TCR engineered T cell therapy from concepts to clinic

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TCR engineered T cell therapy from concepts to clinic

TCR gemanipuleerde T cell therapie van concepten tot kliniek
(met een samenvatting in het Nederlands)

Proefschrift

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door

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geboren op 4 mei 1987 te Venlo

Promotor: Prof.dr. J. Kuball
Copromotor: Dr. Z. Sebestyén

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General Introduction 1

General Introduction

Worldwide cancer incidence has increased with 28% during the past decade and is predicted to further increase due to the aging population (1). Standard of care for most solid tumors has long been surgical resection in combination with radio- and/or chemotherapy. Both radiotherapy and chemotherapy can be highly effective, but do not offer solutions for metastasized disease and are notorious for their side effects due to their aspecific modes of action. Fortunately, recent advances, especially in the field of immuno-oncology, offer patients increasingly better treatment options with less side effects (2). Immuno-oncology studies the interaction between cancer cells and the immune system, how cancer cells suppress the immune system, and how tumor-immune surveillance is evaded (3). Better understanding of these concepts has resulted in the development of antibody therapies, immune-checkpoint inhibitors and cellular therapies for the treatment of cancer. Antibody-based cancer immunotherapy is mainly based on the fact that tumor cells can express antigens, which are not present on healthy cells, and therefore possess a specific binding site for antibodies, which in turn can recruit effector lymphocytes to eliminate the tumor cells. The downside of targeting tumor specific antigens is that they can be downregulated or shed by the tumor, leading to therapy resistance (4). Immune-checkpoint inhibitors function by blocking inhibitory signals between T cells and the tumor, permitting the T cell to be reactivated, reversing exhaustion (5). However, a key prerequisite for checkpoint inhibitors to work is the presence of tumor infiltrating T cells, which are typically only present in tumors with a high mutational load (6, 7). Ideally, novel immunotherapies should target crucial cancer pathways, that cannot be downregulated, irrespective of the mutational load of the tumor.

Cell therapy against cancer

Cellular therapies have been a potential curative option for patients with hematological malignancies since the introduction of HLA-matched allogeneic hematopoietic stem cell transplantation (allo-HSCT) in the 1950s (8). Despite its curative potential, allo-HSCT is infamous for causing graft-versus-host disease (GvHD), leading to morbidity and mortality in the majority of patients (9). Furthermore, allo-HSCT is associated with a delayed immune recovery, making patients prone to opportunistic infections. *In vitro* or *in vivo* T cell depletion after allo-HSCT, in combination with a donor lymphocyte infusion (DLI), has decreased transplantation-

related mortality due to a lower incidence of GvHD and an improved immune reconstitution (10, 11). More recently, the development of an *in vitro* modified DLI product, by the selective removal of alloreactive T cells, has enabled the use of haploidentical donors as a stem cells source for patients without a matched donor (12). Over 50 years after the application of allo-HSCT and the subsequent application of DLI, the approval of the first cellular immunotherapy against a solid tumor, sipuleucel-T (13) against prostate cancer in 2010 (14), and the first engineered T cells expressing chimeric antigen receptors (CAR) in 2017 (15), have paved the way for new cellular interventions against cancer. αβT cell receptor (αβTCR) engineered T cells are a recent addition to cellular interventions against both solid and hematological malignancies (16). In these therapies, allogeneic or autologous T cells are genetically engineered to express a tumor-specific αβTCR, thereby redirecting the donor- or patient-derived T cells towards the tumor. αβTCRs function in an HLA restricted manner, indicating that they are only able to recognize tumor-derived peptides presented by MHC class I molecules on the tumor. Common αβTCRs used to construct αβTCR engineered T cells are directed against cancer/testis antigens, such as MAGE-A3 (17), MART-1 (18), NY-ESO-1 (19), or PRAME (20), which are often overexpressed on tumor cells. A limitation of current αβTCR and CAR engineered T cell therapies is the lack of purity of the final product, which can hamper the therapeutic efficiency. Furthermore, in an allogenic setting, the presence of T cells still expressing the endogenous αβTCR can lead to severe GvHD. A possible way of eliminating the risk of GvHD, is to gene-edit T cells whereby the tumor-specific receptor is introduced in the α-chain locus (21, 22). Unfortunately, increasingly elegant and precise cell therapy approaches are associated with increased manufacturing and regulatory complexity (Figure 1).

γδT cells

The human immune system consists of many proteins and cellular components that offer protection against disease. The T cell subset, which belongs to the lymphocyte class of immune cells, is the most abundant cell subset within the human immune system. T cells play an essential role in the removal of infected or transformed cells, regulation of the immune response and the formation of immunological memory. T cells can be further subdivided into two different populations since they express a receptor composed of a heterodimer of either a conventional α- and β-chain or a non-conventional γ- and δ-chain, in complex with the CD3 protein complex which is responsible for intracellular signaling. αβT cells

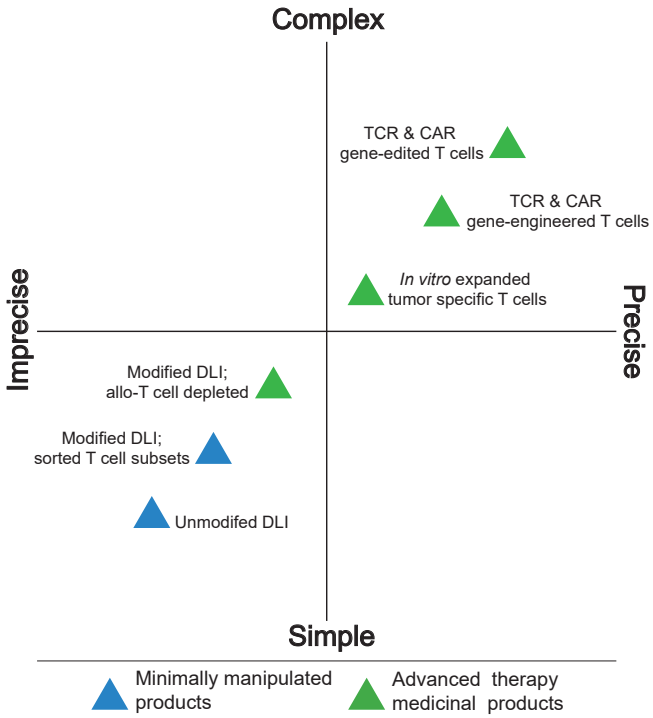

Figure 1. T cell therapy in the era of precision medicine. Complexity of cellular therapies increases proportionally with the precision of the therapy. Donor Lymphocyte Infusions (DLI) after allogeneic hematopoietic stem cell transplantation can benefit patients suffering from hematological malignancies while some T cell Advanced Therapy Medicinal Products (ATMPs) can benefit patients with solid malignancies as well. Adapted from (8).

represent the major subset of T cells in the periphery, while γδT cells are mostly present in certain epithelial tissues (23). It has been shown that these γδT cells are key players of the human immune system, possessing properties of both the adaptive and innate immune system (Figure 2); γδT cells are able to present antigens to CD4+ and CD8+ T cells (24) and can lyse cells which are "stressed", either due to infection (25, 26) or malignant transformation (27). With these multiple modes of action, γδT cells play a major role in tumor immunosurveillance (28, 29). γδT cells are further divided in γδT cells expressing a δ2-chain (Vδ2 positive) or a δ1- or δ3-chain (collectively called Vδ2 negative). Circulating γδT cells mostly belong to the Vγ9Vδ2 subset, where they sense a conformational change of butyrophilin 3A1, caused by an intracellular accumulation of phosphoantigens such as isopentenyl pyrophosphate (30, 31). Most δ2-negative γδT cells are located in epithelial tissues, where they make

up about 50% of all T cells. In contrast to δ2-positive γδT cells, some specific ligands have been identified; δ2-negative γδT cells are activated by stress-related ligands such as EPCR (32), MIC A (33), CD1c, CD1d (34), and Annexin A2 (35). Discovering new tumor-reactive γδT cell clones and identifying their ligands is valuable for the development of novel engineered T cell therapies (36).

T cells engineered to express a defined γδT cell receptor

Given the important role of γδT cells in tumor immunosurveillance and their broad tumor-reactivity, γδT cells are very interesting for therapeutic use. Furthermore, in contrast to checkpoint inhibitors, γδT cells function irrespective of mutational load of the tumor and in contrast to antibody and CAR therapies, γδT cells do not rely on the expression of a specific tumor antigen, which decreases the chance of resistance to the therapy. An increasing amount of pharmaceutical companies are developing γδT cell based therapies and testing these novel approaches in clinical trials (Sebestyén & Kuball, submitted). Unfortunately, most attempts to bring γδT cells to the clinic have not been very successful thus far (38). Failure of therapies using *in vitro* expanded γδT cells can be attributed to the large diversity of the Vγ9Vδ2T cell repertoire and their susceptibility

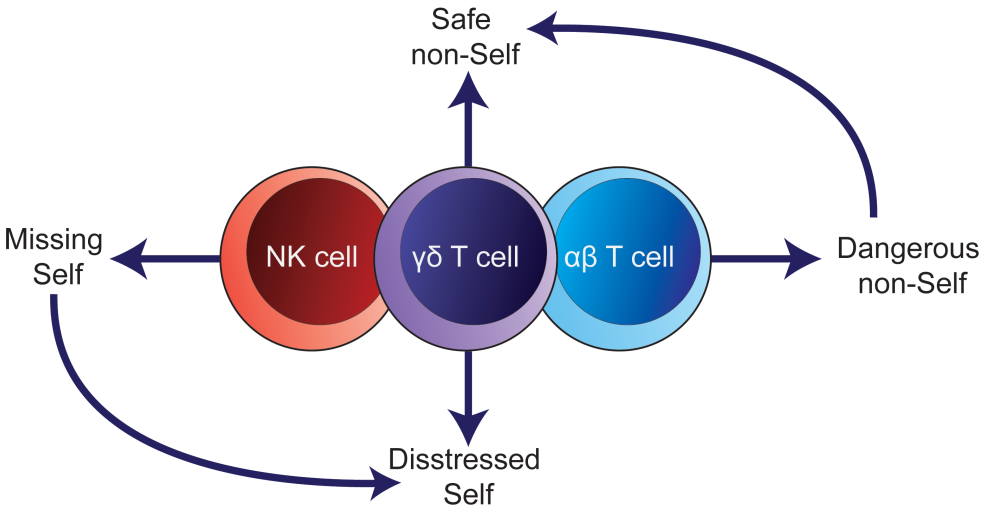

Figure 2. γδT cells possess properties of both innate (such as NK) and adaptive (such as αβT) immune cells. NK cells express a range of receptors primarily sensing "missing self" which is in essence the downregulation of HLA. αβT cells express an αβTCR capable of recognizing non-self peptides presented in HLA. Between these two extremes, γδT cells are able to tolerate "safe non-self" and recognize "distressed self". Adapted from (37).

to exhaustion and activation-induced cell death (39, 40). In addition, γδT cells have a relative limited proliferative capacity (41), and the presence of inhibitory innate receptors can weaken their response (42). To overcome these issues, we have recently developed the concept of T cells engineered to express a defined γδT cell receptor (TEGs) (43). TEGs are autologous T cells engineered to express one defined broadly tumor-reactive γδTCR obtained from a healthy donor. By using αβT cells as a carrier for our defined γδTCR, we take the γδTCR out of the inhibitory innate environment and take advantage of the proliferative capacity of αβT cells (44). Furthermore, the successful introduction of γδTCRs in αβT cells results in a downregulation of the endogenous αβTCRs, enabling the purification of TEGs by the depletion of non- and poorly-engineered T cells in order to further increase activity and definition of the product (43). This purification strategy is very elegant due to the fact that no additional selection genes have to be introduced and the final product is untouched, avoiding potential activation-induced cell death. The anti-human αβTCR antibody used for the purification of TEGs does not cross-react with γδTCR chains and can thereby differentiate between engineered and non-engineered cells. This anti-human αβTCR antibody is commonly used to deplete αβT cells from apheresis products using CliniMACS depletion before allo-SCT and is therefore readily available for clinical use (16, 45).

Outline of the thesis

Cancer immunotherapy is in rapid development ever since the acknowledgement of the important role of the immune system in cancer control. Immune-checkpoint inhibitors can offer a solution to high mutational load tumors, whereas tumors expressing a defined tumor antigen can be targeted by CAR T cells or antibody therapies (46). Despite these major advances in the control and eradication of certain types of tumors, many patients do not respond to any of these therapies. Therefore, this thesis aims to contribute to the development of some of the currently available immunotherapy products, and sheds a light on a whole new generation of cellular immunotherapies with TEGs. Crucial for the development of TEGs is the hunt for γδT cell clones with a tumor specific reactivity. During the past decade several ligands for Vδ2 negative γδT cells have been identified, however, given the vast diversity of Vδ2 negative γδT cells, a potential large number of ligands remains to be identified (47). For that reason, this thesis starts with the characterization of a tumor-reactive Vγ5Vδ1TCR. Most Vδ2 negative γδTCRs are activated by stress-related ligands such as EPCR (32), MIC A (33), CD1c, CD1d

(34), and Annexin A2 (35). However, in sharp contrast to the already described Vδ2 negative γδTCR ligands, **Chapter 2** describes a tumor-specific allo-HLA-restricted γδTCR, named "FE11", which recognizes both solid and hematological tumor cells. Due to its ability to differentiate between healthy and malignant cells, in an HLA-restricted fashion of a common HLA allele, this γδTCR, when applied in the TEGs format, can be a very promising clinical candidate, but also useful for better understanding of successes and failures of haplo-transplantations. **Chapter 3** reviews the immunological and regulatory challenges associated with novel engineered immune cells (16). The focus of this chapter is on αβTCR-, CAR-, and innate receptor-engineered immune interventions and the vast differences in the current landscape of clinical trials in Europe compared to the USA. **Chapter 4** describes a method to counter one of the challenges of cellular immunotherapy by removing impurities from engineered immune cell products, which our group has described before in the context of TEGs (43). To make this purification strategy applicable outside the γδTCR engineered T cell setting, αβTCRs are modified in a way that enables differentiation between introduced and endogenous αβTCRs and subsequent selective depletion of non- and poorly-engineered T cells. Furthermore, this modification of the αβTCR can be used as a safety switch to remove engineered cells *in vivo* when deemed necessary. **Chapter 5** summarizes strategies to increase potency of cellular immunotherapy and emphasizes the importance of reducing costs and time to clinical application of engineered immune cell products, to make these available to a large patient population (48). **Chapter 6** describes the technology transfer of the manufacturing of TEGs in a research environment to GMP-grade manufacturing. Key developmental steps taken in the production of TEG001 for clinical trial NTR6541 are elucidated, in addition, release and potency criteria acceptable for competent authorities are defined (49). This work allows the execution of a clinical trial which aims to investigate the safety and tolerability of TEG001 in patients with high risk hematological malignancies and will be a major step forward in our efforts to bring TEGs to the clinic (50). Finally, in **Chapter 7** we have summarized the abovementioned chapters and placed the respective findings in the context of other studies performed within the field of γδT cell biology and T cell engineering.

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Identification of a tumor-specific allo-HLA-restricted γδTCR

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Abstract

γδT-cells are key players in cancer immune surveillance due to their ability to recognize malignant transformed cells, which makes them promising therapeutic tools in the treatment of cancer. However, the biological mechanisms of how γδT-cell receptors (TCR) interact with their ligands are poorly understood. Within this context we describe a novel allo-HLA-restricted and CD8α-dependent Vγ5Vδ1TCR. In contrast to the previous assumption of the general allo-HLA reactivity of a minor fraction of γδTCRs, we show that classical anti-HLA-directed γδTCR mediated reactivity can selectively act towards hematological and solid tumor cells, while not harming healthy tissues *in vitro* and *in vivo*. We identified the molecular interface with close proximity to the peptide-binding groove of HLA-A*24:02 as the essential determinant for recognition and describe the critical role of CD8αα as co-receptor. We conclude that allo-reactive γδT-cell repertoires provide therapeutic opportunities either within the context of haplo-transplantation or as individual γδTCRs for genetic engineering of tumor reactive T-cells.

Introduction

Human immunity is organized by interacting innate and adaptive immune subsystems that elicit a fast or durable response respectively. γδT-cells are situated between the innate and adaptive immune systems as they share properties of both systems, illustrated by their ability to recognize malignant transformed (1), or infected (2) cells, to clonally expand, and to form memory (3). Recently, the important biological role of γδT-cells in cancer immune surveillance has been further highlighted by the fact that γδT-cells infiltrate various tumors (4, 5). However, the biological understanding of cancer immune surveillance and potential clinical applicability of γδT-cells, or their individual receptors, is substantially hampered by the lack of well-defined γδT-cell receptor (TCR) ligands as well as their precise molecular requirements for recognition (6). γδT-cell ligands that have been identified so far are mostly associated with metabolic changes in stressed cells, e.g. Vγ9Vδ2 T-cells, the major subset of γδT-cells in the periphery, are activated by cells with an increase of intracellular phosphoantigens caused by a dysregulated mevalonate pathway due to transformation or infection (7, 8). γδT-cell that do not express a Vδ2-chain, collectively called Vδ2-negative γδT-cells, are mainly found in tissues and are activated by stress-related ligands such as EPCR (9), MICA (10), and Annexin A2 (11). Furthermore, CD1c and CD1d can present self and foreign lipid antigens to Vδ2-negative γδT-cells in a classical αβT-cell HLA-like fashion (12). Since ligands of both Vδ2-positive and Vδ2-negative γδT-cells are to some extend constitutively expressed on healthy cells, it remains unclear how exactly the balance between self and tumor or infection is orchestrated. Recent data suggests that receptors, such as Vγ9Vδ2TCRs, modulate the delicate line between healthy and diseased tissue by sensing spatial and conformational changes of membrane expressed CD277, which occurs in transformed cells (8, 13). To exploit γδT-cells or their receptors as therapeutical tools, the understanding of the localization and structure of the ligands during stress or transformation needs to be understood. Furthermore, identifying new γδTCR ligands restricted to stressed or transformed cells is valuable for developing therapies for unmet medical needs. Within this context, we aimed to identify a potential ligand of a Vδ1-positive γδT-cell clone, which has been classified as reactive against different tumor cell types, as well as to understand the molecular interaction of this receptor with its ligand (2).

Materials and methods

Cells lines and Flow cytometry
(see supplementary methods)

Generation of γδT-cell clone FE11
Clone FE11 was generated as described in a previous publication (2).

Cloning αβTCRs NEF & WT1
The HLA-A*02:01 restricted $WT1_{126-134}$-specific αβTCR (14) and HLA-A*24:02 restricted NEF_{134-10} αβTCR (Clone C1-28 (15)) were codon-optimized, synthesized at BaseClear (Leiden, The Netherlands) and subcloned into the retroviral pBullet vector.

Retroviral transduction of TCRs
Details are provided in supplementary methods and our previous publication (16).

Retroviral transduction of HLA
Phoenix-ampho retroviral packaging cells were transduced with pLZRS-A*02:01-IRES-NGFR or pLZRS-A*24:02-IRES-NGFR and the retroviral packaging plasmids gag-pol (pHIT60) and env (pCOLT-GALV) using Fugene-HD. The HLA plasmids were kindly provided by Marieke Griffioen (Leiden University Medical Centre, the Netherlands).

CRISPR/Cas genome editing
The β2m gene-specific regions of the gRNA sequence (GAGTAGCGCGAGCACAGCTA) was designed by the CRISPR design tool from the Zhang lab (http://crispr.mit.edu/). As control gRNA, the eGFP gene was targeted (GGAGCGCACCATCTTCTTCA). The pSicoR-CRISPR-Cas9 vector used was a kind gift from Robert Jan Lebbink (University Medical Center Utrecht, The Netherlands). LCL-TM cells were transduced with the viral supernatants, knockdown of β2M was confirmed by flow cytometry.

Functional T cell assays
IFNγ ELISA and ELISPOT were performed as previously described (2, 16) and in supplementary methods.

Flow cytometry FRET
To study dimerization of HLA, cells were labelled with Alexa594-conjugated α-HLA-A (donor) and Alexa647-conjugated α-HLA-A (acceptor), respectively. The donor fluorescence was measured using

a FACS LSRFortessa flow cytometer (BD) where donor fluorescence of the double-labeled healthy samples was compared with that of the double-labeled malignant samples. FRET efficiency was calculated from the fractional decrease of the donor fluorescence in the presence of the acceptor, using the equations as described by Sebestyen and colleagues (17). Correction factors for the spectral overlap between the different fluorescence channels were obtained from data measured on unlabeled and single-labeled cells.

Animal model

The NOD.C*g-Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(HLA-A24)3Dvs/Sz (NSG-A24) mice (18) were kindly provided by Leonard D. Shultz (The Jackson Laboratory, Bar Harbor, ME, USA). C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France). All mice were bred and housed in the specific pathogen-free breeding unit of the Central Animal Facility of Utrecht University. Experiments were conducted according to institutional guidelines after acquiring permission from the local ethical committee and in accordance with current Dutch laws on animal experimentation. NSG-A24 mice received sublethal total body irradiation on day 0 followed by intravenous injection of $5*10^6$ K562 HLA-A*24:02 tumor cells on day 1, after which they were treated with $1*10^7$ TEG011 or Mock TCR transduced T-cells on day 2 and day 9. IL2 ($6x10^5$ IU in 100µl incomplete Freund's adjuvant) was administered subcutaneously once every 3 weeks.

Statistical Analyses

Differences were analyzed using indicated statistical tests in GraphPad Prism 7 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

Tumor specificity of Vγ5Vδ1T-cell clone can be transferred to αβT-cell by transfer of the γδTCR only

To confirm the tumor reactivity of the recently identified tumor specific γδT clone FE11 (2), the clone was co-incubated with SW480 (colorectal adenocarcinoma), EBV-LCL (Epstein-Barr virus-transformed lymphoblastoid cell line), and healthy PBMCs, leading to recognition, as measured by interferon (IFN)γ ELISpot, of the 2 tumor cells lines but not the healthy PBMCs (Figure 1A). Next, both the γ and δ chain of γδT-cell clone FE11 were sequenced, identified as a Vγ5Vδ1 TCR, and cloned into pBullet retroviral vector and subsequently introduced into αβT-cells as previously described (16). Taking the γδTCR out of the innate-like environment enabled us to study the functioning of the receptor without interference of NK-receptors, which are not present on αβT-cells (19, 20). This strategy we have recently described as TEGs (T-cells engineered to express a defined γδT-cell receptor (21, 22)). Introduction of γδTCR-FE11 in αβT-cells (later referred to as TEG011) lead to comparable recognition of target-cells as the original clone FE11 (Figure 1B), indicating that tumor-reactivity is mediated by the γδTCR and independent of (epi)genetic factors exclusively present in the original T cell clone.

γδTCR-like antibodies indicate a role for classical HLA molecules

The observation that the γδTCR-FE11 by itself recognizes multiple tumor cell lines but not healthy PBMCs, was highly interesting. We aimed to identify the ligand of the γδTCR-FE11 by generating TCR-like antibodies by the immunization of C57BL/6 mice with complete tumor cell lines SW480 and LCL-TM that were recognized *in vitro* by TEG011. From the hybridomas generated, 19 clones were isolated that produced antibodies that specifically bound γδTCR-FE11 reactive tumor cells in an antibody binding screen. To further determine the ligand specificity of the antibodies, the FE11 targets SW480 and LCL-TM were pre-incubated with supernatants from these hybridomas and subsequently used in co-cultures to stimulate TEG011. 13 out of the 19 antibodies (i.e. hybridoma supernatants) blocked the activation of the TEG011 substantially, as measured by IFNγ ELISpot (Figure 1C). These data suggest that the majority of the raised antibodies were able to partially or completely prevent the binding of the γδTCR-FE11 to its ligand. In contrast, none of the 19 hybridomas produced an antibody that could block the recognition of $WT1_{126-134}$ (HLA-A*02:01) peptide loaded SW480 by WT1-TCR transduced αβT-cells (Supplementary

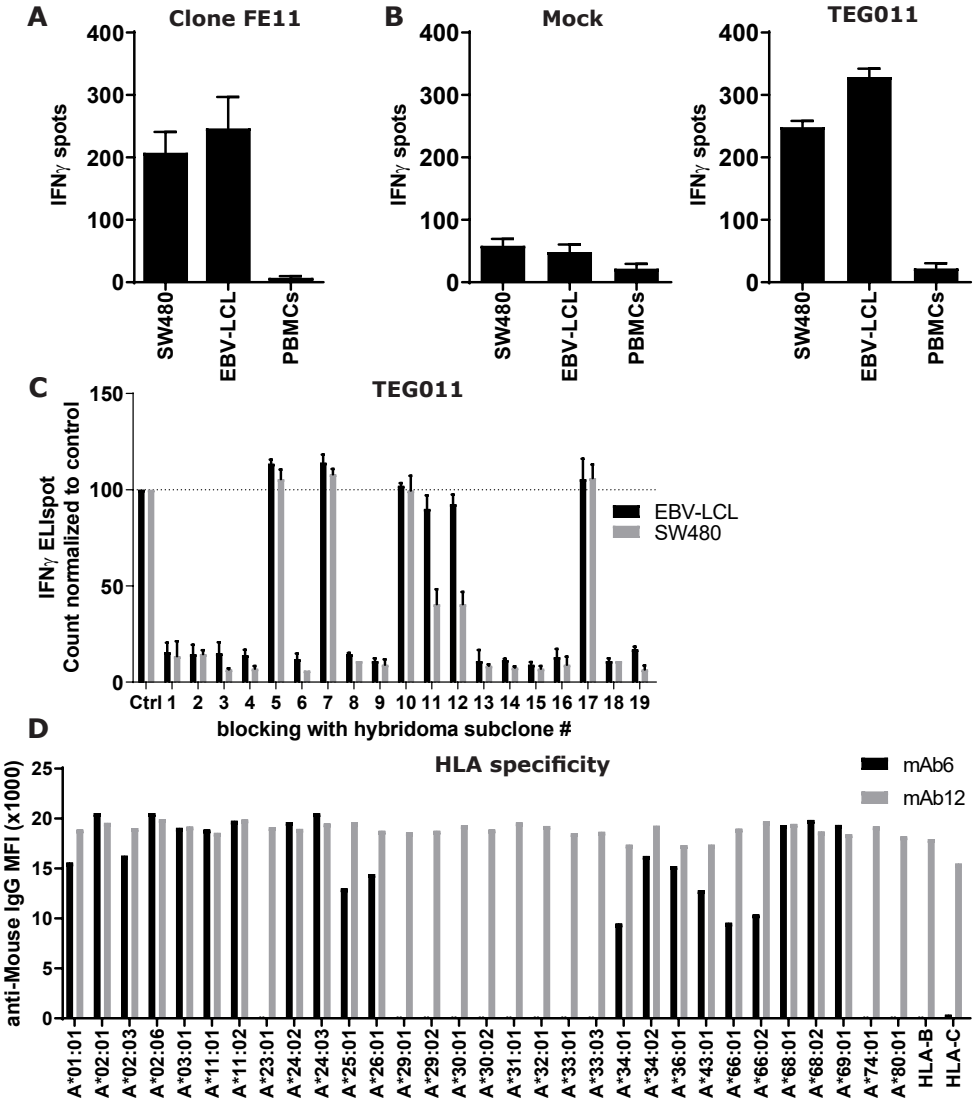


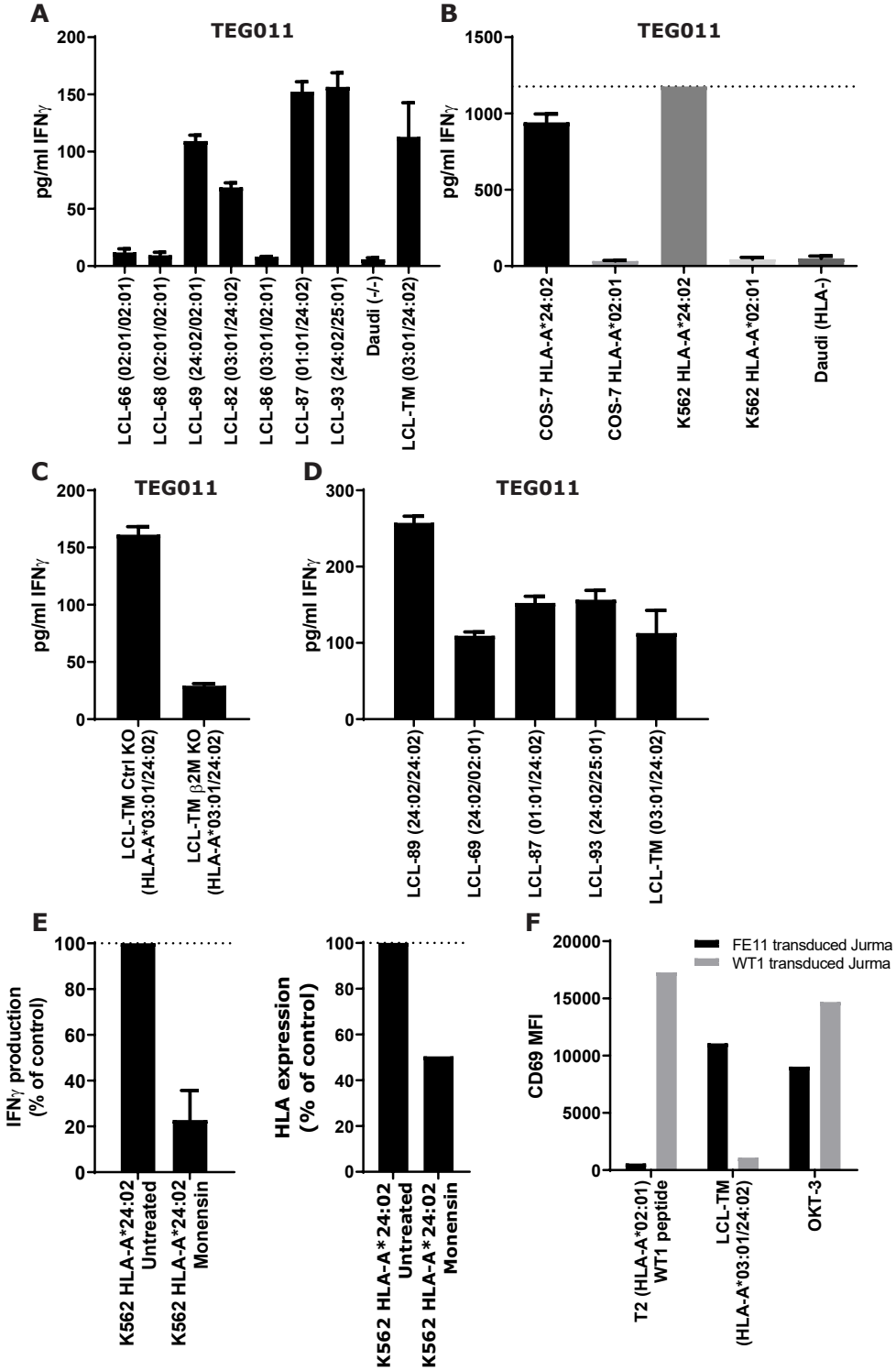
Figure 1. Introduction of γδTCR-FE11 in αβT-cells can re-establish tumor cell recognition of clone FE11. (A) To assess tumor reactivity, FE11 cells were incubated with SW480 or EBV-LCL tumor targets. IFNγ secretion was measured by ELISPOT. Healthy PBMCs served as negative control targets. **(B)** The TCR γ and δ chains of clone FE11 were sequenced and retrovirally transduced into αβT-cells. Transfer of γδTCR-mediated tumor-reactivity was tested by co-incubating γδTCR- or mock-transduced T-cells with indicated target-cells in an IFNγ ELIspot. **(C)** The effect of blocking with FE-11 like hybridoma supernatant on the recognition of SW480 and LCL-TM by γδTCR-FE11 transduced T cells. **(D)** LABScreen Single Antigen HLA class I beads were incubated with antibodies purified from hybrima 6 (mAb 6) or antibodies purified from hybridoma 12 (mAb 12) and secondary α-mIgG-PE and measured using Luminex. Error bars represent SD.

Figure 1A), indicating that the blocking was not induced via binding to e.g. adhesion molecules generally needed for T cell activation (23). From the 19 hybridomas, one antibody that completely blocked activity (clone 6) and one that partially blocked activity (clone 12) (from here on named mAb6 and mAb12) were selected for antibody production and purification. These purified antibodies were coupled to streptavidin beads and subsequently used for ligand-immunoprecipitation in cell lysates of either SW480 or LCL-TM cells. Mass spectrometry analysis resulted in the identification of a panel of mostly classical HLA molecules (Supplementary Table 1) suggesting that, in contrast to the general assumption, classical HLA molecules may be involved in recognition of tumor cells by this particular γδTCR. To confirm that raised antibodies are specific for classical HLA, we incubated LABScreen Single Antigen HLA class I beads(24) with mAb6 and mAb12 and measured the beads by Luminex to determine HLA-specificity. Figure 1D shows that mAb6 has a reactivity to a defined subgroup of HLA-A alleles, while mAb12 displayed no specificity, reacting towards all HLA class I alleles, including HLA-B and HLA-C, present on the LABScreen beads.

Target cell recognition by the γδTCR-FE11 is critically dependent on HLA-A*24:02

The raised antibodies were able to bind a broad range of different HLA types. To further narrow down the type of HLA recognized by γδTCR-FE11, we made use of the library of cell lines from the Centre d'Etude du Polymorphisme Human (CEPH), which contains a large collection of EBV-transformed B-cell lines (EBV-LCLs) obtained from several family pedigrees with a large variety of HLA haplotypes (25) TEG011 was co-incubated with 7 different CEPH EBV-LCLs, covering multiple possible HLA molecules as suggested by the LABScreen beads (Figure 1D), Daudi and LCL-TM, and reactivity was assessed by measuring IFNγ-release.

►**Figure 2. Activation of γδTCR-FE11 transduced T-cells is dependent on expression of HLA-A*24:02**. **(A)** Activation of T-cells, transduced with γδTCR-FE11 by EBV-LCLs with different HLA genotypes. **(B)** Activation of T-cells, transduced with γδTCR-FE11 by HLA-A*24:02 or HLA-A*02:01 target-cells. **(C)** The effect of β2m KO of HLA-A*24:02-positive target-cells on the activation of γδTCR-FE11 transduced T-cells. **(D)** Activation of T-cells, transduced with γδTCR-FE11 by EBV-LCLs with different either homozygous or heterozygous HLA-A*24:02 expression. **(E)** Activation of T-cells, transduced with γδTCR-FE11 by K562 HLA-A*24:02 cells untreated or treated overnight with monensin. **(F)** Activation of Jurma cells, transduced with γδTCR-FE11 or αβTCR WT1 (control) by LCL-TM or A2 restricted WT1 peptide loaded T2 cells. CD3 crosslinking by plate-bound α-CD3 mAb clone OKT-3 served as positive control. Error bars represent SD.


