Untouched GMP-ready purified engineered immune cells to treat cancer

Running title: Purified engineered immune cells

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Although major progress in the quality and efficacy of clinical engineered T cells for adoptive immune therapies against cancer has been achieved in the last decades, no additional purification step for engineered immune cells is being applied due to the lack of suitable tools and strategies. This results in the transfer of also non- and poorly engineered immune cells into patients, which can substantially dampen therapeutic effects and limit additional clinical applications such as the transfer of third party populations. In the present study we therefore developed a system to obtain highly purified $\gamma\delta$TCR engineered immune cells that can be readily translated into a GMP-compliant production process. The engineered cells are superior in efficacy, and provide long-term tumor control in 2 different humanized mouse models without allo-reactivity. This strategy yields a cellular medicine that can be part of an immune intervention strategy in a broad cancer patient population.
ABSTRACT

Purpose: Engineering T cells with receptors to re-direct the immune system against cancer has most recently been described as a scientific breakthrough. However, a main challenge remains the GMP-grade purification of immune cells selectively expressing the introduced receptor in order to reduce potential side effects due to poorly or non-engineered cells.

Experimental design: In order to test a novel purification strategy, we took advantage of a model γδT cell receptor (TCR), naturally interfering with endogenous TCR expression and designed the optimal retroviral expression cassette to achieve maximal interference with endogenous TCR chains. Following retroviral transduction, non-and poorly engineered immune cells characterized by a high endogenous αβTCR expression were efficiently depleted with GMP-grade anti-αβTCR-beads. Next, the engineered immune cells were validated for TCR expression, function against a panel of tumor cell lines and primary tumors and potential allo-reactivity. Engineered immune cells were further validated in two humanized mouse tumor models.

Results: The untouched enrichment of engineered immune cells translated into highly purified receptor-engineered cells with strong anti-tumor reactivity both in vitro and in vivo. Importantly, this approach eliminated residual allo-reactivity of engineered immune cells. Our data demonstrate that even with long-term suboptimal interference with endogenous TCR chains such as in resting cells, allo-reactivity remained absent and tumor control preserved.

Conclusion: We present a novel enrichment method for the production of untouched engineered immune cells, ready to be translated into a GMP-grade method and potentially applicable to all receptor-modified cells even if interference with endogenous TCR chains is far from complete.
INTRODUCTION

Rising clinical data demonstrate the potency of the adoptive transfer of T cells to effectively treat cancer, when T cells are genetically engineered to express tumor-specific receptors (1-4). Although the engineering process of genetically modified T cells has been substantially improved during the last decades, to date all cellular products usually maintain a fraction of non- and poorly transduced immune cells (2, 5). However, non-engineered immune cells have been reported to hamper the activity of adoptively transferred cells in an autologous situation (6, 7) due to the unwanted transfer of regulatory T cells (8) or by competing for homeostatic cytokines (9, 10). In addition, non- or poorly engineered immune cells with high endogenous αβTCR expression have a clear potential to induce graft versus host disease (GVHD) in the context of allogeneic/third party applications (11, 12).

To date, efforts to enhance purity of engineered immune cells mainly focus on positive selection either by expression of an additional transgene such as truncated CD34 (13), truncated nerve growth factor receptor (14) or direct binding of the introduced therapeutic receptor (15, 16). However, these strategies can either result in reduced expression of the desired gene of interest or additional introduction of potentially immunogenic components. In addition, the positive selection process results in so-called ‘touched cells’ and can mediate activation induced cell death (17, 18) directly after isolation or antibody dependent cellular cytotoxicity shortly after transfer into the recipient and may hamper long term persistence of transferred immune cells. An obvious alternative strategy would be the isolation of ‘untouched engineered immune cells’.

Most recently, GMP-grade anti-αβTCR beads became available and are currently used in the context of haploidentical transplantation used by others (19) and us (NTR2463 and NTR3079). Using such selection beads in combination with the knock-down of endogenous αβTCR genes (20) should theoretically result in a population of untouched engineered
immune cells with high purity and substantially reduced “off-target” effects. Despite the fact that various different strategies to knockdown endogenous αβTCR expression have been described most recently including RNA interference (21, 22), Zinc Finger Nucleases (ZFNs) (20), or TALENS (23), clinical application of these elegant techniques is unfortunately far from translation. Off-target integration effects and the need for multiple plasmids to be expressed in one T cell are some of the reasons as to why clinical translation has noticeable obstacles. Alternative simple and cost-effective solutions are clearly still needed. Therefore, classical methods such as the interference with endogenous αβTCR chains by introducing physiological strong αβTCR competitors for the components of the CD3 complex (24-26) such as γδTCR chains (27-29), remain valid alternatives in order to substantially reduce endogenous αβTCR expression. Besides the reduction of endogenous αβTCR expression γδTCRs are promising immune receptors to retarget αβT cells against cancer as reviewed extensively in (30, 31). γδT cells are considered as an innate-like population of immune cells recognizing molecular stress signals on infected or malignant cells. A subset of γδT cells express a TCR composed of Vγ9 and Vδ2 chains that sense accumulated non-peptidic pyrophosphate molecules (phosphoantigens), intermediates of a deregulated mevalonate pathway of isoprenoid synthesis, via BTN3A1 (CD277) and reported by us (27-29) and others (32) to display potent cytotoxicity against a broad range of tumor species. Other intriguing properties of γδTCRs are the absence of forming potentially self-reactive mixed TCR dimers, their HLA-independency, absence of immunogenicity which can either result in reduced therapeutic efficacy or substantial toxicity and avoidance of education and long term tolerance when expressing innate immune receptors out of the context of an innate immune cell (30, 31).

