Untouched GMP-Ready Purified Engineered Immune Cells to Treat Cancer

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Abstract

Purpose: Engineering T cells with receptors to redirect 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 nonengineered 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, nonengineered and poorly engineered immune cells characterized by a high endogenous δβTCR expression were efficiently depleted with GMP-grade anti-δβTCR 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 antitumor 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.

Conclusions: 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. Clin Cancer Res; 21(17): 3957–68. ©2015 AACR.

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 nontransduced and poorly transduced immune cells (2, 5). However, nonengineered 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, nonengineered or poorly engineered immune cells with high endogenous δβTCR 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-δβTCR 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 knockdown of endogenous δβTCR 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 δβTCR expression have been described most recently, including RNA interference (21, 22), zinc finger nucleases (ZENs; ref. 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
Translational Relevance

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 decade, 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 nonengineered 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 this study, we therefore developed a system to obtain highly purified γδTCR 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 two 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.

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 refs. 30 and 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 nonpeptidic pyrophosphate molecules (phosphoanitgens), intermediates of a deregulated mevalonate pathway of isoprenoid synthesis, via BTN3A1 (CD277) and NGFR; kindly provided by Miriam Heemskerk). In brief, packaging cells (phoenix-ampho) were transfected factor receptor in pMP71 was used as control in retroviral transduction experiments (pMP71: -chain-IRES-neomycine, or one retroviral construct (pMP71) containing both -chain-IRES-neomycine or one retroviral construct (pMP71) containing either -chain-IRES-neomycine or -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, the Netherlands) to express both TCR chains simultaneously. A nonsense murine γδTCR, consisting of the α chain derived from the MDM2/ HLA-A2 TCR (35) and the β 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 γδT cells as previously described (27). In brief, packaging cells (phoenix-ampho) were transfected using FugeneHD reagent (Promega) with helper constructs gag-pol (pHT60), env (pCOLT-GALV) (35) and two retroviral constructs (pBullet) containing either γ-chain-δRES-neomycine or δ-chain-RES-puromycine, or one retroviral construct (pMP71)
containing both γδTCR chains separated by a 2A sequence. Human PBMC were preactivated with αCD3 (30 ng/mL; Ortho-clone OKT3; Janssen-Cilag) and IL2 (50 IU/mL; Proleukin, Novartis) and transduced twice with viral supernatant within 48 hours in the presence of 50 IU/mL IL2 and 6 μg/mL polybrene (Sigma-Aldrich). Transduced T cells were expanded by stimulation with

Figure 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 G15 (γ) (28) and F2A (derived from the foot-and-mouth disease virus) and T2A (derived from the Theosa asigna virus) refer to two different 2A ribosomal skipping sequences. B, following retroviral transduction of γδ T cells, γδTCR expression was evaluated by flow cytometry using a pan γδTCR antibody. The percentages γδTCR+ T cells after transduction were calculated as fold increase compared with the percentage γδTCR+ T cells transduced with the δ-F2A-γ transgene. Data are presented as mean fold increase of six independent experiments (+/SEM) in the left panel. Mean fluorescent intensity (MFI) of γδTCR surface expression on γδTCR+ T cells was compared and presented as mean from six independent experiments (+/SEM) in the right panel. C, the four different γδTCR-engineered T-cell populations from B were tested for their antitumor function. Tumor cells Daudi (Burkitt's lymphoma) and OPM2 (multiple myeloma) were loaded with 51Cr and incubated with T cells at indicated E:T ratios for 4 to 5 hours. T cells transduced with a combination of two pBullet vectors containing a single MDM2/HLA-A2 TCRa and a single p53/HLA-A2 TCRb chain were used as control T cells (referred to as pB:aMDM2/pb:p53). 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 10 μmol/L pamidronate. pB:aMDM2/pb:p53 transduced cells were used as control T cells. After 20 hours of 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 by one-way ANOVA; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
anti-CD3/CD28 Dynabeads (0.5 × 10^6 beads/10^6 cells; Life Technologies) and IL2 (50 IU/ml) and in case of pBullet retroviral system selected with 800 μg/ml Geneticin (Gibco) and 5 μg/ml puromycin (Sigma-Aldrich). Next, TCR-transduced T cells were expanded based on a previously described rapid expansion protocol (REP; ref. 27).

Depletion of nonengineered T cells

οβγ T cells were transduced with pMP71: γ-T2A-δ and incubated with a 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 onto an LD column and αβTCR-positive (αβTCR^+) T cells were depleted by MACS cell separation according to the manufacturer’s protocol (Miltenyi Biotec). After depletion, γ8TCR^+ T cells were expanded using T-cell REP.

