Expansion of human γδ T cells for adoptive immunotherapy using a bisphosphonate prodrug

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Cancer immunotherapy with human γδ T cells expressing Vγ2Vδ2 T cell receptor (also termed Vγ9Vδ2) has shown promise because of their ability to recognize and kill most types of tumors in a major histocompatibility complex (MHC) -unrestricted fashion that is independent of the number of tumor mutations. In clinical trials, adoptive transfer of Vγ2Vδ2 T cells has been shown to be safe and does not require preconditioning. In this report, we describe a method for preparing highly enriched human Vγ2Vδ2 T cells using the bisphosphonate prodrug, tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (PTA). PTA stimulated the expansion of Vγ2Vδ2 cells to purities up to 99%. These levels were consistently higher than those observed after expansion with zoledronic acid, the most commonly used stimulator for clinical trials. Cell numbers also averaged more than those obtained with zoledronic acid and the expanded Vγ2Vδ2 cells exhibited high cytotoxicity against tumor cells. The high purity of Vγ2Vδ2 cells expanded by PTA increased engraftment success in immunodeficient NOG mice. Even low levels of contaminating αβ T cells resulted in some mice with circulating human αβ T cells rather than Vγ2Vδ2 cells. Vγ2Vδ2 cells from engrafted NOG mice upregulated CD25 and secreted tumor necrosis factor-α and interferon-γ in response to PTA-treated tumor cells. Thus, PTA expands Vγ2Vδ2 T cells to higher purity than...
zoleadonic acid. The high purities allow the successful engraftment of immunodeficient mice without further purification and may speed up the development of allelogeneic V\textsubscript{γ2}V\textsubscript{δ2} T cell therapies derived from HLA-matched normal donors for patients with poor autologous V\textsubscript{γ2}V\textsubscript{δ2} T cell responses.

**KEYWORDS**
adoptive cancer immunotherapy, bisphosphonate, farnesyl diphosphate synthase, V\textsubscript{γ2}V\textsubscript{δ2} T cells, zoleadonic acid

1 | INTRODUCTION

Recent advances in cancer immunotherapy have revolutionized treatment for a number of cancers. By targeting checkpoint receptors, durable remissions have been achieved in patients with advanced metastatic melanoma, non-small cell lung cancer (NSCLC), bladder cancer and kidney cancer that would have had little chance of survival with conventional chemotherapies or targeted therapies. Similarly, chimeric antigen receptor-T cells (CAR-T) bearing receptors specific for CD19 have successfully treated patients with relapsing B-cell acute lymphoblastic leukemia and diffuse large B-cell lymphoma. However, both these treatments have limitations. Checkpoint blockade targeting PD-1 or its ligand, PD-L1, provides clinical benefits for a minority of the patients; approximately 30% for melanoma and kidney cancer and approximately 20% for lung cancer.\textsuperscript{1,2} The most successful adoptive transfer \γ\δ trials gave the nitrogen-containing bisphosphonate zoleadonic acid (Zol) prior to the transfer of the cells.\textsuperscript{20,21} Bisphosphonates indirectly stimulate V\textsubscript{γ2}V\textsubscript{δ2} T cells by inhibiting farnesyl diphosphate synthase (FDPS), resulting in the subsequent intracellular accumulation of its substrate, isopentenyl pyrophosphate (IPP), which is bound by BTN3A1.\textsuperscript{29,30}

Bisphosphonates enter cells through fluid-phase endocytosis and uptake can be enhanced by Ca\textsuperscript{2+}, suggesting that the negatively charged P-C-P structure limits entry.\textsuperscript{31} To improve cellular uptake and activity, we recently synthesized a bisphosphonate prodrug, tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (PTA), where these negative charges are masked with pivoxil esters.\textsuperscript{32,33} PTA is highly hydrophobic, allowing its efficient entry into cells where intracellular esterases convert it to its active form that blocks FDPS. PTA is a highly potent inhibitor of tumor cell proliferation that is 796-fold more potent against hematopoietic tumors and 27-fold more potent against solid tumors than Zol (the most potent bisphosphonate in current use).\textsuperscript{32} Similarly, PTA efficiently stimulates V\textsubscript{γ2}V\textsubscript{δ2} T cells to secrete TNF-\textalpha with 75 different tumor cell lines, being 903-fold more potent on average than Zol.\textsuperscript{33}

In this study, we examine the effect of the PTA bisphosphonate prodrug on the expansion of peripheral blood V\textsubscript{γ2}V\textsubscript{δ2} T cells ex vivo from patients with prostate cancer and breast cancer and analyze the engraftment success and effector functions of the expanded V\textsubscript{γ2}V\textsubscript{δ2} T cells after adoptive transfer to immunodeficient NOG mice.

