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ORIGINAL ARTICLE

Activated human γδ T cells induce peptide-specific CD8+ T-cell responses to tumor-associated self-antigens

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Abstract Specific cellular immunotherapy of cancer requires efficient generation and expansion of cytotoxic T lymphocytes (CTLs) that recognize tumor-associated self-antigens. Here, we investigated the capacity of human γδ T cells to induce expansion of CD8+ T cells specific for peptides derived from the weakly immunogenic tumor-associated self-antigens PRAME and STEAP1. Coincubation of aminobisphosphonate-stimulated human peripheral blood-derived γδ T cells (Vγ9+Vδ2+), loaded with HLA-A*02-restricted epitopes of PRAME, with autologous peripheral blood CD8+ T cells stimulated the expansion of peptide-specific cytolytic effector memory T cells. Moreover, peptide-loaded γδ T cells efficiently primed antigen-naive CD45RA+ CD8+ T cells against PRAME peptides. Direct comparisons with mature DCs revealed equal potency of γδ T cells and DCs in inducing primary T-cell responses and peptide-specific T-cell activation and expansion. Antigen presentation by γδ T-APCs was not able to overcome the limited capacity of peptide-specific T cells to interact with targets expressing full-length antigen. Importantly, T cells with regulatory phenotype (CD4+ CD25hiFoxP3+) were lower in cocultures with γδ T cells compared to DCs. In summary, bisphosphonate-activated γδ T cells permit generation of CTLs specific for weakly immunogenic tumor-associated epitopes. Exploiting this strategy for effective immunotherapy of cancer requires strategies that enhance the avidity of CTL responses to allow for efficient targeting of cancer.

Keywords Antigen presentation · γδ T cells · Tumor antigens · Self-antigens · Cellular therapy

Introduction

Even though effective treatments now exist for many childhood malignancies, some diseases have remained incurable. For example, advanced stage neuroblastomas and disseminated bone and soft tissue sarcomas are fatal in 60–80% of patients despite modern multimodal treatment regimens involving intensive systemic chemotherapy and optimal local control [1, 2]. Specific immune targeting by cytotoxic T cells (CTLs) may provide an additional treatment modality to eliminate residual disease and prevent relapse in these cancers. Potentially useful target antigens in childhood solid tumors are the cancer testis antigen PRAME ("preferentially expressed antigen in melanoma") [3] and the "six-transmembrane epithelial antigen of the prostate," STEAP1 [4]. PRAME overexpression has been shown in both neuroblastoma and in sarcomas [3, 5, 6], and absence of PRAME in neuroblastoma was restricted to patients with favorable disease [5]. STEAP1 was found to be highly overexpressed in Ewing sarcoma and other high-grade sarcomas [4]. The major challenge for therapeutic T-cell targeting of these tumor-associated self-antigens is the rarity of self-antigen-specific CTLs in the repertoire of normal donors and cancer patients as a consequence of thymic

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depletion. Recruitment, activation, and expansion of these CTLs generally involve priming of rare naive T cells and strictly depend on the quality of the antigen-presenting cell (APC) and on optimized culture conditions [7]. The standard approach to generating tumor-antigen-specific CTLs is based on professional antigen presentation by monocyte-derived dendritic cells (DCs). However, in vitro manipulation of autologous DCs is complicated by the functional heterogeneity and limited life span of this rare cell population, and by their functional impairment in cancer patients [8]. Moreover, DCs have been shown to induce T cells with regulatory phenotype and function, which prevent effective antitumor immune responses [9]. Thus, for more effective and broadly applicable antitumor immunotherapies, robust APCs to induce clinically relevant quantities of functional tumor-specific CTLs are needed.

T cells bearing Vγ2Vδ2+ T-cell receptors have recently emerged as an attractive candidate APC population [10]. Vγ2Vδ2+ T cells are unique to humans and primates and represent the largest subset of $\gamma\delta$ T cells in human peripheral blood (reviewed in [11]). They interact with various protein and non-protein antigens independent of MHC binding and can be efficiently activated by synthetic analogs of endogenous pyrophosphates. These include aminobisphosphonate compounds (pamidronic acid and zoledronic acid) that are used to treat disorders involving excessive bone resorption [12]. Upon activation, $\gamma\delta$ T cells exert potent effector functions including cytolysis and secretion of activating cytokines [13, 14]. Moreover, activated $\gamma\delta$ T cells acquire phenotypic and functional characteristics of professional APCs and can induce T-cell responses to allogeneic, microbial, and viral antigens [10, 15]. In a first attempt to exploit $\gamma\delta$ T cells for antitumor immunotherapy, we have demonstrated that autologous $\gamma\delta$ T cells efficiently stimulate autologous CTLs against the tumor-associated Epstein–Barr virus latency antigen LMP2 in patients with Hodgkin lymphoma [15].

Here, we hypothesized that activated human $\gamma\delta$ T cells can efficiently present PRAME and STEAP1-derived epitopes and allow breaking tolerance against these tumor-associated self-antigens.

Methods

Cells and cell lines

Peripheral blood samples were collected from healthy donors upon approval from the University of Muenster Ethical Board. K-562 is a human leukemia cell line, and RD-ES is a Ewing sarcoma cell line (both from DSMZ, Braunschweig, Germany). Tumor cells were cultured in RPMI 1640 medium (Invitrogen, Darmstadt, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS; Thermo Fisher, Bonn, Germany) and 2 mM L-glutamine, and maintained at 37°C and 5% CO_2. The artificial APC cell line K562 aAPC [7] was derived from the human leukemia cell line K562 by genetic modification to express human HLA-A2, CD80, CD40L, and OX40L, as previously described [7]. PHA blasts were generated by culturing peripheral blood mononuclear cells (PBMC) for 72 h in RPMI 1640 medium containing 3 μg/ml PHA (Sigma-Aldrich, Steinheim, Germany), washed, and resuspended. The amphotropic packaging cell line Phoenix-ampho [16] was provided by Gary P. Nolan (Stanford, CA). The amphotropic FLYRD18 (provided by E. Vanin, Houston, TX) cell line provides viral recombinants with the RD114 envelope.

Plasmids, production of recombinant retrovirus, and transduction of $\gamma\delta$ T cells

The STEAP1 sequence (NM_012449) was amplified by PCR from cDNA derived from the Ewing sarcoma cell line RD-ES and the PRAME sequence (NM_006115) from K-562 cell cDNA and then cloned into the *Age*I and *Xho*I sites of the retroviral vector SFG-IRES-GFP [17] or SFG, respectively. To generate stable retroviral producer cell lines, fresh supernatants collected from transiently transfected Phoenix-ampho cells were used to infect the packaging cell line FLYRD18 by overnight incubation at 37°C in the presence of polybrene (4 μg/ml). The cells were subjected to multiple rounds of infection under the same conditions. Viral supernatants were generated on the resulting bulk producer cell lines by adding Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Taufkirchen, Germany) supplemented with 20% FCS. After 24 h of incubation at 32°C, the supernatants were filtered through a 0.45-μm filter and used to transduce the $\gamma\delta$ T cells.

