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Activated human $\gamma\delta$ T cells as stimulators of specific CD8+ T cell responses to subdominant Epstein Barr virus (EBV) epitopes: Potential for immunotherapy of cancer

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Abstract

The efficacy of current cancer vaccines is limited by the functional heterogeneity and poor availability and expansion of professional antigen-presenting cells (APCs). Besides their potent innate effector properties, $\gamma\delta$ T cells have been suggested to be involved in the initiation and maintenance of adaptive immune responses. Here, we investigated the capacity of human $\gamma\delta$ T cells to induce expansion of virus-specific T cells to Epstein Barr virus (EBV) antigens. Aminobisphosphonate-stimulated human peripheral blood-derived $\gamma\delta$ T cells (V γ 9+V δ 2+) acquired a dual phenotype characteristic for both APCs and effector memory T cells. Coincubation of activated $\gamma\delta$ T cells pulsed with HLA-restricted epitopes of either the highly stimulatory EBV lytic cycle antigen BZLF-1 or the tumor-associated latent EBV antigen LMP2a with autologous peripheral blood lymphocytes induced selective expansion of peptide-specific, fully functional CD3+CD8+ cytolytic effector memory T cells. Furthermore, $\gamma\delta$ T-APCs efficiently processed and presented endogenous antigen, as demonstrated by the capacity of LMP2a gene-transduced $\gamma\delta$ T cells to induce expansion of T cells with broad specificity for various LMP2a peptides. The capacity of autologous $\gamma\delta$ T cells to induce LMP2a-specific autologous CTLs was confirmed in two patients with Hodgkin lymphoma. In summary, bisphosphonate-activated human $\gamma\delta$ T cells stimulate expansion of cytotoxic effector T cells specific for both subdominant and dominant viral epitopes and thus show promise as a novel source of efficient APCs for immunotherapy of viral and malignant disease.

Keywords

Human; T cells; Immunotherapy; Antigen Presentation

INTRODUCTION

Clinical trials of cellular immunotherapy have shown promise for the treatment of viral and malignant disease and revealed the importance of efficient antigen presentation for the induction of therapeutic T cell immunity^{1–3}. Since most tumor cells fail to present antigen, the development of cellular immunotherapies relies on the availability of surrogate antigen-presenting cells (APCs)⁴. The standard approach to generating tumor-specific cytotoxic T cells (CTLs) is based on antigen presentation by dendritic cells (DCs)⁵. While DCs are the most efficient APCs so far, serious drawbacks to their use in adoptive immunotherapy exist, including their scarcity in the peripheral blood, their limited expansion, and their functional heterogeneity. Furthermore, DCs were found to be functionally deficient in cancer patients⁶. These limitations have motivated an intense search for alternative sources of APCs^{7–9}.

Recently, T lymphocytes bearing V γ 2V δ 2+ T cell receptors (referred to as $\gamma\delta$ T cells) have emerged as a promising candidate cell population for the stimulation of antigen-specific immune responses¹⁰. $\gamma\delta$ T cells constitute a small proportion (1–5%) of the lymphocytes that circulate in human blood. In a TCR-dependent manner, they recognize a restricted set of phosphorylated compounds and can be pharmacologically activated by nitrogen-containing bisphosphonates (pamidronate, zoledronate)¹¹. Activated $\gamma\delta$ T cells exert potent and antigen-specific effector functions, including both cytotoxicity and secretion of cytokines¹². Their physiological significance is a matter of intensive investigation. Besides their contribution to the first line defense against microbial pathogens¹³, $\gamma\delta$ T cells have been attributed an important role in autoimmunity¹⁴ and a surveillance function against tumors^{15–17}. An antigen-presenting function of $\gamma\delta$ T cells was suggested by the recent observation that upon activation, these cells acquire phenotypic and functional characteristics of professional APCs, concomitant with the capacity of inducing primary CD4+ and CD8+ $\alpha\beta$ T cell responses to allogeneic and microbial antigens¹⁰.

We hypothesized that the immunostimulatory properties of activated $\gamma\delta$ T cells can be exploited for the induction of therapeutic antiviral and antitumor immunity. We chose T cell immunity to Epstein Barr virus (EBV) as a model system, since EBV antigens are important therapeutic targets in pediatric and adult hematology and oncology^{1–3}. While EBV antigens raise potent and protective T cell responses in healthy individuals¹⁸, EBV infections or reactivations in immunocompromised patients can cause uncontrolled lymphoproliferation². Furthermore, the virus is linked to a range of malignancies, including Hodgkin disease¹⁹, non-Hodgkin lymphomas²⁰ and nasopharyngeal carcinoma²¹. Most EBV-associated tumor cells express a set of latency antigens restricted to LMP1 and LMP2 in the absence of lytic cycle and EBNA3 antigens²². Unfortunately, the generation of LMP-specific T cells is complicated by their limited immunogenicity and is highly dependent on the immunostimulatory quality of the APC^{3,23}.

Here, we addressed whether antigen presentation by $\gamma\delta$ T cells has the capacity for inducing functional effector T cell responses to both highly and weakly immunogenic EBV antigens in an epitope-specific manner. We found that aminobisphosphonate stimulation of human V δ 2+V γ 9+ T cells induces upregulation of multiple costimulatory molecules as well as secretion of high amounts of TNF- α . After pulsing with model peptides derived from both BZLF-1 and LMP2a or genetic modification with the LMP2a gene, activated $\gamma\delta$ T cells induced selective expansion of peptide-specific and fully functional CD8+ CTLs from both EBV-seropositive healthy donors and cancer patients. Their effector functions and pattern and breadth of epitope recognition are comparable to CTLs generated by presentation of the same peptides on DCs or by stimulation with EBV-transformed lymphoblastoid cell lines. These observations pave the way for the use of $\gamma\delta$ T cells as cellular adjuvants for immunotherapy of viral infection and cancer.

METHODS

Cell lines

EBV-immortalized lymphoblastoid cell lines (LCLs) were generated as described previously²⁴. 293T cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The ecotropic and amphotropic packaging cell lines Phoenix-eco and Phoenix-ampho²⁵ were provided by Gary P. Nolan (Stanford, CA). PG-13 (ATCC) is a retrovirus packaging cell line that produces virus pseudotyped with the gibbon-ape leukemia virus (GALV). The amphotropic FLYRD18 (provided by E. Vanin, Houston, TX) cell line provides viral recombinants with the RD114 envelope.

Plasmids and production of recombinant retrovirus

The LMP2a sequence was cloned from LCLs into *AgeI* and *XhoI* sites of the retroviral vector SFG-GFP²⁶ (provided by Martin Pule, London, UK). For control experiments, SFG-14.G2a ζ ²⁷-GFP or SGF-CD19 ζ ²⁸-GFP was used. Stable retroviral producer cell lines were generated as described^{28;29}. Transient production of retroviral supernatant by 293T cells was achieved by cotransfection with the RD114 *env* expression plasmid RDF, the MoMLV *gag/pol* expression plasmid PqPam3(-*env*), the SFG-LMP2a-GFP vector or control vectors respectively, at a ratio of 2:3:3. The supernatant was harvested at days 2 and 3 after transfection.