2 | MATERIALS AND METHODS

2.1 | Reagents

Tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (PTA) and 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (TA) were synthesized as described.\textsuperscript{32} Zoleadonic acid (Zol) was purchased from Novartis AG (Basel, Switzerland). (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP) was synthesized as described.\textsuperscript{34}

In 9 clinical trials involving a total of 213 patients, adoptive transfer of V\textsubscript{γ2}V\textsubscript{δ2} T cells\textsuperscript{18-26} has proven to be safe,\textsuperscript{20,21} does not require pretreatment with cytotoxic agents, and has resulted in a durable remission in a patient with metastatic clear cell renal cancer,\textsuperscript{27} complete and partial responses in patients with breast and cervical cancer,\textsuperscript{20} and stable disease in 50% of patients with advanced NSCLC.\textsuperscript{28} However, most patients progressed, underscoring the need for improvements in the efficacy of this therapy.

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2.2 | Solubilization of PTA

PTA (10 μmoles) was dissolved in 10 mL of either DMSO or ethanol containing 10 mM of trimethyl β-cyclodextrin (TMβCD). TMβCD, a cyclic compound composed of 7 trimethyl-D-glucopyranoside units linked α-1→4, was synthesized as described.35

2.3 | Expansion of Vγ2Vδ2 T cells

Blood was obtained from healthy adult donors and prostate and breast cancer patients following approval by the institutional review board of Kyoto University and the Tokyo Women’s Medical University and written informed consent being provided by patients. Patients’ characteristics are summarized for prostate cancer patients in Table S1 and for breast cancer patients in Table S2. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation. The cells were washed 2 times with PBS, then resuspended in modified Yssel’s medium supplemented with 10% human AB serum (Cosmobio, Koto-ku, Tokyo, Japan) or enriched RPMI 1640 medium.36 They were cultured at 2.5 x 10^6 cells/1.5 mL/well in medium with either 1 μM PTA,
5 μM TA or 5 μM ZOL in the presence of 100 U/mL interleukin-2 (IL-2) (Shionogi Pharmaceutical, Chuo-ku, Osaka, Japan) in 4 wells of a 24-well plate (Corning Incorporated, Corning, NY). The culture medium was replaced every day from day 2 to day 9 with fresh medium containing IL-2. On day 10 for Yssel’s media or on day 14 for RPMI 1640, the cells were harvested. Purity of the V_{α2}V_{δ2} T cells were assessed by flow cytometry and the cells were either directly transferred or frozen in liquid nitrogen for later use.

2.4 | Statistical analysis

Statistical analyses were performed in GraphPad Prism v7.0A using the non-parametric 2-tailed Wilcoxon signed rank test for paired samples. P-values are as given with values <.05 considered significant.

2.5 | Cell aggregation assay

Cell aggregation during the culture was recorded under a microscope (Eclipse TS100, Nikon, Minato-ku, Japan) equipped with a digital camera (CoolPIX L20, Nikon).

2.6 | Flow cytometric analysis

Peripheral blood mononuclear cells before and after expansion were plated out at 2 × 10^5 cells/50 μL in a 96-well plate (Corning Incorporated, Corning, NY). The cells were then treated with 3 μL of FITC-conjugated anti-TCR V_{α2} mAb (clone IMMU 389, Beckman Coulter, Fullerton, CA, USA) and phycoerythrin (PE)-conjugated anti-CD3 mAb (clone SK7, BD Biosciences, San Diego, CA, USA) on ice for 30 min. After being washed 3 times with PBS, the cells were resuspended in 200 μL of 1%