A
TEG011
pg/ml IFNγ
200
150
100
50
0
LCL-66 (02:01/02:01)
LCL-68 (02:01/02:01)
LCL-69 (24:02/02:01)
LCL-82 (03:01/24:02)
LCL-86 (03:01/02:01)
LCL-87 (01:01/24:02)
LCL-93 (24:02/25:01)
Daudi (-/-)
LCL-TM (03:01/24:02)
B
TEG011
pg/ml IFNγ
1500
1000
500
0
COS-7 HLA-A*24:02
COS-7 HLA-A*02:01
K562 HLA-A*24:02
K562 HLA-A*02:01
Daudi (HLA-)
C
TEG011
pg/ml IFNγ
200
150
100
50
0
LCL-TM Ctrl KO (HLA-A*03:01/24:02)
LCL-TM β2M KO (HLA-A*03:01/24:02)
D
TEG011
pg/ml IFNγ
300
200
100
0
LCL-89 (24:02/24:02)
LCL-69 (24:02/02:01)
LCL-87 (01:01/24:02)
LCL-93 (24:02/25:01)
LCL-TM (03:01/24:02)
E
IFNγ production (% of control)
100
80
60
40
20
0
K562 HLA-A*24:02 Untreated
K562 HLA-A*24:02 Monensin
HLA expression (% of control)
100
80
60
40
20
0
K562 HLA-A*24:02 Untreated
K562 HLA-A*24:02 Monensin
F
FE11 transduced Jurma
WT1 transduced Jurma
CD69 MFI
20000
15000
10000
5000
0
T2 (HLA-A*02:01) WT1 peptide
LCL-TM (HLA-A*03:01/24:02)
OKT-3

Correlating reactivity of TEG011 to the different HLA types suggested that uniquely the HLA-A*24:02 haplotype, but not HLA-A*02:01 or HLA-A*03:01 (Figure 2A), was involved in the recognition. To formally confirm HLA-A*24:02 mediated recognition we retrovirally introduced either HLA-A*24:02 or HLA-A*02:01 (as control) into the HLA negative cell lines COS-7 and K562. In both cell lines, introduction of HLA-A*24:02, but not HLA-A*02:01, resulted in strong activation of TEG011 (Figure 2B). Vice versa, a partial CRISPR/Cas9 KO of β2-microglobulin within recognized LCLs, reduced activation of TEG011 (Figure 2C and Supplementary Figure 1B) as expected. Additionally, we found that the density of the ligand HLA-A*24:02 on target-cells was associated with the activity of TEG011, since reactivity of TEG011 was higher against cell lines homozygous for HLA-A*24:02 than against heterozygous cell lines (Figure 2D). In addition, reduction of HLA expression on the cell membrane by monesin, a protein transport inhibitor, resulted in a decreased recognition of target-cells (Figure 2E).
TEGs have been reported to lose allo-reactivity due the down regulation of the endogenous αβTCR due to dominance of the introduced γδTCR (21). However, in order to formally exclude any activity of endogenous αβTCR within the TEG format, we introduced either γδTCR-FE11 or αβTCR-WT1 (as control) into the TCRβ-negative Jurma cell line. The transduced Jurma cells were then co-incubated with WT1 peptide loaded T2 or LCL-TM tumor cells, and target-specific activation of Jurma cells was determined by measuring the activation marker CD69 by flow cytometry. As anticipated, γδTCR-FE11 transduced Jurmas were only activated by the HLA-A*24:02 expressing LCL-TM, while the αβTCR-WT1 transduced Jurmas were only activated by WT1 loaded T2 cells (Figure 2F). In conclusion, target cell recognition by γδTCR-FE11 is critically dependent on and restricted to HLA-A*24:02.

γδTCR-FE11 selectively recognizes HLA-A*24:02 expressed in malignant but not healthy cells

Allo-HLA reactivity is usually a phenomenon restricted to HLA on all cells of an individual (26). To assess if recognition is limited to HLA-A*24:02-positive transformed cells, we co-incubated TEG011 with healthy primary T-cells which were either positive or negative for HLA-A*24:02. In contrast to HLA-A*24:02-positive tumor cells, healthy primary cells were not recognized by TEG011, even when they were positive for HLA-A*24:02 (Figure 3A). Crucially, HLA-A*24:02-positive B-cells immortalized by using EBV transformation did activate TEG011, while non-transformed

PBMCs of the same donor were not recognized, indicating that next to the expression of HLA-A*24:02 allele, malignant transformation is essential for the activation of FE11 (Figure 3B).

Identification of a putative binding-site of γδTCR-FE11

In order to further map the putative binding site of FE11, CEPH EBV-LCLs expressing HLA alleles from different supertypes were tested (i.e. HLA-A*25:01 from supertype HLA-A01 and HLA-A*02:01 from supertype HLA-A02 (27). Additionally, CEPH EBV-LCLs expressing an HLA allele within the same supertype as HLA-A*24:02 (supertype HLA-A24; HLA-A*24:03) was tested. Reactivity of TEG011 could only be observed towards the HLA-A*24:02-positive cells, not towards the strong homologous HLA-A*24:03 present on EBV-LCL-71 (Figure 4A). Sequence alignment (Figure 4B, lower part) revealed that the two amino acids on the α2 helix at position 168 and 169 (asparagine and glycine respectively) are non-homologous between HLA-A*24:02 and the non-recognized HLA alleles, indicating that these residues are key for recognition of HLA-A*24:02 by TEG011. Structural analyses of the putative binding sites at position 168 and 169 indicated a very close proximity to the peptide binding groove (Figure 4B, upper part).

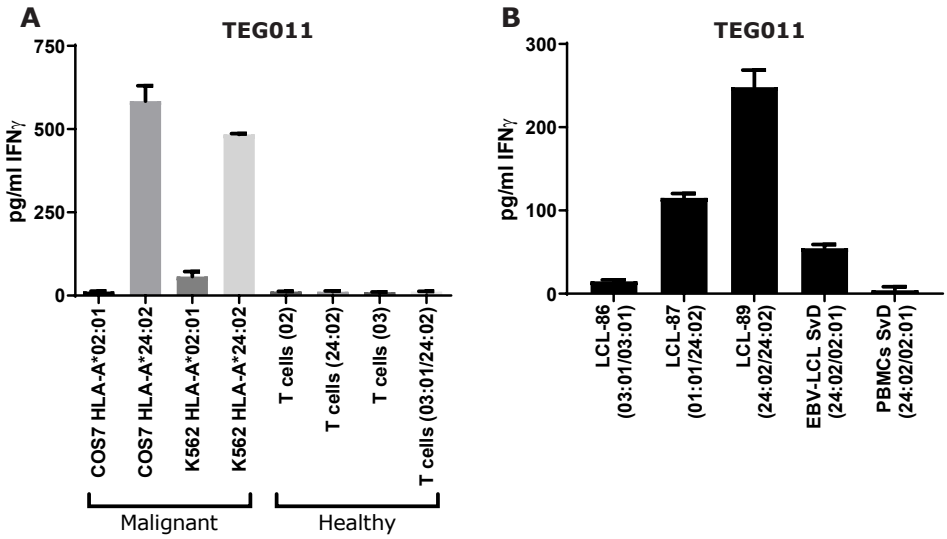
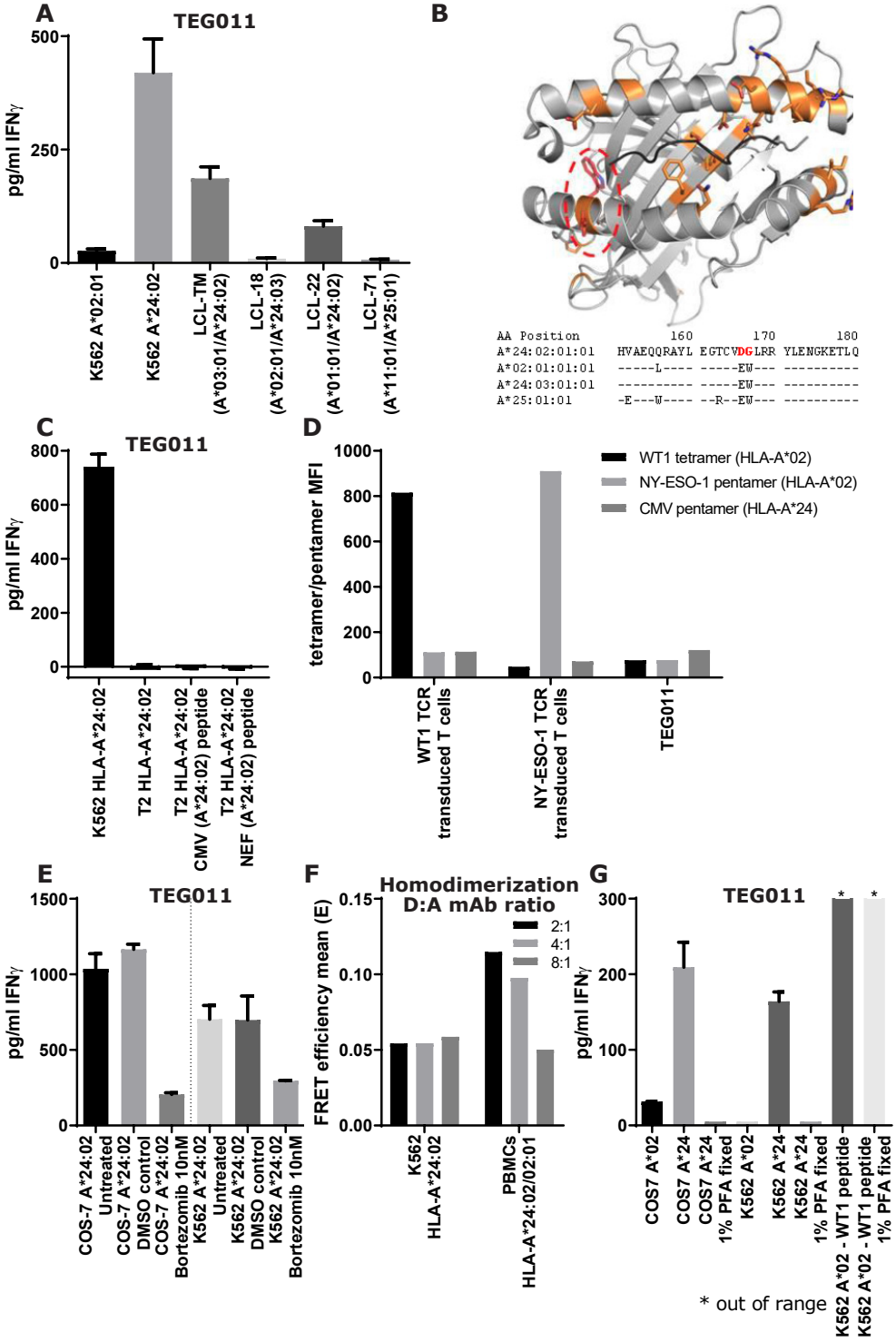


Figure 3. Activation of γδTCR-FE11 transduced T-cells is limited to HLA-A*24:02-positive malignant-cells. (A) Activation of TEG011 by malignant-cells and healthy T-cells. **(B)** Healthy donor B-cells (HD1) were EBV transformed and co-cultured with TEG011. Recognition was assessed by measuring IFNγ secretion using ELISA. Error bars represent SD.


A
TEG011
pg/ml IFNγ
500
250
0
K562 A*02:01
K562 A*24:02
LCL-TM (A*03:01/A*24:02)
LCL-18 (A*02:01/A*24:03)
LCL-22 (A*01:01/A*24:02)
LCL-71 (A*11:01/A*25:01)
B
AA Position 160 170 180
A*24:02:01:01 HVAEQQRAYL EGTCVDGLRR YLENGKETLQ
A*02:01:01:01 -----L---- -----EW--- ----------
A*24:03:01:01 ---------- -----EW--- ----------
A*25:01:01 -E---W---- --R--EW--- ----------
C
TEG011
pg/ml IFNγ
800
600
400
200
0
K562 HLA-A*24:02
T2 HLA-A*24:02
T2 HLA-A*24:02 CMV (A*24:02) peptide
T2 HLA-A*24:02 NEF (A*24:02) peptide
D
tetramer/pentamer MFI
1000
800
600
400
200
0
WT1 tetramer (HLA-A*02)
NY-ESO-1 pentamer (HLA-A*02)
CMV pentamer (HLA-A*24)
WT1 TCR transduced T cells
NY-ESO-1 TCR transduced T cells
TEG011
E
TEG011
pg/ml IFNγ
1500
1000
500
0
COS-7 A*24:02
Untreated
COS-7 A*24:02
DMSO control
COS-7 A*24:02
Bortezomib 10nM
K562 A*24:02
Untreated
K562 A*24:02
DMSO control
K562 A*24:02
Bortezomib 10nM
F
Homodimerization
D:A mAb ratio
2:1
4:1
8:1
FRET efficiency mean (E)
0.15
0.10
0.05
0.00
K562 HLA-A*24:02
PBMCs HLA-A*24:02/02:01
G
TEG011
pg/ml IFNγ
300
200
100
0
COS7 A*02
COS7 A*24
COS7 A*24 1% PFA fixed
K562 A*02
K562 A*24
K562 A*24 1% PFA fixed
K562 A*02 - WT1 peptide
K562 A*02 - WT1 peptide 1% PFA fixed
* out of range

◂**Figure 4. Activation of γδTCR-FE11 transduced T-cells is dependent on the presence of a specific HLA-A*24:02 restricted peptide. (A)** Activation of TEG011 by HLA-A*24:03-positive or negative target-cells. **(B)** The differences between HLA-A*02:01 and HLA-A24:02 mapped on the structure of HLA-A*24:02 (pdb: 3wl9), the two non-homologous amino acids between HLA-A*24:02 and HLA-A*24:03 are show in the red circle (upper panel). Alignment of HLA-A*24:02, 02:01, 24:03, and 25:01 with the two non-homologous amino acids in red (lower panel). **(C)** Activation of T-cells, transduced with γδTCR-FE11, by HLA-A*24:02 transduced TAP deficient T2 cells non-loaded or loaded with the A*24 restricted viral peptides NEF (134-10) or CMV (pp65 341-349). **(D)** WT1 tetramer, NY-ESO1 pentamer and CMV pentamer binding to WT1 TCR, NY-ESO1 TCR and FE11 TCR transduced T-cells. **(E)** The effect of Bortezomib treatment of HLA-A*24:02 transduced target-cells on the activation of γδTCR-FE11 transduced T-cells. **(F)** Homodimerization was assessed on HLA-A*24:02-positive recognized and non-recognized cells by flow cytometry FRET. **(G)** Activation of TEGs, or T-cells transduced with the αβTCR WT1 (control), by HLA-A*24:02 transduced COS-7 and K562 or HLA-A*02:01 (control). Where indicated target-cells were fixed before coincubation. Where indicated target-cells were coincubated with WT1 peptide. Error bars represent SD.

Promiscuous peptides are necessary for HLA-A*24:02 recognition by γδTCR-FE11

Due to this close proximity of the putative binding site to the peptide binding groove, we explored the role of a peptide in the recognition of HLA-A*24:02 by γδTCR-FE11. The cell line T2, which is deficient in TAP-dependent endogenous peptide processing and presentation in HLA molecules, was transduced with HLA-A*24:02 and HLA-A*02:01 (control) and loaded with HLA-A*24:02 restricted NEF and HLA-A*02:01 restricted WT1 peptides respectively. In order to confirm the successful loading of HLA molecules with peptides, stabilization of HLA on the surface of T2 cells was assessed by flow cytometry (Supplementary Figure 2A). HLA-A*24:02 transduced T2 cells externally loaded with NEF or CMV peptide did not lead to activation of TEG011, indicating that the presence of HLA-A*24:02 alone is not sufficient when expressed on T2 cells, but that the presentation of an endogenously processed peptide could be key to establish reactivity (Figure 4C). In order to confirm this hypothesis, we coincubated TEG011 with a CMV-pp65 HLA-A*24:02 restricted pentamer. Whereas the controls, WT1 and NY-ESO-1 αβTCR-transduced T-cells with their respective tetramer or pentamer stained positive (Figure 4D), TEG011 was not stained by the HLA-A*24:02 pentamer. These data suggest that the observed recognition is not caused by classical alloreactivity and most likely involves either promiscuous peptides to stabilize the complex or a specific peptide as a critical determinant for recognition.

To further assess whether endogenously processed peptides are essential

for reactivity, we interfered with the cellular peptide processing machinery by inhibiting the proteasome of recognized tumor cells by pre-treatment with Bortezomib (28). Bortezomib treatment lead to a strong decrease in recognition of both HLA-A*24:02 transduced COS-7 and K562 cells (Figure 4E) by TEG011, suggesting that peptides are at least needed for stabilization of the complex. To explore if transformation-associated peptides are involved in recognition, we selected 15 transformation-associated peptides to load HLA-A*24:02 transduced T2 cells (Supplementary Figure 2B and Supplementary Table 2) and assessed the recognition by TEG011. None of the 15 peptides lead to activation of TEG011 (Figure 5A). Vice versa, we outcompeted the putative endogenous peptide recognized by TEG011 with NEF_{134-10} WT peptide and NEF_{134-10} mutants. NEF_{134-10} mutants were designed by changing the four amino acids which are facing out of the HLA binding groove (Figure 5B). The four amino acids were substituted for the negatively charged amino acid glutamic acid (E), the positively charged amino acid arginine (R), and the smallest amino acid; glycine (G). At least part of these modified peptides could be loaded on LCL-TM cells (Supplementary Figure 3). Next, WT NEF_{134-10} peptide and all different NEF_{134-10} mutants were loaded on LCL-TM after which they were coincubated with TEG011, followed by measurement of IFNγ. None of the peptides were able to decrease the recognition, indicating that the recognition mechanism is more elaborate than a standard αβTCR peptide-HLA interaction (Figure 5C) implying that recognition is not mediated by a specific peptide, but rather promiscuous peptides are involved as stabilizer of the complex.

Conformational change as additional distinguishing factor for recognition

The hypothesis that promiscuous peptides are involved as stabilizer of the complex was supported by the observations that HLA-A*24:02 was also recognized within the context of another species (monkey, COS-7 cell line). In addition, as usually small amounts of endogenously processed and presented peptides are sensed by TCRs, doubling the amount of HLA in a homozygous as compared to heterozygous target should not substantially affect recognition. However, increased amounts of HLA in homozygous individuals nearly doubled functional activity of TEG011, suggesting that rather the HLA-complex than individual peptide-HLA combination was recognized. Therefore, we hypothesized that an additional key-spatial or structural conformational change in HLA-A*24:02, as a result of transformation of a healthy cell into a tumor cell. To elaborate on this

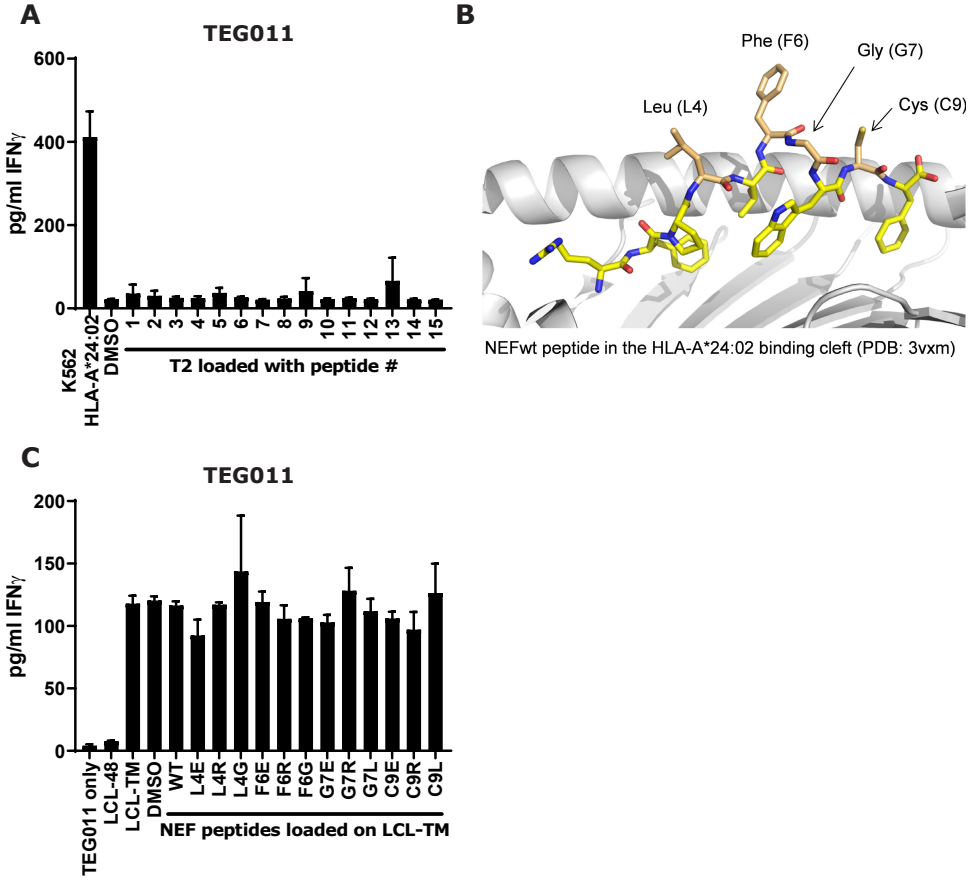


Figure 5. Recognition of LCL-TM cannot be outcompeted by peptides. **(A)** T2 cells were transduced with HLA-A*24:02 and loaded with 10 μM of 15 different transformation-associated peptides (Supplementary Table 1) after which they were coincubated with TEG011. Activation of TEG011 was assessed by measuring IFNγ production. **(B)** The four residues of NEF_{134-10} which are pointed out of the peptide binding groove of HLA-A*24:02 are indicated. **(C)** 10 μM of all generated NEF mutant peptides were loaded on LCL-TM after which they were coincubated with TEG011. Activation of TEG011 was assessed by measuring IFNγ production. Error bars represent SD.

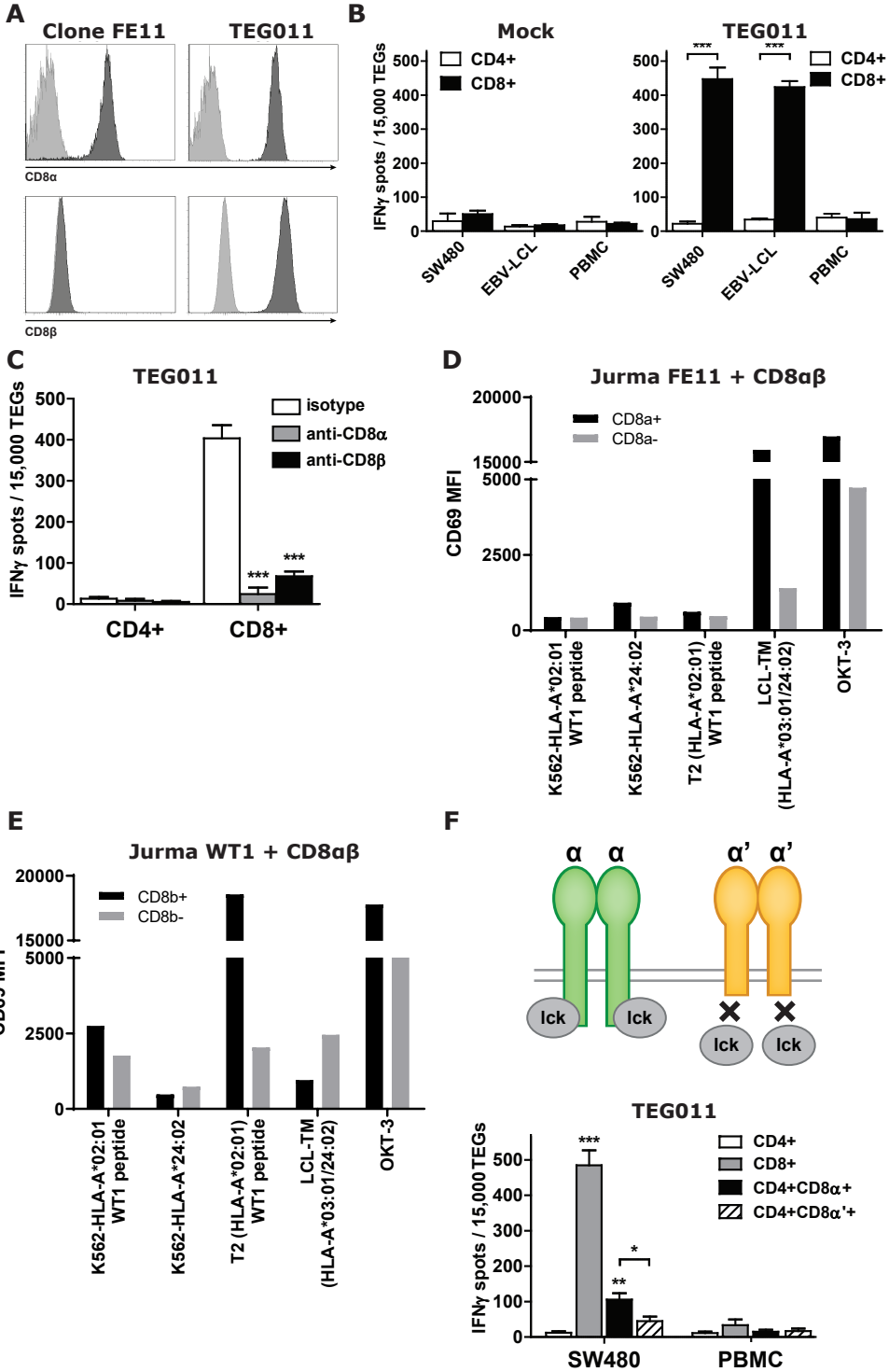
hypothesis we used Förster resonance energy transfer (FRET) based flow cytometry as described before (17), to determine if HLA-A*24:02 clusters in the membrane of tumor cells. In line with this assumption, FRET analysis suggested different behavior of HLA in tumor and healthy tissues with HLA-A*24:02 homodimers on PBMCs and monomers on tumor cells (Figure 4F). In order to formally test if membrane mobility of HLA-A*24:02 is key for recognition by γδTCR-HLA but not αβTCR-HLA, we

assessed the effect of paraformaldehyde fixation on the sensing of target-cells. Whereas the recognition of αβTCR-WT1 transduced T-cells and WT1 peptide loaded target-cells was not affected by fixation, the interaction by TEG011 cells and HLA-A*24:02 transduced target-cells was completely abolished, indicating that there are differences in affinity of the TCR or a key difference in conformation (Figure 4G).

γδTCR-FE11 critically depends on the CD8 co-receptor for tumor recognition

To further support the idea that indeed the γδTCR-HLA interaction differs from classical HLA-αβTCR interactions, we investigated the potential role of co-receptors. One obvious candidate, due to HLA class I restriction, was CD8αα. First, we formally confirmed CD8 expression on the original clone in line with previous reports ((2) and Figure 6A). Next, we assessed whether TEG011 is dependent on the co-expression of CD8, like the original clone, by sorting TEG011 on CD4 and CD8 expression before co-culturing with SW480, LCL-TM or PBMCs (Figure 6B). In contrast to the γδT-cell clone FE11, most αβT-cells express CD8 as a heterodimer of CD8α and CD8β for providing co-stimulation. The role of the CD8αβ heterodimer on TEG011 was assessed by using blocking antibodies for either the CD8α or CD8β chain. Not only CD8α, but also CD8β blocking antibodies completely inhibited recognition of SW480 (Figure 6C), indicating that either CD8αα or CD8αβ is essential for recognition. These data have also been confirmed by comparing CD8-positive and CD8-negative Jurma cells expressing γδTCR-FE11 (Figure 6D) or αβTCR WT1 (control, Figure 6E). For co-stimulation of HLA class I-restricted αβTCRs, CD8αβ can play two different roles; it

►**Figure 6. γδTCR-FE11 critically depends on the CD8 co-receptor for tumor recognition. (A)** CD8α or CD8β expression on clone FE11 and γδTCR-FE11 transduced αβ T-cells. **(B)** CD4+ and CD8+ αβT-cells transduced with the γδTCR-FE11 were sorted and co-cultured with indicated target-cells. **(C)** T cell activation was assessed by IFNγ ELISPOT. CD4+ and CD8+ αβT-cells expressing the γδTCR-FE11 were co-incubated with SW480 target-cells as in (A) but now in the presence of a control antibody or blocking antibodies against CD8α or CD8β. **(D)** Activation of γδTCR-FE11 transduced Jurma cells by HLA-A*24:02-positive and negative target-cells as measured by CD69 upregulation. **(E)** Activation of αβTCR WT1 transduced Jurma cells by HLA-A*02:01-positive target-cells loaded with HLA-A*02 restricted WT1 peptide. CD3 crosslinking by plate-bound α-CD3 mAb clone OKT-3 served as positive control (D+E). **(F)** αβT-cells were transduced with wildtype CD8α or a truncated, signaling-deficient CD8α variant (CD8α'); alongside the γδTCR-FE11, after which CD4+, CD8+, CD4+CD8α+ and CD4+CD8α'+ T-cells populations were sorted. Recognition of healthy PBMCs and SW480 tumor target was assessed by measuring IFNγ secretion using ELISPOT. Error bars represent S.E.M. (*P < 0.05; *P < 0.01; ***P < 0.001).


A
Clone FE11
TEG011
CD8α
CD8β
B
Mock
TEG011
CD4+
CD8+
IFNγ spots / 15,000 TEGs
SW480
EBV-LCL
PBMC
C
TEG011
isotype
anti-CD8α
anti-CD8β
IFNγ spots / 15,000 TEGs
CD4+
CD8+
D
Jurma FE11 + CD8αβ
CD8a+
CD8a-
CD69 MFI
K562-HLA-A*02:01 WT1 peptide
K562-HLA-A*24:02
T2 (HLA-A*02:01) WT1 peptide
LCL-TM (HLA-A*03:01/24:02)
OKT-3
E
Jurma WT1 + CD8αβ
CD8b+
CD8b-
CD69 MFI
K562-HLA-A*02:01 WT1 peptide
K562-HLA-A*24:02
T2 (HLA-A*02:01) WT1 peptide
LCL-TM (HLA-A*03:01/24:02)
OKT-3
F
α α
α' α'
lck
TEG011
CD4+
CD8+
CD4+CD8α+
CD4+CD8α'+
IFNγ spots / 15,000 TEGs
SW480
PBMC

serves as an adhesion molecule that stabilizes the TCR-HLA interaction and it can play an activating role by signaling via LCK (29). On the other hand, CD8αα on αβT-cells has been described as a corepressor rather than a coreceptor by competing with CD8αβ for the LCK signaling molecule (30). To investigate the role of CD8αα for TEG011, we utilized a truncated variant of CD8α which is signaling deficient due to its inability to bind LCK (31). After introducing both γδTCR-FE11 and truncated CD8α (CD8α') in CD4+ αβT-cells we co-cultured the TEGs with SW480. A decrease in the amount of IFNγ spots of the CD8α' variant compared to the CD8α wild type variant was observed (Figure 6F), indicating that CD8αα indeed plays a co-stimulatory role in TEG011.

Improved overall survival by TEG011

To determine safety and effectivity of TEG011 *in vivo* we set up a humanized HLA-A*24:02 transgenic NSG mouse model for adoptive transfer of TEG011. The irradiated mice were injected with HLA-A*24:02 transduced K562 and either TEG011 or LM1 transduced T-cells as control. Mice were taken out of the study when the human endpoint was reached. A relative tumor control of TEG011 compared to LM1 transduced T-cells could be observed (Figure 7). Importantly, pathology showed no histological features of toxicity of TEG011 in the three mice analyzed, indicating that HLA-A*24:02 positive tumor cells but not healthy cells were targeted by TEG011.

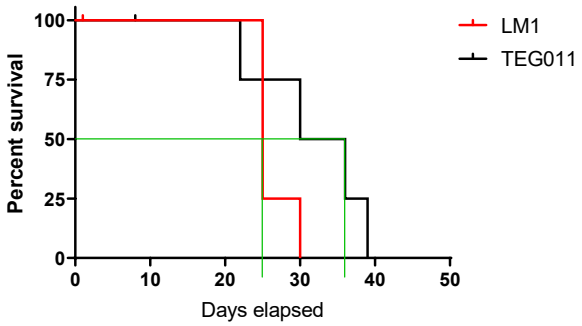


Figure 7. Improved tumor control of TEG011 in NSG-A24 mice. NOD.*Cg-Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(HLA-A24)3Dvs/Sz (NSG-A24) mice were injected with 5x10^{6} K562 HLA-A*24:02 cells on day 1 followed by 1x10^{7} TEG011 or LM1 transduced T-cells on day 2 and day 9 (n=4 per group). Overall survival of treated mice was monitored until the last mouse reached the predefined human endpoint. Comparison of survival curves using the log-rank test, P=0.2.

Discussion

The major finding of the study is that we identified an allo-reactive γδTCR which is able to distinguish between healthy and tumor tissues. Furthermore, we elucidated the molecular interface of the investigated Vγ5Vδ1TCR clone FE11 and provide evidence that, although the binding site is close to the peptide binding groove of HLA-A*24:02, transformation-associated HLA peptides do not dictate recognition between healthy and cancer tissues. Most likely, other key-conformational changes within the membrane selectively occurring to HLA on tumor cells but not on healthy tissues *in vitro* and *in vivo,* are responsible for this.

Allo-HLA type of recognition by γδTCRs has been suggested both for HLA-A*02 (32) and HLA-A*24 (33). However, the underlying molecular mechanism has not been defined so far, in contrast to allo-reactivity of αβTCRs (34-36). Considering this, a γδTCR selectively recognizing tumor cells in an allo-HLA context seems to be plausible, however, our data suggests that the mode of action differs between allo-reactive αβTCRs and allo-reactive γδTCRs. We characterized the essential contact residues of γδTCR-FE11 with HLA-A*24:02, which appeared to be in close proximity to the peptide binding groove. Our data suggests that γδTCR-FE11 is able to recognize amino acids 168 and 169 on the α2-helix of HLA-A*24:02, since these are the only non-homologous amino acids between recognized HLA-A*24:02 and non-recognized HLA-A*24:03 (37). Differences in recognition of the same peptide presented by HLA-A*24:02 and HLA-A*24:03 by CD8+ αβT-cells has been observed before (38). Thus, not only αβTCRs, KIRs, LILRs, and CD8 molecules (39), but also γδTCRs can bind to a specific part of HLA class I. However, our data also imply that, in contrast to recognition of HLA by an allo-peptide reactive αβTCR, promiscuous peptides are involved in mediating recognition by γδTCR-FE11. This assumption would also be an explanation for the activity of TEG011 against a very broad range of tumor cells.

Our FRET data suggests that, instead of a defined tumor-derived peptide, another key spatial or conformational change like differences in clustering of HLA-A*24:02-molecules between tumor cells and healthy cells plays a role in mediating tumor-specificity. HLA clustering has mainly been studied within the context of HLA class II on antigen presenting cells, which does not necessarily depend on the presence of a T-cell (40). Clustering of HLA has been reported to be essential for the recognition of HLA-B*27 recognition through leukocyte immunoglobulin-like receptors B2 and killer cell immunoglobulin-like receptors 3DL2 (41, 42). HLA behavior on tumor

cells has mainly been studied within the context of tumor immune escape by loss or downregulation of HLA class I expression (43). The here suggested preferential monomeric form of HLA at the cell membrane of tumor cells could be part of classical tumor escape mechanisms for αβT-cells, which can however be sensed by the here described allo-reactive γδTCR.

A major concern for using the allo-reactive γδTCR-FE11 in a therapeutic scenario would be that, through the nature of promiscuous peptides as part of the recognition complex, safety is difficult to assess. To partially address this concern, we performed tumor control experiments in HLA-A*24:02 transgenic mice. In histological analyses we could not observe pathological evidence for auto-immunity in the investigated organs. Our observation is also supported by the clinical observation that αβT-cell depleted haplo-transplantations associate with very good tumor control and limited toxicity (44-46). The new wave of haplo-transplantations (47) most likely benefits from allo-tumor reactive γδT-cell immune repertories as described here.