In summary, although major progress in the quality and efficacy of clinical engineered T cells has been achieved in the last decades (1-4), a feasible and cost-effective GMP-
compliant system to obtain highly purified engineered immune cells is not yet available. By utilizing a novel tumor-specific immune receptor naturally interfering with endogenous αβ TCR chains (27-30), an optimized expression system, and clinical grade anti-αβ TCR beads, we developed a highly pure engineered cellular product with improved anti-tumor activity. The production process can be readily translated to meet GMP-grade standards and the resulting cellular medicine can be applied as part of an immune intervention strategy in a broad population of cancer patients in autologous, allogeneic, and third party situations.

MATERIALS AND METHODS

Cells and cell lines

Daudi (CCL-213) and Phoenix-Ampho (CRL-3213) cells were obtained from the American Type Culture Collection at the initiation of the study in 2010 (Authentication by short tandem repeat (STR) profiling/karyotyping/isoenzyme analysis). OPM2, RPMI8226/S, OPM2-Luciferase (OPM2-Luc) and RPMI8226/S-luc (RPMI-Luc) were kindly provided by Anton Martens in 2012 (University Medical Center Utrecht, Utrecht, The Netherlands) and Daudi-Luciferase (Daudi-Luc) by Genmab in 2013 (Utrecht, The Netherlands). The EBV-transformed lymphoblastoid cell lines (EBV-LCL) were a kind gift from Tuna Mutis in 2010 (University Medical Center Utrecht) and not authenticated since receipt. All cells 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, Basel, Switzerland). Phoenix-ampho cells were cultured in DMEM+1%Pen/Strep (Invitrogen) +10%FCS (Bodinco), all other cell lines in RPMI+1%Pen/Strep+10%FCS.

PBMCs were isolated from buffy coats obtained from the Sanquin Blood Bank (Amsterdam,
The Netherlands) or from the Institute for Transfusion Medicine and Immunohematology, Frankfurt, Germany. PBMC samples from AML patients were a kind gift from Matthias Theobald (Mainz, Germany) and from the University Medical Center Utrecht Biobank and were collected according to GCP and Helsinki regulations.

**TCR gene cassettes in retroviral vectors**

The highly tumor reactive γδ2TCR chain genes were obtained via combinatorial-γδTCR chain exchange (28). The TCRγ chain was derived from γδT cell clone G115 (33) and TCRδ chain from clone 5 (28), codon optimized (Geneart Life Technologies, Regensburg, Germany) and cloned into the retroviral vector pBullet as single TCR chain vectors containing either γ-chain-IRES-neomycine or δ-chain-IRES-puromycine. In addition, four different transgene cassettes containing both γ and δTCR chains were designed by exchanging two different 2A peptide linker sequences, F2A and T2A (34), and the order of TCR chains (δ-F2A-γ; γ-F2A-δ; δ-T2A-γ; γ-T2A-δ) (Fig 1A). These γδTCR cassettes were cloned into the optimized retroviral vector pMP71 (kindly provided by Miriam Heemskerk, Leiden University Medical Center, Leiden, Netherlands) to express both TCR chains simultaneously. A nonsense murine αβTCR, consisting of the alpha chain derived from the MDM2/HLA-A2 TCR (35) and the beta chain from the p53/HLA-A2 TCR (36), was used as control TCR in both the pBullet and the pMP71 retroviral vector system. Also truncated Nerve Growth Factor Receptor in pMP71 was used as control in retroviral transduction experiments (pMP71:ΔNGFR) (kindly provided by Miriam Heemskerk).

**Retroviral transduction of T-cells**

γδTCRs were transduced into αβT-cells as previously described (27). In brief, packaging cells (phoenix-ampho) were transfected using FugeneHD reagent (Promega, Madison, Wisconsin,
USA) with helper constructs gag-pol (pHIT60), env (pCOLT-GALV) (35) and two retroviral constructs (pBullet) containing either γ-chain-IRES-neomycin or δ-chain-IRES-puromycine, or one retroviral construct (pMP71) containing both γδ TCR chains separated by a 2A sequence. Human PBMC were pre-activated with αCD3 (30ng/ml) (Orthoclone OKT®3, Janssen-Cilag, Tilburg, The Netherlands) and IL-2 (50 IU/ml) (Proleukin®, Novartis, Arnhem, The Netherlands) and transduced twice with viral supernatant within 48 hours in the presence of 50 IU/ml IL-2 and 6 μg/ml polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands). Transduced T-cells were expanded by stimulation with anti-CD3/CD28 Dynabeads (0.5x10⁶ beads/10⁶ cells) (Life Technologies, Carlsbad, California, USA) and IL-2 (50IU/ml) and in case of pBullet retroviral system selected with 800μg/ml Geneticin (Gibco, Karlsruhe, Germany) and 5μg/ml puromycin (Sigma-Aldrich). Next, TCR-transduced T-cells were expanded based on a previously described rapid expansion protocol (REP) (27).

Depletion of non-engineered T cells

αβT-cells were transduced with pMP71: γ-T2A-δ and incubated with a biotin-labeled anti-αβ TCR antibody (clone BW242/412, Miltenyi Biotec, Germany) followed by incubation with an anti-biotin antibody coupled to magnetic beads (anti-biotin MicroBeads, Miltenyi Biotec). Next, the cell suspension was applied onto an LD column and αβ TCR positive T cells were depleted by MACS cell separation according to the manufacturer’s protocol (Miltenyi Biotec). After depletion, γδ TCR positive T cells were expanded using T-cell REP.