**FIGURE 2** Expansion of V_{γ}2V_{δ}2 T cells by PTA or Zol. PBMC derived from a (A) healthy adult donor or a (B) breast cancer patient (BC37B) were stimulated either by PTA or Zol with interleukin-2 (IL-2) in either enriched RPMI 1640 (panel A) or Yssel’s media (panel B) for 10 days and then analyzed by flow cytometry.
2.7 | TNF-α production by peripheral blood mononuclear cells in response to bisphosphonates

Peripheral blood mononuclear cells derived from a healthy adult donor were cultured for 24 h at $2.5 \times 10^6$ cells/1.5 ml in modified Yssel’s medium with 1 μM PTA, 5 μM TA or 5 μM Zol in the presence of 100 U/mL IL-2 in a 24-well plate. The culture medium was removed and the content of TNF-α was determined using the standard ELISA according to the manufacturer’s protocol (Peprotech, Rocky Hill, NJ, USA).

2.8 | Cytotoxicity assay

Peripheral blood mononuclear cells from 2 healthy adult volunteers were stimulated with 1 μM PTA and IL-2. On day 11, the proportion of Vγ2Vδ2 T cells among CD3 T cells was confirmed to be greater than 99%. Vγ2Vδ2 T cell-mediated cellular cytotoxicity against tumor cells was determined using a non-radioactive cellular cytotoxicity assay according to the manufacturer’s protocol (Techno Suzuta, Heiwa-machi, Nagasaki, Japan). In brief, RPMI8226 (a plasmacytoma cell line that directly stimulates Vγ2Vδ2 T cells), K562 (an erythroleukemic cell line that is sensitive to killing by natural killer (NK) cells and to NK-like killing by T cells) and PTA-pretreated U937 (a monocye-like histiocytic lymphoma cell line) were incubated with the europium-chelate-forming compound, bis(butylroloxy)methyl)-4′, hydroxyxymethyl-2,2′,6,2′-terpyridine-6,6′-dicarboxylate, for 15 min at 37°C. After being washed with RPMI 1640 medium, the tumor cells were incubated with PTA-expanded Vγ2Vδ2 T cells at effector to target ratios of 0, 0.625, 1.25, 2.5, 6, 10, 20 and 40. The cells were centrifuged and after 40 min, the culture supernatants were harvested and mixed with a europium solution to form a europium-chelate complex with released labeling compound. Time-resolved europium fluorescence was measured on a PHERAStar FS multiplate reader (BMG LABTECH GmbH, Allmendgruen, Ortenberg, Germany) or a Berthold multiplate reader (Berthold Technologies GmbH, KG, Bad Wildbad, Germany).

2.9 | Adoptive transfer of Vγ2Vδ2 T cells into immunodeficient NOG mice

Peripheral blood mononuclear cells derived from healthy donors or prostate cancer patients were stimulated with 1 μM of PTA and IL-2 as detailed above and harvested on day 10 for direct transfer (for healthy donors) or frozen for later use (for cancer patients). Vγ2Vδ2 T cells constituted >98% of lymphocytes. Vγ2Vδ2 T cells from healthy donors were transferred directly, whereas Vγ2Vδ2 T cells from cancer patients were thawed, washed and then transferred. For both types of donors, $5 \times 10^7$ Vγ2Vδ2 T cells were i.p. injected into immunodeficient NOD.Cg-Prkdc<sup>scid</sup> IJ/2r<sup>tm1Sug</sup>/Jic (NOG) mice (Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan) that were maintained under specific-pathogen-free conditions. Fourteen or fifteen days later, peripheral blood was obtained and the red blood cells (RBC) were lysed with 1 mL of paraformaldehyde in PBS and analyzed using a FACScalibur or an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).
ACK lysis buffer (8.024 g of NH₄Cl, 1.001 g of KHCO₃ and 3.722 mg of EDTA/Na₂/2H₂O in 1000 mL of Milli-Q water). After washing with PBS/2% FCS, the resulting cells were stained with FITC-conjugated anti-V<sup>d</sup> TCR and PE-conjugated anti-human CD3 mAb. Animal use was approved by the institutional review board of Kyoto University Medical School. All experiments were performed in accordance with the relevant guidelines and regulations of Kyoto University Medical School.

