



TITLE:

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AUTHOR(S):

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ORIGINAL ARTICLE

Expansion of human $\gamma\delta$ T cells for adoptive immunotherapy using a bisphosphonate prodrug

Yoshimasa Tanaka^{1,2,3}  | Kaoru Murata-Hirai¹ | Masashi Iwasaki¹ | Kenji Matsumoto¹ | Kosuke Hayashi¹ | Asuka Kumagai³ | Mohanad H. Nada⁴ | Hong Wang⁴ | Hirohito Kobayashi⁵ | Hiroshi Kamitakahara⁶ | Haruki Okamura⁷ | Tomoharu Sugie⁸ | Nagahiro Minato² | Masakazu Toi⁸ | Craig T. Morita⁴

¹Center for Innovation in Immunoregulative Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

²Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

³Center for Bioinformatics and Molecular Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

⁴Department of Internal Medicine and the Interdisciplinary Graduate Program in Immunology, University of Iowa Carver College of Medicine, Iowa City Veterans Affairs Health Care System, Iowa City, IA, USA

⁵Department of Transfusion Medicine and Cell Processing, Tokyo Women's Medical University, Tokyo, Japan

⁶Department of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

⁷Department of Tumor Immunology and Cell Therapy, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

⁸Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Correspondence

Yoshimasa Tanaka, Center for Bioinformatics and Molecular Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan.

Email: ystanaka@nagasaki-u.ac.jp

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Cancer immunotherapy with human $\gamma\delta$ T cells expressing V γ 2V δ 2 T cell receptor (also termed V γ 9V δ 2) 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 V γ 2V δ 2 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 V γ 2V δ 2 T cells using the bisphosphonate prodrug, tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino)ethylidene-1,1-bisphosphonate (PTA). PTA stimulated the expansion of V γ 2V δ 2 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 V γ 2V δ 2 cells exhibited high cytotoxicity against tumor cells. The high purity of V γ 2V δ 2 cells expanded by PTA increased engraftment success in immunodeficient NOG mice. Even low levels of contaminating $\alpha\beta$ T cells resulted in some mice with circulating human $\alpha\beta$ T cells rather than V γ 2V δ 2 cells. V γ 2V δ 2 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 V γ 2V δ 2 T cells to higher purity than

Toi and Morita authors contributed equally to this work.

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zoledronic acid. The high purities allow the successful engraftment of immunodeficient mice without further purification and may speed up the development of allogeneic V γ 2V δ 2 T cell therapies derived from HLA-matched normal donors for patients with poor autologous V γ 2V δ 2 T cell responses.

KEYWORDS

adoptive cancer immunotherapy, bisphosphonate, farnesyl diphosphate synthase, V γ 2V δ 2 T cells, zoledronic 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.^{1,2} Moreover, the effectiveness of checkpoint blockade correlates with the numbers of nonsynonymous mutations present in the tumors from patients with NSCLC³ and melanoma.⁴ Thus, patients with cancers that have low numbers of mutations, such as many of the pediatric cancers and glioblastomas,⁵ would be predicted to respond infrequently as has been observed.⁶ CAR-T therapy is limited because there are few clearly defined tumor-specific antigens on solid tumors.⁷ Therefore, additional types of immunotherapy are needed to realize the full potential of cancer immunotherapy.

Adoptive immunotherapy with V γ 2V δ 2 T cells (also termed V γ 9V δ 2) is a potential therapy for a variety of cancers and is independent of the mutational status of the tumor. Stimulation of V γ 2V δ 2 T cells is not dependent on peptides presented by MHC proteins and is, therefore, major histocompatibility complex (MHC) - unrestricted.⁸ Instead, V γ 2V δ 2 T cells respond to the presence of small isoprenoid metabolites, such as self isopentenyl pyrophosphate (IPP)⁹ or foreign microbial (*E*-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP)),^{10,11} in a process requiring the butyrophilin 3A1 (BTN3A1) protein, an immunoglobulin superfamily protein present on all normal and tumor cells.¹²⁻¹⁴ The isoprenoid metabolites bind to the B30.2 intracellular domains of BTN3A1,^{15,16} which alters the cell through an unknown process to allow the V γ 2V δ 2 TCR¹⁷ to recognize this intracellular binding. TCR recognition leads to the activation of V γ 2V δ 2 T cells for cytotoxicity and cytokine secretion.

In 9 clinical trials involving a total of 213 patients, adoptive transfer of V γ 2V δ 2 T cells¹⁸⁻²⁶ has proven to be safe,^{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,²⁷ complete and partial responses in patients with breast and cervical cancer,²⁰ and stable disease in 50% of patients with advanced NSCLC.²⁸ However, most patients progressed, underscoring the need for improvements in the efficacy of this therapy.

The most successful adoptive transfer $\gamma\delta$ trials gave the nitrogen-containing bisphosphonate zoledronic acid (Zol) prior to the transfer of the cells.^{20,21} Bisphosphonates indirectly stimulate V γ 2V δ 2 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.^{29,30}

Bisphosphonates enter cells through fluid-phase endocytosis and uptake can be enhanced by Ca²⁺, suggesting that the negatively charged P-C-P structure limits entry.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.^{32,33} PTA is highly hydrophobic, allowing its efficient entry into cells where intracellular esterases convert it to its active acid 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).³² Similarly, PTA efficiently stimulates V γ 2V δ 2 T cells to secrete TNF- α with 75 different tumor cell lines, being 903-fold more potent on average than Zol.³³

In this study, we examine the effect of the PTA bisphosphonate prodrug on the expansion of peripheral blood V γ 2V δ 2 T cells *ex vivo* from patients with prostate cancer and breast cancer and analyze the engraftment success and effector functions of the expanded V γ 2V δ 2 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.³² Zoledronic acid (Zol) was purchased from Novartis AG (Basel, Switzerland). (*E*-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP) was synthesized as described.³⁴