Expansion and transduction of $\gamma\delta$ T-cell cultures

PBMCs (2×10^6) were stimulated with zoledronate (Zometa®, Novartis, Nuernberg, Germany; 1 μg/ml) in RPMI 1640 medium, supplemented with recombinant human interleukin-2 (rhIL-2; Proleukin®, Chiron, Emeryville, CA) at 100 IU/ml, 10% FCS, and 2 mM L-glutamine, in a 24-well plate. Two weekly doses of rhIL-2 (100 IU/ml) were added. Positive selection of $\gamma\delta$ T cells for coincubation experiments was performed on day 7 using TCR-$\gamma\delta$-specific magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 3×10^7 zoledronate-activated cells were labeled with 60 μl Anti-TCR $\gamma\delta$ Hapten antibodies for 10 min at 4°C. Ninety microliters of MACS buffer and 120 μl Anti-Hapten Micro Beads were added for 15 min at 4°C, and then the cells were washed with 6 ml MACS buffer and pelleted by centrifugation (300×*g*, 10 min). The resuspended cells were loaded onto an LS column (Miltenyi Biotec) for magnetic separation.

For retroviral transductions, γδ T cells were pre-stimulated with zoledronate for 48 h as above, transferred to 24-well non-tissue culture-treated plates (Becton–Dickinson, Franklin Lakes, New Jersey) coated with recombinant fibronectin fragment FN CH-296 (Retronectin, Takara Shuzo, Otsu, Japan) at 4 μg/cm^2, and coincubated with viral supernatant for 48 h, followed by further expansion in medium containing rhIL-2 (100 IU/ml). γδ T cells transduced with SFG-IRES-GFP vectors were selected by fluorescence-activated cell sorting of TCRαβ-negative, GFP+ cells.

Peptides and pentamers

To stimulate PRAME-peptide-specific responses, a pool of four previously described HLA-A*02-restricted epitopes, including **VLD**GLDVLL, **ALY**VDSLFFL, and **SLL**Q HLIGL [18], as well as the recently identified 9-mer peptide **NLT**HVLYPV [19] was used. STEAP1-derived, HLA-A*02-restricted peptides were **FLY**TLLREV [20], **GLL**S FFFAV [21], **LLL**GTIHAL [22], and **MIA**VFLPIV [21]. All peptides were synthesized by ProImmune (Oxford, UK), dissolved in DMSO, and used at 5 μM concentrations. Peptide aliquots were stored at −80°C.

Generation of DCs and peptide loading

CD14+ cells were selected from freshly isolated PBMCs by magnetic cell selection using CD14-specific microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), seeded at 10^6/well into a 24-well plate in CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 5% human AB serum (PAN Biotech, Passau, Germany), 2 mM L-glutamine, 800 U/ml GM-CSF, and 1,000 U/ml interleukin (IL)-4 (both cytokines obtained from ImmunoTools, Friesoythe, Germany), and incubated at 37°C and 5% CO_2. For maturation, DCs were harvested on day 5 and replated in medium supplemented with 1 μg/ml IL-6 (Immunotools, Friesoythe, Germany), 1 μg/ml IL-1β (R&D, Wiesbaden-Nordenstadt), 1 μg/ml PGE2 (Sigma-Aldrich), and 1 μg/ml TNF-α (Immunotools). Mature DCs were harvested on day 7. For their use as stimulator cells, DCs or γδ T cells were loaded with peptide pools for 2 h at 37°C.

Flow cytometry

T cells were phenotyped with mAbs against CD3, CD8, CD4, CD56, CD45RO, and CCR7 (all from BD Pharmingen, Heidelberg, Germany). For each sample, 20,000 cells were analyzed with FACS Calibur and BD Cell Quest Software or with FACS Canto and FACS Diva Software. For intracellular FoxP3 staining, cells were fixed with Fixation/Permeabilization buffer (eBioscience, San Diego, USA) for 30 min, washed twice in 1× Permeabilization buffer, and resuspended in 100 μl 1× Permeabilization buffer containing 2% normal rat serum for blocking. After 15 min of incubation at 4°C, fluorochrome-conjugated anti-human FoxP3 antibody (eBioscience) was added for additional 30 min of incubation, followed by two washing steps. The percentage of FoxP3-positive cells was determined after gating on CD4+ cells. For intracellular PRAME staining, 0.5 × 10^6 γδ T cells were washed once in PBS containing 2% FCS (Washing Buffer), permeabilized by 10 min incubation in 300 μl acetone at −20°C, washed again and then resuspended in 100 μl Washing Buffer containing 4 μl unlabeled PRAME-specific mAb clone H-10 (Santa Cruz Biotechnology, Heidelberg, Germany) or 4 μl IgG1 isotype antibody (BD Pharmingen). After 30 min at 4°C, the cells were washed twice, resuspended in 100 μl Washing Buffer containing 1 μl secondary fluorochrome-conjugated goat anti-mouse antibody (BD Pharmingen) for 15 min at 4°C, washed, and analyzed.

Activation and expansion of antigen-specific T cells

A total of 5 × 10^4/well irradiated (30 Gy) peptide-loaded mature DCs or activated γδ T cells were seeded in 24-well plates and coincubated with 1 × 10^6 autologous CD8+ T cells per well (S:R ratio of 1:20) in RPMI medium supplemented with 5% AB serum and 2 mmol of glutamine supplemented with the cytokines IL-6 (100 ng/ml), IL-7 (10 ng/ml), IL-12 (1 ng/ml), IL-15 (2 ng/ml), and IL-21 (10 ng/ml), all from Immunotools, for 7 days at 37°C and 5% CO_2. Positive selection of CD8+ responder T cells from freshly isolated PBMCs was performed by using CD8-specific magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To purify naive CD8+ T cells, we used the Naïve CD8+ T cell Isolation Kit (Miltenyi Biotec). This method is based on antibody labeling and subsequent magnetic depletion of CD45RO-, CD56 CD57-, and CD244-expressing cells, followed by positive selection of the CD8+ effector T-cell population from the pre-enriched cell fraction. After primary stimulation with autologous DCs or γδ T-APCs, T cells were resuspended and stimulated weekly with artificial APCs derived from the human leukemia cell line K562 (K562 aAPCs) by genetic modification to express human HLA-A2, CD80, CD40L, and OX40L [7] and loaded with the respective peptide pools. For the first restimulation, IL-7 (10 ng/ml), IL-12 (1 ng/ml), IL-15 (2 ng/ml), and IL-21 (10 ng/ml) were added, and for subsequent stimulations, IL-2 was supplemented at 50 IU/ml. For the analysis of regulatory T cells, CD3+ T cells were purified using the Pan T Cell Isolation Kit (Miltenyi Biotec). A total of 1.5 × 10^6 CD3+ T cells per well were stimulated with 7.5 × 10^4/well irradiated (30 Gy) peptide-loaded autologous activated γδ T cells or DCs in 24-well plates (stimulator-to-responder ratio 1:20) in RPMI medium

supplemented with AB serum (5%), glutamine (2 mmol), IL-7 (10 ng/ml), IL-12 (1 ng/ml), and IL-15 (2 ng/ml) for 10 days. The proportions of $CD25^{hi}FoxP3+$ cells within the gate of CD4+ T cells were analyzed by flow cytometry.