Flow cytometry

Antibodies used for flow cytometry included: pan-γδ TCR-PE (clone IMMU510), pan-αβ TCR-PE-Cy5 (clone IP26A, both Beckman Coulter, Woerden, The Netherlands), mouse TCRβ-chain-PE (clone H57-597), CD4-FITC (clone RPA-T4, both BD Biosciences, San Francisco, USA).
Jose, USA), CD8-PerCP-Cy5.5 (clone RPA-T8, Biolegend, San Diego, USA), CD20 (Rituximab, Roche, Basel, Switzerland) and Goat-anti-Human-IgG-PE (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). All samples were analyzed on a FACSCanto II using FACSdiva software (BD Biosciences).

Functional T-cell assays

$^{51}$Chromium-release assay for cell-mediated cytotoxicity was previously described (28). In brief, target cells were labeled overnight with 100μCu $^{51}$Cr and incubated for 4-5h with transduced T-cells in five effector-to-target ratios (E:T) between 30:1 and 0.3:1. Percentage of specific lysis was calculated as follows: (experimental cpm – basal cpm)/(maximal cpm – basal cpm) x 100 with maximal lysis determined in the presence of 5% triton and basal lysis in the absence of effector cells.

IFN$\gamma$ ELISPOT was performed using anti-hu IFN$\gamma$ mAb1-D1K (I) and mAb7-B6-1 (II) (Mabtech-Hamburg, Germany) following the manufacturer’s recommended procedure. Target and effector cells (E:T 1:3) were incubated for 24h in the presence of pamidronate (10 μM) (Calbiochem, Germany) where indicated.

IFN$\gamma$ ELISA was performed using ELISA-ready-go! Kit (eBioscience, San Diego, CA, USA) following manufacturer’s instructions. Effector and target cells (E:T 1:1) were incubated for 24h in the presence of pamidronate when indicated.

TCR surface expression assay was performed with T cells that were rested for 21 days and starved of fresh medium and IL-2 supplement for 6 days. T Cells were either stimulated in a 24 wells plate with anti-CD3/CD28 Dynabeads at a bead-to-cell ratio of 1:1 in a final volume of 2 ml medium or with EBV-LCLs in a 96 wells plate at an E:T ratio of 1:3 in a final volume of 200 μl medium supplemented with 30 IU/ml IL-2. After a stimulation period of 4h, 24h and 48h at 37°C and 5% CO$_2$, cells were stained with mAbs against the introduced and/or
endogenous TCR and analyzed by flow cytometry. TCR cell surface expression was calculated as follows: (mean fluorescence intensity (MFI) of T cells stimulated with beads / MFI of unstimulated T cells) x 100 and indicated as % TCR of control.

Animal models:

The RAG2\(^{-/-}\)/γc\(^{-/-}\)-BALB/C mice, originally obtained from AMCAS b.v. (Amsterdam, The Netherlands), were bred and housed in the specific pathogen-free (SPF) 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. For the experiments female mice from 8 to 12 weeks of age were used. \(10^7\) γδTCR or Mock TCR transduced T-cells were i.v. injected together with 0.5\(\times10^6\) Daudi-Luc or 5\(\times10^6\) OPM2-Luc cells. Mice received 0.6\(\times10^6\) IU of IL-2 in IFA s.c. on day 1 and every 21 days till the end of the experiment. Pamidronate (10 mg/kg body weight) was applied in the indicated groups at day 1 i.v. and every 21 days until the end of the experiment. For the OPM2 tumor rechallenge experiment, mice treated with γδTCR T cells that remained tumor free for more than 120 days were rechallenged with 5\(\times10^6\) OPM2-Luc cells without prior irradiation. Naïve and non-irradiated mice were used as control for tumor outgrowth. Tumors were visualized \textit{in vivo} by bioluminescent imaging. Mice were anesthetized by isoflurane before they received an i.p. injection (100\(\mu\)l) of 25mg/ml Beetle Luciferin (Promega). Bioluminescence images were acquired by using a third generation cooled GaAs intensified charge-coupled device camera, controlled by the Photo Vision software and analyzed with M³Vision software (all from Photon Imager; Biospace Laboratory, Paris, France).
Statistical analyses

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

RESULTS

Optimal competition and expression of introduced TCR chains with an individualized transgene cassette

The first goal of the study was to obtain the strongest anti-tumor reactivity and maximal interference with endogenous αβ TCRs (24), by no other means than selectively introducing optimally a strong competitor for components of the CD3 complex such as a novel and highly tumor-reactive γδ2 TCR (γG11/δ5), obtained by combinatorial-γδ TCR-chain exchange (28). General consensus is lacking regarding the optimal TCR transgene cassette for adoptive cell therapy with TCR-engineered T cells (37-41). Therefore, we designed four different γδ TCR transgene cassettes (Fig 1A) to test both the influence of γδ TCR-chain orientation as described for αβ TCR transgene cassettes (37) as well as a particular 2A ribosomal skipping sequence, F2A versus T2A (34). All genes were codon optimized, cloned into the clinical-grade retroviral vector pMP71 (42) and introduced into αβ T cells from multiple donors in order to test general applicability. Placing the γ chain 5’ and the δ chain 3’ of the F2A sequence (γ-F2A-δ) increased transduction efficiency more then 2-fold, depicted as fold-increase relative to transduction efficiency of the δ-F2A-γ cassette and corresponded with significantly higher TCR expression (Fig 1B, left panel). On the other hand the T2A sequence significantly enhanced transduction efficiency and mean fluorescent intensity (MFI) for both γδ TCR chain-orientations (Fig 1B, right panel). Flow cytometric plots from a representative experiment with the four γδ TCR transgene cassettes are shown in...
To determine whether the increased γδTCR expression was associated with increased specific cytolytic capacity, transductants were co-cultured with Daudi (Burkitt’s lymphoma) or OPM2 (multiple myeloma) cells (Fig 1C). T cells transduced with δ-F2A-γ did not induce significant lysis of tumor cells and an opposed orientation of γ- and δ-chain could only minimally increase lysis at the highest effector-to-target (E:T) ratio. In contrast, T cells transduced either with δ-T2A-γ or γ-T2A-δ efficiently lysed target cells. We used T cells transduced with both T2A containing transgene cassettes to examine whether the γδTCR chain-orientation influenced tumor-specific release of effector cytokines (Fig 1D). Although lytic activity was not significantly enhanced, γ-T2A-δ transductants produced significant higher amounts of IFN-γ compared to T cells transduced with δ-T2A-γ and argued for the use of the γ-T2A-δ as our clinical candidate transgene cassette. Given these results, it is worthwhile to individually optimize transgene cassettes for each particular clinical candidate TCR in order to achieve the most efficient competition with endogenous αβTCR chains as well as the strongest expression and activity of introduced receptors.