In summary, we report on the very interesting nature of allo-reactivity of a γδTCR which is able to distinguish between healthy and malignant cells. This observation emphasizes that γδT-cells, within the context of haplo-transplantation, not only possess classical anti-tumor reactivity but also benefit from allo-tumor reactivity (20). In addition, such γδTCR, within the context of TCR gene therapies, can be an interesting addition to tumors expressing HLA-A*24:02.

Conflict of Interest Statement

GK, DB, ZS and JK are inventors on different patents with γδTCR sequences, recognition mechanisms and isolation strategies. GK is employed by Kiadis Pharma (www.kiadis.com). JK is scientific advisor and shareholder of Gadeta (www.gadeta.nl). No potential conflicts of interest were disclosed by the other authors.

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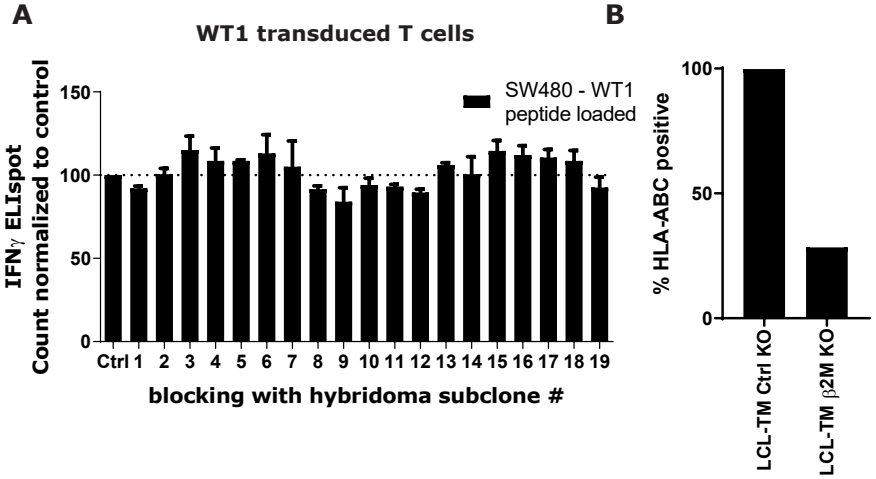
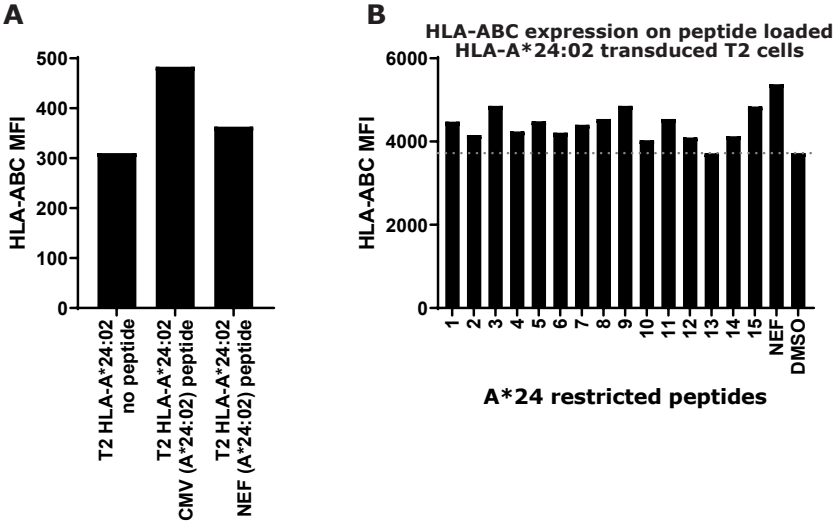
Supplementary figures

Supplementary Table 1. Immunoprecipitation with FE11- like antibodies 6 & 12 indicates that classical HLA molecules are part of the ligand. Antibodies produced by hybridoma 6 and hybridoma 12 were used for immunoprecipitation and subsequent mass spectrometry. Hits with a probability score of 1.0 are displayed.

Sample	Antibody	Accession ID	Gene
LCL-TM	12	P30498	HLA-B
LCL-TM	12	Q29963	HLA-C
LCL-TM	12	P04439	HLA-A
LCL-TM	12	P30508	HLA-C
LCL-TM	12	Q95604	HLA-C
LCL-TM	12	P30499	HLA-C
LCL-TM	12	P05534	HLA-A
LCL-TM	12	P18463	HLA-B
LCL-TM	12	P04222	HLA-C
LCL-TM	12	P01892	HLA-A
LCL-TM	12	P61769	B2M
LCL-TM	12	P30511	HLA-F
LCL-TM	6	P05534	HLA-A
LCL-TM	6	P01892	HLA-A
LCL-TM	6	P04439	HLA-A
LCL-TM	6	Q95604	HLA-C
LCL-TM	6	P30508	HLA-C
LCL-TM	6	Q29963	HLA-C
SW480	12	P05534	HLA-A
SW480	12	P01892	HLA-A
SW480	12	P10321	HLA-C
SW480	12	P01889	HLA-B
SW480	12	P18464	HLA-B
SW480	12	Q95604	HLA-C
SW480	12	P04222	HLA-C
SW480	12	P17693	HLA-G
SW480	12	P30511	HLA-F
SW480	12	P16403	HIST1H1C
SW480	12	P10412	HIST1H1E
SW480	6	P05534	HLA-A



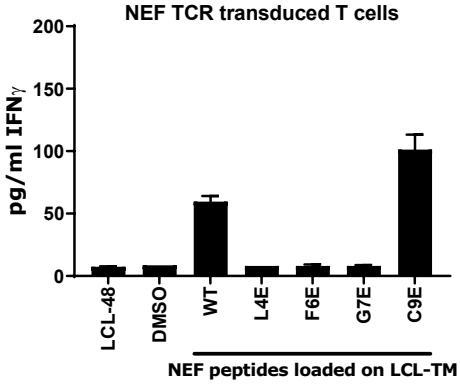
Supplementary Figure 1. (A) The effect of blocking with FE-11 like hybridoma supernatant on the recognition of SW480 loaded with WT1 peptide by αβTCR WT1 transduced T cells. **(B)** LABScreen Single Antigen HLA class I beads were incubated with antibodies purified from hybridoma 6 (mAb 6) or antibodies purified from hybridoma 12 (mAb 12) and secondary α-mIgG-PE and measured using Luminex. Error bars represent SD.



Supplementary Figure 2. (A) HLA-A*24:02 transduced T2 cells were loaded with CMV or NEF HLA-A*24:02 restricted peptides to assess stabilization of HLA on the surface of the T2 cells, as measured by flow cytometry. **(B)** HLA-A*24:02 transduced T2 cells were loaded with 15 HLA-A*24:02 restricted, transformation associated peptides after which stabilization of HLA on the surface of the T2 cells was measured by flow cytometry.

Supplementary Table 2. Transformation-associated peptides

#	AA	Name	Reference
1	DYCNVLNKEF	BRLF1(EBV)	(8)
2	DYNFVKQLF	BMLF1(EBV)	(9)
3	FYTVIPHNF	PARP3 poly (ADP-ribose) polymerase family, member 3	(10)
4	IYNGKLFDL	KIF2C kinesin family member 2C	(10)
5	KFAEEFYSF	CDCA7L cell division cycle associated 7-like	(10)
6	KYPLNLYLL	TMBIM4 transmembrane BAX inhibitor motif containing 4	(10)
7	LYELHVFTF	CTDP1 CTD phosphatase, subunit 1	(10)
8	NYGIYKQDL	HSP105	(11)
9	RYQLDPKFI	EpCAM	(12)
10	RYSIFFDYM	EBNA3A (EBV)	(9)
11	TYGPVFMCL	LMP2 (EBV)	(13)
12	TYPVLEEMF	BRLF1(EBV)	(9)
13	TYSAGIVQI	EBNA3B (EBV)	(14)
14	VFTLKPLEF	HLA-DMA major histocompatibility complex, class II	(10)
15	VYKENLVDGF	NELFE negative elongation factor complex member E	(10)



Supplementary Figure 3. LCL-TM cells were loaded with 10 μM of the WT peptide and the glutamic acid (E)-modified peptides. These loaded cells were coincubated with NEF-TCR transduced T-cells after which activation was assessed by measuring IFNγ production.

Supplementary methods

Cells lines

CEPH EBV-LCL lines (CEU population panel) were a kind gift from Tuna Mutis (VU University Medical Center, Amsterdam, The Netherlands) or ordered from the Coriell Biorepository (Camden, New Jersey, USA). Daudi, K562 (WT), T2, SW480, HEK293, and Phoenix-Ampho cell lines were obtained from ATCC. HEK293FT was obtained from Thermo Fisher Scientific (Breda, The Netherlands). K562 and COS-7 (African green monkey kidney fibroblast-like) transduced with HLA-A*02:01 or HLA-A*24:02 were kindly provided by Fred Falkenburg (Leiden University Medical Centre, the Netherlands). The TCRβ-/- Jurma cell line (a derivate of Jurkat J.RT3-T3.5 cells cells (1), was kindly provided by Hooijberg (VU Medical Center, Amsterdam, The Netherlands), OPM2-Luciferase (OPM2-Luc) was kindly provided by Anton Martens (University Medical Center Utrecht, Utrecht, the Netherlands). LCL-TM (an EBV-LCL line separate from the CEPH panel) was kindly provided by Phil Greenberg (Fred Hutchinson Cancer Research Center, Seattle, U.S.A.). All cell lines were authenticated by short tandem repeat profiling/karyotyping/isoenzyme analysis and were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. All cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using MycoAlert Mycoplasma Kit (Lonza, Breda, The Netherlands). HEK293, Phoenix-Ampho, SW480, and COS-7 cells were cultured in DMEM supplemented with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco, Alkmaar, The Netherlands). All other cell lines were cultured in RPMI with 1% Pen/Strep and 10% FCS. Primary fresh PBMCs were isolated by Ficoll-Paque (GE Healthcare, Eindhoven, The Netherlands) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, The Netherlands).

Functional T cell assays

IFNγ ELISA and ELISPOT were performed as previously described (2, 3). Briefly; 15,000 FE11 TCR-transduced or mock-transduced T-cells and 50,000 target-cells (ratio 0.3:1) were cocultured for 18 hours in nitrocellulose-bottomed 96-well plates (Millipore) precoated with α-IFNγ antibody (clone 1-D1K) (Mabtech). Plates were washed and incubated with a second biotinylated anti-IFNγ antibody (clone 7-B6-1) (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were visualized with TMB substrate (Sanquin) and the number of spots was quantified using ELISPOT Analysis Software (Aelvis). Alternatively, TEG011 and target-

cells were cocultured as above in round-bottom 96-well plates, and IFNγ levels in supernatants were measured by ELISA. For testing stimulation of WT1 αβTCR-transduced T-cells, the HLA-A*02-positive target cells were pulsed with 10 μM $WT1_{126-134}$ (RMFPNAPYL) peptide. For testing stimulation of TEG011, HLA-A*24:02-positive target cells were pulsed with 10 μM $CMV_{341-349}$ (pp65, QYDPVAALF), NEF_{134-10} (RYPLTFGWCF), NEF_{134-10} peptide mutants, or transformation associated peptides as indicated in the supplementary figures.

Flow cytometry

Antibodies used for flow cytometry included: γδTCR-PE (clone IMMU510, Beckman Coulter), CD4-PE-Cy7 (clone RPA-T4, BD), CD8α-APC (clone RPA-T8, BD), CD8α-PerCP-Cy5.5 (clone RPA-T8, Biolegend), CD8α-FITC (clone G42-8, BD), and CD8αβ-PE (clone 2ST8.5H7, BD). NY-ESO1 (HLA-A*02:01 SLLMWITQV) R-PE labelled Pro5 MHC Pentamer (ProImmune, Oxford, United Kingdom) and CMV (HLA-A*24:02 QYDPVAALF) R-PE labelled Pro5 MHC Pentamer (ProImmune) were used according to the manufacturer's instructions. Samples were measured with FACSCanto II and LSRFortessa cytometers (BD) and analyzed with FACSDiva software (BD).

Retroviral transduction of TCRs

The Vγ5Vδ1TCR FE11, an HLA-A*0201 restricted $WT1_{126-134}$-specific αβTCR (4) and an HLA-A*24:02 restricted NEF_{134-10}-specific αβTCR were transduced into αβT cells as described (2, 5). In brief, Phoenix-Ampho packaging cells were transfected with gag-pol (pHIT60), env (pCOLT-GALV) and pBullet retroviral constructs containing TCRγ/β-chain-IRES-neomycine or TCRδ/α-chain-IRES-puromycin, using Fugene-HD (Promega, Leiden, The Netherlands). PBMCs preactivated with α-CD3 (30 ng/ml) (clone OKT3, Miltenyi Biotec) and IL-2 (50 U/ml) were transduced twice with viral supernatant within 48 hours in the presence of 50 U/ml IL-2 and 4μg/ml polybrene (Sigma-Aldrich). Transduced T cells were expanded by stimulation with α-CD3/CD28 Dynabeads (0.5x106 beads/106 cells) (Invitrogen) and IL-2 (50 U/ml) and selected with 800 μg/ml geneticin (Thermo Fisher Scientific) and 5 μg/ml puromycin (Sigma-Aldrich) for one week. CD4+ TCR-transduced T cells were isolated by MACS-sorting using CD4-microbeads (Miltenyi Biotec). Following transduction, transduced T cells were stimulated biweekly according to the REP protocol. Where indicated, CD4+, CD8+, CD4+CD8αα+, CD4+CD8αβ+ and CD8α' (truncated (6)) TCR-transduced T-cells were sorted using a FACSAria II

(BD) flow cytometry to >99% purity. Following selection, TCR-transduced T-cells were stimulated biweekly using the REP protocol. Transgenic TCR expression was routinely assessed by flow cytometry.

Generation of FE11-like mAbs

FE11-like mAbs were generated by immunization of C57BL/6 mice with SW480 and LCL-TM after which standard fusion of spleen cells was performed to generate hybridomas. Monoclonality was achieved by cloning by limiting dilution twice after which isotype determination was determined by flow cytometry using α-mIgG1 APC (Thermo Fisher Scientific), α-mIgG2b RPE (Jackson ImmunoResearch), α-mIgG2c dylight 405 (Jackson ImmunoResearch), and α-m IgG3 PerCP (Jackson ImmunoResearch). For mAb production, hybridomas were cultured 5-8x10^5 cells/ml for 1 week in serum-free hybridoma medium. mAbs were purified using protein G HP SpinTrap columns (GE healthcare) following the manufacturer's instructions.

Protein separation and digestion

Samples were run on a 4-12% Bis-Tris 1D SDS-PAGE gel (BioRad) for 2.5h and stained with colloidal coomassie dye G-250 (Gel Code Blue Stain Reagent, Thermo Scientific). The lane was cut as three bands, which were treated with 6.5 mM dithiothreitol (DTT) for 1 hour at 60°C for reduction and 54 mM iodoacetamide for 30 minutes for alkylation. The proteins were digested overnight with trypsin (Promega) at 37°C. The peptides were extracted with 100% acetonitrile and dried in a vacuum concentrator.

Mass spectrometry: RP-nanoLC-MS/MS

Samples were reconstituted in 10% formic acid and analyzed by nano-LC-MS/MS on a Orbitrap Q-Exactive Plus (Thermo Fisher Scientific) coupled to an Agilent 1290 Infinity System (Agilent Technologies, Middelburg, The Netherlands) operating in reverse phase (C18) equipped with a Reprosil pur C18 (Dr. Maisch, Ammerbuch-Entringen, Germany) trap column (100 µm x 2 cm, 3 µm) and a Poroshell 120 EC C18 (Agilent Technologies) analytical column (75 µm x 50 cm, 2.7 µm). After trapping with 100% solvent A (0.1% FA in H2O) for 10 min, peptides were eluted with an step gradient consisting of 35 min from 13% to 40% and, 3 min from 40% to 100% solvent B (0.1% FA, 80 % ACN). The Q-Exactive Plus was operated in data-dependent acquisition mode using the following settings: full-scan automatic gain control (AGC) target 3e6 at 35 000 resolution; scan range 375–1600 m/z; Orbitrap full-scan maximum injection time 10 ms; MS2 scan AGC target 5e4 at 17 500 resolution; maximum injection 120 ms;

normalized collision energy 25; dynamic exclusion time 10s; isolation window 1.5 m/z; 10 MS2 scans per full scan.

Mass spectrometry data analysis

Raw files were processed using Proteome Discoverer 1.4 (version 1.4.1.14, Thermo Fisher Scientific). Raw files of the 3 bands per sample were combined in one search against a Uniprot database (Homo Sapiens, April 2015). The following parameters were used: carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as enzyme and up to two miss cleavages were allowed. A false discovery rate of 0.01 was used. Datasets processed by Proteome Discoverer were submitted to the Contaminant Repository for Affinity Purification (CRAPome) and proteins identified were sorted by Significance Analysis of INTeractome (SAINT) score, and the fold change scores FC-A or FC-B. The controls used were taken from the control immunoprecipitations performed with unspecific antibodies in each cell line. Proteins with SAINT probability greater than 0.9 were considered high-scoring interactions (7).

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Title:
(EN) HUMAN LEUKOCYTE ANTIGEN RESTRICTED GAMMA DELTA T CELL RECEPTORS AND METHODS OF USE THEREOF

(FR) RÉCEPTEURS DE LYMPHOCYTES T GAMMA DELTA RESTREINTS À L'ANTIGÈNE LEUCOCYTAIRE HUMAIN ET LEURS MÉTHODES D'UTILISATION

Abstract:
(EN) Disclosed herein are compositions and methods of treating a subject with cancer. The compositions and methods utilize immunoresponsive cells to effect killing of tumor cells.

(FR) L'invention concerne des compositions et des méthodes de traitement d'un sujet atteint d'un cancer. Les compositions et les méthodes font appel à des cellules immunoréactives pour tuer les cellules tumorales.

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Orchestrating an immune response against cancer with engineered immune cells expressing αβTCRs, CARs, and innate immune receptors: an immunological and regulatory challenge

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Abstract

Over half a century ago, the first allogeneic stem cell transplantation (allo-SCT) initiated cellular immunotherapy. For several decades, little progress was made, and toxicity of allo-SCT remained a major challenge. However, recent breakthroughs have opened new avenues to further develop this modality and to provide less toxic and equally efficient interventions for patients suffering from hematological or solid malignancies. Current novel cellular immune interventions include ex vivo expansion and adoptive transfer of tumor-infiltrating immune cells or administration of drugs which antagonize tolerizing mechanisms. Alternatively, transfer of immune cells engineered to express defined T cell receptors (TCRs) and chimeric antigen receptors (CARs) has shown its potential. A valuable addition to 'engineered' adaptive immunity has emerged recently through the improved understanding of how innate immune cells can attack cancer cells without substantial side effects. This has enabled the development of transplantation platforms with limited side effects allowing early immune interventions as well as the design of engineered immune cells expressing innate immune receptors. Here, we focus on innate immune interventions and their orchestration with TCR- and CAR- engineered immune cells. In addition, we discuss how the exploitation of the full potential of cellular immune interventions is influenced by regulatory frameworks. Finally, we highlight and discuss substantial differences in the current landscape of clinical trials in Europe as compared to the USA. The aim is to stimulate international efforts to support regulatory authorities and funding agencies, especially in Europe, to create an environment that will endorse the development of engineered immune cells for the benefit of patients.

Introduction

With the first allogeneic stem cell transplantation (allo- SCT) more than 50 years ago, a new era of therapeutic intervention was born, namely cellular immunotherapy. In particular, donor lymphocyte infusions (DLI) provided early and important insights into the potency and mode of action of immunotherapy, as it has the potential to induce sustainable remissions even in patients with advanced hematological malignancies. Also, solid malignancies can be targeted by T lymphocytes, both from an allogeneic stem cell source (1) and by endogenously derived tumor- infiltrating lymphocytes (TIL) (2). Analyzing failure and success of immunotherapies in hematological malignancies, and in solid cancers, frequently elucidated the same requirements for an efficient therapy, i.e., the immune system seems to be most effective when mounting a complex immune response against a defined intruder. Yet, limiting immune interventions to one or two antigens in therapeutic interventions may increase the likelihood of tumor escape. This might explain the success of allo-SCT and TILs but also the recent clinical success of antibodies designed to act on inhibiting regulatory components of the immune system such as anti-PD1 or anti-CTLA4 (3, 4). However, as 'releasing the brake' from all T cells does not only affect tumor-specific immune responses, unwanted 'off-target' reactivity is frequently observed, like graft-versus-host disease (GVHD) or immune-related side effects (4). Consequently, ideal future designs of immune therapeutic interventions should broaden tumor antigen-specific immune responses, but without substantial toxicity.

A recent and promising intervention is the controlled enlargement of the immune repertoire by transferring tumor specificity. This transfer is accomplished by redirecting T cells with a receptor-recognizing defined antigens on a cancer cell (5). Receptors explored to date have been either isolated from cancer reactive αβT cells (6) or engineered by fusing tumor-reactive antibodies with signaling domains of T cells, so-called chimeric antigen receptor (CAR) (7). Landmark clinical trials with an TCR specific for MART-1 melanocyte differentiation antigen (8) or an anti-CD19 CAR (9) have shown the great potential of this approach, leading to an impressive number of ongoing clinical trials (Table 1 and Supplementary Table 1 insert 'in ESM'). However, the number of antigens that can be safely targeted in patients is—at least at this stage—still relatively limited. In this view, the transfer of an alternative set of immune cells and receptors will be discussed. This includes the prospects of 'low-GVHD' transplantation protocols, based on the preservation of innate immune

cells early after transplantation, which can serve as platform for additional immune interventions, as well as the transfer of immune cells designed to express highly selected (innate) immune receptors originated from the innate immune system such as NKG2D or γδTCRs (reviewed in detail (10)). These innate receptors are a less utilized type of immune receptors but possess some appealing and unique advantages as compared to TCRs and CARs. Finally, the prospects and limitations of broadening the application of this exciting and potent therapeutic strategy are discussed.

Table 1. Ongoing clinical trials with TCR- or CAR-modified T cells.

	Targeted antigens	Stem cell source	Number of trials in USA	Number of trials in EU
TCR based (n=13)	NY-ESO-1 (n=6); MAGE-A3 (n=2); WT-1 (n=2); MART-1 (n=1); miscellaneous (n=2)	Autologus (12); unknown (1)	12	1
CAR based (n=52)	CD19 (n=27); GD2 (n=4); mesothelin HER2 (n=3); miscellaneous (n=14)	Autologus (49); allogenic (4)	47	5

Innate allo-SCT as a novel immune platform for early immune interventions

Allo-SCT substantially increases the overall survival for many patients with high-risk hematological diseases. Nevertheless, the outcome for most patients is still poor, due to the high risk of developing severe life-threatening GVHD, encountering relapse, or substantial long-term toxicity with a reduced quality of life. Adoptive transfer of genetically modified T cells with a tumor-specific TCR is therefore an attractive strategy to skew the T cell compartment toward a more defined anti-tumor repertoire post-allo-SCT (11). As such, there is a need for less toxic transplantation regimens which have a substantially reduced incidence of GVHD, do not require long-term immune suppression, and allow for early additional immune interventions. This may be achieved by separating the initial engraftment of stem cells from the application of immune cells via partial or complete removal of immune cells from the allograft prior to transplantation (Figure 1). These transplantation strategies, with either a delayed endogenous T cell reconstitution or a T cell add back via DLI, have resulted in a decreased transplantation-related mortality (TRM) due to a lower incidence of GVHD, as compared to T cell replete allo-SCT (12). This is a consequence of separation of the inflammation mediated by the required conditioning from the infusion of αβT cells. For instance, in a recent prospective and randomized phase III clinical trial, Pasquine

et al. (13) have demonstrated that complete elimination of immune cells by enrichment of the CD34+ cells lowers long-term morbidity as a result of a substantially reduced chronic GVHD, without negatively impacting relapse rates in patients with acute myeloid leukemia (AML). Selective depletion of αβT cells has been suggested as an alternative approach (14). This strategy maintains NK cells and γδ T cells in the graft, which have an intrinsic activity against tumors and infections, without detrimental reactivity toward healthy tissue (10). As this regimen favors the early reconstitution of the innate immune system, such a strategy should theoretically result in an improved control of the tumor and infections. The feasibility of such an approach has been shown most recently by Bettiana et al. (15), in which 23 children with non-malignant disorders received a HLA-haploidentical hematopoietic stem cell transplantation (haplo-HSCT) after ex vivo elimination of T cells and CD19+ B cells. In this cohort, none of the patients developed a GVHD grade III, and the cumulative incidence of TRM was 9.3 %. However, the impact on malignancies could not be assessed, as only those patients with benign disorders received

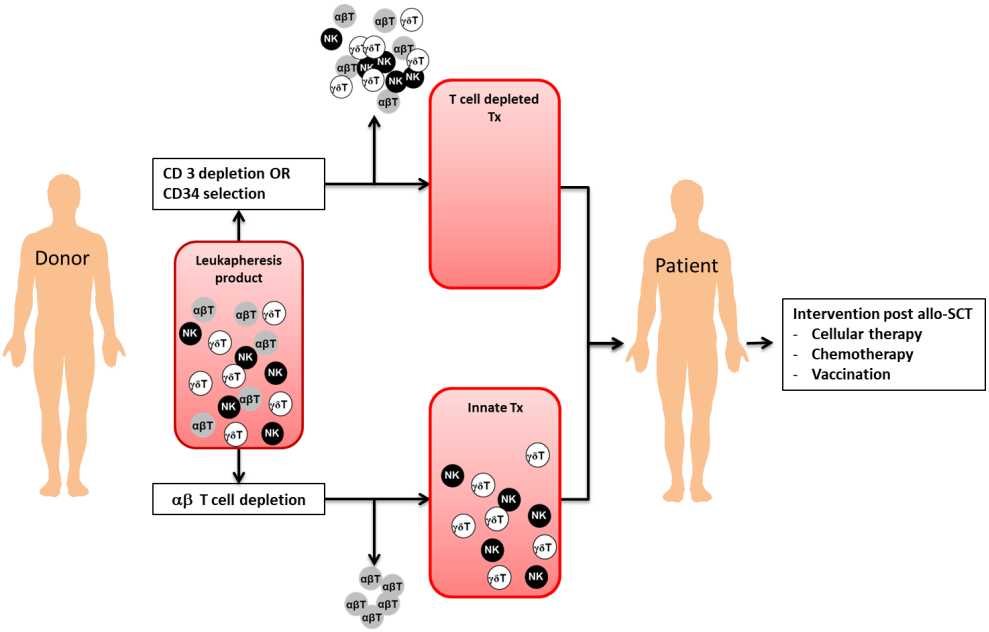

Figure 1. Low GVHD allo-SCT platforms can be achieved by complete removal of the T cell repertoire via CD34+ selection (13) (*upper part*). Alternatively, a so-called innate allo-SCT can be generated by specific removal of T cells from the graft, rendering γδT cells and NK cells within the leukapheresis product (*lower part*) (14, 15).

transplantations. Currently, T cell depletion (Figure 1) is evaluated both in the setting of haplo-SCT and in the MRD/MUD in patients suffering from hematological malignancies. A potential drawback of these strategies is that innate immune system reconstitution early after transplantation is very diverse and does not necessarily contain all the components required to control tumors and infections (for review see (16)). In addition, even with an optimally selected donor, the innate immune system quickly becomes accustomed to its new host environment (education), resulting most likely in a loss of efficacy of the innate donor immune system after a couple of months post-allo-SCT (17). This creates the need for additional immune interventions that do not increase GVHD after an innate allo-SCT, in particular within the context of poor risk hematological malignancies, for which the race against relapse is difficult to win, and GVHD remains a substantial threat.

Moving from DLI to genetically engineered T cells: aiming for a diverse repertoire with multiple and complementary defined receptors

To date, the most potent immune intervention after allo- SCT is a DLI. DLI has already for some decades been appreciated as a curative treatment in relapsing disease after allo-SCT, especially in patients with chronic myeloid leukemia, and to a lesser extend for AML (18, 19). Also for hematological diseases, originally thought to be less sensitive to DLI—like acute lymphoblastic leukemia (ALL) and multiple myeloma (MM)—some recent reports have shown a beneficial effect of DLI (20, 21). Effects are usually observed 4–6 weeks post-application, after doses administered with a range between $1x10^5$ and $1x10^7$ T cells per kg. However, a DLI does not always provide tumor control, and DLI can be associated with substantial GVHD. Again the unpredictable diversity in the repertoire of a DLI is the major hurdle, given that the dose of the tumor-reactive T cells within the DLI is most likely just a fraction of the total T cell pool, as the frequency of allo- reactive T cells is reported to be between 1–10% (16). In order to further increase efficacy of a DLI while reducing toxicity, limitation of the diversity of transferred cells is needed. Most likely, relatively low doses will be sufficient, given they have the correct specificity and are available within a defined immunological subtype which can expand, contract, and provide long-term memory. In addition, some variety must be preserved to allow a diverse repertoire to tackle cancer cells at different targets and to prevent tumor escape mechanisms, such as antigen loss. This goal can potentially be accomplished by taking advantage of T cells

genetically engineered to express a single or a variety of diverse tumor-specific immune receptors (Figure 2a). Already over a decade ago, it has been demonstrated in animal models that the specificity of a T cell can be transferred between T cells by introduction of and TCR genes (22). These and other observations have been translated to the first series of clinical trials with TCR- transduced T cells with different target antigens for solid malignancies (23). Tumor-reactive TCRs were classically either isolated from TILs, from peripheral blood of patients responding to immune therapy (MAGE vaccination studies for instance), or from mouse origin. As a consequence, isolated anti-tumor TCRs are restricted toward a limited pool of HLA molecules. To further extend this method to have broader application, it may be technically feasible to generate cellular products harboring multiple tumor- specific immune receptors extracted from a given patient. With innovative techniques, in which the cancer exome is analyzed in a high throughput fashion (24), it is now possible to identify tumor-specific T cells directed against unique tumor antigens in individual patients (25) and as such fully exploit the cancer 'antigenome' and overcome HLA barriers. In addition to neo-antigen TCR transfer, novel treatment concepts may arise from identifying highly abundant TCR pairs from TILs, as they seem to be enriched for tumor mutation-specific antigens (unpublished data presented by S. A. Rosenberg at AACR 2014 in San Diego). Although transfer of neo-antigen-specific TCRs and TCR gene capture may bear a huge potential, such a personalized treatment concept will face major medical, regulatory, logistical, and financial challenges, as it creates the need to individualize genetic engineering to multiple (known and unknown) targets varying for every given patient. CARs—which can be applied irrespective of HLA type—seem a highly attractive alternative for clinical implementation by pharmaceutical companies. The first clinical studies with a CD19-specific CAR have shown very promising results in ALL (26) and CLL (9) and led to an impressive amount of clinical trials (Table 1 and reviewed in (27)). The results of these studies will provide valuable information which is likely to contribute to the improvement in cellular therapy. However, the number of antigens for which antigen-specific receptors are tested in current clinical trials is frequently redundant and thus very limited (as shown in Table 1). To expand cellular therapy to a broader range of tumors or to enlarge the TCR- or CAR- redirected T cell repertoire, alternative targets and receptors are needed. Receptors of the innate immune system might provide an interesting alternative (Figure 2) (10, 16).

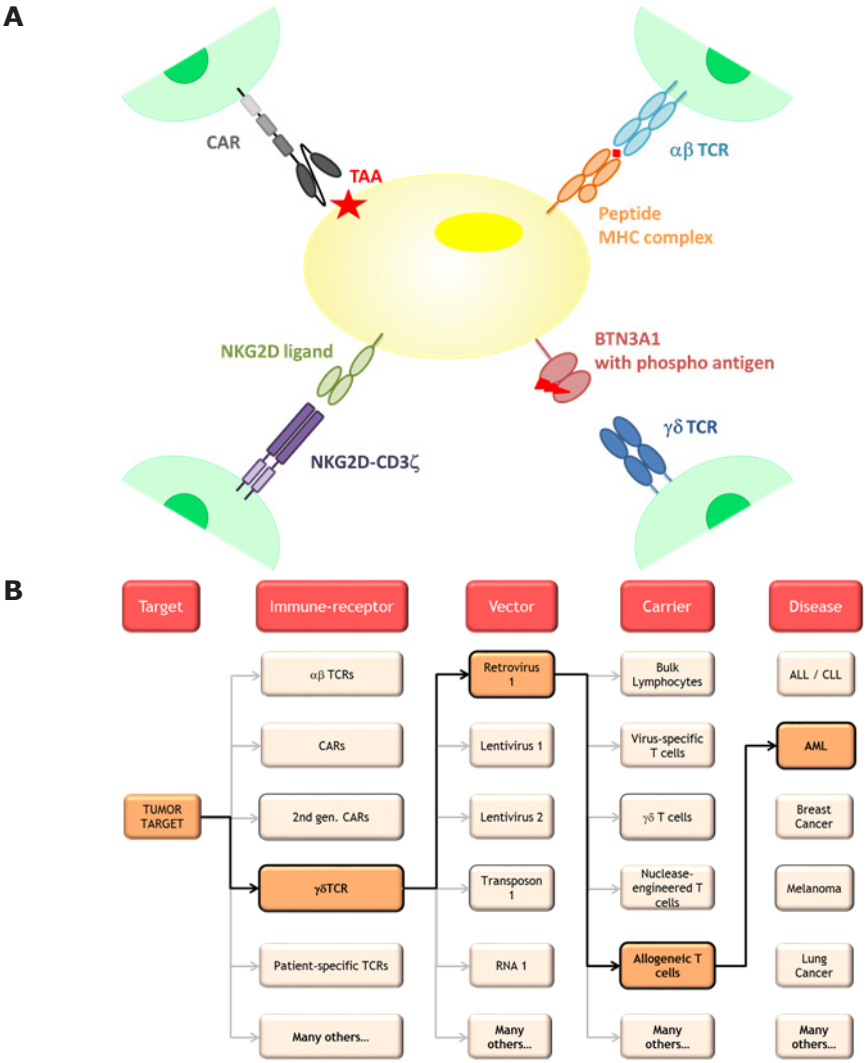

Figure 2. (A) Utilization of T cell receptors, T cell receptors, CARs, and NK cell receptors to transfer desired immune specificities to donor T cells. *TAA* tumor associated antigen. **(B)** Toolbox of immune receptors, vectors for gene transfer, and carrier cells that can be combined with each other to treat different malignancies.

Innate immune receptors with unique features leading to comprehensive tumor recognition

Natural killer (NK) cells are the most widely studied subset of innate immune cells in the context of anti-tumor responses. NK cells express an array of activating and inhibitory receptors, which collectively discriminate healthy cells from diseased cells by sensing self-'stress'

molecules on diseased target cells, including tumor cells (28). NKG2D is the best known of these receptors. NKG2D recognizes stress-induced self-MHC class I-related proteins, which have a selectively increased surface expression on transformed cells from both hematological and solid origins (29). To harness NK cell-mediated toxicity, chimeric receptors linking NKG2D to the cytoplasmic domain of CD3ζ have been constructed, and T cells equipped with such an NKG2D receptor display anti-tumor reactivity in both hematological and solid tumor models (30). Also, bi-specific antibodies of a NK cell receptor fused to a single-chain fragment have shown tumor reactivity in various tumor models (31). Inhibitory receptors impede NK cell reactivity by sensing the presence of MHC class I molecules constitutively expressed on almost all healthy cells. Killer cell immunoglobulin-like receptors (KIRs) are a well-studied example of such inhibitory molecules. For example, it has been reported that NK cells can kill allogeneic cells when their inhibitory KIRs are not engaged due to mismatched HLA alleles (32). Two recent phase I studies in AML (33) and MM (34) have shown that an antiKIR antibody can be safely administered to patients, and as such, full KIR saturation can be achieved, supporting subsequent trials to test for clinical activity.