2.10 | TNF-α production by adoptively transferred V<sub>γ</sub>2V<sub>δ</sub>2 T cells in vitro

NOG mice were i.p. injected with 5 × 10⁷ V<sub>γ</sub>2V<sub>δ</sub>2 T cells from a breast cancer patient (BC21). Two weeks later peripheral blood was taken from the mice, RBC were lysed with ACK buffer, and the resulting peripheral blood cells were stimulated with 1 μM of HMBPP at a cell concentration of 1 × 10⁵ cells/100 μL in vitro. After 24 h, the culture supernatants were harvested and TNF-α levels were determined in triplicate by ELISA (Peprotech, Rocky Hill, NJ, USA).

2.11 | CD25 expression on adoptively transferred V<sub>γ</sub>2V<sub>δ</sub>2 T cells stimulated in vivo

NOG mice were i.p. injected with 1 × 10⁶ EJ-1 cells. Four weeks later the mice were i.p. injected with 1 × 10⁷ V<sub>γ</sub>2V<sub>δ</sub>2 T cells and 1 μg of PTA. V<sub>γ</sub>2V<sub>δ</sub>2 T cells were harvested 24 h after the injection and stained with PE-conjugated anti-human CD25 mAb (clone BC96, Biolegend, San Diego, CA, USA).

2.12 | Determination of IFN-γ mRNA in adoptively transferred V<sub>γ</sub>2V<sub>δ</sub>2 T cells stimulated in vivo

NOG mice were i.p. injected with 1 × 10⁶ EJ-1 cells. Four weeks later, the mice were i.p. injected with 1 × 10⁷ V<sub>γ</sub>2V<sub>δ</sub>2 T cells with or without 1 μg of PTA. Peripheral blood was taken from the mice 4 h and 24 h after the challenge, and human IFN-γ mRNA levels measured in the harvested cells by quantitative PCR. Total RNA was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and complementary DNA was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase kit and random hexamers.
and 24 h after the injection, serum was prepared, and serum IFN-γ levels were measured by ELISA (Peprotech, Rocky Hill, NJ).

3 RESULTS

3.1 Delivery of PTA in an inclusion complex with trimethyl β-cyclodextrin

We recently described a new thiazole bisphosphonate prodrug, PTA, that is highly active relative to its acid form, TA, and to Zol (the most potent bisphosphonate in clinical use) (structures shown in Figure 1A).\(^{32,33}\) PTA is highly hydrophobic and is insoluble in water or ethanol. Although PTA can be dissolved in DMSO for research use, few drugs solubilized in DMSO have been approved for use in patients. Therefore, we tested a number of nonionic detergents for their ability to solubilize PTA in ethanol. A trimethyl derivative of β-cyclodextrin (TMβCD) had the best ability to solubilize PTA in ethanol (Figure S1). Cyclodextrin compounds can form inclusion complexes with drugs where hydrophobic drugs bind to their central hydrophobic cavity and where the polar surface of cyclodextrin makes the complex soluble in ethanol.\(^{38,39}\) PTA could be dissolved up to a concentration of 1 mM in ethanol containing 10 mM of TMβCD. Importantly, PTA solubilized in TMβCD/ethanol exhibited identical bioactivity to PTA solubilized in DMSO. For example, PTA in TMβCD/ethanol inhibited the proliferation of the EJ-1 bladder carcinoma and the U937 histiocytic lymphoma cell lines identically to PTA in DMSO (Figure S1C,D). Thus, TMβCD/ethanol can be used as the solvent for PTA, as was done in this study.

3.2 Stimulation of Vγ2Vδ2 T cells by PTA

PTA potently inhibits the growth of tumor cell lines in vitro\(^{32}\) and selectively activates Vγ2Vδ2 T cells in PBMC to proliferate, secrete cytokines and kill tumor cells.\(^{33}\) Consistent with these results, when we compared the activity of Zol to the PTA prodrug and its TA acid form, PTA was strongly active. Zol and TA stimulated Vγ2Vδ2 T cell-dependent cell aggregation at concentrations of approximately 10 μM, whereas PTA caused aggregation at approximately 0.1 μM (Figure 1B). Similarly, PTA stimulated TNF-α production by Vγ2Vδ2 T cells at an EC\(_{50}\) of 3 nM, whereas TA and Zol stimulated at an EC\(_{50}\) of 3000 nM (Figure 1C). Thus, PTA was 1000-fold more potent than TA and Zol in stimulating Vγ2Vδ2 T cells in PBMC.