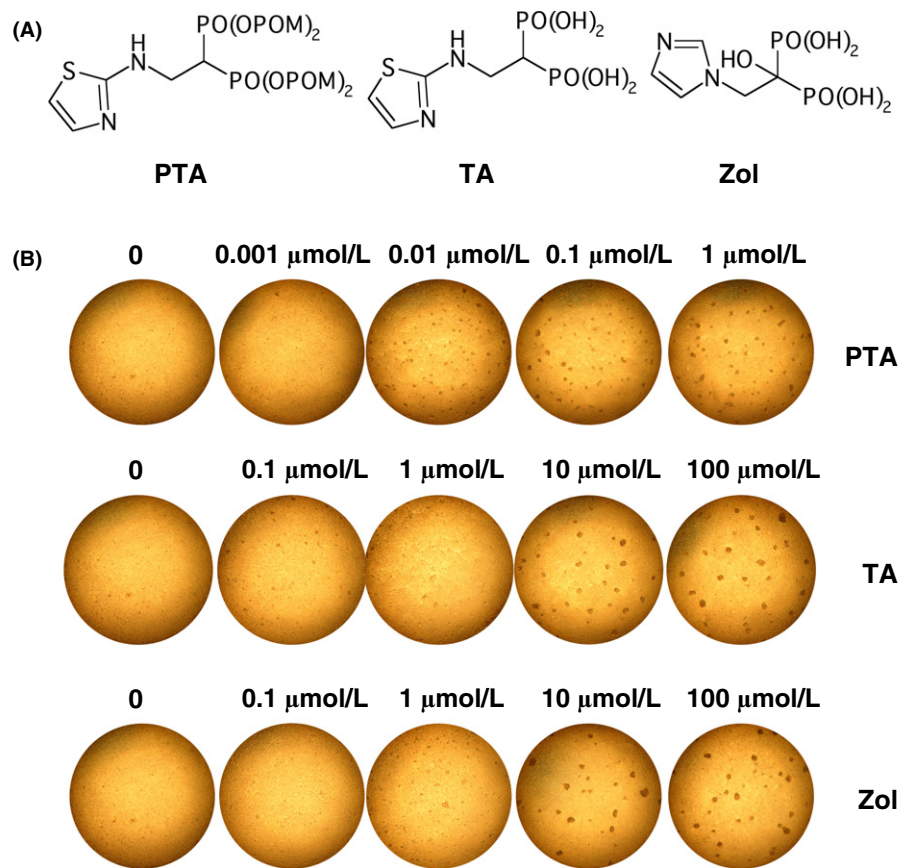


FIGURE 1 Structure and immunological activity of bisphosphonates. A, Structure of the bisphosphonate prodrug, PTA, its active acid form, TA, and zoledronic acid (Zol). B, Effect of bisphosphonates on the aggregation of V γ 2V δ 2 T cells in peripheral blood mononuclear cells (PBMC). PBMC from a healthy donor where V δ 2 T cells constituted 20.2% of total T cells, were exposed to varying concentrations of the bisphosphonates for 2 days, and cell aggregation was monitored under a microscope. C, TNF- α production by PBMC in response to bisphosphonates. After incubation of PBMC with bisphosphonates for 2 days, supernatants were harvested and TNF- α levels measured by ELISA

2.2 | Solubilization of PTA

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

2.3 | Expansion of V γ 2V δ 2 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 2.5×10^6 cells/1.5 mL/well in medium with either 1 μ M PTA,