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

Peripheral blood mononuclear cells (PBMCs) (2×10^6 /well) were stimulated with zoledronate (1 μ g/ml; kindly provided by Novartis Deutschland, Nürnberg, Germany) 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 coinubation experiments was performed on day 9 using TCR $\gamma\delta$ -specific magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For retroviral transductions, $\gamma\delta$ T cells were prestimulated as above, then 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 μ g/cm², and coinubated with viral supernatant for 48 hours.

Generation of DCs

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 RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 800 U/mL GM-CSF and 1,000 U/mL IL-4 (both cytokines from ImmunoTools, Friesoythe, Germany), and incubated at 37°C and 5% CO₂. Medium supplemented with IL-4 and GM-CSF was replaced on day 3. For maturation, DCs were harvested on day 6 and replated in medium supplemented with IL-6 (100 ng/ml), IL-1 β (10 ng/ml), PGE2 (1 μ g/ml) and TNF- α (10 ng/ml). Mature DCs were harvested on day 9 and their phenotype was assessed by flow cytometry.

Peptides and pentamers

For BZLF-1, the HLA-B8 binding epitope **RAKFKQLL**³⁰, hereafter named RAK, and for LMP2a, the HLA-A2 epitope **FLYALALL**³¹ (FLY) were used. All peptides and pentamers were synthesized by ProImmune (Oxford, UK). A peptide library consisting of 122 15-mer peptides with 11-aa overlap covering the complete sequence of LMP2a (B95-8 strain; Swiss prot access P13285) was purchased from D. Stoll (Natural and Medical

Sciences Institute, University of Tuebingen, Germany) and pooled as described previously³. To determine the minimal recognized LMP2 epitope sequence, additional peptides, varying in length from 9 to 14 aa, were obtained from Genemed Synthesis and reconstituted at 10 mg/ml in DMSO. For their use as stimulator cells, DCs or $\gamma\delta$ T cells were pulsed with 5 μ g/ml of peptide for 1 hour at 37°C, followed by three washing steps with phosphate-buffered saline (PBS).

Flow cytometry

For immunophenotyping, cells were stained with fluorescein-conjugated monoclonal antibodies (Becton Dickinson, San Jose, CA) directed against CD3, CD4, CD8, TCR $\alpha\beta$, TCR $\gamma\delta$, HLA-DR, CD11c, CD40, CD54, CD80, CD83, CD86, CDw137L (4-1BBL), CD45RA, CD45RO, CCR7, CD27, anti-IL-12 p40/p70 (clone C11.5), anti-IFN- γ and anti-TNF- α proteins. Anti-V γ 2V δ 2 (clone IMMU 389) was purchased from Beckman Coulter (Karlsruhe, Germany). For each sample, 20,000 cells were analyzed with FACSCalibur and Cell Quest Software or FACSCanto and Diva Software (Becton Dickinson, San Jose, CA). For pentamer staining, T cells (1×10^6) were incubated with 1 μ l of unlabeled pentameric complex at room temperature for 15 minutes, followed by staining with 4 μ l PE-labeled fluorotag, anti-CD8 FITC and anti-CD3 PerCP.

Activation and expansion of antigen-specific T cells

5×10^4 /well irradiated (30 Gy) peptide-pulsed APCs were seeded into 24-well plates and coincubated with 1×10^6 autologous PBMCs per well (S:R ratio of 1:20) in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine without the addition of cytokines for 10–12 days. When CD8⁺ T cells were used as responder cells, magnetic cell selection from freshly isolated PBMCs was performed by using CD8-specific microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and rhIL-2 (25 IU/ml) and rhIL-7 (20 ng/ml) were added to the cocultures on day 1 and 7. SFG-LMP2a-GFP or SFG-14.G2a ζ -GFP (control) transduced $\gamma\delta$ T cells were sorted for TCR $\alpha\beta$ ⁺/GFP⁺ cells (>95% purity) before coincubation with autologous CD8⁺ T cells at a S:R ratio of 1:20.

Cytotoxicity assays

Cytotoxic specificity was determined in standard ⁵¹Cr release assays. Various numbers of T effector cells were coincubated in triplicate with 2500 target cells labeled with 100 μ Ci ⁵¹Cr/ 0.5×10^6 cells (PE Applied Biosystems) in a total volume of 200 μ l in a V-bottomed 96-well plate. At the end of a 4-hour incubation period at 37°C and 5% CO₂, supernatants were harvested, and radioactivity was counted in a gamma counter. Maximum release was determined by lysis of target cells with Triton X.

Measurement of cytokine production

Triplicate samples of effector T cells (5×10^4 /well) were cocultured with peptide-pulsed $\gamma\delta$ T cells or LCLs at a stimulator-to-effector ratio of 3:1 in the presence of rhIL-2 (25 IU/ml) in 96-well round bottomed plates. After 72 hours, the supernatants were harvested and analyzed for human IFN- γ by enzyme-linked immunosorbent assay (Hözel Diagnostika, Köln). For intracellular cytokine detection, cytokine secretion was blocked with 10 μ g/ml brefeldin A (BFA; Sigma, Deisenhofen, Germany) for 4 hours, and the cells were permeabilized using a proprietary solution (Becton Dickinson, San Jose, CA), then stained according to the manufacturer's recommendations.

ELISPOT assay

ELISPOT analysis was used to determine the epitope specificities of expanded CTLs in response to LMP2 pepmix, which contains all 15-mer peptides of LMP2 in one pool, and

overlapping peptide pools for LMP2, as previously described^{1,3}. IFN- γ ELISPOT assay was also used for the detection of LMP2a-specific T cells in a patient with Hodgkin lymphoma. Irradiated (30 Gy) SFG-LMP2a-GFP transduced and GFP-positive selected $\gamma\delta$ T cells were used as stimulator cells in 11 day cocultures with positive-selected autologous CD8⁺ T cells in RPMI medium containing 8% autologous serum. For restimulation, LMP2a-transduced CD8-negative T cells were used. For this purpose, the negative cell fraction after sorting of CD8⁺ T cells was prestimulated at 1×10^6 /ml for 48 hours in 24-well plates precoated with OKT-3 (1 μ g/ml; Ortho Pharmaceuticals, Raritan, NJ) and anti-CD28 antibody (1 μ g/ml; Pharmingen, San Diego, CA), then transduced with either SFG-LMP2a-GFP or an irrelevant control vector (SFG-LMP2a-CD19 ζ), and GFP-positive cells were selected by FACS. To determine antigen-specific cytokine secretion, LMP2a-prestimulated cells (2×10^4 cells/well) were restimulated with 8×10^4 /well autologous transduced CD8-negative T cells in triplicate wells of 96-well filtration plates (Millipore, Schwalbach, Germany) precoated with IFN- γ -specific antibody clone 1-D1K (Mabtech, Hamburg, Germany; 15 μ g/ml). Control wells contained LMP2a-prestimulated CD8⁺ T cells coincubated with autologous non-transduced or control vector-transduced CD8-negative T cells, or with medium alone. After 20 hours, the plates were washed and incubated with detector mAb 7-B6-1 biotin (Mabtech) followed by Streptavidin-AP (Mabtech). Spots were detected by adding AP substrate BCIP/NBT (Moss, Pasadena, U.S.A.) for 15 min and enumerated with a BIOSYS BIOREADER 3000 PRO.