3.3 Ex vivo expansion by PTA of Vγ2Vδ2 T cells for adoptive immunotherapy

To determine whether PTA can be used to expand Vγ2Vδ2 T cells for adoptive immunotherapy, PMBC from cancer patients and from healthy donors were stimulated with PTA and IL-2 and the resulting Vγ2Vδ2 T cells were assessed for purity and biological activity after 10 days. Similar to what we have previously
shown for Zol. PTA stimulated maximum expansion within a relatively narrow dose range when PBMC were continuously exposed to PTA as it was slowly diluted (Figure 2). Thus, V\textsubscript{d}2V\textsubscript{d}2 T cell expansion was maximal at a PTA concentration of 1 lM, while a PTA concentration of 2 lM was highly toxic (Figure 2A).

PTA stimulation resulted in highly enriched populations of V\textsubscript{d}2V\textsubscript{d}2 T cells that approached 100% of CD3 T cells, whereas Zol stimulation was consistently less (Figure 2A,B). To determine the effect of PTA on V\textsubscript{d}2V\textsubscript{d}2 T cells from cancer patients, PBMC from 8 cancer patients with prostate or breast cancer were stimulated with either PTA or Zol (Figure 3). PTA stimulation resulted in significantly higher enrichment of the V\textsubscript{d}2V\textsubscript{d}2 T cell population as compared with Zol stimulation (95.6 ± 5.7% of total cells vs 90.3 ± 10.0%, P = .0078, Figure 3A). All donors exhibited higher enrichment with PTA compared to Zol. In addition, the number of V\textsubscript{d}2V\textsubscript{d}2 T cells was 20% higher on average for stimulation with PTA as compared with Zol (6.3 × 10\textsuperscript{8} ± 5.5 × 10\textsuperscript{8} cells with PTA vs 5.7 × 10\textsuperscript{8} ± 4.3 × 10\textsuperscript{8} cells with Zol, P = .38, Figure 3B), although this was observed for only 6 out of 8 patients and was not statistically significant. The increase in V\textsubscript{d}2V\textsubscript{d}2 cell numbers varied widely ranging from 959 to 28 722-fold (6344-fold for PTA vs 4302-fold for Zol, P = .31) and was not significantly different (Figure 3C). Examples of V\textsubscript{d}2V\textsubscript{d}2 T cell expansion in response to PTA are shown for prostate cancer patients (Figure 4) and healthy donors (Figure S2). Thus, ex vivo PTA stimulation of V\textsubscript{d}2V\textsubscript{d}2 T cells in PBMC from

FIGURE 6 Adoptively transferred human V\textsubscript{d}2V\textsubscript{d}2 T cells from prostate cancer patients persist in the blood of NOG mice. V\textsubscript{d}2V\textsubscript{d}2 T cells (>98%) were expanded from prostate cancer patient 2 (PC02) (left panels) and patient 4 (PC04) (right panels) and frozen for later use. 5 × 10\textsuperscript{7} thawed V\textsubscript{d}2V\textsubscript{d}2 T cells were then adoptively transferred into each NOG mice. Fifteen days after transfer, peripheral blood was obtained. After RBC lysis, the resulting cells were stained and then analyzed by flow cytometry for expression of V\textsubscript{d}2 TCR and human CD3. Values shown are % of total cells. Mean ± SD PC02 2.5% ± 0.9%, n = 3 PC04 6.4% ± 2.4%, n = 3
3.4 | Cytotoxic activity of $\gamma$2Vδ2 T cells expanded by PTA

Adult $\gamma$2Vδ2 T cells are highly cytotoxic\(^{40}\) and exhibit cytototoxicity for most tumor cell lines.\(^{8,33}\) $\gamma$2Vδ2 T cells expanded with PTA were, therefore, assessed for their cytotoxic activity against an NK-sensitive cell line, a stimulatory plasmacytoma and a tumor cell line treated with PTA. PTA-expanded $\gamma$2Vδ2 T cells exhibited strong NK-like cytotoxic activity efficiently lysing the NK target cell line, K562, which lacks MHC class I (Figure 5A). PTA-expanded $\gamma$2Vδ2 T cells also lysed the RPMI 8226 plasmacytoma cell line that is directly stimulatory for $\gamma$2Vδ2 T cells through a TCR-dependent mechanism (Figure 5A).\(^{17,41}\)

Finally, PTA-expanded $\gamma$2Vδ2 T cells efficiently lysed U937 tumor cells that had been treated with PTA (Figure 5B). Therefore, expansion of $\gamma$2Vδ2 T cells by PTA preserves their cytotoxic activity.