ELISPOT assay

Ninety-six-well filter plates (MultiScreen, MAHAS4510, Billerica, MA) were coated overnight with 10 μg/ml anti-IFNγ Ab (catcher mAB91-DIK; Mabtech, Nacka Strand, Sweden). T cells were plated at 0.5 to 1 $\times$ 10^5 cells/well and stimulated with PRAME or STEAP1 peptide pools (5 μM each peptide) or individual peptides as indicated. Non-specific stimulation with PMA/ionomycin was used as a control. Experiments were done in triplicates. After 18–24 h, the plates were washed and incubated with the secondary biotin-conjugated anti-IFN-γ mAb (detector mAb (7-B6-1 biotin); Mabtech). After incubation with avidin-biotinylated HRP complex (Vectastain Elite ABC Kit (standard), PK6100; Vector Laboratories, Burlingame, CA), plates were developed with AEC substrate (Sigma-Aldrich). Spot-forming cells (SFCs) and input cell numbers were plotted after plates were evaluated using Bioreader 3000 Pro, Biosys, Karben, Germany.

Cytotoxicity assays

Cytotoxic specificity was determined in standard ^{51}Cr release assays. Various numbers of T effector cells were coincubated in triplicate with 2,500 target cells labeled with 100 μCi $^{51}Cr/0.5 \times 10^6$ cells (PE Applied Biosystems) in a total volume of 200 μl in a V-bottomed 96-well plate. At the end of a 4-h incubation period at 37°C and 5% CO_2, supernatants were harvested, and radioactivity was counted in a gamma counter. Maximum release was determined by lysis of target cells with Triton X.

Statistical analysis

The Student's *t* test was used to test for significance in each set of values, assuming equal variance. Mean values ± SD are given unless otherwise stated.

Results

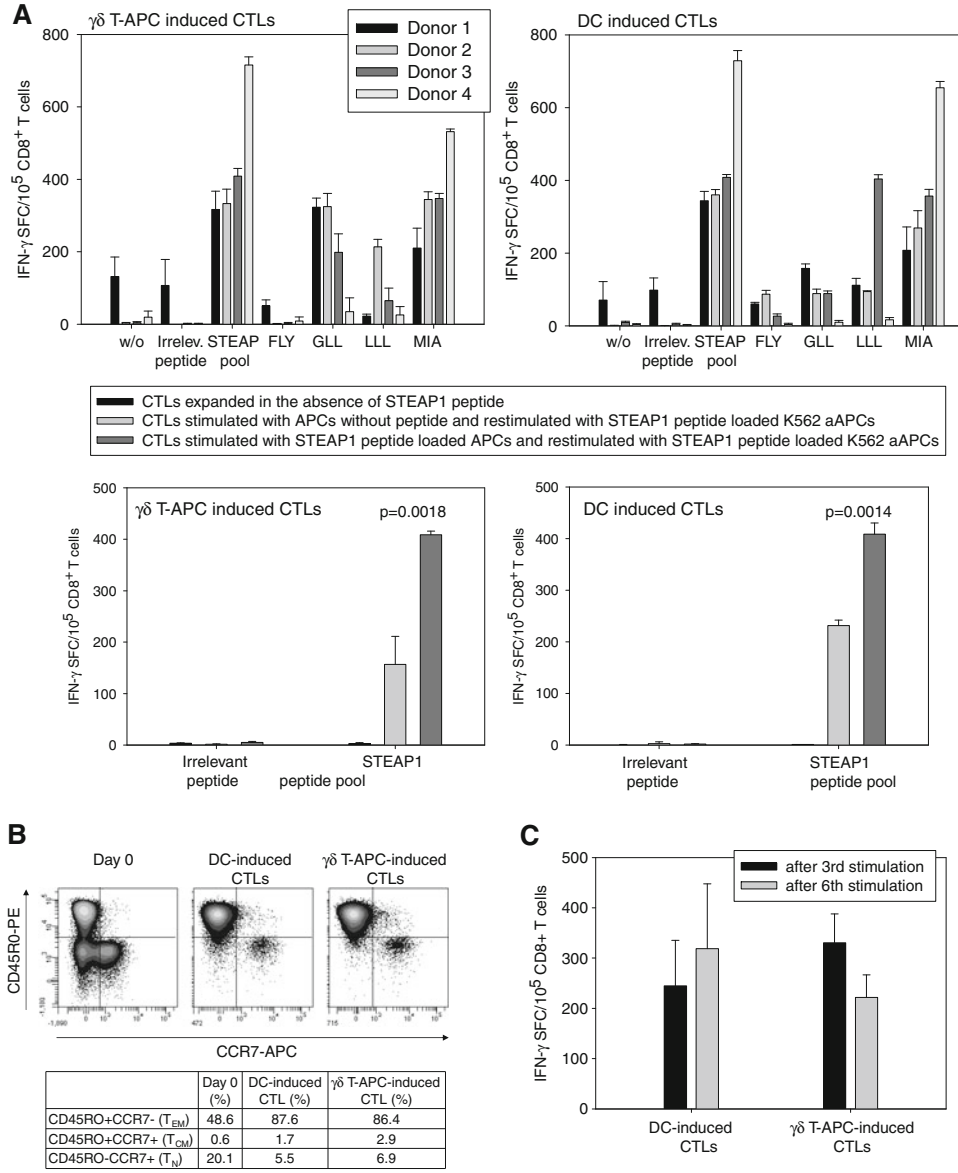
Zoledronate-activated $\gamma\delta$ T cells induce the expansion of STEAP1 peptide-specific CTLs from CD8+ autologous T cells