Statistical analysis

The student T test was used to test for significance in each set of values, assuming equal variance. Mean values $\pm$ SD are given unless otherwise stated.

RESULTS

Zoledronate-activated $\gamma\delta$ T cells acquire a combined phenotype characteristic for both APCs and effector memory cells and secrete TNF- α

In all seven healthy donors, in vitro stimulation of PBMCs with a single dose of zoledronate (1 μ g/ml) induced significant expansion of $\gamma\delta$ T cells, resulting in a relative increase of $\gamma\delta$ TCR (V γ 2V δ 2)-expressing T cells. On day 8, the majority ($87.1 \pm 6.8\%$) of the cultured cells coexpressed CD3 and V γ 2V δ 2, corresponding to an overall $\gamma\delta$ T cell expansion of 150.6 ± 54.5 -fold. In absolute numbers, a mean of $39.8 \pm 5.7 \times 10^6$ $\gamma\delta$ T cells (range $32.3 - 47.2 \times 10^6$) were obtained from 10×10^6 PBMCs. These numbers compare favorably to the numbers of immature DCs obtained from the same amount of blood in 5 donors ($0.6 \times 10^6 \pm 0.3 \times 10^6$, range $0.5 \times 10^6 - 1.1 \times 10^6$). $\gamma\delta$ T cells were also efficiently expanded from the peripheral blood of four patients with Hodgkin lymphoma, with 10.8 to 103.1-fold (43.5 ± 41.6 -fold) overall $\gamma\delta$ T cell expansion within 8 days, yielding cultures containing 28.5 to 93.5% ($64.5 \pm 27.5\%$) $\gamma\delta$ TCR⁺ T cells on day 8. By positive selection using TCR $\gamma\delta$ -specific magnetic beads, $>95\%$ $\gamma\delta$ T cell purity was routinely obtained.

To evaluate the potential of bisphosphonate-activated $\gamma\delta$ T cells to serve as APCs, we assessed the pattern and kinetics of surface expression of immunostimulatory molecules, which can be used as a morphological readout for APC function³². During zoledronate-induced expansion, $\gamma\delta$ T cells acquired a phenotype characteristic for APCs, including upregulation of HLA-DR as well as the costimulatory ligands CD80, CD86, CD83, CD11c, CD54, CD40, and CDw137L (4-1BBL) (Figure 1A). Whereas expression of most markers decreased after peak levels were reached on day 5, CD86 persisted for >10 days, and HLA-DR remained upregulated on $>70\%$ of the cells for prolonged culture periods of >2 to 3 weeks (Figure 1B).

On the basis of expression of the surface markers CD45RA, CD45RO and CD27, $\gamma\delta$ T cells can be further subclassified into distinct effector cell populations with varying functional properties³³. We found that during zoledronate-induced cell culture, the proportion of effector memory $\gamma\delta$ T cells (T_{EM} ; CD27⁻/CD45RO⁺/CD45RA⁻) increased while central memory T cells (T_{CM} ; CD27⁺/CD45RO⁺/CD45RA⁻) were found at decreasing percentages (Figure 1C). Neither naïve nor fully differentiated, RA⁺ effector memory T cells (T_{EMRA}) were found after 9 days of in vitro culture. CCR7 remained downregulated on the majority of $\gamma\delta$ T cells after stimulation with zoledronic acid and during prolonged in vitro culture (not shown). The T-cell stimulatory function of professional APCs is largely determined by secretion of TNF- α and IL-12. Whereas IL-12 was not detected in activated $\gamma\delta$ T cells, an intracellular cytokine detection assay revealed production of TNF- α at median intensities about 10-fold inferior to lipopolysaccharide (LPS)-stimulated mature DCs (Figure 1D).

Thus, in response to aminobisphosphonate stimulation, $\gamma\delta$ T cells differentiate towards potent effector T cells and at the same time acquire some of the typical phenotypic and functional properties of antigen-presenting cells.

Zoledronate-activated $\gamma\delta$ T cells induce epitope-specific CTL expansion to EBV lytic cycle antigen BZLF-1

To investigate the stimulatory properties of bisphosphonate-activated $\gamma\delta$ T cells, we analyzed their ability to induce expansion of $\alpha\beta$ effector T cells specific for an HLA-B8-restricted peptide derived from the EBV lytic cycle antigen BZLF-1 (RAK). Purified populations of $\gamma\delta$ T cells were obtained from the peripheral blood of four HLA-B8 positive healthy donors by zoledronate stimulation and subsequent positive selection using $\gamma\delta$ TCR-specific beads. The $\gamma\delta$ T cells were pulsed with RAK peptide at 5 μ g/ml, irradiated with 30 Gy and coincubated with autologous PBMCs at a 1:20 stimulator-to-responder ratio. In parallel cocultures, peptide-pulsed autologous DCs were used as stimulators cells. 10–12 days of coculture with peptide-pulsed $\gamma\delta$ T cells resulted in selective expansion of RAK-pentamer-positive CD3⁺ CD8⁺ T cells from <1% to a mean of 51.1% $\pm$ 26.2% (range 8.3%–89.7%) (Figure 2A, Table I). This increase in the percentage of RAK peptide-specific T cells was accompanied by numerical expansion by a mean of 40.8-fold (range 15.9 to 80.1) within 12 days of stimulation. The percentages of pentamer-positive CD8⁺ T cells in cocultures with peptide-pulsed DCs were equal or superior in a donor-dependent manner (mean of 64.5% $\pm$ 35.4%, range 10.6–96.0%) (Figure 2A, Table I). Peptide-specific T cell expansion in response to $\gamma\delta$ T cell or DC stimulation in the absence of peptide was minimal (mean of 3.3% $\pm$ 2.3%, range 0.9%–7.0% for $\gamma\delta$ T-APCs and 2.2% $\pm$ 0.9%, range 0.9–3.2% for DCs).