3.5 | Purity of $\gamma$2Vδ2 T cells helps to determine their engraftment success after adoptive transfer in NOG mice

To assess their potential use for cancer immunotherapy, $\gamma$2Vδ2 T cells expanded by PTA were adoptively transferred into immunodeficient NOG mice. $\gamma$2Vδ2 T cells expanded from prostate cancer patient 2 (PC02) and 4 (PC04) using PTA with IL-2 (shown in Figure 4) were i.p. injected into immunodeficient NOG mice and continued to circulate 15 days later. Mice receiving cells from prostate cancer patient 2 (PC02) averaged 2.5% $\gamma$2Vδ2 T cells, whereas mice receiving cells from prostate cancer patient 4 (PC04) averaged 6.4% $\gamma$2Vδ2 T cells of total cells (Figure 6). Similarly, NOG mice receiving $\gamma$2Vδ2 T cells derived from a normal donor averaged 18.8% in Experiment 1 and 12.6% in Experiment 2 (Figure S3), whereas breast cancer patients averaged 8.8% for BC36 and 4.8% for BC35 (Figure S4). The higher proportions of $\gamma$2Vδ2 T cells observed with the normal donor were likely due to the transfer of freshly expanded $\gamma$2Vδ2 T cells instead of the previously frozen cells used from prostate and breast cancer patients.

Transfer of highly enriched $\gamma$2Vδ2 T cells (>98% of total cells) reduced the proportion of NOG mice with elevated levels of $\gamma$β T cells (Figure 7A,B). For example, transfer of $\gamma$2Vδ2 T cells expanded using Zol from patient BC35 which were 90% pure resulted in only 1 out of 9 NOG mice having $\gamma$2Vδ2 T cells that constituted greater than 80% of circulating T cells while transfer of $\gamma$2Vδ2 T cells which were 97% pure resulted in only 1 out of 5 NOG mice (Figure S4). In contrast, transfer of $\gamma$2Vδ2 T cells with purities >98% resulted in 29 out of 32 NOG mice with $\gamma$2Vδ2 T cells constituting greater than 80% of circulating T cells (Figure 7A, lower panel). Thus, the high purity of $\gamma$2Vδ2 T cells expanded using PTA helped to ensure successful engraftment of NOG mice without $\gamma$β T cell outgrowth.

3.6 | Adoptively transferred $\gamma$2Vδ2 T cells from NOG mice are fully functional

To assess the functional capabilities of $\gamma$2Vδ2 T cells expanded by PTA in vivo, we tested various immunological functions of the cells.
after their adoptive transfer to immunodeficient NOG mice. First, recovered V\textsubscript{γ}2V\textsubscript{δ}2 T cells were tested for their ability to secrete TNF-α. Despite 2 weeks in NOG mice without exogenous human IL-2 or IL-15, recovered V\textsubscript{γ}2V\textsubscript{δ}2 T cells secreted TNF-α upon in vitro stimulation with the bacterial metabolite, HMBPP (Figure 8A). Note that murine cells and other human T cells do not respond to HMBPP or its analogs, as we have shown in earlier studies.\textsuperscript{11,42}