Enrichment of untouched engineered T cells using a GMP-grade antibody

Although retroviral transduction efficiencies have been greatly increased over the past decades and clinical αβTCR-engineered T cell products contain up to 60-85% TCR positive T cells, the inter-patient variation remains significant (2, 43). To overcome this problem and obtain a pure γδTCR-engineered T cell product for clinical application we introduce a new procedure for the depletion of non- or poorly transduced T cells, taking advantage of the observation that upon introduction of a γδTCR, the endogenous αβTCR expression is decreased (27, 29) (and Fig S1 and Fig 2A). This procedure is based on a monoclonal antibody against the αβTCR complex (clone BW242/412, Miltenyi Biotec) currently used to deplete αβT cells from stem cell grafts resulting in a mean 4 logs αβT cell depletion.
(NTR2463, NTR3079 and (19)). In short, αβT cells were transduced with pMP71:γ-T2A-δ and incubated with the biotin-labeled anti-αβTCR antibody followed by incubation with an anti-biotin antibody coupled to magnetic beads for the depletion of αβTCR positive T cells by MACS cell separation. After depletion, γδTCR T cells were expanded utilizing a previously described T-cell expansion protocol (44). This procedure resulted in near complete depletion of single αβTCR positive T cells (from 51% to 0.4%) and a dramatic increase in γδTCR single positive T cells (from 20% to 77%) (Fig 2A). Importantly, the αβTCR/γδTCR double positive T cells that remained were characterized by relative low surface expression of the endogenous αβTCR. This phenotype was stable until day 5 after stimulation, when T-cells were highly activated and proliferative. However, the phenotype changed at day 9 towards a major population of αβTCR/γδTCR double positive T-cells (68%) and a decreased percentage of γδTCR single positive cells (25%) when T cells reside in a more resting phase (Fig 2A).

The reappearance of endogenous αβTCR chains in more resting cells allowed us to test whether early αβTCR T cell depletion still provides a functional advantage when interference with endogenous TCR chains is far from complete. The functionality of the purified engineered immune cell product was therefore tested 10 days after selection and expansion and compared to a bulk engineered cell product without αβTCR depletion. Indeed, αβTCR T cell depletion significantly increased specific lysis of Daudi cells (p<0.01) (Fig 2B) as well as IFN-γ production in response to three different tumor cell lines (p<0.001) (Fig 2C). These results demonstrate that αβTCR T cell depletion increased the anti-tumor potential of the engineered cell product.

In order to test whether highly purified engineered immune cells in a resting state with suboptimal expression of introduced γδTCR chains still provide a therapeutic advantage compared to primary γδT cells with optimal γδTCR expression but polyclonal γ9δ2TCR usage, anti-tumor activity of primary bulk γ9δ2T cells and GMP-grade T cells engineered to...
express an optimized γδ2TCR were tested at day 10 against a panel of primary leukemic cells from acute myeloid leukemia (AML) patients. Treatment of leukemic cells with pamidronate to block the mevalonate pathway downstream to induce accumulation of isopentenyl pyrophosphate resulted in IFNγ secretion by T cells in response to 9 out of 16 AML samples. In 5 out of 8 tested samples the optimized γδTCR-engineered T cells produced significantly enhanced levels of IFNγ compared to primary polyclonal γδ2T cells isolated from a healthy donor (Fig 2D). This result suggests that transfer of optimized engineered γδTCR T cells, as part of a clinical immune intervention strategy, is preferred over a polyclonal γδT cell product. Together, we propose here a depletion procedure using a GMP-grade antibody for the enrichment of untouched γδTCR engineered T cells resulting in a highly tumor-reactive clinical cell product even in the absence of a complete interference with the expression of endogenous αβTCR chains.