Following NK receptors, the γδTCR has recently drawn attention as an alternative anti-tumor immune receptor with some unique appealing features (reviewed in (10)). γδ T cells express a somatically recombined γδ TCR, but behave like innate cells in a way that they—like NK cells–become activated by 'stressed cells,' The γδTCR is just one of the multiple proteins on the surface of a γδ T cell which can sense molecular stress signatures. A significant subset of γδ T cells express a TCR composed of V9 and V2 chains, which can recognize multiple targets on malignant cells, such as the complex of apolipoprotein A1 (ApoA1) and F1-ATPase. In addition, they can sense accumulated non-peptidic pyrophosphate molecules (phosphoantigens), intermediates of a deregulated mevalonate pathway of isoprenoid synthesis, via BTN3A1 (CD277). As such, γ9δ2T cells can mount immune responses against tumor cells derived from both hematological and solid malignancies (35, 36). Unfortunately, translating these in vitro observations into effective clinical protocols remains challenging, since—despite substantial evidence in vivo in mice (37)— adoptively transferred autologous γδ T cells demonstrate anti-tumor reactivity only at modest and variable response rates (16). The moderate success of these responses seems to be critically determined by the composition of the γδ T cell repertoire. Diversity in the γδTCR as

well as the coreceptor repertoire leads to a diverse function and activation status of an individual γδ T cell. This makes 'the γδ T cell repertoire' a very heterogeneous population with anti-tumor activity that is difficult to predict. For instance, in vitro analysis of individual γ9δ2T cell clones revealed a highly differential anti-tumor reactivity (38), which did not appear to be explained by the different repertoire of inhibitory and activating receptors on individual cells (39), but by the small variations in the CDR3 region of the γ9δ2 TCR (38).

Therapeutic concepts with engineered 'innate receptors' fall in two categories: single proteins and membrane-bound receptors, and both may complement other types of cellular therapies. For instance, NKG2D fused to anti-CD3 variable fragment (scFv-NKG2D) has been shown to engage tumor cells with T cells (34). Also antibodies directed against KIRs (see above) and soluble MHC class I-related pro tein A (sMICA; a ligand for NKG2D which in its soluble form is associated with NK inactivation) have been demonstrated to stimulate T cell-specific responses (40). Concomitant administration of such proteins, with DLI or an engineered cellular product, may very well result in synergistic responses. The group of membrane-bound engineered innate receptors can either consist of (optimized) wild-type protein (38), fusion proteins (CARs) or single-chain receptors. Transfer of such receptors into T cells may complement attractive features of both the innate and adaptive immune system. T cells are easy to collect and are fully equipped to proliferate and activate upon antigen recognition. The introduced innate receptor is not MCH-restricted. In addition, the formation of mixed dimers does not occur. As such, a cellular product can be engineered containing sufficient numbers of effector cells with desired and uniform specificity.

For example, our group is preparing a phase I trial for γδTCR gene transfer. To 'pick the most effective γδTCRs' for future clinical applications, we have developed the 'combinatorial γδTCR chain exchange.' This allows for selection of the γ9δ2TCRs with the highest affinity (38). Like their counterparts, γδTCR genes can be retrovirally transduced into both CD4+ and CD8+ T cells. These γδTCR-engineered T cells can recognize a broad panel of tumor cell lines both in vitro and in vivo as well as a variety of primary AML blasts, but they ignore non-transformed cells (41). Interestingly, introduction of the γδTCR leads to down-regulation of the endogenous TCR, likely due to competition for components of the CD3 complex. This competition can be used to negatively select non-engineered cells with TCR bright cells from a transduced T cell bulk, resulting in an

end product containing almost 100 % γδTCR-engineered T cells with increased anti-tumor function *in vitro* as well as *in vivo* (Straetemans et al, unpublished data). These results have led to the design of the first clinical trial in which γδ TCR-engineered T cells will be tested in patients with AML, who will receive an T cell- depleted stem cell transplantation from a MRD/MUD (Figure 1) followed by an infusion of γδ TCR-engineered T cells 3–6 months post-allo-SCT.

A space of endless choices: How to develop the best cellular immunotherapy?

Further work is needed to establish genetically modified T cells as a widely accepted mode of treatment for hematological malignancies. Preclinical and small phase I studies—in the past mainly initiated by academic institutions, but now increasingly promoted by young and innova tive biotech companies—are essential to broaden existing concepts and to develop new concepts. While the field is moving from allo-SCT to more engineered cellular products that are enriched for anti-tumor activity and depleted of unwanted cross-reactive T cells, many challenges remain for the translation of novel concepts from the lab to the clinical setting.

Engineered cellular therapies constitute a new class of products that on one hand bear a huge potential for benefit (as shown by unprecedented effect sizes in a large fraction of treated patients) but on the other hand bear a risk of serious (even fatal) side effects (42). As outlined above, earlier progress in science and technologies has equipped us with a huge toolbox of immune receptors, vectors for gene transfer, and carrier cells that can now be combined with each other (or with additional non-cellular compounds) in multiple ways and therefore give rise to countless different permutations of products for the treatment of various hematological and solid cancers (Figure 2b).

Altering one component of the toolbox could lead to increased benefit or increased toxicity (or both) for patients. Due to the species specificity of antigen expression, antigen processing, antigen presentation, and immune recognition, the available non-clinical in vivo models are not predictive for the outcome in patients (43). Although non-clinical in vitro studies to predict off-target cross-reactivity have been proposed (44), it is clear that the final answers can only be obtained in clinical trials. A researcher may feel inclined to utilize an empiric approach and iteratively test multiple permutations in a series of small-scale trials, to identify the more toxic products as early failures and to select the best therapies for advanced clinical testing. Given that each permutation of the tool box is considered

as a novel compound, and due to the high regulatory requirements associated with clinical testing of each permutated product, the described "empiric approach" will simply not be feasible. Due to limitations in time and available resources, this approach will only allow testing of one (or very few) permutations in the clinic.

In this light, the existing regulatory framework requires a lively discussion of the field on what might be done to facilitate the access to innovative cellular therapies without increasing the risk for patients. Some investigators have raised the criticism that the field of cellular immunotherapy (in particular therapies that include engineering of lymphocytes with TCRs or CARs) is sometimes held to higher standards as compared to other and more established groups of products. One seemingly well documented example is the requirement to test for replication competent retrovirus (RCR) which sponsors have to do on the master cell banks, retroviral supernatant lots as well as on the actual T cell product (45). FDA guidelines further require follow-up analysis for RCRs to be performed at 3, 6, and 12 months and yearly following treatment. The required RCR testing is labor-intensive, costly, and time consuming, which limits the translation of such innovative approaches. Currently, available data from more than 500 patient-years of clinical implementation did not show any evidence for secondary malignancies due to insertional mutagenesis form retroviral gene transfer to lymphocytes in patients (46). It seems difficult to understand why developers of gene/cell therapies have to meet this high level of testing requirements despite the availability of decade-long safety experience, while chemotherapeutic agents that have a documented rate of induction of secondary malignancies may be used in thousands of patients every day without similar testing requirements. In addition, there is great uncertainty how to best design nonclinical programs to support clinical trial applications. Given the potentially low predictive value of animal models and the availability of an increasing repertoire of in vitro tests to predict potential toxicity in humans (e.g., alanine scans for novel TCRs or use of complex tissue cell cultures), now may be the time to delineate minimum requirements for non-clinical programs that both developers and regulators may agree upon. Such efforts cannot be achieved by single investigators but require field-wide efforts and a consensus process that can only happen in larger networks with broad representation of the different stakeholders.

Challenges are even more complex when considering the novel personalized treatment concepts with patient-specific neo-antigen-

specific TCRs and TCR genes captured from TILs. These strategies can be regarded as personalized therapies (similar to mutanome vaccines) where the specificity of the drug product will change between patients (47). If the same regulatory requirements for these personalized products are applied as for defined products, these new treatment concepts will never become feasible. Thus, a novel regulatory blueprint is needed for such personalized TCR approaches. A first justification for a less stringent handling of such personalized products could arise from the position that (similar to a DLI) no new specificity is added to the immune system. Still, a series of unresolved regulatory questions exists and needs to be solved prior to testing these novel therapies in the clinic. As mentioned earlier, the delineation of applicable principles for such a novel and disruptive type of personalized medicine needs to be accomplished through scientific discussions among the various stakeholders and incorporates a balance between the patient's interest for safe but also timely access to novel treatments. A similar strategy has also been proposed to define a first regulatory blueprint for mutanome vaccines (47).

In summary, there is an urgent need for innovative strategies to reduce the amount of time and resources for bringing a novel cell therapy into the clinic without inappropriately increasing the risk for patients. Such strategies would allow for testing more combinations of novel technologies and realization of their full potential. We propose to enter an open discussion about the generation of flexible regulatory frameworks tailored to the unique properties of immune receptor-engineered cells. The major challenge will be to balance the need for safety within the context of urgent medical need, which requires continuous innovation.

A tenfold difference in clinical trials with genetically engineered T cells, what is causing the gap?

To determine which types of products are currently developed, a literature search was performed for ongoing clinical trials with TCR- or CAR-modified T cells. We identified an encouraging number of 65 studies, of which only a disappointing 10 % were enlisted in the European Union (EU) (Table 1). The remaining 90 % were listed in the USA.

One may only speculate on the reasons for this striking difference. Critical success factors that are often discussed but that never seem to be systematically addressed are large clinical/academic infrastructures, access to funding for innovation not only in the early stage but also for clinical trials, and concentration of talent which typically moves to the most attractive environments. Regarding the first, a variety of lists are

published yearly, ranking the top universities around the world. The bulk of the top 10 is situated in the USA. Many of the currently developed cellular immunotherapies emerged from large institutions such as the NIH, the University of Pennsylvania, or the MD Anderson Cancer center that combine access to patients, scientific infrastructure, medical expertise, manufacturing capacities, and funding opportunities in a way that is difficult to match at most European institutions which are much smaller in size. Comparing (financial) resources between the USA and Europe seems virtually impossible as well. Within Europe, many national and international governmental, private, and commercially sponsored programs exist. As an example, since 1984, the European Commission has launched seven framework programs, which are dedicated to research and innovation. Since 1998, 351.1 million EUR has been donated to programs involving gene therapy projects (48). This year, the eighth framework program Horizon 2020 opened, which is the biggest EU research and innovation program ever, with nearly 80 billion of funding available over 7 years up to 2020. It aims to secure Europe's global competiveness by ensuring that Europe can produce world-class science, remove barriers to innovation, and make it easier for the public and private sectors to work together in delivering innovation. However, as impressive as the numbers might be at a first sight, initiatives like these seem very modest when compared to resources available in the USA, where young and innovative companies such as Juno or Kite can raise several hundred million dollars within a very short time. Another example is the number of family foundations in the USA that has grown from about 3200 in 2001 to more than 40,000 in 2015, with total annual grants for academic research and translation of more than $21.3 billion, according to the Foundation Center. In the USA, federal tax breaks encourage the funding through foundations, which give philanthropists more control over their donations. A third—and perhaps more fundamental—hurdle in initiating clinical trials in Europe with genetically modified cells is the bureaucratic burden imposed by regulation. In 2004, the European Clinical Trials Directive 2001/20/EC (EU-CTD) was introduced in order to protect clinical trial subjects by establishing quality, safety, and ethical criteria of initiated trials. In current practice, this implies that a trial needs to be reviewed in each individual member state by both a research ethic committee and national competent authority. This process turned out to be suboptimal in daily practice. First, one could argue that a scientific and ethical judgment should be integrated in one review body, since the scientific merit of clinical research cannot be judged without an ethical evaluation and vice versa. In addition, leaving the organization of

the review bodies to the individual member states leads to large inter-country differences and subsequent inconsistent evaluations (reviewed in (49)). Consequently, between 2007 and 2011, the number of clinical trials conducted in the EU fell by 25 %, and the number of clinical trials applied for in 2007 (5000) dropped to 3800 by 2011, with most studies being limited to one country. So despite a potential additional protection of subjects, the European Clinical Trial Directive seems to prohibit European citizens from accessing innovative therapies. In the USA, there is a more streamlined approach, with one nationally appointed service, namely the Department of Health and Human Services (DHHS) providing oversight of clinical trials. All investigators must comply with these regulations when conducting clinical gene therapy trials. Although precise numbers are absent, the general feeling among researchers in the field is that the time to approve a gene therapy protocol is shorter in the USA than it is in the EU. This may also be reflected by the already mentioned difference in numbers of clinical trials in the EU as compared to the USA. Also, a trend toward increased clinical trials in areas with emerging economies such as Asia, South America, and Russia is acknowledged by the EU.

To diminish the time-consuming bureaucracy in the EU, a new legislation 'Clinical Trial Regulation EU No 536/2014' was adopted on April 2014 and is scheduled to be implemented in May 2016. The objective is 'to restore European Union's competitiveness in clinical research and the development of new and innovative treatments and medicines for the ultimate benefit of patients.' Indeed, they claim to make the process more transparent and faster, but the notion that a single research protocol still has to be judged both by a scientific committee of a reporting member state and separately by a scientific committee of each individual member state makes one wonder whether this new legislation will truly lead to a reduction 'in red tape' and increase in trials. Obviously, the efficacy of this new legislation has to be evaluated—which will at least take another year—, but the lack of centralization of the process feels like a missed opportunity, not only with regard to the speed of the process, but also as an incentive for European researches to act as a united team.

Regardless of the reasons for the striking differences in the processes for clinical trials initiated in the USA versus Europe, it is clear that researchers who intend to translate their science into novel therapies for patients should still mainly focus on the science around engineered cellular products. Additionally, researchers should also contribute to improving the regulatory, structural and financial cornerstones for these novel treatments.

Concluding remarks

Designing novel concepts of allo-SCT by promoting the early reconstitution of innate immune cells complemented subsequently with 'genetically engineered immunity' by utilizing both, innate and adaptive, receptors has the potential to substantially reduce toxicity and provide a profound short- and long-term protection against cancer. Components of this concept can be utilized not only for hematological but also for solid malignancies and allow engineering of a diverse immune response against cancer. Bearing in mind that the associated treatment-related morbidity and mortality of the current state-of-the-art treatment for many poor risk hematological malignancies are substantial, regulatory, and financial requirements, for the implementation of novel innovative cellular designer drugs seem to be completely out of balance. With a treatment-related mortality of up to 30 %, frequently accepted for many routinely performed allo-SCTs in 2015 worldwide, one might question whether current regulatory and financial hurdles compromise rather than protect the lives of our patients. Thus, controlling cancer needs not only an orchestrated action of immune cells expressing defined CARs, αβTCRs, γδTCRs, and other innate immune receptors, but also a well-balanced discussion about regulatory and financial needs with all involved groups: the academic research community, pharmaceutical companies, authorities, and patients and their families—a major immunological and societal challenge.

Conflict of Interest Statement

Jürgen Kuball receives clinical trial support for the innate transplantation platform by Miltenyi Biotec as well as holds two patents dealing with innate immune receptors and selection procedures of engineered immune receptors. The other authors declare that they have no conflict of interest.

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Supplementary table 1. Current and planned clinical trials with either aβ TCR or CAR based gene therapy studies, both in an autologous as well as in an allogeneic setting within the USA and EU. Search method: Open studies in USA and EU, excluding studies with unknown status. Queries: TCR, CAR, chimeric antigen receptor. Excluding non-oncology conditions and excluding non-TCR/CAR methods. Search date: December 1st 2014.

Sponsor	Country	ID	Phase	Type	Target	Method	Engineering	Start date	End date	Condition	Enrollment
Jonsson Comprehensive Cancer Center	USA	NCT00910650	II	TCR	MART-1	Autologous PBMCs + MART-1.26-35 peptide-pulsed dendritic cell (DC) vaccines	Retroviral vector	OCT09	DEC16	Metastatic Melanoma	22
Jonsson Comprehensive Cancer Center	USA	NCT02070406	I	TCR	NY-ESO-1	Autologous T-cells + ipilimumab + NY-ESO-1157-165 peptide pulsed DC vaccine	Retroviral vector	MAR14	FEB19	Unspecified Adult Solid Tumor	12
Jonsson Comprehensive Cancer Center	USA	NCT01697527	II	TCR	NY-ESO-1	Autologous PBMCs + NY-ESO-1157-165 peptide pulsed DC vaccine	Retroviral vector	NOV12	OCT18	Malignant Neoplasm	22
NCI	USA	NCT02111850	I/II	TCR	MAGE-A3	Autologous $CD4^+$ T-cells	Retroviral vector	JAN14	JAN17	Metastatic Cancer	107
NCI	USA	NCT02153905	I/II	TCR	MAGE-A3	Autologous T-cells	Retroviral vector	MAY14	NOV18	Metastatic Cancer, Metastatic Melanoma	102
NCI	USA	NCT02062359	II	TCR	NY-ESO-1	CD62L+ lymphocytes	Retroviral vector	FEB14	JUN19	Metastatic Cancer, Metastatic Melanoma	22
Abramson Cancer Center of the University of Pennsylvania	USA	NCT02030847	II	CAR	CD19	Autologous T-cells	Lentiviral vector	JAN14	JUL15	B Cell ALL	24
Adaptimmune	USA	NCT01350401	I/II	TCR	NY-ESO-1/ LAGE	Autologous T-cells	Lentiviral vector	MAY11	FEB15	Melanoma	6
Adaptimmune	USA	NCT01892293	I/II	TCR	NY-ESO-1c259	Autologous T-cells	Unknown	SEP13	APR15	MM	10

Sponsor	Country	ID	Phase	Type	Target	Method	Engineering	Start date	End date	Condition	Enrollment
NCI	USA	NCT01967823	II	mTCR	NY-ESO-1	Autologous PBMCs	Retroviral vector	SEP13	MAR15	Metastatic Cancers Other Than Melanoma	43
Adaptimmune	USA	NCT01352286	I/II	TCR	MAGE-A3/6 NY-ESO-1	Autologous T-cells Syngeneic T-cells	Unknown	APR11	APR14	MM	26
Loyola University	USA	NCT01586403	I	TCR	TIL 1383I	Autologous T-cells	Unknown	JUL12	SEP13	Melanoma	15
Abramson Cancer Center of the University of Pennsylvania	USA	NCT02135406	I	CAR	CD19 scFv TCR ζ	Autologous T-cells	Lentiviral vector	MAY14	MAY16	MM	15
Fred Hutchinson Cancer Research Center	USA	NCT01640301	I/II	TCR	WT1	Unknown	Unknown	DEC12	MAR17	myelodysplastic syndrome, AML, CML	55
Abramson Cancer Center of the University of Pennsylvania	USA	NCT01747486	II	CAR	CD19	Autologous T-cells	Unknown	DEC12	DEC15	chronic lymphocytic leukemia, small lymphocytic leukemia	32
Memorial Sloan-Kettering Cancer Center	USA	NCT01430390	I	CAR	CD19	Allogeneic Epstein-Barr Virus Specific Cytotoxic T-Lymphocytes	Unknown	SEP11	SEP15	ALL	26
Abramson Cancer Center of the University of Pennsylvania	USA	NCT02030834	II	CAR	CD19	Autologous T-cells	Unknown	JAN14	JUL15	Non-Hodgkin Lymphoma, Patients with CD19+B Cell Lymphomas	55
Abramson Cancer Center of the University of Pennsylvania	USA	NCT01355965	I	CIR (CAR)	mesothelin	Autologous T-cells	Unknown	MAY11	MAY14	Malignant Pleural Mesothelioma	6
Baylor College of Medicine	USA	NCT01822652	I	CAR	GD2	Autologous T-cells	Retroviral vector	AUG13	AUG15	Neuroblastoma	14
NCI	USA	NCT02107963	I	CAR	GD2	Autologous T-cells	Retroviral vector	FEB14	DEC16	Sarcoma, Osteosarcoma, Rhabdomyosarcoma, Ewing Sarcoma, Melanoma	72

Sponsor	Country	ID	Phase	Type	Target	Method	Engineering	Start date	End date	Condition	Enrollment
Abramson Cancer Center of the University of Pennsylvania	USA	NCT02209376	I	CAR	EGFRvIII	Autologous T-cells	Lentiviral vector	JUL14	JUL16	Glioma	12
NCI	USA	NCT01087294	I	CAR	CD19	Allogeneic T-cells	Retroviral vector	FEB10	OCT15	B-cell Leukemia, Hodgkin Lymphoma, Non-Hodgkin Lymphoma, B-Cell Lymphoma	36
Seattle Children's Hospital	USA	NCT01683279	I	CAR	CD19	Autologous T-cells	Lentiviral vector	DEC12	JAN15	B Cell Leukemia	18
Baylor College of Medicine	USA	NCT00902044	I	CAR	HER2	Autologous T-cells	Unknown	JUL09	JUL15	Sarcoma	36
Celdara Medical, LLC	USA	NCT02203825	I	CAR	NKG2D-Ligands	Autologous T-cells	Unknown	OCT14	OCT15	AML, MM, Advanced Myelodysplastic Syndrome	21
Memorial Sloan-Kettering Cancer Center	USA	NCT01840566	I	CAR	CD19	Autologous T-cells	Unknown	APR13	APR15	Non-Hodgkin Lymphoma	18
Baylor College of Medicine	USA	NCT02050347	I	CAR	CD19	Autologous T-cells Allogeneic T-cells	Retroviral vector	APR14	MAR18	Non-Hodgkin's Lymphoma, B-Cell ALL, B-Cell CLL	56
Baylor College of Medicine	USA	NCT01953900	I	CAR	GD2	Autologous VZV T-cells	Retroviral vector	APR14	APR18	Sarcoma	26
Seattle Children's Hospital	USA	NCT02028455	I/II	CAR	CD19	Autologous T-cells	Lentiviral vector	JAN14	JAN17	CD19+ Leukemia	80
Baylor College of Medicine	USA	NCT01109095	I	CAR	HER2	Autologous CMV T-cells	Unknown	JAN14	JAN17	Glioblastoma Multiforme	18
Baylor College of Medicine	USA	NCT00881920	I	CAR	kappa immuno-globulin	Autologous T-cells	Retroviral vector	JUL09	JUL19	Lymphoma, Myeloma, Leukemia	54
Baylor College of Medicine	USA	NCT01316146	I	CAR	CD30	Autologous T-cells	Unknown	DEC11	DEC14	Non-Hodgkin's Lymphoma, Hodgkin's Lymphoma	18

Sponsor	Country	ID	Phase	Type	Target	Method	Engineering	Start date	End date	Condition	Enrollment
NCI	USA	NCT00924326	I	CAR	CD19	Autologous T-cells	Retroviral vector	FEB09	JAN17	Small Lymphocytic Lymphoma, Mantle Cell Lymphoma, Follicular Lymphoma, Large Cell Lymphoma, CLL	40
Abramson Cancer Center of the University of Pennsylvania	USA	NCT02159716	I	CAR	mesothelin	Autologous T-cells	Lentiviral vector	JUN14	JUN16	Metastatic Pancreatic (Ductal) Adenocarcinoma, Epithelial Ovarian Cancer, Malignant Epithelial Pleural Mesothelioma	24
NCI	USA	NCT01454596	I/II	CAR	EGFRvIII	Autologous T-cells	Retroviral vector	SEP11	SEP19	Malignant Glioma, Glioblastoma, Brain Cancer	160
Fred Hutchinson Cancer Research Center	USA	NCT01865617	I/II	CAR	CD19	Autologous T-cells	Lentiviral vector	MAY13	APR29	Leukemia, Lymphoma	54
City of Hope Medical Center	USA	NCT02159495	I	CAR	CD123	Autologous T-cells	Lentiviral vector	OCT14	OCT17	Adult AML	24
Baylor College of Medicine	USA	NCT01853631	I	CAR	CD19	Autologous T-cells	Retroviral vector	FEB10	FEB17	Non-Hodgkin Lymphoma, CLL	14
Baylor College of Medicine	USA	NCT00889954	I	CAR	HER2	Autologous EBV T-cells	Retroviral vector	MAY09	JUL15	HER2 Positive Malignancies	18
NCI	USA	NCT01593696	I	CAR	CD19	Autologous T-cells	Retroviral vector	APR12	JAN20	ALL, B Cell Lymphoma, Leukemia, Large Cell Lymphoma, Non-Hodgkin Lymphoma	48
Memorial Sloan-Kettering Cancer Center	USA	NCT01860937	I	CAR	CD19	Autologous T-cells	Unknown	MAY13	MAY16	Relapsed B-Cell ALL	24
Children's Mercy Hospital Kansas City	USA	NCT01460901	I	CAR	GD2	Allogeneic T-cells	Retroviral vector	OCT11	OCT13	Neuroblastoma	3
Children's Hospital of Philadelphia	USA	NCT01626495	I	CAR	CD19	Autologous T-cells	Lentiviral vector	AUG11	AUG16	B Cell Leukemia, B Cell Lymphoma	20

Sponsor	Country	ID	Phase	Type	Target	Method	Engineering	Start date	End date	Condition	Enrollment
NCI	USA	NCT02215967	I	CAR	BCMA	Autologous T-cells	Unknown	AUG14	APR18	Myeloma, Plasma-Cell, Myeloma-Multiple	38
City of Hope Medical Center	USA	NCT02208362	I	CAR	CD19	Autologous T-cells	Lentiviral vector	DEC14	DEC18	Glioma	44
Memorial Sloan-Kettering Cancer Center	USA	NCT01044069	I	CAR	CD19	Autologous T-cells	Unknown	JAN10	JAN15	Leukemia, ALL	40
Memorial Sloan-Kettering Cancer Center	USA	NCT00466531	I/II	CAR	CD19	Autologous T-cells	Lentiviral & retroviral vector	MAR07	DEC15	Leukemia	30
Baylor College of Medicine	USA	NCT01192464	I	CAR	CD30	Autologous EBV+ T-cells	Retroviral vector	MAR11	OCT18	Hodgkin's Lymphoma, Non-Hodgkin's Lymphoma	18
M.D. Anderson Cancer Center	USA	NCT02194374	I	CAR	ROR1	Autologous T-cells	Unknown	DEC14	DEC8	Leukemia	48
Baylor College of Medicine	USA	NCT00586391	I	CAR	CD19	Autologous T-cells	Retroviral vector	FEB09	FEB18	B Cell Lymphoma, Chronic Lymphocytic Leukemia	54
City of Hope Medical Center	USA	NCT02051257	I	CAR	CD19	Autologous T-cells	Lentiviral vector	MAY14	JUN21	Leukemia, Lymphoma	24
City of Hope Medical Center	USA	NCT02146924	I	CAR	CD19	Autologous T-cells	Lentiviral vector	JUL14	JUL18	B-cell Adult Acute Lymphoblastic Leukemia, Recurrent Adult Acute Lymphoblastic Leukemia	24
City of Hope Medical Center	USA	NCT02153580	I	CAR	CD19	Autologous T-cells	Lentiviral vector	SEP14	SEP17	Leukemia, Lymphoma	48
Abramson Cancer Center of the University of Pennsylvania	USA	NCT01837602	I	CAR	cMet	Autologous T-cells	Unknown	APR13	APR17	Metastatic Breast Cancer, Triple Negative Breast Cancer	15
National Cancer Institute (NCI)	USA	NCT01218867	I/II	CAR	VEGFR2	Autologous CD8+ T-cells	Retroviral vector	OCT10	OCT18	Metastatic Cancer, Metastatic Melanoma, Renal Cancer, Colorectal Cancer, Ovarian Cancer, Lung Cancer	118

Sponsor	Country	ID	Phase	Type	Target	Method	Engineering	Start date	End date	Condition	Enrollment
Baylor College of Medicine	USA	NCT00840853	I/II	CAR	CD19	CMV, adenovirus, and EBV+ Autologous T-cells	Retroviral vector	APR09	APR16	ALL, CLL, Non-Hodgkin's Lymphoma	36
National Cancer Institute (NCI)	USA	NCT01583686	I/II	CAR	mesothelin	Autologous T-cells	Retroviral vector	MAR12	MAR19	Metastatic Cancer, Pancreatic Cancer, Mesothelioma, Ovarian	136
Abramson Cancer Center of the University of Pennsylvania	USA	NCT01897415	I	CAR	mesothelin	Autologous T-cells	Unknown	JUL13	JAN15	Metastatic Pancreatic Ductal Adenocarcinoma	10
Memorial Sloan-Kettering Cancer Center	USA	NCT01140373	I	CAR	PSMA	Autologous T-cells	Unknown	JUN10	JUN15	Prostate Cancer	18
University College, London	EU	NCT01621724	I/II	TCR	WT1	Autologous T-cells	Retroviral vector	APR12	APR16	AML, CML	18
Uppsala University	EU	NCT02132624	I/II	CAR	CD19	Autologous T-cells	Retroviral vector	APR14	APR17	B Cell Lymphoma, B Cell Leukemia	15
University of Zurich	EU	NCT01722149	I	CAR	FAP	Autologous CD8+ T-cells	Retroviral vector	APR13	OCT14	Malignant Pleural Mesothelioma	6
King's College London	EU	NCT01818323	I	CAR	ErbB1	Autologous CD4+ T-cells	Unknown	JUN13	JUN15	Head and Neck Cancer	30
Professor Robert Hawkins	EU	NCT01493453	I	CIR (CAR)	CD19	Autologous T-cells	Retroviral vector	MAR08	MAY14	Non-Hodgkin Lymphoma	24
University College, London	EU	NCT01195480	I/II	CAR	CD19	Autologous T-cells	Retroviral vector	MAY12	DEC14	ALL	30

Select - kill strategy for untouched αβTCR-gene engineered T cell products

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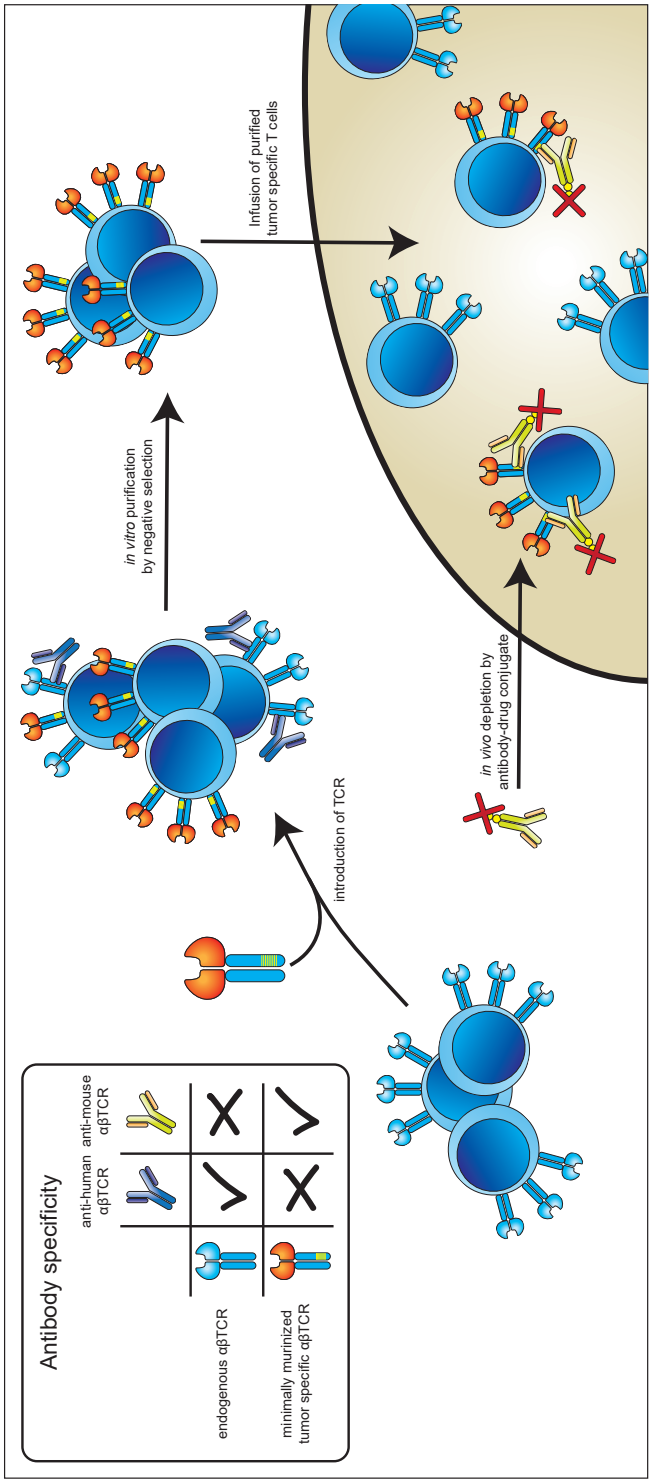
Abstract

T cell engineering strategies, which incorporate a method for the purification of genetically modified T cells, as well as engineered T cell deletion after transfer into patients, are needed to increase efficacy, reduce potential side effects, and improve safety. By characterizing the antigen binding side of a GMP-grade anti-αβTCR antibody, usually used for clinical grade depletion of αβT cells from stem cell transplantation products, we developed a strategy which allows for the purification of untouched αβTCR engineered immune cells by changing only two amino acids in the TCR β chain constant domain of introduced TCR chains. Vice versa, we engineered an antibody, which targets an extended mutated region of nine amino acids in the TCR β chain constant domain, in order to allow for later depletion of engineered immune cells. This strategy can be applied to any T cell engineering strategy that interferes with the endogenous αβTCR chains.

Graphical Abstract



The authors identified the antigen binding site of a GMP-grade anti-human αβTCR antibody and developed a strategy for purification of TCR engineered T cells by murinization of this epitope. In addition, the authors showed that this murinized epitope on the human αβTCR can be used to specifically deplete engineered T cells with an anti-mouse αβTCR antibody. These two properties combined lead to an elegant select-kill mechanism without the introduction of additional selection or depletion genes.

