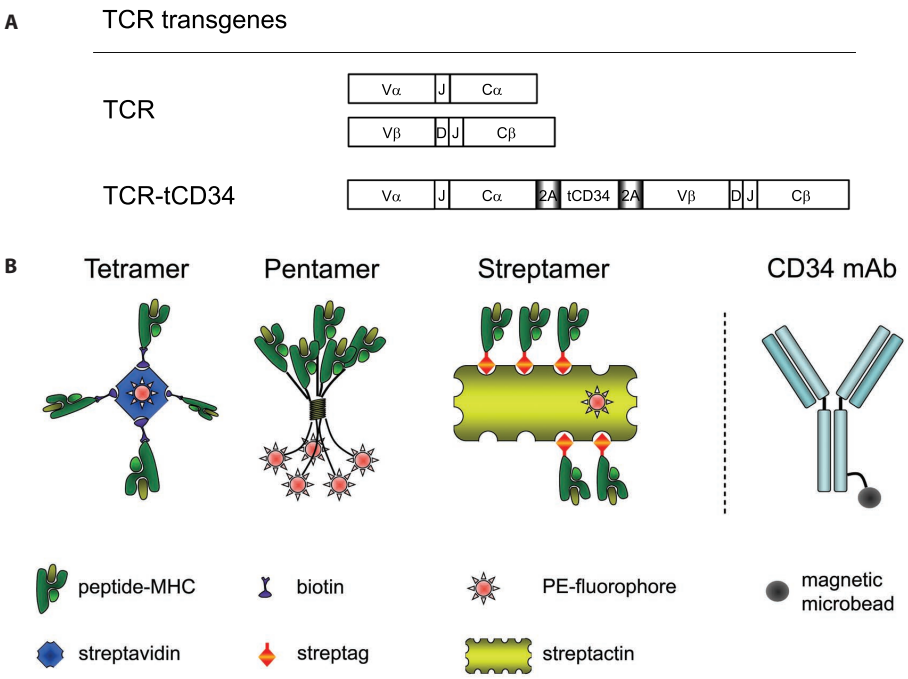

Figure 6.1. Gene-constructs and sorting reagents to MACSort TCR-engineered human T cells. (A) Schematic representation of TCRα, TCRβ and TCRα-2A-tCD34-2A-TCRβ transgenes used to gene-engineer primary human T cells. TCR specific for gp100/A2 comprised TRAV13-1*02/J52*01/CA and TRBV27*01/J2-7*01/D2*02/CB2 (132). tCD34 represents a truncated and functionally inert variant of CD34. T cells were transduced either with pBullet:TCRα + pBullet:TCRβ and termed TCR T cells or pBullet:TCRα-2A-tCD34-2A-TCRβ and termed TCR-tCD34 T cells. Abbreviations used: V: TCRαβ-variable domain; C: TCRαβ-constant domain; D: TCRβ-diversity domain; J: TCRαβ-joining domain; 2A: 2A sequence coding for a self-cleaving peptide; tCD34: truncated CD34 molecule. **(B)** Schematic illustration of reagents used to MACSort TCR-engineered human T cells. From left to right: tetramer (180), pentamer (63), streptamer (255, 256); and CD34 mAb (258). Tetramers consist of 4 gp100 peptide (YLEPGPVTA)/HLA-A2 (gp100/A2) monomers that were biotinylated and multimerized with streptavidin-Phycoerythrin; pentamers consist of 5 gp100/A2 monomers that were linked to Phycoerythrin and multimerized by a self-assembling, coiled-coil structure; and streptamers consist of 8-12 gp100/A2 monomers that were streptagged and multimerized with streptactin-Phycoerythrin (depicted with 5 monomers). Anti-CD34 mAbs consist of heavy and light-chains providing 2 antigen-binding sites and are directly coupled to magnetic microbeads.

the packaging cell line Phoenix-A, and the melanoma cell lines BLM and FM3 were cultured in DMEM medium (BioWhittaker) supplemented with 10% fetal bovine serum (FBS, Stonehouse, Gloucestershire, UK), 2 mM L-glutamine, non-essential amino acids and antibiotics. Monoclonal Abs used in this study are: anti-CD34 microbeads (clone: QBEND/10, Miltenyi Biotec, Bergisch Gladbach, Germany); anti-PE microbeads (Miltenyi Biotec); FITC- and PE-conjugated anti-TCR-Vβ27 (Beckman Coulter, Marseille, France); APC- and non-conjugated anti-CD3ε (OKT3, BD Biosciences, San Jose, USA, and Beckman Coulter, respectively); APC-conjugated anti-CD8α (RPA-T8, BD Biosciences); FITC-conjugated anti-CD34 (AC136, Miltenyi Biotec, Bergisch Gladbach, Germany); PE-conjugated anti-CD107a (H4A3, BD Biosciences); FITC-conjugated anti-IFNγ (B27, BD Biosciences); FITC-conjugated anti-IL-2 (MQ1-17H12, BD Biosciences); and FITC-conjugated anti-IL2Rα (M-A251, BD Biosciences). Other reagents included: Retronectin (human fibronectin fragments CH-296, Takara Shuzo Co. Ltd., Otsu, Japan); PMA (Sigma-Aldrich, St. Louis, USA); PHA (Remel Europe, Dartford, England); gp100 peptide (YLEPGPVTA) (Proimmune); Golgistop (BD Biosciences); Golgiplug (BD Biosciences); and PFA (Brunschwig, Amsterdam, the Netherlands).

TCR and tCD34 transgenes

TCRαβ specific for gp100/A2 was derived from CTL clone 296 and uses the TCR-V genes TRAV13-1*02/J52*01/C and TRBV27*01/J2-7*01/D2*02/C2 (nomenclature according to http://imgt.org) (132). TCRα and TCRβ chains were cloned in pBullet vectors either as single TCR chains (i.e., pBullet:TCRα + pBullet:TCRβ (132)) or combined TCRα and TCRβ chains and linked by 2A sequences and truncated CD34 (i.e., pBullet:TCRα-2A-tCD34-2A-TCRβ). See figure 6.1A for a schematic representation of TCR transgenes. Truncated CD34, a naturally occurring splice variant of human CD34 that lacks Protein Kinase C binding sites in its cytoplasmic tail (257), was flanked by 2A sequences (kindly provided by Dr. Gilham, University of Manchester, Manchester, UK), and introduced into pBullet vector via PCR and a *Nco*I ligation. Primers used to amplify 2A-tCD34-2A are: tCD34 5′ primer: attcggccatggcggggcgcgcccgctcgagcgagtgaaacagactttgaat; tCD34 3′ primer: acgcgtccaagcttggcaattgattccctggcccggggttggactc, and introduced additional restriction sites that allowed subsequent introduction of TCRα and TCRβ chains in pBullet:tCD34. TCRα and TCRβ were sub-cloned via *Nco*I/*Xho*I and *Mfe*I/*Mlu*I into pBullet:tCD34, respectively.

Retroviral gene transfer into T cells

Moloney Murine Leukemia retroviruses, positive for TCR and TCR-tCD34, were produced by co-cultures of the packaging cells 293T and Phoenix-A. Packaging cells were calcium phosphate-transfected with pBullet:TCRα + pBullet:TCRβ or pBullet:TCRα-2A-tCD34-2A-

TCRβ, and the helper vectors: pHIT60 MLV GAG/POL and pCOLT-GALV ENV. Transduction of human T cells was performed as described previously (71).

Peptide-MHC complexes

Complexes of $gp100_{280-288}$/HLA-A*0201 (gp100/A2) peptide-MHC used in this study are tetramers, pentamers and streptamers. Tetramers were generated by multimerization of 5 µg biotinylated monomers (Sanquin, Amsterdam, Netherlands) with 12.5 µl streptavidin-PE (BD Biosciences), which were mixed and incubated for 40 min at 4 °C. Streptavidin-PE was added and mixed in steps of 2.5 µl that were followed by incubations of 8 min; Pentamers were purchased as PE-conjugated multimers (ProImmune, Oxford, UK); Streptamers were generated by multimerization of 1 µg of streptagged monomers (IBA, Göttingen, Germany) with 0.75 µg streptactin-PE (IBA) in 50 µl buffer (PBS with 0.5% BSA, 2 mM EDTA, and pH 7.4), which were mixed and incubated for 45 min. Sort reagents, during preparations and once ready, were kept at 4 °C and protected from light. Concentrations of tetramers, pentamers and streptamers for use in flow cytometry were determined per batch by serial dilutions and set at 1:100, 1:20, and undiluted, respectively. See figure 6.1 for a schematic representation of peptide-MHC reagents, and table 6.1 for properties and usage of peptide-MHC multimers in MACS.

MACS to enrich gene-engineered T cells

Human T cells were labeled either with PE-conjugated peptide-MHC multimers and microbead-conjugated PE mAb or microbead-conjugated CD34 mAb according to the manufacturer's instructions (Miltenyi Biotec). In case of peptide-MHC multimer stainings, reagents were added to cell pellets (see table 6.1 for final concentrations) and incubated for 45 min (*all* solutions were ice-cold and *all* incubations were performed at 4 °C and protected from light). T cells were washed twice with PBS-0.5% BSA (pH 7.4), re-suspended in PBS-0.5% BSA with PE mAb microbeads (volume ratio 4:1) and incubated for 15 min. In case of CD34 mAb stainings, T cells were washed with PBS, re-suspended in PBS-0.5% BSA with CD34 mAb microbeads and FcR-blocking reagent (volume ratio 5:1:1) and incubated for 30 min. Microbead-labeled T cells were washed, re-suspended in PBS-0.5% BSA, passed over a MACS pre-separation filter and separated in MACS separation columns that were exposed to a magnetic field. Sorted T cells were washed and subsequently flushed from the column with PBS-0.5% BSA. In case of streptamers, sorted T cells were treated twice with 1 mM biotin-D (Invitrogen, Carlsbad, USA) for 20 min and washed repeatedly. Finally, sorted T cells were counted and cultured on feeder cells ($1x10^4$ T cells / 200 µl feeder medium (179)).

T cell counts

T cells were counted directly after MACS, 1 week after MACS, and weekly up to 6 weeks after MACS. T cell viability was determined by trypan blue exclusion and viable T cells were counted microscopically using Bürker chambers and a Leitz Laburlux 12 microscope (Leica Geosystems BV, Rijswijk, The Netherlands). T cell yields and expansions are represented as absolute numbers or fold increases in cell numbers, respectively.

Flow cytometry

All T cell stainings were performed according to standard protocols. T cells ($1x10^4$ cells) were incubated with peptide-MHC multimers for 15 min at RT or mAbs for 30 min on ice, and fixed with 1% PFA. To measure antigen-specific T cell responses, T cells were stimulated with target cells and assessed for surface expression of CD107a and intracellular expression of IFNγ and IL-2. Target cells used were BLM (without or with 10^{-5} M gp100 peptide) and FM3 cells. CD107a expression was detected as described in chapter 3. Briefly, T cells were re-suspended in a mixture of T cell medium, Golgistop, and CD107a mAb-PE. Next, target cells and T cells were mixed in a 1:1 ratio and incubated for 2h at 37 °C and 5% CO_2. T cells were subsequently stained with CD3ε mAb to allow distinction of T cells from target cells at the time of analysis. Intracellular cytokine levels were detected in T cells ($2x10^5$) that were stimulated with target cells ($6x10^4$) in the presence of Golgiplug for 6 h at 37 °C and 5% CO_2. Next, T cells were washed and stained with CD8α mAb-APC, after which T cells were washed again, permeabilized (permeabilization solution 2, BD Biosciences) for 10 min at RT, and stained with IFNγ mAb-FITC or IL-2 mAb-FITC. Samples were measured on a FACSCalibur dual-laser flow cytometer (Beckton Dickinson, Alphen a/d Rijn, the Netherlands). Data analysis was performed on viable and in some cases CD3-positive T cells using Cellquest software (BD Biosciences), and data was displayed either as dotplots or histograms.

IFNγ production

T cells were assayed for their IFNγ production as described previously (131). BLM melanoma cells, without or with titrated amounts of gp100 peptide (between 10^{-10} and 10^{-5} M) and FM3 melanoma cells were used to stimulate T cells and O/N supernatants were tested in triplicate. T cell IFNγ levels were determined using ELISA (Sanquin) and a Titertek plus reader (Merlin, Breda, Netherlands) according to the manufacturer's instructions.

Statistical analyses

Student's *t*-tests (unpaired; two-tailed) and Graphpad Prism 4 software were used to test the various sort reagents with respect to *in vitro* properties of T cells. Differences with p-values <0.05 were considered significant.

RESULTS

MACS with streptamers or CD34 mAb results in enhanced T cell yield and expansion

Primary human T cells were transduced with gp100/A2-specific TCR and TCR-tCD34 genes and MACSorted with tetramers, pentamers, streptamers or CD34 mAb. Flow cytometry analyses showed that pre-sort TCR T cells labeled similarly with the various peptide-MHC multimers, which extends an earlier report by Yao and colleagues (260). In addition, TCR and TCR-tCD34 T cells showed comparable binding of peptide-MHC multimers (data not shown). MACS of TCR T cells or TCR-tCD34 T cells with peptide-MHC multimers (input for all labeling conditions: $10x10^6$ T cells) resulted in comparable numbers of T cells directly after MACS (output: 0.07-0.41x10^6 T cells), whereas MACS of TCR-tCD34 T cells with CD34 mAb (input again: $10x10^6$ T cells) resulted in significantly enhanced numbers of T cells (output: $1.0x10^6$ T cells; fig. 6.2A). MACS of TCR-tCD34 T cells with CD34 mAb also resulted in the highest yield of T cells at 1 week after MACS ($22.7x10^6$ T cells), which was significantly higher when compared to MACS of TCR-tCD34 T cells with streptamers ($2.5x10^6$ T cells; fig. 6.2B). The increased yield of CD34-mAb sorted T cells was due to both enhanced T cell numbers directly after MACS and enhanced T cell expansion in the 1st week after MACS (figs. 6.2A and C). MACS of TCR T cells with streptamers resulted in a significantly enhanced yield of T cells at 1 week after MACS ($9.7x10^6$ T cells) when compared to tetramers and pentamers (3.1 and $0.9x10^6$ T cells, respectively; fig. 6.2B). The increased yield of streptamer-sorted T cells was primarily due to enhanced T cell expansion in the 1st week after MACS (fig. 6.2C) rather than enhanced T cell numbers directly after MACS (fig. 6.2A). Notably, streptamer-sorted TCR-tCD34 T cells, when compared to streptamer-sorted TCR T cells, yielded lower T cell numbers, which appeared related to a reduced T cell expansion in the 1st week after MACS (figs. 6.2A and B). When analyzing T cell yields at later time points, i.e. between 3 to 6 weeks after MACS, we observed clear T cell expansion rates (weekly T cell expansion > 10 fold) with no differences observed between the sort reagents (fig. 6.2C).

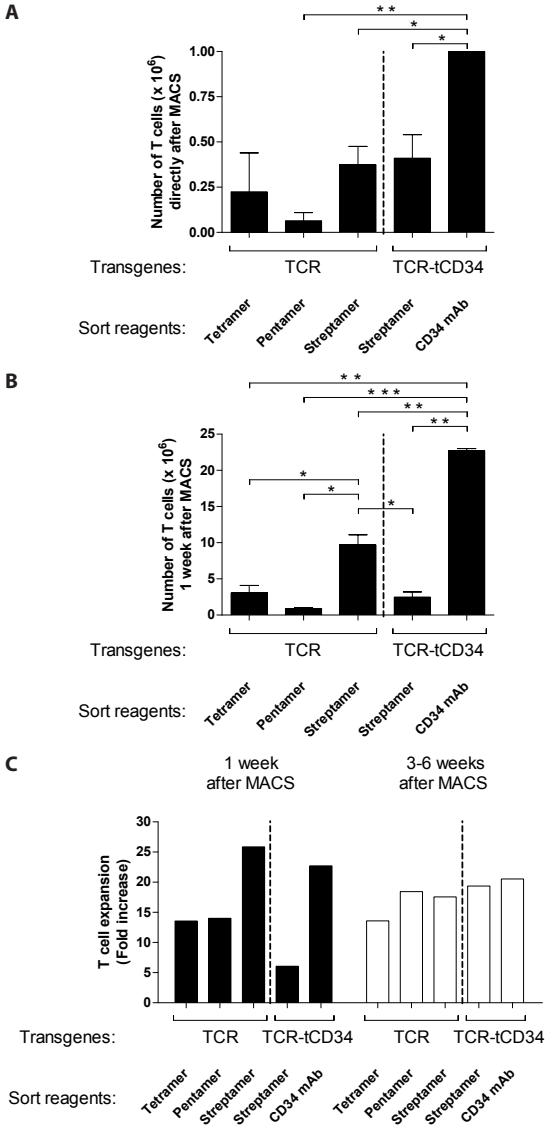

Figure 6.2. MACS with streptamer or CD34 mAb improves T cell yield. Primary human T cells were transduced either with TCR or TCR-tCD34 transgenes as depicted in figure 6.1A. TCR T cells were MACSorted with tetramers, pentamers, and streptamers, whereas TCR-tCD34 T cells were MACSorted with streptamers and CD34 mAbs. Following MACSort, T cell numbers were counted microscopically and T cells were expanded using feeder cultures (as described in materials and methods section). Mean T cell numbers + SEM **(A)** directly after MACS or **(B)** 1 week after MACS are from 2-6 repeat experiments with T cells from 2-4 healthy donors. (**C**) T cell expansion at 1 week (black bars) or 3-6 week(s) (white bars) after MACS expressed as fold increase in T cell numbers when comparing 1 week and directly after MACS and 3-6 weeks and 1 week after MACS, respectively. T cell expansions are from representative T cell cultures out of 2-4 repeat experiments with T cells from 2-4 healthy donors with similar results. Statistically significant differences are calculated with Student's *t*-tests: *: $p<0.05$, **: $p<0.005$.

MACS with streptamers, but not CD34 mAb, results in T cell populations significantly enriched for peptide-MHC binding

MACS of TCR T cells with tetramers, pentamers or streptamers resulted in clear enrichments of peptide-MHC-binding T cells (2.2, 1.8, and 3.1 fold increase, respectively; fig. 6.3A). Streptamers, however, were the only peptide-MHC multimers that resulted in percentages of peptide-MHC-binding T cells (> 65%) that were significantly enhanced when compared to pre-sort T cells (20%). Please note that limited output of T cell numbers directly after MACSort with tetramers and pentamers (in some cases < 50,000 cells) did not allow for flow cytometry with peptide-MHC multimers since the total number of T cells was expanded to provide a yield at 1 week after MACSort necessary for downstream assays. Generally, in our experience, peptide-MHC enrichment at 1 week after MACS or FACSort correlates well with the actual sorting efficiency. Percentages of streptamer-sorted T cells that bind peptide-MHC proved stable over a culture period of up to 6 weeks (fig. 6.3A). In contrast, percentages of pentamer-sorted T cells that bind peptide-MHC decreased to pre-sort values in a 6-week time period.

MACS of TCR-tCD34 T cells with either CD34 mAb or streptamers resulted in a maximal enrichment of tCD34 expressing T cells, near to 100 %, which was stable over time (fig. 6.3B). Unexpectedly, MACS of TCR-tCD34 T cells with CD34 mAb did not result in an enrichment of peptide-MHC-binding T cells (fig. 6.3A). Also, streptamer MACS of TCR-tCD34 T cells resulted in an enrichment of peptide-MHC-binding T cells that was 2-fold less and not stable over time when compared to TCR T cells (fig. 6.3A).

MACS with CD34 mAb yields T cells with enhanced CD107a mobilization and IFNγ production in response to antigen-positive melanoma cells

To analyze T cell functions, we decided to compare streptamers, which among the peptide-MHC multimers tested showed the best T cell yield and enrichment for peptide-MHC-binding T cells, with CD34 mAb as sort reagents. Following 3-6 weeks after MACS, TCR and TCR-tCD34 T cells were stimulated with peptide-loaded BLM or native FM3 melanoma cells and analyzed for surface expression of CD107a and IFNγ production. To better compare T cell functions, values were standardized for peptide-MHC binding (see s*upplementary figure 6.1A*). Streptamer-sorted TCR-tCD34 T cells, when compared to similarly sorted TCR T cells, revealed a higher level of CD107a expression and similar levels of IFNγ production in response to FM3 melanoma cells (figs. 6.4A and B). Strikingly, CD34 mAb-sorted T cells showed the highest level of CD107a expression and IFNγ production in response to melanoma cells (figs. 6.4A and B). To assess T cell sensitivity, we have tested IFNγ responses towards titrated amounts of gp100 peptide. CD34 mAb-sorted T cells generally produced about 2-fold more IFNγ in response to 10^{-9} to

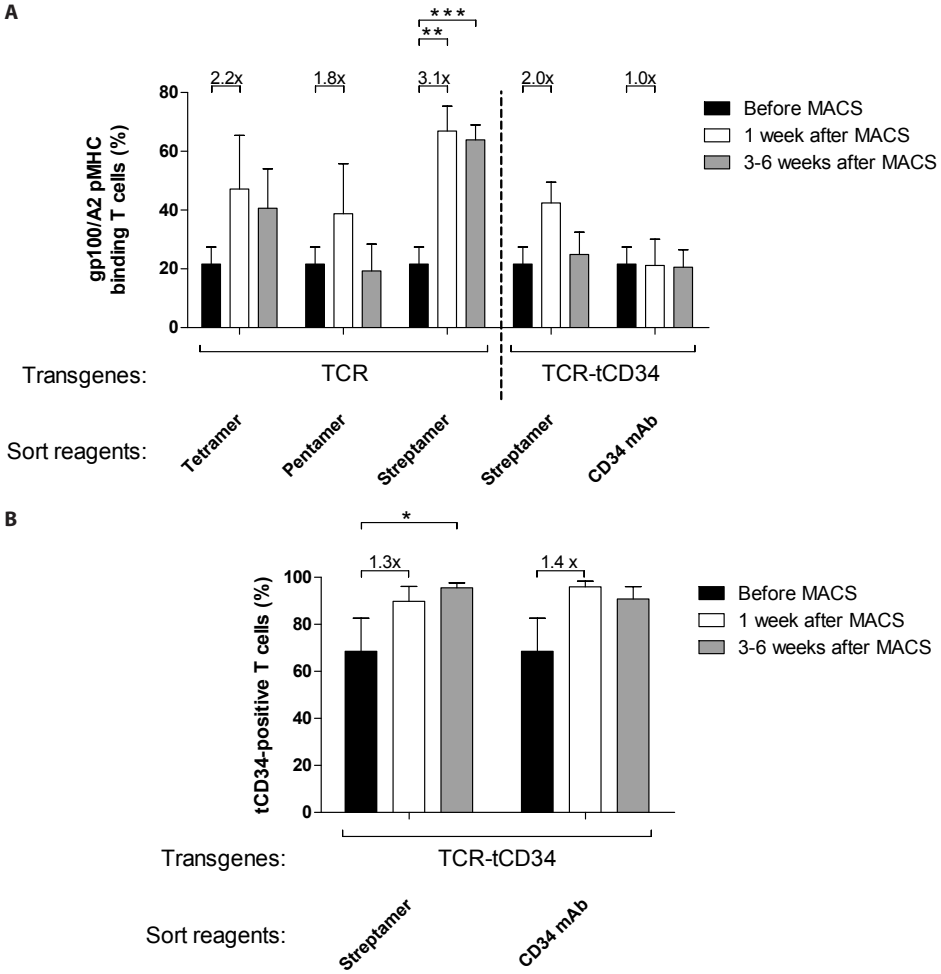

Figure 6.3. MACS of TCR T cells with streptamers results in enhanced peptide-MHC binding that is stable over time. Primary human T cells were transduced and MACSorted as described in the legend to figure 6.2. Sorted T cells were analyzed by flow cytometry for **(A)** peptide-MHC binding (using gp100/A2 pentamer) and **(B)** surface expression of tCD34 (using CD34 mAb) at various time points: before MACS (black bars), 1 week after MACS (white bars); and 3-6 weeks after MACS (gray bars). Bars represent mean % + SEM of 2-4 repeat experiments with T cells from 2-4 healthy donors. Binding of peptide-MHC or CD34 mAb before MACS was 20 and 70%, respectively. Enrichment of T cells that bind peptide-MHC or CD34 mAb is presented as fold difference between T cells at 1 week after and before MACS, and indicated above corresponding bars. Statistically significant differences are calculated with Student's *t*-tests: *: $p<0.05$; **: $p<0.005$.

10^{-5} M peptide, however equal EC_{50}'s of approximately 0.04 µM peptide were observed (*supplementary fig. 6.1B*).