To assess whether $\gamma\delta$ T cells can induce autologous CTL responses to peptides derived from tumor-associated self-antigens, peripheral blood mononuclear cells from healthy donors were stimulated with a single dose of zoledronic acid (1 μg/ml) and IL-2 (100 IU/ml). On day 7, TCR$\gamma\delta$+ T cells represented 65.4 ± 23.2% (range 15.9–93.9%) of cultured cells. $\gamma\delta$ T cells were positively selected by magnetic cell sorting, resulting in purities of 99.2 ± 1.1 (range 96.7–99.9, $n = 11$ cell cultures) (Supplementary Figure available on-line). Purified $\gamma\delta$ T cells were loaded with a pool of four HLA-A*02-restricted STEAP1 peptides and cocultured with autologous responder CD8+ T cells in the presence of IL-6, IL-7, IL-15, IL-21, and IL-12. To avoid background peptide presentation by alternative cells with APC function (CD4+ T cells, monocytes, and B cells), CD8+ responder T cells were purified by positive selection (94.7±3.5%, range 89.7–99.3%). Comparative experiments were performed using peptide-loaded autologous mature DCs as stimulator cells. Cultures were expanded by two to five rounds of restimulation using peptide-loaded K562 aAPCs [7]. ELISPOT analysis after expansion demonstrated the presence of T cells specifically secreting IFN-γ in response to restimulation with the STEAP1 peptide pool used for initial stimulation (Fig. 1a). Moreover, the expanded T cells efficiently recognized individual STEAP1 peptides present within the pool (Fig. 1a, upper panel), with variable patterns of peptide-specific responses among donors and with the use of either $\gamma\delta$ T-APCs or DCs. Irrelevant peptide did not induce any background cytokine secretion. Importantly, $\gamma\delta$ T-APCs were equally effective to autologous DCs in stimulating epitope-specific T cells against STEAP1 ($P = 0.9$). While K562 aAPCs alone cannot prime peptide-specific CTLs(7), we found that they contribute to the generation of STEAP1 peptide-specific CTLs from CD8+ T lymphocytes from both of two donors; however, responses were significantly enhanced by primary stimulation with peptide-loaded DCs or $\gamma\delta$ T cells (Fig. 1a, lower panel).

T-cell populations expanded after initial stimulation with either DCs or $\gamma\delta$ T cells contained comparable proportions of effector memory (T_{EM}; CD45RO+CCR7-), central memory (T_{CM}; CD45RO+CCR7+), and naive (T_N; CD45RO-CCR7+) T-cell subsets (Fig. 1b). During several rounds of expansion in the presence of K562 aAPCs, T cells maintained epitope specificity (Fig. 1c). Thus, aminobisphosphonate-stimulated human peripheral blood-derived $\gamma\delta$ T cells efficiently stimulate peptide-specific T cells against the tumor-associated antigen STEAP1.

Zoledronate-activated $\gamma\delta$ T cells efficiently induce the expansion of PRAME-peptide-specific CTLs and prime peptide-specific CTLs from antigen-naive precursor cells

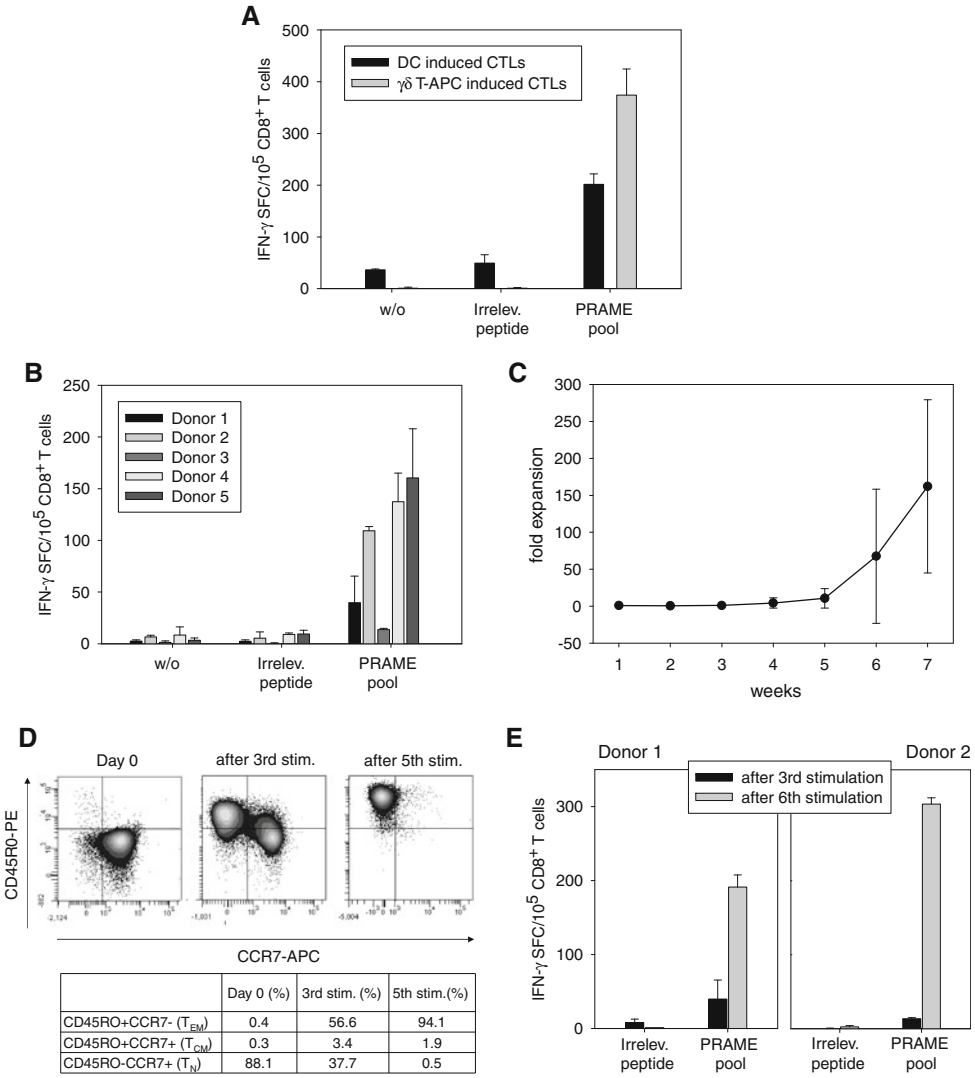
To reproduce our findings with an alternative tumor-associated self-antigen, we used activated $\gamma\delta$ T cells and DCs to



	Day 0 (%)	DC-induced CTL (%)	γδ T-APC-induced CTL (%)
CD45RO+CCR7- (T_{EM})	48.6	87.6	86.4
CD45RO+CCR7+ (T_{CM})	0.6	1.7	2.9
CD45RO-CCR7+ (T_N)	20.1	5.5	6.9