Introduction

The FDA approval of the first engineered T cells expressing chimeric antigen receptors has paved the way for new cellular interventions in the clinic (1). A next wave of receptors will come with T cell receptor (TCR) engineered T cells specific for targets on both solid and hematological malignancies (2). Most clinical trials using αβTCR engineered T cells are directed against cancer/testis antigens, such as MAGE-A3 (3), MART-1 (4), NY-ESO-1 (5) or PRAME (6). Although the clinical response rates are very encouraging, only a small proportion of the patients benefit from these novel treatments (3, 7). Disappointing response rates can be partially attributed to the presence of non- and poorly- engineered T cells in the administered cell product (8). These non- and poorly-engineered T cells can hamper the therapeutic efficiency because of e.g. insufficient expression of the introduced receptor, or by competition for homeostatic cytokines (8, 9). Furthermore, in an allogenic setting, the presence of T cells still expressing the endogenous αβTCR can lead to severe graft versus host disease. Purification of engineered T cells before infusion can overcome these hurdles, ultimately resulting in enhanced *in vivo* activity. Current methods for purification of engineered T cells often depend on the expression of artificial molecules like truncated CD34 (10) or truncated NGFR (11), in addition to the tumor specific receptor. However, bigger transgene cassettes used to introduce multiple proteins are relatively difficult to express, and additional transgenes can add immunogenic properties to the engineered cell product (12). Besides purification of engineered T cells to increase effectivity, elimination of engineered T cells after adoptive transfer might be needed in case of cytokine release syndrome (13) or off-target toxicities due to peptide mimicry (3, 14). Current solutions for eliminating transferred cells are e.g. the co-expression of HSV-TK along with the transgene of interest (15), mainly limited by the immunogenicity and relatively large size of the HSV-TK gene (16). An alternative elegant solution is to introduce a myc-tag into the αβTCR sequence itself followed by *in vivo* depletion through myc-specific antibodies (17). However, introducing artificial genes into the αβTCR might alter downstream signaling through modifying e.g. its glycosylation (18). Selection of engineered T cells and subsequent *in vivo* elimination achieved with a single marker, which has previously been described for CD20 (19), would be favorable, due to the relatively small transgene cassette and therefore better expression. Even more optimal would be a method where the introduced tumor specific TCR could also be

used for both purification and *in vivo* depletion, and thereby combines all three properties in one gene: tumor specificity, a selection opportunity of cells expressing the transgene at high levels, as well as an *in vivo* depletion option which allows for the elimination of the engineered immune cells in case of toxicities caused by the introduced receptor. Within this context we have explored a strategy based on the recent development of purified T cells engineered to express a defined γδT cell receptor (TEGs) (20, 21). In this strategy we took advantage of the observation that an anti-human αβTCR antibody used for the purification of TEGs does not cross-react with γδTCR chains and can thereby differentiate between engineered and non-engineered cells. This anti-human αβTCR antibody is routinely used to deplete αβTCR T cells from apheresis products using CliniMACS depletion before allogeneic stem cell transplantation (2, 22). Here we describe the translation of the TEG purification procedure into a purification procedure for αβTCR engineered T cells, with the additional opportunity to eliminate engineered T cells, resulting in a complete "select – kill strategy" for αβTCR engineered T cell products.

Materials and methods

Cells and cell lines

Phoenix-Ampho cells (CRL-3213) were obtained from ATCC and cultured in DMEM (Thermo Fisher Scientific, Breda, The Netherlands) containing 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco, Alkmaar, The Netherlands). The TCRβ-/- Jurma cell line (a derivate of Jurkat J.RT3-T3.5 cells (45)), a kind gift from Erik Hooijberg (VU Medical Center, Amsterdam, The Netherlands), TCRβ-/- Jurkat-76, a kind gift from Edite Antunes (Johannes Gutenberg-University, Mainz, Germany) and the T2 cell line (ATCC CRL-1992) were cultured in RPMI 1640 + GlutaMAX (Thermo Fisher Scientific) containing 1% Pen/Strep and 10% FCS. Cell lines were authenticated by short tandem repeat profiling/karyotyping/isoenzyme analysis. All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. In addition, all cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using MycoAlert Mycoplasma Kit (Lonza, Breda, The Netherlands). Peripheral Blood Mononuclear Cells (PBMCs) were obtained from Sanquin Blood Bank (Amsterdam, the Netherlands) and isolated by Ficoll-Paque (GE Healthcare, Eindhoven, The Netherlands) from buffy coats. PBMCs were cultured using the previously described Rapid Expansion Protocol (REP; (31)) in RPMI containing 5% non-typed human serum (Sanquin Blood Bank), 1% Pen/Strep, and 50 μM β-Mercaptoethanol (collectively called HuRPMI).

Cloning of TCR chains into single retroviral vectors

The "minimally murinized" Vα16.1 and Vβ4.1 chains from an NY-ESO1$_{157-165}$/HLA-A*02 specific TCR, respectively named M2.2.3 and M1.KA,4.1, were generated as previously described (27). Additional partially murinized (regions or single residues) TCR chains were ordered from GeneArt (Thermo Fisher Scientific) or constructed via mutagenesis PCR. Cysteine modified chains were designed as reported previously (30). Variants of chimeric αβ/γδ TCRs were composed using the IMGT database (46). Sequences were codon optimized and ordered in an industrial resistance-gene harboring vector or as DNA strings (Geneart Life Technologies). DNA strings were processed using the TA TOPO cloning kit (Thermo Fisher Scientific) and cloned into the pCR™2.1-TOPO® vector, according to the manufacturer's protocol. All TCR chains were cloned separately into the retroviral vector pMP71 between the EcoRI and NotI restriction sites,

using the indicated restriction enzymes and T4 DNA ligase (all from New England Biolabs, Ipswich MA, United States). Transformation of ligated constructs was performed in JM109 competent E. Coli (Promega, Leiden, The Netherlands), and subsequent plasmid DNA isolation was conducted using Nucleobond® PC500, according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany).

Retroviral transduction of primary T cells and T cell lines

Phoenix-Ampho packaging cells were transfected using Fugene-HD (Promega) with env (pCOLT-GALV), gagpol (pHIT60), and separate pMP71 constructs containing α or β chains from an NY-ESO1$_{157-165}$/HLA-A*02 specific TCR (isolated from clone ThP2 (47)) kindly provided by Wolfgang Uckert (23), or containing TCRγ(G115)-T2A-TCRδ(G115) LM1 (20). PBMCs (preactivated with 50 IU/ml IL-2 (Proleukin, Novartis, Arnhem, The Netherlands) and 30 ng/ml anti-CD3 (clone OKT-3, Miltenyi Biotec, Bergisch Gladbach, Germany)), Jurma or Jurkat-76 cells were transduced twice within 48 hours with viral supernatant in 6-well plates (4x10^6 cells/well) in the presence of 50 IU/ml IL-2 (PBMCs only) and 6 µg/ml polybrene (Sigma-Aldrich). After transduction, primary T cells were expanded by the addition of 50 µl/well anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific) and 50 IU/ml IL-2.

Purification of engineered T cells by MACS depletion of poorly and non-engineered immune cells

Transduced primary T cells were incubated with biotin-labeled anti-human αβTCR antibody (clone BW242/412; Miltenyi Biotec), followed by incubation with an anti-biotin antibody coupled to magnetic beads (anti-biotin MicroBeads; Miltenyi Biotec) (20). Next, the cell suspension was applied to an LD column in a QuadroMACS™ Separator. αβTCR-positive T cells were depleted by MACS cell separation according to the manufacturer's protocol (Miltenyi Biotec).

In silico TCR modelling

The structure of different murinized constant domains was predicted using SWISS-MODEL (48) on the modeled template of the β chain of the human JKF6 T-cell receptor (PDB entry code: 4ZDH). The structure of the murinized constant domains when binding H57-597 was modeled on the template of the β chain of the murine N15 T-cell receptor (PDB entry code: 1NFD) (49). Structure visualizations were performed using PyMol Molecular Graphics System (50).

Chimeric antibody production and purification

Armenian hamster Anti murine β-chain antibody H57-597 was de-novo sequenced (Rapid Novor, Kitchener, ON, Canada). Hamster-human (IgG1) chimeric H57-597 antibody was generated using Lonza expression vectors (pEE14·4-kappaLC, pEE14·4-IgG1) (51, 52). The antibody was produced by transient transfection of HEK293F cells with the heavy chain coding plasmid, the light chain coding plasmid and pAdVAntage (Accession Number U47294; Promega), using 293fectin transfection reagent (Invitrogen) following the manufacturer's instructions. Antibody-containing supernatant was harvested 4 days after transfection and purified by affinity chromatography using HiTrap Protein G HP antibody purification columns (GE Healthcare).

Sequencing

DNA sequences of cloning intermediates and final constructs in pMP71 were verified by Barcode Sequencing (Baseclear, Leiden, The Netherlands). 75 µg plasmid DNA and 25 pmol primer specific for the pCR™2.1-TOPO® vector or pMP71 vector were premixed in a total of 20 µl and sent to Baseclear for Sanger sequencing.

Flow cytometry

Cells were stained with Vβ4-FITC (TRBV29-1, clone WJF24; Beckman Coulter), αβTCR-PE (clone BW242/412; Miltenyi Biotec), CD3-PB (clone UCHT1; BD), CD4-PeCy7 (clone RPA-T4; eBioscience, Thermo Fisher Scientific), CD8-APC (clone RPA-T8; BD), CD8-PB (clone SK1; Biolegend), or RPE-conjugated NY-ESO-$1_{157-165}$ HLA-A*02:01 (SLLMWITQV) pentamer (ProImmune, Oxford, United Kingdom). Samples were fixed using 1% PFA in PBS, measured on a FACSCanto-II flow cytometer (BD), and analyzed using FACSDiva (BD) or FlowJo (Three Star Inc.) software.

ELISA

Effector and target cells (E:T 50,000:50,000) were incubated for 16 hours after which supernatant was harvested. IFNγ ELISA was performed using ELISA-ready-go! Kit (eBioscience) following the manufacturer's instructions.

MMAE ADC construction

Chimeric H57-MC-VC-PAB-MMAE was constructed using a kit from CellMosaic, (Woburn, MA, United States) following the manufacturer's instructions.

Results

Anti-human αβTCR binds an epitope on the TCRβ chain of human αβT cells

The GMP-grade anti-human αβT cell receptor (TCR) monoclonal antibody clone BW242/412 (from now on referred to as anti-human αβTCR) recognizes a common determinant of the human TCRα/β-CD3 complex, which has not been characterized yet. In order to allow further epitope mapping, we first tested the antibody's ability to bind to murine αβTCRs. Therefore, Jurma T cells, a TCR-deficient T cell line, were transduced with human αβTCRs directed against the cancer/testis antigen NY-ESO-$1_{157-165}$ (23) or with a murine nonsense αβTCR composed of the TCRα chain of an MDM2-specific αβTCR (24) and the TCRβ chain of a p53-specific αβTCR (25). Specific binding of anti-human αβTCR was only observed to the human (αHuHu/βHuHu) but not the murine (αMuMu/βMuMu) TCR transduced Jurma cells (Figure 1A). To exclude that parts of the human variable domain of the used anti-human αβTCR are involved in binding, the human NY-ESO-1 αβTCR variable domain was grafted on the murine constant domain to create a chimeric αβTCR (αHuMu/βHuMu). Replacing only the human TCRα and TCRβ constant domains by murine equivalents completely abrogated binding of anti-human αβTCR, to levels resembling binding to a fully murine αβTCR (αMuMu/βMuMu). This indicates that the human constant domain contains the binding epitope. Comparable transgenic expression of murine and human TCRs was confirmed by anti-MuTCRβ and anti-Vβ4 respectively (Figure 1A). Infusion of T cells expressing TCRs with complete murine constant domains into patients can generate immunogenic effects and lead to a decreased persistence of the engineered cells *in vivo* (26). To minimize these undesirable effects, we aimed to map the minimal amount of murine residues needed to disrupt binding of anti-human αβTCR, by making use of previously described chimeric-TCRα and β chains, with mutational blocks covering all amino acid differences between the constant regions of human and mouse αβTCRs (23). We tested three NY-ESO-1 TCRα chain variants and four NY-ESO-1 TCRβ chain variants, each containing one murine domain flanked by complete human amino acid sequences. Every TCRα chain was paired with the fully human TCRβ chain (βHuHu) (Figure 1B) and every TCRβ chain was paired with the fully human TCRα chain (αHuHu) (Figure 1C) and introduced into Jurma cells, after which binding of anti-human αβTCR was determined by flow cytometry. Transduction efficiency of the constructs was measured by anti-Vβ4 and was comparable in all conditions

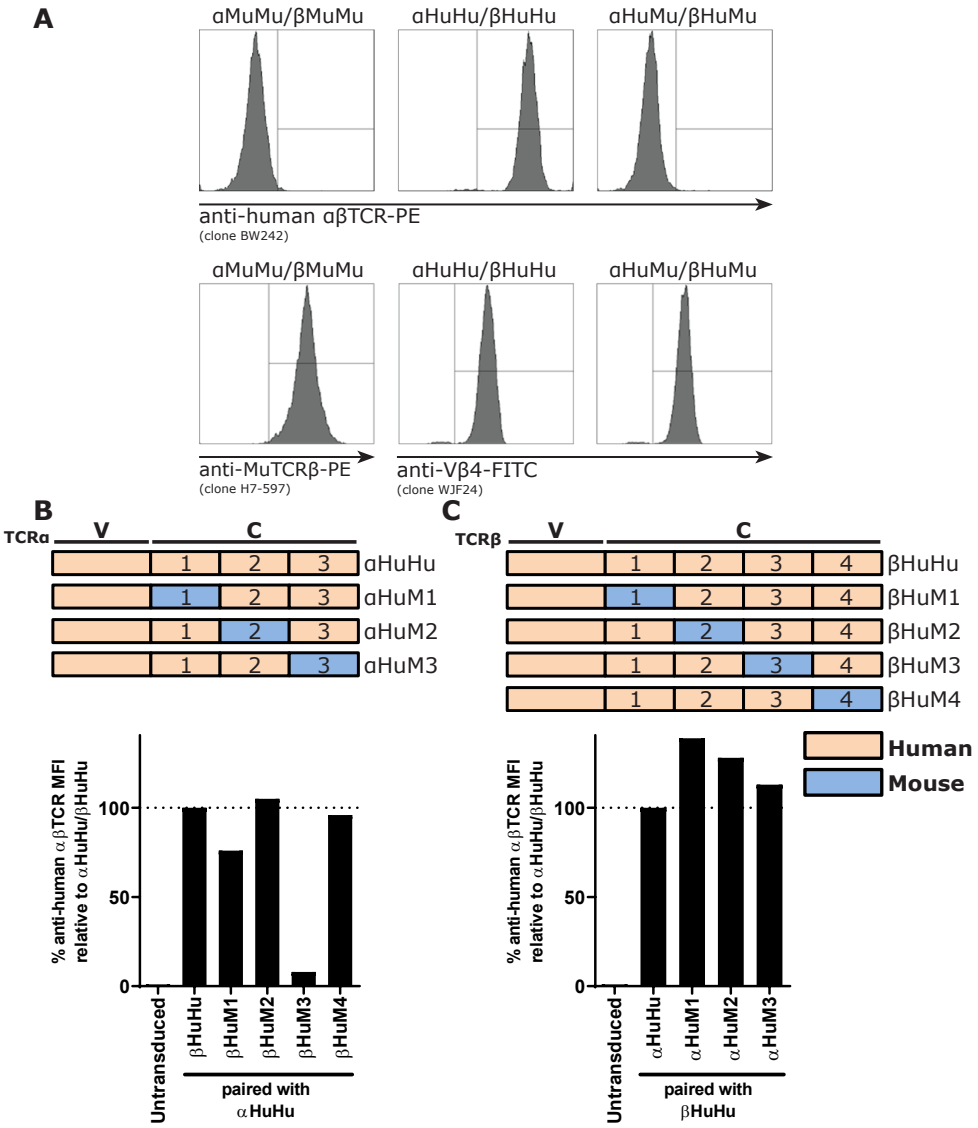


Figure 1. Partial murinization of the TCRβ chain constant domain abrogates binding of the anti-human αβTCR antibody clone BW242/412. (A) Jurma cells were transduced with fully murine (αMuMu/βMuMu), fully human NY-ESO-1 specific (αHuHu/βHuHu) or chimeric αβTCR, in which the α- and β- constant domains were murine, and the variable domains were human NY-ESO-1 specific. Binding of anti-human αβTCR, anti-MuTCRβ and Vβ4 was assessed by flow cytometry. **(B)** Schematic representation of the constructed αβTCRs that cover all amino acid differences in the TCRα chain and **(C)** TCRβ chain (upper panels). Jurma cells were transduced with the different murinized αβTCRs after which anti-human αβTCR antibody binding was assessed by flow cytometry (B&C lower panels). Untransduced Jurma cells served as a negative control.

(data not shown). Antibody binding was significantly impaired in T cells expressing the αβTCR which includes murine domain 3 (βHuM3), while none of the other chimeric αβTCRs substantially impaired anti-human αβTCR binding (Figure 1B and C). These results indicate that domain 3 of the TCRβ chain (βHuM3) dictates the binding of anti-human αβTCR.

Anti-human αβTCR binding can be abrogated by mutating 2 residues

Analysis of the sequence of domain 3 of the TCRβ chain constant domain revealed eleven residues which are non-homologous between murine and human species (Supplemental Figure 1A). To determine which residues are essential for anti-human αβTCR binding, we constructed eleven variants of the TCRβ chain in which each one of the non-homologous amino acids was replaced by the murine counterpart. These eleven constructs were paired with the completely human αTCR chain (αHuHu), introduced in Jurma cells and tested for binding by the anti-human αβTCR antibody. Of the eleven generated mutants, the substitutions of 'human' glutamic acid (E108) to the 'murine' lysine (K), 'human' threonine (T110) to the 'murine' proline (P), and 'human' aspartic acid (D112) to the 'murine' glycine (G) showed a substantial abrogation of anti-human αβTCR binding (Figure 2A). However, none of these substitutions was sufficient to induce total abrogation as shown by the TCR consisting of αHuHu/βHuM3. Therefore we constructed TCRβ chains with a combination of the aforementioned mutations. The TCRβ chains with a D112G mutation combined with E108K or T110P were both effective in abrogating binding of the anti-human αβTCR antibody (Figure 2B), which can be explained by a substantial decrease in bulkiness, thus a decrease in size of these residues (Figure 2C and Supplemental Figure 1B). For further engineered T cell experiments the combination of T110P and D112G murinization was selected.

Purification of αβTCR engineered T cells using anti-human αβTCR MACS

Due to the competition of introduced αβTCR chains with endogenous αβTCR chains in primary T cells, the introduction of foreign αβTCRs is frequently impaired when compared to αβTCR deficient Jurma cells. Murine αβTCRs, or residues derived from murine αβTCRs introduced into human αβTCRs, and expressed in human T cells, have been reported to outcompete endogenous human TCR chains (27-29). Furthermore, these murine and murinized αβTCRs preferentially pair with each other, thereby decreasing the occurrence of mispairing with endogenous human αβTCRs.

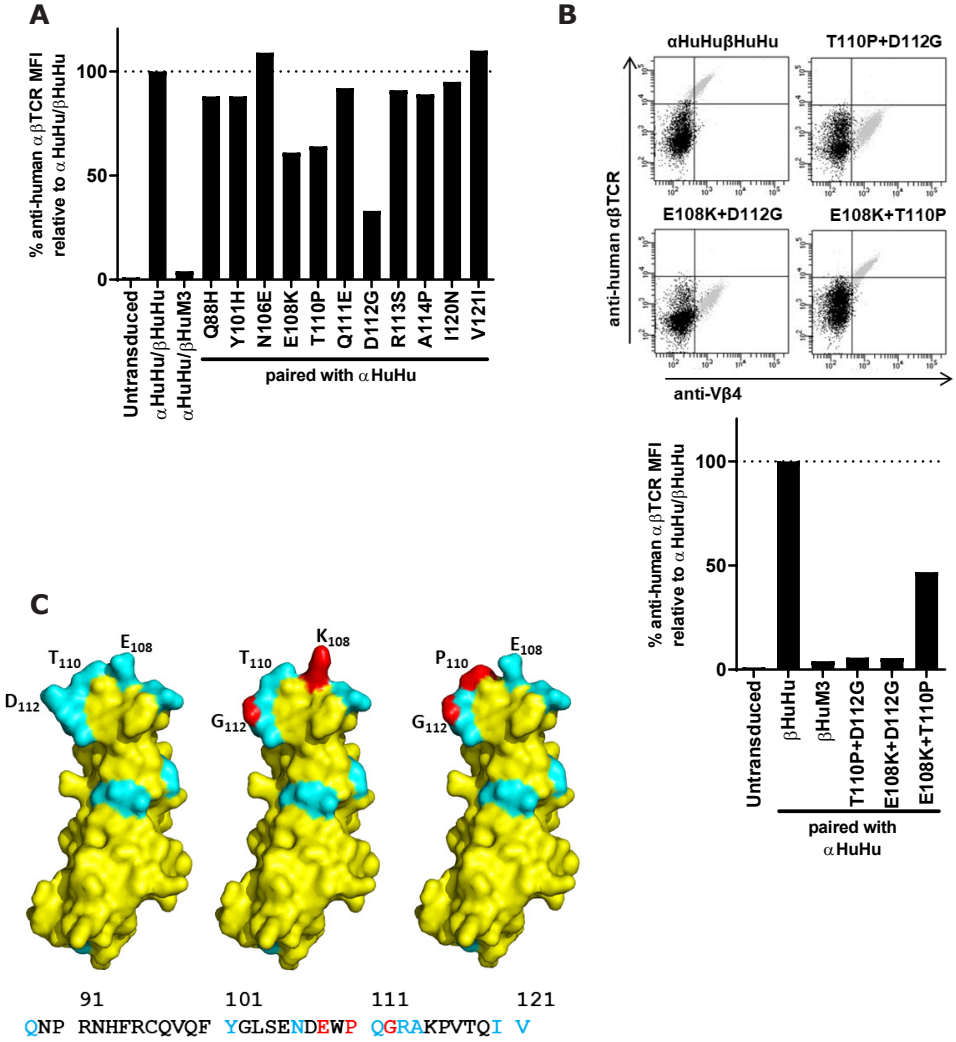


Figure 2. A combination of two specific murine amino acids in the TCRβ chain constant domain is sufficient to abrogate binding of the anti-human αβTCR antibody clone BW242/412. (A) Jurma cells were transduced with αβTCRs containing single murine amino acid substitutions in the 3rd domain of the β chain after which binding of anti-human αβTCR antibody was assessed using flow cytometry. Untransduced Jurma cells served as a negative control while fully human αβTCR transduced Jurma cells served as a positive control. **(B)** Jurma cells were transduced with αβTCRs containing combinations of murine amino acids in the 3rd domain of the β chain, after which binding of anti-human αβTCR antibody was assessed using flow cytometry. **(C)** Visualization of the eleven non-homologous amino acids between human and mouse β chain 3rd domain in cyan using SWISS-MODEL (48) on the modeled template of the β chain of the human JKF6 T-cell receptor (PDB entry code: 4ZDH). Effective single murine amino acid substitutions are displayed in red.

Therefore, we utilized single murine amino acids to enhance the expression of introduced TCRs (27). These "minimally murinized" constant domain variants (from now on referred to as mm) contain murine amino acids which are both critical and sufficient to improve pairing between the two chains (27). Next, we introduced the above-identified murine residues (T110P+D112G) in the TCRβ chain constant domain in order to test whether this indeed was sufficient to disrupt the binding of anti-human αβTCR in human primary T cells. To test this concept, healthy donor T cells were transduced with mm NY-ESO-1 specific αβTCRs as a negative control or mm NY-ESO-1 specific αβTCRs, including the two identified mutations T110P+D112G. The αβTCR and Vβ4 expression after transduction were assessed by flow cytometry. The fraction of cells positive for anti-Vβ4, but negative for anti-human αβTCR is the fraction of interest (Figure 3A, middle plot). Magnetic-activated cell sorting (MACS) depletion using anti-human αβTCR resulted in a significant increase of the Vβ4 engineered T cell fraction which was still visible after 2 weeks of expansion (Figure 3A ,right plot, right quadrants). The T cell fraction present in the upper left quadrant are αβTCR positive and Vβ4 negative, likely due to the re-expression of the endogenous TCR. All surviving residual non-T cells which are still present at the moment of MACS depletion are not removed by our method and therefore visible in the lower left quadrant. A comparison of purity directly after isolation, and after two weeks of expansion, and investigation of not only the introduced Vβ4 chain but also of pairing, thus the specificity of introduced chains by anti-Vβ4 and NY-ESO-$1_{157\text{-}165}$ HLA-A*02:01 pentamer staining, demonstrated that the introduced new mutations do not interfere with the used mm-pairing strategy (Figure 3B) and that purity of the engineered cells is maintained two weeks after expansion (Figure 3C).

Enrichment strategy within the context of alternative stabilization procedures

Multiple alternative strategies to prevent αβTCR chain mispairing and thereby increasing the expression of the introduced tumor specific αβTCR have been reported. E.g., adding an additional cysteine residue, to introduce a disulfide bridge between the α and β chains, has been shown to increase expression and decrease mispairing (30). Also, human γδTCRs introduced in human T cells do not pair with endogenous αβTCRs (31), therefore it is attractive to use γδTCR constant domains for engineering αβT cells in a similar way. We therefore tested whether our enrichment strategy could also be combined with these alternative pairing solutions.

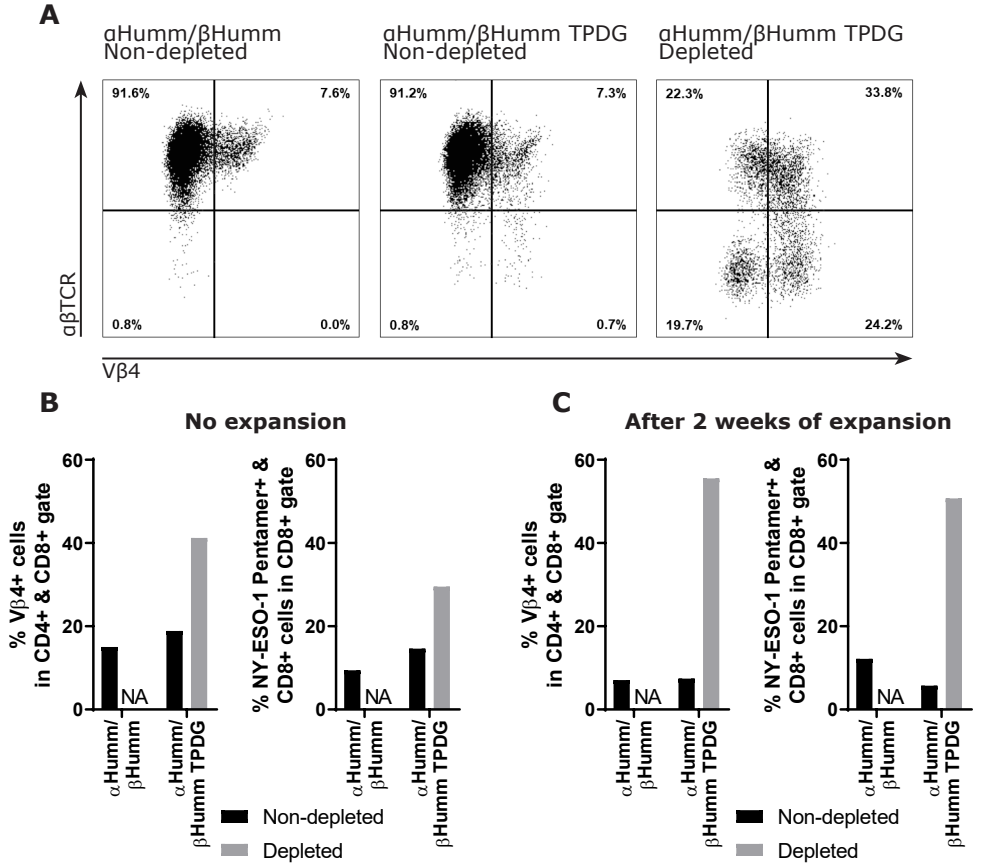


Figure 3. Primary αβT cells engineered with murinized αβTCRs can be successfully depleted from non- and poorly-engineered immune cells by using anti-human αβTCR antibody clone BW242/412. (A) Primary αβT cells were transduced with minimally murinized αβTCRs with (middle panel) and without (left panel) the "TPDG" mutations. Primary αβT cells with the "TPDG" mutations were MACS-depleted (right-panel). Endogenous αβTCR expression and expression of the introduced αβTCR without the "TPDG" mutations were determined by flow cytometry using anti-human αβTCR antibody. Expression of the introduced βTCR chain was assessed with an anti-Vβ4 antibody (CD4/CD8+) and expression of correctly paired αβTCR chains by NY-ESO-1 pentamers (CD8+) **(B)** directly after purification and **(C)** 2 weeks after expansion.

Firstly, we constructed an NY-ESO-1 specific TCR with an additional disulfide bridge by the mutation of one specific residue in each chain; T48C in TCRCα and S57C in TCRCβ (30). Secondly, we constructed an NY-ESO-1 specific TCR with the same additional disulfide bridge and with a human γδTCR trans-membrane domain. A schematic representation of all three approaches is displayed in Figure 4A. To later make use of the αβTCR depletion method, we introduced the mutations T110P+D112G in

the β chains. We then assessed the expression of the different TCRs in primary T cells by measuring the percentage of Vβ4+ and NY-ESO-$1_{157-165}$ HLA-A*02:01 pentamer+ cells within the CD8+ population (Figure 4B). All three conditions resulted in a NY-ESO-$1_{157-165}$ HLA-A*02:01 pentamer+ CD8+ fraction almost as big as the Vβ4+ CD8+ fraction, indicating that all TCRs are preferentially paired. The three different conditions were αβTCR depleted in the same way as before, and the percentage of Vβ4+ cells (Figure 5A) and NY-ESO-$1_{157-165}$ HLA-A*02:01 pentamer+ cells within the CD8+ population (Figure 5B) was measured by flow cytometry.

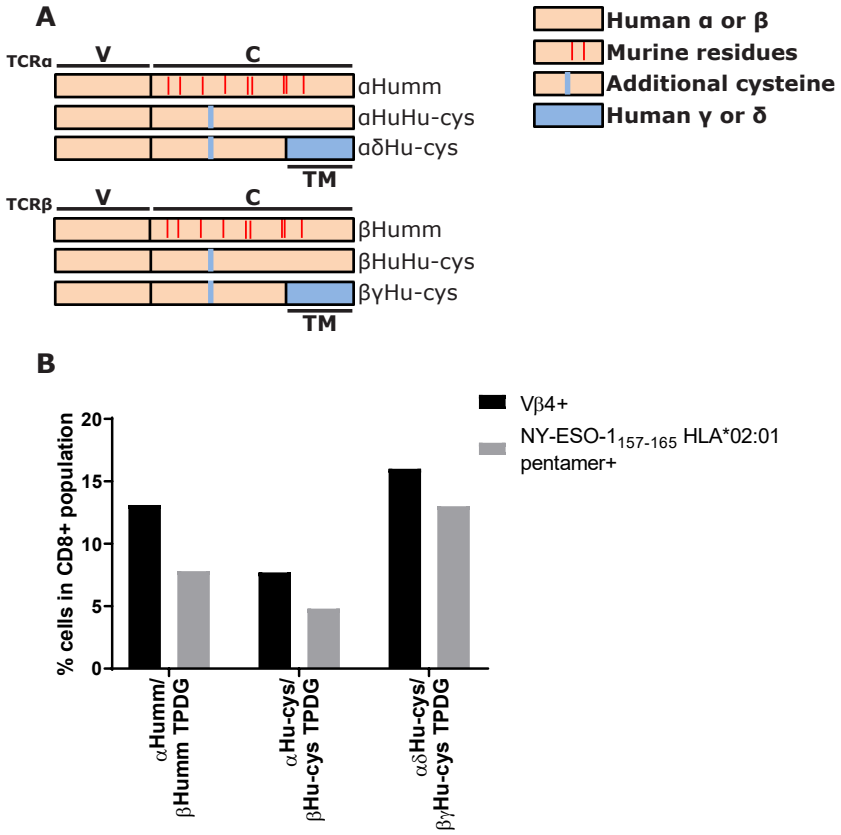


Figure 4. Efficacy of different strategies to induce preferential pairing of introduced α and βTCR chains. (A) Schematic representation of the three different methods for creating preferential pairing between the introduced α and βTCR chains. TM indicates the transmembrane domain. **(B)** Primary αβT cells were transduced with the 3 differentially modified αβTCRs as indicated in (A) and expression of the introduced βTCR was determined by an anti-Vβ4 antibody. Pairing of the introduced α and βTCR chains were assessed by NY-ESO-1 pentamers.

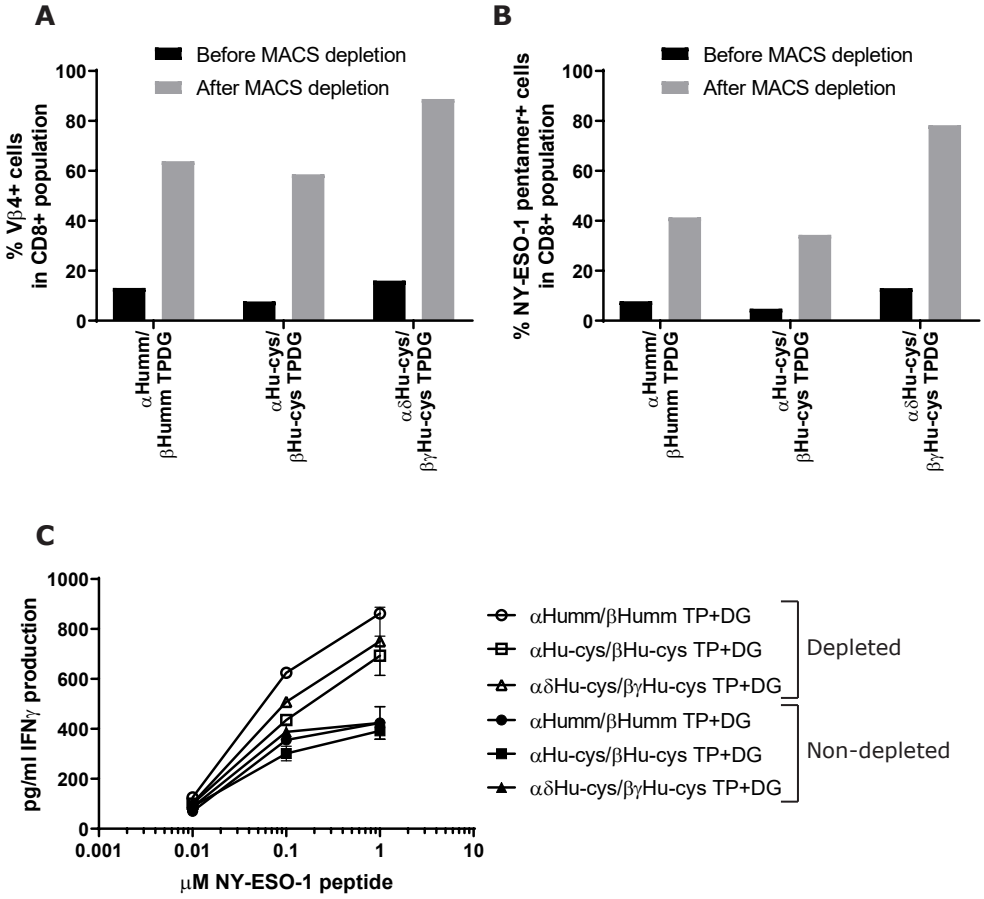


Figure 5. Depletion of non- and poorly- engineered T cells within the context of different preferential αβTCR pairing strategies. Primary αβT cells were transduced with the 3 differently modified αβTCRs as indicated in Figure 4A and depleted with the anti-human αβTCR antibody clone BW242/412. **(A)** Directly after depletion, expression of the introduced βTCR was determined by an anti-Vβ4 antibody. **(B)** Expression of appropriately paired introduced α and βTCR chains were determined by NY-ESO-1 pentamers. **(C)** Functionality of purified or non-purified engineered immune cells was assessed in a stimulation assay after co-incubation with NY-ESO-$1_{157-165}$ peptide pulsed T2 cells. IFNγ production was measured in the supernatant by ELISA.