Abolished allo-reactivity following enrichment of engineered T cells

The enrichment procedure that resulted in a pure γδTCR transduced T cell population with low or absent expression of αβTCR may reduce the allo-reactive potential of the T cell product in an allogeneic setting. However, this advantage could theoretically be counterbalanced by the latter up-regulation of endogenous αβTCRs in resting engineered immune cells (Fig 2A). To further simulate a resting T cell following in vivo transfer with substantial reoccurring expression of endogenous αβTCR chains, we pushed the system to a greater extend by using engineered T cells that lacked stimulus for more than 20 days and were starved of IL-2 for 6 days. Mock (ΔNGFR transduced), γδTCR-engineered and γδTCR-engineered αβTCR depleted T cells were tested against a panel of 13 mismatched EBV-LCL cell lines or healthy donor derived PBMCs in an IFNγ ELISPOT assay (Fig 3A and B) (see
Fig 3C upper panels for γδTCR versus endogenous αβTCR expression in rested T cells). Whereas Mock T cells produced IFNγ in response to 9 out of 13 EBV-LCL cell lines, allo-reactivity of γδTCR engineered bulk T cells was greatly reduced (significant reduction for 8 out of 9 EBV-LCL lines) and more importantly even completely abolished in the γδTCR-engineered αβTCR depleted T cell population. The reduced allo-reactivity of γδTCR engineered T cells was even more apparent when the different T cell populations were tested against a panel of 20 different healthy donor derived PBMCs (Fig 3B). No allo-reactivity was detected in the γδTCR transduced T cell populations, but Mock T cells produced IFNγ in response to 9 out of 10 PBMC donor combinations. Importantly, while allo-reactivity was abolished, both γδTCR engineered populations maintained their anti-tumor reactivity (Fig 3A and B).

One possible explanation for the reduced allo-reactivity in γδTCR transduced T cells despite their significantly recovered endogenous αβTCR may be the preferential hit of memory T cells and not allo-reactive naïve T cells in the transduction procedure. However, this explanation could be excluded since CD45RA depleted (memory) and CD45RO depleted (naïve) T cells were transduced with identical transduction efficiencies (data not shown). Meanwhile, reduced surface expression of the endogenous αβTCR in γδTCR transduced T cells may partially account for reduced allo-reactivity. In unstimulated resting cells, the total amount of αβTCR, as indicated by the mean fluorescence intensity (MFI), in γδTCR engineered cells was reduced for bulk cells by 4% (MFI: 12546) and for αβTCR depleted cells by 14% (MFI: 11334) when compared to ΔNGFR transduced cells (MFI: 13117) (Fig 3C, upper panel). Finally, a preferential recovery of introduced as compared to endogenous TCR chains upon antigen encounter may also contribute to this effect. Indeed, upon stimulation of resting T cells with anti-CD3/CD28 beads the expression of the retrovirally
introduced γδTCR decreased less profoundly, was restored within 24 hours and reached higher levels than before T cell stimulation within 48 hours (Fig 3C, lower panel and Fig S2A-D). This quick restoration and high level of TCR surface expression following T cell re-activation was not restricted to γδTCRs, thus most likely a property of the expression system used here, since expression kinetics of a retrovirally introduced αβTCR were comparable (Fig S2). These results suggest that although the endogenous αβTCR is re-expressed in resting engineered T cells, re-activation preferentially decreases endogenous αβTCR expression (Fig 3C, lower panel). To formally address this hypothesis in a more physiologically relevant setting we co-cultured resting γδTCR-engineered αβTCR depleted T cells with an HLA-mismatched EBV-LCL that induced an allo-reactive IFNγ response by ΔNGFR transduced cells (LCL1 from Fig 3A). Co-culturing these cells resulted in a down-regulated endogenous αβTCR expression, which reached a minimal level after 48 hours, while the γδTCR expression remained largely stable over time (Fig S2E). Thus, we confirm that in γδTCR engineered T cells T cell re-activity, such as allo-reactivity, is prevented and the expression of retrovirally introduced tumor-specific TCRs is favored and anti-tumor reactivity maintained.

These data question the need of a complete elimination of endogenous αβTCR chains in purified engineered immune cells when expressing strong αβTCR competitors with an optimal expression system.

Improved in vivo tumor control by optimized engineered T cell product

We evaluated the clinical potency of the optimized γδTCR T cell product in relation to γδTCR-engineered T cells produced with the extensively used pBullet retroviral transduction and antibiotic selection system (45), referred to as pB:γδTCR T cells. Following transduction of peripheral blood αβT cells and selection with antibiotics (pBullet) or enrichment with
αβ TCR depletion beads (pMP71) and subsequent T cell expansion, both immune products were evaluated. Interestingly, not only the percentage γδ TCR positive T cells was higher for the αβ TCR depleted γδ TCR T cell product, but also the number of γδ TCR complexes per cell increased more than 2-fold compared to pB:γδ TCR T cells as measured by MFI (Fig 4A). In addition, lysis of three tested tumor cell lines was significantly enhanced by αβ TCR depleted γδ TCR T cells transduced with the pMP71:γ-T2A-δ vector cassette as compared to pB:γδ TCR T cells (Fig 4B). To test if the superior anti-tumor activity of αβ TCR depleted γδ TCR T cells is reflected in vivo we used a humanized mouse tumor model for adoptive transfer of γδ TCR-engineered T cells. Irradiated RAG2−/−γc−/− double-knockout mice were injected with Luciferase-positive Daudi tumor cells and either with γδ TCR or Mock TCR engineered T cells and tumor growth was evaluated by bioluminescence imaging. Both γδ TCR-engineered T cell products significantly inhibited tumor growth compared to Mock TCR T cells, but αβ TCR depleted γδ TCR T cells further delayed tumor outgrowth and significantly increased survival compared to pB:γδ TCR T cells (Fig 4C).

In a second tumor model of multiple myeloma the anti-tumor activity of the optimized αβ TCR depleted γδ TCR engineered cell product was assessed. Irradiated Rag2−/−γc−/− double-knockout mice were injected with Luciferase-positive OPM2 cells and γδ TCR or Mock TCR engineered T cells and tumor growth was evaluated by bioluminescence imaging. Interestingly, tumor growth was completely prevented by clinical-grade γδ TCR T cells in 4 out of 7 mice (Fig S3). 120 days after first tumor and T cell injections, tumor free mice were re-challenged with a second injection of tumor cells without prior irradiation and non-irradiated naïve mice were used as control for tumor-outgrowth. Selectively re-challenged mice remained tumor free indicating that adoptive transfer of engineered immune cells can mediate long-term tumor protection in vivo (Fig 4D). In summary, these data underscore the
potency of a cost-effective purified engineered immune cell product for clinical application
taking advantage of an optimal expression system, strong competitors for endogenous TCR
chains such as a γδ TCR, and GMP-grade anti-αβ TCR beads.