Fig. 1 Zoledronate-activated $\gamma\delta$ T cells activate STEAP1 peptide-specific CTLs. **a** MACS-purified autologous CD8+ T cells from four donors were cocultured with either DCs or $\gamma\delta$ T-APCs pulsed with four HLA-A*02-restricted peptides (5 μM each) derived from STEAP1, followed by two stimulations with peptide-pulsed K562 aAPCs. IFN-γ production was measured by ELISPOT analysis in response to restimulation with STEAP1 peptide pool or with the individual peptides as indicated. As controls (*lower panel*), CD8+ T cells were coincubated with either DCs or $\gamma\delta$ T-APCs in the absence of STEAP1 peptides, followed by two to three stimulations with peptide-pulsed K562 aAPCs, or in the absence of peptide (representative experiment of two). IFN-γ production was measured by ELISPOT analysis in response to restimulation with the STEAP1 peptide pool or with the individual peptides as indicated. Experiments were done in triplicates. **b** The effector phenotypes of CTL prior to and following STEAP1-peptide-specific expansion was determined by staining with CCR7- and CD45RO-specific mAbs by flow cytometry analysis on day 6 after 3rd stimulation. Since CD45RA was not directly analyzed, and the functional relevance of RA-positive T_{EM} cells is not clear, cells with T_{EMRA} phenotype (presumably *lower left* quadrant) were not included into the analysis. **c** STEAP1-specific IFN-γ production of CD8+ T cells after primary stimulation with either DCs or $\gamma\delta$ T-APCs was determined by ELISPOT analysis after two versus five rounds of in vitro restimulation with STEAP1-peptide-loaded K562 aAPCs

induce the expansion of PRAME-specific CTLs. Primary stimulation with both types of APCs loaded with a PRAME peptide pool, followed by 2 rounds of expansion, induced T cells specifically secreting IFN-γ in response to restimulation with PRAME peptides (Fig. 2a). To investigate whether activated $\gamma\delta$ T cells are capable to prime antigen-naive T cells, we performed another series of experiments using purified CD45RA+CD8+ T cells as responder cells. Magnetic cell selection resulted in purities of 98.7 ± 1.2% (96.8–99.8%) CD45RA+CD8+ T cells in 5 donors.



	Day 0 (%)	3rd stim. (%)	5th stim.(%)
CD45RO+CCR7- (T_{EM})	0.4	56.6	94.1
CD45RO+CCR7+ (T_{CM})	0.3	3.4	1.9
CD45RO-CCR7+ (T_N)	88.1	37.7	0.5

Fig. 2 Zoledronate-activated $\gamma\delta$ T-APCs activate PRAME-peptide-specific CTLs and efficiently prime antigen-naïve CD45RA+ CTL. MACS-purified autologous CD8+ T cells (**a**) or CD8+ CD45RA+ naive T cells (**b**) were cocultured with either DCs (**a**) or $\gamma\delta$ T-APCs (**a**, **b**) loaded with four HLA-A*02-restricted peptides (5 μM each) derived from PRAME, followed by two stimulations with peptide-pulsed K562 aAPCs. IFN-γ production was measured by ELISPOT analysis in response to restimulation with the PRAME peptide pool. **c** Expansion of CTLs by initial stimulation with peptide-loaded autologous $\gamma\delta$ T-APCs and subsequent restimulation with peptide-loaded K562 aAPCs. **d** The effector phenotype of PRAME-CTL was determined by flow cytometry prior to and after 3 and 5 rounds of peptide-specific expansion. **e** PRAME-peptide-specific CTL responses by IFN-γ ELISPOT analysis after initial priming of CD45RA+ naive CD8+ T cells with peptide-loaded autologous $\gamma\delta$ T-APCs were compared after two and five subsequent weekly restimulations with peptide-pool-loaded K562 aAPCs

Cocultures of PRAME-peptide-loaded activated $\gamma\delta$ T cells with CD45RA+ naive T cells efficiently induced peptide-specific IFN-γ secreting CTLs (Fig. 2b). Subsequent restimulations with peptide-loaded K562 aAPCs resulted in efficient T-cell expansion of 68 ± 91-fold after a total of 5 stimulations ($n = 3$), and 79- and 244-fold after 6 stimulations ($n = 2$) (Fig. 2c). During expansion, the T-cell cultures acquired an effector memory phenotype, with the majority of T cells after 5 stimulations representing CD45RO+CCR7- effector memory (T_{EM}) T cells (Fig. 2d). Expanding CTLs maintained specificity to PRAME peptide pools, as demonstrated by ELISPOT analysis after a total of three versus six rounds of stimulation that revealed an increase in peptide-specific CTLs during prolonged culture periods in both donors (Fig. 2e). Thus, $\gamma\delta$ T-APCs efficiently prime naive CD8+ T-cell precursors against self-antigen-derived peptides and induce robust expansion of peptide-specific CTLs.

Reduced proportions of T cells with regulatory phenotypes arise during peptide-specific CTL expansion with $\gamma\delta$ T-APCs versus DCs

CD4+ T cells with regulatory phenotype characterized by intracellular FoxP3 expression and high CD25 surface expression (CD4+CD25^{hi}FoxP3+ cells, T_{reg} cells) have been shown to interfere with protective antitumor immune responses and are considered a major impediment to effective cellular immune targeting. Therefore, we compared the proportions of T_{reg} cells in peptide-specific CTL cultures following peptide presentation by either autologous DCs or $\gamma\delta$ T cells. Decreased proportions of CD25^{hi}FoxP3+ cells within the gate of CD4+ T cells were found in all experiments, including both PRAME and STEAP1 peptide stimulation (Fig. 3). Thus, compared to professional antigen presentation by DCs, $\gamma\delta$ T-APCs have

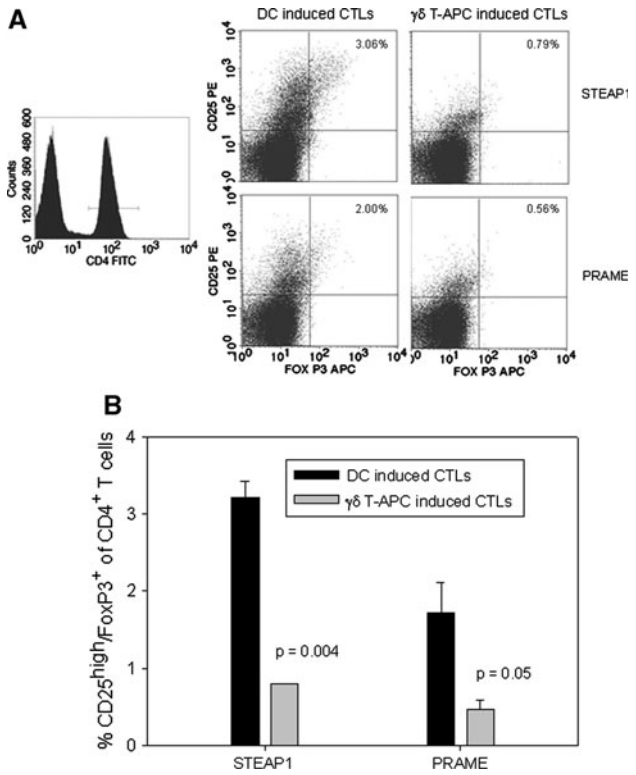


Fig. 3 Peptide-specific CTL expansion by $\gamma\delta$ T-APCs induces lower proportions of T cells with Treg phenotypes than analogous stimulation with DCs. Quantification of CD4+ CD25hi FoxP3+ T_{reg} cells by flow cytometry (**a**) and relative proportions of CD4+ CD25hi FoxP3+T_{reg} cells as a fraction of CD4+ T cells (**b**) during expansion of STEAP1 and PRAME-peptide-specific CTLs after priming of CD3+ T cells with autologous $\gamma\delta$ T-APCs or DCs, respectively

a lower propensity to induce T cells with regulatory phenotype along with peptide-specific CTLs.

