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Large-scale expansion of Vγ9Vδ2 T cells with engineered K562 feeder cells in G-Rex vessels and their use as chimeric antigen receptor–modified effector cells

LIN XIAO[1,*], CAN CHEN[2,*], ZHENDONG LI[1], SUMIN ZHU[1], JOHAN CK TAY[1], XI ZHANG[1], SHIJUN ZHA[1], JIEMING ZENG[3], WEE KIAT TAN[2], XIN LIU[4], WEE JOO CHNG[4,5,6] & SHU WANG[1,3]

[1]Department of Biological Sciences, National University of Singapore, Singapore, [2]Tessa Therapeutics, Pte Ltd., Singapore, [3]Institute of Bioengineering and Nanotechnology, Singapore, [4]Department of Haematology-Oncology, National University Cancer Institute Singapore, National University Health System, Singapore, [5]Cancer Science Institute of Singapore, National University of Singapore, Singapore, and [6]Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Abstract

Vγ9Vδ2 T cells are a minor subset of lymphocytes in the peripheral blood that has been extensively investigated for their tolerability, safety and anticancer efficacy. A hindrance to the broad application of these cells for adoptive cellular immunotherapy has been attaining clinically appropriate numbers of Vγ9Vδ2 T cells. Furthermore, Vγ9Vδ2 T cells exist at low frequencies among cancer patients. We, therefore, sought to conceive an economical method that allows for a quick and robust large-scale expansion of Vγ9Vδ2 T cells. A two-step protocol was developed, in which peripheral blood mononuclear cells (PBMCs) from healthy donors or cancer patients were activated with Zometa and interleukin (IL)-2, followed by co-culturing with gamma-irradiated, CD64-, CD86- and CD137L-expressing K562 artificial antigen-presenting cells (aAPCs) in the presence of the anti-CD3 antibody OKT3. We optimized the co-culture ratio of K562 aAPCs to immune cells, and migrated this method to a G-Rex cell growth platform to derive clinically relevant cell numbers in a Good Manufacturing Practice (GMP)-compliant manner. We further include a depletion step to selectively remove αβ T lymphocytes. The method exhibited high expansion folds and a specific enrichment of Vγ9Vδ2 T cells. Expanded Vγ9Vδ2 T cells displayed an effector memory phenotype with a concomitant down-regulated expression of inhibitory immune checkpoint receptors. Finally, we ascertained the cytotoxic activity of these expanded cells by using nonmodified and chimeric antigen receptor (CAR)–engrafted Vγ9Vδ2 T cells against a panel of solid tumor cells. Overall, we report an efficient approach to generate highly functional Vγ9Vδ2 T cells in massive numbers suitable for clinical application in an allogeneic setting.

Key Words: *antibody-dependent cell-mediated cytotoxicity, artificial antigen-presenting cells, chimeric antigen receptor, gamma delta T cells*

Introduction

Vγ9Vδ2 T cells are a subset of γδ T cells that make up approximately 0.5–5% of peripheral blood T cells [1]. Unlike classical αβ T cells, Vγ9Vδ2 T cells express Vγ9 and Vδ2 of T-cell receptor (TCR) chains that recognize and interact with antigens in a major histocompatibility complex (MHC)–independent fashion. γδ TCRs can be activated by a set of tumor-associated antigens, including phosphoantigens that are produced during metabolic dysregulation in tumor cells, lipids presented by CD1 family members and cell stress markers [2–4]. Like innate natural killer (NK) lymphocytes, γδ T cells also express the NKG2D receptor and killer-cell immunoglobulin-like receptors (KIRs) that can play either co-stimulatory or inhibitory roles. As such, γδ T cells are considered to play important roles in immune surveillance against

*These two authors contributed equally to the work.

Correspondence: **Shu Wang**, PhD, Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117543, Singapore. E-mail: dbsws@nus.edu.sg

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tumors. Importantly for cancer treatment, γδ T cells are capable of infiltrating a range of human solid tumors, including renal, bladder, ovarian, colorectal, breast and nasopharyngeal carcinomas, to interact with and kill cancer cells [5].