Zoledronate-activated $\gamma\delta$ T cells efficiently propagate expansion of specific CTLs to EBV latency antigen LMP2a

Due to the immunogenicity of BZLF-1, the APC requirements for inducing CTLs specific for this antigen are limited³⁴. To obtain further evidence for the antigen-presenting qualities of activated $\gamma\delta$ T cells, we assessed their ability to support T cell expansion to a less immunogenic antigen. The choice of the latency antigen LMP2a was based on its low T-cell stimulatory properties as well as its importance as a target for tumor immunotherapy of Hodgkin's lymphoma and nasopharyngeal carcinoma^{1;3}. Starting from <0.2% pentamer-positive T cells at the onset of the cultures, stimulation of autologous PBMCs with $\gamma\delta$ T cells pulsed with the HLA-A2-restricted LMP2a epitope FLY resulted in a substantial increase to a mean of 12.6% $\pm$ 10.6% (range: 1.6–33.2%) in 6 donors, corresponding to 36.6-fold (range 27.7 to 60.0) numerical expansion, and comparable to stimulation by peptide-pulsed autologous DCs (mean of 16.7 $\pm$ 11.3% pentamer-positive cells, range 1.2–29.0%) (Figure

2B, Table I). Again, $\gamma\delta$ T cell stimulation in the absence of peptide failed to induce substantial FLY-specific T cell proliferation (mean of $0.9 \pm 0.4\%$; 0.2–1.6%).

Within cocultures relying on unselected PBMCs as responder cells, alternative cells with APC function, including CD4⁺ T cells, B cells and monocytes, may be partially responsible for peptide-specific CTL stimulation. To separate the antigen-presenting role of $\gamma\delta$ T cells from this potential background, we performed additional experiments using purified CD8⁺ T cells as responder cells. Cocultures with purified $\gamma\delta$ T cells and purified CD8⁺ T cells consistently yielded high proportions of FLY-specific T cell populations (mean of $6.6 \pm 7.7\%$, range 1.3–17.7%) comparable to those obtained with unselected responder cells (Figure 2B). Taken together, $\gamma\delta$ T cells efficiently stimulate peptide-specific CD8⁺ CTL expansion in response to both high and moderately immunogenic antigens.

CTLs expanded in response to antigen presented by $\gamma\delta$ T cells have an effector memory phenotype and exert potent and peptide-specific effector functions

Suboptimal antigen presentation may result in the selective expansion of T cell populations with inappropriate phenotypic and functional properties³⁵. Therefore, we compared the immunophenotypes of pentamer-positive CTLs on day 15 after initial stimulation with $\gamma\delta$ T-APCs with those generated in the presence of mature DCs. Among gated CD8⁺ pentamer-positive T cells, mature effector memory T cells (T_{EM}), defined by expression of CD45RO in the absence of CCR7, dominated under both culture conditions (Figure 3A), while a minority of the T cells were T_{CM} (CD45RO⁺, CCR7⁺). No significant difference was found between the proportions of TCM cells in $\gamma\delta$ T cell-induced versus DC-induced cultures ($2.3 \pm 0.9\%$ versus $2.6 \pm 1.6\%$ cultures).

To assess the functional properties of the expanded T cells, we determined their specific cytokine secretion and target cytotoxicity. CD8⁺ T cells expanded in the presence of FLY peptide-pulsed activated $\gamma\delta$ T cells efficiently and specifically lysed peptide-pulsed LCLs or autologous $\gamma\delta$ T cells (Figure 3B), and secreted IFN- γ and TNF- α in response to peptide-pulsed targets (Figure 3C, D). While the capacity of CTLs expanded in the presence of peptide-pulsed autologous DCs versus $\gamma\delta$ T cells to perform peptide-specific cytotoxicity and cytokine secretion slightly varied within individual donors, no consistent superiority of either of the two CTL populations was apparent (not shown). The peptide specificity and MHC restriction of target recognition were confirmed in control experiments using HLA-mismatched allogeneic LCLs or unpulsed APCs as stimulator cells of cytokine release (Figure 3D), as well as in MHC class I blocking assays (not shown). Lysis of autologous LCLs expressing wild-type LMP2 by CTLs expanded in the presence of either kind of antigen-presenting cells was above the background of unpulsed $\gamma\delta$ T cells, but in a range not exceeding 15% in any of the donors. Likewise, the flow cytometry based IFN- γ secretion assay failed to demonstrate significant functional responses of CD8⁺ T cells to unpulsed LCLs. By contrast, assessment of IFN- γ secretion by the more sensitive ELISA revealed efficient interaction of FLY-specific CTLs with autologous but not allogeneic LCLs, likely representing endogenous presentation of the LMP2a antigen at low densities (Figure 3D). These data demonstrate that peptide presentation by $\gamma\delta$ T cells induces robust expansion of fully functional and antigen-specific effector memory CTLs.

$\gamma\delta$ T cells efficiently present endogenous full-length antigen and induce expansion of CTLs with broad epitope specificity

To investigate whether $\gamma\delta$ T cells are capable of endogenously processing full-length antigens, followed by transport to the surface and presentation on class I MHC molecules, we introduced the full sequence of the LMP2a gene into activated $\gamma\delta$ T cells from 4 individual EBV-seropositive donors by retroviral gene transfer. The transduction efficiency

as assessed by GFP expression was $13.8 \pm 6.4\%$ (range 4.7–20.0%). For coincubation experiments, LMP2a-expressing $\gamma\delta$ T APCs were selected by flow cytometry cell sorting of GFP-positive cells (purity >95%). In the presence of LMP2a-gene-transduced autologous $\gamma\delta$ T cells, T cells specific for the LMP2a peptide FLY were successfully expanded from polyclonal T cells ($1.1 \pm 0.7\%$, range 0.4–2.2%) (Figure 4A). Thus, activated $\gamma\delta$ T cells efficiently process and present endogenous antigen.

In additional experiments, the epitope specificities of LMP2a-specific CTLs generated from HLA-A2-positive donors by stimulation with autologous LCLs versus LMP2a-transduced autologous $\gamma\delta$ T cells were compared by screening against a variety of known and unknown LMP2 peptides. CTLs expanded in response to LMP2a full-length antigen presentation secreted IFN- γ in response to several different LMP2 peptides, representing discrete epitopes on the LMP2 antigen. For each of the two donors tested, the pattern of epitope reactivity was comparable to that obtained by stimulation with autologous LCL (Figure 4B). Thus, the breadth of the antigen response induced by $\gamma\delta$ T cells is not inferior to natural antigen presentation.

Autologous $\gamma\delta$ T cells induce LMP2a-specific T cell expansion in EBV-seropositive cancer patients

To investigate the feasibility of the approach in cancer patients, we analysed the specific stimulatory capacity of $\gamma\delta$ T cells from two pediatric patients undergoing chemotherapy for Hodgkin lymphoma. In the Hodgkin lymphoma patient who was HLA-A2-positive, FLY pentamer-positive T cells were efficiently induced in cocultures of non-selected PBMCs with FLY peptide pulsed autologous $\gamma\delta$ T cells (Figure 5A). In the second patient who was HLA-A2-negative, SFG-LMP2a-GFP-transduced $\gamma\delta$ T cells were used as stimulator cells. As a read-out for the induction of LMP2a-specific T cell responses, we assessed the number of spot-forming cells in an IFN- γ ELISPOT assay. Comparable to the healthy donors, retroviral transduction of aminobisphosphonate-activated T cells resulted in 14% GFP-expressing $\gamma\delta$ T cells. Stimulation of autologous CD8+ T cells with positively selected SFG-LMP2a-GFP $\gamma\delta$ T cells induced a strong increase of IFN- γ producing cells compared to those exposed to non-transduced $\gamma\delta$ T cells or $\gamma\delta$ T cells transduced with SFG-GFP containing an irrelevant gene insert (Figure 5B).