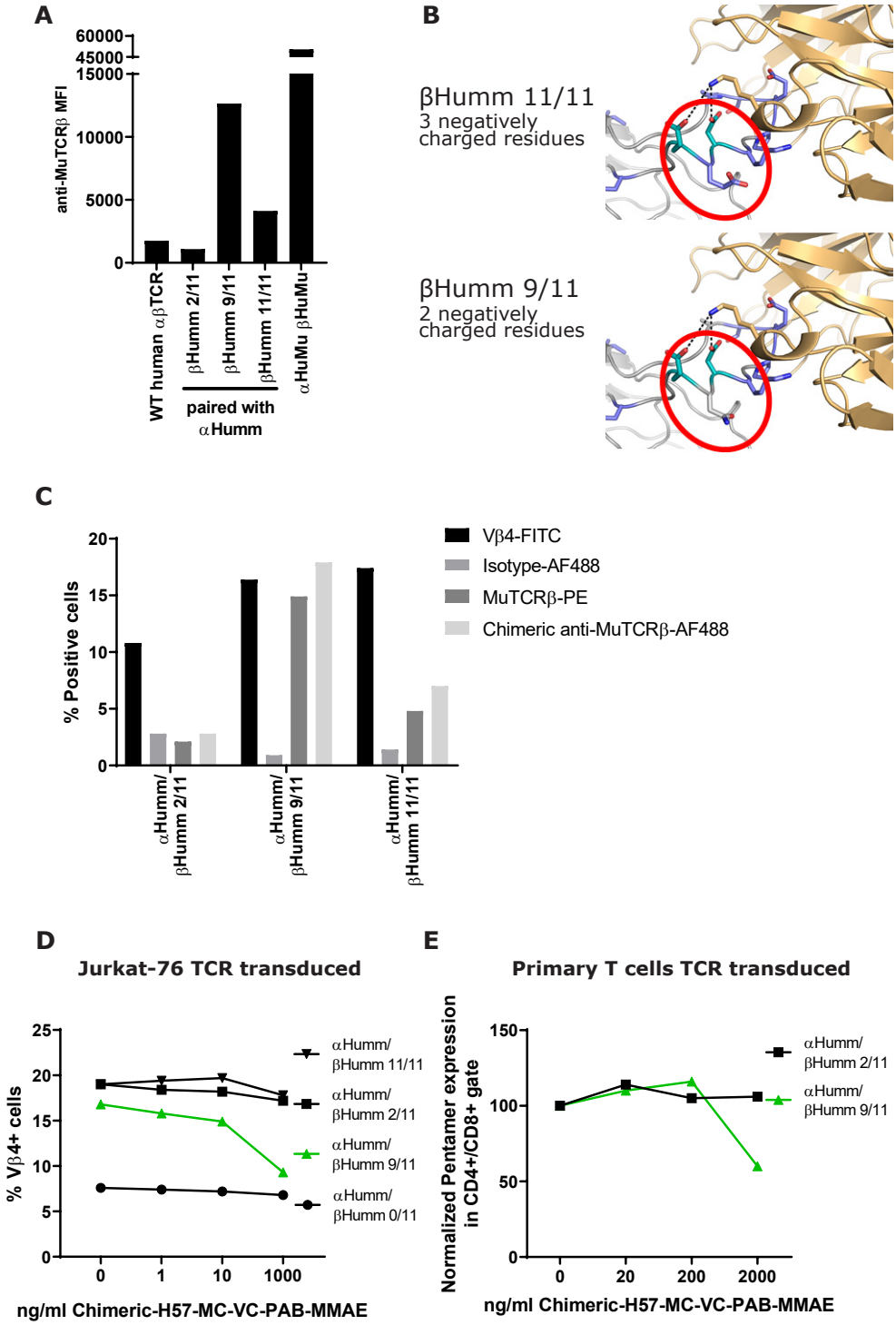
All three described methods were suitable for creating preferential pairing and subsequent purification by our αβTCR depletion method. Thus, partial murinization or stabilization through cysteines are equally potent and adding γ and δ domains did not significantly enhance expression or pairing.

Augmented *in vitro* tumor cell recognition by purified engineered T cells

To determine if purified NY-ESO-$1_{157-165}$ αβTCR engineered T cells were superior in target cell recognition compared to non-purified cells, we pulsed T2 cells with multiple concentrations of NY-ESO-$1_{157-165}$ peptide. Purified engineered T cells showed a stronger response to the peptide loaded T2 cells than the non-purified cells. However, no substantial differences between the three pairing strategies could be observed (Figure 5C). In further engineered T cell experiments, the mm approach was used to prevent mispairing and increase expression.

Developing an antibody recognizing the introduced mutated region

The infusion of engineered T cells can potentially be toxic, due to the occurrence of cytokine release syndrome (13) or off-target toxicity of the receptor used (14). To be able to deplete infused engineered T cells *in vivo* when deemed necessary, we first aimed to raise an antibody specific for the T110P+D112G murinized variant of the αβTCR by immunizing three Wistar rats with a human-mouse chimeric peptide. Despite the fact that antibodies were formed against the chimeric peptide (Supplemental Figure 2A), no antibody binding against surface-expressed αβTCRs could be detected (Supplemental Figure 2B). Therefore we assessed if the commercially available anti-murine TCRβ chain antibody clone H57-597 (from now on referred to as anti-MuTCRβ), was able to bind the murinized αβTCRs on Jurkat-76 cells generated so far. Jurkat-76 cells expressing the T110P+D112G murinized variant of the αβTCR (indicated by βHumm 2/11; two out of the eleven non-homologous amino acids in the 3rd domain are murinized) were not bound by anti-MuTCRβ, however, Jurkat-76 cells expressing the βHummM3 murinized variant of the αβTCR (indicated by βHumm 11/11; all eleven non-homologous amino acids in the 3rd domain are murinized) were bound by anti-MuTCRβ. To limit the amount of murine amino acids introduced, we also constructed a variant in which 9/11 non-homologous amino acids in the 3rd domain are murinized (Supplemental Figure 2C). Both 11/11 and 9/11 non-homologous murine amino acids in β chain of domain 3 were sufficient to reestablish binding of anti-MuTCRβ, however, not to the same extent as the complete murine αβTCR (Figure 6A), while fewer murinized mutants, including the T110P+D112G (2/11) mutations did not allow binding of anti-MuTCRβ. Surprisingly, 9/11 caused a higher MFI than 11/11. Structural analyses suggested that this differential binding could be a consequence of the fact that 9/11 contains one less negatively charged residue and therefore results in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αHuMu βHuMu
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βHumm 9/11
2 negatively charged residues
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Vβ4-FITC
Isotype-AF488
MuTCRβ-PE
Chimeric anti-MuTCRβ-AF488
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αHumm/ βHumm 2/11
αHumm/ βHumm 9/11
αHumm/ βHumm 0/11
E
Primary T cells TCR transduced
Normalized Pentamer expression in CD4+/CD8+ gate
150
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0
0
20
200
2000
ng/ml Chimeric-H57-MC-VC-PAB-MMAE
αHumm/ βHumm 2/11
αHumm/ βHumm 9/11

a more focused electrostatic potential to attract the lysine on CDR1 of anti-MuTCRβ (Figure 6B). Since the clone of anti-MuTCRβ antibody is of Armenian Hamster origin and presumably induces severe side effects once administered to humans, like anti-thymocyte globulin (32), we aimed to generate a humanized variant of anti-MuTCRβ. Therefore we generated chimeric variants of anti-MuTCRβ (H57-597, PDB entry code: 1NFD) by exchanging the hamster IgG2 constant domain for the human IgG1 constant domain (referred to as chimeric anti-MuTCRβ). We tested binding of this newly constructed antibody in engineered Jurkat-76 cells, which resulted in specific antibody binding to the 9/11 murinized TCRβ chain expressed on Jurkat-76 (Supplemental Figure 3). To determine the capacity of the chimeric anti-MuTCRβ antibody to bind to primary T cells expressing the murinized αβTCRs, we conjugated this antibody and an isotype control to Alexa Fluor 488 (AF488) and determined binding by flow cytometry. The chimeric anti-MuTCRβ antibody was able to bind both 9/11 and 11/11 murinized TCRs and, as observed in Figure 6A, the binding to 9/11 was stronger than to 11/11 (Figure 6C).

Depletion of engineered immune cells through a mutation-specific antibody

To assess if the chimeric variant of anti-MuTCRβ was able to selectively deplete engineered T cells *in vitro,* the antibody was coupled to monomethyl auristatin E (MMAE), a cell cycle inhibitor, using the protease cleavable linker VC-PAB (33), to create an antibody-drug conjugate (ADC). Jurkat-76 cells transduced with different murinized TCRs were incubated with multiple

◂**Figure 6. Depletion of engineered T cells by using a mutation-specific antibody. (A)** Jurkat-76 cells were transduced with 5 different murinized αβTCRs to assess binding of anti-MuTCRβ. Wild-type (WT) αβTCR transduced Jurkat-76 cells served as a negative control, while Jurkat-76 transduced with a TCR containing a complete murine constant domain served as a positive control. **(B)** The structure of the murinized constant domains (βHumm 11/11 and βHumm 9/11) when binding of H57-597 was modeled on the template of the β chain of the murine N15 T-cell receptor (PDB entry code: 1NFD) (49). **(C)** Primary αβT cells were transduced with 3 different murinized αβTCRs to assess binding of wild-type and chimeric anti-MuTCRβ. anti-Vβ4 and anti-Human IgG1-AF488 isotype were included as positive and negative control respectively. **(D)** Jurkat-76 were transduced with 4 different murinized αβTCRs and incubated with chimeric H57-MC-VC-PAB-MMAE for 24 hours and then stained with an anti-Vβ4 antibody. **(E)** Primary αβT cells were transduced with 2 differently murinized αβTCRs, depleted for poorly and non-engineered T cells, expanded using our REP protocol and subsequently incubated with the mutation-specific chimeric antibody H57-MC-VC-PAB-MMAE for 24 hours. Surviving engineered immune cells were determined by NY-ESO-1 pentamer staining.

concentrations of the ADC. The highest concentration of chimeric H57-MC-VC-PAB-MMAE led to a decrease of Vβ4 positivity in the 9/11 condition only (Figure 6D). This specific decrease indicates that the ADC is able to selectively deplete 9/11, and not 11/11 αβTCR engineered Jurkat-76 *in vitro,* most likely due to the weaker binding of the engineered antibody to the 11/11 αβTCR (Figure 6C). To assess whether this mechanism is also effective if introduced TCRs need to compete with endogenous TCRs, primary T cells transduced with the 2/11 and 9/11 murinized αβTCRs were αβTCR depleted with the antibody selectively recognizing wild type αβTCR, expanded using our REP protocol and incubated with the ADC for 24 hours. As observed for αβTCR transduced Jurkat-76 cells, the 9/11 murinized αβTCR engineered cells were selectively depleted, as indicated by a substantial decrease in NY-ESO-$1_{157-165}$ HLA-A*02:01 pentamer positivity (Figure 6E). Although the concentrations of chimeric H57-MC-VC-PAB-MMAE needed to be effective *in vitro* are higher than one would expect from an MMAE-ADC, this is potentially irrelevant *in vivo* due to additional cleavage of the VC-PAB linker by extracellular proteases (34).

Discussion

The main findings of our study are that replacing only two amino acids within the constant domain of the TCR β chain allows for the purification of αβTCR engineered T cells with GMP-ready tools, which are currently used in daily clinical practice for purification of hematopoietic transplants from αβT cells (35). The very same region on the TCR β chain can also serve as target for antibodies, which can deplete engineered immune cells. This select-kill mechanism is a novel and unique strategy for increasing purity and augmenting safety of αβTCR engineered T cells with minor engineering steps, after transfer into patients.

A sufficient down-regulation of the endogenous αβTCR chains by the introduced αβTCR chains is essential for this method to work. Therefore, strategies interfering with endogenous αβTCRs or utilizing knock out of the α or β locus to enhance expression of introduced αβTCRs (36) will benefit from this strategy. However, engineering of T cells via ZFN, CRISPR or TALENs (37) require additional engineering steps and therefore are an additional hurdle for GMP grade production. We accomplished dominance of the introduced receptors by using a previously described method where human residues are replaced by key murine counterparts (23). Furthermore, we successfully assessed whether the introduction of an additional disulfide bridge (38) or the exchange of the human αβTCR transmembrane domain for the human γδTCR counterpart (20) could also lead to enhanced expression. Thus, we found, in line with our recently published solution for TEGs (20), an elegant and minimalistic strategy to purify αβTCR engineered T cells. This is particularly important in the light of the current practice that infused engineered products harbor only between 15-55% of engineered immune cells (39, 40). The lack of purity can become a major clinical obstacle in terms of efficacy (20) as well as toxicity (13, 41).

Many tumor-associated tumor antigens targeted by αβTCR gene therapy are not exclusively expressed on tumor cells (42). Thus, depending on the type of the antigen targeted by the introduced αβTCR, depletion strategies can be useful. This is illustrated by multiple clinical trials, which have led to devastating results caused by off-target or on-target but off-tumor toxicities (3, 14). Preclinical strategies to predict off-target toxicities by affinity enhanced TCRs provide an important tool to minimize these risks (43). However, these strategies are not infallible and therefore it is extremely valuable to be able to deplete engineered immune cells with affinity matured receptors, or when targeting novel antigens or antigens

which are also partially expressed on healthy tissues. Methods described so far for introducing a safety switch in engineered T cell products rely on the introduction of additional genes for the expression of (truncated) targetable proteins, the introduction of inducible caspase proteins (44) or sensitivity to ganciclovir in the case of the widely used HSV-TK suicide gene (16). The method described here, using minimal murine amino acid substitutions, is not only suitable for creating an untouched population of purified T cells, but also allows for *in vivo* depletion when needed. The identified two murine amino acids to enable αβTCR depletion need to be expanded with an additional seven, to create a chimeric TCR β chain with a total of nine murine amino acids. The major advantage of our strategy, as compared to strategies using e.g. myc-tags introduced into the TCR α chain (17), is its combined property as a selection and safeguard system, as well as the usage of natural αβTCR domains which do most likely not affect signaling or impair pairing.

In conclusion, the murinization of two specific residues in the TCRβ constant domain allows for the untouched isolation of αβTCR engineered T cell products. When a safeguard of engineered immune cells is required, mutating additional seven human amino acids to murine residues in the TCRβ constant domain allows binding of an antibody, which then selectively recognizes engineered T cells. Ultimately, this chimeric receptor design and subsequent purification can be rapidly implemented in any engineering procedure for TCRs used for targeting hematological or solid malignancies. This will allow for further enhancement, efficacy and reduction of adverse effects caused by non- and poorly- engineered T cells. With the additional safety switch, engineered T cells can be depleted at a later time point.

Conflict of Interest Statement

GK, DB, ZS and JK are inventors on different patents with γδTCR sequences, recognition mechanisms and isolation strategies. GK is employed by Kiadis Pharma (www.kiadis.com). JK is scientific advisor and shareholder of Gadeta (www.gadeta.nl). No potential conflicts of interest were disclosed by the other authors.

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Supplementary figures

A

TCRα

```
                    1                                                   
   1         11        21        31        41        51        61
Hu IQNPDPAVYQ LRDSKSSDKS VCLFTDFDSQ TNVSQSKDSD VYITDKTVLD MRSMDFKSNS AVAWSNKSDF
Mu ****E***** *K*PR*O*ST L********* I**PKTME*G TF******** *KA**S***G *I****OTS*

                2                   3
   71        81        91        101       111       121       131
Hu ACANAFNNSI IPEDTFFPSP ESSCDVKLVE KSFETDTNLN FQNLSVIGFR ILLLKVAGFN LLMTLRLWSS
Mu T*QDI*K--- -ETNATY**S DVP**AT*T* ******M*** ******M*L* ********** **********
```

TCRβ

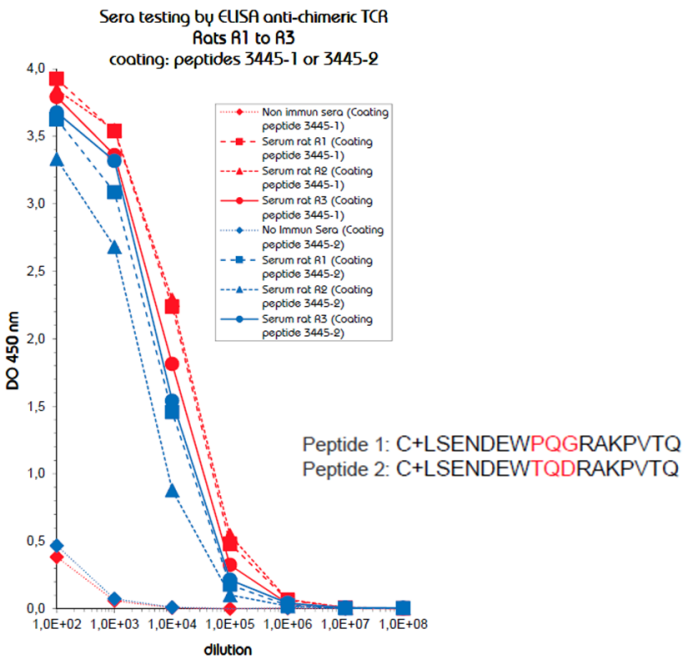
```
                    1                                                   2
   1         11        21        31        41        51        61
Hu EDLKNVFPPE VAVFEPSEAE ISHTQKATLV CLATGFYPDH VELSWWVNGK EVHSGVSTDP QPLKEQPALN
Mu ***R**T**K *SL****K** *ANK****** ***R**F*** ********** ********** *AY**---S*

                                    3
   71        81        91        101       111       121       131
Hu DSRYCLSSRL RVSATFWQNP RNHFRCQVQF YGLSENDEWT QDRAKPVTQI VSAEAWGRAD CGFTSESYQQ
Mu Y*-******* *******H** ********** H****E*K*P EGSP*****N I********* **I**A**H*

                4
   141       151       161       171
Hu GVLSATILYE ILLGKATLYA VLVSALVLMA MVKRKDSRG
Mu ********** ********** ****G***** ***K*N*--
```

B

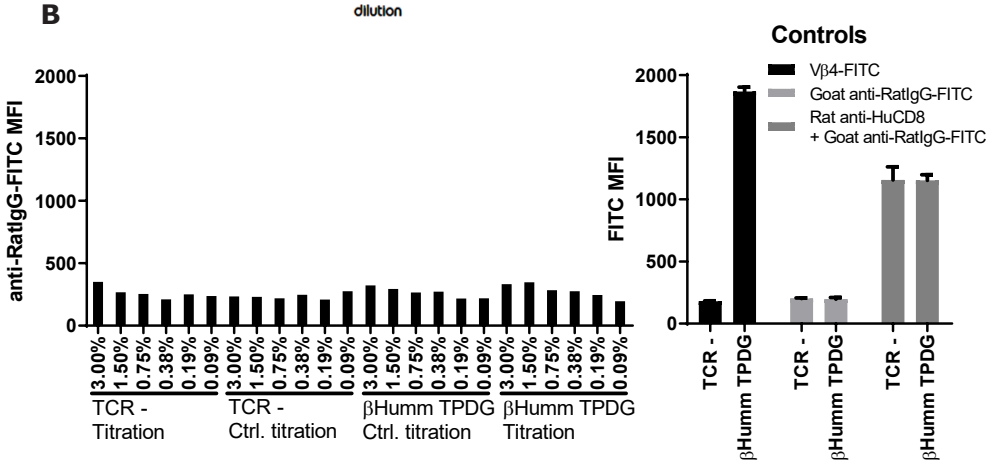
	Change in…			
	Size	**Charge**		**Hydrophobicity**
Q_{88} H	-	+	Uncharged to positive charge	Hydrophilic to hydrophobic
Y_{101} H	-	+	Uncharged to positive charge	-
N_{106} E	-	+	Uncharged to negative charge	-
E_{108} K	**-**	**+**	**Negative to positive charge**	**Slightly more hydrophilic**
T_{110} P	**less bulky**	**-**		**-**
Q_{111}E	-	+	Uncharged to negative charge	-
D_{112} G	**less bulky**	**+**	**Negative to uncharged**	**Less hydrophilic**
R_{113} S	less bulky	+	Positive to uncharged	-
A_{114} P	-	-		Less hydrophobic
I_{120} N	-	-		Hydrophobic to hydrophilic
V_{121} I	-	-		-

Supplemental Figure 1. Alignment of human and murine TCR α and β chains. (A) There is extensive homology between human and murine TCR chains. **(B)** The differences between the eleven non-homologous amino acids in the 3rd domain of the β chain (βM3).

A



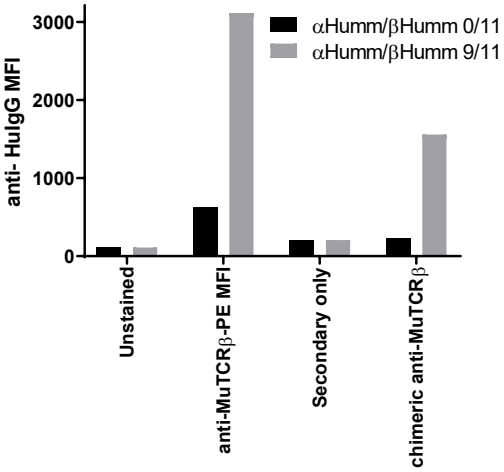
B



C

```
                          91          101         111         121
Human            QNP RNHFRCQVQF YGLSENDEWT QDRAKPVTQI V
11/11 (Murine)   H.. .......... H....E.K.P EGSP.....N I
2/11             ... .......... .........P .G........ .
9/11             H.. .......... H......K.P EGS......N I
```

◂**Supplemental Figure 2. Attempting to raise an antibody specific for the T110P+D112G murinized variant of the αβTCR by immunizing 3 Wistar rats with a human-mouse chimeric peptide. (A)** Determining the presence of peptide-specific antibodies in the serum of the immunized rats. **(B)** Assessing the ability of the generated antibodies to bind surface-expressed TCRs. aHumm/βHumm TPDG transduced or non-transduced Jurkat-76 cells were incubated with the indicated percentage of rat serum, after which flow cytometry using anti-RatIgG-FITC was performed. In the controls panel, the functionality of this secondary antibody was confirmed by staining the Jurkat-76 cells with rat anti-HuCD8 followed by anti-RatIgG-FITC. Expression of the TCR was confirmed using anti-Vβ4-FITC. **(C)** Sequence alignment of the human and murine 3rd domain of the TCRβ chain and the constructed 2/11 and 9/11 murinized variants.



Supplemental Figure 3. Chimeric anti-MuTCRβ antibody binds to primary T cells expressing the murinized TCR containing 9 out of 11 murine residues in the 3rd domain of the β chain. Jurkat-76 cells were transduced with 2 different αβTCRs, containing 0/11 or 9/11 murine residues in the 3rd domain of the β chain, to assess binding of the newly generated chimeric and CDR grafted anti-MuTCRβ antibodies. As negative controls, unstained and secondary antibody only conditions were used. As a positive control, wild-type PE-conjugated anti-MuTCRβ was used.

The next step towards GMP-grade production of engineered immune cells

5

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Oncoimmunology, 2015

Abstract

Removing less potent T cell subsets as well as poorly- or non-engineered cells can optimize effectiveness of engineered T cell therapy against cancer. We have recently described a novel, GMP-ready method for the purification of engineered immune cells that might further boost the clinical success of cancer immunotherapy.

Adoptive transfer of genetically engineered T cells is a promising strategy in the fight against cancer. An increasing number of clinical trials show the high potential of cancer immunotherapy using immune cells engineered to express tumor specific immune receptors, which most recently attracted interest from patients and "big pharma". This is reflected by the impressive number of clinical trials currently recruiting patients for treatment with genetically modified T cells (1). To further exploit this potent application of cancer immunotherapy, various possibilities may be considered.

In addition to defining the best immune receptor (1), optimizing the composition of the engineered T cell graft is likely to contribute to the success of clinical outcome. Currently, the engineered T cell graft contains a very heterogeneous population of T cells that are engineered, caused (A) by the vast variety of CD3+ subsets in peripheral blood mononuclear cells, as well as (B) the fact that current protocols only redirect a fraction of immune cells. Thus, usually most clinical trials administer a very diverse product including many different immune cell subsets as well as engineered, poorly- and non-engineered immune cells.

The presence of multiple T cell subsets in an infused cell product can lead to dampening of the immune response by e.g. regulatory T cells, or in the context of an allogeneic stem cell transplantation (allo-SCT) induce graft versus host disease (GvHD). In order to overcome such obstacles and to increase long-term memory, transfer of selected immune subsets has been proposed (2). For example, in an elegant primate model Berger et al. demonstrated that central memory T cells, as defined by a CD62L+ phenotype, show an increased capacity to persist after adoptive transfer (3). However, in the context of an allo-SCT the very same subsets might be harmful. When the CD62L+ T cell population was depleted from the graft in mice, GvHD was significantly reduced (4). Therefore the right choice of subset does not only depend on the desired immunological phenotype of engineered immune cells, such as central memory T cells, but also on the context of clinical application. Cells appropriate for use in an autologous setting might be harmful when used for allo-SCT. In the context of allo-SCT, downregulation of endogenous receptors might be an additional important engineering step (5). Regardless of the desired subset, processing cell fractions in a good-manufacturing-practices (GMP) certified environment is usually cumbersome and expensive due to the fact that sequential isolation steps using multiple GMP-grade antibodies are necessary.

A second important step towards a more defined product is selecting

immune cells with maximal receptor expression in order to reduce unwanted bystander activity by poorly- or non-engineered immune cells. At present, efforts to increase the purity of the engineered immune cells mainly utilize positive selection, which can result in unwanted activation of T cell subsets. Furthermore, this method is often based on the expression of an additional transgene like truncated CD19 or proteins like epidermal growth factor receptor which are normally absent in the hematological cell lineage (6). These strategies do not only interfere with the expression of the introduced immune receptor, which can be detrimental when affinity of used receptors is low (J. Kuball, unpublished observation), but more importantly also lead to immunogenicity, altered homing or the rejection of engineered immune cells.

Therefore, we propose in a recent issue of *Clinical Cancer Research*, a novel GMP-ready strategy to remove poorly- and non-engineered T cells from a cellular product based -in contrast to recent efforts- on negative selection (5). We demonstrate that interference with endogenous αβTCRs combined with GMP-grade anti-αβTCR beads can provide highly purified untouched engineered immune cells without the additional need for selection markers. We used a tumor specific γδTCR (7, 8) to naturally interfere with endogenous αβTCRs, a readily translatable strategy. A T cell editing technique such as RNAi, TALENs, Zinc Finger Nucleases or CRISPR/Cas9, to knock out the endogenous TCR chains, provides an alternative approach for the introduction of a tumor specific receptor lacking natural interference with endogenous αβTCRs. However, clinical translation of these techniques might still need some years. GMP-grade anti-αβTCR beads recently became available and are typically used in the context of hematopoietic stem cell transplantations by others (9) and us (10).

Furthermore, clinical devices for apheresis and magnetic cell sorting are well established in daily routine, therefore the combination of such techniques opens a new avenue towards the broader application of engineered immune cells in men with more purified products. This has the potential to significantly reduce "off-target" effects. In addition, we observed increased anti-tumor responses, both *in vitro* and *in vivo*, of the αβTCR depleted cells as compared to a bulk engineered cellular product. Even though our approach resulted in a gradual re-expression of the endogenous αβTCR over time, allo-reactivity remained absent and tumor control preserved. Our method can be applied to virtual any engineered immune product in which competition with endogenous αβTCRs takes place. Choosing the most potent subset of T cells and increasing the purity of an

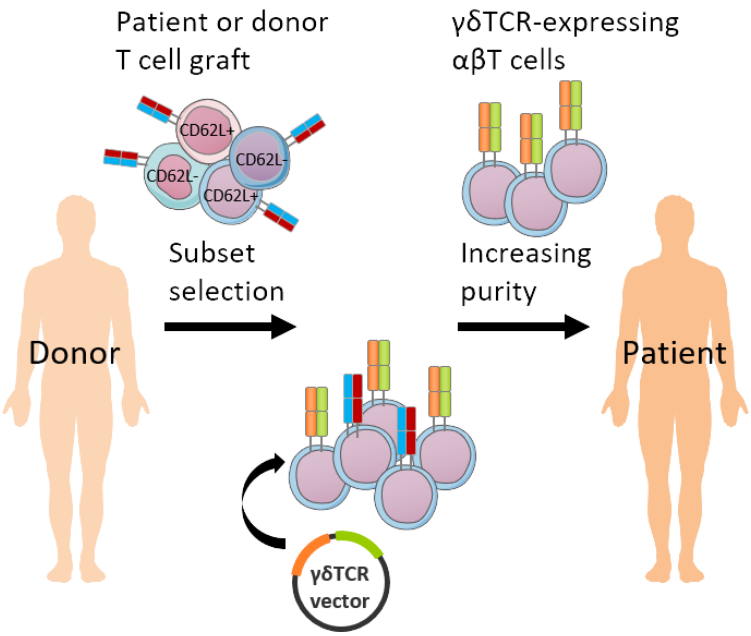

Figure 1. Choosing the most potent subset of T cells and increasing the purity of an engineered cellular product. The most potent subset of T cells will be selected from leukapheresis material, followed by the gene transfer of a tumor specific immune receptor, e.g. γδTCR and subsequent downregulation or additional knock-out of the endogenous αβTCR. Poorly- or non-transduced immune cells will be depleted from the cellular product before infusion in the patient.

engineered cellular product (Figure 1) are two complementary strategies that can bring cancer immunotherapy to the next level. Key is to exploit readily available GMP-grade methods to reduce costs and time to clinical application, in order to democratize implementation.

Conflict of Interest Statement
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GMP-Grade Manufacturing of T cells Engineered to Express a Defined γδTCR

6

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Abstract

γ9δ2T cells play a critical role in daily cancer immune surveillance by sensing cancer- mediated metabolic changes. However, a major limitation of the therapeutic application of γ9δ2T cells is their diversity and regulation through innate co-receptors. In order to overcome natural obstacles of γ9δ2T cells, we have developed the concept of T cells engineered to express a defined γδT cell receptor (TEGs). This next generation of chimeric antigen receptor engineered T (CAR-T) cells not only allows for targeting of hematological but also of solid tumors and, therefore, overcomes major limitations of many CAR-T and γδT cell strategies. Here, we report on the development of a robust manufacturing procedure of T cells engineered to express the high affinity Vγ9Vδ2T cell receptor (TCR) clone 5 (TEG001). We determined the best concentration of anti-CD3/CD28 activation and expansion beads, optimal virus titer, and cell density for retroviral transduction, and validated a Good Manufacturing Practice (GMP)-grade purification procedure by utilizing the CliniMACS system to deplete non- and poorly-engineered T cells. To the best of our knowledge, we have developed the very first GMP manufacturing procedure in which αβTCR depletion is used as a purification method, thereby delivering untouched clinical grade engineered immune cells. This enrichment method is applicable to any engineered T cell product with a reduced expression of endogenous αβTCRs. We report on release criteria and the stability of TEG001 drug substance and TEG001 drug product. The GMP-grade production procedure is now approved by Dutch authorities and allows TEG001 to be generated in cell numbers sufficient to treat patients within the approved clinical trial NTR6541. NTR6541 will investigate the safety and tolerability of TEG001 in patients with relapsed/refractory acute myeloid leukemia, high-risk myelodysplastic syndrome, and relapsed/refractory multiple myeloma.

Introduction

Chimeric antigen receptor engineered T (CAR-T) cells are currently entering clinical practice with remarkable response rates resulting in multiple FDA approvals in 2017 (1). Major limitations of current clinical strategies are, however, that CAR-T cells rarely offer solutions to solid tumors. Another restriction of current CAR-T approaches is that target antigens are often present on healthy tissues. Therefore, we introduced the concept of metabolic cancer targeting through a defined high-affinity Vγ9Vδ2T cell receptor (TCR) (2) and proposed to utilize T cells engineered to express a defined γδT cell receptor (TEGs) as the next generation of CAR-T. Vγ9Vδ2TCRs sense spatial and conformational changes of butyrophilin 3A1 (CD277) and RhoB mediated by intracellular phosphoantigen accumulation (PAg). Transformed cells often have accumulated PAg due to a dysregulated mevalonate pathway, enabling γ9δ2T cells to recognize them (3, 4). The TEG concept allows for selecting the most potent Vγ9Vδ2TCR and targeting of liquid and solid tumors (5). TEGs also overcome the diversity of natural γ9δ2T cells (6) and avoid negative regulation of the Vγ9Vδ2TCR through innate receptors of γ9δ2T cells (7). In addition, as Vγ9Vδ2TCR are introduced in both CD8 effector and CD4 helper cells, TEGs can deliver professional help through, e.g., maturing dendritic cells (5). For clinical testing of the TEG concept, we recently selected a highly tumor reactive Vγ9Vδ2TCR clone (clone 5) from the natural repertoire of a healthy individual (2). This particular Vγ9Vδ2TCR showed a strong reactivity toward a broad range of tumor cells within the TEG format, including primary leukemic blasts (8) as well as primary multiple myeloma cells (9). Due to the selection of a highaffinity Vγ9Vδ2TCR, TEGs also outperform natural γ9δ2T cells in terms of direct tumor recognition (2). For administration of TEGs in human, we recently proposed a purification step of TEGs by depletion of non- and poorly-engineered cells in order to further increase activity and definition of the product (8). However, a Good Manufacturing Practice (GMP)-grade procedure for a TEG drug product has not yet been defined. In this article, we describe the developmental process from a "research method" (8) to a manufacturing procedure that is fully compliant with GMP. Given that this process requires connecting two completely different worlds, a flexible research environment with a rigid GMP environment, the reported developmental process can be of high interest to researchers who aim at translating research findings to the clinic.

Materials and Methods

Production of Master Cell Bank (MCB) and Viral Vector Stock

The retroviral vector supernatant was produced in 293Vec-RD114 cells, a 293SF-based packaging cell clone producing RD114 pseudotyped viral particles containing MP71:TCRγ5-T2A-TCRδ5 transgene cassette, by BioNTech (Idar-Oberstein, Germany) (10, 11). To establish this packaging clone, first, a primary seed clone was established in a two-step transfection–transduction protocol. Candidate monoclonal cells were tested for the presence of the TCR transgene using qPCR. Transgene-positive clones were expanded to 14 cm Petri dishes in order to harvest supernatant. Primary seed clones were screened for virus titer production and the most productive cell clone (the producer cell line) was selected to grow a MCB. The MCB was released according to predefined criteria and stored in liquid nitrogen. Sequence integrity of the transgene was confirmed by sequence analysis of the MCB and TEG001 drug product samples.

Preparation of Leukapheresis Material

Patient-derived mononuclear cells obtained by leukapheresis were cryopreserved in freezing medium [sodium chloride (NaCl) 0.9% with 10% dimethylsulfoxide and 5% human albumin (HA)]. The material was thawed at 37°C and mixed with five volumes of leukapheresis thaw medium [X-VIVO 15 chemically defined medium without gentamicin and phenol red (Lonza, Breda, The Netherlands), hereafter, called X-VIVO 15, supplemented with 10% HA]. After washing, cells were resuspended in culture medium with cytokines (X-VIVO 15 medium with 5% human serum), 1.7×10^3 IU/ml of MACS GMP Recombinant Human interleukin (IL)-7 (Miltenyi Biotec, Bergisch Gladbach, Germany), and 1.5×10^2 IU/ml MACS GMP Recombinant Human IL-15 (Miltenyi Biotec).