Within the larger peripheral blood mononuclear cell (PBMC) population, the Vγ9Vδ2 T-cell subset can be specifically expanded with the treatment of phosphoantigens such as isopentenyl pyrophosphate (IPP) or its synthetic analogue of bromohydrin pyrophosphate (BrHPP). Zoledronate or zoledronic acid (Zometa), a Food and Drug Administration (FDA)-approved and commercially available bisphosphonate drug, which has been used to treat patients with postmenopausal osteoporosis, has also been used to artificially raise the intracellular levels of IPP by inhibiting farnesyl pyrophosphate synthase (FPPS), an enzyme acting downstream of IPP in the mevalonate pathway [2–4]. Initial Zometa/Vγ9Vδ2 T-cell immunotherapy used an *in vivo* expansion approach through direct administration of Zometa into patients. Vγ9Vδ2 T cells have also been tested for adoptive cancer immunotherapy, a fast-developing field that involves the isolation of immune cells, *ex vivo* cell expansion and re-infusion of the expanded lymphocytes into patients to treat cancer. The expansion of Vγ9Vδ2 T cells *ex vivo* with Zometa together with cytokines allows for the reproducible generation of large numbers of effector cells, the optimization and control of cell population purity and the maintenance and augmentation of cell function and survival. In several early-phase clinical trials of adoptive immunotherapy for cancer, treatments with Zometa-expanded peripheral blood Vγ9Vδ2 T cells are very well tolerated and have yielded some encouraging positive clinical outcomes [5–10]. A meta-analysis of gene expression signatures of a panel of 39 tumor types has identified the presence of intratumoral γδ T cells as the most significant indicator for favorable prognosis that is positively correlated with patient survival [11].

Advances in adoptive immunotherapy with Vγ9Vδ2 T cells can be improved significantly through developing methods for large-scale, clinically relevant cell expansion over a short period of time. There have been major advances in this regard over the last several years [12–16]. Artificial antigen-presenting cells (aAPCs) generated through genetic engineering of human erythroleukemia cell line K562 have been used to support robust *ex vivo* expansion of several types of immune cells [17,18]. Deniger *et al.* [19] have reported the use of K562-derived aAPCs that express membrane-*bound IL* (mIL)-15, CD86 and CD137L for polyclonal expansions of heterogeneous gamma-delta T subsets after the isolation of γδ T cells from PBMCs. A recent publication reports the use of K562-aAPCs expressing CD83, CD137L and CD32 for the expansion of Vγ9Vδ2 T cells freshly isolated from PBMCs [20]. In this respect, we have developed another K562 line expressing CD64, CD86 and CD137L and recently reported the use of the K562-aAPCs for the co-expansion of cytokine-induced killer cells and Vγ9Vδ2 T cells [21].

In the current study, we focused on the use of K562 aAPCs in G-Rex cell culture vessels for Vγ9Vδ2 T-cell expansion. A two-step protocol was developed in which PBMCs were first activated with Zometa and interleukin (IL)-2 for 7 days and the activated cells were then numerically expanded by co-culturing with K562-aAPCs, anti-human CD3 monoclonal antibody OKT3, Zometa and IL-2 in G-Rex cell culture vessels for 10 days. The Good Manufacturing Practice (GMP)-compliant G-Rex vessels contain a gas-permeable membrane at the base and are unique in supporting large media volumes without compromising gas exchange [22–30]. Our results demonstrate that the new method enables the generation, from relatively small amounts of peripheral blood cells, of large clinically relevant quantities of functional Vγ9Vδ2 T cells in 17 days and the expanded Vγ9Vδ2 T cells are suitable for use as either unmodified or chimeric antigen receptor (CAR)–engrafted effector cells.

Results

Ex vivo *expansion of Vγ9Vδ2 T cells with a two-step protocol*

Vγ9Vδ2 T cells are commonly cultured and expanded via stimulation with Zometa. In our two-step protocol (Figure 1A), PBMCs were first treated with Zometa in combination with IL-2 in a serum-supplemented growth medium for 7 days to enrich Vγ9Vδ2 T cells. Although the total number of cells did not increase significantly during this 7-day period, the Vγ9Vδ2 T-cell population could increase from an initial 1–5% in the original untreated PBMCs (day 0) to 70–80% after Zometa treatment for 7 days. In the second step, Zometa-treated cells were mixed with irradiated K562-aAPCs stably expressing three genes that encode the high affinity Fc receptor CD64 and co-stimulatory molecule ligands CD86 and CD137L (4-1BBL) [21] (Supplementary Figure S1). This co-culture was performed for 10 days with the serum-supplemented medium containing OKT3, Zometa and IL-2.