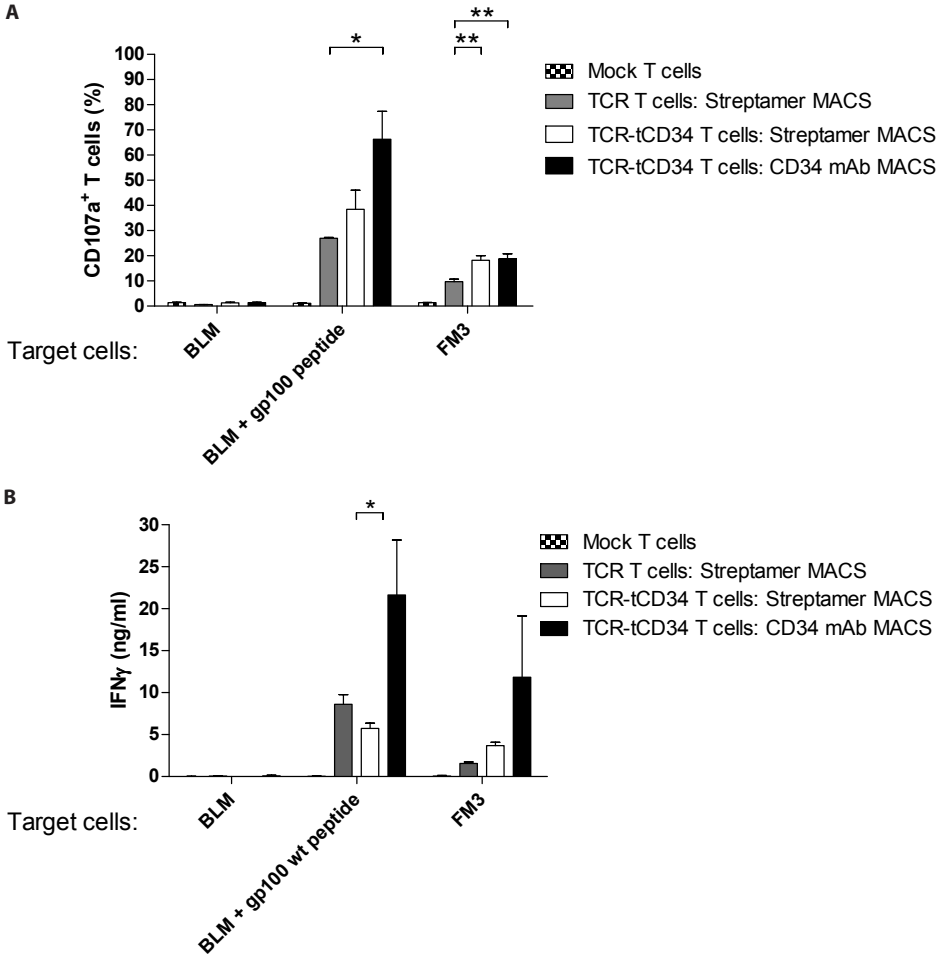

Figure 6.4. Human T cells sorted with CD34 mAb demonstrate increased antigen-specific responses. Primary human T cells were transduced with empty virus particles (Mock T cells), TCR (TCR T cells) or TCR-tCD34 transgenes (TCR-tCD34 T cells) and were either not MACSorted (Mock T cells, blocked bars) or MACSorted with streptamers (TCR T cells, gray bars; TCR-tCD34 T cells, white bars) or anti-CD34 mAbs (TCR-tCD34 T cells, black bars). T cells sorted with CD34 mAb show up-regulated **(A)** surface expression of T cell CD107a and **(B)** IFNγ production upon stimulation with antigen-positive melanoma cells. T cells were stimulated with the following target cells: BLM cells (gp100-/A2+) loaded without or with 10^{-5} M gp100 peptide, and FM3 cells (gp100+/A2+). CD107a expression (%) was measured by flow cytometry, gating on viable and CD3-positive T cells, following a 2 hrs stimulation, and IFNγ production (ng/ml) was measured following O/N stimulation in conditioned supernatants by ELISA. Bars represent mean values + SEM of 2 independent measurements from two healthy donors. CD107a and IFNγ data were corrected for differences in peptide-MHC binding, with peptide-MHC binding of CD34 mAb-sorted T cells set to 100 % (see *supplementary figure 6.1A* for details). Statistically significant differences are calculated with Student's *t*-tests: *: $p<0.05$, **: $p<0.005$, ***: $p<0.0005$.

MACS with CD34 mAb, but not streptamers, results in CD4 and CD8 T cells that both contribute to antigen-specific IFNγ production

To follow up on the enhanced function of CD34 mAb-sorted T cells, we performed multi-parameter flow cytometry to study the contribution of CD4 and CD8 T cell subsets to IFNγ production. Besides intracellular IFNγ, sorted T cells were analyzed for peptide-MHC binding, and surface expression of TCR-Vβ27, tCD34 and CD8α. Percentages of CD4 T cells in pre-sorted T cells are 67% (fig. 6.5A; non-sorted Mock T cells), whereas CD4 T cell percentages in TCR T cells sorted with streptamers or TCR-tCD34 T cells sorted with either streptamers or CD34 mAb were 5%, 1% and 38%, respectively (fig. 6.5A). Expectedly, MACS with streptamers yielded a T cell population that was biased for CD8 T cells. In contrast, MACS with CD34 mAb yielded T cells that contained significant populations of both CD4 and CD8 T cells. CD4 T cells within CD34 mAb-sorted T cells expressed TCR-Vβ27 and tCD34 at levels equal to those observed in CD8 T cells, yet CD4 T cells did not bind peptide-MHC (figs. 6.5A-C). Stimulation of T cells with peptide-loaded BLM cells resulted in enhanced intracellular levels of IFNγ in CD8 T cells irrespective of the MACS reagent used. Notably, in CD34 mAb-sorted T cells both CD4 and CD8 T cell subsets demonstrated antigen-specific IFNγ production (fig. 6.5D). Similarly, although to a smaller extent, CD4 T cells contributed to antigen-specific IL-2 production of CD34 mAb-sorted T cells (s*upplementary fig. 6.2A* and *B*). Table 6.2 provides a summary of our data with respect to yield, expansion, enrichment for peptide-MHC binding and tCD34, and antigen-specific functions of T cells MACSorted with tetramers, pentamers, streptamers or CD34 mAb.

DISCUSSION

In this study, we have performed a head-to-head comparison of tetramers, pentamers, streptamers and CD34 mAb as sort reagents to obtain a population of MACS-enriched TCR-engineered T cells. First, we compared the different peptide-MHC multimers and noted that streptamers provided a significantly improved output of T cell numbers, which was primarily due to enhanced T cell expansion in the 1st week after MACS. This observation is in line with a previous report that showed that dissociation of streptamers into monomers, as we have done following MACS, reduces activation-induced cell death (AICD) and improves T cell proliferation when compared to non-dissociated multimers (256). In fact, multimeric peptide-MHC is reported to result in up-regulated expression of FasL, which in turn results in FasL/Fas mediated T cell death (261). In contrast, peptide-MHC monomers do not lead to full and endured TCR-mediated T cell activation, most likely due to relatively low affinity interactions with TCRs and high off kinetics of mono-

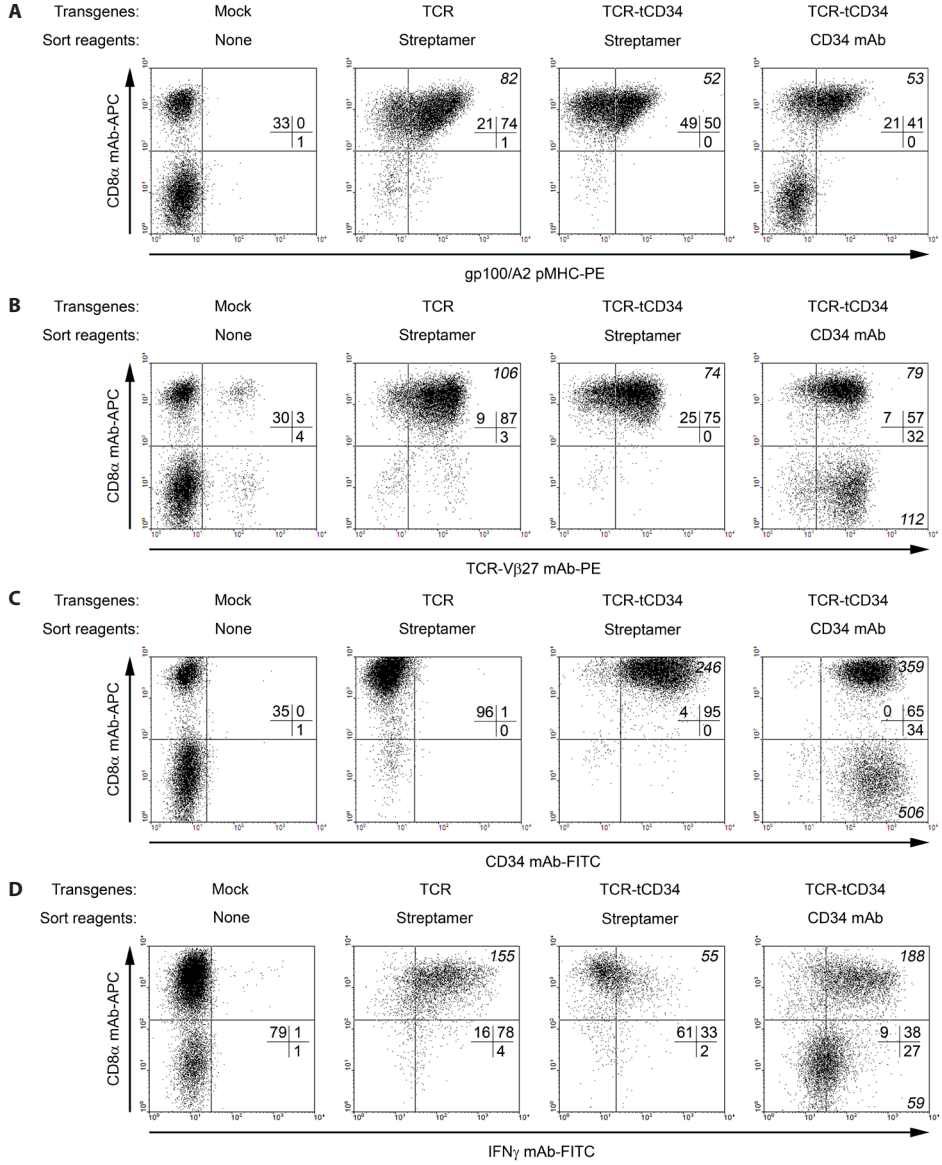

Figure 6.5. IFNγ production of CD34 mAb-sorted T cells, but not peptide-MHC-sorted T cells, depends on both CD8 and CD4 T cells. Primary human T cells were transduced and MACSorted as described in legend to figure 6.4. T cells were analyzed by flow cytometry after staining with APC-conjugated anti-CD8α mAb in combination with one of the following reagents: (**A**) PE-conjugated gp100/A2 tetramer (n=12); **(B)** PE-conjugated anti-TCR-Vβ27 mAb (n=6); **(C)** FITC-conjugated anti-CD34 mAb (n=14); and (**D**) FITC-conjugated anti-IFNγ mAb (n=2). In case of IFNγ stainings, T cells were stimulated for 6 hrs with BLM (gp100-/A2+) cells loaded with 10^{-5} M gp100 peptide, permeabilized and stained with anti-IFNγ mAb, as described in more detail in the materials and methods section. The figures show representative dotplots, indicating percentages (all quadrants) and MFIs (upper and lower right quadrants when % ≥ 5, in italic) of stained T cells.

Table 6.2. *In vitro* **evaluation of T cells that are MACSorted with peptide-MHC multimers or CD34 mAb[a]**

	TCR T cells			TCR-tCD34 T cells	
	T	P	S	S	CD34
T cell output numbers (directly after MACS)[b]	±	–	+	+	++
T cell yield (1 week after MACS)[c]	±	–	+	±	++
T cell expansion (1 week after MACS)[d]	±	±	++	–	++
T cell expansion (3-6 weeks after MACS)[d]	±	+	+	+	++
pMHC binding (1 week after MACS)[e]	+	+	++	+	–
pMHC binding (3-6 weeks after MACS)[e]	+	–	++	±	–
tCD34 expression (1 week after MACS)[f]	N.A.	N.A.	N.A.	+	++
tCD34 expression (3-6 weeks after MACS)[f]	N.A.	N.A.	N.A.	++	++
Antigen-specific CD107a mobilization[g]	N.D.	N.D.	±	±	++
Antigen-specific IFNγ secretion[h]	N.D.	N.D.	±	±	++

[a] Primary human T cell populations were transduced with TCR or TCR-tCD34 genes, MACSorted with gp100/A2 tetramers (T), pentamers (P) or streptamers (S), or with CD34 mAb (CD34), and evaluated for various *in vitro* parameters. See materials and methods section for details. N.A.: Not Applicable; N.D.: Not Determined.
[b] T cell numbers directly after MACS (counted microscopically): – = 0-0.10x10^6; ± = 0.11-0.25x10^6; + = 0.26-0.75x10^6; ++ = >0.75x10^6.
[c] T cell numbers at 1 week after MACS (counted microscopically): – = 0-1x10^6; ± = 2-5x10^6; + = 6-15x10^6; ++ = >15x10^6.
[d] T cell expansion at 1 or 3-6 week(s) after MACS (fold increase in T cell numbers at 1 week versus directly after MACS and 3-6 weeks versus 1 week after MACS, respectively): – = 0-10; ± = 11-15; + = 16-20; ++ = >20.
[e] Peptide-MHC binding at 1 and 3-6 week(s) after MACS (measured by flow cytometry with gp100/A2 pentamer): – = ≤ 20% (value before MACS); ± = 21-30; + = 31-50; ++ = >50%.
[f] tCD34 expression at 1 and 3-6 week(s) after MACS (measured by flow cytometry with CD34 mAb): – = ≤ 70% (value before MACS); ± = 71-80; + = 81-90; ++ = >90%.
[g] T cell CD107a mobilization at 3-6 weeks after MACS (measured by flow cytometry with CD107a mAb upon peptide-specific stimulation): – = 0-20; ± = 21-40; + = 41-60; ++ = >60%.
[h] T cell IFNγ production at 3-6 weeks after MACS (measured by ELISA upon peptide-specific stimulation): – = 0-5; ± = 6-10; + = 11-20; ++ = >20 ng/ml.

meric peptide-MHC (31, 262). Besides streptamers, the use of a mAb directed against a truncated CD34 molecule represents another means of circumventing AICD. When comparing peptide-MHC multimers to CD34 mAb, we observed that CD34 mAb-sort of gene-marked T cells provided the highest output of T cell numbers, which was due to both enhanced T cell numbers directly after MACS and enhanced T cell expansion in the 1st week after MACS. T cell populations all expanded at similar rates up to 6 weeks after MACS, implying that T cells bound to sort reagents such as tetramers and pentamers are ultimately 'diluted out' or lost (fig. 6.2).

With respect to enrichment for peptide-MHC binding, streptamer-based MACS of TCR T cells resulted in the most effective enrichment, which proved stable over time. The reversibility of streptamer binding, which, as discussed above, potentially aids T cell expansion of sorted T cells, may have contributed to the observed T cell enrich-

ment. MACS of TCR-tCD34 T cells with CD34 mAb resulted in a T cell population that was nearly completely positive for tCD34 for at least 6 weeks but did not show an improved binding of peptide-MHC. Similar results were obtained with streptamer MACS of TCR-tCD34 T cells, showing enrichment of tCD34 but only a weak and transient enrichment for peptide-MHC-binding T cells. Findings with TCR-tCD34 T cells suggest that a CD34 mAb sort does not result in concomitant enrichment of peptide-MHC-binding T cells, whereas a peptide-MHC multimer sort does result in enrichment of tCD34-positive T cells. This observation may be related to the fact that surface expression of TCRαβ chains, but not tCD34, is prone to competition for endogenous proteins and TCR mis-pairing (see chapter 2).

When comparing TCR-tCD34 and TCR T cells with respect to peptide-MHC binding, we postulate that the TCRα-2A-tCD34-2A-TCRβ cassette may compromise gene expression of TCRαβ. Although we cannot exclude that the products of TCR genes intervened by tCD34 are more prone to mis-pair with endogenous TCR chains, TCR genes expressed from a single construct are generally less prone to mis-pair when compared to TCR genes expressed from separate constructs (65). Sizes of transgenes in pBullet retroviral vectors may adversely affect gene expression (TCR-tCD34: 2867 nt; TCRα or β: 827 and 923 nt) (data not shown). More specifically, surface expression of the TCRα gene, positioned 5′ of 2A sequences, may be reduced as a consequence of the 2A amino acids added to the 3′ end of the TCRα protein (263, 264). Addition of 2A amino acids to the intracellular domain of TCRα potentially decreases the stability of immunoglobulin constant region-containing proteins. Since TCR-Vα13 mAbs are commercially not available, we cannot formally proof the decreased surface expression of TCRα. However, two lines of evidence support a decreased expression of TCRα by TCR-tCD34 T cells. First, peptide-MHC binding by TCR-tCD34 T cells is decreased compared to TCR T cells whereas TCRβ surface expression is not (fig. 6.3A, *supplementary fig. 6.3*, and figs. 6.5A and B). Second, Mean Fluorescence Intensities of peptide-MHC binding by streptamer-sorted TCR-tCD34 T cells was 1.6-fold lower when compared to similarly sorted TCR T cells (fig. 6.5A). Along these same lines, binding of peptide-MHC monomers (upon reversal of streptamers into peptide-MHC monomers) is expected to persist less long in case of TCR-tCD34 T cells, potentially providing a decreased T cell proliferation signal in the setting of feeder cultures (with T cell co-stimulation; fig. 6.2C). TCRβ expression appears generally less sensitive for additional 3′ amino acids (95, 234, 265), suggesting that a TCRβ-2A-tCD34-2A-TCRα or tCD34-2A-TCRβ-2A-TCRα configuration, in particular in those vectors that can carry large sized transgenes may be more favorable for stable TCRα expression and peptide-MHC multimer binding by TCR-tCD34 T cells.

When analyzing T cell functions, CD34 mAb-sorted T cells revealed enhanced CD107a mobilization and IFNγ production in response to melanoma target cells. The enhanced functional avidity of these T cells did not correlate with the surface expression of TCRβ,

which is in line with the aforementioned discrepancy between TCRβ expression and peptide-MHC binding. *Supplementary figure 6.3* demonstrates that MACSort enhanced the percentages of TCRβ in a manner that appeared independent of T cell population and sort reagent used. Within our panel of MACSorted T cells, CD34 mAb-sorted T cells are unique with respect to the presence of CD4 T cells (fig. 6.5A) and these CD4 T cells contributed to higher antigen-specific T cell IFNγ responses (fig. 6.5D). It is noteworthy that besides T cell function, it is not expected that CD4 T cells contributed to differences observed with respect to either T cell output numbers, T cell yield or enrichment for peptide-MHC binding (figs. 6.2 and 6.3). First, the relative presence of CD4 T cells was identical in pre-sort T cells from individual donors (as in non-sorted Mock T cells; fig. 6.5A). Second, T cell expansion rates did not differ among all sorted T cell populations tested (fig. 6.2C) suggesting that differences in T cell yield and enrichment for peptide-MHC binding at 1 week after MACS (figs. 6.2 and 6.3) were not due to the presence of CD4 T cells. Somewhat unexpected, CD4 T cells did not contribute to peptide-MHC binding of CD34 mAb-sorted T cells. We hypothesize that MACS procedures, in contrast to for instance FACS procedures, may apply additional forces to the TCR:peptide-MHC interactions, such as those caused by downward flows of washing buffers (249). Consequently, MACS of T cells may select for T cells with the highest avidity for peptide-MHC, in which case CD8 T cells generally have an advantage over CD4 T cells. Downward flow may not affect MACS with CD34 mAb since antibodies generally have a 10,000 fold higher ligand-binding affinity than TCRs (262). The absence of peptide-MHC binding by TCR-positive CD4 T cells is an observation that seemingly contradicts a previous report, which showed that the same gp100/A2 TCR acts independently of the CD8α co-receptor (206). In fact, in the latter study we have shown that CD4 and CD8 T cells revealed comparable peptide-MHC binding and nearly comparable T cell cytotoxicity, but T cells were transduced with TCR genes and obtained via peptide-MHC/CD8α mAb-based FACSort (206). The lowered and unstable TCRα expression in TCR-tCD34 T cells may reduce T cell avidity and hamper peptide-MHC binding, resulting, as discussed, in a lowered peptide-MHC binding MFI of CD8 T cells and potentially, in this study, in absent peptide-MHC binding by CD4 T cells (fig. 6.5A).

Despite the absence of peptide-MHC binding, the CD4 T cell population in CD34 mAb-sorted TCR-tCD34 T cells contributed to antigen-specific responses as shown by intracellular IFNγ and, to a lesser extent, IL-2 production (fig. 6.5D and s*upplementary fig. 6.2A*). This observation is in agreement with the notion that T cell function only requires a few peptide-MHC molecules, which may be well below the detection limit of T cell peptide-MHC binding by standard flow cytometry methods (266). IFNγ and IL-2 production by CD4 T cells is important for recruitment and mobilization of CD8 T cells to the tumor site (267, 268). In addition, CD4 T cells aid the induction of CD8 T cell-mediated immune responses, targeting of tumor stroma, and formation of T cell memory (269-

276). Findings on IFNγ and IL-2 production by CD34 mAb-sorted CD4 T cells extend a previous study, where we observed an antigen-specific production of high levels of IFNγ and low levels of IL-2 in gp100/A2 TCR-positive CD4 T cells (206). In another study, CD8 T cells transduced with a ERBB2-specific Chimeric Antigen Receptor (CAR) revealed the best T cell expansion and protection against metastases when co-cultured with Th1-type rather than Th2-type T cells (277). Thus, the anti-tumor activity of CD34 mAb-sorted T cells may be further exploited by skewing CD4 T cells towards a Th1 phenotype.

Our study has focused on three types of peptide-MHC multimers to enrich TCR-transduced T cells with MACS. To date, other peptide-MHC multimers have been designed that contain up to 200 peptide-MHC monomers (278-282). Although the use of these novel peptide-MHC reagents needs to be tested for T cell sorting, the enhanced valencies of these peptide-MHC multimers may allow enrichment for CD4 T cells, yet the accompanying lower 'off-rates' and inability to revert binding are likely to result in AICD.

In short, MACSort of T cells with detachable streptamers improves T cell yield and enrichment for peptide-MHC binding T cells when compared to tetramers and pentamers. Truncated CD34 proves a valid surrogate marker to MACSort T cells, resulting in a further enhancement of T cell yield and maximal CD34 enrichment, yet does not enrich T cells for peptide-MHC binding. CD34 mAb sort yields CD4 T cells that significantly contribute to antigen-specific T cell functions. Therefore, we conclude that CD34 mAb-based MACSorting of T cells has benefits towards applications of T cell therapy, especially those that require CD4 T cells.

ACKNOWLEDGEMENTS

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SUPLEMENTARY FIGURES

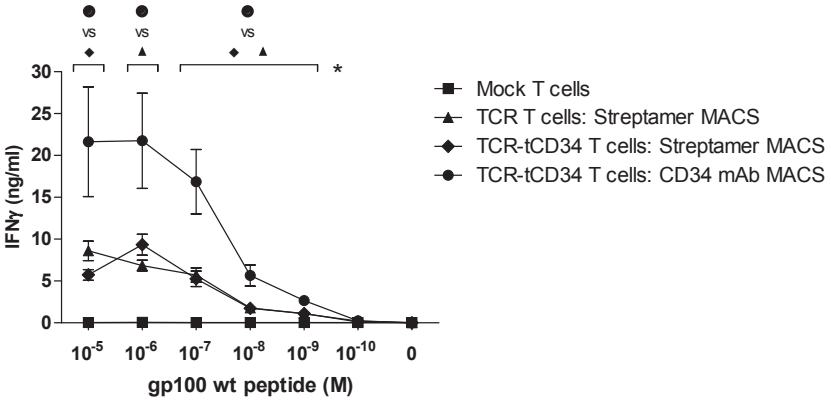
A

Transgenes:	Mock	TCR	TCR-tCD34	TCR-tCD34
Sort reagents:	None	Streptamer	Streptamer	CD34 mAb
pMHC binding (%):	2 ± 0	85 ± 1	54 ± 6	40 ± 5

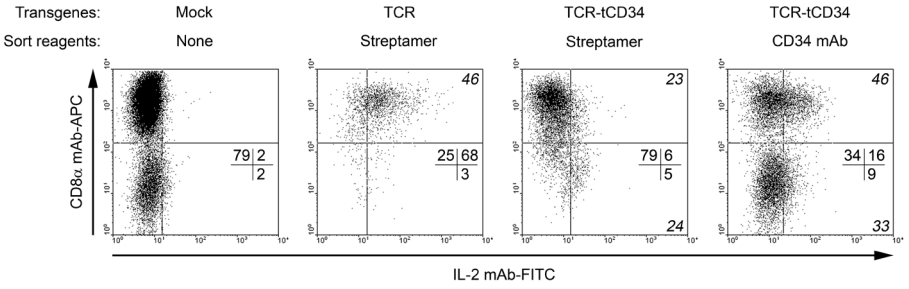
**

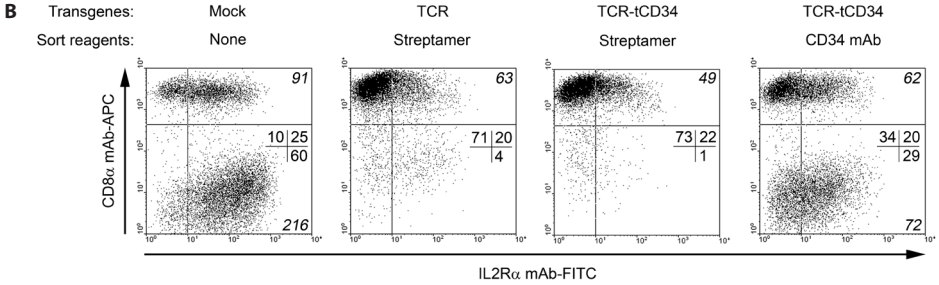
***

B

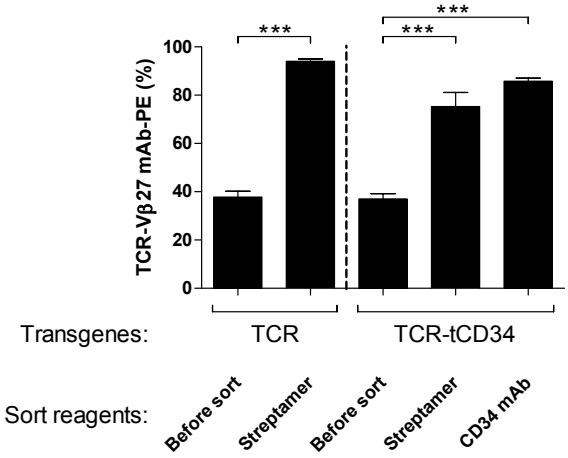


Supplementary figure 6.1. CD34 mAb-sorted T cells demonstrated an enhanced antigen-specific IFNγ production. Primary human T cells were transduced and MACSorted as described in legend to figure 6.4. **(A)** T cells were analyzed for peptide-MHC binding 3-6 weeks after MACS by flow cytometry (using gp100/A2 tetramer). Numbers represent mean % +/- SEM of 4 repeat experiments with T cells from 2 healthy donors. **(B)** Sorted T cells from (A) were analyzed for IFNγ production following antigen-specific stimulation. T cells were stimulated with BLM cells loaded with titrated amounts of gp100 peptide and O/N supernatants were tested for IFNγ by ELISA. Graphs represent mean IFNγ values (in ng/ml) +/- SEM of 2 independent measurements from 2 healthy donors. IFNγ data were corrected for differences in peptide-MHC-binding, with peptide-MHC-binding of CD34 mAb-sorted T cells (as in A) set to 100%. EC_{50} values of streptamer and CD34 mAb-sorted T cells (0.05 and 0.03 µM peptide, respectively) were calculated using Masterplex ReaderFit software. Statistically significant differences are calculated with Student's *t*-tests: * = p<0.05; ** = p<0.005; *** = p<0.0005.