DISCUSSION

In this paper, we propose to exploit the immunostimulatory qualities of human $\gamma\delta$ T cells for establishing more effective immunotherapeutic strategies in viral and malignant disease. Such strategies rely on the potent ex vivo or in vivo expansion of effector memory T cells with defined specificities for subdominant antigens and adequate functional properties. We show that aminobisphosphonate-activated human peripheral blood $\gamma\delta$ (V γ 9V δ 2) T cells acquire surface expression of various T-cell-stimulatory molecules and secrete substantial amounts of TNF- α as major prerequisites for efficient T cell stimulation. In an EBV model, $\gamma\delta$ T cells stimulate potent and specific CD8+ T cell responses to even subdominant, tumor-associated antigens. Importantly, they efficiently process and present endogenous antigen, demonstrated by induction of T cell responses to a broad spectrum of LMP2 peptides following transduction with the gene encoding full-length LMP2 antigen. Thus, $\gamma\delta$ T cells emerge as another cell population to induce powerful cytolytic T cell responses to weak viral and tumor-associated antigens and may thus provide useful tools for generating effector cells for immunotherapy.

Immunity to EBV was previously shown to provide an excellent model for the induction of therapeutic T cell memory responses in humans². Due to the expression of EBV antigens in virus-associated tumor cells^{19–21}, their significance extends beyond viral disease towards

cancer. EBV antigens with varying T cell stimulatory capacities have been identified. The immediate early protein BZLF-1 has a high capacity for inducing potent T cell responses, resulting in efficient immune control of viral reactivation³⁶. Here, it was used as a prototype for a highly immunogenic antigen with marginal requirements for professional antigen presentation. The viral latency antigen LMP2a, by contrast, is a weak stimulator of the immune response and thus more representative of the majority of tumor-associated antigens. Its consistent expression on the malignant cells in EBV-positive Hodgkin's lymphoma and other EBV-associated diseases has stimulated ongoing efforts in therapeutic targeting of this protein, and the first clinical studies have now demonstrated the feasibility and promise of this approach^{1,3}. The dependence of LMP2a-specific CTL expansion on the potency of antigen presentation is well defined and thus allowed us to evaluate the immunostimulatory quality of $\gamma\delta$ T cells.

The ideal APC for clinical adoptive immunotherapy will permit selective expansion of potent T cells with a cytolytic effector phenotype and exclusive specificity for a given tumor antigen. We show here that bisphosphonate-activated $\gamma\delta$ T cells fulfill this requirement. While the stimulation of T cells with suboptimal APCs was shown to induce anergy or apoptosis³⁵, presentation of viral epitopes to memory CD8⁺ T cells by $\gamma\delta$ T cells induced robust expansion of peptide-specific, functionally competent CTLs. Importantly, potent CD8⁺ T cell stimulation was achieved both by short-term presentation of exogenous peptides and after retroviral delivery of full-length antigen, which more closely mimics the physiological presentation of viral and tumor antigens. In direct comparison with DCs, neither the epitope specificity nor the effector functions of $\gamma\delta$ T-cell induced CTLs were compromised. LMP2 epitope-specific CD8 cells did not kill wild-type LCL targets in short-term cytotoxicity assays, however, specific and functional interactions with autologous LCLs became apparent in a high sensitivity IFN- γ ELISA, confirming previous observations with EBV-latent cycle antigen-specific T cells^{1,37}. Importantly, $\gamma\delta$ T cells expressing full-length antigen elicited an equally broad epitope-specific T cell response as natural EBV antigen presentation provided by autologous LCLs.

From a practical point of view, a therapeutic APC should be available in large numbers and easily expandable. This is an important disadvantage of DCs, since their expansion is a time-consuming process that involves expensive cytokines which are often unavailable as clinical-grade reagents. Another significant obstacle to the therapeutic use of DCs is their functional heterogeneity and unpredictable characteristics after ex vivo manipulation, as well as their lack of adequate function in many cancer patients⁶. To facilitate clinical immunotherapy, various sources of alternative APCs have been investigated. Schultze et al. were the first to propose the use of B cells activated via their CD40 receptor as alternative APCs for inducing tumor antigen-specific T cell responses⁹. More recently, contact-activated monocytes were shown to function as potent APCs for the stimulation of recall responses against subdominant viral antigens³⁸. Furthermore, artificial APCs (AAPCs), generated by modification of a scaffold cell with the desired costimulatory molecules, were effective stimulators of CTLs specific for tumor-associated peptides^{7,8}. Antigen-presenting function was also found in activated CD4⁺ T cells³⁹⁻⁴¹, though comparative experiments with DCs have revealed a substantially inferior capacity to induce CTL proliferation¹⁰.

Exploiting $\gamma\delta$ T cells to generate antigen-specific CTLs for adoptive immunotherapy has a number of potential advantages over existing strategies. An important point is the reported ease of $\gamma\delta$ T cell expansion and ex vivo manipulation. As we confirm in this study, high numbers of $\gamma\delta$ T cells, superior to iDCs, can be obtained by ex vivo culture of peripheral blood lymphocytes from both normal donors and immunocompromised cancer patients in the presence of nitrogen-containing bisphosphonates¹¹. The APC properties of $\gamma\delta$ T cells are induced by stimulation with pharmacologically relevant doses of bisphosphonates, which

activate $\gamma\delta$ T cells by interference with the mevalonate pathway and subsequent intracellular enrichment of their natural TCR ligand isopentenyl pyrophosphate (IPP)¹⁶. Thus, by drug-induced restimulation in vivo, $\gamma\delta$ T cells may become a persistent and renewable source of APCs. In contrast to “off the shelf” artificial APCs, which may trigger allogeneic immune responses, autologous $\gamma\delta$ T-APCs are immunologically inert, which allows to focus the immune activation on the desired, generally weakly immunogenic tumor antigens. As shown here, the susceptibility of $\gamma\delta$ T cells to genetic modification allows for stable expression of full-length antigen, enabling autologous presentation of the entire spectrum of relevant peptides for a certain virus or tumor protein. This is an important advantage over DCs which, due to their failure to divide and proliferate, do not allow for stable integration of foreign genes. Stable gene transfer into $\gamma\delta$ T cells may further be a useful tool to manipulate their natural characteristics and improve their APC function, homing and survival by artificial expression of costimulatory, cytokine or chemokine genes. For instance, transgenic expression of IL-2 and IL-15 in T-cell based APCs was recently shown to increase the expansion of CTLs both in vitro and in vivo in a strictly antigen-dependent manner⁴² and may thus be useful to prolong the in vivo survival of therapeutic $\gamma\delta$ T cells.