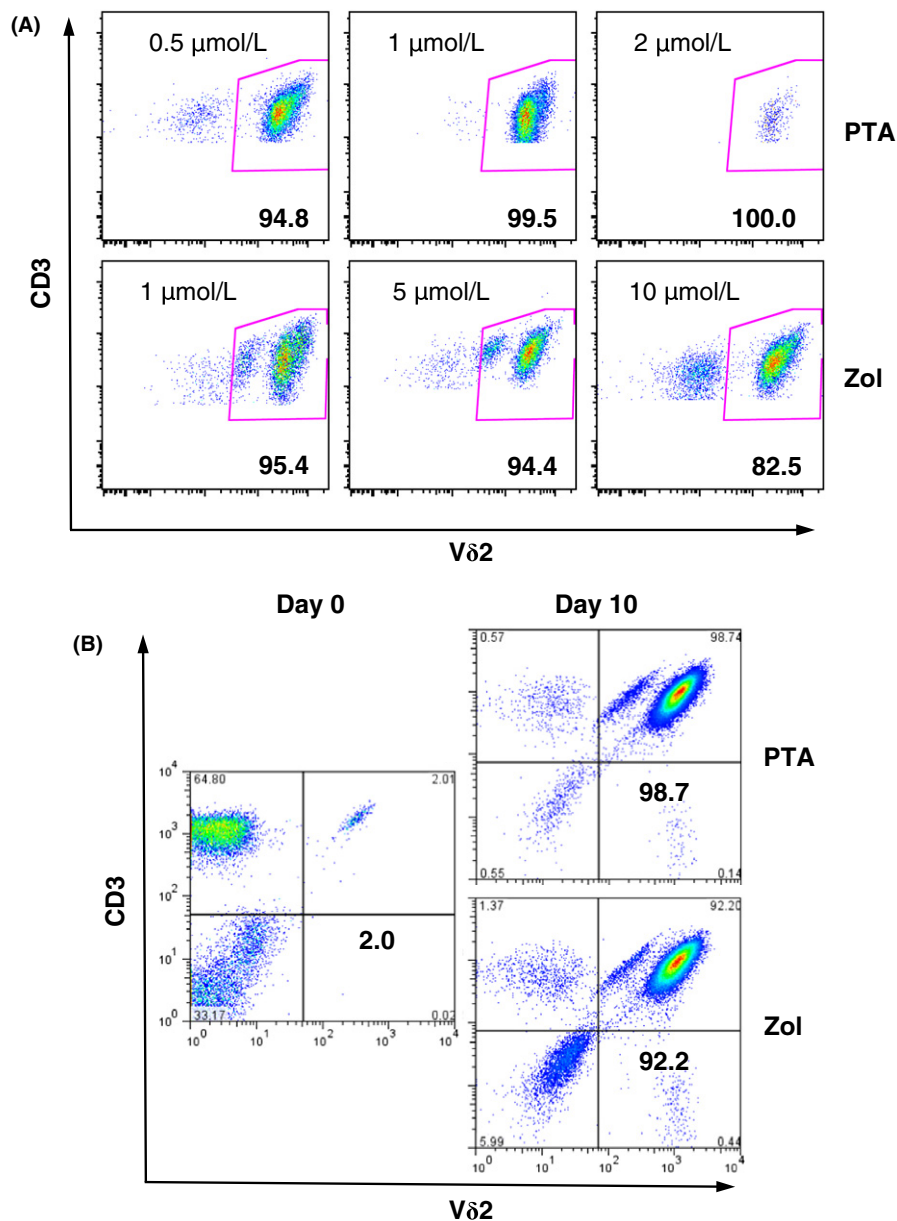


FIGURE 2 Expansion of V γ 2V δ 2 T cells by PTA or Zol. PBMC derived from a (A) healthy adult donor or a (B) breast cancer patient (BC37B) were stimulated either by PTA or Zol with interleukin-2 (IL-2) in either enriched RPMI 1640 (panel A) or Yssel's media (panel B) for 10 days and then analyzed by flow cytometry

5 μ M TA or 5 μ M 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 media, the cells were harvested. Purity of the V γ 2V δ 2 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 μ L in a 96-well plate (Corning Incorporated, Corning, NY). The cells were then treated with 3 μ L of FITC-conjugated anti-TCR V δ 2 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 μ L of 1%

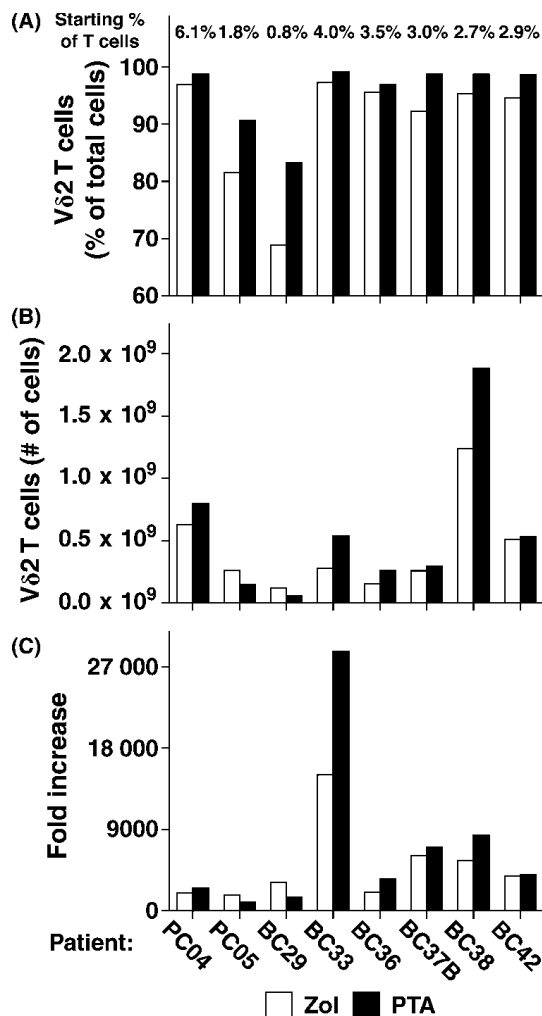


FIGURE 3 Expansion of $V\gamma 2V\delta 2$ T cells by PTA increases their purity and number compared with expansion by Zol. $V\gamma 2V\delta 2$ T cells were expanded from peripheral blood mononuclear cells (PBMC) derived from patients with prostate or breast cancer by either PTA or Zol. PBMC were cultured with PTA or Zol in the presence of interleukin-2 (IL-2) for 10 days and then analyzed before and after by flow cytometry. A, Purity of $V\gamma 2V\delta 2$ T cells expanded by PTA or Zol. $V\gamma 2V\delta 2$ T cells averaged 5.3% higher purity (ranging from 1.4 to 14.4%) ($95.6 \pm 5.7\%$ of total cells for PTA vs $90.3 \pm 10.0\%$ for Zol, $P = .0078$). The starting percentage of $V\gamma 2V\delta 2$ T cells of total T cells is listed for each patient at the top of the panel. The mean \pm SD was $3.1 \pm 1.6\%$ for the patients versus $2.9 \pm 3.9\%$ for normal adults as determined in an earlier study.⁴⁹ B, Number of $V\gamma 2V\delta 2$ T cells expanded by PTA or Zol. Cell numbers averaged 30% higher with PTA compared to Zol. $V\gamma 2V\delta 2$ T cells expanded on average to $5.6 \times 10^8 \pm 5.8 \times 10^8$ cells with PTA versus $4.3 \times 10^8 \pm 3.7 \times 10^8$ cells with Zol ($P = .20$, not significant). (c) Fold increase of $V\gamma 2V\delta 2$ T cells expanded by PTA or Zol. PTA expansion compared to Zol ranged from 0.5 to 1.9-fold different (7085-fold for PTA vs 4936-fold for Zol, $P = .25$, not significant). BC, breast cancer; PC, prostate cancer

paraformaldehyde in PBS and analyzed using a FACSCalibur or an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

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 2.5×10^6 cells/1.5 mL in modified Yssel's medium with 1 μ M PTA, 5 μ M TA or 5 μ M 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 μ M PTA and IL-2. On day 11, the proportion of $V\gamma 2V\delta 2$ T cells among CD3 T cells was confirmed to be greater than 99%. $V\gamma 2V\delta 2$ 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 $V\gamma 2V\delta 2$ 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 monocyte-like histiocytic lymphoma cell line) were incubated with the europium-chelate-forming compound, bis(butyryloxymethyl) 4'-hydroxymethyl-2,2':6',2''-terpyridine-6,6''-dicarboxylate, for 15 min at 37°C.³⁷ After being washed with RPMI 1640 medium, the tumor cells were incubated with PTA-expanded $V\gamma 2V\delta 2$ 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 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 $V\gamma 2V\delta 2$ T cells into immunodeficient NOG mice