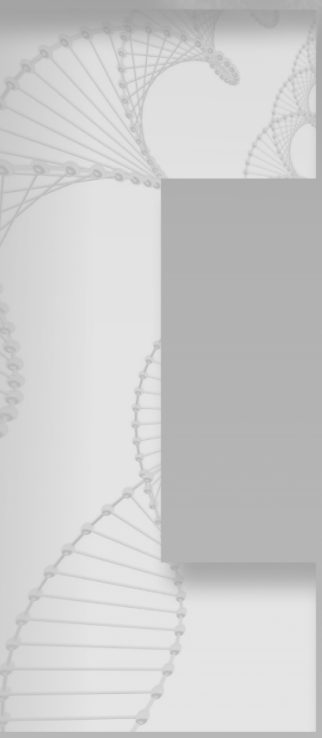




Supplementary figure 6.2. CD34 mAb-sorted CD4 T cells express IL-2 and IL-2Rα. Primary human T cells were transduced and MACSorted as described in legend to figure 6.4. T cells were stained with APC-conjugated anti-CD8α mAb in combination with one of the following antibodies: (**A**) FITC-conjugated anti-IL-2 mAb (n=2); or **(B)** FITC-conjugated anti-CD25 mAb (n=10). In case of IL-2 stainings, T cells were stimulated for 6h with BLM (gp100-/A2+) cells loaded with 10^{-5} M gp100 peptide, permeabilized, and stained with anti-IL-2 mAb, as described in more detail in the materials and methods section. Stainings were analyzed by flow cytometry and figures show representative dotplots, indicating % (all quadrants) and MFI of stained T cells (upper and lower right quadrants when % ≥ 5, in italic).



Supplementary figure 6.3. MACSort with streptamer or CD34 mAb yields T cell populations with significantly enhanced percentages of TCRβ expression. Primary human T cells were transduced and MACSorted as described in legend to figure 6.4. T cells were stained with PE-conjugated anti-TCR-Vβ27 mAb and analyzed by flow cytometry. Bars represent mean % + SEM of 2-4 repeat experiments with T cells from 2 healthy donors. Statistically significant differences are calculated with Student's *t*-tests: *** = p<0.0005.



7

General discussion

In preceding chapters, I have presented the following three strategies to enhance the functional avidity of TCR-engineered T cells without compromising their target specificity:

1. equipment of TCR transgenes with signaling cassettes (chapters 3 and 4);
2. enhancement of TCR affinity (chapter 5); and
3. enrichment of TCR T cells using MACS technology (chapter 6).

In chapters 7.1 to 7.3, I discuss these strategies with respect to their impact on function and self-reactivity of gene-engineered T cells. In chapter 7.4, I will discuss the feasibility of combining these strategies and how this will potentially advance clinical TCR gene therapy.

7.1 EQUIPMENT OF TCR TRANSGENES WITH SIGNALING CASSETTES

In chapters 3 and 4 we have extended our previous studies on the equipment of TCR genes with a signaling cassette consisting of the complete human accessory molecule CD3ζ. This signaling cassette was coupled to both TCRα and TCRβ chains (i.e., TCR:ζ), and resulted in enhanced surface expression of TCR:ζ that was independent of endogenous CD3 and prevented mis-pairing between introduced and endogenous TCR chains (see chapters 1 and 2 for details). In chapter 3 we attempted to minimize the incorporation of CD3ζ sequences with the intent to make minimal TCR:ζ variants with less or no immunogenicity. With the use of two techniques, we have generated 8 minimal TCR:ζ variants either by replacing extracellular, transmembrane and/or intracellular CD3ζ domains by corresponding wild-type (wt) TCR sequences (domain-exchange technique) or transplanting a limited number of CD3ζ transmembrane amino acids onto structurally similar positions in wt TCR (3D-modeling technique). Ideally, minimal TCR:ζ variants needed to preserve the ability of parental TCR:ζ to prevent TCR mis-pairing and at the same time introduce the ability to associate with endogenous CD3 accessory molecules, and potentially enhance the functional T cell responses. Our findings are summarized in top half of box 7.1. In short, replacing the intracellular CD3ζ domain with corresponding intracellular wt TCR sequences resulted in a minimal TCR:ζ with non-functional surface expression. When replacing the CD3ζ transmembrane domain, we demonstrated that this domain was required for TCR:ζ's cell surface expression, prevention of TCR mis-pairing, and lack of association with endogenous CD3. The ability of CD3ζ transmembrane domain to induce homodimerization, as reported for monomeric IL-2Rα chains coupled to CD3ζ (188), is probably critical to preserve the above-mentioned properties of parental TCR:ζ. When replacing the CD3ζ extracellular domain we observed that this domain was dispensable for functional expression of TCR:ζ in Jurkat T cells but not primary human T cells. The lack of minimal TCR:ζ without the extracellular CD3ζ domain to become

Box 7.1. Key findings from chapters 3 and 4

- CD3ζ's intracellular domain: this domain is dispensable for TCR:ζ cell surface expression but not for downstream signaling and T cell activation
- CD3ζ's transmembrane domain: this domain
  - is required for cell surface expression
  - prevents TCR mis-pairing
  - prevents association with endogenous TCR/CD3
  - the above mentioned properties cannot be recapitulated by transplanting a limited number of transmembrane amino acids of CD3ζ onto TCRαβ
- CD3ζ's extracellular domain: this domain is dispensable for functional surface expression in Jurkat T cells, but not primary human T cells. Expression of TCR:ζ without the extracellular CD3ζ domain by primary human T cells cannot be rescued by codon-optimization and an optimal vector configuration
- TCR:28ε does not mis-pair with endogenous TCR chains nor associate with endogenous CD3 molecules
- Compared to wild-type TCR, TCR:28ε demonstrated enhanced:
  - cell surface expression and peptide-MHC binding
  - antigen-specific responses
  - accumulation of cell surface and intracellular effector molecules in immune-synapses
- TCR:28ε does not reveal antigen-independent T cell activation

expressed on primary human T cells could not be rescued by codon optimization of TCR genes and optimal vector configuration. Results with a minimal TCR:ζ that lacked the extracellular CD3ζ domain but now included TCR sequences that encompass the connecting peptide motifs (CPMs) were somewhat unexpected. CPM sequences normally interact with CD3γδε and CD8α (115, 116, 191, 283), and its interaction with CD8α stabilizes the complex between TCR and peptide-MHC, and increases the T cell's dwell time and responses towards low-affinity ligands (238). However, sensitive measurements with Fluorescence Resonance Energy Transfer (FRET) did not confirm enhanced association between this minimal TCR:ζ (without the extracellular domain of CD3ζ) and endogenous CD3. Our observations may be limited to a TCR:ζ specific for MAGE-A1/HLA-A1 since a John Cunningham virus (JCV)/HLA-A2 specific TCR fused to transmembrane and intracellular CD3ζ did demonstrate functional expression by primary human T cells (133). In total, we conclude that exclusion of intracellular, transmembrane and/or extracellular domains of CD3ζ results in functionally compromised TCRs and that properties of TCR:ζ are best preserved when incorporating a complete CD3ζ molecule. Notably, recent clinical trials demonstrate that anti-CAR immune responses were directed against murine idiotypes, but never against boundaries between genetically introduced human molecules (223, 284), making it unlikely that parental TCR:ζ, or TCRs with other human signaling cassettes such as TCR:28ε, will demonstrate enhanced immunogenicity.

In chapter 4 we designed and tested a co-stimulatory signaling cassette that combined the transmembrane and intracellular domains of CD28 with the intracellular domain of CD3ε (CD28-CD3ε). This co-stimulatory domain proved most optimal with respect to functional expression of single-chain TCRs when comparing cassettes that contained different accessory molecules with or without the CD28 co-stimulatory molecule. Findings with TCRα and TCRβ both containing this cassette (TCR:28ε) are summarized in bottom half of box 7.1. TCR:28ε demonstrated enhanced cell surface expression and binding of peptide-MHC when compared to wt TCR. Flow cytometric FRET revealed that TCR:28ε does neither mis-pair with endogenous TCR, does not associate with endogenous CD3 nor affects the CD8α dependency of a tested gp100 specific TCR. T cells expressing TCR:28ε mediated similar CD107a mobilization as wt TCR in response to antigen-positive melanoma cells but significantly enhanced CD107a mobilization in response to peptide-loaded target cells. Importantly, genetic incorporation of the CD28-CD3ε signaling cassette retains the TCR's antigen specificity as evidenced by a non-changed response towards altered peptide ligands and lack of T cell responses in the absence of cognate antigen. The latter findings argue that the CD28 sequence in TCR:28ε does not inhibit, but perhaps even accommodates shielding of the Immunoreceptor Tyrosine-based Activation Motif (ITAM) that is present in CD3ε to prevent unwanted T cell activity. A basic rich stretch (BRS), also present in the CD3ε molecule, normally enables the embedding of ITAM in the plasma membrane' phospholipid bilayer (285). Other studies have suggested that positioning of the BRS more distal from the plasma membrane may render ITAM accessible for phosphorylation and lead to antigen-independent T cell activation (286). In line with our hypothesis that CD28 accommodates rather than disturbs shielding of ITAM is the high level of similarity between the relative number of basic residues in CD3 and CD28 molecules, on average 30% in CD3's BRS and 24% in CD28. In future experiments we would like to test this hypothesis using a FRET system, similar to the one used to determine embedding of the CD3ε intracellular tail (286), with a membrane dye as acceptor and TCR:28ε C-terminal fluorophore as donor. In addition to effector T cell responses, chapter 4 studies have demonstrated that TCR:28ε initiates highly active synapses that contain high levels of TCR:28ε, endogenous TCR/CD3 and CD8α, as well as phosphorylated LCK (pLCK) and ERK (pERK). The enhanced levels of TCR:28ε and CD8α, both containing LCK docking sites, may well be responsible for the observed increase of synaptic levels of pLCK, and consequently the enhanced function of TCR:28ε T cells. LCK activation is an early event in antigen-induced downstream signaling as indicated in figure 1.1, and induces signal amplification and diversification via activation of ZAP70, LAT and ERK, resulting in full T cell activation.

Introduction of CD28 into Chimeric Antigen Receptors (CARs) resulted in increased T cell persistence, which in turn positively correlated with clinical activity (75, 76, 78, 79, 173, 287). Current *in vitro* data with TCR:28ε-transduced T cells make this receptor

a candidate TCR for *in vivo* testing of T cell persistence and anti-tumor activities. Using CARs, CD28 co-stimulation was shown to overcome TGF-β-mediated repression of T cell proliferation, the latter representing an often-observed defense mechanism in tumors to counteract immune responses (288). Other members of the CD28 family of co-stimulatory molecules such as CD134 or CD137, either as single molecules or combined with CD28, have also been incorporated into CARs (77, 199). T cells gene-engineered with a CAR that incorporated CD137 and CD3ζ persisted for 6 months and provided complete clinical responses in two and a partial response in one out of three patients suffering from chronic lymphocytic leukemia (CLL) (77). Treatment of an additional 7 patients with CLL revealed another complete response and three partial responses (Carl June, presentation at the Cellular Therapy against Cancer Symposium, London, Febr. 27th - March 2nd, 2013). CARs containing both CD28 and CD134, mediate decreased activation-induced cell death of effector T cells, prolonged peripheral persistence of T cells, and selectively decreased production of the repressive cytokine IL-10 (199, 289). Such newly designed signaling cassettes, whether incorporated into CARs or TCRs, may represent interesting candidates to test for their clinical anti-tumor activity. Alternatively, as described in more detail in chapter 1, co-stimulation of T cells can be provided *ex vivo* by artificial antigen presenting cells expressing co-stimulatory molecule ligands. In one study, in which patients with metastatic melanoma were treated with T cells that were educated with such co-stimulatory cells, four out of seven evaluable patients showed a clinical response (85).

Conclusion:
Modifying TCRs with signaling cassettes containing CD3ζ or a combination of CD28 and CD3ε successfully prevents TCR mis-pairing. Functional expression of TCR:ζ depends on the complete CD3ζ molecule including its intracellular, transmembrane and extracellular domain. TCR:28ε conveys antigen-specific responses, and does not result in a loss of peptide-fine specificity nor mediates antigen-independent T cell responses. We believe TCR:28ε is a promising receptor format and warrants further testing in pre-clinical TCR gene therapy settings.

7.2 ENHANCEMENT OF TCR AFFINITY

In chapter 5, we have collaborated with Adaptimmune Ltd., Abingdon, UK, to investigate whether affinity-enhanced TCRs selectively lead to improved T cell function without increase in self-recognition. In case readers are not familiar with the terms TCR affinity and T cell avidity, I would like to refer to a detailed description in box 1.2. In this study, phage-display was used to select gp100/HLA-A2-specific TCRs with amino acid substitu-

Box 7.2. Key findings from chapter 5

- TCR specific for gp100/HLA-A2 containing CDR3α (K_D >4 μM) and CDR2β (K_D <1 μM), but not CDR3β (K_D 4-1 μM), amino acid substitutions displayed enhanced peptide-MHC binding
- Enhanced functionality without loss of peptide-fine specificity was only mediated by TCRs with K_D values >4 μM and containing CDR3α amino acid substitutions
- TCRs with K_D values <1 μM and amino acid substitutions in CDR2β displayed HLA-A2-dependent reactivity against melanoma target cells, but also HLA-A2 positive donor T cells

tions that were restricted to either Complementarity Determining Region (CDR) 3α, 2β or 3β, and having K_D values ranging from 18.5 μM to 26 nM. These TCRs were transduced into primary human T cells and findings with these T cells are summarized in box 7.2. We observed that antigen binding and T cell function did not correlate with K_D values, which confirmed an earlier notion that affinities of soluble TCRs do not accurately represent the performance of TCRs when expressed on T cells (92). When looking at production of multiple cytokines as a measure of functionally avid T cells, we identified a hierarchy in the K_D values of our gp100/HLA-A2-specific TCRs. In case TCRs have K_D values between 4 and 18.5 μM, which in our panel of TCRs were limited to those with amino acid substitutions in CDR3α, we observed gain of antigen-specific T cell function when compared to wt TCR (K_D=18.5 μM). In case TCRs have K_D values between 1 and 4 μM, in our panel limited to amino acid substitutions in CDR3β, we observed reduced T cell function and some loss of antigen-specificity. And in case TCRs have K_D values lower than 1 μM, in our panel limited to amino acid substitutions in CDR2β, we observed enhanced yet significant antigen-independent T cell functions. T cell activation may require an optimal dwell time in order to allow serial triggering (239, 290, 291), and enhancing TCR affinity beyond a certain threshold, in particular with significantly lowered K_{off} values (see also table 5.1), may adversely affect T cell function. It is hypothesized that affinities above a pre-set threshold, reflected by K_D values around 4-6 μM, induce T cell activation at a similar rate resulting in an 'all-or-nothing' quality of TCR/CD8 associations and TCR-mediated signaling (238). Initial findings with thymocytes were confirmed with primary human T cells expressing affinity-enhanced TCRs directed against NY-ESO1/HLA-A2 (92, 240) or gp100/HLA-A2 (chapter 5). In contrast, K_D values in the nanomolar range may allow enhanced dwell times that are primarily guided by binding to HLA-A2, not binding to peptide:HLA-A2. To what extent affinity thresholds for either T cell sensitivity or specificity are of a universal nature needs to be confirmed with additional studies. CDR3 regions are responsible for peptide:TCR interaction, whereas CDR1 and CDR2 are mainly responsible for TCR:HLA interaction (292) (see also box 1.2). Along this line, findings from chapter 5 suggest that amino acid substitutions in CDR3α are less prone, while those in CDR2β are more prone, to result in loss of peptide specificity. On the one hand, the clinical use of an affinity-enhanced NY-ESO-1/HLA-A2 specific TCR with a K_D value of 730 nM

and harboring CDR3α mutations showed significant anti-tumor effects with no evidence of side-effects (46). On the other hand, we currently cannot exclude that the specificity of TCR:peptide-MHC interactions is restricted not only by the site of mutations but also by a limited window of affinity enhancement. Holler and colleagues have shown that a QL9-L^d specific TCR with CDR3α mutations and an enhanced K_D value of 9 nM resulted in loss of self-reactivity (228). Moreover, a NY-ESO-1/HLA-A2 specific TCR resulted in loss of antigen-specificity when the TCR harbored amino acid substitutions in CDR2β and had a K_D value of 280 nM, but not a K_D value of 1.1 μM (224). To formally prove that CDR3α but not CDR2β may be a preferred site for mutagenesis, it would be advisable to test the antigen sensitivity and specificity of a panel of TCRs, preferably covering multiple antigen specificities, with mutations in either CDR3α or CDR2β and having K_D's that ranged from nM to μM.

Conclusion:
Limited affinity-enhancement of TCRs with K_D values in the (sub)micromolar range, potentially directed by amino acid substitutions in CDR3, may result in highly functional TCRs that show no loss of peptide specificity. In contrast, affinity-enhancement of TCRs with K_D values in the nanomolar range, potentially directed by amino acid substitutions in CDR2, may result in TCRs with enhanced self-reactivity and argues against its use to generate TCRs to be tested in clinical trials.

7.3 ENRICHMENT OF TCR T CELLS USING MACS TECHNOLOGY

In chapter 6 we used Magnetic-Activated Cell Sorting (MACS) to enrich TCR-engineered T cells and compared various peptide-MHC complexes (i.e., tetramers, pentamers, and streptamers) and anti-CD34 mAb as sorting reagents. Our findings are summarized in box 7.3. Primary human T cells were transduced with TCRα and TCRβ genes on separate

Box 7.3. Key findings from chapter 6

- MACSorting with streptamer, when compared to tetramers and pentamers, resulted in increased:
  - T cell yield
  - T cell proliferation within 1 week after cell sorting
  - enrichment of TCR-engineered T cells that remains stable in time (6 weeks)
- MACSorting with anti-CD34 mAb, when compared to streptamers, resulted in:
  - further increased T cell yield
  - similar T cell proliferation within 1 week after cell sorting
  - negligible enrichment for TCR transgene-expressing T cells
  - a T cell population that consists of both CD8 and CD4 T cells and demonstrates enhanced functional responses

vectors and when MACSorted with peptide-MHC multimers, we observed that streptamers yielded the largest T cell population with most significant and stable enrichment of TCR transgene expression. In accordance with the design of streptamers, which allows release of peptide-MHC monomers from the streptamer scaffold and prevents activation-induced cell death, the acquired T cell population displayed the highest fold of T cell expansion. In subsequent experiments, T cells were transduced with TCRα and TCRβ genes on a single vector interspersed by 2A coupled to truncated CD34 (tCD34) and MACSorted with CD34 mAb. These T cells showed similar T cell proliferation rates but an increased T cell yield when compared to the above-mentioned streptamers. Notably, tCD34-enriched T cell populations showed negligible enrichment for peptide-MHC binding, but we did observe enhanced CD107a cell surface mobilization and IFNγ release in response to antigen-positive tumor cells when compared to streptamer-enriched T cells. With respect to the lack of enrichment for peptide-MHC binding of T cells, we argue that this binding was below the detection limit of standard flow cytometry, potentially due to low expression levels of TCRα as a consequence of TCRα transgene being positioned 3' of the 2A gene segment, but sufficient to induce functional responses . Multi-parameter flow analysis suggested that the observed improvement of functional T cell responses were related to the unique presence of CD4 T cells in the CD34 mAb MACSorted T cell population. These CD4 T cells contributed to the observed IFNγ responses by producing IFNγ themselves and by enhancing the IFNγ production of CD8 T cells.

In addition to the applied peptide-MHC multimers, alternative complexes to enrich for TCR-engineered T cells have been described. For instance, a low valency complex consisting of soluble TCRs dimerized via IgG molecular scaffolds (280) can be used, but these TCR dimers generally show a less-robust staining than tetrameric peptide-MHC complexes (279). Several high valency complexes are also available, such as dextramers (valency of 24) (282), peptide-MHC loaded quantum dots and peptide-MHC presented by biotinylated lipids (valency between 60 and 200; 278, 281). Although usefulness of these novel peptide-MHC reagents needs to be tested for T cell sorting, the high valency may be beneficial and, in analogy to our findings with CD34 mAb, result in sorting of CD4 T cells thereby circumventing a bias towards CD8 T cells as observed with lower valency streptamers. However, a downside to irreversible high valency binding is increased risk of activation-induced cell death.

The magnetic activated cell sorting (MACS) technology has been developed over the past 30 years from a technique to assist cytometric cell separations to a faster, but equally effective alternative for Fluorescence-Activated Cell Sorting (FACS; (248, 249)). FACS may represent a more flexible platform as it does allow the simultaneous detection and selection of multiple TCR specificities, which in some instances is preferred. In example, two-dimensional combinatorial coding with peptide-MHC multimers using 8 fluorochromes allows simultaneous detection and isolation of 28 different tumor

specific T cell populations (37). MACS technology, however, in the format of CliniMACS allows rapid and high quality isolation at Good Manufacturing Practices (GMP) level. CD34 mAb-based CliniMACS has proven its robustness and reproducibility in multi-center clinical trials to prevent graft-versus-host responses (293). We propose to further develop CD34 mAb-based MACS for clinical T cell therapy since the co-presence of CD4 T cells may be beneficial towards CD8 T cell function *in vivo* (269-271, 273-276, 294).

Conclusion:
We demonstrate that the use of truncated CD34 as a surrogate marker to enrich for TCR-engineered T cells constitutes an attractive alternative to the more standard peptide-MHC based sorting protocols. In fact, in a MACS setting we show that CD34 mAb-based enrichment results in enhanced function of the T cell population due to sorting of both CD4 and CD8 T cells.

7.4 WHAT HAVE FINDINGS LEARNED US FOR FUTURE CLINICAL TRIALS?

We have set out to increase safety and efficacy of TCR gene therapy through gene-engineering of TCR and vectors along the lines discussed in chapters 7.1 to 7.3. To take most advantage of each of these strategies, I propose to incorporate various genetic enhancements into a single expression vector (fig 7.1). The combination of the CD28-CD3ε signaling cassette and affinity-matured TCRs, within the earlier defined perimeters, together with enrichment of T cell populations via CD34 mAb-based MACSort, may further enhance the functional avidity of infused T cells without compromising their antigen specificity. Below, I will discuss how such a combined effort may also enhance TCR gene therapy efficacy by addressing an immune-suppressive tumor environment and safety by addressing more general treatment related toxicities.

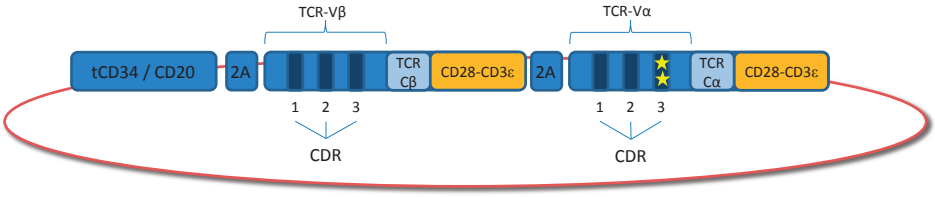


Figure 7.1. Genetic content of a single vector that combines affinity-enhanced TCR chains, CD28-CD3ε signaling cassettes and a tCD34 or CD20 surrogate marker. TCRα and TCRβ variable and constant domains are indicated by Vα, Vβ, Cα and Cβ, respectively. Amino acid substitutions in CDR3α are indicated by stars. Abbreviations used in this figure: CDR, complementarity determining region; tCD34, truncated CD34; TCR-C, T cell receptor constant domain; TCR-V, TCR variable domain.