Flow cytometry

Antibodies used for flow cytometry included: pan-γ8TCR-PE (clone IMM510), pan-οβTCR-PE-Cy5 (clone IP26A; both Beckman Coulter), mouse TCRβ-chain-PE (clone H57-597), CD4-FITC (clone RPA-T4; both BD Biosciences), CD8a-PerCP-Cy5.5 (clone RPA-T8; Biolegend), CD20 (Rituximab; Roche), and Goat-anti-Human-IgG-PE (Jackson ImmunoResearch Laboratories). All samples were analyzed on a FACSscan 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 100 μCi 51Cr and incubated for 4 to 5 h 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) × 100 with maximal lysis determined in the presence of 5% triton and basal lysis in the absence of effector cells.

IFNy ELISPOT was performed using anti-hu IFNy mAb1-D1K (I) and mAb7-B6-1 (II) (Mabtech) following the manufacturer’s recommended procedure. Target and effector cells (E:T 1:1) were incubated for 24 hours in the presence of pamidronate (10 μmol/L; Calbiochem) where indicated.

IFNy ELISA was performed using ELISA-ready-go! kit (eBioscience) following manufacturer’s instructions. Effector and target cells (E:T 1:1) were incubated for 24 hours 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 IL2 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 IL2. After a stimulation period of 4, 24, and 48 hours at 37°C and 5% CO2, 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 stained with beads/MFI of unstimulated T cells) × 100 and indicated as % TCR of control.

Animal models

The RAG2^−/−γc^−/−BALB/c mice, originally obtained from AMCAS b.v., 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 γ8TCR or Mock TCR-transduced T cells were intravenously injected together with 0.5 × 10^6 Daudi-Luc or 5 × 10^6 OPM2-Luc cells. Mice received 0.6 × 10^6 IU of IL2 in IFA subcutaneously 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 intravenously and every 21 days until the end of the experiment. For the OPM2 tumor rechallenge experiment, mice treated with γ8TCR T cells that remained tumor free for more than 120 days were rechallenged with 5 × 10^6 OPM2-Luc cells without prior irradiation. Naive and non-irradiated mice were used as control for tumor outgrowth. Tumors were visualized in vivo by bioluminescence imaging. Mice were anesthetized by isoflurane before they received an intraperitoneal injection (100 μl) of 25 mg/ml Beetle Luciferin (Promega). Bioluminescence images were acquired using a third-generation cooled GaAs intensified charge-coupled device camera, controlled by the Photo Vision software and analyzed with M^2 Vision software (all from Photon Imager; Biospace Laboratory).

Statistical analyses

Differences were analyzed using indicated statistical tests in GraphPad Prism (GraphPad Software Inc.).

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 antitumor reactivity and maximal interference with endogenous οβTCRs (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 γ82TCR (γc11/β5), obtained by combinatorial-γ8TCR-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 γ8TCR transgene cassettes (Fig. 1A) to test both the influence of γ8TCR-chain orientation as described for οβγTCR 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 οβT 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 than 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). However, the T2A sequence significantly enhanced transduction efficiency and MFI for both γ8TCR chain orientations (Fig. 1B, right). Flow cytometric plots from a representative experiment with the four γ8TCR transgene cassettes are shown in Supplementary Fig. S1. To determine whether the increased γ8TCR expression was associated with increased specific cytolytic

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capacity, transductants were cocultured with Daudi (Burkitt's lymphoma) or OPM2 (multiple myeloma) cells (Fig. 1C). T cells transduced with δ-T2A-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 IFNγ compared with 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% to 85% TCR+ T cells, the interpatient 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 nontransduced or poorly transduced T cells, taking advantage of the observation that upon introduction of a γδTCR, the endogenous αβTCR expression is decreased (refs. 27 and 29; and Supplementary 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 cells grafting in a mean 4 logs αβT-cell depletion (NTR2463, NTR3079, and ref. 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+ 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+ 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 with 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 IFNγ production in response to three different tumor cell lines (P < 0.001; Fig. 2C). These results demonstrate that αβTCR T-cell depletion increased the antitumor 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 with primary γδT cells with optimal γδTCR expression but polyclonal γδ2TCR usage, antitumor activity of primary bulk γδ2T cells and GMP-grade T cells engineered to express an optimized γδ2TCR were tested at day 10 against a panel of primary leukemia cells from AML patients. Treatment of leukemia 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 of 16 AML samples. In five of eight tested samples, the optimized γδ2TCR-engineered T cells produced significantly enhanced levels of IFNγ compared with primary polyclonal γδ2T cells isolated from a healthy donor (Fig. 2D). This result suggests that transfer of optimized engineered γδ2TCR 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 γδ2TCR 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 γδ2TCR-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 upregulation of endogenous αβTCR 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 extent by using engineered T cells that lacked stimulus for more than 20 days and were starved of IL2 for 6 days. Mock (ΔNGFR transduced), γδ2TCR-engineered, and γδ2TCR-engineered γδ2TCR-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, top, for γδTCR vs. endogenous αβTCR expression in rested T cells). Although Mock T cells produced IFNγ in response to 9 out of 13 EBV-LCL cell lines, allo-reactivity of γδ2TCR-engineered bulk T cells was greatly reduced (significant reduction for eight of nine EBV-LCL lines) and more importantly even completely abolished in the γδ2TCR-engineered γδ2TCR-depleted T-cell population. The reduced allo-reactivity of γδ2TCR-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 γδ2TCR-transduced T-cell populations, but Mock T cells produced IFNγ in response to 9 out of 10 PBMC donor combinations. Importantly, although allo-reactivity was abolished, both γδ2TCR-engineered populations maintained their antitumor reactivity (Fig. 3A and B).