Peripheral blood mononuclear cells derived from healthy donors or prostate cancer patients were stimulated with 1 μ M 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). $V\gamma 2V\delta 2$ T cells constituted >98% of lymphocytes. $V\gamma 2V\delta 2$ T cells from healthy donors were transferred directly, whereas $V\gamma 2V\delta 2$ T cells from cancer patients were thawed, washed and then transferred. For both types of donors, 5×10^7 $V\gamma 2V\delta 2$ T cells were i.p. injected into immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/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

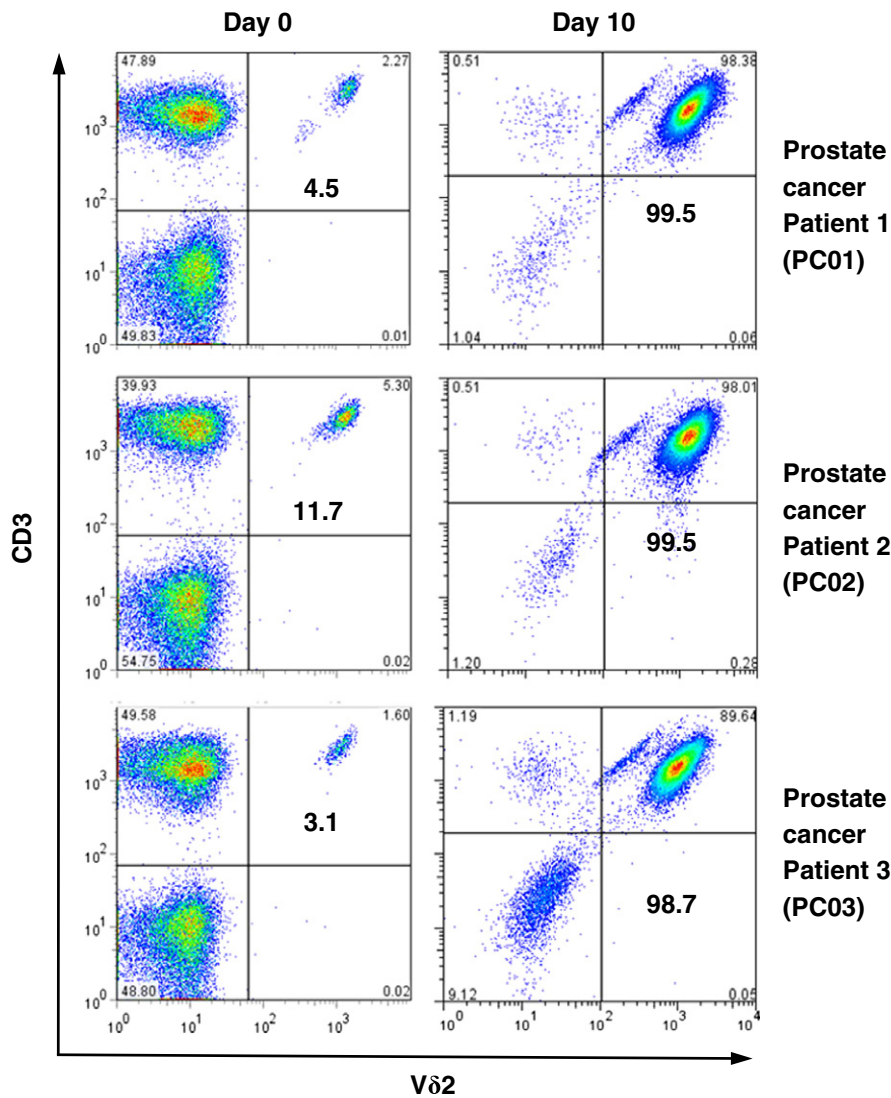


FIGURE 4 Expansion of V γ 2V δ 2 T cells from prostate cancer patients by PTA leads to high purity. Peripheral blood mononuclear cells (PBMC) isolated from prostate cancer patients were analyzed by flow cytometry before (left panels) and after (right panels) stimulation with PTA and interleukin-2 (IL-2) for 10 days. Values shown are % of CD3 T cells. The absolute number of V δ 2 T cells increased from 2.0×10^5 to 2.4×10^8 for PC01, 5.7×10^5 to 6.4×10^8 for PC02, and 1.7×10^5 to 7.6×10^8 for PC03, with the expansion rate being 1175-fold for PC01, 1118-fold for PC02, and 4398-fold for PC03

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 δ 2 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 γ 2V δ 2 T cells in vitro

NOG mice were i.p. injected with 5×10^7 V γ 2V δ 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 μ M of HMBPP at a cell concentration of 1×10^5 cells/100 μ L 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 γ 2V δ 2 T cells stimulated in vivo

NOG mice were i.p. injected with 1×10^6 EJ-1 cells. Four weeks later the mice were i.p. injected with 1×10^7 V γ 2V δ 2 T cells and 1 μ g of PTA. V γ 2V δ 2 T cells were harvested 24 h after the injection and stained with PE-conjugated anti-human CD25 mAb (clone BC96, Biologend, San Diego, CA, USA).

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

NOG mice were i.p. injected with 1×10^6 EJ-1 cells. Four weeks later, the mice were i.p. injected with 1×10^7 V γ 2V δ 2 T cells with or without 1 μ g 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 μ g of total RNA using SuperScript III reverse transcriptase kit and random hexamers