7.4.1 Immune-suppressive tumor environments

Tumors are well capable to compromise anti-tumor T cell responses by several mechanisms as reviewed by Zou and colleagues (295). In example, tumor stroma, most likely through a lack of Toll-Like Receptor ligands and IFNγ production, may keep antigen-presenting cells in an immature state (296, 297). In addition, intra-tumoral T cells, often as a consequence of antigen stimulation in the absence of T cell co-stimulation, express co-inhibitory molecules such as CTLA-4, PD-1 and TIM-3 (298, 299) and show a block in expression of effector molecules such as IFNγ. Furthermore, presence of suppressive immune cells such as regulatory T cells and myeloid-derived suppressor cells, depletion of key amino acids such as arginine and tryptophan (300-302), and production of anti-inflammatory cytokines such as TGF-β, IL-4 and IL-13 (303) all contribute to the inhibition of effector T cells. Interestingly, immune suppressed effector T cells show a reduced expression of the effector molecules pLCK and pFYN and abrogated expression of CD3ζ (304). We propose that a combination of CD28-mediated T cell co-stimulation, TCR affinity-enhancement and T cell enrichment may counteract the immune suppressive nature of the tumor milieu. First, inclusion of a CD28-CD3ε signaling cassette provides a TCR that does not rely on endogenous CD3 proteins for its surface expression or initiation of T cell activation, and is able to mediate accumulation of enhanced levels of synaptic pLCK. In addition, inclusion of a CD28-containing cassette in a CAR conveys resistance to TGF-β-mediated repression of T cell function (288). A potential risk of CD28-containing receptors is enhanced proliferation of regulatory T cells due to LCK-mediated IL-2 production (205), which, however, can be addressed by mutating the LCK-binding site of CD28 or by using cassettes that harbor additional co-stimulatory molecules such as CD134 (205, 289). Second, affinity-matured TCRs (within defined perimeters) provide optimal T cell responses. Studies with CD28-containing CARs revealed that enhanced T cell co-stimulation does not alter the activation threshold of T cells (305), which argues that combined use of affinity-matured TCRs and a CD28-CD3ε signaling cassette does not compromise advantages of either one component. In addition, affinity-matured TCRs lead to enhanced production of multiple effector cytokines, such as IFNγ, TNFα and IL-2, which may revert an anti-inflammatory tumor environment into a more pro-inflammatory environment. Importantly, enhanced production of pro-inflammatory T cell cytokines may not only contribute to T cell-mediated anti-tumor immunity, but may also involve innate immunity and the eradication of antigen-negative tumor cells (306). Third, enrichment of the frequencies of TCR-engineered T cells via MACSort, in particular when enriching both CD4 and CD8 T cells, will further enhance the functional avidity of therapeutic T cells. The enrichment of multiple pools of T cells engineered with TCRs directed against different target antigens may further advance T cell therapy and limit the risk of tumor recurrence (307, 308).

7.4.2 Treatment related toxicities

Patients treated with T cells are generally pre-conditioned with cytotoxic drugs such as cyclophosphamide and fludarabine and receive high-dose IL-2 injections directly following T cell infusions to enhance the clinical effectiveness of T cell administration (24). However, administration of cytotoxic drugs may result in neutropenia and thrombocytopenia (46), and high-dose IL-2 also is reported to cause off-target toxicities (309, 310). T cell co-stimulation has been demonstrated to make T cell therapy less dependent on patient pre-conditioning and supportive IL-2 treatments, which became evident from two seminal studies. First, T cells gene-engineered with a CD19-specific CAR:CD137 provided complete and partial clinical responses in three and four out of ten patients suffering from chronic B cell leukemia, respectively, all without high-dose IL-2 administration (77). Second, MART-1/A2-specific T cells that were stimulated with artificial antigen presenting cells positive for CD28 ligands mediated clinical responses in four out of seven evaluable patients suffering from metastatic melanoma without the need for chemotherapy or IL-2 support (85). Most likely, autologous IL-2 production is enhanced following T cell co-stimulation and constitutes an important factor in explaining the benefits of T cell co-stimulation. Along this line, the use of CD28 signaling cassettes and affinity-matured TCRs may also represent strategies to make T cell therapy less dependent on additional patient treatments.

With respect to toxicities that are a direct consequence of administered T cells, one can make the distinction between on and off-target toxicities as described in detail in chapter 1. Ideal T cell target antigens would be neo-antigens, which are exclusively expressed by tumor and not healthy cells. These antigens, however, are generally expressed by only single or limited numbers of patients. With the development of second generation sequencing techniques, the targeting of neo-antigens may become feasible in the near future (50, 51). In addition, some CTA show highly restricted expression by tumor cells when compared to healthy cells and may constitute safe and effective target antigens (see chapter 1 for details). CTA expression can be up-regulated and become more homogeneously expressed within tumors by the use of epigenetic modulating compounds, such as the 5-Aza 2'deoxyazacytidine (AZA) demethylation agent and/or the histone deacetylase Valproate (VPA) (311-315). Phase I and II clinical studies that target acute myeloid leukemia, myelodysplasia, multiple myeloma and renal cell carcinoma report enhanced anti-tumor responses due to up-regulated expression of CTA antigens and no evidence of adverse events related to epigenetic modulation (311, 312, 316, 317). Notably, recent data from clinical TCR gene therapy studies have pointed to the necessity to carefully screen target antigens, including MAGEs, with respect to safety. In one study, in which MAGE-A3/HLA-A2 (KVA epitope) was targeted in metastatic melanoma patients, 2 patients lapsed into coma and died, most likely because of T cell recogni-

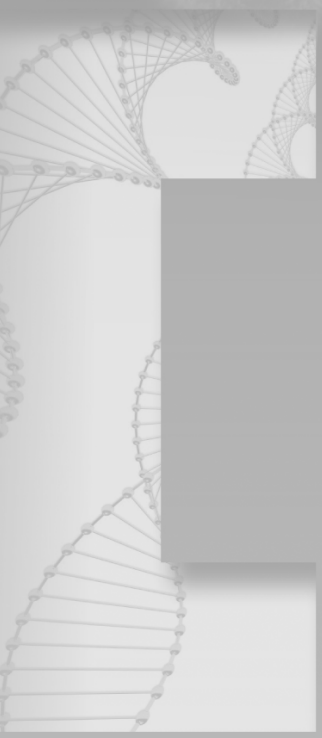
tion of rare neurons positive for MAGE-A12 and possibly MAGE-A9 and expressing the shared KVA epitope (62). In another study, in which MAGE-A3/HLA-A1 (EVD epitope) was targeted in metastatic melanoma and multiple myeloma patients, 2 patients suffered from cardiotoxicity and died, possibly because of T cell recognition of a similar but not identical peptide from the muscle protein Titin (Dr. Bent Jakobsen, Cellular Therapy against Cancer Symposium, London, Febr. 27th - March 2nd, 2013). These studies clearly warrant for stringent preclinical screening to reduce the risk of toxicities in future trials. In addition, such unexpected toxicities can be addressed by incorporating suicide genes into therapeutic T cells such as the drug-induced suicide switches HSV-TK or iCasp9, or the tag-mediated suicide switch CD20 (see chapter 2). In this respect, truncated CD34 cannot be used as a suicide marker because CD34 is widely expressed on hematopoietic stem cells (318). CD20, however, could represent a candidate switch to combine antibody-based enrichment of T cells with antibody-mediated depletion of T cells (319).

Conclusion:

The combined use of affinity-matured TCRs within defined perimeters, CD28-CD3ε signaling cassettes, and truncated CD34 as a marker for T cell enrichment may result in safe and highly avid T cells for use in TCR gene therapy studies. These strategies are preferably combined with TCRs specific for neo-antigens or CTAs, such as MAGE-C2 or NY-ESO-1, to prevent on-target target toxicities. In addition, the additional inclusion of a suicide switch, such as CD20, may further enhance safety of clinical TCR gene therapy.

"I have a friend who's an artist and has sometimes taken a view which I don't agree with very well. He'll hold up a flower and say "look how beautiful it is," and I'll agree. Then he says "I as an artist can see how beautiful this is but you as a scientist take this all apart and it becomes a dull thing," and I think that he's kind of nutty. First of all, the beauty that he sees is available to other people and to me too, I believe. Although I may not be quite as refined aesthetically as he is … I can appreciate the beauty of a flower. At the same time, I see much more about the flower than he sees. I could imagine the cells in there, the complicated actions inside, which also have a beauty. I mean it's not just beauty at this dimension, at one centimeter; there's also beauty at smaller dimensions, the inner structure, also the processes. The fact that the colors in the flower evolved in order to attract insects to pollinate it is interesting; it means that insects can see the color. It adds a question: does this aesthetic sense also exist in the lower forms? Why is it aesthetic? All kinds of interesting questions which the science knowledge only adds to the excitement, the mystery and the awe of a flower. It only adds. I don't understand how it subtracts."

Richard P. Feynman



References

1. Hollestein, L. M., S. A. van den Akker, T. Nijsten, H. E. Karim-Kos, J. W. Coebergh, and E. de Vries. 2012. Trends of cutaneous melanoma in The Netherlands: increasing incidence rates among all Breslow thickness categories and rising mortality rates since 1989. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 23: 524-530.
2. Middleton, M. R., J. J. Grob, N. Aaronson, G. Fierlbeck, W. Tilgen, S. Seiter, M. Gore, S. Aamdal, J. Cebon, A. Coates, B. Dreno, M. Henz, D. Schadendorf, A. Kapp, J. Weiss, U. Fraass, P. Statkevich, M. Muller, and N. Thatcher. 2000. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 18: 158-166.
3. Atkins, M. B., M. T. Lotze, J. P. Dutcher, R. I. Fisher, G. Weiss, K. Margolin, J. Abrams, M. Sznol, D. Parkinson, M. Hawkins, C. Paradise, L. Kunkel, and S. A. Rosenberg. 1999. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 17: 2105-2116.
4. Hodi, F. S., S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbe, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos, and W. J. Urba. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine* 363: 711-723.
5. Prieto, P. A., J. C. Yang, R. M. Sherry, M. S. Hughes, U. S. Kammula, D. E. White, C. L. Levy, S. A. Rosenberg, and G. Q. Phan. 2012. CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 18: 2039-2047.
6. Chapman, P. B., A. Hauschild, C. Robert, J. B. Haanen, P. Ascierto, J. Larkin, R. Dummer, C. Garbe, A. Testori, M. Maio, D. Hogg, P. Lorigan, C. Lebbe, T. Jouary, D. Schadendorf, A. Ribas, S. J. O'Day, J. A. Sosman, J. M. Kirkwood, A. M. Eggermont, B. Dreno, K. Nolop, J. Li, B. Nelson, J. Hou, R. J. Lee, K. T. Flaherty, and G. A. McArthur. 2011. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *The New England journal of medicine* 364: 2507-2516.
7. Herndon, T. M., S. G. Demko, X. Jiang, K. He, J. E. Gootenberg, M. H. Cohen, P. Keegan, and R. Pazdur. 2012. U.S. Food and Drug Administration Approval: peginterferon-alfa-2b for the adjuvant treatment of patients with melanoma. *The oncologist* 17: 1323-1328.
8. Topalian, S. L., F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, P. D. Leming, D. R. Spigel, S. J. Antonia, L. Horn, C. G. Drake, D. M. Pardoll, L. Chen, W. H. Sharfman, R. A. Anders, J. M. Taube, T. L. McMiller, H. Xu, A. J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G. D. Kollia, A. Gupta, J. M. Wigginton, and M. Sznol. 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine* 366: 2443-2454.
9. Wolchok, J. D., H. Kluger, M. K. Callahan, M. A. Postow, N. A. Rizvi, A. M. Lesokhin, N. H. Segal, C. E. Ariyan, R. A. Gordon, K. Reed, M. M. Burke, A. Caldwell, S. A. Kronenberg, B. U. Agunwamba, X. Zhang, I. Lowy, H. D. Inzunza, W. Feely, C. E. Horak, Q. Hong, A. J. Korman, J. M. Wigginton, A. Gupta, and M. Sznol. 2013. Nivolumab plus Ipilimumab in Advanced Melanoma. *The New England journal of medicine*.
10. Hamid, O., C. Robert, A. Daud, F. S. Hodi, W. J. Hwu, R. Kefford, J. D. Wolchok, P. Hersey, R. W. Joseph, J. S. Weber, R. Dronca, T. C. Gangadhar, A. Patnaik, H. Zarour, A. M. Joshua, K. Gergich, J. Elassaiss-Schaap, A. Algazi, C. Mateus, P. Boasberg, P. C. Tumeh, B. Chmielowski, S. W. Ebbinghaus, X. N. Li,

S. P. Kang, and A. Ribas. 2013. Safety and Tumor Responses with Lambrolizumab (Anti-PD-1) in Melanoma. *The New England journal of medicine*.

11. Kirkwood, J. M., L. H. Butterfield, A. A. Tarhini, H. Zarour, P. Kalinski, and S. Ferrone. 2012. Immunotherapy of cancer in 2012. *CA: a cancer journal for clinicians* 62: 309-335.
12. Zito, C. R., and H. M. Kluger. 2012. Immunotherapy for metastatic melanoma. *Journal of cellular biochemistry* 113: 725-734.
13. Kirkwood, J. M., S. Lee, S. J. Moschos, M. R. Albertini, J. C. Michalak, C. Sander, T. Whiteside, L. H. Butterfield, and L. Weiner. 2009. Immunogenicity and antitumor effects of vaccination with peptide vaccine+/-granulocyte-monocyte colony-stimulating factor and/or IFN-alpha2b in advanced metastatic melanoma: Eastern Cooperative Oncology Group Phase II Trial E1696. *Clinical cancer research : an official journal of the American Association for Cancer Research* 15: 1443-1451.
14. Aarntzen, E. H., G. Schreibelt, K. Bol, W. J. Lesterhuis, A. J. Croockewit, J. H. de Wilt, M. M. van Rossum, W. A. Blokx, J. F. Jacobs, T. Duiveman-de Boer, D. H. Schuurhuis, R. Mus, K. Thielemans, I. J. de Vries, C. G. Figdor, C. J. Punt, and G. J. Adema. 2012. Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cells responses in stage III and IV melanoma patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* 18: 5460-5470.
15. de Vries, I. J., W. J. Lesterhuis, N. M. Scharenborg, L. P. Engelen, D. J. Ruiter, M. J. Gerritsen, S. Croockewit, C. M. Britten, R. Torensma, G. J. Adema, C. G. Figdor, and C. J. Punt. 2003. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* 9: 5091-5100.
16. Tel, J., E. H. Aarntzen, T. Baba, G. Schreibelt, B. M. Schulte, D. Benitez-Ribas, O. C. Boerman, S. Croockewit, W. J. Oyen, M. van Rossum, G. Winkels, P. G. Coulie, C. J. Punt, C. G. Figdor, and I. J. de Vries. 2013. Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. *Cancer research* 73: 1063-1075.
17. Restifo, N. P., M. E. Dudley, and S. A. Rosenberg. 2012. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature reviews. Immunology* 12: 269-281.
18. Muranski, P., A. Boni, P. A. Antony, L. Cassard, K. R. Irvine, A. Kaiser, C. M. Paulos, D. C. Palmer, C. E. Touloukian, K. Ptak, L. Gattinoni, C. Wrzesinski, C. S. Hinrichs, K. W. Kerstann, L. Feigenbaum, C. C. Chan, and N. P. Restifo. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112: 362-373.
19. Nishimura, T., K. Iwakabe, M. Sekimoto, Y. Ohmi, T. Yahata, M. Nakui, T. Sato, S. Habu, H. Tashiro, M. Sato, and A. Ohta. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *The Journal of experimental medicine* 190: 617-627.
20. Malyguine, A. M., S. L. Strobl, and M. R. Shurin. 2012. Immunological monitoring of the tumor immunoenvironment for clinical trials. *Cancer immunology, immunotherapy : CII* 61: 239-247.
21. Nguyen, L. T., P. H. Yen, J. Nie, N. Liadis, D. Ghazarian, A. Al-Habeeb, A. Easson, W. Leong, J. Lipa, D. McCready, M. Reedijk, D. Hogg, A. M. Joshua, I. Quirt, H. Messner, P. Shaw, M. Crump, E. Sharon, and P. S. Ohashi. 2010. Expansion and characterization of human melanoma tumor-infiltrating lymphocytes (TILs). *PloS one* 5: e13940.
22. Horowitz, M. M., R. P. Gale, P. M. Sondel, J. M. Goldman, J. Kersey, H. J. Kolb, A. A. Rimm, O. Ringden, C. Rozman, B. Speck, and et al. 1990. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 75: 555-562.
23. Kolb, H. J., G. Socie, T. Duell, M. T. Van Lint, A. Tichelli, J. F. Apperley, E. Nekolla, P. Ljungman, N. Jacobsen, M. van Weel, R. Wick, M. Weiss, and H. G. Prentice. 1999. Malignant neoplasms in long-term

survivors of bone marrow transplantation. Late Effects Working Party of the European Cooperative Group for Blood and Marrow Transplantation and the European Late Effect Project Group. *Annals of internal medicine* 131: 738-744.

24. Klebanoff, C. A., H. T. Khong, P. A. Antony, D. C. Palmer, and N. P. Restifo. 2005. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends in immunology* 26: 111-117.
25. Rosenberg, S. A., J. C. Yang, R. M. Sherry, U. S. Kammula, M. S. Hughes, G. Q. Phan, D. E. Citrin, N. P. Restifo, P. F. Robbins, J. R. Wunderlich, K. E. Morton, C. M. Laurencot, S. M. Steinberg, D. E. White, and M. E. Dudley. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* 17: 4550-4557.
26. Itzhaki, O., E. Hovav, Y. Ziporen, D. Levy, A. Kubi, D. Zikich, L. Hershkovitz, A. J. Treves, B. Shalmon, D. Zippel, G. Markel, R. Shapira-Frommer, J. Schachter, and M. J. Besser. 2011. Establishment and large-scale expansion of minimally cultured "young" tumor infiltrating lymphocytes for adoptive transfer therapy. *J Immunother* 34: 212-220.
27. Besser, M. J., R. Shapira-Frommer, A. J. Treves, D. Zippel, O. Itzhaki, L. Hershkovitz, D. Levy, A. Kubi, E. Hovav, N. Chermoshniuk, B. Shalmon, I. Hardan, R. Catane, G. Markel, S. Apter, A. Ben-Nun, I. Kuchuk, A. Shimoni, A. Nagler, and J. Schachter. 2010. Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 16: 2646-2655.
28. Dudley, M. E., C. A. Gross, M. M. Langhan, M. R. Garcia, R. M. Sherry, J. C. Yang, G. Q. Phan, U. S. Kammula, M. S. Hughes, D. E. Citrin, N. P. Restifo, J. R. Wunderlich, P. A. Prieto, J. J. Hong, R. C. Langan, D. A. Zlott, K. E. Morton, D. E. White, C. M. Laurencot, and S. A. Rosenberg. 2010. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16: 6122-6131.
29. Konig, R. 2002. Interactions between MHC molecules and co-receptors of the TCR. *Current opinion in immunology* 14: 75-83.
30. Riha, P., and C. E. Rudd. 2010. CD28 co-signaling in the adaptive immune response. *Self/nonself* 1: 231-240.
31. Minguet, S., M. Swamy, B. Alarcon, I. F. Luescher, and W. W. Schamel. 2007. Full activation of the T cell receptor requires both clustering and conformational changes at CD3. *Immunity* 26: 43-54.
32. Morgan, R. A., M. E. Dudley, J. R. Wunderlich, M. S. Hughes, J. C. Yang, R. M. Sherry, R. E. Royal, S. L. Topalian, U. S. Kammula, N. P. Restifo, Z. Zheng, A. Nahvi, C. R. de Vries, L. J. Rogers-Freezer, S. A. Mavroukakis, and S. A. Rosenberg. 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314: 126-129.
33. Carrasco, J., A. Van Pel, B. Neyns, B. Lethe, F. Brasseur, N. Renkvist, P. van der Bruggen, N. van Baren, R. Paulus, K. Thielemans, T. Boon, and D. Godelaine. 2008. Vaccination of a melanoma patient with mature dendritic cells pulsed with MAGE-3 peptides triggers the activity of nonvaccine anti-tumor cells. *J Immunol* 180: 3585-3593.
34. Germeau, C., W. Ma, F. Schiavetti, C. Lurquin, E. Henry, N. Vigneron, F. Brasseur, B. Lethe, E. De Plaen, T. Velu, T. Boon, and P. G. Coulie. 2005. High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. *The Journal of experimental medicine* 201: 241-248.
35. Lurquin, C., B. Lethe, E. De Plaen, V. Corbiere, I. Theate, N. van Baren, P. G. Coulie, and T. Boon. 2005. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *The Journal of experimental medicine* 201: 249-257.

36. Schuler-Thurner, B., E. S. Schultz, T. G. Berger, G. Weinlich, S. Ebner, P. Woerl, A. Bender, B. Feuerstein, P. O. Fritsch, N. Romani, and G. Schuler. 2002. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *The Journal of experimental medicine* 195: 1279-1288.
37. Hadrup, S. R., A. H. Bakker, C. J. Shu, R. S. Andersen, J. van Veluw, P. Hombrink, E. Castermans, P. Thor Straten, C. Blank, J. B. Haanen, M. H. Heemskerk, and T. N. Schumacher. 2009. Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nature methods* 6: 520-526.
38. Sadovnikova, E., L. A. Jopling, K. S. Soo, and H. J. Stauss. 1998. Generation of human tumor-reactive cytotoxic T cells against peptides presented by non-self HLA class I molecules. *European journal of immunology* 28: 193-200.
39. Li, L. P., J. C. Lampert, X. Chen, C. Leitao, J. Popovic, W. Muller, and T. Blankenstein. 2010. Transgenic mice with a diverse human T cell antigen receptor repertoire. *Nature medicine* 16: 1029-1034.
40. Parkhurst, M. R., J. C. Yang, R. C. Langan, M. E. Dudley, D. A. Nathan, S. A. Feldman, J. L. Davis, R. A. Morgan, M. J. Merino, R. M. Sherry, M. S. Hughes, U. S. Kammula, G. Q. Phan, R. M. Lim, S. A. Wank, N. P. Restifo, P. F. Robbins, C. M. Laurencot, and S. A. Rosenberg. 2011. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Molecular therapy : the journal of the American Society of Gene Therapy* 19: 620-626.
41. Savage, P., L. Gao, K. Vento, P. Cowburn, S. Man, N. Steven, G. Ogg, A. McMichael, A. Epenetos, E. Goulmy, and H. J. Stauss. 2004. Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen. *Blood* 103: 4613-4615.
42. Wilde, S., C. Geiger, S. Milosevic, B. Mosetter, S. Eichenlaub, and D. J. Schendel. 2012. Generation of allo-restricted peptide-specific T cells using RNA-pulsed dendritic cells: A three phase experimental procedure. *Oncoimmunology* 1: 129-140.
43. Straetemans, T., M. van Brakel, S. van Steenbergen, M. Broertjes, J. Drexhage, J. Hegmans, B. N. Lambrecht, C. Lamers, P. van Der Bruggen, P. G. Coulie, and R. Debets. 2012. TCR gene transfer: MAGE-C2/HLA-A2 and MAGE-A3/HLA-DP4 epitopes as melanoma-specific immune targets. *Clinical & developmental immunology* 2012: 586314.
44. Gilham, D. E., R. Debets, M. Pule, R. E. Hawkins, and H. Abken. 2012. CAR-T cells and solid tumors: tuning T cells to challenge an inveterate foe. *Trends in molecular medicine* 18: 377-384.
45. Johnson, L. A., R. A. Morgan, M. E. Dudley, L. Cassard, J. C. Yang, M. S. Hughes, U. S. Kammula, R. E. Royal, R. M. Sherry, J. R. Wunderlich, C. C. Lee, N. P. Restifo, S. L. Schwarz, A. P. Cogdill, R. J. Bishop, H. Kim, C. C. Brewer, S. F. Rudy, C. VanWaes, J. L. Davis, A. Mathur, R. T. Ripley, D. A. Nathan, C. M. Laurencot, and S. A. Rosenberg. 2009. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114: 535-546.
46. Robbins, P. F., R. A. Morgan, S. A. Feldman, J. C. Yang, R. M. Sherry, M. E. Dudley, J. R. Wunderlich, A. V. Nahvi, L. J. Helman, C. L. Mackall, U. S. Kammula, M. S. Hughes, N. P. Restifo, M. Raffeld, C. C. Lee, C. L. Levy, Y. F. Li, M. El-Gamil, S. L. Schwarz, C. Laurencot, and S. A. Rosenberg. 2011. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 29: 917-924.
47. Vujanovic, L., and L. H. Butterfield. 2007. Melanoma cancer vaccines and anti-tumor T cell responses. *Journal of cellular biochemistry* 102: 301-310.
48. Lucas, S., and P. G. Coulie. 2008. About human tumor antigens to be used in immunotherapy. *Seminars in immunology* 20: 301-307.