A strong argument supporting the use of $\gamma\delta$ T cells in cancer therapy is their effector function and native antitumor activity^{15;16}, which are thought to contribute to tumor immune surveillance in vivo and provide the rationale for current concepts of using bisphosphonate-activated $\gamma\delta$ T cells for immunotherapy of cancer^{28;43}. Indeed, two independent studies have shown a correlation between increased numbers of $\gamma\delta$ T cells and antitumor control in patients with advanced cancer treated with aminobisphosphonates and IL-2^{11;43}. As demonstrated in Figure 1C, we confirm that aminobisphosphonate activation of $\gamma\delta$ T cells induces not only upregulation of immunostimulatory molecules, but also selective expansion of CD45RO+CD27- T_{EM} cells, which have previously been shown to exert potent cytolytic effector functions⁴⁴.

Combined effector and stimulatory properties have recently been identified in individual immune cell populations in mice and humans^{45–47}. $\gamma\delta$ T cells apparently represent a further example for a multifunctional cell receiving integrated signals that lead to both APC and immediate effector activities. An unresolved question regards the extent to which $\gamma\delta$ T cells can mimic the superb qualities of DCs in lymph node homing, processing of phagocytosed external antigen, and subsequent priming of CD4+ T cell responses. While first evidence was provided that $\gamma\delta$ T cells can stimulate primary T-cell responses to allogeneic and microbial antigens¹⁰, the detailed role of $\gamma\delta$ T cells in inducing adaptive immune responses will have to be subject of further investigations. The most relevant question from a therapeutic point of view is whether T-cell priming is an absolute requirement for obtaining clinical anti-cancer immune responses. Numerous reports illustrate that antigen-experienced memory CTLs to tumor-associated antigens preexist in the bone marrow and peripheral blood of many cancer patients^{48–50}. Their selective in vitro expansion for subsequent adoptive transfer or in vivo stimulation by effective immunization strategies are primary goals of current immunotherapy trials. As demonstrated here, $\gamma\delta$ T cells are attractive candidates for inducing superior and sustained effector CTL responses to subdominant antigens in vivo and may thus serve as potent cellular vaccines in viral disease and malignancy.

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Reference List

1. Bollard CM, Gottschalk S, Leen AM, et al. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood*. 2007; 110:2838–45. [PubMed: 17609424]
2. Heslop HE, Ng CYC, Li CF, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nature Medicine*. 1996; 2:551–5.
3. Straathof KC, Leen AM, Buza EL, et al. Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. *Journal of Immunology*. 2005; 175:4137–47.
4. Huang AYC, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of Bone-Marrow-Derived Cells in Presenting Mhc Class I-Restricted Tumor-Antigens. *Science*. 1994; 264:961–5. [PubMed: 7513904]
5. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. *Nature Reviews Immunology*. 2005; 5:296–306.
6. Maecker B, Mougiakakos D, Zimmermann M, et al. Dendritic cell deficiencies in pediatric acute lymphoblastic leukemia patients. *Leukemia*. 2006; 20:645–9. [PubMed: 16498391]
7. Maus MV, Thomas AK, Leonard DGB, et al. Ex vivo expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4-1BB. *Nature Biotechnology*. 2002; 20:143–8.
8. Oelke M, Maus MV, Didiano D, June CH, Mackensen A, Schneck JP. Ex vivo induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig-coated artificial antigen-presenting cells. *Nature Medicine*. 2003; 9:619–24.
9. Schultze JL, Michalak S, Seamon MJ, et al. CD40-activated human B cells: An alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *Journal of Clinical Investigation*. 1997; 100:2757–65. [PubMed: 9389740]
10. Brandes M, Willmann K, Moser B. Professional antigen-presentation function by human gamma delta T cells. *Science*. 2005; 309:264–8. [PubMed: 15933162]
11. Kunzmann V, Bauer E, Wilhelm M. Gamma/delta T-cell stimulation by pamidronate. *N Engl J Med*. 1999; 340:737–8. [PubMed: 10068336]
12. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature*. 1995; 375:155–8. [PubMed: 7753173]
13. Boismenu R, Havran WL. Gammadelta T cells in host defense and epithelial cell biology. *Clin Immunol Immunopathol*. 1998; 86:121–33. [PubMed: 9473374]
14. Hoshino T, Ohta A, Nakao M, et al. TCR gamma delta + T cells in peripheral blood of patients with adult Still's disease. *J Rheumatol*. 1996; 23:124–9. [PubMed: 8838520]
15. Fisch P, Malkovsky M, Kovats S, et al. Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science*. 1990; 250:1269–73. [PubMed: 1978758]
16. Gober HJ, Kistowska M, Angman L, Jenö P, Mori L, De Libero G. Human T cell receptor gamma delta cells recognize endogenous mevalonate metabolites in tumor cells. *Journal of Experimental Medicine*. 2003; 197:163–8. [PubMed: 12538656]
17. Nanno M, Seki H, Mathioudakis G, et al. Gamma/delta T cell antigen receptors expressed on tumor-infiltrating lymphocytes from patients with solid tumors. *Eur J Immunol*. 1992; 22:679–87. [PubMed: 1312472]
18. Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. *Annual Review of Immunology*. 1997; 15:405–31.
19. Herbst H, Dallenbach F, Hummel M, et al. Epstein-Barr-Virus Latent Membrane-Protein Expression in Hodgkin and Reed-Sternberg Cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1991; 88:4766–70. [PubMed: 1647016]
20. Kuppers R. B cells under influence: Transformation of B cells by Epstein-Barr virus. *Nature Reviews Immunology*. 2003; 3:801–12.