In trying to define an optimized K562-aAPC co-culture condition, we tested the effects of different cell ratios of immune cells to K562-aAPCs in the second step, from 1:0 to 1:200, on Vγ9Vδ2 T-cell expansion in cell culture flasks. While the cells that were continually treated with Zometa for 10 days, without adding K562-aAPCs (ratio = 1:0), were expanded on an average of 72-fold, co-culturing with K562-aAPCs provided expansion folds from 376-fold at 1:2 ratio to

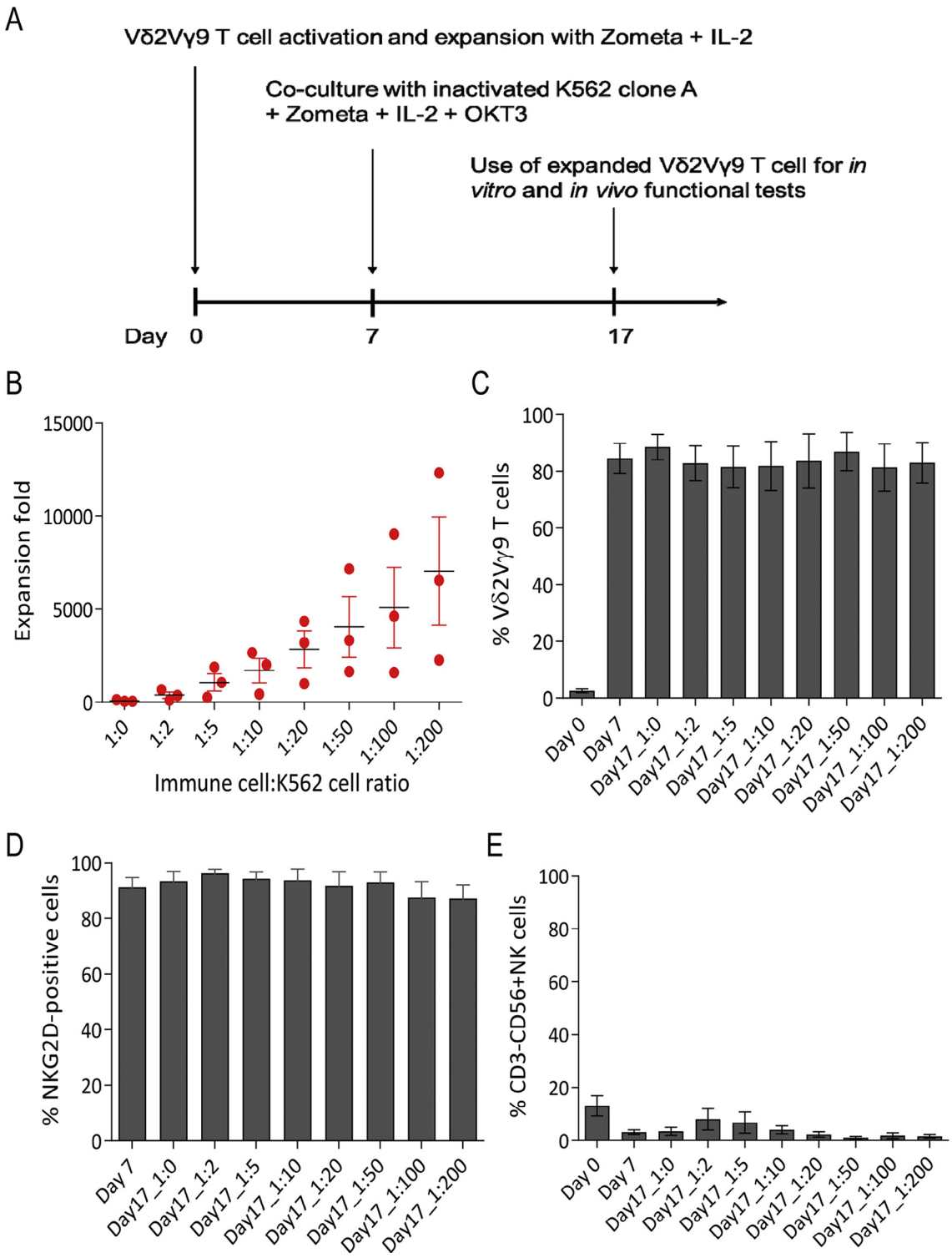

Figure 1. Optimization of Vγ9Vδ2 T-cell expansion protocol. (A) Schematic illustration of γδ T-cell expansion using Zometa treatment and K562 aAPC Clone A co-culturing. PBMCs were activated for 7 days with 5 μmol/L Zometa and 300 IU/mL IL-2, followed by co-culturing with inactivated K562 Clone A cells in G-Rex with AIM-V supplemented with 1% human plasma for another 10 days. Five μmol/L Zometa, 60 ng/mL OKT3 and 300 IU/mL IL-2 were added on day 7 and 300 IU/mL IL-2 were refreshed every 2–3 days during the co-culture. (B) Effects of the ratios between immune cells and K562 Clone A cells (from 1:2 to 1:200) on γδ T-cell expansion. Starting with 2×10^6 PBMCs at day 0, the total expansion folds at day 17 are shown. The data from three healthy donors were collected and shown as mean ± SEM. (C) Flow cytometric analysis to demonstrate the Vγ9Vδ2 T-cell frequency on day 17, as compared with the original untreated PBMCs (Day 0). (D) Flow cytometric analysis to demonstrate the NKG2D-positive cell frequency on day 17. (E) Flow cytometry analysis to demonstrate the CD3-CD56$^+$NK cell frequency on day 17. Day17_Ctrl in B, C, D and E: γδ T-cell expansion without K562 Clone A aAPCs.

Table I. Expression of Vγ9Vδ2 T cells in G-Rex[a].

ID no.	Vγ9Vδ2 T cell: initial percentage	Vγ9Vδ2 T cell: purity after expansion	Total cell expansion fold	Vγ9Vδ2 T-cell expansion fold	Serum supplemented
ID804, healthy	1.8%	86.7%	3885	187,040	1% plasma
ID805, healthy	0.2%	84.6%	2838	1,195,671	1% plasma
ID857, healthy	1.2%	93.3%	2448	191,017	1% plasma
ID920, healthy	1.5%	93.8%	7898	496,089	1% plasma
ID1832, healthy	6.8%	85.6%	2770	88, 16	1% plasma
ID975, healthy	1.3%	93.2%	3484	43, 33	1% plasma
ID976, healthy	3.3%	89.5%	5915	439,615	1% plasma
ID337, healthy	7.9%	82.7%	9701	101,554	1% plasma