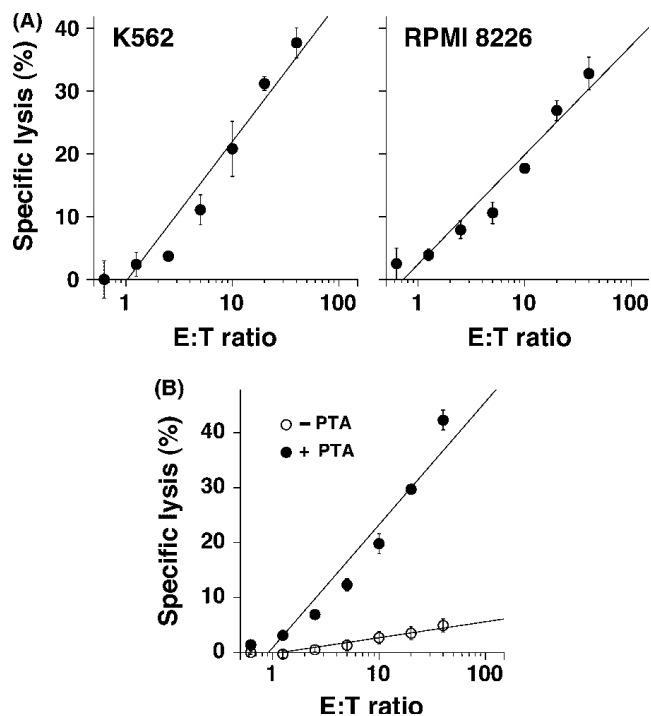


FIGURE 5 Cytotoxic activity of PTA-expanded $V_{\gamma}2V_{\delta}2$ T cells against tumor cells. A, Cytotoxicity of PTA-expanded $V_{\gamma}2V_{\delta}2$ T cells against K562 and RPMI 8226 tumor cells. K562 erythroleukemia cells (right panel) and RPMI 8226 plasmacytoma cells (left panel) were labeled with a europium-chelate-forming procompound, washed, and then incubated with PTA-expanded $V_{\gamma}2V_{\delta}2$ T cells at effector to target ratios of 0, 0.625, 1.25, 2.5, 5, 10, 20 and 40 for 40 min at 37°C in a 5% CO_2 incubator. The culture supernatants were combined with an europium solution and the levels of the europium-chelate complex determined by measuring time-resolved fluorescence. Specific lysis (%) was calculated as [(experimental release – spontaneous release)/(maximum release – spontaneous release)] \times 100. All assays were performed in triplicate. B, Cytotoxicity of PTA-expanded $V_{\gamma}2V_{\delta}2$ T cells against PTA-pulsed U937 histiocytic lymphoma cells. U937 cells were either not treated (open circles) or treated with 500 nM PTA (solid circles) at 37°C with 5% CO_2 for 2 h followed by labeling and testing for specific lysis as detailed in (A)

(Invitrogen, Carlsbad, CA, USA). Then, RT-PCR was performed using a 0.2 μ M concentration of both forward and reverse primers and iTaq SYBR Green Supermix with ROX (Bio Rad, Hercules, CA, USA). The quantity of each cDNA was normalized by GAPDH. The following PCR primers were used for amplification: IFN- γ , 5'-TGACCAGAGCATCCAAAAGA-3' and 5'-CTCTTCGACCTCGAAA CAGC-3'; GAPDH, 5'-CGACCACTTTGTCAAGCTCA -3' and 5'-AGG GGAGATTCAGTGTGGTG -3'.

2.13 | IFN- γ production by adoptively transferred $V_{\gamma}2V_{\delta}2$ T cells stimulated in vivo

NOG mice were i.p. injected with 1×10^6 EJ-1 cells. Four weeks later the mice were further i.p. injected with 1×10^7 $V_{\gamma}2V_{\delta}2$ T cells with or without PTA. Peripheral blood was taken from the mice 4 h

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 (TM β CD) 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 TM β CD. Importantly, PTA solubilized in TM β CD/ethanol exhibited identical bioactivity to PTA solubilized in DMSO. For example, PTA in TM β CD/ethanol inhibited the proliferation of the EJ-1 bladder carcinoma and the U937 histiocytoma cell lines identically to PTA in DMSO (Figure S1C,D). Thus, TM β CD/ethanol can be used as the solvent for PTA, as was done in this study.

3.2 | Stimulation of $V_{\gamma}2V_{\delta}2$ T cells by PTA

PTA potently inhibits the growth of tumor cell lines in vitro³² and selectively activates $V_{\gamma}2V_{\delta}2$ 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 $V_{\gamma}2V_{\delta}2$ T cell-dependent cell aggregation at concentrations of approximately 10 μ M, whereas PTA caused aggregation at approximately 0.1 μ M (Figure 1B). Similarly, PTA stimulated TNF- α production by $V_{\gamma}2V_{\delta}2$ 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 $V_{\gamma}2V_{\delta}2$ T cells in PBMC.

3.3 | Ex vivo expansion by PTA of $V_{\gamma}2V_{\delta}2$ T cells for adoptive immunotherapy

To determine whether PTA can be used to expand $V_{\gamma}2V_{\delta}2$ T cells for adoptive immunotherapy, PMBC from cancer patients and from healthy donors were stimulated with PTA and IL-2 and the resulting $V_{\gamma}2V_{\delta}2$ T cells were assessed for purity and biological activity after 10 days. Similar to what we have previously