**DISCUSSION**

Adoptive cell therapy using gene-engineered lymphocytes has moved towards a
feasible and effective treatment modality for cancer as demonstrated by exciting clinical
results during the last decade (1-5). However, large-scale clinical implementation of this
promising immunotherapy still awaits some critical hurdles to be taken such as the generation
of a purified engineered immune product. We provide evidence that the combination of an
optimized and individualized expression system with strong αβ TCR competitors and clinical
anti-αβ TCR beads is a highly efficient system, even if interference with endogenous αβ TCR
expression is far from complete. Thus, we present to our best knowledge the first enrichment
procedure using a clinical grade anti-αβ TCR mAb for the production of untouched gene-
engineered immune cells suitable for autologous, allogeneic or third party immune
intervention platforms which can be valuable for expression-receptor formats interfering with
endogenous αβ TCR chains.

Optimal gene expression is crucial when interference with αβ TCR chains is not
mediated via siRNA (21), ZFN (20), or TALENS (23). Therefore, an individualized testing of
existing expression systems was essential when utilizing a natural occurring strong competitor
for the CD3 complex (27). Although virus-derived 2A ‘ribosomal skipping sequences’ are
widely used to obtain equimolar expression of introduced genes, there are a handful of
different 2A sequences available. A T2A sequence was superior to the F2A and we can
unfortunately not provide a reasonable explanation for this observation. Our data revealed a
preferred position of the TCRδ chain downstream of the 2A cleavage element and this is in line with an optimal αβ TCR gene cassettes described by Leisegang and colleagues (37).

Our selection procedure utilizes a GMP-grade clinical bead, which is currently employed in the stem cell transplantation field by others (19) and us (NTR2463 and NTR3079) in order to deplete αβT cells from the donor. This procedure resulted in removal of non- and poorly transduced bystander cells, improved γδ TCR surface expression levels and resulted in almost 100% γδ TCR-engineered cells in the end product, which was translated into an increased anti-tumor function both in vitro and in vivo. Interestingly, when compared to a non-engineered polyclonal primary γδT cell population, selected γδ TCR-engineered T cells not only recognize an increased number of primary AML patient samples but also clearly produce higher amounts of IFNγ in case the tumor cells are recognized. This encouraging feature of our engineered immune cells is likely due to the choice for a high avidity γδ TCR in contrast to a polyclonal usage of γδ TCRs in a primary γδT cell population in combination with high γδ TCR surface expression as a result of the selection procedure.

The substantial up-regulation of endogenous αβ TCR chains in resting engineered immune cells allowed us to thoroughly investigate pitfalls if interference with endogenous αβ TCR chains is not complete. Even after 3 weeks, tumor control remained preserved in engineered immune cells in vitro and in vivo and allo-reactivity could not be observed. This is most likely due to (A) a still reduced surface expression of αβ TCR when compared to non-engineered cells and (B) faster and higher up-regulation of introduced TCR surface expression after antigen encounter. This observation is in line with previous data showing that in contrast to the endogenous TCR, introduced TCR expression under the control of an optimized viral promoter is restored and increased shortly after antigen-specific stimulation of either the endogenous or introduced TCR (46). The observed down-modulation of the
endogenous αβ TCR upon introducing a tumor-specific immune receptor paves the way towards the broader applicability of our strategy: the combination of siRNA (47), ZFNs (20) or TALENS (23) to knock down endogenous TCRs with the introduction of other innate receptors followed by the depletion with anti-αβ TCR beads. This would yield cellular end products with pure populations of engineered cells that do not express their endogenous receptor and significantly increase the efficacy and safety both in an autologous and allogeneic scenario. However, our data stress also that a complete long-term interference with endogenous αβ TCR chains is most likely not necessary for safety and efficacy. Residual expression of endogenous TCR chains might be even needed for an optimal long-term homeostatic proliferation (48).

Surprisingly, we also observed a relatively low allo-reactivity in non-engineered bystander cells, questioning whether our read-out systems have been sensitive enough. We excluded a preferential transduction of naïve T cells and subsequent down-regulation of endogenous αβ TCRs in naïve T cells which have been reported as more potent GVHD mediators than memory T cells (49, 50). However, our observation is also in line with the recent clinical observation that the transfer of non-selected CAR-engineered donor T-cells with intact endogenous αβ TCR chains in human did not associate with substantial GVHD (51) and might reflect a rather reduced αβ TCR expression in such extensively cultured and rapidly expanded cells prior infusion. In line with this observation and in contrast to reports by others (11, 20) we did not observe substantial GVHD in different humanized mouse models after adoptive transfer of engineered immune cells despite the fact that some models have employed similar mouse strains (11, 20) (TS & JK unpublished observations).

All together, we provide a novel, feasible and cost-effective strategy that is ready to be translated into a GMP-grade procedure to generate untouched engineered immune cells by taking advantage of an individualized expression system in combination with a strong
competitor for endogenous αβTCR chains and clinical-grade αβTCR beads. We also
demonstrate that the interference with endogenous αβTCR chains does not necessarily need
to be complete in terms of long-term competition in order to obtain a safer and more efficient
product. We conclude that the by us developed depletion strategy is therefore applicable to
nearly any engineered immune product interfering temporarily or permanently with
endogenous αβTCR chains.