Second, we examined the ability of transferred V\textsubscript{γ}2V\textsubscript{δ}2 T cells to respond to bisphosphonate stimulation in vivo. For these experiments, NOG mice bearing human EJ-1 bladder carcinoma cells were i.p. injected with V\textsubscript{γ}2V\textsubscript{δ}2 T cells with or without PTA and V\textsubscript{γ}2V\textsubscript{δ}2 T cells recovered from the mice 4 h and 24 h later. After 24 h, V\textsubscript{γ}2V\textsubscript{δ}2 T cells upregulated expression of the CD25 IL-2α receptor when stimulated with PTA (right panel, Figure 8B) but not if PTA was omitted (left panel, Figure 8B). Moreover, IFN-γ mRNA expression in V\textsubscript{γ}2V\textsubscript{δ}2 T cells was upregulated, reaching maximum levels at 4 h and declining thereafter (Figure 8C). Serum human IFN-γ protein levels increased later such that levels were higher at 24 h compared with 4 h (Figure 8D), consistent with the requirement for translation of IFN-γ to produce the cytokine rather than its release from preformed stores as has been demonstrated for in vivo responses to lymphocytic choriomeningitis virus.\textsuperscript{43} IFN-γ production was dependent on V\textsubscript{γ}2V\textsubscript{δ}2 T cells given that NOG mice bearing EJ-1 cancer cells with PTA but no V\textsubscript{γ}2V\textsubscript{δ}2 T cells or receiving only PTA alone failed to upregulate IFN-γ expression at either the mRNA or protein levels. These findings clearly demonstrate that adoptively transferred V\textsubscript{γ}2V\textsubscript{δ}2 T cells that have been expanded by PTA stimulation can be specifically activated in vivo with bisphosphonate stimulation to upregulate CD25 for proliferation and IFN-γ for its myriad effector functions.
DISCUSSION

In the present study, we show that PTA, a novel bisphosphonate prodrug, expands peripheral blood V\(_2\)V\(_6\)\(_2\) T cells up to several thousand-fold in 10 days, with very high purity in both cancer patients and healthy adult donors. PTA is, thus, ideal for the preparation of large numbers of highly homogeneous V\(_2\)V\(_6\)\(_2\) T cells for use in adoptive immunotherapy for cancer. These expanded V\(_2\)V\(_6\)\(_2\) T cells exhibited full immunological functions when tested in preclinical immunodeficient mouse models and their high purity helped to limit the outgrowth of human \(\alpha\beta\) T cells in these mice.

The pivaloyloxymethyl groups of PTA mask the hydrophilic phosphonate moieties, allowing cell entry but also making PTA hydrophobic such that it dissolves in DMSO but not in ethanol. Because of the possible toxicity of DMSO and its chemical properties (high freezing point and garlic taste), few intravenous drugs are solubilized in DMSO for patient use. However, because none of the conventional detergents used in clinical practice allowed PTA to dissolve in ethanol, we tested TM\(_{jICD}\). TM\(_{jICD}\) is highly soluble in both water and ethanol and its parent compound, JCD, has been used for drug delivery. The less hydrophilic interiors of these compounds allow them to form complexes with hydrophobic drugs. Accordingly, TM\(_{jICD}\) solubilizes PTA in ethanol. This TM\(_{jICD}/\)ethanol solubilization method could be applicable to other hydrophobic therapeutics, such as alkoxymethyl derivatives of anionic compounds. PTA dissolved in TM\(_{jICD/}\)ethanol exhibited identical biological activity to PTA dissolved in DMSO, suggesting that PTA preparation in TM\(_{jICD}/\)ethanol could be used for both preclinical and clinical studies.

In previous clinical studies, it was not clear whether IL-2 infusion was essential for adoptive immunotherapy with \(\gamma\delta\) T cells because some studies gave IL-2 in vivo. This study clearly shows that V\(_2\)V\(_6\)\(_2\) T cells continued to circulate in the peripheral blood even 2 weeks after i.P. injection into NOG mice in the absence of exogenous IL-2. In addition, the transferred V\(_2\)V\(_6\)\(_2\) T cells were functionally active, as evidenced by the expression of TNF-\(\alpha\), CD25 and IFN-\(\gamma\) in response to PTA-sensitized tumor cells or HMBPP stimulation. Although IL-2 is absolutely required for the maintenance of V\(_2\)V\(_6\)\(_2\) T cells in vivo, these findings suggest that IL-2 is not absolutely necessary in adoptive immunotherapy with V\(_2\)V\(_6\)\(_2\) T cells. Thus, IL-2 infusion might be omitted or reduced in patients undergoing adoptive cancer immunotherapy with V\(_2\)V\(_6\)\(_2\) T cells.