V δ 2 T cells in NOG mouse blood

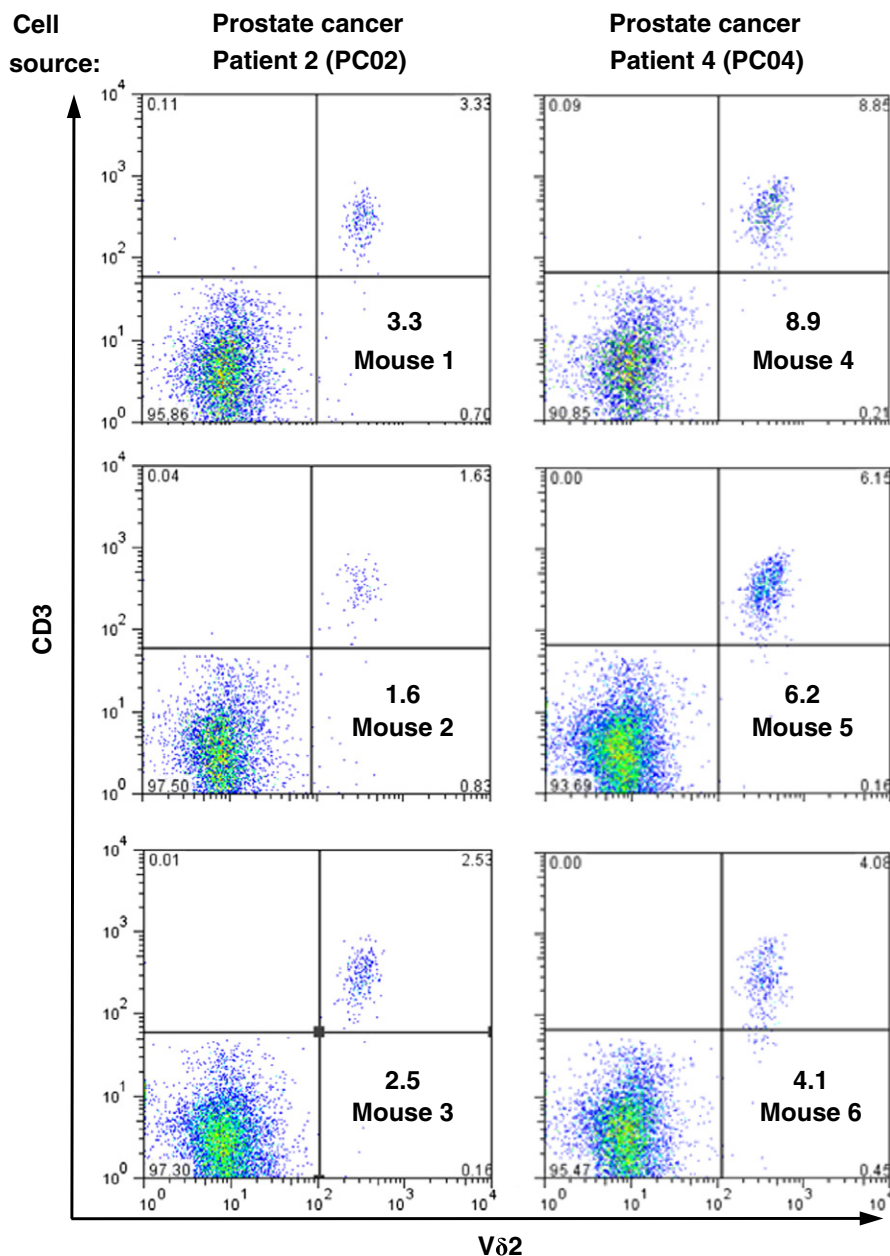


FIGURE 6 Adoptively transferred human V γ 2V δ 2 T cells from prostate cancer patients persist in the blood of NOG mice. V γ 2V δ 2 T cells (>98%) were expanded from prostate cancer patient 2 (PC02) (left panels) and patient 4 (PC04) (right panels) and frozen for later use. 5×10^7 thawed V γ 2V δ 2 T 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 δ 2 TCR and human CD3. Values shown are % of total cells. Mean \pm SD PC02 2.5% \pm 0.9%, n = 3 PC04 6.4% \pm 2.4%, n = 3

shown for Zol,³⁶ 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 γ 2V δ 2 T cell expansion was maximal at a PTA concentration of 1 μ M, while a PTA concentration of 2 μ M was highly toxic (Figure 2A). PTA stimulation resulted in highly enriched populations of V γ 2V δ 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 γ 2V δ 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 γ 2V δ 2 T cell population as compared with Zol stimulation (95.6 \pm 5.7% of

total cells vs 90.3 \pm 10.0%, $P = .0078$, Figure 3A). All donors exhibited higher enrichment with PTA compared to Zol. In addition, the number of V γ 2V δ 2 T cells was 20% higher on average for stimulation with PTA as compared with Zol ($6.3 \times 10^8 \pm 5.5 \times 10^8$ cells with PTA vs $5.7 \times 10^8 \pm 4.3 \times 10^8$ 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 γ 2V δ 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 γ 2V δ 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 γ 2V δ 2 T cells in PBMC from