REFERENCES


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Author contributions: T.S., C.G., S.Heijhuurs and S.H. performed experiments and analyzed data; H.B. provided vital reagents; T.S., C.G., I.S., Z.S. and J.K., designed research and interpreted data; T.S., Z.S and J.K. wrote the manuscript.
LEGENDS TO FIGURES

Fig 1. The γ-T2A-δ TCR transgene cassette improves γδTCR expression and function

A. Schematic overview of four different γδTCR transgene cassettes in the retroviral vector pMP71. TCRδ chain was derived from clone 5 (δ) and TCRγ from clone G115 (γ) (28) and F2A (derived from the foot-and-mouth disease virus) and T2A (derived from the Thosea asigna virus) refer to two different 2A ribosomal skipping sequences. B. Following retroviral transduction of αβ T cells, γδTCR expression was evaluated by flow cytometry using a pan γδTCR antibody. The percentages γδTCR positive T cells after transduction were calculated as fold increase compared to the percentage γδTCR positive T cells transduced with the δ-F2A-γ transgene. Data is presented as mean fold increase of 6 independent experiments (+SEM) in the left panel. Mean Fluorescent Intensity (MFI) of γδTCR surface expression on γδTCR positive T cells was compared and presented as mean from 6 independent experiments (+SEM) in the right panel. C. The four different γδTCR-engineered T cell populations from B were tested for their anti-tumor function. Tumor cells Daudi (Burkitt’s Lymphoma) and OPM2 (Multiple Myeloma) were loaded with 51Cr and incubated with T cells at indicated E:T ratio’s for 4-5 hours. T cells transduced with a combination of two pBullet vectors containing a single MDM2/HLA-A2 TCRα and a single p53/HLA-A2 TCRβ chain were used as control T cells (referred to as pB:αMDM2/βp53). γδTCR expression at the time of assay was the following: γ-T2A-δ: 46%; δ-T2A-γ: 35%; γ-F2A-δ: 5%; δ-F2A-γ: 3.5%. Percentage of specific lysis is shown as mean of triplicates (+/- SD). D. T cells transduced with the δ-T2A-γ (20% γδTCR+) or γ-T2A-δ (30% γδTCR+) transgene cassette were incubated with Daudi, OPM2, RMI/S8226S tumor cells or healthy donor derived PBMCs in the presence of 10μM pamidronate. pB:αMDM2/βp53 transduced cells were used as control T cells. After 20 hours...
incubation supernatants were harvested and analyzed for IFNγ secretion by ELISA. Data are presented as mean IFNγ production in pg/ml (+ SD). Statistical significances were calculated with by one-way anova; *p<0.05; **p<0.01; ***p<0.001.

Fig 2. Improved anti-tumor activity of pure γδTCR-engineered T cells after αβTCR T cell depletion

Enrichment of γδTCR engineered T cells by GMP grade depletion of αβTCR positive T cells.

A. Flow cytometric representation of pMP71:γ-T2A-δ transduced αβT cells before and directly after αβTCR T cell depletion (αβTCR T cell depleted). Depleted T cells were followed up during T cell expansion for their γδTCR transgene as well as endogenous αβTCR expression using a pan-γδTCR and a pan-αβTCR antibody for flow cytometry analysis.

Percentages of cells in each quadrant are indicated. B. γδTCR transduced T cells (bulk, 9% γδTCR+) and αβTCR depleted (41% γδTCR+) T cells were incubated with 51Cr loaded Daudi cells at indicated E:T ratio’s for 4-5 hours. pB:αMDM2/βp53 transduced cells were used as control T cells. Percentage of specific lysis is shown as mean of triplicates (+/-SD). Statistical significances were calculated by two-way anova; **p<0.01; ***p<0.001. C. γδTCR transduced bulk (6 % γδTCR+) and αβTCR depleted (51 % γδTCR positive) T cells were incubated with different tumor target cells as indicated and IFNγ secretion was measured by IFNγ ELISPOT. pMP71:ΔNGFR transduced T cells were used as control T cells. IFNγ spots per 15.000 T cells are shown as mean of triplicates (+SD). T cells only did not produce any significant levels of IFNγ. Statistical significances were calculated by two-way anova;

* p<0.05; ** p<0.01; *** p<0.001. D. Primary AML tumor samples (AML 1-16) were incubated with γδTCR transduced T cells that were αβTCR depleted (>70% γδTCR+) or with a bulk population of primary γδT cells (only AML 1-9) with or without 10μM pamidronate (PAM) and IFNγ secretion was measured by ELISPOT. IFNγ spots per 15.000 T cells is
shown as mean of triplicates (+SD). 50 spots/15,000 cells were considered as a positive anti-tumor response and indicated by the black horizontal line.