Using PTA to expand V\(_2\)V\(_6\)\(_2\) T cells for adoptive immunotherapy has potential advantages over Zol. The most commonly used stimulator for clinical trials. PTA stimulation consistently resulted in highly enriched populations of V\(_2\)V\(_6\)\(_2\) T cells that were of significantly higher purity than those expanded by Zol. In many cases, V\(_2\)V\(_6\)\(_2\) T cells were >99% of total T cells and it may be possible to further increase cell yields by using pulse PTA stimulation, as we have shown for pulse stimulation with Zol. The ability to generate highly enriched V\(_2\)V\(_6\)\(_2\) T cells would be useful for preclinical studies in immunodeficient mice. Our findings show that relatively low levels of \(\alpha\beta\) T cell contamination can lead to the outgrowth of \(\alpha\beta\) T cells in the mice. This contamination could lead to xenogeneic graft-versus-host-disease that is observed when human PBMC are xenotransplanted into NSG mice and that leads to their deaths with a median survival of 40 days.\(^{44,45}\) The contamination could also compromise tumor immunity by V\(_2\)V\(_6\)\(_2\) T cells through competition with \(\gamma\delta\) T cells. Our findings suggest that V\(_2\)V\(_6\)\(_2\) T cells should generally be purified prior to use in long-term tumor studies in immunodeficient NSG or NOG mice. Using PTA-expanded V\(_2\)V\(_6\)\(_2\) T cells as a starting population would ensure that purification would give highly enriched V\(_2\)V\(_6\)\(_2\) T cells with few \(\gamma\delta\) T cells.

Another potential use of such highly enriched populations would be allogeneic adoptive transfer of V\(_2\)V\(_6\)\(_2\) T cells for tumor immunotherapy. Thus far, all clinical trials have used syngeneic V\(_2\)V\(_6\)\(_2\) T cells. However, because V\(_2\)V\(_6\)\(_2\) T cells are not alloreactive and because the BTN3A1 is not polymorphic, V\(_2\)V\(_6\)\(_2\) T cells can be stimulated by allogeneic tumor cells treated with PTA or a prenyl pyrophosphate. Because they are not alloreactive, transfer of V\(_2\)V\(_6\)\(_2\) T cells will not cause graft-versus-host disease. Note that in natural infections, very high numbers of V\(_2\)V\(_6\)\(_2\) T cells can be observed (up to 50% of circulating T cells) without autoimmunity or other toxicity (reviewed in Morita et al.\(^8\)).

The ability to generate highly enriched V\(_2\)V\(_6\)\(_2\) T cells preparations without contaminating \(\gamma\delta\) T cells would allow their use as "off-the-shelf" reagents for cancer immunotherapy. This would result in major cost savings and greatly increase the feasibility of such treatments because normal donors that expand particularly well could be selected and these individuals can be repeatedly leukopheresed to obtain starting PBMC. To avoid host-versus-graft disease, the donors could be HLA typed and donors selected based on their degree of HLA match to the recipient. If host-versus-graft responses develop, the donor for the V\(_2\)V\(_6\)\(_2\) T cells could be switched. If necessary, \(\gamma\delta\) T cells could be depleted using anti-\(\gamma\delta\) TCR magnetic beads as this process would be very efficient because of their low abundance. Such an approach is being investigated commercially for CAR-NKT cells. Moreover, infusion of HLA-matched allogeneic virus-specific cytotoxic lymphocytes derived by in vitro stimulation is already being used to treat patients undergoing bone marrow transplantation who have developed severe viral infections with Epstein-Barr virus or cytomegalovirus.\(^{47}\) In one study, the transferred cells could be detected in the blood for a median period of 10 weeks and there was minimal toxicity.\(^{48}\) The ability to generate large numbers of highly enriched V\(_2\)V\(_6\)\(_2\) T cells that can be banked and used as "off-the-shelf" reagents could greatly facilitate the development of adoptive V\(_2\)V\(_6\)\(_2\) T cell therapies.

CONFLICT OF INTEREST

Y.T. is a co-inventor of novel terpyridine-derivative proligands for measuring cytotoxicity: PCT/JP2015/059838. C.T.M. is a co-inventor of US Patent 8,012,466 on the development of live bacterial vaccines for activating \(\gamma\delta\) T cells and has no other financial or non-financial conflict of interest. The other authors declare no financial or non-financial conflicts of interest.
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