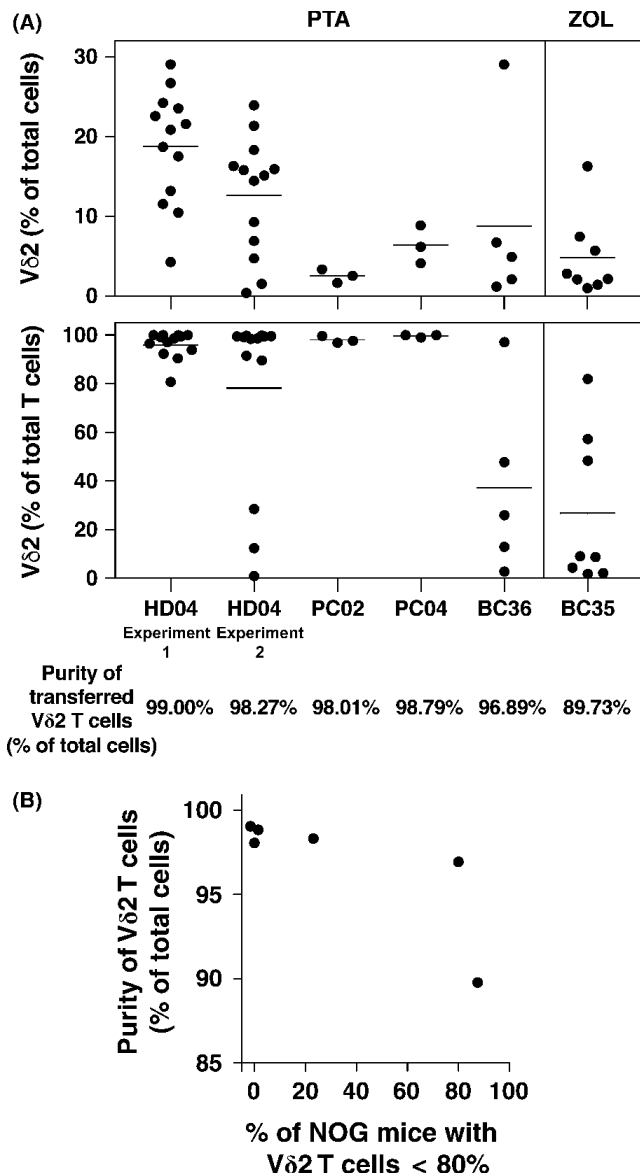


FIGURE 7 Purity of $V\gamma 2V\delta 2$ T cells helps to determine their engraftment success after adoptive transfer in NOG mice. A, Summary of the results of NOG mouse engraftment with expanded $V\gamma 2V\delta 2$ T cells. Top panel: Levels of $V\gamma 2V\delta 2$ T cells as a percentage of total cells for each transfer experiment. Bottom panel: Levels of $V\gamma 2V\delta 2$ T cells as a percentage of T cells for each transfer experiment. Each point represents 1 NOG mouse. $V\gamma 2V\delta 2$ T cells were expanded using PTA except for BC35 that was expanded using Zol. Thawed $V\gamma 2V\delta 2$ T cells were used for cells derived from cancer patients whereas freshly expanded $V\gamma 2V\delta 2$ T cells were used for the healthy donor. The purity of the starting populations as a percent of total cells is listed at the bottom. B, Purity of the starting population of $V\gamma 2V\delta 2$ T cells helps to determine engraftment success. The purity of the transferred $V\gamma 2V\delta 2$ T cells is correlated with the percentage of NOG mice with predominant $V\gamma 2V\delta 2$ T cell populations (>80%) at 2 weeks. Each data point represents 1 transfer experiment

cancer patients results in $V\gamma 2V\delta 2$ T cell populations that were of significantly higher purity and slightly higher number than when Zol was used for stimulation.

3.4 | Cytotoxic activity of $V\gamma 2V\delta 2$ T cells expanded by PTA

Adult $V\gamma 2V\delta 2$ T cells are highly cytotoxic⁴⁰ and exhibit cytotoxicity for most tumor cell lines.^{8,33} $V\gamma 2V\delta 2$ 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 $V\gamma 2V\delta 2$ 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 $V\gamma 2V\delta 2$ T cells also lysed the RPMI 8226 plasmacytoma cell line that is directly stimulatory for $V\gamma 2V\delta 2$ T cells through a TCR-dependent mechanism (Figure 5A).^{17,41} Finally, PTA-expanded $V\gamma 2V\delta 2$ T cells efficiently lysed U937 tumor cells that had been treated with PTA (Figure 5B). Therefore, expansion of $V\gamma 2V\delta 2$ T cells by PTA preserves their cytotoxic activity.

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

To assess their potential use for cancer immunotherapy, $V\gamma 2V\delta 2$ T cells expanded by PTA were adoptively transferred into immunodeficient NOG mice. $V\gamma 2V\delta 2$ 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% $V\gamma 2V\delta 2$ T cells, whereas mice receiving cells from prostate cancer patient 4 (PC04) averaged 6.4% $V\gamma 2V\delta 2$ T cells of total cells (Figure 6). Similarly, NOG mice receiving $V\gamma 2V\delta 2$ 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 $V\gamma 2V\delta 2$ T cells observed with the normal donor were likely due to the transfer of freshly expanded $V\gamma 2V\delta 2$ T cells instead of the previously frozen cells used from prostate and breast cancer patients.

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

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

To assess the functional capabilities of $V\gamma 2V\delta 2$ T cells expanded by PTA in vivo, we tested various immunological functions of the cells