**Fig 3. Abolished allo-reactivity but preserved anti-tumor activity of rested γδTCR-engineered T cells after αβTCR T cell depletion.**

T cells were retrovirally transduced with pMP71:γ-T2A-δ, enriched for γδTCR transduced T cells (αβTCR depleted and 65% γδTCR +) or not (bulk and 9% γδTCR+) or pMP71:ΔNGFR as control and expanded as described. T cells were cultured without stimulus for more than 20 days and starved of IL-2 for the last 6 days and considered to be resting T cells. **A.** Resting T cells were co-cultured with OPM2 tumor cells and a panel of HLA-mismatched EBV-LCLs for 24 hours and IFNγ ELISPOT assay was used to measure both anti-tumor activity and allo-reactivity. IFNγ spots per 15,000 T cells is shown as mean of triplicates (+SD). **B.** A panel of 20 HLA-mismatched healthy donor derived PBMCs were irradiated and cells from 2 different donors were mixed, indicated by the donor numbers, and used as allo-reactive target cells. Resting T cells were co-cultured with Daudi tumor cells and the allo-reactive target cells for 24 hours and IFNγ ELISPOT assay was used to measure both anti-tumor activity and allo-reactivity. IFNγ spots per 15,000 T cells is shown as mean of triplicates (+SD). **C.** T cells described in A and B, were either left untreated (rested T cells, upper panel) or incubated with anti-CD3/CD28 beads for 4 days (αCD3/CD28 stimulated T cells, lower panel). T cells were stained with a pan αβTCR- and pan γδTCR-specific antibody and analyzed by flow cytometry. Percentages of cells in each quadrant and the MFI of αβTCR+ cells are indicated. Statistical significances were calculated with by two-way anova; *p<0.05; **p<0.01; ***p<0.001.
Fig 4. Improved tumor control of purified γδTCR T cell product

T cells were retrovirally transduced with pMP71:γ-γ2A-δ, pB:γ/δ and pB:αMDM2/βp53 as control TCR and underwent αβTCR T cell depletion in case of pMP71:γ-γ2A-δ or antibiotic selection in case of pBullet vector-engineered T cells prior to expansion. pMP71:γ-γ2A-δ refers to αβTCR T cell depleted engineered cells A. γδTCR expression was evaluated by flow cytometry and histogram plots illustrate γδTCR expression and MFI values are indicated. B. T cells were incubated for 4-5 hours with ⁵¹Cr loaded Daudi, OPM2 or RPMI tumor cells at indicated E:T ratio’s. Percentage of specific lysis is shown as mean of triplicates (+/-SEM). Statistical significances of pB:γ/δ versus pMP71:γ-γ2A-δ T cells were calculated by one-way anova; *p<0.05; **p<0.01, ***p<0.001. C. RAG2⁻/⁻ γc⁻/⁻ double knock-out mice were irradiated on day 0 and injected i.v. with 0.5x10⁶ Daudi-Luc cells and 10⁷ γδTCR engineered or control T cells at day 1. Mice were injected s.c. with 6x10⁵ IU IL-2 in IFA and i.v. with pamidronate (10 mg/kg body weight) at day 1 and every 3 weeks until the end of the experiment. Bioluminescence Imaging was used to monitor tumor growth every 7 days. Data represent mean of all mice per group (n=4-7) (+/-SEM) and is presented in the left panel. Statistical significances were calculated by one-way anova; *p<0.05; **p<0.01. Overall survival of treated mice was monitored until the end of the experiment and is presented in the right panel. Statistical significances were calculated by log-rank (Mantel-Cox) test; *p<0.05; **p<0.01. D. RAG2⁻/⁻ γc⁻/⁻ double knock-out mice were irradiated on day 0 and injected i.v. with 5x10⁶ OPM2-Luc cells and 10⁷ γδTCR-engineered or control T cells at day 1 (see result cancer free survival in Fig S3). Mice that remained tumor free until day 120 (4 out of 7) were re-challenged with 5x10⁶ OPM2-Luc cells without prior irradiation and 5 non-irradiated naïve mice were used as control mice. Tumor growth was measured as in C and presented in the left panel. Statistical significances were calculated by two-way anova; ***p<0.01. Cancer free
survival of rechallenged mice was monitored and presented in the right panel. Statistical significances were calculated by log-rank (Mantel-Cox) test; **p<0.01
Figure 2

A) pMP71:γ-T2A-δ transduced T cells

αβTCR T cell depletion

Rapid T cell expansion

B) Daudi

specific lysis (%)

0 10 20 30 40 50

0.3:1 1:1 3:1 10:1 30:1

E:T

pMP71:γ-T2A-δ αβTCR depleted

pMP71:γ-T2A-δ

pB:αMDM2/βp53

C) IFNγ (spots/1500 cells)

0 50 100 150 200

Daudi OP92 RPM1 T cells only

pMP71:ΔNGFR

pMP71:γ-T2A-δ

pMP71:γ-T2A-δ αβTCR depleted

D) IFNγ (spots/5000 cells)

0 100 200 300 400 500 600 700 800

T cells only Daudi AML-1 AML-2 AML-3 AML-4 AML-5 AML-6 AML-7 AML-8 AML-9 AML-10 AML-11 AML-12 AML-13 AML-14 AML-15 AML-16

γδT cells γδT cells + PAM

pMP71:γ-T2A-δ αβTCR depleted + PAM

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Figure 4

A

- pMP71:γ-T2A-δ
- pB:γ/δ
- pB:αMDM2/βp53

Counts

γδTCR

MFI: 2038

MFI: 1007

MFI: 67

B

- pMP71:γ-T2A-δ
- pB:γ/δ
- pB:αMDM2/βp53

% specific lysis

E:T ratio

Daudi

OMP2

RPMI

C

- pB:αMDM2/βp53
- pB:γ/δ
- pMP71:γ-T2A-δ

counts/min

0 7 14 21 28 35 42 49

days after Daudi / T cell injection

Overall survival (%)

0 10 20 30 40 50 60 70 80 90 100

days after Daudi / T cell injection

D

- naive mice
- MP71:γ-T2A-δ

counts/min

0 7 14 21 28 35 42 49 56

days after 2nd OMP2 cell injection

Cancer free survival (%)

0 20 40 60 80 100

days after 2nd OMP2 cell injection

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Untouched GMP-ready purified engineered immune cells to treat cancer

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