49. Cheever, M. A., J. P. Allison, A. S. Ferris, O. J. Finn, B. M. Hastings, T. T. Hecht, I. Mellman, S. A. Prindiville, J. L. Viner, L. M. Weiner, and L. M. Matrisian. 2009. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clinical cancer research : an official journal of the American Association for Cancer Research* 15: 5323-5337.
50. Meyerson, M., S. Gabriel, and G. Getz. 2010. Advances in understanding cancer genomes through second-generation sequencing. *Nature reviews. Genetics* 11: 685-696.
51. Zhao, J., and S. F. Grant. 2011. Advances in whole genome sequencing technology. *Current pharmaceutical biotechnology* 12: 293-305.
52. Stratton, M. R. 2011. Exploring the genomes of cancer cells: progress and promise. *Science* 331: 1553-1558.
53. Wood, L. D., D. W. Parsons, S. Jones, J. Lin, T. Sjoblom, R. J. Leary, D. Shen, S. M. Boca, T. Barber, J. Ptak, N. Silliman, S. Szabo, Z. Dezso, V. Ustyanksky, T. Nikolskaya, Y. Nikolsky, R. Karchin, P. A. Wilson, J. S. Kaminker, Z. Zhang, R. Croshaw, J. Willis, D. Dawson, M. Shipitsin, J. K. Willson, S. Sukumar, K. Polyak, B. H. Park, C. L. Pethiyagoda, P. V. Pant, D. G. Ballinger, A. B. Sparks, J. Hartigan, D. R. Smith, E. Suh, N. Papadopoulos, P. Buckhaults, S. D. Markowitz, G. Parmigiani, K. W. Kinzler, V. E. Velculescu, and B. Vogelstein. 2007. The genomic landscapes of human breast and colorectal cancers. *Science* 318: 1108-1113.
54. Lee, W., Z. Jiang, J. Liu, P. M. Haverty, Y. Guan, J. Stinson, P. Yue, Y. Zhang, K. P. Pant, D. Bhatt, C. Ha, S. Johnson, M. I. Kennemer, S. Mohan, I. Nazarenko, C. Watanabe, A. B. Sparks, D. S. Shames, R. Gentleman, F. J. de Sauvage, H. Stern, A. Pandita, D. G. Ballinger, R. Drmanac, Z. Modrusan, S. Seshagiri, and Z. Zhang. 2010. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. *Nature* 465: 473-477.
55. Heemskerk, B., P. Kvistborg, and T. N. Schumacher. 2012. The cancer antigenome. *The EMBO journal* 32: 194-203.
56. Hofmann, O., O. L. Caballero, B. J. Stevenson, Y. T. Chen, T. Cohen, R. Chua, C. A. Maher, S. Panji, U. Schaefer, A. Kruger, M. Lehvaslaiho, P. Carninci, Y. Hayashizaki, C. V. Jongeneel, A. J. Simpson, L. J. Old, and W. Hide. 2008. Genome-wide analysis of cancer/testis gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 105: 20422-20427.
57. de Carvalho, F., A. L. Vettore, and G. W. Colleoni. 2012. Cancer/Testis Antigen MAGE-C1/CT7: new target for multiple myeloma therapy. *Clinical & developmental immunology* 2012: 257695.
58. Caballero, O. L., and Y. T. Chen. 2009. Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer science* 100: 2014-2021.
59. Schultz, E. S., B. Schuler-Thurner, V. Stroobant, L. Jenne, T. G. Berger, K. Thielemanns, P. van der Bruggen, and G. Schuler. 2004. Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 172: 1304-1310.
60. Gure, A. O., R. Chua, B. Williamson, M. Gonen, C. A. Ferrera, S. Gnjatic, G. Ritter, A. J. Simpson, Y. T. Chen, L. J. Old, and N. K. Altorki. 2005. Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11: 8055-8062.
61. Costa, F. F., K. Le Blanc, and B. Brodin. 2007. Concise review: cancer/testis antigens, stem cells, and cancer. *Stem Cells* 25: 707-711.
62. Morgan, R. A., N. Chinnasamy, D. Abate-Daga, A. Gros, P. F. Robbins, Z. Zheng, M. E. Dudley, S. A. Feldman, J. C. Yang, R. M. Sherry, G. Q. Phan, M. S. Hughes, U. S. Kammula, A. D. Miller, C. J. Hessman, A. A. Stewart, N. P. Restifo, M. M. Quezado, M. Alimchandani, A. Z. Rosenberg, A. Nath, T. Wang, B. Bielekova, S. C. Wuest, N. Akula, F. J. McMahon, S. Wilde, B. Mosetter, D. J. Schendel, C. M. Laurencot,

and S. A. Rosenberg. 2013. Cancer Regression and Neurological Toxicity Following Anti-MAGE-A3 TCR Gene Therapy. *J Immunother* 36: 133-151.

63. Sebestyen, Z., E. Schooten, T. Sals, I. Zaldivar, E. San Jose, B. Alarcon, S. Bobisse, A. Rosato, J. Szollosi, J. W. Gratama, R. A. Willemsen, and R. Debets. 2008. Human TCR that incorporate CD3zeta induce highly preferred pairing between TCRalpha and beta chains following gene transfer. *J Immunol* 180: 7736-7746.
64. Willemsen, R. A., M. E. Weijtens, C. Ronteltap, Z. Eshhar, J. W. Gratama, P. Chames, and R. L. Bolhuis. 2000. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene therapy* 7: 1369-1377.
65. Bendle, G. M., C. Linnemann, A. I. Hooijkaas, L. Bies, M. A. de Witte, A. Jorritsma, A. D. Kaiser, N. Pouw, R. Debets, E. Kieback, W. Uckert, J. Y. Song, J. B. Haanen, and T. N. Schumacher. 2010. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nature medicine* 16: 565-570, 561p following 570.
66. van Loenen, M. M., R. de Boer, A. L. Amir, R. S. Hagedoorn, G. L. Volbeda, R. Willemze, J. J. van Rood, J. H. Falkenburg, and M. H. Heemskerk. 2010. Mixed T cell receptor dimers harbor potentially harmful neoreactivity. *Proceedings of the National Academy of Sciences of the United States of America* 107: 10972-10977.
67. Ochi, T., H. Fujiwara, S. Okamoto, J. An, K. Nagai, T. Shirakata, J. Mineno, K. Kuzushima, H. Shiku, and M. Yasukawa. 2011. Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. *Blood* 118: 1495-1503.
68. Okamoto, S., J. Mineno, H. Ikeda, H. Fujiwara, M. Yasukawa, H. Shiku, and I. Kato. 2009. Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR. *Cancer research* 69: 9003-9011.
69. Provasi, E., P. Genovese, A. Lombardo, Z. Magnani, P. Q. Liu, A. Reik, V. Chu, D. E. Paschon, L. Zhang, J. Kuball, B. Camisa, A. Bondanza, G. Casorati, M. Ponzoni, F. Ciceri, C. Bordignon, P. D. Greenberg, M. C. Holmes, P. D. Gregory, L. Naldini, and C. Bonini. 2012. Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nature medicine* 18: 807-815.
70. Lamers, C. H., R. A. Willemsen, B. A. Luider, R. Debets, and R. L. Bolhuis. 2002. Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer. *Cancer gene therapy* 9: 613-623.
71. Lamers, C. H., R. A. Willemsen, P. van Elzakker, B. A. van Krimpen, J. W. Gratama, and R. Debets. 2006. Phoenix-ampho outperforms PG13 as retroviral packaging cells to transduce human T cells with tumor-specific receptors: implications for clinical immunogene therapy of cancer. *Cancer gene therapy* 13: 503-509.
72. Gattinoni, L., D. J. Powell, Jr., S. A. Rosenberg, and N. P. Restifo. 2006. Adoptive immunotherapy for cancer: building on success. *Nature reviews. Immunology* 6: 383-393.
73. Hombach, A. A., A. Holzinger, and H. Abken. 2012. The weal and woe of costimulation in the adoptive therapy of cancer with chimeric antigen receptor (CAR)-redirected T cells. *Current molecular medicine*.
74. Riley, J. L., and C. H. June. 2005. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood* 105: 13-21.
75. Savoldo, B., C. A. Ramos, E. Liu, M. P. Mims, M. J. Keating, G. Carrum, R. T. Kamble, C. M. Bollard, A. P. Gee, Z. Mei, H. Liu, B. Grilley, C. M. Rooney, H. E. Heslop, M. K. Brenner, and G. Dotti. 2011. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *The Journal of clinical investigation* 121: 1822-1826.

76. Brentjens, R. J., I. Riviere, J. H. Park, M. L. Davila, X. Wang, J. Stefanski, C. Taylor, R. Yeh, S. Bartido, O. Borquez-Ojeda, M. Olszewska, Y. Bernal, H. Pegram, M. Przybylowski, D. Hollyman, Y. Usachenko, D. Pirraglia, J. Hosey, E. Santos, E. Halton, P. Maslak, D. Scheinberg, J. Jurcic, M. Heaney, G. Heller, M. Frattini, and M. Sadelain. 2011. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 118: 4817-4828.
77. Kalos, M., B. L. Levine, D. L. Porter, S. Katz, S. A. Grupp, A. Bagg, and C. H. June. 2011. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Science translational medicine* 3: 95ra73.
78. Kochenderfer, J. N., M. E. Dudley, S. A. Feldman, W. H. Wilson, D. E. Spaner, I. Maric, M. Stetler-Stevenson, G. Q. Phan, M. S. Hughes, R. M. Sherry, J. C. Yang, U. S. Kammula, L. Devillier, R. Carpenter, D. A. Nathan, R. A. Morgan, C. Laurencot, and S. A. Rosenberg. 2012. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 119: 2709-2720.
79. Kershaw, M. H., J. A. Westwood, L. L. Parker, G. Wang, Z. Eshhar, S. A. Mavroukakis, D. E. White, J. R. Wunderlich, S. Canevari, L. Rogers-Freezer, C. C. Chen, J. C. Yang, S. A. Rosenberg, and P. Hwu. 2006. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12: 6106-6115.
80. Lamers, C. H., S. C. Langeveld, C. M. Groot-van Ruijven, R. Debets, S. Sleijfer, and J. W. Gratama. 2007. Gene-modified T cells for adoptive immunotherapy of renal cell cancer maintain transgene-specific immune functions in vivo. *Cancer immunology, immunotherapy : CII* 56: 1875-1883.
81. Park, J. R., D. L. Digiusto, M. Slovak, C. Wright, A. Naranjo, J. Wagner, H. B. Meechoovet, C. Bautista, W. C. Chang, J. R. Ostberg, and M. C. Jensen. 2007. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Molecular therapy : the journal of the American Society of Gene Therapy* 15: 825-833.
82. Pule, M. A., B. Savoldo, G. D. Myers, C. Rossig, H. V. Russell, G. Dotti, M. H. Huls, E. Liu, A. P. Gee, Z. Mei, E. Yvon, H. L. Weiss, H. Liu, C. M. Rooney, H. E. Heslop, and M. K. Brenner. 2008. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nature medicine* 14: 1264-1270.
83. Till, B. G., M. C. Jensen, J. Wang, E. Y. Chen, B. L. Wood, H. A. Greisman, X. Qian, S. E. James, A. Raubitschek, S. J. Forman, A. K. Gopal, J. M. Pagel, C. G. Lindgren, P. D. Greenberg, S. R. Riddell, and O. W. Press. 2008. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 112: 2261-2271.
84. Singh, H., M. J. Figliola, M. J. Dawson, H. Huls, S. Olivares, K. Switzer, T. Mi, S. Maiti, P. Kebriaei, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2011. Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. *Cancer research* 71: 3516-3527.
85. Butler, M. O., P. Friedlander, M. I. Milstein, M. M. Mooney, G. Metzler, A. P. Murray, M. Tanaka, A. Berezovskaya, O. Imataki, L. Drury, L. Brennan, M. Flavin, D. Neuberg, K. Stevenson, D. Lawrence, F. S. Hodi, E. F. Velazquez, M. T. Jaklitsch, S. E. Russell, M. Mihm, L. M. Nadler, and N. Hirano. 2011. Establishment of antitumor memory in humans using in vitro-educated CD8+ T cells. *Science translational medicine* 3: 80ra34.
86. Aleksic, M., N. Liddy, P. E. Molloy, N. Pumphrey, A. Vuidepot, K. M. Chang, and B. K. Jakobsen. 2012. Different affinity windows for virus and cancer-specific T-cell receptors: implications for therapeutic strategies. *European journal of immunology* 42: 3174-3179.

87. Zeh, H. J., 3rd, D. Perry-Lalley, M. E. Dudley, S. A. Rosenberg, and J. C. Yang. 1999. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J Immunol* 162: 989-994.
88. Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by alpha beta T cell receptors. *Annual review of immunology* 16: 523-544.
89. Morris, G. P., and P. M. Allen. 2012. How the TCR balances sensitivity and specificity for the recognition of self and pathogens. *Nature immunology* 13: 121-128.
90. Williams, C. B., D. L. Engle, G. J. Kersh, J. Michael White, and P. M. Allen. 1999. A kinetic threshold between negative and positive selection based on the longevity of the T cell receptor-ligand complex. *The Journal of experimental medicine* 189: 1531-1544.
91. Wilde, S., D. Sommermeyer, M. Leisegang, B. Frankenberger, B. Mosetter, W. Uckert, and D. J. Schendel. 2012. Human antitumor CD8+ T cells producing Th1 polycytokines show superior antigen sensitivity and tumor recognition. *J Immunol* 189: 598-605.
92. Zhao, Y., A. D. Bennett, Z. Zheng, Q. J. Wang, P. F. Robbins, L. Y. Yu, Y. Li, P. E. Molloy, S. M. Dunn, B. K. Jakobsen, S. A. Rosenberg, and R. A. Morgan. 2007. High-affinity TCRs generated by phage display provide CD4+ T cells with the ability to recognize and kill tumor cell lines. *J Immunol* 179: 5845-5854.
93. Engels, B., H. Cam, T. Schuler, S. Indraccolo, M. Gladow, C. Baum, T. Blankenstein, and W. Uckert. 2003. Retroviral vectors for high-level transgene expression in T lymphocytes. *Human gene therapy* 14: 1155-1168.
94. Schambach, A., H. Wodrich, M. Hildinger, J. Bohne, H. G. Krausslich, and C. Baum. 2000. Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* 2: 435-445.
95. Leisegang, M., B. Engels, P. Meyerhuber, E. Kieback, D. Sommermeyer, S. A. Xue, S. Reuss, H. Stauss, and W. Uckert. 2008. Enhanced functionality of T cell receptor-redirected T cells is defined by the transgene cassette. *J Mol Med* 86: 573-583.
96. Scholten, K. B., D. Kramer, E. W. Kueter, M. Graf, T. Schoedl, C. J. Meijer, M. W. Schreurs, and E. Hooijberg. 2006. Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. *Clin Immunol* 119: 135-145.
97. Coccoris, M., T. Straetemans, C. Govers, C. Lamers, S. Sleijfer, and R. Debets. 2010. T cell receptor (TCR) gene therapy to treat melanoma: lessons from clinical and preclinical studies. *Expert opinion on biological therapy* 10: 547-562.
98. Sussman, J. J., J. S. Bonifacino, J. Lippincott-Schwartz, A. M. Weissman, T. Saito, R. D. Klausner, and J. D. Ashwell. 1988. Failure to synthesize the T cell CD3-zeta chain: structure and function of a partial T cell receptor complex. *Cell* 52: 85-95.
99. Haga-Friedman, A., M. Horovitz-Fried, and C. J. Cohen. 2012. Incorporation of transmembrane hydrophobic mutations in the TCR enhance its surface expression and T cell functional avidity. *J Immunol* 188: 5538-5546.
100. Kuball, J., B. Hauptrock, V. Malina, E. Antunes, R. H. Voss, M. Wolfl, R. Strong, M. Theobald, and P. D. Greenberg. 2009. Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *The Journal of experimental medicine* 206: 463-475.
101. Ahmadi, M., J. W. King, S. A. Xue, C. Voisine, A. Holler, G. P. Wright, J. Waxman, E. Morris, and H. J. Stauss. 2011. CD3 limits the efficacy of TCR gene therapy in vivo. *Blood* 118: 3528-3537.
102. Cobbold, M., N. Khan, B. Pourgheysari, S. Tauro, D. McDonald, H. Osman, M. Assenmacher, L. Billingham, C. Steward, C. Crawley, E. Olavarria, J. Goldman, R. Chakraverty, P. Mahendra, C. Craddock, and P. A. Moss. 2005. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *The Journal of experimental medicine* 202: 379-386.

103. Dudley, M. E., J. R. Wunderlich, P. F. Robbins, J. C. Yang, P. Hwu, D. J. Schwartzentruber, S. L. Topalian, R. Sherry, N. P. Restifo, A. M. Hubicki, M. R. Robinson, M. Raffeld, P. Duray, C. A. Seipp, L. Rogers-Freezer, K. E. Morton, S. A. Mavroukakis, D. E. White, and S. A. Rosenberg. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298: 850-854.

104. Yee, C., J. A. Thompson, D. Byrd, S. R. Riddell, P. Roche, E. Celis, and P. D. Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* 99: 16168-16173.

105. Bollard, C. M., L. Aguilar, K. C. Straathof, B. Gahn, M. H. Huls, A. Rousseau, J. Sixbey, M. V. Gresik, G. Carrum, M. Hudson, D. Dilloo, A. Gee, M. K. Brenner, C. M. Rooney, and H. E. Heslop. 2004. Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J Exp Med* 200: 1623-1633.

106. Comoli, P., P. Pedrazzoli, R. Maccario, S. Basso, O. Carminati, M. Labirio, R. Schiavo, S. Secondino, C. Frasson, C. Perotti, M. Moroni, F. Locatelli, and S. Siena. 2005. Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes. *J Clin Oncol* 23: 8942-8949.

107. Rooney, C. M., C. A. Smith, C. Y. Ng, S. K. Loftin, J. W. Sixbey, Y. Gan, D. K. Srivastava, L. C. Bowman, R. A. Krance, M. K. Brenner, and H. E. Heslop. 1998. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92: 1549-1555.

108. Savoldo, B., J. A. Goss, M. M. Hammer, L. Zhang, T. Lopez, A. P. Gee, Y. F. Lin, R. E. Quiros-Tejeira, P. Reinke, S. Schubert, S. Gottschalk, M. J. Finegold, M. K. Brenner, C. M. Rooney, and H. E. Heslop. 2006. Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood* 108: 2942-2949.

109. Savoldo, B., M. H. Huls, Z. Liu, T. Okamura, H. D. Volk, P. Reinke, R. Sabat, N. Babel, J. F. Jones, J. Webster-Cyriaque, A. P. Gee, M. K. Brenner, H. E. Heslop, and C. M. Rooney. 2002. Autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for the treatment of persistent active EBV infection. *Blood* 100: 4059-4066.

110. Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 333: 1038-1044.

111. Dudley, M. E., J. R. Wunderlich, J. C. Yang, R. M. Sherry, S. L. Topalian, N. P. Restifo, R. E. Royal, U. Kammula, D. E. White, S. A. Mavroukakis, L. J. Rogers, G. J. Gracia, S. A. Jones, D. P. Mangiameli, M. M. Pelletier, J. Gea-Banacloche, M. R. Robinson, D. M. Berman, A. C. Filie, A. Abati, and S. A. Rosenberg. 2005. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23: 2346-2357.

112. Dudley, M. E., J. C. Yang, R. Sherry, M. S. Hughes, R. Royal, U. Kammula, P. F. Robbins, J. Huang, D. E. Citrin, S. F. Leitman, J. Wunderlich, N. P. Restifo, A. Thomasian, S. G. Downey, F. O. Smith, J. Klapper, K. Morton, C. Laurencot, D. E. White, and S. A. Rosenberg. 2008. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 26: 5233-5239.

113. Balch, C. M., S. J. Soong, J. E. Gershenwald, J. F. Thompson, D. S. Reintgen, N. Cascinelli, M. Urist, K. M. McMasters, M. I. Ross, J. M. Kirkwood, M. B. Atkins, J. A. Thompson, D. G. Coit, D. Byrd, R. Desmond, Y. Zhang, P. Y. Liu, G. H. Lyman, and A. Morabito. 2001. Prognostic factors analysis of

17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* 19: 3622-3634.

114. Call, M. E., J. Pyrdol, M. Wiedmann, and K. W. Wucherpfennig. 2002. The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell* 111: 967-979.
115. Backstrom, B. T., E. Milia, A. Peter, B. Jaureguiberry, C. T. Baldari, and E. Palmer. 1996. A motif within the T cell receptor alpha chain constant region connecting peptide domain controls antigen responsiveness. *Immunity* 5: 437-447.
116. Xu, C., M. E. Call, and K. W. Wucherpfennig. 2006. A membrane-proximal tetracysteine motif contributes to assembly of CD3deltaepsilon and CD3gammaepsilon dimers with the T cell receptor. *The Journal of biological chemistry* 281: 36977-36984.
117. Kuhns, M. S., and M. M. Davis. 2007. Disruption of extracellular interactions impairs T cell receptor-CD3 complex stability and signaling. *Immunity* 26: 357-369.
118. Tailor, P., S. Tsai, A. Shameli, P. Serra, J. Wang, S. Robbins, M. Nagata, A. L. Szymczak-Workman, D. A. Vignali, and P. Santamaria. 2008. The proline-rich sequence of CD3epsilon as an amplifier of low-avidity TCR signaling. *J Immunol* 181: 243-255.
119. Call, M. E., and K. W. Wucherpfennig. 2007. Common themes in the assembly and architecture of activating immune receptors. *Nat Rev Immunol* 7: 841-850.
120. Exley, M., C. Terhorst, and T. Wileman. 1991. Structure, assembly and intracellular transport of the T cell receptor for antigen. *Semin Immunol* 3: 283-297.
121. Gil, D., W. W. Schamel, M. Montoya, F. Sanchez-Madrid, and B. Alarcon. 2002. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* 109: 901-912.
122. Bendle, G. M., J. B. Haanen, and T. N. Schumacher. 2009. Preclinical development of T cell receptor gene therapy. *Curr Opin Immunol* 21: 209-214.
123. Cohen, C. J., Y. Zhao, Z. Zheng, S. A. Rosenberg, and R. A. Morgan. 2006. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res* 66: 8878-8886.
124. Voss, R. H., J. Kuball, R. Engel, P. Guillaume, P. Romero, C. Huber, and M. Theobald. 2006. Redirection of T cells by delivering a transgenic mouse-derived MDM2 tumor antigen-specific TCR and its humanized derivative is governed by the CD8 coreceptor and affects natural human TCR expression. *Immunol Res* 34: 67-87.
125. Thomas, S., S. A. Xue, M. Cesco-Gaspere, E. San Jose, D. P. Hart, V. Wong, R. Debets, B. Alarcon, E. Morris, and H. J. Stauss. 2007. Targeting the Wilms tumor antigen 1 by TCR gene transfer: TCR variants improve tetramer binding but not the function of gene modified human T cells. *J Immunol* 179: 5803-5810.
126. Boulter, J. M., M. Glick, P. T. Todorov, E. Baston, M. Sami, P. Rizkallah, and B. K. Jakobsen. 2003. Stable, soluble T-cell receptor molecules for crystallization and therapeutics. *Protein engineering* 16: 707-711.
127. Kuball, J., M. L. Dossett, M. Wolfl, W. Y. Ho, R. H. Voss, C. Fowler, and P. D. Greenberg. 2007. Facilitating matched pairing and expression of TCR chains introduced into human T cells. *Blood* 109: 2331-2338.
128. Cohen, C. J., Y. F. Li, M. El-Gamil, P. F. Robbins, S. A. Rosenberg, and R. A. Morgan. 2007. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. *Cancer Res* 67: 3898-3903.

129. Voss, R. H., R. A. Willemsen, J. Kuball, M. Grabowski, R. Engel, R. S. Intan, P. Guillaume, P. Romero, C. Huber, and M. Theobald. 2008. Molecular design of the Calphabeta interface favors specific pairing of introduced TCRalphabeta in human T cells. *J Immunol* 180: 391-401.
130. Schaft, N., B. Lankiewicz, J. W. Gratama, R. L. Bolhuis, and R. Debets. 2003. Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT. *Journal of immunological methods* 280: 13-24.
131. Schaft, N., B. Lankiewicz, J. Drexhage, C. Berrevoets, D. J. Moss, V. Levitsky, M. Bonneville, S. P. Lee, A. J. McMichael, J. W. Gratama, R. L. Bolhuis, R. Willemsen, and R. Debets. 2006. T cell re-targeting to EBV antigens following TCR gene transfer: CD28-containing receptors mediate enhanced antigen-specific IFNgamma production. *Int Immunol* 18: 591-601.
132. Schaft, N., R. A. Willemsen, J. de Vries, B. Lankiewicz, B. W. Essers, J. W. Gratama, C. G. Figdor, R. L. Bolhuis, R. Debets, and G. J. Adema. 2003. Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR alpha beta genes into primary human T lymphocytes. *J Immunol* 170: 2186-2194.
133. Yang, W., E. L. Beaudoin, L. Lu, R. A. Du Pasquier, M. J. Kuroda, R. A. Willemsen, I. J. Koralnik, and R. P. Junghans. 2007. Chimeric immune receptors (CIRs) specific to JC virus for immunotherapy in progressive multifocal leukoencephalopathy (PML). *International immunology* 19: 1083-1093.
134. Chung, S., K. W. Wucherpfennig, S. M. Friedman, D. A. Hafler, and J. L. Strominger. 1994. Functional three-domain single-chain T-cell receptors. *Proc Natl Acad Sci U S A* 91: 12654-12658.
135. van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254: 1643-1647.
136. Zhang, T., X. He, T. C. Tsang, and D. T. Harris. 2004. Transgenic TCR expression: comparison of single chain with full-length receptor constructs for T-cell function. *Cancer Gene Ther* 11: 487-496.
137. Eshhar, Z., T. Waks, G. Gross, and D. G. Schindler. 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A* 90: 720-724.
138. Annenkov, A. E., S. P. Moyes, Z. Eshhar, R. A. Mageed, and Y. Chernajovsky. 1998. Loss of original antigenic specificity in T cell hybridomas transduced with a chimeric receptor containing single-chain Fv of an anti-collagen antibody and Fc epsilonRI-signaling gamma subunit. *J Immunol* 161: 6604-6613.
139. Gross, G., and Z. Eshhar. 1992. Endowing T cells with antibody specificity using chimeric T cell receptors. *Faseb J* 6: 3370-3378.
140. Abken, H., A. Hombach, U. Reinhold, and S. Ferrone. 1998. Can combined T-cell- and antibody-based immunotherapy outsmart tumor cells? *Immunol Today* 19: 2-5.
141. Sadelain, M., R. Brentjens, and I. Riviere. 2009. The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 21: 215-223.
142. Denkberg, G., and Y. Reiter. 2006. Recombinant antibodies with T-cell receptor-like specificity: novel tools to study MHC class I presentation. *Autoimmun Rev* 5: 252-257.
143. Willemsen, R., P. Chames, E. Schooten, J. W. Gratama, and R. Debets. 2008. Selection of human antibody fragments directed against tumor T-cell epitopes for adoptive T-cell therapy. *Cytometry A* 73A: 1093-1099.
144. Chames, P., S. E. Hufton, P. G. Coulie, B. Uchanska-Ziegler, and H. R. Hoogenboom. 2000. Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. *Proc Natl Acad Sci U S A* 97: 7969-7974.