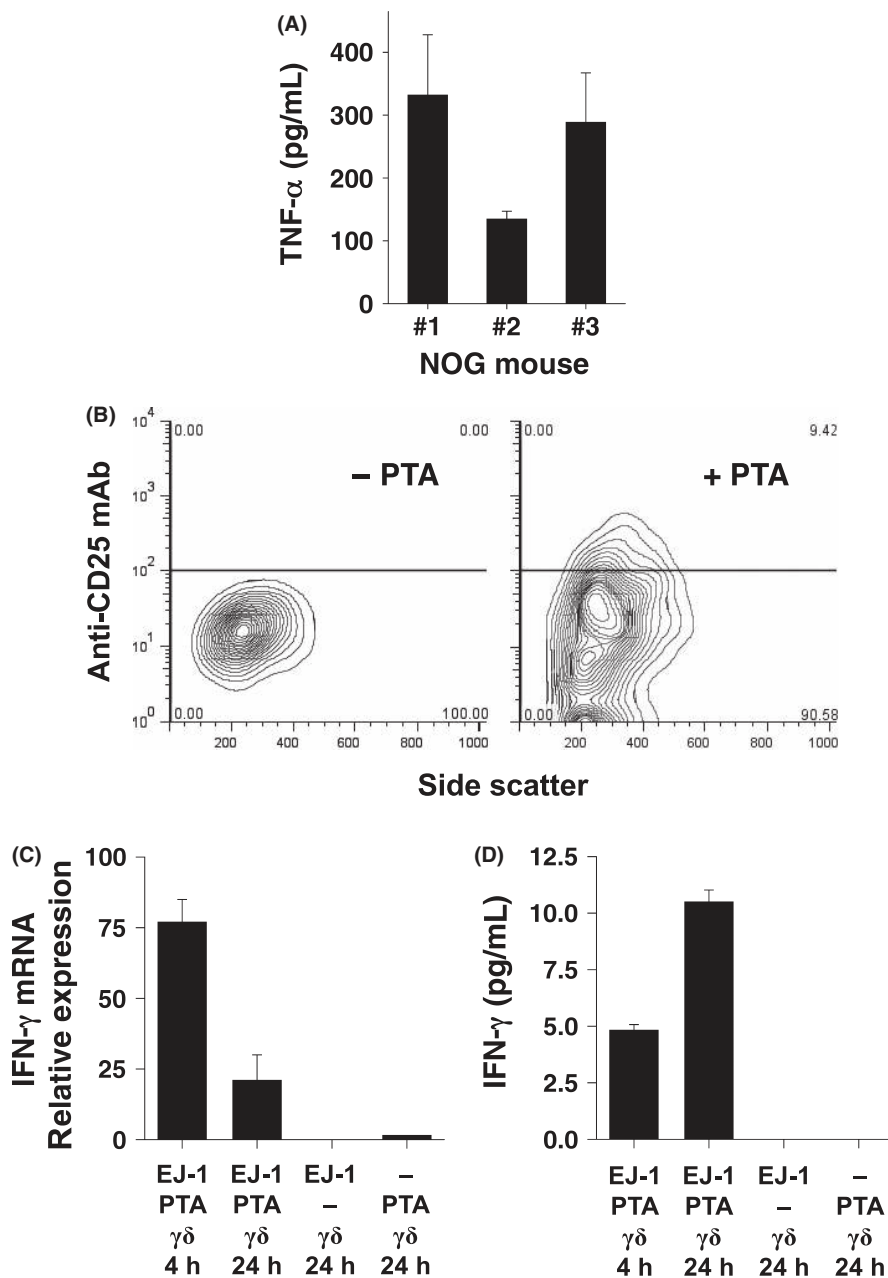


FIGURE 8 Functional analyses of V γ 2V δ 2 T cells adoptively transferred into NOG mice. A, TNF- α production by adoptively transferred V γ 2V δ 2 T cells from NOG mice. Three NOG mice were each i.p. injected with 2×10^7 V γ 2V δ 2 T cells derived from a healthy donor. Two weeks later, peripheral blood cells were collected and V γ 2V δ 2 T cells stimulated by HMBPP in vitro. After 24 h of incubation, culture supernatants were collected and TNF- α levels assessed in triplicate. B, Expression of CD25 on V γ 2V δ 2 T cells adoptively transferred into NOG mice bearing EJ-1 bladder cancer tumors treated with PTA. NOG mice were i.p. injected with EJ-1 tumor cells. Four weeks later, the mice were i.p. injected with 1×10^7 V γ 2V δ 2 T cells with or without PTA. V γ 2V δ 2 T cells were harvested 24 h later and CD25 expression assessed by flow cytometry. C, IFN- γ mRNA expression by V γ 2V δ 2 T cells adoptively transferred into NOG mice bearing EJ-1 tumors treated with PTA. NOG mice were i.p. injected with EJ-1 tumor cells. Four weeks later, the mice were i.p. injected with 1×10^7 V γ 2V δ 2 T cells with or without PTA or PTA alone. Peripheral blood samples were obtained 4 h and 24 h after the challenge and IFN- γ mRNA levels determined by quantitative PCR. D, IFN- γ production by V γ 2V δ 2 T cells adoptively transferred into NOG mice bearing EJ-1 tumors treated with PTA. NOG mice were treated as described in (C). Blood samples were collected 4 h and 24 h after PTA injection, serum prepared and serum IFN- γ levels measured by ELISA

after their adoptive transfer to immunodeficient NOG mice. First, recovered V γ 2V 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 γ 2V 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.^{11,42}

Second, we examined the ability of transferred V γ 2V 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 γ 2V δ 2 T cells with or without PTA and V γ 2V δ 2 T cells recovered from the mice 4 h and 24 h later. After 24 h, V γ 2V δ 2 T cells upregulated expression of the CD25 IL-2 α 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 γ 2V 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.⁴³ IFN- γ production was dependent on V γ 2V δ 2 T cells given that NOG mice bearing EJ-1 cancer cells with PTA but no V γ 2V 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 γ 2V 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.

4 | DISCUSSION

In the present study, we show that PTA, a novel bisphosphonate prodrug, expands peripheral blood V γ 2V δ 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 γ 2V δ 2 T cells for use in adoptive immunotherapy for cancer. These expanded V γ 2V δ 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 β CD. TM β CD is highly soluble in both water and ethanol and its parent compound, β CD, has been used for drug delivery. The less hydrophilic interiors of these compounds allow them to form complexes with hydrophobic drugs. Accordingly, TM β CD solubilizes PTA in ethanol. This TM β CD/ethanol solubilization method could be applicable to other hydrophobic therapeutics, such as alkoxymethyl derivatives of anionic compounds. PTA dissolved in TM β CD/ethanol exhibited identical biological activity to PTA dissolved in DMSO, suggesting that PTA preparation in TM β CD/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 γ 2V δ 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 γ 2V δ 2 T cells were functionally active, as evidenced by the expression of TNF- α , CD25 and IFN- γ in response to PTA-sensitized tumor cells or HMBPP stimulation. Although IL-2 is absolutely required for the maintenance of V γ 2V δ 2 T cells in vitro, these findings suggest that IL-2 is not absolutely necessary in adoptive immunotherapy with V γ 2V δ 2 T cells. Thus, IL-2 infusion might be omitted or reduced in patients undergoing adoptive cancer immunotherapy with V γ 2V δ 2 T cells.

Using PTA to expand V γ 2V δ 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 γ 2V δ 2 T cells that were of significantly higher purity than those expanded by Zol. In many cases, V γ 2V δ 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.³⁶ The ability to generate highly enriched V γ 2V δ 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 γ 2V δ 2 T cells through competition with $\alpha\beta$ T cells. Our findings suggest that V γ 2V δ 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 γ 2V δ 2 T cells as a starting population would ensure that purification would give highly enriched V γ 2V δ 2 T cells with few $\alpha\beta$ T cells.

Another potential use of such highly enriched populations would be allogeneic adoptive transfer of V γ 2V δ 2 T cells for tumor immunotherapy. Thus far, all clinical trials have used syngeneic V γ 2V δ 2 T cells. However, because V γ 2V δ 2 T cells are not alloreactive and because the BTN3A1 is not polymorphic,⁴⁶ V γ 2V δ 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 γ 2V δ 2 T cells will not cause graft-versus-host disease. Note that in natural infections, very high numbers of V γ 2V δ 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 γ 2V δ 2 T cells preparations without contaminating $\alpha\beta$ 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 leukaphoresed 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 γ 2V δ 2 T cells could be switched. If necessary, $\alpha\beta$ T cells could be depleted using anti- $\alpha\beta$ 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 γ 2V δ 2 T cells that can be banked and used as “off-the-shelf” reagents could greatly facilitate the development of adoptive V γ 2V δ 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.

ORCID

Yoshimasa Tanaka  <http://orcid.org/0000-0002-5024-0614>

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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