One possible explanation for the reduced allo-reactivity in γδ2TCR-transduced T cells despite their significantly recovered endogenous αβTCR may be the preferential hit of memory T...
Figure 2.
Improved antitumor activity of pure γδTCR-engineered T cells after αβTCR T-cell depletion. Enrichment of γδTCR engineered T cells by GMP grade depletion of αβTCR T cells. A, flow cytometric representation of pMP71:γT2A-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+) cells were incubated with 51Cr-loaded Daudi cells at indicated E:T ratios for 4 to 5 hours. pB:αxMDM2/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+) T cells were incubated with different tumor target cells as indicated and IFNγ secretion was measured by IFNγ ELISPOT. pMP71:ΔNGFGR-transduced T cells were used as control T cells. IFNγ spots per 15,000 T cells are shown as mean of triplicates (±SD). Statistical significances were calculated by two-way ANOVA; **, 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 10 μmol/L pamidronate (PAM) and IFNγ secretion was measured by ELISPOT. IFNγ spots per 15,000 T cells are shown as mean of triplicates (±SD). Fifty spots/15,000 cells were considered as a positive antitumor response and indicated by the black horizontal line.
cells and not allo-reactive naïve T cells in the transduction procedure. However, this explanation could be excluded because 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 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 with ΔNGFR-transduced cells (MFI: 13117; Fig. 3C, top). Finally, a preferential recovery of introduced as compared with

Figure 3.
Abolished allo-reactivity but preserved antitumor 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 IL2 for the last 6 days and considered to be resting T cells. A, resting T cells were cocultured with OPM2 tumor cells and a panel of HLA-mismatched EBV-LCLs for 24 hours and IFNγ ELISPOT assay was used to measure both antitumor activity and allo-reactivity. IFNy 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 two different donors were mixed, indicated by the donor numbers, and used as allo-reactive target cells. Resting T cells were cocultured with Daudi tumor cells and the allo-reactive target cells for 24 hours and IFNγ ELISPOT assay was used to measure both antitumor activity and allo-reactivity. IFNy 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, top) or incubated with anti-CD3/CD28 beads for 4 days (αCD3/CD28-stimulated T cells, bottom). T cells were stained with a panγδTCR- 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 by two-way ANOVA: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
endogenous TCR chains upon antigen encounter may also con-tribute to this effect. Indeed, upon stimulation of resting T cells with anti-CD3/CD28 beads, the expression of the retrovirally introduced γδ TCR decreased less profoundly, was restored within 24 hours and reached higher levels than before T-cell stimulation within 48 hours (Fig. 3C, bottom, and Supplementary Fig. S2A–S2D). This quick restoration and high level of TCR surface expression following T-cell reactivation was not restricted to γδ TCRs, thus most likely a property of the expression system used here, because expression kinetics of a retrovirally introduced γδ TCR were comparable (Supplementary Fig. S2). These results suggest that although the endogenous γδ TCR is reexpressed in resting engineered T cells, reactivation preferentially decreases endogenous γδ TCR expression (Fig. 3C, bottom). To formally address this hypothesis in a more physiologically relevant setting, we cocultured resting γδ TCR-engineered γδ TCR-depleted T cells with an HLA-matched EBV-LCL that induces an allo-reactive IFNγ response by αβ TCR-transduced cells (LCL1 from Fig. 3A). Co-culturing these cells resulted in a down-regulated endogenous γδ TCR expression, which reached a minimal level after 48 hours, whereas the γδ TCR expression remained largely stable over time (Supplementary Fig. S2E). Thus, we confirm that in γδ TCR-engineered T cells T-cell reac-tivity, such as allo-reactivity, is prevented and the expression of retrovirally introduced tumor-specific TCRs is favored and antitumor reactivity maintained. These data question the need of a complete elimination of endogenous γδ TCR chains in purified engineered immune cells when expressing strong γδ TCR competitiors with an optimal expression system.