145. Willemsen, R. A., R. Debets, E. Hart, H. R. Hoogenboom, R. L. Bolhuis, and P. Chames. 2001. A phage display selected fab fragment with MHC class I-restricted specificity for MAGE-A1 allows for retargeting of primary human T lymphocytes. *Gene therapy* 8: 1601-1608.
146. Weijtens, M. E., R. A. Willemsen, D. Valerio, K. Stam, and R. L. Bolhuis. 1996. Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J Immunol* 157: 836-843.
147. Stewart-Jones, G., A. Wadle, A. Hombach, E. Shenderov, G. Held, E. Fischer, S. Kleber, F. Stenner-Liewen, S. Bauer, A. McMichael, A. Knuth, H. Abken, A. A. Hombach, V. Cerundolo, E. Y. Jones, and C. Renner. 2009. Rational development of high-affinity T-cell receptor-like antibodies. *Proc Natl Acad Sci U S A* 106: 5784-5788.
148. Hulsmeyer, M., P. Chames, R. C. Hillig, R. L. Stanfield, G. Held, P. G. Coulie, C. Alings, G. Wille, W. Saenger, B. Uchanska-Ziegler, H. R. Hoogenboom, and A. Ziegler. 2005. A major histocompatibility complex-peptide-restricted antibody and t cell receptor molecules recognize their target by distinct binding modes: crystal structure of human leukocyte antigen (HLA)-A1-MAGE-A1 in complex with FAB-HYB3. *J Biol Chem* 280: 2972-2980.
149. Newrzela, S., B. Gunda, and D. von Laer. 2009. T cell culture for gammaretroviral transfer. *Methods Mol Biol* 506: 71-82.
150. Ross, J. 1995. mRNA stability in mammalian cells. *Microbiol Rev* 59: 423-450.
151. Jorritsma, A., R. Gomez-Eerland, M. Dokter, W. van de Kasteele, Y. M. Zoet, Doxiadis, II, N. Rufer, P. Romero, R. A. Morgan, T. N. Schumacher, and J. B. Haanen. 2007. Selecting highly affine and well-expressed TCRs for gene therapy of melanoma. *Blood* 110: 3564-3572.
152. de Witte, M. A., A. Jorritsma, A. Kaiser, M. D. van den Boom, M. Dokter, G. M. Bendle, J. B. Haanen, and T. N. Schumacher. 2008. Requirements for effective antitumor responses of TCR transduced T cells. *J Immunol* 181: 5128-5136.
153. Varela-Rohena, A., P. E. Molloy, S. M. Dunn, Y. Li, M. M. Suhoski, R. G. Carroll, A. Milicic, T. Mahon, D. H. Sutton, B. Laugel, R. Moysey, B. J. Cameron, A. Vuidepot, M. A. Purbhoo, D. K. Cole, R. E. Phillips, C. H. June, B. K. Jakobsen, A. K. Sewell, and J. L. Riley. 2008. Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nature medicine* 14: 1390-1395.
154. Chames, P., R. A. Willemsen, G. Rojas, D. Dieckmann, L. Rem, G. Schuler, R. L. Bolhuis, and H. R. Hoogenboom. 2002. TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity. *J Immunol* 169: 1110-1118.
155. Geiger, T. L., P. Nguyen, D. Leitenberg, and R. A. Flavell. 2001. Integrated src kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes. *Blood* 98: 2364-2371.
156. Orentas, R. J., S. J. Roskopf, G. P. Nolan, and M. I. Nishimura. 2001. Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide. *Clin Immunol* 98: 220-228.
157. Willemsen, R. A., C. Ronteltap, P. Chames, R. Debets, and R. L. Bolhuis. 2005. T cell retargeting with MHC class I-restricted antibodies: the CD28 costimulatory domain enhances antigen-specific cytotoxicity and cytokine production. *J Immunol* 174: 7853-7858.
158. Hombach, A., and H. Abken. 2007. Costimulation tunes tumor-specific activation of redirected T cells in adoptive immunotherapy. *Cancer Immunol Immunother* 56: 731-737.
159. Rossig, C., C. M. Bollard, J. G. Nuchtern, C. M. Rooney, and M. K. Brenner. 2002. Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. *Blood* 99: 2009-2016.

160. Teague, R. M., P. D. Greenberg, C. Fowler, M. Z. Huang, X. Tan, J. Morimoto, M. L. Dossett, E. S. Huseby, and C. Ohlen. 2008. Peripheral CD8+ T cell tolerance to self-proteins is regulated proximally at the T cell receptor. *Immunity* 28: 662-674.
161. Heemskerk, M. H., M. Hoogeboom, R. Hagedoorn, M. G. Kester, R. Willemze, and J. H. Falkenburg. 2004. Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. *J Exp Med* 199: 885-894.
162. van der Veken, L. T., M. Coccoris, E. Swart, J. H. Falkenburg, T. N. Schumacher, and M. H. Heemskerk. 2009. Alpha beta T cell receptor transfer to gamma delta T cells generates functional effector cells without mixed TCR dimers in vivo. *J Immunol* 182: 164-170.
163. Lamers, C. H., S. Sleijfer, A. G. Vulto, W. H. Kruit, M. Kliffen, R. Debets, J. W. Gratama, G. Stoter, and E. Oosterwijk. 2006. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24: e20-22.
164. Chomez, P., O. De Backer, M. Bertrand, E. De Plaen, T. Boon, and S. Lucas. 2001. An overview of the MAGE gene family with the identification of all human members of the family. *Cancer research* 61: 5544-5551.
165. Gotter, J., B. Brors, M. Hergenhahn, and B. Kyewski. 2004. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *The Journal of experimental medicine* 199: 155-166.
166. Bonini, C., G. Ferrari, S. Verzeletti, P. Servida, E. Zappone, L. Ruggieri, M. Ponzoni, S. Rossini, F. Mavilio, C. Traversari, and C. Bordignon. 1997. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 276: 1719-1724.
167. Straathof, K. C., M. A. Pule, P. Yotnda, G. Dotti, E. F. Vanin, M. K. Brenner, H. E. Heslop, D. M. Spencer, and C. M. Rooney. 2005. An inducible caspase 9 safety switch for T-cell therapy. *Blood* 105: 4247-4254.
168. Kieback, E., J. Charo, D. Sommermeyer, T. Blankenstein, and W. Uckert. 2008. A safeguard eliminates T cell receptor gene-modified autoreactive T cells after adoptive transfer. *Proceedings of the National Academy of Sciences of the United States of America* 105: 623-628.
169. Serafini, M., M. Manganini, G. Borleri, M. Bonamino, L. Imberti, A. Biondi, J. Golay, A. Rambaldi, and M. Introna. 2004. Characterization of CD20-transduced T lymphocytes as an alternative suicide gene therapy approach for the treatment of graft-versus-host disease. *Human gene therapy* 15: 63-76.
170. Huang, J., H. T. Khong, M. E. Dudley, M. El-Gamil, Y. F. Li, S. A. Rosenberg, and P. F. Robbins. 2005. Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. *J Immunother* 28: 258-267.
171. Kaneko, S., S. Mastaglio, A. Bondanza, M. Ponzoni, F. Sanvito, L. Aldrighetti, M. Radrizzani, S. La Seta-Catamancio, E. Provasi, A. Mondino, T. Nagasawa, K. Fleischhauer, V. Russo, C. Traversari, F. Ciceri, C. Bordignon, and C. Bonini. 2009. IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood* 113: 1006-1015.
172. Pouw, N., E. Treffers-Westerlaken, J. Kraan, F. Wittink, T. ten Hagen, J. Verweij, and R. Debets. 2010. Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells. *Cancer Immunol Immunother* 59: 921-931.
173. Berger, C., M. C. Jensen, P. M. Lansdorp, M. Gough, C. Elliott, and S. R. Riddell. 2008. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *The Journal of clinical investigation* 118: 294-305.

174. Hinrichs, C. S., Z. A. Borman, L. Cassard, L. Gattinoni, R. Spolski, Z. Yu, L. Sanchez-Perez, P. Muranski, S. J. Kern, C. Logun, D. C. Palmer, Y. Ji, R. N. Reger, W. J. Leonard, R. L. Danner, S. A. Rosenberg, and N. P. Restifo. 2009. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A* 106: 17469-17474.

175. Rosenberg, S. A., and M. E. Dudley. 2009. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Current opinion in immunology* 21: 233-240.

176. Kohlmeyer, J., M. Cron, J. Landsberg, T. Bald, M. Renn, S. Mikus, S. Bondong, D. Wikasari, E. Gaffal, G. Hartmann, and T. Tuting. 2009. Complete regression of advanced primary and metastatic mouse melanomas following combination chemoimmunotherapy. *Cancer Res* 69: 6265-6274.

177. Roszik, J., Z. Sebestyen, C. Govers, Y. Guri, A. Szoor, Z. Palyi-Krekk, G. Vereb, P. Nagy, J. Szollosi, and R. Debets. 2011. T-cell synapse formation depends on antigen recognition but not CD3 interaction: studies with TCR:zeta, a candidate transgene for TCR gene therapy. *European journal of immunology* 41: 1288-1297.

178. Sommermeyer, D., and W. Uckert. 2010. Minimal amino acid exchange in human TCR constant regions fosters improved function of TCR gene-modified T cells. *J Immunol* 184: 6223-6231.

179. Van de Griend, R. J., B. A. Van Krimpen, S. J. Bol, A. Thompson, and R. L. Bolhuis. 1984. Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. *Journal of immunological methods* 66: 285-298.

180. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94-96.

181. Szentesi, G., G. Horvath, I. Bori, G. Vamosi, J. Szollosi, R. Gaspar, S. Damjanovich, A. Jenei, and L. Matyus. 2004. Computer program for determining fluorescence resonance energy transfer efficiency from flow cytometric data on a cell-by-cell basis. *Computer methods and programs in biomedicine* 75: 201-211.

182. Aarnoudse, C. A., M. Kruse, R. Konopitzky, N. Brouwenstijn, and P. I. Schrier. 2002. TCR reconstitution in Jurkat reporter cells facilitates the identification of novel tumor antigens by cDNA expression cloning. *Int J Cancer* 99: 7-13.

183. Schroten, C., R. Kraaij, J. L. Veldhoven, C. A. Berrevoets, M. A. den Bakker, Q. Ma, M. Sadelain, C. H. Bangma, R. A. Willemsen, and R. Debets. 2010. T cell activation upon exposure to patient-derived tumor tissue: a functional assay to select patients for adoptive T cell therapy. *Journal of immunological methods* 359: 11-20.

184. Lissina, A., K. Ladell, A. Skowera, M. Clement, E. Edwards, R. Seggewiss, H. A. van den Berg, E. Gostick, K. Gallagher, E. Jones, J. J. Melenhorst, A. J. Godkin, M. Peakman, D. A. Price, A. K. Sewell, and L. Wooldridge. 2009. Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers. *J Immunol Methods* 340: 11-24.

185. Luton, F., M. Buferne, J. Davoust, A. M. Schmitt-Verhulst, and C. Boyer. 1994. Evidence for protein tyrosine kinase involvement in ligand-induced TCR/CD3 internalization and surface redistribution. *J Immunol* 153: 63-72.

186. Zhao, Y., Q. J. Wang, S. Yang, J. N. Kochenderfer, Z. Zheng, X. Zhong, M. Sadelain, Z. Eshhar, S. A. Rosenberg, and R. A. Morgan. 2009. A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *J Immunol* 183: 5563-5574.

187. Orlando, L., L. Accomasso, P. Circosta, V. Turinetto, E. Lantelme, P. Porcedda, V. Minieri, M. Pautasso, R. A. Willemsen, A. Cignetti, and C. Giachino. 2011. TCR transfer induces TCR-mediated tonic inhibition of RAG genes in human T cells. *Mol Immunol* 48: 1369-1376.
188. Rutledge, T., P. Cosson, N. Manolios, J. S. Bonifacino, and R. D. Klausner. 1992. Transmembrane helical interactions: zeta chain dimerization and functional association with the T cell antigen receptor. *The EMBO journal* 11: 3245-3254.
189. Call, M. E., J. R. Schnell, C. Xu, R. A. Lutz, J. J. Chou, and K. W. Wucherpfennig. 2006. The structure of the zetazeta transmembrane dimer reveals features essential for its assembly with the T cell receptor. *Cell* 127: 355-368.
190. Weijtens, M. E., R. A. Willemsen, E. H. Hart, and R. L. Bolhuis. 1998. A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes. *Gene therapy* 5: 1195-1203.
191. Mallaun, M., D. Naeher, M. A. Daniels, P. P. Yachi, B. Hausmann, I. F. Luescher, N. R. Gascoigne, and E. Palmer. 2008. The T cell receptor's alpha-chain connecting peptide motif promotes close approximation of the CD8 coreceptor allowing efficient signal initiation. *J Immunol* 180: 8211-8221.
192. Ulivieri, C., A. Peter, E. Orsini, E. Palmer, and C. T. Baldari. 2001. Defective signaling to Fyn by a T cell antigen receptor lacking the alpha -chain connecting peptide motif. *J Biol Chem* 276: 3574-3580.
193. Willemsen, R., C. Ronteltap, M. Heuveling, R. Debets, and R. Bolhuis. 2005. Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. *Gene Ther* 12: 140-146.
194. Krieger, E., G. Koraimann, and G. Vriend. 2002. Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. *Proteins* 47: 393-402.
195. Rosenberg, S. A. 2012. Raising the bar: the curative potential of human cancer immunotherapy. *Science translational medicine* 4: 127ps128.
196. Purbhoo, M. A., J. M. Boulter, D. A. Price, A. L. Vuidepot, C. S. Hourigan, P. R. Dunbar, K. Olson, S. J. Dawson, R. E. Phillips, B. K. Jakobsen, J. I. Bell, and A. K. Sewell. 2001. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor zeta chain. *The Journal of biological chemistry* 276: 32786-32792.
197. Roszik, J., J. Szollosi, and G. Vereb. 2008. AccPbFRET: an ImageJ plugin for semi-automatic, fully corrected analysis of acceptor photobleaching FRET images. *BMC bioinformatics* 9: 346.
198. Li, Z., W. Wu, O. Kemp, M. Stephen, and N. Manolios. 1998. The interchain disulfide linkage of T-cell antigen receptor-alpha and -beta chains is a prerequisite for T-cell activation. *Cellular immunology* 190: 101-111.
199. Hombach, A. A., and H. Abken. 2011. Costimulation by chimeric antigen receptors revisited the T cell antitumor response benefits from combined CD28-OX40 signalling. *International journal of cancer. Journal international du cancer* 129: 2935-2944.
200. Ahmed, F., S. Friend, T. C. George, N. Barteneva, and J. Lieberman. 2009. Numbers matter: quantitative and dynamic analysis of the formation of an immunological synapse using imaging flow cytometry. *Journal of immunological methods* 347: 79-86
201 Bunnell, S. C., D. I. Hong, J. R. Kardon, T. Yamazaki, C. J. McGlade, V. A. Barr, and L. E. Samelson. 2002. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *The Journal of cell biology* 158: 1263-1275.
202. Fernandez-Miguel, G., B. Alarcon, A. Iglesias, H. Bluethmann, M. Alvarez-Mon, E. Sanz, and A. de la Hera. 1999. Multivalent structure of an alphabetaT cell receptor. *Proceedings of the National Academy of Sciences of the United States of America* 96: 1547-1552.

203. Kuhns, M. S., A. T. Girvin, L. O. Klein, R. Chen, K. D. Jensen, E. W. Newell, J. B. Huppa, B. F. Lillemeier, M. Huse, Y. H. Chien, K. C. Garcia, and M. M. Davis. 2010. Evidence for a functional sidedness to the alphabetaTCR. *Proceedings of the National Academy of Sciences of the United States of America* 107: 5094-5099.
204. Schamel, W. W., I. Arechaga, R. M. Risueno, H. M. van Santen, P. Cabezas, C. Risco, J. M. Valpuesta, and B. Alarcon. 2005. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *The Journal of experimental medicine* 202: 493-503.
205. Kofler, D. M., M. Chmielewski, G. Rappl, A. Hombach, T. Riet, A. Schmidt, A. A. Hombach, C. M. Wendtner, and H. Abken. 2011. CD28 costimulation Impairs the efficacy of a redirected t-cell antitumor attack in the presence of regulatory t cells which can be overcome by preventing Lck activation. *Molecular therapy : the journal of the American Society of Gene Therapy* 19: 760-767.
206. Willemsen, R. A., Z. Sebestyen, C. Ronteltap, C. Berrevoets, J. Drexhage, and R. Debets. 2006. CD8 alpha coreceptor to improve TCR gene transfer to treat melanoma: down-regulation of tumor-specific production of IL-4, IL-5, and IL-10. *J Immunol* 177: 991-998.
207. Stefanova, I., B. Hemmer, M. Vergelli, R. Martin, W. E. Biddison, and R. N. Germain. 2003. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nature immunology* 4: 248-254.
208. Kim, H. H., M. Tharayil, and C. E. Rudd. 1998. Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. *The Journal of biological chemistry* 273: 296-301.
209. August, A., S. Gibson, Y. Kawakami, T. Kawakami, G. B. Mills, and B. Dupont. 1994. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proceedings of the National Academy of Sciences of the United States of America* 91: 9347-9351.
210. DeFord-Watts, L. M., J. A. Young, L. A. Pitcher, and N. S. van Oers. 2007. The membrane-proximal portion of CD3 epsilon associates with the serine/threonine kinase GRK2. *The Journal of biological chemistry* 282: 16126-16134.
211. Gallo, E. M., K. Cante-Barrett, and G. R. Crabtree. 2006. Lymphocyte calcium signaling from membrane to nucleus. *Nature immunology* 7: 25-32.
212. Schneider, H., and C. E. Rudd. 2008. CD28 and Grb-2, relative to Gads or Grap, preferentially co-operate with Vav1 in the activation of NFAT/AP-1 transcription. *Biochemical and biophysical research communications* 369: 616-621.
213. Lin, J., and A. Weiss. 2001. T cell receptor signalling. *Journal of cell science* 114: 243-244.
214. Tammana, S., X. Huang, M. Wong, M. C. Milone, L. Ma, B. L. Levine, C. H. June, J. E. Wagner, B. R. Blazar, and X. Zhou. 2010. 4-1BB and CD28 signaling plays a synergistic role in redirecting umbilical cord blood T cells against B-cell malignancies. *Human gene therapy* 21: 75-86.
215. Pule, M. A., K. C. Straathof, G. Dotti, H. E. Heslop, C. M. Rooney, and M. K. Brenner. 2005. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Molecular therapy : the journal of the American Society of Gene Therapy* 12: 933-941.
216. Milone, M. C., J. D. Fish, C. Carpenito, R. G. Carroll, G. K. Binder, D. Teachey, M. Samanta, M. Lakhal, B. Gloss, G. Danet-Desnoyers, D. Campana, J. L. Riley, S. A. Grupp, and C. H. June. 2009. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy* 17: 1453-1464.
217. Carpenito, C., M. C. Milone, R. Hassan, J. C. Simonet, M. Lakhal, M. M. Suhoski, A. Varela-Rohena, K. M. Haines, D. F. Heitjan, S. M. Albelda, R. G. Carroll, J. L. Riley, I. Pastan, and C. H. June. 2009. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28

and CD137 domains. *Proceedings of the National Academy of Sciences of the United States of America* 106: 3360-3365.

218. Cole, D. K., N. J. Pumphrey, J. M. Boulter, M. Sami, J. I. Bell, E. Gostick, D. A. Price, G. F. Gao, A. K. Sewell, and B. K. Jakobsen. 2007. Human TCR-binding affinity is governed by MHC class restriction. *J Immunol* 178: 5727-5734.
219. Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America* 93: 4102-4107.
220. Yee, C., P. A. Savage, P. P. Lee, M. M. Davis, and P. D. Greenberg. 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J Immunol* 162: 2227-2234.
221. Dutoit, V., V. Rubio-Godoy, P. Y. Dietrich, A. L. Quiqueres, V. Schnuriger, D. Rimoldi, D. Lienard, D. Speiser, P. Guillaume, P. Batard, J. C. Cerottini, P. Romero, and D. Valmori. 2001. Heterogeneous T-cell response to MAGE-A10(254-262): high avidity-specific cytolytic T lymphocytes show superior antitumor activity. *Cancer research* 61: 5850-5856.
222. Stanislawski, T., R. H. Voss, C. Lotz, E. Sadovnikova, R. A. Willemsen, J. Kuball, T. Ruppert, R. L. Bolhuis, C. J. Melief, C. Huber, H. J. Stauss, and M. Theobald. 2001. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nature immunology* 2: 962-970.
223. Davis, J. L., M. R. Theoret, Z. Zheng, C. H. Lamers, S. A. Rosenberg, and R. A. Morgan. 2010. Development of human anti-murine T-cell receptor antibodies in both responding and nonresponding patients enrolled in TCR gene therapy trials. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16: 5852-5861.
224. Robbins, P. F., Y. F. Li, M. El-Gamil, Y. Zhao, J. A. Wargo, Z. Zheng, H. Xu, R. A. Morgan, S. A. Feldman, L. A. Johnson, A. D. Bennett, S. M. Dunn, T. M. Mahon, B. K. Jakobsen, and S. A. Rosenberg. 2008. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *J Immunol* 180: 6116-6131.
225. Zoete, V., and O. Michielin. 2007. Comparison between computational alanine scanning and per-residue binding free energy decomposition for protein-protein association using MM-GBSA: application to the TCR-p-MHC complex. *Proteins* 67: 1026-1047.
226. Chervin, A. S., D. H. Aggen, J. M. Raseman, and D. M. Kranz. 2008. Engineering higher affinity T cell receptors using a T cell display system. *Journal of immunological methods* 339: 175-184.
227. Dunn, S. M., P. J. Rizkallah, E. Baston, T. Mahon, B. Cameron, R. Moysey, F. Gao, M. Sami, J. Boulter, Y. Li, and B. K. Jakobsen. 2006. Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide-MHC without increasing apparent cross-reactivity. *Protein science : a publication of the Protein Society* 15: 710-721.
228. Holler, P. D., L. K. Chlewicki, and D. M. Kranz. 2003. TCRs with high affinity for foreign pMHC show self-reactivity. *Nature immunology* 4: 55-62.
229. Kessels, H. W., M. D. van Den Boom, H. Spits, E. Hooijberg, and T. N. Schumacher. 2000. Changing T cell specificity by retroviral T cell receptor display. *Proceedings of the National Academy of Sciences of the United States of America* 97: 14578-14583.
230. Li, Y., R. Moysey, P. E. Molloy, A. L. Vuidepot, T. Mahon, E. Baston, S. Dunn, N. Liddy, J. Jacob, B. K. Jakobsen, and J. M. Boulter. 2005. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nature biotechnology* 23: 349-354.
231. Holler, P. D., and D. M. Kranz. 2003. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity* 18: 255-264.