Self-peptide-specific CTLs lyse peptide-loaded target cells but fail to functionally interact with antigen-expressing targets

To further assess the functional properties of the expanded CTLs, we determined their specific target cytolysis. CD8+ T cells expanded upon primary stimulation with STEAP1 or PRAME-peptide-loaded activated $\gamma\delta$ T cells efficiently and specifically lysed peptide-loaded autologous PHA blasts or $\gamma\delta$ T cells (Fig. 4a, b). The question remains whether expanded populations of peptide-pool-specific T cells functionally interact with tumor targets that naturally overexpress the target antigen. Previous studies have demonstrated low T-cell receptor avidity and failure to lyse tumor targets of CTLs generated by stimulation with peptides derived from tumor-associated self-antigens [23–25]. To explore the capacity of STEAP1 CTLs to lyse target cells via endogenous STEAP1 expression, we engineered activated $\gamma\delta$ T cells to express full-length STEAP1 by retroviral gene transfer. Lysis of autologous $\gamma\delta$ T cells expressing wild-type STEAP1 by CTLs induced by either DCs (left panel) or $\gamma\delta$ T-APCs (right panel) was not above the background of unmodified $\gamma\delta$ T cells (Fig. 4c). Thus, while $\gamma\delta$ T-APC induced CTLs are not inferior to those stimulated with professional APCs, antigen presentation by $\gamma\delta$ T-APCs does not overcome the failure of peptide-specific CTLs against the self-antigen STEAP1 to functionally interact with targets endogenously expressing antigen.

One strategy to extend the functional capacity of peptide-specific CTLs toward antigen-expressing targets is to stimulate antigen-specific precursor cells with full-length antigen, representing the entire range of antigenic epitopes. We have previously shown that activated $\gamma\delta$ T cells are capable of endogenous processing and MHC class I presentation of antigens [15]. Thus, we set out to investigate whether the introduction of the full-length tumor-associated self-antigen DNA sequence into activated $\gamma\delta$ T cells by retroviral gene transfer allows them to stimulate expansion of fully functional antigen-specific CTL. Since the GFP marker gene is immunogenic and likely to skew CTL responses to STEAP-GFP (or PRAME-GFP) transgenes toward GFP, we first established flow cytometry-based intracellular detection methods of PRAME and STEAP1 using the commercially available antibodies anti-PRAME clone H-10, and anti-STEAP1 clone XY-16, respectively. While detection of STEAP1 above background was not reliable (not shown), an optimized intracellular staining protocol allowed to reliably determine and quantify PRAME expression in $\gamma\delta$ T cells after retroviral insertion of full-length PRAME (Fig. 5a, c). The efficiencies of transduction as assessed by intracellular staining on days 1–3 after retroviral gene transfer in 12 donors were $6.3 \pm 2.4\%$ (range 1.5–10.9%). However, repeated attempts at using PRAME gene-modified $\gamma\delta$ T cells to generate CTLs against full-length PRAME failed (not shown). In a previous study, transient overexpression of PRAME in cultured cell lines was shown to induce a caspase-independent cell death [26]. To exclude potential toxicity of the PRAME gene product in $\gamma\delta$ T cells, $\gamma\delta$ T-APCs were stained with annexinV and 7AAD after transduction. PRAME-transduced $\gamma\delta$ T cells were not prone to increased apoptosis (Fig. 5b). By contrast, repeated intracellular staining of gene-modified $\gamma\delta$ T cells revealed rapid downregulation of PRAME expression during in vitro culture to expression levels not exceeding those of GFP-transduced control cells (Fig. 5c). Thus, in contrast to the EBV-associated latency antigen LMP2a [15], the self-antigen PRAME cannot be expressed in $\gamma\delta$ T cells to allow for the presentation of full-length antigen.

In conclusion, $\gamma\delta$ T cells are potent APCs capable of priming naive CD8+ T cells against tumor-associated self-antigens, with comparable efficiency to the current gold