21. Niedobitek G. Epstein-Barr virus infection in the pathogenesis of nasopharyngeal carcinoma. *Journal of Clinical Pathology-Molecular Pathology*. 2000; 53:248–54.
22. Niedobitek G, Kremmer E, Herbst H, et al. Immunohistochemical detection of the Epstein-Barr virus-encoded latent membrane protein 2A in Hodgkin's disease and infectious mononucleosis. *Blood*. 1997; 90:1664–72. [PubMed: 9269787]
23. Gahn B, Siller-Lopez F, Pirooz AD, et al. Adenoviral gene transfer into dendritic cells efficiently amplifies the immune response to LMP2A antigen: A potential treatment strategy for Epstein-Barr virus-positive Hodgkin's lymphoma. *International Journal of Cancer*. 2001; 93:706–13.
24. Rooney CM, Smith CA, Ng CY, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet*. 1995; 345:9–13. [PubMed: 7799740]
25. Kinsella TM, Nolan GP. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther*. 1996; 7:1405–13. [PubMed: 8844199]
26. Riviere I, Brose K, Mulligan RC. Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc Natl Acad Sci U S A*. 1995; 92:6733–7. [PubMed: 7624312]
27. Rossig C, Bollard CM, Nuchtern JG, Merchant DA, Brenner MK. Targeting of G(D2)-positive tumor cells by human T lymphocytes engineered to express chimeric T-cell receptor genes. *Int J Cancer*. 2001; 94:228–36. [PubMed: 11668503]
28. Rischer M, Pscherer S, Duwe S, Vormoor J, Jurgens H, Rossig C. Human gamma delta T cells as mediators of chimaeric-receptor redirected anti-tumour immunity. *British Journal of Haematology*. 2004; 126:583–92. [PubMed: 15287953]
29. Landmeier S, Altwater B, Pscherer S, et al. Gene-engineered varicella-zoster virus-reactive CD4+ cytotoxic T cells exert tumor-specific effector function. *Cancer Research*. 2007; 67:8335–43. [PubMed: 17804749]
30. Hislop AD, Annels NE, Gudgeon NH, Leese AM, Rickinson AB. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J Exp Med*. 2002; 195:893–905. [PubMed: 11927633]
31. Lee SP, Thomas WA, Murray RJ, et al. HLA A2.1-restricted cytotoxic T cells recognizing a range of Epstein-Barr virus isolates through a defined epitope in latent membrane protein LMP2. *J Virol*. 1993; 67:7428–35. [PubMed: 7693972]
32. Croft M, Dubey C. Accessory molecule and costimulation requirements for CD4 T cell response. *Crit Rev Immunol*. 1997; 17:89–118. [PubMed: 9034725]
33. Dieli F, Vermijlen D, Fulfaro F, et al. Targeting human gamma delta T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Research*. 2007; 67:7450–7. [PubMed: 17671215]
34. Pudney VA, Leese AM, Rickinson AB, Hislop AD. CD8(+) immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. *Journal of Experimental Medicine*. 2005; 201:349–60. [PubMed: 15684323]
35. Tsang JYS, Chai JG, Lechler R. Antigen presentation by mouse CD4(+) T cells involving acquired MHC class II : peptide complexes: another mechanism to limit clonal expansion? *Blood*. 2003; 101:2704–10. [PubMed: 12433692]
36. Laichalk LL, Thorley-Lawson DA. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *Journal of Virology*. 2005; 79:1296–307. [PubMed: 15613356]
37. Lee SP, Brooks JM, Al Jarrah H, et al. CD8 T cell recognition of endogenously expressed Epstein-Barr virus nuclear antigen 1. *Journal of Experimental Medicine*. 2004; 199:1409–20. [PubMed: 15148339]
38. Leen A, Ratnayake M, Foster A, et al. Contact-activated monocytes: efficient antigen presenting cells for the stimulation of antigen-specific T cells. *J Immunother*. 2007; 30:96–107. [PubMed: 17198088]
39. Adamopoulou E, Diekmann J, Tolosa E, et al. Human CD4(+) T cells displaying viral epitopes elicit a functional virus-specific memory CD8(+) T cell response. *Journal of Immunology*. 2007; 178:5465–72.

40. Atanackovic D, Matsuo M, Ritter E, et al. Monitoring CD4(+) T cell responses against viral and tumor antigens using T cells as novel target APC. *Journal of Immunological Methods*. 2003; 278:57–66. [PubMed: 12957396]
41. Foster AE, Leen AM, Lee T, et al. Autologous designer antigen-presenting cells by gene modification of T lymphocyte blasts with IL-7 and IL-12. *Journal of Immunotherapy*. 2007; 30:506–16. [PubMed: 17589291]
42. Quintarelli C, Vera JF, Savoldo B, et al. Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. *Blood*. 2007; 110:2793–802. [PubMed: 17638856]
43. Wilhelm M, Kunzmann V, Eckstein S, et al. gamma delta T cells for immune therapy of patients with lymphoid malignancies. *Blood*. 2003; 102:200–6. [PubMed: 12623838]
44. Angelini DF, Borsellino G, Poupot M, et al. Fc gamma RIII discriminates between 2 subsets of V gamma 9V delta 2 effector cells with different responses and activation pathways. *Blood*. 2004; 104:1801–7. [PubMed: 15178578]
45. Chan CW, Crafton E, Fan HN, et al. Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. *Nature Medicine*. 2006; 12:207–13.
46. Strydom G, Bangert C, Tauber M, Strohal R, Kopp T, Stingl G. Tumorcidal activity of TLR7/8-activated inflammatory dendritic cells. *Journal of Experimental Medicine*. 2007; 204:1441–51. [PubMed: 17535975]
47. Taieb J, Chaput N, Menard C, et al. A novel dendritic cell subset involved in tumor immunosurveillance. *Nature Medicine*. 2006; 12:214–9.
48. Feuerer M, Beckhove P, Bai L, et al. Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. *Nat Med*. 2001; 7:452–8. [PubMed: 11283672]
49. Greiner J, Schmitt M, Li L, et al. Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. *Blood*. 2006; 108:4109–17. [PubMed: 16931630]
50. Molldrem JJ, Lee PP, Wang C, et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat Med*. 2000; 6:1018–23. [PubMed: 10973322]

Figure 1. Zoledronate-activated $\gamma\delta$ T cells acquire a phenotype characteristic for APCs
 (A) Expression of various surface molecules was assessed by flow cytometry on gated CD3⁺ TCR $\gamma\delta$ ⁺ T cells on days 0 and 5 after stimulation with zoledronate. (B) Kinetics of expression of HLA-DR, CD80 and CD86 on gated CD3⁺TCR $\gamma\delta$ ⁺ T cells were determined by flow cytometry on days 0, 2, 5, 8, 12 and 15 after stimulation. (C) To discriminate between distinct effector cell populations within zoledronate-stimulated $\gamma\delta$ T cells, gated CD3⁺ TCR $\gamma\delta$ ⁺ T cells were costained with fluorescence-labeled CD45RO- and CD27-specific antibodies on days 0, 2 and 9 after stimulation. Shown are representative experiments of three. (D) Intracellular IL-12 and TNF- α secretion by gated CD3⁺ TCR $\gamma\delta$ ⁺ T cells within cultures of unstimulated or zoledronic-acid (22 hrs) stimulated PBMCs. Gated DCs within cultures of PBMCs stimulated with LPS (1 μ g/ml) for 22 hrs served as positive controls. Shown are representative experiments of three.

Figure 2.

(A) Activated $\gamma\delta$ T cells induce epitope-specific CTL expansion to EBV lytic cycle antigen BZLF-1. PBMCs from healthy donors were coincubated with autologous stimulator cells (upper panel, DCs; lower panel, $\gamma\delta$ T-APCs), either unpulsed or pulsed with the BZLF-1 epitope RAK. The percentages of RAK-specific CTLs prior to (day 0) and on day 12 after stimulation were quantified by pentamer staining followed by flow cytometry analysis within a gate set on CD3+/CD8+ T cells. Shown is one representative experiment of seven. **(B) Activated $\gamma\delta$ T cells propagate expansion of epitope-specific CTLs to EBV latency antigen LMP2a.** Healthy donor PBMCs were coincubated with autologous stimulator cells (upper panel, DCs; middle panel, $\gamma\delta$ T cells), either unpulsed or pulsed with the LMP2a epitope FLY. In further experiments, CD8+ sorted T cells were used as responder cells and coincubated with $\gamma\delta$ T-APCs in the presence or absence of FLY peptide (lower panel). The percentages of FLY-specific CTLs prior to (day 0) and on day 12 after stimulation were quantified by pentamer staining followed by flow cytometry analysis within a gate set on CD3+/CD8+ T cells. Shown is one representative experiment of six.