232. van de Griend, R. J., and R. L. Bolhuis. 1984. Rapid expansion of allospecific cytotoxic T cell clones using nonspecific feeder cell lines without further addition of exogenous IL2. *Transplantation* 38: 401-406.
233. O'Callaghan C, A., M. F. Byford, J. R. Wyer, B. E. Willcox, B. K. Jakobsen, A. J. McMichael, and J. I. Bell. 1999. BirA enzyme: production and application in the study of membrane receptor-ligand interactions by site-specific biotinylation. *Analytical biochemistry* 266: 9-15.
234. Yang, S., C. J. Cohen, P. D. Peng, Y. Zhao, L. Cassard, Z. Yu, Z. Zheng, S. Jones, N. P. Restifo, S. A. Rosenberg, and R. A. Morgan. 2008. Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. *Gene therapy* 15: 1411-1423.
235. Appay, V., R. A. van Lier, F. Sallusto, and M. Roederer. 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 73: 975-983.
236. Straetemans, T., M. Coccoris, C. Berrevoets, E. Treffers-Westerlaken, C. E. Scholten, D. Schipper, T. L. Ten Hagen, and R. Debets. 2012. T-cell receptor gene therapy in human melanoma-bearing immune-deficient mice: human but not mouse T cells recapitulate outcome of clinical studies. *Human gene therapy* 23: 187-201.
237. Sykulev, Y., M. Joo, I. Vturina, T. J. Tsomides, and H. N. Eisen. 1996. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* 4: 565-571.
238. Palmer, E., and D. Naeher. 2009. Affinity threshold for thymic selection through a T-cell receptor-co-receptor zipper. *Nature reviews. Immunology* 9: 207-213.
239. Kalergis, A. M., N. Boucheron, M. A. Doucey, E. Palmieri, E. C. Goyarts, Z. Vegh, I. F. Luescher, and S. G. Nathenson. 2001. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nature immunology* 2: 229-234.
240. Schmid, D. A., M. B. Irving, V. Posevitz, M. Hebeisen, A. Posevitz-Fejfar, J. C. Sarria, R. Gomez-Eerland, M. Thome, T. N. Schumacher, P. Romero, D. E. Speiser, V. Zoete, O. Michielin, and N. Rufer. 2010. Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *J Immunol* 184: 4936-4946.
241. Sami, M., P. J. Rizkallah, S. Dunn, P. Molloy, R. Moysey, A. Vuidepot, E. Baston, P. Todorov, Y. Li, F. Gao, J. M. Boulter, and B. K. Jakobsen. 2007. Crystal structures of high affinity human T-cell receptors bound to peptide major histocompatibility complex reveal native diagonal binding geometry. *Protein engineering, design & selection : PEDS* 20: 397-403.
242. Carreno, L. J., E. M. Riquelme, P. A. Gonzalez, N. Espagnolle, C. A. Riedel, S. Valitutti, and A. M. Kalergis. 2010. T-cell antagonism by short half-life pMHC ligands can be mediated by an efficient trapping of T-cell polarization toward the APC. *Proceedings of the National Academy of Sciences of the United States of America* 107: 210-215.
243. Zehn, D., S. Y. Lee, and M. J. Bevan. 2009. Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458: 211-214.
244. McMahan, R. H., J. A. McWilliams, K. R. Jordan, S. W. Dow, D. B. Wilson, and J. E. Slansky. 2006. Relating TCR-peptide-MHC affinity to immunogenicity for the design of tumor vaccines. *The Journal of clinical investigation* 116: 2543-2551.
245. Corse, E., R. A. Gottschalk, M. Krogsgaard, and J. P. Allison. 2010. Attenuated T cell responses to a high-potency ligand in vivo. *PLoS biology* 8.
246. Weijtens, M. E., E. H. Hart, and R. L. Bolhuis. 2000. Functional balance between T cell chimeric receptor density and tumor associated antigen density: CTL mediated cytolysis and lymphokine production. *Gene Ther* 7: 35-42.

247. Cooper, L. J., M. Kalos, D. A. Lewinsohn, S. R. Riddell, and P. D. Greenberg. 2000. Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes. *J Virol* 74: 8207-8212.
248. Molday, R. S., S. P. Yen, and A. Rembaum. 1977. Application of magnetic microspheres in labelling and separation of cells. *Nature* 268: 437-438.
249. Miltenyi, S., W. Muller, W. Weichel, and A. Radbruch. 1990. High gradient magnetic cell separation with MACS. *Cytometry* 11: 231-238.
250. Feuchtinger, T., C. Richard, S. Joachim, M. H. Scheible, M. Schumm, K. Hamprecht, D. Martin, G. Jahn, R. Handgretinger, and P. Lang. 2008. Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. *J Immunother* 31: 199-206.
251. Hammer, M. H., G. Brestrich, A. Mittenzweig, A. Roemhild, S. Zwinger, M. Subklewe, C. Beier, A. Kurtz, N. Babel, H. D. Volk, and P. Reinke. 2007. Generation of EBV-specific T cells for adoptive immunotherapy: a novel protocol using formalin-fixed stimulator cells to increase biosafety. *J Immunother* 30: 817-824.
252. Rauser, G., H. Einsele, C. Sinzger, D. Wernet, G. Kuntz, M. Assenmacher, J. D. Campbell, and M. S. Topp. 2004. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood* 103: 3565-3572.
253. Newell, E. W., L. O. Klein, W. Yu, and M. M. Davis. 2009. Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. *Nat Methods* 6: 497-499.
254. Labarriere, N., N. Gervois, A. Bonnin, R. Bouquie, F. Jotereau, and F. Lang. 2008. PBMC are as good a source of tumor-reactive T lymphocytes as TIL after selection by Melan-A/A2 multimer immunomagnetic sorting. *Cancer Immunol Immunother* 57: 185-195.
255. Knabel, M., T. J. Franz, M. Schiemann, A. Wulf, B. Villmow, B. Schmidt, H. Bernhard, H. Wagner, and D. H. Busch. 2002. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med* 8: 631-637.
256. Neudorfer, J., B. Schmidt, K. M. Huster, F. Anderl, M. Schiemann, G. Holzapfel, T. Schmidt, L. Germeroth, H. Wagner, C. Peschel, D. H. Busch, and H. Bernhard. 2007. Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* 320: 119-131.
257. Fehse, B., A. Richters, K. Putimtseva-Scharf, H. Klump, Z. Li, W. Ostertag, A. R. Zander, and C. Baum. 2000. CD34 splice variant: an attractive marker for selection of gene-modified cells. *Mol Ther* 1: 448-456.
258. Stull, R. A., W. C. Hyun, and M. G. Pallavicini. 2000. Simultaneous flow cytometric analyses of enhanced green and yellow fluorescent proteins and cell surface antigens in doubly transduced immature hematopoietic cell populations. *Cytometry* 40: 126-134.
259. Preuss, E., A. Treschow, S. Newrzela, D. Brucher, K. Weber, U. Felldin, E. Alici, G. Gahrton, D. von Laer, M. S. Dilber, and B. Fehse. 2010. TK.007: A novel, codon-optimized HSVtk(A168H) mutant for suicide gene therapy. *Hum Gene Ther* 21: 929-941.
260. Yao, J., C. Bechter, M. Wiesneth, G. Harter, M. Gotz, L. Germeroth, P. Guillaume, F. Hasan, S. von Harsdorf, T. Mertens, D. Michel, H. Dohner, D. Bunjes, M. Schmitt, and A. Schmitt. 2008. Multimer staining of cytomegalovirus phosphoprotein 65-specific T cells for diagnosis and therapeutic purposes: a comparative study. *Clin Infect Dis* 46: e96-105.
261. Xu, X. N., M. A. Purbhoo, N. Chen, J. Mongkolsapaya, J. H. Cox, U. C. Meier, S. Tafuro, P. R. Dunbar, A. K. Sewell, C. S. Hourigan, V. Appay, V. Cerundolo, S. R. Burrows, A. J. McMichael, and G. R. Screaton.

2001. A novel approach to antigen-specific deletion of CTL with minimal cellular activation using alpha3 domain mutants of MHC class I/peptide complex. *Immunity* 14: 591-602.

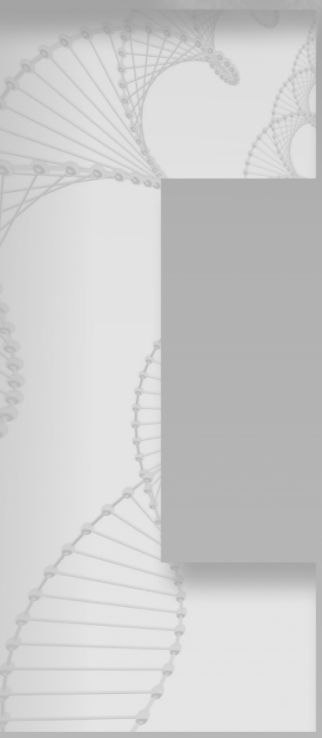
262. Matsui, K., J. J. Boniface, P. A. Reay, H. Schild, B. Fazekas de St Groth, and M. M. Davis. 1991. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science* 254: 1788-1791.
263. Garcia, K. C., M. Degano, R. L. Stanfield, A. Brunmark, M. R. Jackson, P. A. Peterson, L. Teyton, and I. A. Wilson. 1996. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science* 274: 209-219.
264. Rudolph, M. G., R. L. Stanfield, and I. A. Wilson. 2006. How TCRs bind MHCs, peptides, and coreceptors. *Annual review of immunology* 24: 419-466.
265. Hart, D. P., S. A. Xue, S. Thomas, M. Cesco-Gaspere, A. Tranter, B. Willcox, S. P. Lee, N. Steven, E. C. Morris, and H. J. Stauss. 2008. Retroviral transfer of a dominant TCR prevents surface expression of a large proportion of the endogenous TCR repertoire in human T cells. *Gene Ther* 15: 625-631.
266. Irvine, D. J., M. A. Purbhoo, M. Krogsgaard, and M. M. Davis. 2002. Direct observation of ligand recognition by T cells. *Nature* 419: 845-849.
267. Bos, R., and L. A. Sherman. 2010. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. *Cancer Res* 70: 8368-8377.
268. Nakanishi, Y., B. Lu, C. Gerard, and A. Iwasaki. 2009. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* 462: 510-513.
269. Bennett, S. R., F. R. Carbone, F. Karamalis, J. F. Miller, and W. R. Heath. 1997. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *The Journal of experimental medicine* 186: 65-70.
270. Gao, F. G., V. Khammanivong, W. J. Liu, G. R. Leggatt, I. H. Frazer, and G. J. Fernando. 2002. Antigen-specific CD4+ T-cell help is required to activate a memory CD8+ T cell to a fully functional tumor killer cell. *Cancer research* 62: 6438-6441.
271. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of CD4(+) T cells in the antitumor immune response. *The Journal of experimental medicine* 188: 2357-2368.
272. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421: 852-856.
273. Jennings, S. R., R. H. Bonneau, P. M. Smith, R. M. Wolcott, and R. Chervenak. 1991. CD4-positive T lymphocytes are required for the generation of the primary but not the secondary CD8-positive cytolytic T lymphocyte response to herpes simplex virus in C57BL/6 mice. *Cellular immunology* 133: 234-252.
274. Keene, J. A., and J. Forman. 1982. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *The Journal of experimental medicine* 155: 768-782.
275. Schietinger, A., M. Philip, R. B. Liu, K. Schreiber, and H. Schreiber. 2010. Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *The Journal of experimental medicine* 207: 2469-2477.
276. Smith, C. M., N. S. Wilson, J. Waithman, J. A. Villadangos, F. R. Carbone, W. R. Heath, and G. T. Belz. 2004. Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. *Nature immunology* 5: 1143-1148.
277. Moeller, M., M. H. Kershaw, R. Cameron, J. A. Westwood, J. A. Trapani, M. J. Smyth, and P. K. Darcy. 2007. Sustained antigen-specific antitumor recall response mediated by gene-modified CD4+ T helper-1 and CD8+ T cells. *Cancer Res* 67: 11428-11437.

278. Chattopadhyay, P. K., D. A. Price, T. F. Harper, M. R. Betts, J. Yu, E. Gostick, S. P. Perfetto, P. Goepfert, R. A. Koup, S. C. De Rosa, M. P. Bruchez, and M. Roederer. 2006. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nature medicine* 12: 972-977.
279. Davis, M. M., J. D. Altman, and E. W. Newell. 2011. Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. *Nature reviews. Immunology* 11: 551-558.
280. Lebowitz, M. S., S. M. O'Herrin, A. R. Hamad, T. Fahmy, D. Marguet, N. C. Barnes, D. Pardoll, J. G. Bieler, and J. P. Schneck. 1999. Soluble, high-affinity dimers of T-cell receptors and class II major histocompatibility complexes: biochemical probes for analysis and modulation of immune responses. *Cellular immunology* 192: 175-184.
281. Mallet-Designe, V. I., T. Stratmann, D. Homann, F. Carbone, M. B. Oldstone, and L. Teyton. 2003. Detection of low-avidity CD4+ T cells using recombinant artificial APC: following the antiovalbumin immune response. *J Immunol* 170: 123-131.
282. Scholler, J., M. Singh, L. Bergmeier, K. Brunstedt, Y. Wang, T. Whittall, D. Rahman, J. Pido-Lopez, and T. Lehner. 2010. A recombinant human HLA-class I antigen linked to dextran elicits innate and adaptive immune responses. *Journal of immunological methods* 360: 1-9.
283. Naeher, D., I. F. Luescher, and E. Palmer. 2002. A role for the alpha-chain connecting peptide motif in mediating TCR-CD8 cooperation. *J Immunol* 169: 2964-2970.
284. Lamers, C. H., R. Willemsen, P. van Elzakker, S. van Steenbergen-Langeveld, M. Broertjes, J. Oosterwijk-Wakka, E. Oosterwijk, S. Sleijfer, R. Debets, and J. W. Gratama. 2011. Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. *Blood* 117: 72-82.
285. Deford-Watts, L. M., T. C. Tassin, A. M. Becker, J. J. Medeiros, J. P. Albanesi, P. E. Love, C. Wulfing, and N. S. van Oers. 2009. The cytoplasmic tail of the T cell receptor CD3 epsilon subunit contains a phospholipid-binding motif that regulates T cell functions. *J Immunol* 183: 1055-1064.
286. Xu, C., E. Gagnon, M. E. Call, J. R. Schnell, C. D. Schwieters, C. V. Carman, J. J. Chou, and K. W. Wucherpfennig. 2008. Regulation of T cell receptor activation by dynamic membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif. *Cell* 135: 702-713.
287. Robbins, P. F., M. E. Dudley, J. Wunderlich, M. El-Gamil, Y. F. Li, J. Zhou, J. Huang, D. J. Powell, Jr., and S. A. Rosenberg. 2004. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 173: 7125-7130.
288. Koehler, H., D. Kofler, A. Hombach, and H. Abken. 2007. CD28 costimulation overcomes transforming growth factor-beta-mediated repression of proliferation of redirected human CD4+ and CD8+ T cells in an antitumor cell attack. *Cancer research* 67: 2265-2273.
289. Hombach, A. A., J. Heiders, M. Foppe, M. Chmielewski, and H. Abken. 2012. OX40 costimulation by a chimeric antigen receptor abrogates CD28 and IL-2 induced IL-10 secretion by redirected CD4(+) T cells. *Oncoimmunology* 1: 458-466.
290. Thomas, S., S. A. Xue, C. R. Bangham, B. K. Jakobsen, E. C. Morris, and H. J. Stauss. 2011. Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen. *Blood* 118: 319-329.
291. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375: 148-151.
292. Rudolph, M. G., and I. A. Wilson. 2002. The specificity of TCR/pMHC interaction. *Current opinion in immunology* 14: 52-65.
293. Keever-Taylor, C. A., S. M. Devine, R. J. Soiffer, A. Mendizabal, S. Carter, M. C. Pasquini, P. N. Hari, A. Stein, H. M. Lazarus, C. Linker, S. C. Goldstein, E. A. Stadtmauer, and R. J. O'Reilly. 2012. Characteristics of CliniMACS(R) System CD34-enriched T cell-depleted grafts in a multicenter trial for acute

myeloid leukemia-Blood and Marrow Transplant Clinical Trials Network (BMT CTN) protocol 0303. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 18: 690-697.

294. Friedman, K. M., P. A. Prieto, L. E. Devillier, C. A. Gross, J. C. Yang, J. R. Wunderlich, S. A. Rosenberg, and M. E. Dudley. 2012. Tumor-specific CD4+ melanoma tumor-infiltrating lymphocytes. *J Immunother* 35: 400-408.
295. Zou, W. 2005. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nature reviews. Cancer* 5: 263-274.
296. Diamond, M. S., M. Kinder, H. Matsushita, M. Mashayekhi, G. P. Dunn, J. M. Archambault, H. Lee, C. D. Arthur, J. M. White, U. Kalinke, K. M. Murphy, and R. D. Schreiber. 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *The Journal of experimental medicine* 208: 1989-2003.
297. Fuertes, M. B., A. K. Kacha, J. Kline, S. R. Woo, D. M. Kranz, K. M. Murphy, and T. F. Gajewski. 2011. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *The Journal of experimental medicine* 208: 2005-2016.
298. Fourcade, J., Z. Sun, M. Benallaoua, P. Guillaume, I. F. Luescher, C. Sander, J. M. Kirkwood, V. Kuchroo, and H. M. Zarour. 2010. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *The Journal of experimental medicine* 207: 2175-2186.
299. Sakuishi, K., L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo, and A. C. Anderson. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *The Journal of experimental medicine* 207: 2187-2194.
300. Munn, D. H., M. D. Sharma, D. Hou, B. Baban, J. R. Lee, S. J. Antonia, J. L. Messina, P. Chandler, P. A. Koni, and A. L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *The Journal of clinical investigation* 114: 280-290.
301. Rodriguez, P. C., C. P. Hernandez, D. Quiceno, S. M. Dubinett, J. Zabaleta, J. B. Ochoa, J. Gilbert, and A. C. Ochoa. 2005. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *The Journal of experimental medicine* 202: 931-939.
302. Rodriguez, P. C., A. H. Zea, J. DeSalvo, K. S. Culotta, J. Zabaleta, D. G. Quiceno, J. B. Ochoa, and A. C. Ochoa. 2003. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. *J Immunol* 171: 1232-1239.
303. Vesely, M. D., M. H. Kershaw, R. D. Schreiber, and M. J. Smyth. 2011. Natural innate and adaptive immunity to cancer. *Annual review of immunology* 29: 235-271.
304. Mizoguchi, H., J. J. O'Shea, D. L. Longo, C. M. Loeffler, D. W. McVicar, and A. C. Ochoa. 1992. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 258: 1795-1798.
305. Chmielewski, M., A. A. Hombach, and H. Abken. 2011. CD28 cosignalling does not affect the activation threshold in a chimeric antigen receptor-redirected T-cell attack. *Gene therapy* 18: 62-72.
306. Chmielewski, M., C. Kopecky, A. A. Hombach, and H. Abken. 2011. IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer research* 71: 5697-5706.
307. Kaluza, K. M., T. Kottke, R. M. Diaz, D. Rommelfanger, J. Thompson, and R. Vile. 2012. Adoptive transfer of cytotoxic T lymphocytes targeting two different antigens limits antigen loss and tumor escape. *Human gene therapy* 23: 1054-1064.

308. Pulido, J., T. Kottke, J. Thompson, F. Galivo, P. Wongthida, R. M. Diaz, D. Rommelfanger, E. Ilett, L. Pease, H. Pandha, K. Harrington, P. Selby, A. Melcher, and R. Vile. 2012. Using virally expressed melanoma cDNA libraries to identify tumor-associated antigens that cure melanoma. *Nature biotechnology* 30: 337-343.
309. Rosenberg, S. A., M. T. Lotze, L. M. Muul, A. E. Chang, F. P. Avis, S. Leitman, W. M. Linehan, C. N. Robertson, R. E. Lee, J. T. Rubin, and et al. 1987. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *The New England journal of medicine* 316: 889-897.
310. Rosenberg, S. A., M. T. Lotze, J. C. Yang, P. M. Aebersold, W. M. Linehan, C. A. Seipp, and D. E. White. 1989. Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Annals of surgery* 210: 474-484; discussion 484-475.
311. Atanackovic, D., T. Luetkens, B. Kloth, G. Fuchs, Y. Cao, Y. Hildebrandt, S. Meyer, K. Bartels, H. Reinhard, N. Lajmi, S. Hegewisch-Becker, G. Schilling, U. Platzbecker, G. Kobbe, T. Schroeder, C. Bokemeyer, and N. Kroger. 2011. Cancer-testis antigen expression and its epigenetic modulation in acute myeloid leukemia. *American journal of hematology* 86: 918-922.
312. Chou, J., L. N. Voong, C. L. Mortales, A. M. Towlerton, S. M. Pollack, X. Chen, C. Yee, P. F. Robbins, and E. H. Warren. 2012. Epigenetic modulation to enable antigen-specific T-cell therapy of colorectal cancer. *J Immunother* 35: 131-141.
313. Coral, S., L. Sigalotti, M. Altomonte, A. Engelsberg, F. Colizzi, I. Cattarossi, E. Maraskovsky, E. Jager, B. Seliger, and M. Maio. 2002. 5-aza-2'-deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinoma: immunotherapeutic implications. *Clinical cancer research : an official journal of the American Association for Cancer Research* 8: 2690-2695.
314. Pollack, S. M., Y. Li, M. J. Blaisdell, E. A. Farrar, J. Chou, B. L. Hoch, E. T. Loggers, E. Rodler, J. F. Eary, E. U. Conrad, 3rd, R. L. Jones, and C. Yee. 2012. NYESO-1/LAGE-1s and PRAME are targets for antigen specific T cells in chondrosarcoma following treatment with 5-Aza-2-deoxycitabine. *PloS one* 7: e32165.
315. Wargo, J. A., P. F. Robbins, Y. Li, Y. Zhao, M. El-Gamil, D. Caragacianu, Z. Zheng, J. A. Hong, S. Downey, D. S. Schrump, S. A. Rosenberg, and R. A. Morgan. 2009. Recognition of NY-ESO-1+ tumor cells by engineered lymphocytes is enhanced by improved vector design and epigenetic modulation of tumor antigen expression. *Cancer immunology, immunotherapy : CII* 58: 383-394.
316. Goodyear, O., A. Agathanggelou, I. Novitzky-Basso, S. Siddique, T. McSkeane, G. Ryan, P. Vyas, J. Cavenagh, T. Stankovic, P. Moss, and C. Craddock. 2010. Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. *Blood* 116: 1908-1918.
317. Toor, A. A., K. K. Payne, H. M. Chung, R. T. Sabo, A. F. Hazlett, M. Kmieciak, K. Sanford, D. C. Williams, W. B. Clark, C. H. Roberts, J. M. McCarty, and M. H. Manjili. 2012. Epigenetic induction of adaptive immune response in multiple myeloma: sequential azacitidine and lenalidomide generate cancer testis antigen-specific cellular immunity. *British journal of haematology* 158: 700-711.
318. Siena, S., M. Bregni, B. Brando, F. Ravagnani, G. Bonadonna, and A. M. Gianni. 1989. Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. *Blood* 74: 1905-1914.
319. Marin, V., E. Cribioli, B. Philip, S. Tettamanti, I. Pizzitola, A. Biondi, E. Biagi, and M. Pule. 2012. Comparison of different suicide-gene strategies for the safety improvement of genetically manipulated T cells. *Human gene therapy. Part B. Methods* 23: 376-386.



Summary

Samenvatting

SUMMARY

Standard treatment options for melanoma, such as dacarbazine or high-dose IL-2, demonstrate limited objective and complete response rates. Currently, new immune therapies are being developed of which adoptive transfer of Tumor Infiltrating Lymphocytes (TILs) demonstrates most impressive clinical results. Therapy with TCR gene-engineered T cells entails a variant of TIL therapy that may be more flexible and applicable to multiple forms of cancer and is based on gene-manipulation of peripheral blood T cells to express tumor-specific TCRα and TCRβ transgenes.

Preclinical research described in this thesis focusses on modifications of TCR transgenes, in particular it addresses:

1. Fusing TCR with signaling cassettes
2. Enhancing TCR affinity
3. Enriching TCR-engineered T cells

Chapter 1 provides an overview of Food and Drug Administration-approved as well as experimental therapies for treating melanoma. Advantages of T cells include their ability to self-replicate, kill tumor cells via different mechanisms, and form immunological memory, which most likely have contributed to the high objective and complete response rates observed with adoptive T cell therapy. Clinical trials with TCR-engineered T cells revealed objective responses rates of up to about 70%, but at the same time revealed treatment-related toxicities. In this chapter strategies to further increase treatment efficacy and prevent or limit toxicities are described in more detail, emphasizing those strategies that rely on the engineering of TCR transgenes. With respect to therapeutic efficacy, three critical parameters have been introduced: T cell co-stimulation, TCR affinity and frequency of TCR-engineered T cells. In addition, I have discussed how these parameters can be exploited to enhance the efficacy of T cell therapy. Toxicities include on and off-target toxicities. The former is often a consequence of targeting an antigen that is expressed on tumor tissue but also, albeit it at low levels, on healthy tissues. The latter may be a consequence of TCR mis-pairing, i.e., unwanted combinations of endogenous and introduced TCR chains that yield new TCR heterodimers of unknown and potentially anti-self specificity. Moreover, recent trials pointed out that off-target toxicities may also be a consequence of TCRs recognizing similar yet not identical peptides (Dr. Bent Jakobsen, Cellular Therapy of Cancer Symposium, London, Febr 27th-March 2nd 2013). With respect to these toxicities, I have discussed different groups of antigens and concluded that some cancer testis antigens and neo-antigens may constitute safe targets for T cell therapy, but at the same time would like to recommend stringent safety testing and use of suicide switches for future trials.

A review of genetic TCR modifications to prevent or reduce TCR mis-pairing is given in **chapter 2**. We divided genetically modified TCRs according to their dependency on CD3 proteins for surface expression and downstream signaling. CD3-dependent strategies include TCR murinization and/or cysteine modification. CD3-independent strategies include single or two-chain TCRs that are coupled to signaling cassettes, typically consisting of CD3ζ or Fc(ε)RIγ. Reviewing reports on differently modified TCRs suggest that TCR mis-pairing is best prevented by CD3-independent strategies, such as the use of a CD3ζ signaling cassette (i.e., TCR:ζ) which, by not depending on the limited availability of endogenous CD3, also enhanced T cell avidity. More recently, techniques were developed that focus on preventing the expression of endogenous TCR chains rather than preventing TCR mis-pairing, such as the use of zinc-finger nucleases or silencing RNA.