Improved in vivo tumor control by optimized engineered T-cell product

We evaluated the clinical potency of the optimized γδ TCR-engineered T cells produced with the extensively used pBullet retroviral transduction and antibiotic selection system (45), referred to as pB-γδ TCR T cells. Following transduction of peripheral blood αβ T cells and selection with antibiotics (pBullet) or enrichment with γδ TCR depletion beads (pMP71) and subsequent T-cell expansion, both immune products were evaluated. Interestingly, not only the percentage γδ TCR+ T cells was higher for the γδ TCR-depleted γδ TCR T-cell product, but also the number of γδ TCR complexes per cell increased more than 2-fold compared with pB-γδ TCR T cells as measured by MFIs (Fig. 4A). In addition, lysis of three tested tumor cell lines was significantly enhanced by γδ TCR-depleted γδ TCR T cells transduced with the pMP71:γδ T2A-Δ vector cassette as compared with pB-γδ TCR T cells (Fig. 4B). To test if the superior antitumor activity of γδ 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−/−γδ TCR−/− 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 with Mock TCR T cells, but γδ TCR-depleted γδ TCR T cells further delayed tumor outgrowth and significantly increased survival compared with pB-γδ TCR T cells (Fig. 4C).

In a second tumor model of multiple myeloma, the antitumor activity of the optimized γδ TCR-depleted γδ 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 four of seven mice (Supplementary Fig. S3). One hundred and twenty days after first tumor and T-cell injections, tumor free mice were rechallenged with a second injection of tumor cells without prior irradiation and nonirradiated naive mice were used as control for tumor outgrowth. Selectively rechallenged 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-γδ 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
alternative TCR competitors and clinical anti-β7TCR beads is a highly efficient system, even if interference with endogenous β7TCR expression is far from complete. Thus, we present to our best knowledge the first enrichment procedure using a clinical-grade anti-β7TCR 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 β7TCR chains.

Optimal gene expression is crucial when interference with β7TCR chains is not mediated via siRNA (21), ZFNs (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 TCR8 chain downstream of the 2A cleavage element and this is in line with an optimal β7TCR 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 γδT cells from the donor. This procedure resulted in removal of nontransduced and poorly transduced bystander cells, improved γδTCR surface expression levels, and resulted in almost 100% γδTCR-engineered cells in the end product, which was translated into an increased antitumor function both in vitro and in vivo. Interestingly, when compared with a nonengineered polyclonal primary γδT-cell population, selected γδTCR-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 γδTCR in contrast to a polyclonal usage of γδTCRs in a primary γδT-cell population in combination with high γδTCR surface expression as a result of the selection procedure.

The substantial upregulation of endogenous β7TCR chains in resting engineered immune cells allowed us to thoroughly investigate pitfalls, if interference with endogenous β7TCR 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 (i) a still reduced surface expression of γδTCR when compared with nonengineered cells and (ii) faster and higher upregulation 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 downmodulation of the endogenous β7TCR upon introducing a tumor-specific immune receptor paves the way towards the broader applicability of our strategy: the combination of siRNA (22), ZFNs (20), or TALENS (23) to knockdown endogenous TCRs with the introduction of other innate receptors followed by the depletion with anti-β7TCR 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 β7TCR 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 (47).

Surprisingly, we also observed a relatively low allo-reactivity in nonengineered 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 β7TCRs in naïve T cells, which have been reported as more potent GVHD mediators than memory T cells (48, 49). However, our observation is also in line with the recent clinical observation that the transfer of nonselected CAR-engineered donor T cells with intact endogenous β7TCR chains in human did not associate with substantial GVHD (50) and might reflect a rather reduced β7TCR 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 (refs. 11 and 20; T. Straetemans and J. Kuball, unpublished data).

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 β7TCR chains and clinical-grade β7TCR beads. We also demonstrate that the interference with endogenous β7TCR 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 developed depletion strategy is therefore applicable to nearly any engineered immune product interfering temporarily or permanently with endogenous β7TCR chains.

Disclosure of Potential Conflicts of Interest
J. Kuball reports receiving commercial research grants from Miltenyi and is listed as an inventor on a patent, owned by University Medical Center Utrecht, on the use of antibodies for enrichment of engineered T cells with exogenous immune receptors and antibodies for use in depletion of engineered T cells. No potential conflicts of interest were disclosed by the other authors.

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