Figure 3. CTLs expanded in response to antigen presentation by $\gamma\delta$ T cells have an effector memory phenotype and exert potent and peptide-specific effector functions

(A) The effector phenotypes of FLY-specific CTLs generated by stimulation with peptide-pulsed autologous $\gamma\delta$ T-APCs were compared with CTLs expanded in response to peptide presentation by mature autologous DCs by staining of gated CD8+ FLY pentamer-positive cells with CD45RO- and CCR7-specific antibodies (representative experiment of four). (B) Cytotoxicity of FLY peptide-pulsed and unpulsed targets by autologous CTLs induced by coincubation with peptide-pulsed autologous DCs or $\gamma\delta$ T cells (representative experiment of three). Unprimed $\gamma\delta$ T cells provide the negative control in this experiment. (C) Intracellular IFN- γ and TNF- α secretion by CD8+ pentamer+ CTLs induced by coincubation with peptide-pulsed autologous $\gamma\delta$ T cells in response to stimulation with either unpulsed or peptide-pulsed LCLs (lower panel) or autologous $\gamma\delta$ T cells (upper panel), as assessed by flow cytometry (representative experiment of two). (D) IFN- γ secretion by CTLs induced by coincubation with FLY peptide-pulsed autologous DCs or $\gamma\delta$ T-APCs in response to either unpulsed or pulsed autologous (auto) or allogeneic mismatched (allo; negative control) LCLs, as assessed by ELISA (representative experiment of three, means and standard deviations of triplicate samples).

Figure 4. LMP2a gene-transduced $\gamma\delta$ T APCs induce peptide-specific CTLs within a population of CD8+ autologous responder T cells

(A) Purified CD8+ T cells from a normal donor (HLA type A2;25/B15(62)) were coincubated with autologous $\gamma\delta$ T cells retrovirally transduced with either control (14.G2a ζ) or LMP2a gene for 12 days, followed by quantification of HLA A2-restricted FLY pentamer-positive cells by flow cytometry. (B) The breadth of the LMP2 epitope specificity of a CTL line initiated using $\gamma\delta$ T cells transduced with retrovirus LMP2 (LMP2-CTL) was compared to a CTL line initiated with an autologous lymphoblastoid cell line (EBV-CTL). CTLs (1×10^5 /well) (HLA-A2;25/B15(62)) were stimulated with an LMP2-peptide library pooled into 20 pools. Responses were measured in an 18-hour IFN- γ ELISPOT assay. All peptides were divided into 20 pools in such a way that each peptide is present in 2 pools. Shown is one of two experiments with two donors. (C) Responses to these peptide pools could subsequently be mapped to 3 different epitopes (HLA-A2- restricted **FLYALALL**, HLA-A25- restricted **TVMSNTLLSAW** and a further epitope the restriction of which is yet to be determined **LALSLLLLAAVASSY**) demonstrating that the breadth of the LMP2-specific T cell response is similar in both EBV-CTL and LMP-CTL. Shown is mean and standard deviation of duplicate wells.

Figure 5. Autologous $\gamma\delta$ T cells induce LMP2a-specific CTL expansion in cancer patients
 (A) PBMCs from an HLA-A2-positive patient with Hodgkin lymphoma were coincubated with autologous $\gamma\delta$ T cells as stimulator cells, either unpulsed (left panel) or pulsed with the LMP2a epitope FLY (right panel). The percentage of FLY-specific CTLs 10 days after stimulation was assessed by pentamer staining followed by flow cytometry analysis within a gate set on CD3+/CD8+ T cells. (B) $\gamma\delta$ T cells from an HLA-A2-negative patient with Hodgkin lymphoma were retrovirally transduced with full-length LMP2a (SFG-LMP2a-GFP), and GFP-expressing cells were positively selected by FACS. Autologous CD8+ T cells purified from peripheral blood lymphocytes by FACS were prestimulated with LMP2a-transduced $\gamma\delta$ T cells for 11 days. The negative cell fraction after sorting of CD8+ T cells was prestimulated with CD3- and CD28-specific antibodies, then transduced with either SFG-LMP2a-GFP or an irrelevant control vector (SFG-CD19 ζ -GFP). To determine antigen-specific cytokine secretion, triplicate samples of LMP2a-prestimulated cells (2×10^4 cells/well) were restimulated with 8×10^4 /well autologous transduced CD8-negative T cells, and IFN- γ secretion was quantified by ELISPOT analysis. Control wells contained LMP2a-prestimulated CD8+ T cells coincubated with autologous non-transduced or control vector-transduced CD8-negative T cells, or with medium alone.

Activated $\gamma\delta$ T cells induce epitope-specific CTL expansion to EBV antigens BZLF-1 (RAK) and LMP2a (FLY). The table summarizes the percentages of pentamer-positive T cells in response to stimulation with peptide-pulsed or unpulsed $\gamma\delta$ T cells and DCs, respectively, from multiple experiments using effector cells obtained from eight individual donors.

Table 1

Donor-Cell line	peptide	$\gamma\delta$ w/o peptide	$\gamma\delta$ + peptide	DCs w/o peptide	DCs + peptide
A-1	RAK	7.0%	60.0%	n.d.	82.0%
A-2	RAK	n.d.	67.7%	n.d.	83.0%
A-3	RAK	6.5%	89.7%	2.0%	89.2%
B-1	RAK	6.0%	80.0%	2.0%	96.0%
B-2	RAK	3.1%	63.0%	3.2%	93.0%
B-3	RAK	2.8%	55.5%	n.d.	n.d.
C-1	RAK	2.4%	53.0%	n.d.	n.d.
D-1	RAK	2.0%	41.9%	n.d.	10.6%
E-1	RAK	1.3%	8.3%	3.0%	13.4%
E-2	RAK	0.9%	8.5%	n.d.	n.d.
E-3	RAK	0.9%	35.0%	0.9%	49.0%
mean		3.3%	51.1%	2.2%	64.5%

C-1	FLY	0.7%	10.0%	1.3%	22.8%
C-2	FLY	0.2%	15.2%	n.d.	n.d.
C-3	FLY	1.4%	13.3%	1.3%	21.6%
C-4	FLY	1.0%	30.2%	n.d.	29.0%
C-5	FLY	1.6%	14.6%	n.d.	n.d.
F-1	FLY	1.0%	4.3%	n.d.	n.d.
F-2	FLY	1.1%	4.9%	n.d.	n.d.
F-3	FLY	0.4%	8.1%	n.d.	9.0%
G-1	FLY	0.8%	33.2%	n.d.	n.d.
H-1	FLY	1.0%	5.8%	n.d.	n.d.
H-2	FLY	0.7%	2.1%	n.d.	n.d.
H-3	FLY	0.7%	1.6%	0.5%	1.2%
mean		0.9%	12.6%	1.0%	16.7%