Activation of T Cells

The cell suspension was diluted to a concentration of 1×10^6 T cells/ml with culture medium containing cytokines. T cells were activated by adding anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific, Etten-Leur, the Netherlands) to the cell suspension at a bead to cell ratio of 1:5 or otherwise indicated, homogenizing for 30 min at room temperature (RT) under rocking conditions, and subsequently incubating for 40–50 h at 37°C/5% CO_2. At Day 2, activated T cells were harvested by centrifugation and subsequently resuspended in culture medium containing cytokines. Manual cell count using trypan blue exclusion was performed and the cell

suspension was further diluted with culture medium containing cytokines to a target concentration of 0.5 × 10^6 viable cells/ml.

Transduction

Non-tissue culture treated 24-well plates (Thermo Fisher Scientific) were coated with Retronectin (Takara Bio, SaintGermain-en-Laye, France) at saturating conditions and incubated for 40–50 h at 2–8°C. At the day of transduction, the coated plates were incubated for 30 min at 37°C with 0.4% HA in NaCl, 0.9% to block unspecific binding. Next, viral supernatant was thawed at RT and diluted 1:1 with X-VIVO 15 medium, or as described in the relevant figure. RetroNectin-coated plates were coated with 2.0 ml/well diluted viral supernatant by spinning for 90 min at 500 × *g* at RT (one-spin hit transduction). The remaining supernatant was aspirated and discarded. Subsequently, 1 × 10^6 activated cells were added per well of the viral-supernatant-coated plates (2.0 ml cell suspension of 0.5 × 10^6 cells/ml) and incubated for 16–24 h at 37°C/5% CO_2.

At Day 3, transduced cells were harvested from the 24-well plate, centrifuged, and subsequently resuspended in culture medium with cytokines. Manual cell count was performed and the cell suspension was further diluted with culture medium with cytokines to a final target concentration of 0.25 × 10^6 viable cells/ml. The cell suspension was transferred to MACS GMP Cell Differentiation Bag(s) (Miltenyi Biotec) and incubated for 60–80 h at 37°C/5% CO_2.

Expansion of Transduced Cells

Transduced cells were cultured from Day 3 to Day 13. At Day 6, samples from cell suspension were taken to determine the concentration of viable cells by trypan blue exclusion. Transduction efficiency was determined by flow cytometry (% γδTCR positive T cells). The cell suspension was centrifuged and cultured in fresh culture medium supplemented with cytokines to a target concentration of 0.25 × 10^6 viable cells/ml and incubated for 36–48 h at 37°C/5% CO_2.

At Day 8, manual cell count was performed to determine the concentration of viable cells by trypan blue exclusion. The cell suspension, if applicable, was diluted to a target viable cell concentration of 1 × 10^6 cells/ml with fresh culture medium without cytokines. The total volume of cell suspension was then supplemented with half the cytokine concentration. The cell suspension was incubated for 36–48 h at 37°C/5% CO_2.

At Day 10, manual cell count was performed to determine the concentration of viable cells by trypan blue exclusion. The cell suspension was

centrifuged and further diluted with fresh culture medium supplemented with cytokines to a target viable cell concentration of about 1 × 10^6 cells/ml. The cell suspension was incubated for 60–80 h at 37°C/5% CO_2.

Purification of TEG001 by Research MACS Depletion of Non- and Poorly-Engineered Immune Cells

pMP71: γTCR-T2A-δTCR-transduced T cells were incubated with biotin-labeled anti-αβTCR antibody (clone BW242/412; Miltenyi Biotec), followed by incubation with an anti-biotin antibody coupled to magnetic beads (anti-biotin MicroBeads; Miltenyi Biotec). Next, the cell suspension was applied to an LD column in a QuadroMACS™ Separator. αβTCR-positive T cells were depleted by MACS cell separation according to the manufacturer's protocol (Miltenyi Biotec).

Purification of TEG001 by CliniMACS Depletion of Non- and Poorly-Engineered Immune Cells

At Day 13, the cell suspension volume was reduced, when necessary, to 150–200 ml by removing supernatant after centrifugation. Anti-CD3/CD28 beads were removed from the cell suspension of transduced T cells using a magnet (Dynamag Cell Therapy Systems magnet). The cell suspension was processed as follows:

a) Washed with phosphate buffered saline/ethylenediaminetet-raacetic Acid/HA buffer (PBS/EDTA buffer with 0.5% HA) and adjusted to a volume of 95 ml with PBS/EDTA/HA buffer. b) Incubated with 7.5 ml of TCRαβ-Biotin reagent (biotinlabeled anti αβTCR antibody (clone BW242/412; Miltenyi Biotec)) for 30 min on a swivel plate. c) Washed with 600 ml PBS/EDTA/HA buffer and after centrifugation, the volume was adjusted to 190 ml with PBS/EDTA/ HA buffer. d) Incubated with 15 ml of anti-Biotin reagent (anti biotin antibody coupled to magnetic beads) for 30 min on a swivel plate. e) Washed by adding PBS/EDTA/HA buffer to a volume of about 600 ml and removing supernatant after centrifugation. Subsequently, PBS/EDTA/HA buffer was added to a volume of about 200 ml and the αβTCR-expressing T cells (non- and poorly-engineered cells) were depleted using a CliniMACS Plus instrument (Magnetic Activated Cell Sorting) cell separation, program "depletion 3.1." f) Washed twice with infusion medium (NaCl 0.9% for infusion with 4% HA) and resuspended in infusion medium to obtain 25 ml TEG001 drug substance.

Cells and Cell Lines

Daudi (CCL-213) was obtained from the American Type Culture Collection and ML-1 was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Cell lines were authenticated by short tandem repeat profiling/karyotyping/isoenzyme analysis. All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. In addition, all cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using MycoAlert Mycoplasma Kit. Daudi and ML-1 were cultured in RPMI + 1% Pen/Strep + 10% FCS (Bodinco, Alkmaar, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or apheresis material obtained from the Sanquin Blood Bank (Amsterdam, the Netherlands).

Flow Cytometry

Antibodies used for flow cytometry include: pan-γδTCR-PE (clone IMMU510; Beckman Coulter, Woerden, the Netherlands), pan-αβTCR-APC (clone IP26; eBioscience, Thermo Fisher Scientific), CD4-V450 (clone RPA-T4; BD Biosciences), CD8αPerCP-Cy5.5 (RPA-T8; Biolegend), CD3-eFluor 450 (OKT-3; eBioscience), CD45-FITC (2D1; BD Biosciences), CD16-FITC (3G8; BD Biosciences), CD56-FITC (MY31; BD Biosciences), CD27-APC-eFluor780 (O323; eBioscience), CD45RO-PE-Cy7 (UCHL-1; BD Biosciences). All samples were analyzed on a BD LSRFortessa using FACSdiva software (BD Biosciences).

ELISPOT and ELISA Assays

IFNγ ELISPOT was performed as previously described (5, 6). Briefly, 15,000 TCR-transduced or mock-transduced (TEG-LM1) T cells and 50,000 target cells (ratio 0.3:1) were cocultured for
24 h in nitrocellulose-bottomed 96-well plates (Merck, SchipholRijk, the Netherlands), pre-coated with anti-IFNγ antibody (clone 1-D1K) (Mabtech, Nacka Strand, Sweden). Plates were washed and incubated with a second biotinylated anti-IFNγ antibody (clone 7-B6-1) (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were visualized with tetramethylbenzidine substrate (Sanquin) and the number of spots was quantified using ELISPOT Analysis Software (Aelvis, Hannover, Germany). IFNγ ELISA was performed using ELISA-ready-go! Kit (eBioscience) following manufacturer's instructions. Effector and target cells (E:T 15,000:15,000) were incubated for 24 h in the presence of pamidronate when indicated.

Statistical Analyses

Differences were analyzed using indicated statistical tests in GraphPad Prism 7 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

Defining Optimal Activation of Primary T Cells With Anti-CD3/CD28 Coated Beads

Stimulation of T cells with immobilized anti-CD3 and anti-CD28 antibodies provides both an antigen stimulus and co-stimulation for optimal T cell activation and expansion (12). Consequently, anti-CD3/CD28-coated beads are widely applied to engineer cellular products for different types of adoptive αβT cell therapies (13–16). The optimal anti-CD3/28 bead to CD3+ T cell ratio for activation, transduction with viral supernatant, and expansion of engineered immune cells is, however, frequently dependent on the specific transgene and production process. In order to define the best anti-CD3/28 bead to CD3+ T cell number for engineering TEGs, PBMCs were first analyzed for CD3+ T cell content by flow cytometry, and then incubated with various ratios of anti-CD3/CD28 beads in the presence of the cytokines interleukin (IL)-7 and IL-15. As a control stimulus, soluble OKT-3 and IL-2 was used. Next, T cells were transduced with non-GMP grade retroviral supernatant and expanded as described in the Section "Materials and Methods." After 10 days, the total number of TEGs, defined as double positive TEGs when expressing γδTCRs and αβTCRs or single positive TEGs when expressing γδTCRs only, were assessed by flow cytometry. The mean total cell number of single and double positive TEGs ranged from 2 to 6 × 10^6 cells when stimulated with anti-CD3/28 beads, and peaked at an anti-CD3/28 bead to T cell ratio of 1:5, while our standard OKT3 research protocol delivered 3 × 10^6 cells (Figure 1). Due to the limited number of replicates, when performing a Mann–Whitney *U*-test, the difference between none of the conditions was significant ($p > 0.05$). A 1:5 bead to T cell ratio was chosen for the activation of T cells in all following TEG manufacturing procedures. This ratio is sufficient to activate T cells and is substantially lower in numbers than advised by the manufacturer and, therefore, saves costs during the future production procedures.

Selection of a GMP-Grade Retroviral Producer Cell Clone

One of the most critical raw materials of the TEG manufacturing process is the viral vector supernatant used to transduce the T cells. Selection of a potent GMP-grade cell clone that produces the retroviral vector encoding the γδTCR is, therefore, critical for the success of the GMP-grade transduction process. The retroviral vector supernatant was produced using 293SF-based packaging cells, 293Vec-RD114, by BioNTech (10, 11).

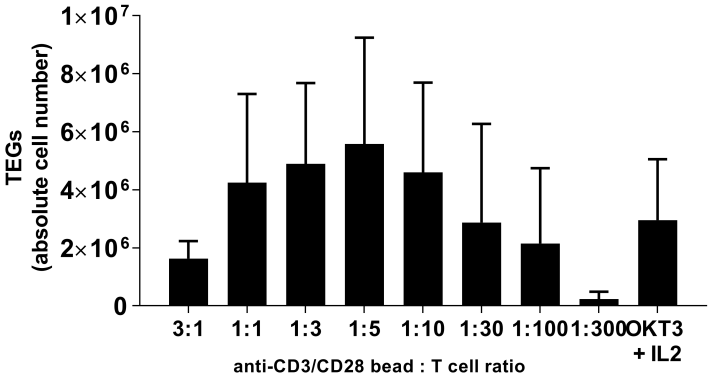

Figure 1. TEG yield depends on the optimal anti-CD3/CD28 bead to T cell ratio at the day of T cell activation. Multiple anti-CD3/CD28 bead to T cell ratios were tested and compared with respect to total TEG yield at day 10. After activation, transduction and expansion TEG numbers were defined by combining viable cell count with flow cytometry for γδTCR+ T cell percentage. OKT3 + IL2 served as a control activation and expansion stimulus. Mean absolute cell number + SD is shown, n = 2–3. The differences according to Mann–Whitney U tests are not significant ($p > 0.05$).

A large number of engineered producer cell clones were generated and the virus titer in the supernatant was assessed by titration experiments on Jurkat cells and analysis of γδTCR positive cells by flow cytometry (Figure 2A). The 8 best clones were selected for the second round of testing. Two additional clones from the upper midfield (#8, #62) were added to confirm the ranking (Figure 2B). Clone #73 was selected as the best GMP-grade retroviral producer cell line and was, therefore, further expanded and the titer was assessed before and after 0.45 µm filtration of the supernatant from different harvesting runs (Figure 2C). Filtration was performed in order to eliminate cell debris, a key step for generating GMP grade viral supernatant. This associated, however, with an up to sixfold reduction in viral particles in different harvesting runs (Figure 2C).

Virus Titer Impacts Transduction Efficiency of Primary T Cells

In the manufacturing of genetically modified cellular medicines, there is a strong relationship between transduction efficiency and the ability to produce sufficient cell numbers that meet predefined quality criteria (17). To optimize the production process we, therefore, assessed the amount of virus needed for optimal efficiency in a one-hit transduction. Viral supernatant generated in different pre-GMP proof runs from producer cell line clone #73 was used with virus titers ranging from 8.7×10^3 to 2.7

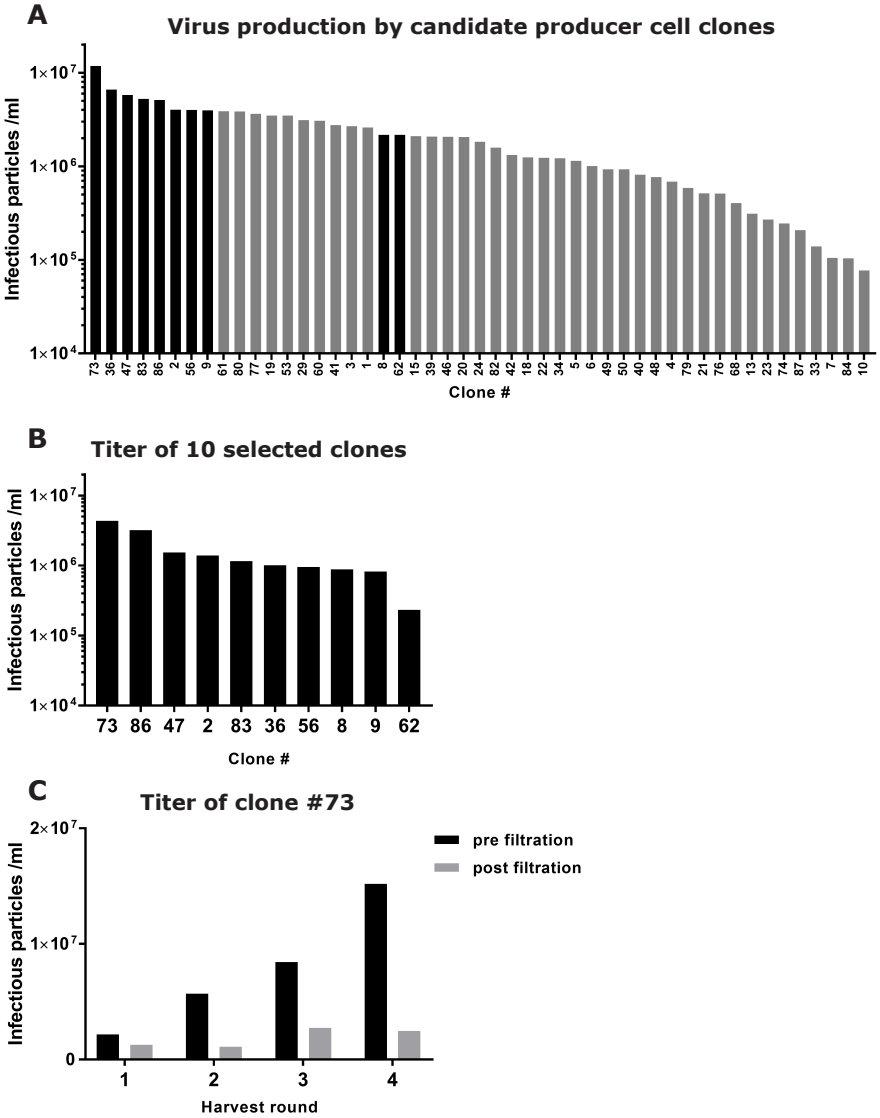

Figure 2. Selection of a viral vector producer cell clone. Retroviral supernatant was produced in 293vec-RD114 packaging cells. (A) The titer produced by the different clones was assessed in Jurkat cells. The clones depicted by the black bars were selected for a second round of testing **(B)**. **(C)** Clone #73 was picked for production of the TEG001 Good Manufacturing Practice retroviral supernatant. The titer was assessed after four rounds of harvest, before, and after filtration.

× 10^6 infectious particles (ip) per milliliters. Transduction efficiency was evaluated after 7 and 10 days of expansion with the optimized 1:5 anti-CD3/28 bead to T cell ratio. The percentage of TEGs was determined by flow cytometry using a pan-γδTCR antibody. The total number of both single and double positive TEGs was determined. Viral supernatant containing 1 × 10^6 ip/ml provided transduction efficiencies of 60–70% TEGs (Figure 3A). The majority of TEGs showed high expression levels of γδTCR while being negative for αβTCR due to the successful competition of the introduced γδTCR chains against endogenous αβTCRs for components of the CD3 complex as reported (8). Thus, virus titers around 1 × 10^6 ip/ml are sufficient for the generation of TEGs.

Impact of T cell Density on Transduction and Expansion Efficiency

It has been suggested that the density of activated T cells during the transduction procedure influences the transduction efficiency (18). Therefore, the optimal cell concentration during transduction was investigated within the context of four different virus titers (range 0.03–1.5 × 10^6 ip/ml), and five different T cell densities (range 0.1–2.0 × 10^6/ml). During the expansion phase, T cell densities were adjusted to defined concentrations at day 3 and 6 (both 0.25 × 10^6/ml), and day 8 and 10 (both 1.0 × 10^6/ml). The percentage of γδTCR-positive T cells was determined after 7 and 10 days by flow cytometry using a pan-γδTCR antibody (Figure 3B). Differences in TEG transduction efficiencies were only observed for very low virus titer conditions (0.03 × 10^6 ip/ml). A cell concentration of 0.5 × 10^6/ml was selected as the standard cell density during transduction for TEG001 manufacturing process.

Impact of GMP-Grade Virus Titer From Clone #73 on Transduction Efficiency

Due to procedural differences between the manufacturing of research-grade and GMP-grade retroviral supernatant, the relationship between virus titer and transduction efficiency for the final GMP viral supernatant batch, which will be used for the production of TEGs for the clinical trial, was further investigated. T cells were transduced at a density of 0.5 × 10^6 cells/ml with different dilutions of GMP-grade viral supernatant in a onespin hit transduction procedure. The transduction efficiency and total γδTCR positive cell numbers were evaluated after 6, 9, and 13 days by flow cytometry using a pan-γδTCR antibody (Figure 3C). The percentage of TEGs with the different pre-GMP titers was highest between day 6 (Figure 3C) and day 10 (data not shown) followed by a small decrease,

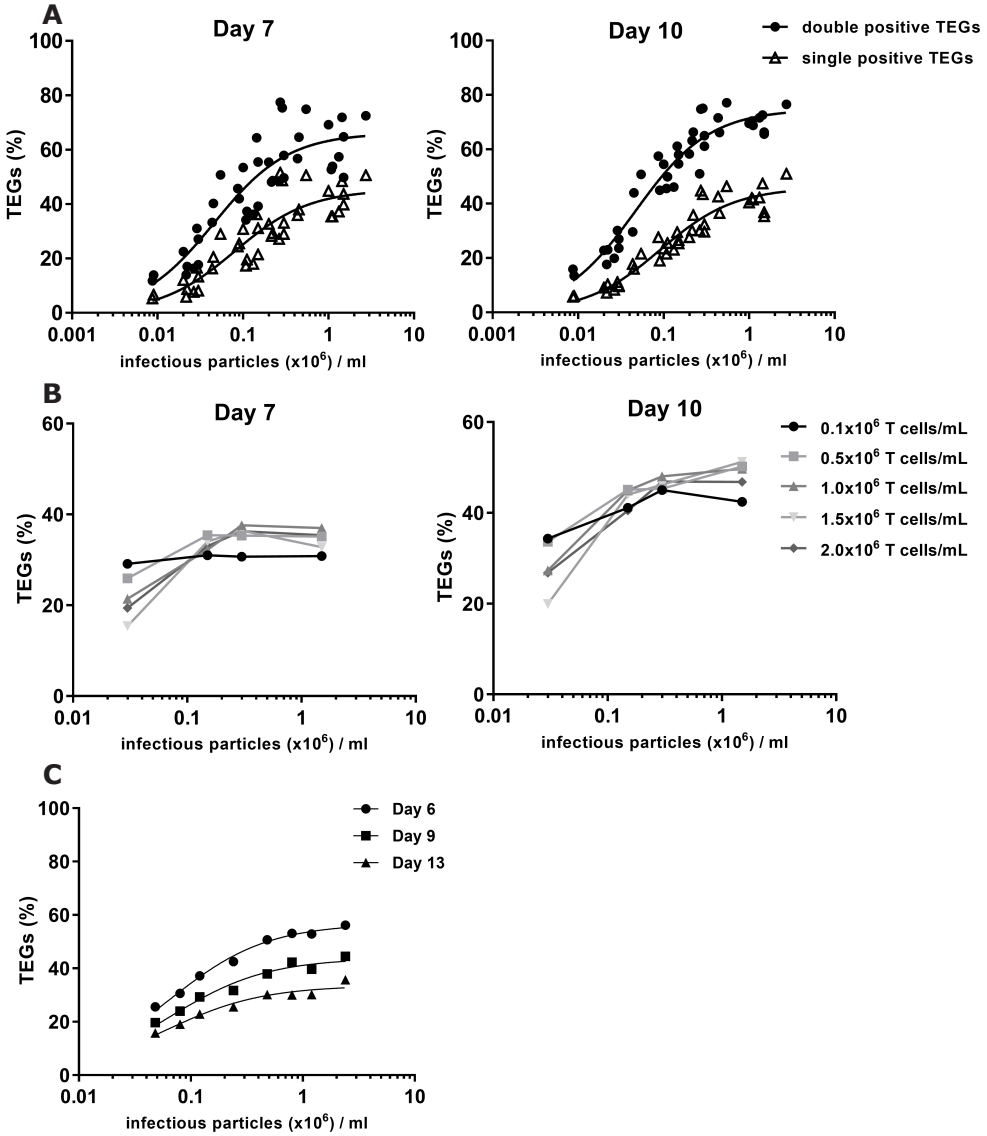

Figure 3. Transduction efficiency depends on the virus titer. T cells were activated with 1:5 CD3/CD28 bead to cell ratio followed by retroviral transduction with the γδTCR and expansion. (A) 0.5 × 10^6 cells per ml were transduced with different concentrations of pre-Good Manufacturing Practice (GMP) viral supernatant. The % TEGs are shown as total double positive TEGs (black circles) and single positive TEGs (open triangles) at day 7 and day 10 after activation. **(B)** The relationship between T cell concentration and transduction efficiency was investigated. Transduction efficiency was evaluated after a 7- and 10-day expansion period, for a range of T cell concentrations during transduction and four different virus titers. **(C)** 0.5 × 10^6 cells/ml were transduced with different concentrations of GMP viral supernatant to determine the relationship between virus titer and transduction efficiency after a 6-, 9-, and 13-day expansion period.

most likely in line with our previous observation that engineered immune cells have a slight disadvantage in proliferative capacity early after transduction when compared to non engineered immune cells (19). In line with the observation for pre-GMP-grade viral supernatant, the final GMP-grade viral supernatant that will be used for the clinical study provided transduction efficiencies of up to 60% TEGs when utilizing a titer of 1.2×10^6 ip/ml (Figure 3C).

Higher γδTCR Expression Increases Antitumor Activity

Defining a potency assay for a medicinal product is critical for assessing whether the final product is biologically active. Previous reports suggested that γδTCR expression levels correlate with activity of TEGs (5, 8). To formally confirm that indeed γδTCR expression is key for TEG activity, we used a defined CD4+ T cell clone (20), which underwent the transduction procedure but remained untransduced (T cells A) or was transduced with the MP71:TCRγ5-T2A-TCRδ5 retroviral vector, resulting in low and intermediate γδTCR single positive cell lines (T cells B and C, respectively). Primary T cells from a GMP proof run were used to generate the cell line with a high single positive γδTCR fraction that was further purified for CD4+ T cells by CD4 MACS selection after transduction (T cells D) (Figure 4A). Next, activity of theses γδTCR cell lines with different amounts of single γδTCR-positive T cells was compared side by side in the presence of pamidronate against Daudi as a positive, or ML1 as a negative tumor target (Figure 4B). CD4+ TEGs with higher γδTCR expression had a higher activity in terms of IFNγ cytokine secretion when compared to CD4+ T cell clones with lower or literally absent γδTCR expression. As control, T cells A–C, which express an endogenous allogeneic HLA-DPB1*04:01-reactive αβTCR, were coincubated with an HLA-DPB1*04:01 expressing B cell line. This resulted in cytokine levels equivalent or higher than from T cells D, indicating that T cells A–C were highly functional when triggered by the endogenous TCR (data not shown). These data are in line with previous reports from our group (8) and support the rationale to enrich in the GMP process only for TEGs with highest γδTCR expression. Therefore, we defined γδTCR-positive expression as a potency assay for TEGs and γδTCR single positive TEGs defines the functionally most active population.

Enrichment of TEGs by CliniMACS Through Depletion of Non- and Poorly-Engineered Immune Cells

Non- and poorly-engineered cell fractions are usually present in genetically engineered T cell products and associate with little or no activity as shown

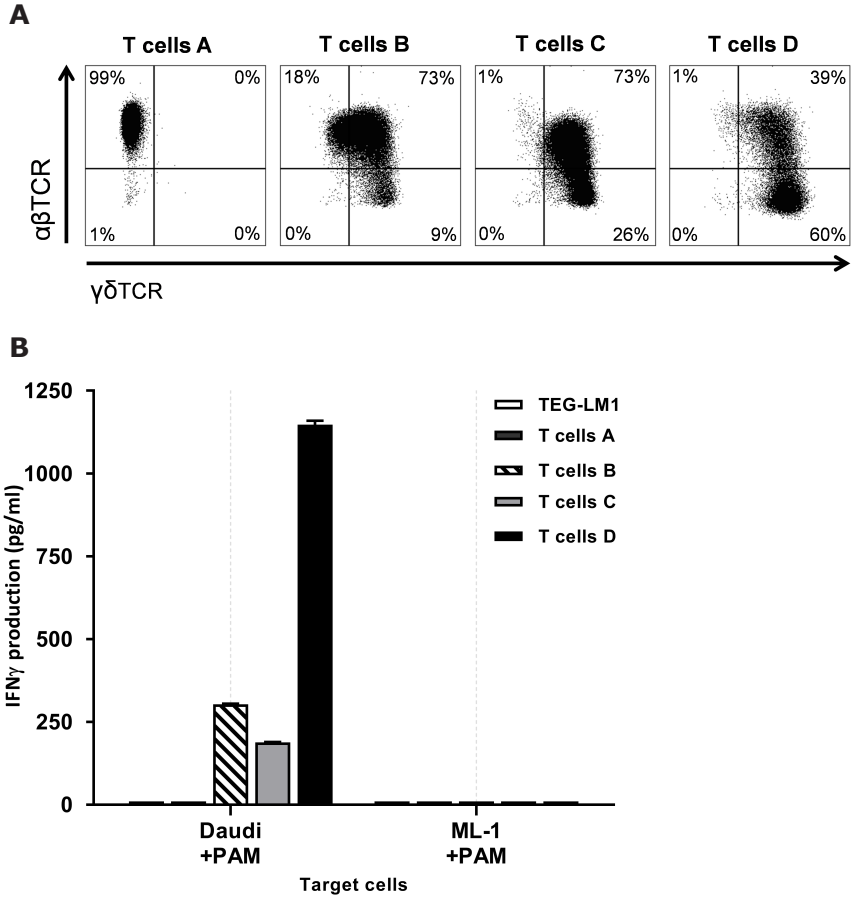

Figure 4. γδTCR expression defines functional activity. (A) A defined CD4+ T cell clone (20) underwent the transduction procedure but remained untransduced (T cells A) or was transduced with the MP71:TCRγ5-T2A-TCRδ5 retroviral vector, resulting in low and intermediate γδTCR single positive cell lines (T cells B and C, respectively). Primary T cells were used to generate the cell line with a high single positive γδTCR fraction that was further purified for CD4+ T cells by CD4 MACS selection after transduction (T cells D). In all cases, after one cycle of expansion, γδTCR and αβTCR expression was measured in the viable CD45+ gate by flow cytometry after which the cells were used in a function assay. **(B)** Different T cells were coincubated with the indicated tumor target cell lines in the presence of pamidronate in triplicate. Daudi is the prototypic TEG001 positive target, ML-1 is the negative control target. TEG-LM1 served as the negative control effector. After 20 h at 37°C, supernatant was harvested and analyzed by IFNγ ELISA. Mean IFNγ production + SD is shown.

also for TEG001 (Figures 4A,B). In addition, such cell fractions could even be harmful as they might harbor unwanted specificities. To avoid this potential drawback, a procedure for the depletion of non- and poorly-engineered T cells was developed by taking advantage of the observation that upon introduction of a γδTCR, the endogenous αβTCR expression is substantially decreased or even absent (2, 5). First, we defined "in process controls." During six large-scale proof runs, the percentage of γδTCR-positive cells was, therefore, assessed at day 6 (in process control), before and after the CliniMACS depletion procedure. Between 31 and 63% of all cells were positive for γδTCR at day 6, and 73–92% at day 13 after CliniMACS depletion (Figure 5A). In addition, we assessed the robustness of the observation that introducing γδTCR substantially outcompetes endogenous αβTCRs during different GMP runs. The ratio of single positive to double positive TEGs ranged between 1.6 and 2.5 and was above 1.5 for all runs (Figure 5B). Then, we aimed to assess whether a depletion of non- and poorly-engineered TEGs could not only be performed with research devices (8), but also with GMP-grade αβTCR beads on a CliniMACS device. Therefore, we compared side by side the research-scale and largescale depletion of non- and poorly-engineered TEGs through αβTCR depletion on a CliniMACS. After the depletion procedure, we observed a comparable purity of single positive TEGs after both procedures (research-grade: 73% versus clinical-grade: 76%; Figure 5C), while the remaining αβTCR positive T cell fraction was very low for both research-grade and GMP-grade (0.3 and 0.0%, respectively). The αβTCR negative γδTCR negative populations present in both research-grade and GMP-grade depleted products (24 and 27%, respectively) mainly consisted of NK cells (data not shown). This double negative population was present at the end of all manufacturing runs (n = 6, range 8–27%), and was donor and batch dependent. The recovery after CliniMACS αβTCR depletion, indicated as percentage of γδTCR + T cell output of the respective input, varied between 19 and 33% (n = 6, Figure 5D). This procedure allowed us to produce TEGs in numbers up to 2×10^9 cells and is, therefore, sufficient to deliver dosages needed for the planned clinical study. The complete manufacturing schedule is depicted in Figure 5E.

Immunological Phenotype of TEG001 Drug Substance

Next, we characterized the immunological phenotype of the drug substance TEG001, as the *in vivo* proliferation capacity and function of genetically modified cell therapy products is not only determined by the introduced receptor but also by the differentiation phenotype of the individual T cells.

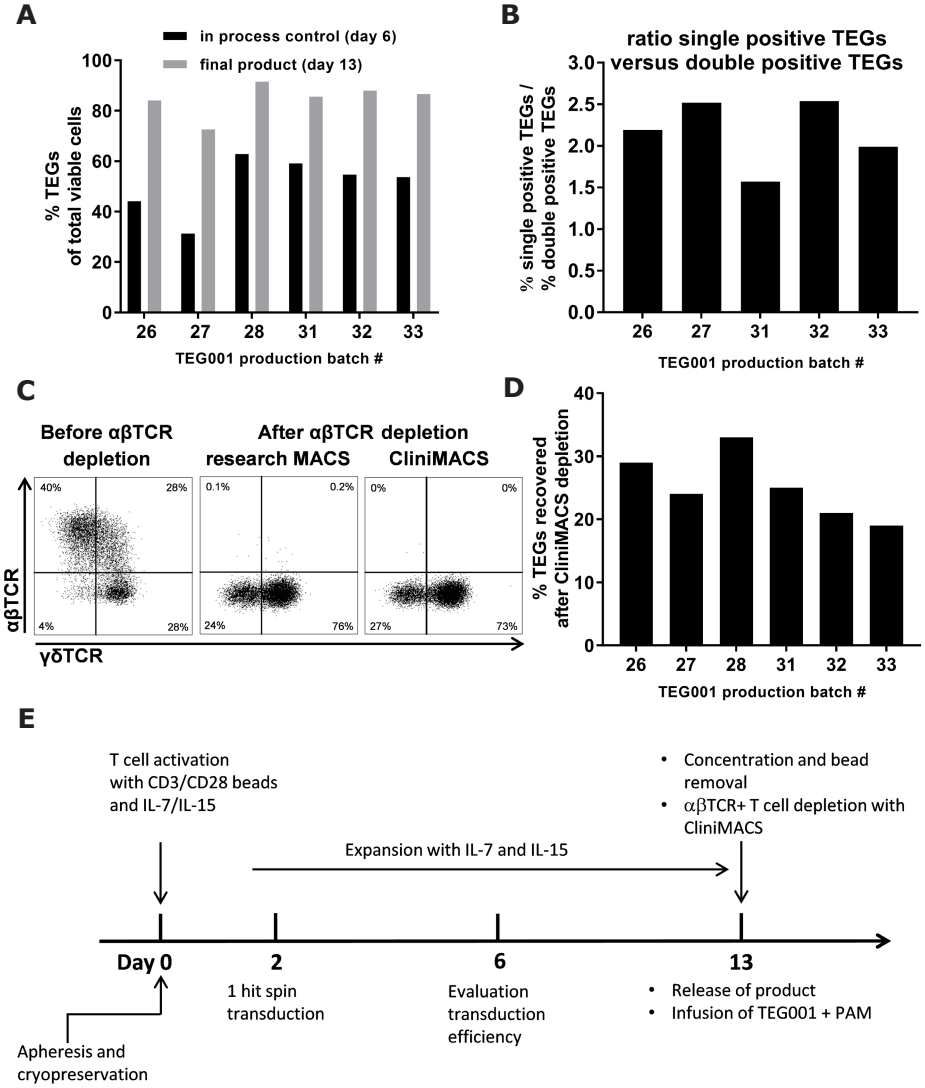

Figure 5. Successful enrichment of TEGs by CliniMACS depletion. (A) Comparison of TEGs transduction efficiency during production (in-process control, day 6) and at the end of production (final product) of six different production batches. **(B)** After introduction of pMP71:γTCR-T2A-δTCR and expansion of the T cells both γδTCR+αβTCR− T cells as γδTCR+αβTCR+ T cells are present. **(C)** During one of the research scale production batches, the cells at Day 13 were split and the non-transduced T cells were depleted using the research MACS or CliniMACS cell separation systems. **(D)** γδTCR+ cell recovery as percentage of the γδTCR+ cell input was measured after each αβTCR CliniMACS depletion. **(E)** Overview of the Good Manufacturing Practice TEG001 production process.

The differentiation phenotype of TEGs was determined by measuring the expression of CD27 and CD45RO (21) of five pre-GMP production runs. The major subset of TEGs was, after a 2-week expansion period, T cells with an effector (T_{eff}) and effector memory (T_{em}) phenotype (Figure 6A). In addition, all products contained engineered central memory T cells (T_{cm}), an immune subset enabling potential long-term persistence of TEG *in vivo* (22).