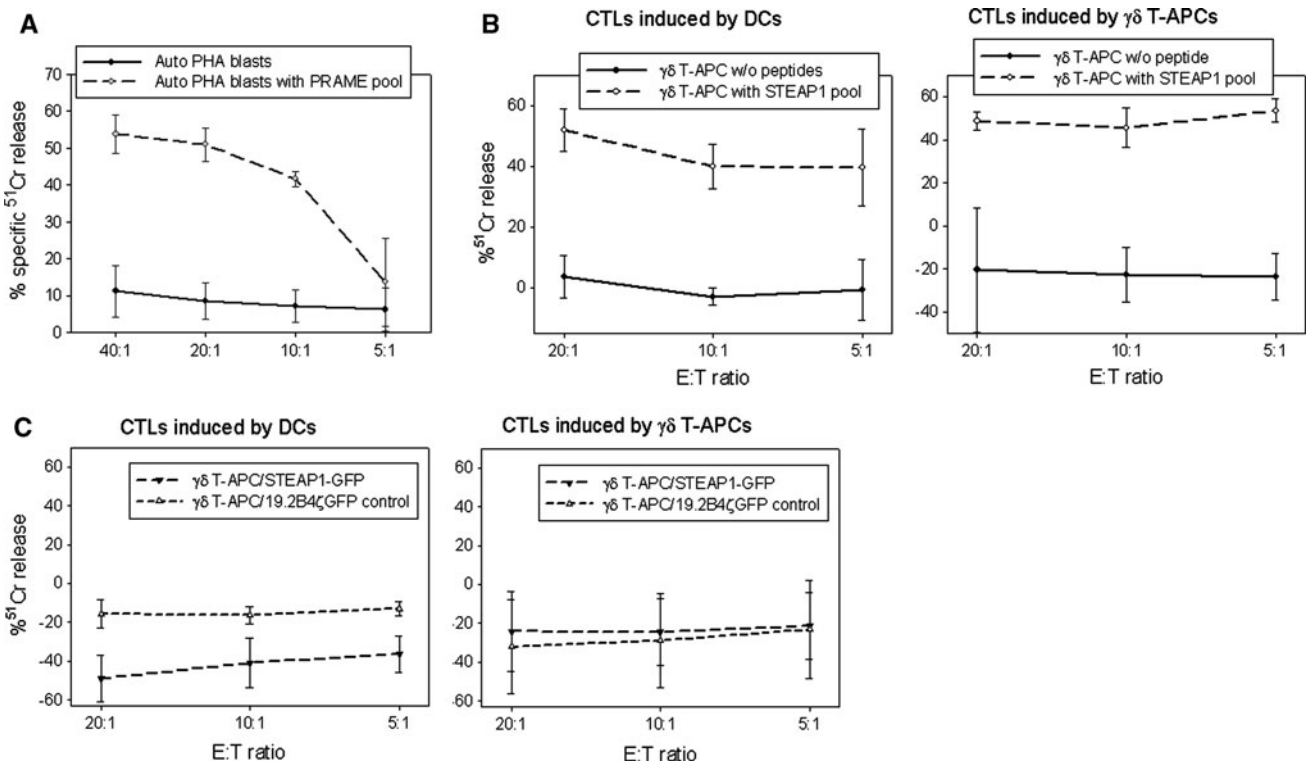


Fig. 4 Peptide-specific CTLs specifically and efficiently lyse peptide-loaded targets but fail to interact with endogenous antigen. Cytolytic activity of PRAME (**a**) or STEAP1 (**b**, **c**)-peptide-specific CTL generated by primary activation of naive CD8+ T cells with $\gamma\delta$ T-APCs or with DCs, as indicated, toward autologous PHA blasts or $\gamma\delta$ T cells loaded with PRAME peptide pool or in the absence of peptide (**a**, **b**) or toward $\gamma\delta$ T cells retrovirally transduced to express STEAP1 or the control construct 19.2B4ζ (**c**) in ^{51}Cr release assays. CTLs were tested after a total of 6 stimulations (representative experiment of two).

standard of antigen presentation via DCs. They induce lower proportions of T cells with regulatory phenotypes and may thus be a superior source of APCs for adoptive immunotherapy of cancer. Failure of in vitro generated peptide-specific CTLs to interact with antigen-expressing tumor cells remains a substantial obstacle to adoptive T-cell strategies and cannot easily be overcome by full-length antigen expression in APCs since these may be subject to downregulation in non-malignant cells.

Discussion

Efforts to generate tumor-antigen-specific CTLs for immunotherapy of cancer are limited by the requirement for autologous professional APCs that allow to prime and expand rare subpopulations of T cells with native antigen specificity. Here, we demonstrate that $\gamma\delta$ T cells under optimized culture conditions are equivalent to DCs in stimulating epitope-specific CTL responses against weakly immunogenic tumor-associated self-antigens.

Previous interest in exploiting $\gamma\delta$ T cells for anticancer immunotherapy has mainly relied on a potential role of this T-cell subset in tumor immunosurveillance. Indeed, in a recent in vitro study, $\gamma\delta$ T cells were cytotoxic to cancer-initiating subsets in solid tumors [27]. Clinical trials have been started to address whether bisphosphonate-activated autologous peripheral blood $\gamma\delta$ T cells have relevant anti-tumor activity in cancer patients [28, 29]. More recently, the capacity of $\gamma\delta$ T cells to act as APCs has been explored. Activation of $\gamma\delta$ T cells with their TCR ligand IPP or with aminobisphosphonates indeed induces expression of various (co)stimulatory molecules [10, 15]. Moreover, activated $\gamma\delta$ T cells can promote potent CD4+ and CD8+ $\alpha\beta$ T-cell responses to allogeneic, microbial, viral, and tumor model antigens [10, 30], including subdominant viral epitopes [15], cancer testis antigens [30], and primary responses by naive CD4+ and CD8+ $\alpha\beta$ TCR+ T cells [10, 30]. Here, we extend these observations to the weakly immunostimulatory self-antigens PRAME and STEAP1 that are overexpressed in pediatric solid cancers and thus provide attractive target antigens in refractory disease. Due to the low immunogenicity of most tumor-associated antigens, tumor-antigen-specific T cells must be derived from low-frequency precursor cells within the naive autologous repertoire, challenging the quality of the APC used for in vitro stimulation. We demonstrate that autologous $\gamma\delta$ T cells can indeed prime and expand rare CD8+ T

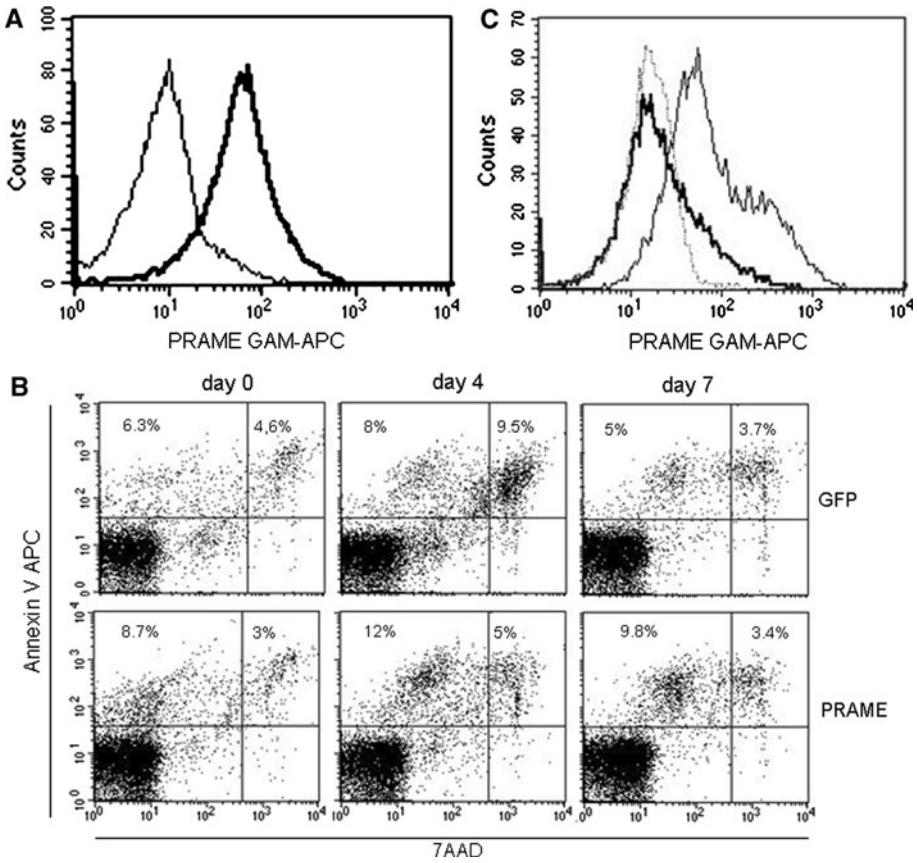


Fig. 5 PRAME full-length antigen expression in $\gamma\delta$ T-APCs. **a** Intracellular staining of K-562 cells with PRAME-specific mAb (*thick line*) versus isotype control antibody (*thin line*). **b** Fluorescence-labeled Annexin V and 7AAD staining of PRAME versus GFP-transduced $\gamma\delta$ T cells(one experiment of two). **c** Intracellular staining of $\gamma\delta$ T-APCs following retroviral gene transfer of full-length PRAME on the day of transduction (*thin line*) and on day 7 after transduction (*thick line*). GFP-transduced $\gamma\delta$ T-APCs served as negative control (*dashed line*)

cells from the naive T-cell compartment to tumor-associated peptides with an efficiency similar to that of DCs.