In **chapter 3** we attempted to minimize the content of CD3ζ in TCR:ζ without compromising TCR:ζ's ability to prevent mis-pairing and be functionally expressed, thereby potentially also lowering this receptor's immunogenicity.We have generated a panel of TCR:ζ variants in which extracellular, transmembrane, intracellular domains or combinations thereof were replaced with natural TCR domains (a domain exchange strategy), and in which a limited number of CD3ζ transmembrane amino acids were transplanted onto structurally similar positions within TCRα and TCRβ (a 3D-modelling strategy). TCR transgenes were transduced in human T cells and our studies have demonstrated that the complete CD3ζ transmembrane domain, but not a limited number of CD3ζ transmembrane amino acids, is critical for cell surface expression and prevention of TCR mis-pairing. For T cell activation, both the CD3ζ transmembrane and intracellular domains were required. Notably, replacing the extracellular CD3ζ domain with natural TCR sequences compromised cell surface expression and antigen-specific functions in primary human T cells. We conclude that various TCR:ζ properties can be attributed to defined CD3ζ domains and that the complete CD3ζ content is required for functional expression of TCR:ζ on primary human T cells. Immunogenicity of TCR:ζ, no otherwise modified TCRs, is expected to be non-existent since clinical trials have indicated that immune responses occurring against receptor-engineered T cells are directed against murine variable domains but not against boundaries between human molecules.

In **chapter 4** we have built on the CD3ζ signaling cassette and included various accessory molecules and/or the CD28 co-stimulatory molecule, along the lines previously explored for Chimeric Antigen Receptors (CARs). Using a single-chain TCR platform, we have compared signaling cassettes consisting of Fc(ε)RIγ, CD3ε or CD3ζ with or without CD28 and selected a combination of CD28 and CD3ε for optimal receptor expression and function. When translating this signaling cassette to a two-chain TCR (i.e., TCR:28ε), we observed enhanced binding of peptide-MHC and no evidence for TCR mis-pairing.

TCR:28ε does not show off-target recognition, as evidenced by a non-changed peptide-fine specificity when compared to wt TCR, no loss of CD8 co-receptor dependency and no induction of T cell responses towards antigen-negative target cells. Notably, TCR:28ε induces the formation of immune synapses with significantly enhanced accumulations of TCR transgene and evidence of early TCR signaling. In conclusion, TCR:28ε mediates safe and functional avid T cell responses which merits further studies to formally investigate this receptor's potential to advance TCR gene therapy.

TCR affinity, the second focus of this thesis, has been covered in **chapter 5**. We analyzed whether perimeters of TCR affinities that determine gain of function differ from those that determine gain of self-reactivity. To this end, we have used phage display to obtain gp100/A2 specific TCRs that were affinity-enhanced (K_D value parental TCR: 18.5 μM) and observed that improved antigen-specific T cell function was only achieved with minor affinity enhancements (K_D values >4 μM), which in our panel of TCRs was restricted to amino acid substitutions in CDR3α. A further increase in TCR affinity was related to loss of peptide-fine specificity. In fact, TCRs of high affinity (K_D values <1 μM), with amino acid substitutions restricted to CDR2β, displayed gp100 peptide-independent reactivity. We conclude that TCR affinity-enhancement to enhance functional avidity of therapeutic T cells without compromising their safety should be limited to the lower micromolar range and is potentially best achieved through amino acid substitutions in CDR3α.

Enrichment of TCR-engineered T cells, the third focus of this thesis, was addressed in **chapter 6**. We compared several sorting reagents to enhance the frequency of TCR-engineered T cells. T cells were transduced with TCRs and sorted using Magnetic Activated Cell Sorting (MACS) on the basis of peptide-MHC multimers or an antibody against a co-transduced truncated CD34 (tCD34). When comparing different peptide-MHC multimers (i.e., tetramers, pentamers and streptamers), we observed that T cell yield, expansion and stability of enrichment was mostly improved when using streptamers. When using MACS with anti-CD34 antibody, we observed an improved T cell yield (compared to MACS with streptamers) and a clear enrichment for tCD34-positive T cells. Unfortunately, tCD34-directed sort did not enrich for TCR transgene, which was possibly related to the vector positioning of the TCRα gene 3' of the 2A gene resulting in low levels of TCRα expression. Unexpectedly, tCD34-sorted T cells revealed enhanced T cell function, which we have attributed to the unique presence of CD4 T cells in the T cell population when compared to streptamer-sorted T cells. We postulate that downward flow forces applied during the MACS procedure may select for high affinity interactions, providing an advantage of CD8 over CD4 T cells in multimer but not antibody-based sorting. Consequently, the use of a surrogate-marker, such as tCD34, may have benefits for *in vivo* anti-tumor responses of therapeutic T cell populations following *in vitro* enrichment.

Finally, in **chapter 7**, the results of previous chapters have been discussed in light of on-going developments in the field of TCR gene therapy. I have proposed to combine the above-described strategies and discuss how this may address challenges such as the immune suppressive tumor environment and treatment-related toxicities. For future studies, I propose in this chapter to use a single vector that encompasses TCR:28ε transgenes, TCR-V genes that are affinity-enhanced within defined perimeters, and a surrogate marker that allows sorting and potentially acts as a suicide switch.

SAMENVATTING

Op dit moment groeit het aantal mensen dat jaarlijks wordt geconfronteerd met plaveiselcelkanker, ook wel melanoom genoemd, in Nederland nog altijd sterk. De kans op deze ziekte neemt ook toe met de leeftijd. Met de huidige vergrijzing houdt dat in dat het aantal patiënten zal blijven stijgen. Als het melanoom uitzaait daalt de 10-jaar overlevingskans naar minder dan 10%. Ondanks dat de huidige en meest toegepaste medicijnen (goedgekeurd volgens de Amerikaanse 'Federal Drug Administration') in ongeveer de helft van de patiënten weliswaar een effect hebben (geremde groei van melanoom), zorgen deze medicijnen slechts in maximaal 1 tot 6% van de patiënten voor genezing. De meeste medicijnen gaan ook nog eens gepaard met zeer ernstige neveneffecten bij 30 tot 40% van de patiënten. In dit opzicht is het vermeldenswaardig dat er 6% minder gevallen van melanoom zouden zijn wanneer er géén gebruik meer gemaakt zou worden van zonnebanken. Dit zou een verbod op zonnebanken dus even effectief maken als de huidige medicijnen tegen melanoom.

Sinds kort zijn er twee nieuwe klassen medicijnen, waarvan de eerste resultaten bij patiënten met melanoom een duidelijk toegenomen effectiviteit laten zien en, onder de juiste condities, een verantwoorde veiligheid. Beide klassen medicijnen resulteren in een actief immuunsysteem. Zo zijn er antistoffen beschikbaar die 'remmende moleculen' op immuuncellen blokkeren (zie **hoofdstuk 1** voor details). De tweede klasse medicijnen is gebaseerd op het toedienen van immuuncellen. Een voorbeeld hiervan is T-cel receptor gen therapie. Deze therapie liet in eerste studies bij wel 67% van behandelde patiënten een effect op melanoomgroei laat zien (verkleining van tumorgrootte) en bij wel 18% van de patiënten genezing. De immuuncellen die worden gebruikt bij T-cel receptor gen therapie zijn de zogenaamde T-cellen. Iedereen heeft ongeveer 250 miljoen T-cellen. Deze cellen verschillen in de samenstelling van hun T-cel receptor (TCR). Deze receptor bindt aan zogenaamde 'Major Histocompatibility Complex (MHC)'-moleculen, die op het celoppervlak van alle cellen in ons lichaam (met slechts enkele uitzonderingen) vóórkomen. MHC-moleculen presenteren fragmenten (peptiden) van willekeurige eiwitten uit de cel en de combinatie tussen peptiden en MHC-moleculen zijn daardoor een afspiegeling van wat er in een cel gebeurt, dus ook als deze cel een infectie doorloopt of 'ontspoort' in geval van een kankercel. Elke TCR heeft een peptide-MHC specificiteit en alleen wanneer de TCR het gehele peptide-MHC-complex herkent leidt dit tot T-cel activatie en kan de T-cel overgaan tot celdoding. Voor T-cel activatie, maar ook voor expressie op de T-cel, is de TCR afhankelijk van CD3-eiwitten (CD staat voor 'Cluster of Differentiation' wat een algemene aanduiding voor oppervlakte-eiwitten is). Een TCR komt tot expressie in een complex met 6 CD3-eiwitten, en het geheel van TCR/CD3 eiwitten geeft de T-cel de mogelijkheid peptide-MHC-herkenning te vertalen naar activatiesignalen in de T-cel.

In geval van een tumor zijn T-cellen met de gewenste TCR vaak niet of in onvoldoende aantallen aanwezig in de patiënt of heeft de tumor deze T-cellen juist in 'slaap gesust' oftewel geïnactiveerd. T-cel gen therapie pakt deze problemen aan door T-cellen uit het lichaam van een patiënt te halen, in het laboratorium te activeren en genen in deze T-cellen te introduceren die coderen voor de gewenste TCR (TCR gen transfer), namelijk een die een peptide-MHC-combinatie herkent dat specifiek is voor de melanoom-cellen. Vervolgens worden deze T-cellen weer aan de patiënt terug gegeven waarna deze cellen, samen met de reeds in de patiënt aanwezige immuuncellen, het melanoom kunnen aanvallen.

In **hoofdstuk 1** worden de huidige resultaten, behaald met TCR gen therapie, in meer detail beschreven en belangrijke verbeteringen voor deze therapie voorgesteld, zoals het voorkòmen van neveneffecten en het verbeteren van anti-melanoom effecten door de T-cellen.

Therapie-gerelateerde neveneffecten kunnen zich uiten in zogenaamde 'on-target' en 'off-target' toxiciteit. On-target toxiciteit kan ontstaan door het gebruik van TCRs die peptide-MHC herkennen die ook tot expressie komen op gezonde cellen. Dit kan worden voorkòmen door TCRs te kiezen die peptide-MHC herkennen die enkel en alleen vóórkomen op melanoom en niet op gezond weefsel, zoals (I) peptide-MHC met een melanoom-gerelateerde mutatie in het peptide of (II) peptide-MHC waarvan het peptide afkomstig is van geselecteerde en tumor-gerelateerde eiwitten die, behalve in melanoomcellen, niet of nauwelijks in gezonde cellen tot expressie komen. In geval van off-target toxiciteit herkent de T-cel andere peptide-MHC-moleculen, welke op gezond weefsel kunnen vóórkomen, als gevolg van zogenaamde TCR mis-paring. Een TCR bestaat uit een alpha(α)-keten en een beta(β)-keten waarbij de combinatie de specifieke peptide-MHC herkenning mogelijk maakt. Ná TCR gen transfer heeft een T-cel dus 2 sets van TCRαβ ketens, nl. de reeds aanwezige set en de geïntroduceerde set TCR ketens, hetgeen kan leiden tot onwenselijke combinaties van TCRαβ ketens. Dit proces noemen we TCR mis-paring. Nieuw gecombineerde TCRαβ kan mogelijk op zijn beurt peptide-MHC herkennen die op gezonde cellen tot expressie komt.

Het verbeteren van anti-melanoom effecten door de T-cellen kan op verschillende manieren. Ten eerste kunnen T celeffecten via T-cel-co-stimulatie, een signaal welke T-cellen in de tumor vaak niet ontvangen, verbeterd worden omdat dit signaal zorgt voor overleving van de T-cel in de patiënt. Co-stimulatie signalen kunnen verzorgt worden door verschillenden moleculen, zoals CD28, en deze signalen kunnen er ook voor zorgen dat effecten langdurig behouden blijven en dat T-cellen eenmaal aanwezig in de tumor niet in slaap gesust worden. Ten tweede kan het versterken van de binding tussen TCR en peptide-MHC, ook wel affiniteit genoemd, de T-celeffecten verbeteren. TCR-affiniteit

voor peptide-MHC wordt bepaald door 3 belangrijke regio's in de α- en β-ketens. Deze regio's worden 'Complementarity Determining Regions (CDR)' genoemd en het is bekend dat CDR1 en CDR2 voornamelijk de binding van TCR aan het MHC molecuul verzorgen en CDR3 de binding van TCR aan het gepresenteerde peptide. Door het veranderen van sommige aminozuren in deze CDRs kan de affiniteit van de TCR voor het peptide-MHC worden verhoogd. Ten derde kunnen T-celeffecten in patiënten ook worden versterkt door meer T-cellen met de gewenste TCR aan de patiënt te geven. Door de verkregen T-celpopulatie ná TCR gentransfer te verrijken, wordt het percentage T-cellen dat deze TCR tot expressie brengt verhoogd, hetgeen resulteert in een grotere populatie T -cellen met de gewenste TCR.

Wat betreft de TCR gen therapie-gerelateerde toxiciteit heeft ons onderzoek zich toegespitst op het voorkòmen van TCR mis-paring en daardoor potentiële off-target toxiciteit. Er zijn verschillende methodes ontwikkeld op basis van genetische modificatie van de TCR om dit te doen en deze worden uitgebreid in **hoofdstuk 2** beschreven. Hier delen we de gemodificeerde TCRs in op basis van CD3-afhankelijkheid of –onafhankelijkheid voor expressie. We stellen dat een genetische aanpak die leidt tot een CD3-onafhankelijk TCR het probleem van TCR mis-paring het best aanpakt en zorgt voor een verhoogde functionele TCR-expressie. Een voorbeeld van een CD3-onafhankelijke methode is de genetische fusie tussen TCRα en TCRβ ketens en het CD3ζ molecuul, welke we de naam TCR:ζ hebben gegeven. Deze TCR bevat zelf weliswaar een CD3-molecuul maar vormt geen complex met de andere reeds aanwezige CD3 moleculen zoals een gewone TCR. Eerdere studies van onze onderzoeksgroep hebben aangetoond dat TCR:ζ resulteert in gewenste T-celfuncties zonder het optreden van TCR mis-paring. In **hoofdstuk 3** genereerden we nieuwe TCR:ζ varianten om te onderzoeken welk minimaal deel van CD3ζ in TCR:ζ noodzakelijk is voor de genoemde eigenschappen (de gewenste T-celfuncties zonder het optreden van TCR mis-paring). Ons werk toont aan dat het intracellulaire deel van CD3ζ cruciaal is voor T-celactivatie en dat het transmembrane deel van CD3ζ essentieel is voor expressie en het voorkòmen van TCR mis-paring. Echter, het verwijderen van het extracellulaire deel van CD3ζ in TCR:ζ verlaagt functionele expressie. Onze conclusie luidt daarom dat de complete TCR:ζ in alle gevallen beter is dan minimale varianten van TCR:ζ.

Voor het verbeteren van T-celeffecten hebben we onderzoek gedaan naar T-cel-co-stimulatie (**hoofdstuk 4**), het verhogen van TCR-affiniteit voor peptide-MHC (**hoofdstuk 5**) en het verrijken van T-celpopulaties voor T-cellen met de gewenste TCR (**hoofdstuk 6**). Ten eerste gaan we in **hoofdstuk 4** verder in op het fuseren van moleculen met een TCR om, behalve het voorkòmen van TCR mis-paring, de anti-tumor activiteit te verhogen. In dit hoofdstuk beschrijven we het fuseren van de TCR met CD28, dat normaal voor T-cel-

co-stimulatie zorgt en daardoor een verbeterd effect geeft. Aan de TCR:CD28 voegen we ook domeinen van andere signaleringsmoleculen toe, waarbij uitgebreid onderzoek laat zien dat TCR:CD28:CD3ε (TCR:28ε) de meest geschikte kandidaat is. TCR:28ε lijkt niet alleen veilig wat betreft TCR mis-paring en niet-specifieke effecten, maar lijkt ook de potentie van T-celeffecten te verhogen.

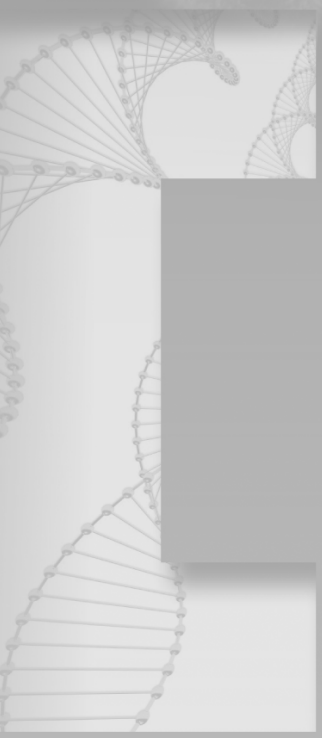
Ten tweede hebben we onderzocht hoe aminozuurveranderingen in verschillende CDRs kan leiden tot verhoogde-TCR-affiniteiten voor peptide-MHC en een verbeterde T-celeffect. De TCR-affiniteit voor peptide-MHC wordt uitgedrukt in de dissociatieconstante 'K_D'. Hoe lager deze waarde, hoe hoger de affiniteit. In **hoofdstuk 5** testen we verschillende TCRs met aminozuurveranderingen in CDRs en 2 tot 712 keer verhoogde affiniteit ten opzichte van de originele TCR, met een K_D waarde van 18.5 μM. Experimenten toonden aan dat een minimale affiniteitsverhoging, met een grens van K_D waardes rond de 4 μM, een verbeterde T-cel-reactiviteit tegen melanoomcellen tot gevolg heeft. TCRs die hieraan voldeden hadden aminozuurveranderingen in CDR3α.

Als laatste hebben we ons gericht op het verrijken van populaties van T-cellen die de gewenste TCR tot expressie brengen. Een van die verrijkingsmethoden, Magnetic Activated Cell Sorting (MACS), maakt gebruik van magneten en het labelen van T-cellen met kleine metalen bolletjes. In **hoofdstuk 6** vergelijken we verschillende types peptide-MHC-complexen en een genetische marker wat betreft hun bruikbaarheid voor verrijking van T-cellen. Peptide-MHC-complexen zijn beschikbaar in verschillende formaten die vaak 4, 5 of 10 peptide-MHC-complexen bevatten. Het gen dat we gebruikt hebben voor de markering van T-cellen codeerde voor een gemuteerd, en daardoor inactief, celoppervlakmolecuul CD34. Van de verschillende peptide-MHC-complexen bleek dat met de zogenaamde streptameren (met zo'n 10 peptide-MHC-complexen) T-cellen het meest efficiënt werden geïsoleerd en ook het beste overleefden. De T-celpopulatie die werd verkregen via isolatie op basis van CD34 toont echter de sterkste functionaliteit. Dit komt doordat isolatie via CD34, in tegenstelling tot streptameren, niet selecteert voor één type T-celsubpopulatie (de CD8-T-cellen), maar ook voor een tweede T-celsubpopulatie (de CD4-T-cellen) die elkaar versterken in functionele experimenten.

In **hoofdstuk 7** worden de resultaten van hoofdstukken 3, 4, 5 en 6 kort samengevat en in een breder perspectief geplaatst van recente ontwikkelingen in het veld van TCR gen therapie. Door het combineren van de resultaten van deze hoofdstukken worden, naar mijn mening, de huidige uitdagingen van TCR gen therapie in de breedte aangepakt. Ik stel uiteindelijk dan ook voor om:

1. TCR-genen te introduceren die gefuseerd zijn met CD28 en CD3ε (TCR:28ε) om TCR mis-paring te voorkòmen en T-cel co-stimulatie te versterken.

2. aminozuurveranderingen toe te passen in CDR3α-domeinen en de TCR-affiniteit beperkt te verhogen om herkenning van peptide-MHC en activatie van T-cellen te verbeteren zonder verlies van peptide-specificiteit.
3. om een markergen te introduceren naast de TCR-genen om zo via verrijking van TCR-expressie de functionaliteit van de T-celpopulatie het sterkst te verhogen.



Curriculum vitae

List of publications

PhD portfolio

Dankwoord

CURRICULUM VITAE

Coen Govers was born on the 18th of October 1983 in Oss, the Netherlands, and raised in Heesch. He attended secondary school at the Maasland College in Oss and graduated in 2002. He went to college at the Radboud University Nijmegen and studied Molecular Life Sciences. His first graduation project entitled 'Expression and activity of LFA-1 on mature dendritic cells' was performed at the Tumor Immunology department of the Nijmegen Centre for Molecular Life Sciences under the supervision of Drs. Ben Joosten, Dr. Alessandra Cambi, and Prof.dr. Carl G. Figdor. His second graduation project was performed at the Target Discovery department of Organon under the supervision of Dr. Edwin Janssen. The project was entitled 'Target-finding of a male fertility inhibitor' and also included a literature study entitled 'Refractory Disease in Cancer'. In addition, the project included a theoretical component which was performed under the supervision of Dr. Peter de Boer and was entitled 'Resume of genes affecting male fertility as disclosed by "knock-out" technology'. Coen obtained his MSc degree in 2007 and thereafter started as a PhD student at the Laboratory of Experimental Tumor Immunology in the department of Medical Oncology at the Erasmus MC University. His research, under the supervision of Dr. Reno Debets and Prof.dr. Jaap Verweij, focussed on engineering TCR transgenes to prevent TCR mis-pairing and enhance functional therapeutic T cell avidity. As of 2012, Coen is employed as junior researcher at Wageningen UR Food & Biobased Research investigating immune-modulatory effects of food (components).

LIST OF PUBLICATIONS

Coen Govers, Zsolt Sebestyén, Miriam Coccoris, Ralph A. Willemsen and Reno Debets. 2010. T cell receptor gene therapy: strategies for optimizing transgenic TCR pairing and T cell avidity. *Trends in Molecular Medicine 16(2):77-87*.

Coen Govers, Zsolt Sebestyén, Cor Berrevoets, Hanka Venselaar and Reno Debets. 2011. T cell receptor fused to human CD3ζ: Transmembrane domain of CD3ζ prevents TCR mis-pairing, whereas complete CD3ζ directs functional TCR expression. *The Open Gene Therapy Journal 4(1):11-22*.

Coen Govers, Cor Berrevoets, Elike Treffers-Westerlaken, Marieke Broertjes and Reno Debets. 2012. Magnetic-activated cell sorting of TCR-engineered T cells using tCD34 as a gene marker, but not peptide-MHC multimers, results in significant numbers of functional CD4 and CD8 T cells. *Human Gene Therapy Methods 23(3):213-224*.

Coen Govers, Zsolt Sebestyén, János Roszik, Árpád Szöőr, Konstantina Panoutsopoulou, Mandy van Brakel, Marieke Broertjes, György Vereb, János Szöllősi and Reno Debets. T cell receptors genetically linked to CD28 and CD3ε do not mis-pair with endogenous TCRs and mediate enhanced T cell activation. *Manuscript submitted*.

Coen Govers, Mandy van Brakel, Andrew Gerry, Alan D. Bennett, Nicholas J. Pumphrey, Nathaniel Liddy, Anouschka Minderhout, Sabine van Steenbergen-Langeveld, Jaap Verweij, Bent K. Jakobsen and Reno Debets. CDR3α mutations in gp100/HLA-A2 TCR causing minor affinity-enhancement demonstrate improved T cell function without gain in self-reactivity. *Manuscript under revision*.

Miriam Coccoris, Trudy Straetemans, **Coen Govers**, Cor Lamers, Stefan Sleijfer and Reno Debets. 2010. TCR gene therapy to treat melanoma: lessons from clinical and preclinical studies. *Expert Opinion in Biological Therapy 10(4):547-562*.

János Roszik, Zsolt Sebestyén, **Coen Govers**, Yakir Guri, Árpád Szöőr, Zsuzsanna Pályi-Krekk, György Vereb, Peter Nagy, János Szöllősi and Reno Debets. 2011. T cell synapse formation depends on antigen recognition but not CD3 interaction: studies with TCR:ζ, a candidate transgene for TCR gene therapy. *European Journal of Immunology 41(5):1288-1297*.

PhD PORTFOLIO

Erasmus MC
University Medical Center Rotterdam

General information:

Name: Coen Govers
Research school: Molecular Medicine
Period: October 2007 until December 2011
Supervisor: Dr. Reno Debets
Promotor: Prof.dr. Jaap Verweij

1. PhD training (1 credit = 28hrs workload)	Year	credits
1.1 General Academic skills		
– Biomedical English Writing and Communication	2010	4
– Integrity in Medical Research	2010	2
1.2 Research skills		
– Workshop on bioinformatic analysis, tools and services	2008	1
1.3 In-depth courses (e.g. Research school, medical training)		
– Molmed course "Basic and Translational Oncology"	2007	1
– Annual course "Molecular Medicine"	2008	1
– Course "Molecular Immunology"; SMBWO Theoretical Immunology exam passed	2010	5
1.4a Oral presentations		
– JNI Scientific Research Meetings, Erasmus Medical Center	2009 – 2011	3
– Cellular Therapy of Cancer Symposium, ATTACK, Montpellier, France	2010	1
– Annual Molecular Medicine Day, Erasmus Medical Center	2011	1
– Annual Dutch Tumor Immunology Meeting, Breukelen, The Netherlands	2011	1
– T cell consortium meeting, Erasmus Medical Center	2011	1
– Annual Meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2011	1
– Science day, Dept. Medical Oncology, Erasmus Medical Center	2011	1
1.4b Poster presentations		
– Annual Molecular Medicine Day, Erasmus Medical Center	2008 - 2010	3
– Annual meeting of the European Society for Cellular and Gene Therapy, Bruges, Belgium	2008	1

– Annual Meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2008 / 2011	1
– Cellular Therapy of Cancer Symposium, ATTACK, Milano, Italy and Montpellier, France	2009 / 2010	1
1.5 International conferences		
– Annual meeting of the European Society for Cellular and Gene Therapy, Bruges, Belgium	2008	1
– Cellular Therapy of Cancer Symposium, ATTACK, Milano, Italy and Montpellier, France	2009 - 2010	2
1.6 National conferences		
– Annual Meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2007 – 2009 / 2011	4
– Annual Dutch Tumor Immunology Meeting, Breukelen, The Netherlands	2008 - 2011	4
1.7 Seminars and Workshops		
– Bioinformatics workshop 'Browsing genes and genomes with Ensembl'	2009	0.5
– Photoshop CS3 workshop	2010	0.5
2. Teaching activities		
2.1 Supervising thesis		
– Bachelor student	2011	3
Total credits		44

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