Release Specifications of TEG001 Drug Substance and TEG001 Drug Product, Batch Analyses, and Stability

Release specifications are an essential component of quality assurance and protect the patient from receiving a suboptimal cellular product. Based on our small-scale runs, we defined, therefore, product release specifications. To avoid overly stringent product definition criteria, which could result in the discarding of a product for clinical use, we defined release criteria for the TEG001 drug substance as ≥50% γδTCR positive T cells, ≥70% viability, and ≤10% αβTCR positive T cells in addition to standard microbiological release criteria (Table 1). All six large-scale runs met these release criteria as indicated in Table 2. In addition, after formulation of the drug product, stability of TEG001 drug product was assessed. This is essential for clinical practice as products are frequently administered for logistical reasons within 1 day after production is complete. Therefore, a fraction of TEG001 drug product was stored for 16 and 20 h at 4°C and tested for viability over time. All three tested batches remained stable

Table 1. Release specifications TEG001 drug substance.

Parameter	Method	Acceptance criteria
Identity		
- TEG001	Flow cytometry	Identity confirmed
Purity		
- % γδTCR-positive T cells	Flow cytometry	≥50%
- Viability	Manual cell count	≥70%
Impurities		
- % αβTCR-positive T cells[a]	Flow cytometry	≤10%
Microbiology		
- Sterility	Ph.Eur	Negative
- Mycoplasma	Ph.Eur	Negative
- Endotoxins	Ph.Eur	<2.0 IU/ml

The TEG001 release specifications as defined in the investigational medicinal product dossier.
[a]Only applicable for patients who previously received an allogeneic hematopoietic stem cell transplantation.

over the tested time period (Table 3). Next, we tested whether GMP-grade TEG001 drug product is functional after storage for 20 h at 4°C, by co-incubation of TEG001 and TEG-LM1 (mock control) with the reference target cell line Daudi, in the presence of pamidronate (2, 8). TEG001 was effective in recognizing Daudi, while there was no recognition by mock TEGs when assessed by IFNγ ELISPOT (Figure 6B).

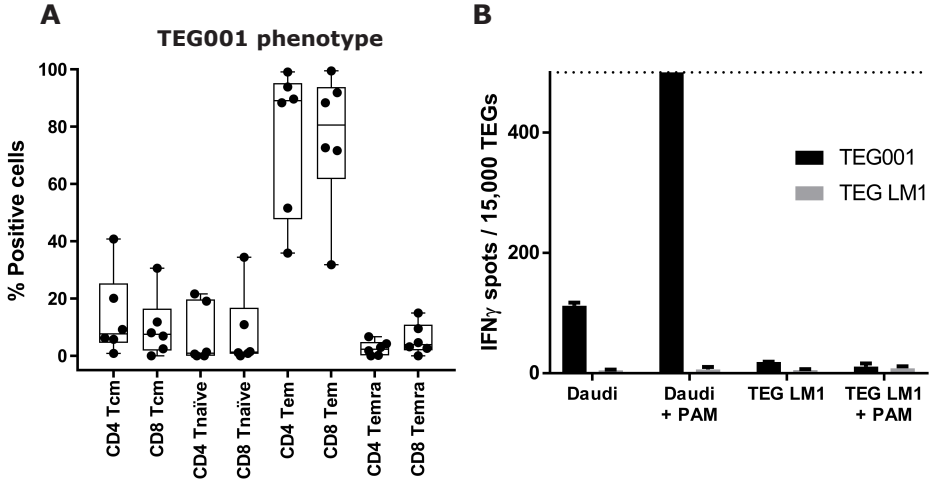

Figure 6. TEGs have a predominant effector-memory/effector phenotype. (A) The phenotype of TEGs from four different donors was determined by measuring CD45RO in combination with CD27 expression on day 13, after the CliniMACS depletion. CD45RO+/CD27+ is considered as T_{cm}, CD45RO−/CD27+ as T_n, CD45RO+/CD27− as T_{em}, and CD45RO−/CD27− as T_{emra} (21). **(B)** TEGs were produced according to the described procedure after which they were stored at 2–8°C. After 20 h, the TEGs were coincubated with Daudi in the absence and presence of pamidronate (PAM) as a positive target, or TEG LM1 in the absence and presence of PAM as a negative target. TEG LM1 as effector served as the negative control. The maximum assay sensitivity was set at 500 spots (dashed line).

Table 2. TEG001 drug substance batch analysis data of large scale runs.

Parameter	Acceptance criteria	Run					
		26	27	28	31	32	33
Identity							
- TEG001	Identity confirmed	Pass	Pass	Pass	Pass	Pass	Pass
Purity							
- γδTCR-positive T cells	≥50%	84%	73%	92%	86%	88%	87%
- Viability	≥70%	99%	98%	99%	97%	100%	100%
Impurities							
- αβTCR-positive T cells[a]	≤10%	0.0%	0.0%	0.2%	0.5%	0.4%	0.1%
Microbiology							
- Sterility	Negative	ND	ND	negative	negative	ND	ND
- Mycoplasma	Negative	ND	ND	negative	negative	ND	ND
- Endotoxins	<2.0 IU/ml	ND	ND	Pass	Pass	ND	ND

TEG001 was produced using our Good Manufacturing Practice large scale production protocol in multiple large scale manufacturing runs. TEG001 was formulated after which the identity, purity, and viability was assessed using trypan blue exclusion and flow cytometry.
[a]Only applicable for patients who previously received an allogeneic hematopoietic stem cell transplantation.

Table 3. Stability data of TEG001 cell suspension for infusion stored at 2–8°C.

Time point	Parameter	Run (content TEG001 cells per 100 ml drug product)						
		31	31	32	32	32	33	33
		69×10^6	12×10^7	71×10^6	14×10^7	26×10^7	73×10^7	31×10^7
	Viability	97%	97%	100%	100%	100%	100%	99%
T = 0 h	Viable cell number recovery	100%	100%	100%	100%	100%	100%	100%
Storage	Viability	96%	97%	99%	100%	99%	100%	100%
T = 16 h	Viable cell number recovery	93%	94%	99%	94%	90%	100%	97%
Storage	Viability	94%	94%	100%	100%	99%	100%	100%
T = 20 h	Viable cell number recovery	84%	94%	99%	94%	90%	97%	97%

TEG001 was produced using our Good Manufacturing Practice large scale production protocol in multiple large scale manufacturing runs. TEG001 was formulated in NaCl 0.9% for infusion with 4% HA at different cellular concentrations, to study the effect of TEGs density on cell viability and viable cell recovery at T = 0 and after storage at 2–8°C for 16 and 20 h.

Discussion

We have developed a robust GMP-grade TEG production protocol, which not only includes a conventional transduction and expansion step but also a very stringent CliniMACS enrichment procedure to guarantee high purity of the drug substance. This purification procedure can be used for any engineered immune cell product, which associates with a reduced expression of the αβTCR, like CAR-T introduced in the αTCR locus (23, 24). By utilizing this protocol, we have been able to produce and enrich TEGs in numbers, which are sufficient to reach the highest dose level of our upcoming phase I trial NTR6541. Furthermore, we have shown that γδTCR expression can be used as potency assay for TEG001, and that the TEG001 drug product is stable for at least 20 h at 4°C, which allows for provisional release and transportation to the location of the infusion.

In current manufacturing processes of CAR-T cells, purification steps are often not included. As a consequence, final products currently infused into patients harbor only between 15 and 55% of engineered immune cells (18, 25, 26). The lack of purity can become a major clinical obstacle, in particular, when engineering T cells from patients who relapse after allogeneic stem cell transplantation. Re-infusion of CAR-T cells in patients after allogenic stem cell transplantation has been reported to associate with incidences of acute and chronic graft versus host disease (GvHD), up to 10% (27). GvHD after infusion of CAR-T cells is most likely a consequence of endogenous αβTCRs still expressed at physiological levels in CAR-T cells, as well as the presence of non-engineered immune cells within the product. With our TEG concept, we provide comprehensive solutions to these problems. First, as suggested from our previous data, not only in the research environment (8) but also with our presented GMP manufacturing process, endogenous αβTCRs are substantially downregulated in TEGs. Reduced expression of endogenous αβTCRs is most likely due to the efficient competition of the introduced γδTCR against endogenous αβTCR for CD3 components of a T cell. Second, the additional GMP enrichment procedure utilizing the CliniMACS system achieves purity of TEGs, which can exceed 90%. The removal of non- and poorly-engineered cells from the final drug substance has another advantage, in addition to the reduced risk of GvHD and a more potent product due to enrichment of γδTCR-positive cells; the improved competition for homeostatic cytokines (28, 29). Other strategies for the refinement of engineered immune cells have been developed recently. However, alternative purification strategies frequently depend on the introduction of an additional truncated protein

such as CD19 or epidermal growth factor receptor, which are normally absent in the T cell lineage (30). Using a transgene cassette with an additional sequence for selection purposes can lead to lower transduction efficiencies and reduced expression of the introduced immune receptor or alternative homing (J. Kuball, unpublished observation) and associates frequently with unwanted T cell activation and immunogenicity (31). To the best of our knowledge, we have developed the very first GMP manufacturing procedure in which αβTCR depletion is used as a purification method, thereby delivering untouched clinical-grade engineered immune cells. Despite an efficient elimination of non- and poorly-engineered αβT cells, our procedure also enriches for NK cells. An additional purification step before T cell engineering might, therefore, be intriguing for the next generation of TEG-manufacturing, such as αβTCR+ or CD3+ cell selection before T cell activation (32), as proposed by others.

Advanced therapy medicinal products (ATMPs), such as TEGs, are individualized and complex biological products that require careful consideration of their nature in order to define adequate "in process" and "release" tests. ATMPs are also frequently freshly prepared and directly infused into patients after production, limiting the possibilities for extensive safety and release testing for an individual product. Despite the limited possibilities, regulatory authorities oblige a potency assay before batch release. Potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" (33). Potency must be measured in a robust and biologically relevant way, which reflects the mechanism of action. Thus, defining valid potency assays can be a major challenge for ATMPs. For CAR019, expression of the introduced receptor as a potency assay has been proposed by vendors and accepted by the FDA (17, 34). We provide now evidence that for TEGs, γδTCR expression levels are an adequate potency assay. However, expression levels of receptors are rather simplified and surrogate methods to assess for activity will not predict efficacy *in vivo*. Therefore, alternative methods are needed. Highthroughput characterization of TEGs on single cell levels could be interesting alternatives, as previously also reported for CAR-T (35).

In conclusion, we have developed a GMP-grade manufacturing strategy for TEGs incorporating an αβTCR depletion to obtain a final product substantially enriched for TEGs. The described process can also be valuable for any CAR-T product interfering with endogenous αβTCR expression. We also

defined release and potency criteria acceptable for competent authorities. TEG001 will be used for an upcoming phase I dose escalation clinical trial registered as NTR6541. This trial aims to investigate the safety and tolerability of TEG001 in patients with relapsed/refractory acute myeloid leukemia, high-risk myelodysplastic syndrome, and relapsed/refractory multiple myeloma.

Conflict of Interest Statement

GK, ZS, and JK are inventors on different patents with γδTCR sequences, recognition mechanisms, and isolation strategies. JK is CSO and shareholder of Gadeta (www.gadeta.nl). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Summarizing Discussion 7

Summarizing discussion

We are entering an era in which the standard of care for cancer patients is shifting from chemotherapy to more specific and truly personalized medicine. Ever since the first results of autologous *ex vivo* expanded tumor infiltrating lymphocytes (TILs) for the treatment of advanced melanoma, the enormous potency of the immune system to treat cancer has become evident (1, 2). Unfortunately, TILs can only be obtained from cancer patients with relatively large tumor lesions and even then is only effective in a small percentage of these patients. This limited success rate can be attributed to the fact that the tumor has a low mutational load (3) or is devoid of T cell infiltrate (4). The latter could be a consequence of the complete absence of tumor-specific T cells, which will therefore not enable T cell infiltration within the tumor tissue. However, also the presence of infiltrated T cells in itself is not a prognostic marker since these can be true bystander T cells, which are ineffective in recognizing the tumor cells (5). An additional reason for lack of immune infiltration can be the fact that the tumor protects itself from immune infiltration (6).
More recently, adoptive transfer of engineered T cells has gained a lot of attention since chimeric antigen receptor (CAR) T cells have been approved for therapeutic use due to their promising clinical results. Both FDA and EMA approved CAR T cell therapies, Kymriah & Yescarta, and virtually all CAR T cell therapies in clinical trials are directed towards CD19 (7). CD19 is a B cell marker expressed on all peripheral B cells, but not the progenitor B cells in the bone marrow, and therefore an ideal target in case of B cell malignancies such as acute lymphoblastic leukemia (8) and diffuse large B-cell lymphoma (9). CD19 CAR T cell products do not necessarily have to be able to differentiate between healthy and malignant CD19 expressing cells due to the fact that hematopoietic progenitors are not targeted and temporary B cell aplasia is tolerated (10). Other CAR-based immunotherapies and antibody therapies also rely on the expression of extracellular tumor specific antigens, which can be downregulated or shed by the tumor, leading to therapy resistance (11).

αβTCR engineered T cells for the treatment of cancer

The before mentioned TIL therapy can be very effective in the scenario where tumor-reactive T cells are available within the patient and can be isolated from the tumor tissue. Since the applicability of TIL therapy is very limited, scientists have attempted to use the reactivity

of tumor-specific αβT cells by isolating the αβT cell receptor (αβTCR). When identified, these tumor-reactive αβTCRs can be used as tumor recognizing moieties by introducing them in other αβT cells (12). However, in contrast to CAR-engineered T cells therapies, no αβTCR engineered T cell therapy has been approved just yet. One probable reason is that many tumor-associated antigens targeted by αβTCRs are not exclusively expressed on tumor cells (13) and can therefore cause off-target or on-target off-tumor toxicities, as illustrated in several clinical trials with devastating results (14, 15). We have attempted to further potentiate αβTCR engineered T cell therapies by developing an elegant select-kill mechanism by the introduction of a limited number of murine amino acids on a specific location of the TCR β-chain. This allows for the purification of the product *in vitro* and depletion of the engineered cells *in vivo*. Purification of αβTCR engineered T cells is important to increase the efficacy and decrease potential toxicity of these products (16-18). Currently, engineered T cell products only have a purity of 15-55% (19, 20), indicating there is ample room for improvement. Elimination of engineered T cells after adoptive transfer might be needed in case of cytokine release syndrome (18) or off-target toxicities due to peptide mimicry (14). The presented approach can be applied to engineered T cell products based both on TCRs recognizing the common cancer/testis antigens or the so-called neo-antigens, which are a direct consequence of genetic alterations in tumor cells (21).

γδTCRs as broadly tumor specific receptors for novel immunotherapies

Most γδT cell receptors (γδTCRs) recognize stress induced ligands or metabolic alterations and are thereby able to differentiate between healthy and malignant cells. In contrast to antibody and CAR-based immunotherapies, most γδT cells are not restricted to one specific antigen and are therefore broadly tumor reactive. Another clear benefit of using γδT cells or their γδTCRs for therapy is that their effectivity does not depend on the presence of tumor-reactive T cells, which is the case for both checkpoint-inhibitors and TIL-therapy (22). However, to use γδT cells or their γδTCRs as therapeutical tools in the clinic, regulatory hurdles need to be taken. For example, the poor characterization of many ligands complicates efficacy and toxicity testing. These tests are needed for approval of clinical studies by authorities as well as for a better patients selection.

In addition to TCRs derived from Vδ2 positive γδT cells (23-25), also

TCRs extracted from Vδ2 negative γδT cells are promising for use as receptors in the "T-cells engineered to express a defined γδT cell receptor (TEG) format" (16, 26), since these γδTCRs provide a myriad of potential new targets. Crucially, we have shown to be also able to transfer the tumor-specificity of these γδTCRs to αβT cells (27). These δ2 negative TCRs, containing a δ1 or δ3 variable TCR chain frequently recognize stress-induced ligands, which often belong to the MHC-like family of proteins (28). While others have recently described a γδTCR recognizing tumor-antigens within classical HLA molecules (29), we are among the first to have characterized a γδTCR which recognizes target cells in an allo-MHC class I restricted fashion and is able to discriminate between healthy and tumor tissues. The described γδTCR clone FE11, and more importantly the FE11 γδTCR in the TEG format (TEG011), only recognizes HLA-A*24:02 positive malignant cells while leaving HLA-A*24:02 healthy cells untouched. Allo-HLA reactivity of γδT cells has been suggested before (30, 31), however, it had been considered as a general phenomenon, not restricted to malignant transformed cells. Thus, our observation of a γδTCR acting in an HLA-restricted fashion is not based on classical allogenicity, since, in contrast to earlier A*24 reactive γδT cells described (30), healthy cells are not recognized by the FE11 γδTCR. However, it could also be that previous reports missed this intriguing property, as they did not analyze healthy and malignant cells in detail. Our data suggest that, in contrast to the classical αβTCR peptide-MHC recognition and the recently described γδTCR recognizing MART-1 on HLA-A*02:01 (29), not one specific peptide is involved in recognition. It is postulated that peptides are essential to support the structure which is recognized by γδTCR FE11. In addition, a different organization of the HLA at the cell membrane seems to be a discriminating factor, though formal proof for this hypothesis is still pending.

Science in translation focusing on TEGs

Recent advances in the field of tumor immunology and immunotherapy have offered new treatment modalities to cancer patients. However, the swift entry of cellular immunotherapy into the clinic is hampered by the extensive regulatory requirements (7). When comparing cellular therapies to the more established monoclonal antibody therapies, it is evident that cellular products are heterogeneous and therefore far more complex. This complexity, likely combined with some fear for this new class of therapies, makes regulatory authorities hesitant in the approval for new advanced therapy medicinal products (ATMPs), such as CAR- and TCR-engineered T

cells. There seems to be a one to one correlation between the precision of ATMPs and the complexity regarding both manufacturing and regulatory procedures (32). To enhance the implementation of cell therapy products we propose to exploit readily available GMP-grade methods (33). A key example of how we are making use of readily available methods is by using GMP-grade anti-αβTCR beads which are typically used in the context of hematopoietic stem cell transplantations (4, 34). Unfortunately, when manufacturing is becoming more and more complex, these methods are increasingly unavailable.

All clinical trials using γδT cells thus far have relied on *in vitro* or *in vivo* expanded bulk γδT cells (35). The major subset of γδT cells used in these trials are Vγ9Vδ2T cells, due to their relatively large abundance in the periphery and the ability to expand these cells by using aminobisphosphonates such as zoledronate or pamidronate (36), which are both approved drugs (37). Multiple trials have relied on the *in vitro* expansion of autologous or haploidentical Vγ9Vδ2T cells, while others have tried to expand γδT cells *in vivo* by the injection of pamidronate (38) or zoledronate (39) in combination with interleukin (IL)-2. The expansion of γδT cells in patients seems to be less potent than in healthy controls, which could attribute to the disappointing clinical response observed (38). Multiple other reasons can attribute to these unsatisfactory clinical trial results, such as the fact that expanded bulk γδT cells, both *in vitro* and *in vivo*, are characterized by their large diversity in terms of specificity and avidity (40). Furthermore, the exhaustion or even death of effector cells due aminobisphosphonate stimulation can be detrimental to the therapy (38). Clinical trials using γδTCRs may therefore benefit from the use of our TEG concept, in which one defined high affinity γδTCR is used, resulting in a product with a homogenous specificity. Since αβT cells are the carriers for our defined γδTCR, *in vitro* expansion using aminobisphosphonates is not required, preventing the potential exhaustion by these compounds. Our first clinical TEG product (TEG001), in which a defined high affinity Vγ9Vδ2TCR is used, the *in vitro* expansion is based on the use of anti-CD3/CD28 beads (41) in the presence of the cytokines IL-7 and IL-15 (26). Patients do not receive IL-2 injections, preventing the side effects of this cytokine (42). Testing TEG001 in our first clinical trial (NTR6541) will be a major step forward in our efforts to make TEGs available to the patient (43).

Concluding remarks

The increased understanding of the human immune system and its ability to detect and eradicate tumor cells has led to a promising wave of novel therapeutics which have the potency to become the new standard of care. This thesis aims to contribute to the development of novel cell therapy products and the GMP manufacturing thereof.

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Appendices &

Nederlandse Samenvatting

Het immuunsysteem van de mens is in staat te voorkomen dat mensen ziek worden van micro-organismen zoals bacteriën en virussen. In de afgelopen jaren is het steeds duidelijker geworden dat het immuunsysteem ook betrokken is bij het voorkomen en bestrijden van kanker. Kanker kan ontstaan wanneer een cel in het menselijk lichaam mutaties oploopt, door bijvoorbeeld ultraviolette straling of roken, waardoor deze ongeremd kan gaan delen. Het immuunsysteem blijkt deze cellen in een vroeg stadium te kunnen herkennen en opruimen. Indien het immuunsysteem hier niet meer toe in staat is, kunnen de tumorcellen uitgroeien waardoor er kanker kan ontstaan. Er zijn grofweg vier methoden om kanker te behandelen; een chirurgische ingreep, radiotherapie, chemotherapie en immunotherapie. Onze onderzoeksgroep doet onderzoek naar immunotherapie, die in deze thesis nader beschreven wordt.

Cellulaire immunotherapie tegen kanker

Kanker ontstaat wanneer lichaamseigen cellen ongeremd gaan delen en zo uiteindelijk een tumor vormen. Medicijnen die gebruikt worden om kanker te behandelen zijn vaak gericht tegen alle snel delende cellen en niet specifiek gericht tegen de tumor cellen. Het gevolg daarvan is dat er vaak bijwerkingen ontstaan. Een voorbeeld hiervan is haarverlies, aangezien er ook in de haarzakjes van nature snel delende cellen aanwezig zijn. Het doel van immunotherapie is om het immuunsysteem van de patiënt zelf te gebruiken om de kanker te bestrijden. Dit kan door het immuunsysteem te stimuleren met zogenaamde checkpoint inhibitoren, door het gebruik van tumor-specifieke monoklonale antilichamen die de tumorcellen zichtbaar maken voor het immuunsysteem of door gebruik te maken van de cellen van het immuunsysteem zelf. Aangezien onze onderzoeksgroep specifiek werkt aan therapieën gebaseerd op de immuuncellen van de patiënt zelf, spreken we over cellulaire immunotherapie tegen kanker. Kenmerkend voor alle vormen van immunotherapie is dat ze meer specifiek gericht zijn tegen de tumor, waardoor ze effectief kunnen zijn met minder bijwerkingen.

γδT cellen

Het immuunsysteem bestaat uit verschillende immuuncellen, ook wel lymfocyten genoemd, die allemaal hun eigen rol vervullen in het voorkomen en genezen van infecties. Lymfocyten bestaan uit drie groepen cellen; de T cellen, B cellen en NK cellen. De T cellen kunnen verder onderverdeeld

worden in de veel voorkomende conventionele αβ T cellen en de minder voorkomende γδ T cellen. γδ T cellen zijn T cellen die een γδ T cel receptor (TCR) tot expressie brengen op de buitenkant van de cel, waarmee ze andere cellen aftasten op zoek naar geïnfecteerde cellen. We zijn specifiek in deze γδ T cellen geïnteresseerd omdat aangetoond is dat γδ T cellen een belangrijke rol vervullen in het voorkomen en bestrijden van kanker. γδTCRs blijken in staat te zijn subtiele veranderingen in het metabolisme van tumorcellen ten opzichte van normale cellen te herkennen. γδ T cellen zijn vervolgens in staat deze tumorcellen zelf op te ruimen, maar kunnen ook andere onderdelen van het immuunsysteem aansporen de kanker te verwijderen.

TEGs; genetische modificatie van T cellen

Alle mensen hebben een klein percentage γδ T cellen in het bloed. Deze cellen zijn zeer eenvoudig te isoleren na het afnemen van een kleine hoeveelheid bloed. In ons laboratorium hebben we, uit het bloed van een aantal gezonde vrijwilligers, meerdere γδ T cellen geïdentificeerd die veel verschillende solide tumorcellen (afkomstig van bijvoorbeeld long- of borstkanker) en hematologische tumorcellen (afkomstig van leukemie) kunnen herkennen. Door het uitvoeren van moleculair biologische technieken hebben we de DNA sequenties van deze γδTCRs achterhaald. Met behulp van de gevonden DNA sequenties zijn we in staat om andere T cellen te herprogrammeren met een van deze γδTCRs. Hierdoor gaan deze T cellen allemaal dezelfde tumor-specifieke γδTCR tot expressie brengen. Om dit te bereiken, maken we gebruik van een aangepast virus dat de veel voorkomende αβ T cellen infecteert met de γδTCR. In theorie kunnen we daardoor de T cellen van de patiënt in het laboratorium herprogrammeren en deze na expansie terug spuiten zodat de tumor door de genetisch gemanipuleerde T cellen wordt opgeruimd. Deze genetisch gemodificeerde cellen noemen we TEGs, verwijzend naar het Engelse "T cells engineered to express a defined γδTCR".

Hoofdstuk II beschrijft de zoektocht naar het ligand van één van de tumor specifieke γδTCRs die we hebben geïdentificeerd. De herkenningsmechanismen van een αβTCR en een γδTCR zijn normaal gesproken zeer verschillend. αβ T cellen herkennen kleine stukjes eiwit die gepresenteerd worden door zogenaamde Humane Leukocyten Antigenen (HLA) moleculen op de oppervlakte van bijna alle cellen in het menselijk lichaam. Indien deze stukjes eiwit afkomstig zijn van bijvoorbeeld een virus of bacterie, zal de αβ T cel de geïnfecteerde cel

opruimen. γδ T cellen herkennen normaal gesproken moleculen die verhoogd tot expressie komen indien een cel geïnfecteerd is, maar ook wanneer een cel een tumorcel is geworden en daardoor een licht veranderd metabolisme heeft. Dit gebeurt onafhankelijk van de HLA moleculen, waardoor er een duidelijk verschil in herkenningsmechanisme is. In dit hoofdstuk bestuderen we een γδTCR met de naam FE11, die in tegenstelling tot de meeste andere γδTCRs, wel afhankelijk is van een specifiek HLA molecuul. We proberen in dit hoofdstuk het ligand te identificeren en het herkenningsmechanisme te ontrafelen. Aangezien FE11 in staat is om zeer veel verschillende tumoren te herkennen, is het aantrekkelijk om deze γδTCR uiteindelijk als immunotherapie te gebruiken. De sequentie van FE11 en de mogelijke toepassingen daarvan zijn door onze groep gepatenteerd.

Hoofdstuk III bestaat uit een review artikel waarin we de ontwikkelingen binnen het veld van de immunotherapie bespreken en specifiek kijken naar de klinische trials zoals die in Europa en de Verenigde Staten worden uitgevoerd. Immunotherapie is relatief nieuw en fundamenteel anders dan klassieke therapieën zoals chemotherapie. Waar chemotherapie in grote hoeveelheden gemaakt kan worden, voor de behandeling van veel verschillende patiënten, wordt cellulaire immunotherapie voor iedere patiënt specifiek geproduceerd. Daarnaast is de houdbaarheid van cellulaire immunotherapie vaak zeer kort. Dit komt doordat, in tegenstelling tot andere medicijnen, cellulaire immunotherapie uit een levend cel product bestaat, dat buiten het menselijk lichaam snel in kwaliteit achteruit gaat. Deze grote verschillen tussen de klassieke therapieën en immunotherapie, alsook het feit dat immunotherapie nog vrij nieuw en onbekend is, zorgen ervoor dat de regelgeving een stuk uitgebreider is. Deze regelgeving met betrekking tot het testen van nieuwe therapieën is zeer belangrijk om de patiënt te beschermen, maar remt helaas ook de snelle introductie van nieuwe behandelmethoden.

In **hoofdstuk IV** bespreken we een methode om cellulaire immunotherapie met behulp van αβTCRs te verbeteren. Zoals eerder aangegeven zijn αβ T cellen in staat om tumorcellen te herkennen doormiddel van de herkenning van een stukje tumor-eiwit in HLA. Het is mogelijk om cellulaire immunotherapie te maken die bestaat uit αβ T cellen gemanipuleerd om een tumor-specifieke αβTCR tot expressie te brengen. Het proces dat daarbij wordt toegepast, staat bekend als retrovirale transductie, een proces dat zelden 100% effectief is. Het gevolg hiervan is dat een bepaald

percentage van de gemanipuleerde T cellen de tumor-specifieke TCR tot expressie brengt, maar dat ook een groot percentage van de T cellen nog steeds de oorspronkelijke (endogene) αβTCR bevat. Dit laatste kan er voor zorgen dat het product minder effectief is en bijwerkingen veroorzaakt. In dit hoofdstuk beschrijven we een manier om deze αβTCR gemanipuleerde T cellen te purificeren, zodat er een beter en meer homogeen product ontstaat. Deze purificatie is gebaseerd op een klein aantal mutaties dat aangebracht wordt in de geïntroduceerde αβTCRs.
De behandeling van patiënten met cellulaire immunotherapie kan voor bijwerkingen zorgen indien de αβTCR onverwacht meer dan alleen het tumor materiaal herkent. In dit geval is het nuttig om de αβTCR gemanipuleerde cellen na een tijdje uit de patiënt te verwijderen. Om dit te verwezenlijken hebben we een zeer elegante methode bedacht, die gebruikmaakt van de specifieke mutaties die ook van belang zijn voor de purificatie. Kort samengevat beschrijven we hoe tumor-specifieke αβTCRs zo aan te passen dat, nadat deze in T cellen zijn geïntroduceerd, deze T cellen gepurificeerd kunnen worden en indien nodig later verwijderd kunnen worden uit de patiënt. De sequentie van de gemuteerde αβTCR en de mogelijke toepassingen zijn door onze groep gepatenteerd.

Hoofdstuk V bestaat uit een korte review waarin we bespreken hoe cellulaire immunotherapie verbeterd kan worden. Uiteraard is de keuze voor de te gebruiken tumor-specifieke receptor zeer belangrijk voor het succes van de therapie. Echter, T cellen staan er om bekend om verschillende activatie-statussen te kunnen hebben. Onderzoek wijst uit dat bepaalde T cellen, wanneer geïnfuseerd in de patiënt, veel langer leven dan andere T cellen en daardoor uiteindelijk veel effectiever zijn. Niet alleen de effectiviteit, maar ook de kosten van nieuwe therapieën zijn van belang. Therapieën moeten betaalbaar blijven om beschikbaar te zijn voor een grote patiëntenpopulatie. Echter, door de patiënt-specifieke productie zijn de kosten van immunotherapie vaak vele male hoger dan die van klassieke therapieën. Door gebruik te maken van reeds geïmplementeerde methoden en technieken moeten de kosten relatief laag gehouden kunnen worden.

In **Hoofdstuk VI** bespreken we in detail het productieproces van onze cellulaire immunotherapie TEG001. We laten zien hoe bepaalde parameters in het productieproces tot stand zijn gekomen en wat de specificaties van het eindproduct zijn. In onze eerste klinische trial testen

we TEG001 bij patiënten met acute myeloïde leukemie, multiple myeloom en myelodysplastisch syndroom.

In het laatste hoofdstuk van dit proefschrift, **hoofdstuk VII**, worden de hierboven beschreven resultaten in een breder perspectief geplaatst en vergeleken met de resultaten die momenteel in de literatuur bekend zijn.

Dankwoord

Dit proefschrift zou er niet zijn geweest zonder de hulp van een heleboel mensen. Graag wil ik iedereen bedanken die op welke manier dan ook aan dit werk heeft bijgedragen. Minstens zo belangrijk waren de mensen die niet direct een bijdrage hebben geleverd, maar er wel voor hebben gezorgd dat ik een bijzonder leuke promotieperiode heb gehad!

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Dear Dr. Zsolt, despite probably being the most sophisticated man on earth, you still had chili-kruidnoten in August; that is not OK! Thanks for the many discussions we had and allowing me to express my frustrations whenever I needed to. I will never forget our gold leaf & gin-tonic conference and the fact that we almost didn't get there because you don't really know left and right.

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List of Publications

This thesis

Kierkels GJ, Scheper W, Schiffler M, Aarts-Riemens T, Beringer DX, Kramer L, Straetemans T, Leusen JHW, San José E, Vargas-Diaz D, Altelaar M, Heck AJR, Shultz LD, Ishikawa F, Nishimura MI, Sebestyén Z, Kuball J. Identification of a tumor-specific allo-HLA-restricted γδTCR. *Submitted* 2018

- Patent: PCT/EP2017/064323
  Human leukocyte antigen restricted gamma delta T cell receptors and methods of use thereof.

Kierkels GJ, Scheper W, Frijlink E, de Bruin A, Aarts-Riemens T, Beringer DX, Kramer L, Heijhuurs S, Straetemans S, Uckert W, Sebestyén Z, Kuball J. Select - kill strategy for untouched αβTCR-gene engineered T cell products. *Submitted* 2018

- Patent: PCT/EP2015/077286
  Use of antibodies for enrichment of engineered T cells with exogenous immune receptors and antibodies for use in depletion of engineered T cells.
- Revised patent submitted.

Straetemans T*, Kierkels GJ*, Doorn R, Jansen K, Heijhuurs S, Dos Santos JM, van Muyden ADD, Vie H, Clemenceau B, Raymakers R, de Witte M, Sebestyén Z, Kuball J. GMP-Grade Manufacturing of T Cells Engineered to Express a Defined γδTCR. *Front Immunol.* 2018 May 30;9:1062.

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Others

Janssen A*, Villacorta Hidalgo J*,... Kierkels GJ... Fisch P#, Kuball J#. T-cell receptors with public γ or δ chains derived from breast cancer-infiltrating T lymphocytes mediate antitumor reactivity. *Submitted* 2018

Houben F, De Vos WH, Krapels IP, Coorens M, Kierkels GJ, Kamps MA, Verstraeten VL, Marcelis CL, van den Wijngaard A, Ramaekers FC, Broers JL. Cytoplasmic localization of PML particles in laminopathies. *Histochem Cell Biol*. 2013 Jan;139(1):119-34.

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Curriculum Vitae

Guido Kierkels was born on May 4th 1987 in Venlo, the Netherlands. In 2005 he completed his secondary education (VWO - bilingual Atheneum) at the Bisschoppelijk College Broekhin, Roermond. In 2006 he started with a bachelor's degree in Molecular Life Sciences at Maastricht University, followed by a master's degree in Oncology & Developmental Biology at the same university. His master's thesis "Developing targeted combination therapies for the treatment of HER2 positive breast cancer" was written at the British Columbia Cancer Research Centre, Vancouver Canada, at the department of Experimental Therapeutics under the supervision of prof. Marcel Bally. After obtaining his master's degree he started working at the contract manufacturing agency PharmaCell (now Lonza) in Maastricht, where he worked on the European introduction of the first cellular immunotherapy against cancer; sipuleucel-T. In 2014 Guido decided to pursue his cancer immunotherapy interest within a different setting and started his PhD at the Laboratory of Translational Immunology, Utrecht University Medical Center, under the supervision of Prof. Jürgen Kuball and Dr. Zsolt Sebestyén. Here he worked on both αβTCR and γδTCR gene therapy against cancer, with an emphasis on target identification and GMP manufacturing. The results of Guido's work on TCR gene therapy are described in this thesis.
Currently, Guido works as a Technical Operations Specialist at Kiadis Pharma, Amsterdam. Here he supports the clinical manufacturing of ATIR101, a host-alloreactive T cell depleted donor lymphocyte infusion for the treatment of acute myeloid leukemia, acute lymphoblastic leukemia, and myelodysplastic syndrome.