The use of autologous $\gamma\delta$ T cells as professional APCs has a number of potential advantages over the current gold standard of DCs. Firstly, the cytolytic effector functions and native antitumor activity by $\gamma\delta$ T cells may allow for MHC-independent tumor targeting and may thus amplify peptide-specific immune responses to $\gamma\delta$ T-APC-based tumor vaccines. Secondly, compared to DC expansion that is time-consuming and expensive and involves cytokines often unavailable as clinical-grade reagents, $\gamma\delta$ T cells are easily expanded to high numbers in vitro by culture of peripheral blood lymphocytes in the presence of aminobisphosphonates [12]. Moreover, while autologous DC culture and function are negatively affected by the extensive pretreatment of many cancer patients with immunosuppressive medications, $\gamma\delta$ T cells are effectively activated and expanded in cancer patients [15, 28, 29].

Thirdly, we show that $\gamma\delta$ T cells are significantly less active in inducing FoxP3+ T_{reg} cells compared to DCs. The immunosuppressive properties of DCs are a serious concern for their therapeutic use. Besides their role in effector T-cell activation, human DCs are also specialized to limit unrestrained immune responses, e.g., by upregulation of the immunomodulating enzyme indoleamine 2,3-dioxygenase. Specifically, mature human DCs efficiently support the expansion of autologous FoxP3+ T_{reg} cells [9, 31], which in turn suppress DC-stimulated autologous and allogeneic T-cell proliferation. Thus, DC-mediated expansion of T_{reg} cells may severely counteract the activity of tumor-specific T cells. Reduced T_{reg} expansion in response to $\gamma\delta$ T-APC stimulation therefore provides an additional rationale for the choice of this cell population as tumor APC.

Finally, an attractive feature of $\gamma\delta$ T cells is their sensitivity to genetic modification [15, 32]. DCs, due to their failure to divide and proliferate, do not allow for stable integration of foreign genes. Gene transfer may be a useful tool to manipulate APC function, homing, and survival by expression of costimulatory, cytokine, or chemokine genes [33, 34] and to express the desired antigens for the stimulation of autologous tumor-antigen-specific CTLs. Indeed, in an Epstein–Barr virus model, we have confirmed that $\gamma\delta$ T cells gene-modified to express full-length antigen elicited the same breadth of LMP2 epitope-specific T-cell responses as CTL lines generated by stimulation with autologous LCLs [15]. Here, we show that aberrant expression of tumor-associated self-antigens in $\gamma\delta$ T cells is not always feasible, as expression of full-length PRAME was rapidly downregulated after gene transfer. This observation reveals a potential dilemma for therapeutic applications of these strategies: Antigens that contribute to the malignant

phenotype are the most attractive targets but may be especially active in mediating apoptosis of non-malignant cells or be prone to downregulation. Analogous to viral proteins that are toxic when expressed at high levels [35], functionally inactive, non-toxic mutants retaining immunogenicity may have to be designed express PRAME for efficient antigen presentation.

Although activated $\gamma\delta$ T cells permitted selective and robust expansion of T cells with a cytolytic effector phenotype and exclusive peptide specificity for both tumor-associated self-antigens, the failure of the CTLs to lyse targets endogenously expressing these antigens seriously compromises their therapeutic value. Importantly, this failure was not restricted to CTLs expanded using $\gamma\delta$ T cells, but extended to those generated by professional peptide presentation by DCs. This problem represents a major challenge to effective tumor immunotherapy since it reflects a physiological mechanism to prevent autoimmune CTL responses to self-antigens by clonal deletion or anergy of high-affinity T cells from the circulating T-cell compartment [36]. While similar observations have been made by others [23–25], some previous studies report expansion of high-avidity peptide-specific CTLs against self-antigens, including PRAME and STEAP1, that interact not only with peptide-loaded cells but also with tumor cells naturally expressing these targets [7, 19–22, 37, 38]. There are several potential explanations for these discrepancies. Besides the primary activation stimulus, culture conditions including cytokine combinations, stimulator cells, and peptide concentrations are critical to allow for optimal CTL expansion. However, at least two of the previous reports have used very similar expansion conditions, based on K562 aAPCs and cytokine combinations [7, 19]. Moreover, the choice of the target cell naturally expressing the respective target antigen may affect results. Difficulties in obtaining tumor cell cultures as well as autologous PBMCs from pediatric solid tumor patients limit the application of autologous coincubation experiments. Since CTL responses to established tumor cell lines overexpressing the respective antigens may in part result from alloreactivity, we chose autologous $\gamma\delta$ T cells expressing full-length antigen by retroviral gene transfer, along with the respective antigen-negative controls, as targets for these experiments. Although this type of control was not included in most previous publications, efficient MHC class I blocking of antitumor CTL responses and cytokine secretion in response to autologous tumor cells in some previous reports leaves little doubt that peptide-specific CTLs indeed interacted with at least some of the native targets [7, 19]. The explanation remains that whether or not high-avidity CTLs against specific peptides survive thymic deletion may be donor-dependent.

Robust methods are needed that allow expanding fully functional, high-avidity CTLs against autologous tumor cells from all cancer patients. Potential solutions include the use of T cells from HLA-A*0201 negative individuals to expand CTLs recognizing the respective peptides in the context of HLA-A*0201 as pioneered by Stauss et al. [23]. Moreover, compared to individual peptides chosen on the basis of epitope prediction models, presentation of full-length antigen may allow extending the CTL response toward further specificities. One approach is based on cross-presentation of exogenous tumor antigens on class I MHC molecules. $\gamma\delta$ T cells can indeed acquire antigen via professional phagocytosis [39] and can cross-present soluble proteins to antigen-specific CD8+ T cells. However, antigen cross-presentation by both $\gamma\delta$ T cells and DCs was limited by rapid degradation within the immunoproteasome in both cell types [30]. While our attempts to aberrantly express endogenous PRAME in $\gamma\delta$ T cells by genetic modification failed due to downregulation of antigen expression, the use of protein-spanning peptide mixtures may provide a means to induce broader responses including individual high-avidity CTL clones.

In conclusion, $\gamma\delta$ T cells deserve further investigation with regard to their potential use as APCs for cancer immunotherapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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