ID390, healthy	3.2%	93.1%	2602	75 702	1% plasma
ID0606-01, lymphoma	0.4%	93.3%	9062	2,113,712	5% AB
ID0612, lymphoma	1.7%	71%	8906	371,956	5% AB
ID0518, lymphoma	0.8%	87.6%	10 532	1,153,254	5% AB +1% plasma[b]
ID0610, Lymphoma	2.7%	92%	15 480	527,467	5% AB +1% plasma[b]
Mean	2.52%	88.18%	6579	537,340	
± SD	± 2.36%	± 6.44%	± 4047	± 607,389	

ID, identification; AB, human serum from type AB donors.
[a]ID804, 805, 857 and 920 were expanded in G-Rex10 and the rest of the samples were in G-Rex100. PBMCs were treated with Zometa and expanded with inactivated K562-aAPCs as illustrated in Figure 1A.
[b]ID0518 and ID0610 were cultured with 5% AB serum during PBMC activation and with 1% plasma during co-culture.

7042-fold at 1:200 ratio (Figure 1B). The immune cell and K562-aAPC ratios did not affect the frequencies of expanded Vγ9Vδ2 T cells, which remained around 80% across all the tested ratios (Figure 1C). The varying ratios also did not affect the expression of NKG2D, an activating receptor of Vγ9Vδ2 T cells, which reached 90% by the end of the co-culture (Figure 1D). When the CD3-CD56+ natural killer (NK) cell population was examined, we observed that the frequency of NK cells decreased from 8% at 1:2 ratio to 1.6% at 1:200 ratio (Figure 1E), possibly caused by the significant increase of the Vγ9Vδ2 T-cell population. Since our K562-aAPCs express green fluorescent protein (GFP), we examined residue K562-aAPCs with flow cytometric analysis after the expansion and observed that K562-aAPCs were totally eliminated after co-culture for 10 days at the cell ratio of 1:100 (Supplementary Figure S2), but very low percentages of residual K562-aAPCs (0.1–0.4%) were still detected when the cell ratio of 1:200 was used. Taking into account the various factors of Vγ9Vδ2 T-cell expansion fold, the residual K562-aAPCs and the cost of preparing the aAPCs, the cell ratio of 1:100 was used for the following expansion experiments in G-Rex.

The G-Rex cell culture vessels allow gas exchange across the base of the culture, thus supporting higher cell number per unit surface area and increased cell survival and expansion rates. The G-Rex vessels have been tested for the expansion of several types of immune cells [22–30], but not for the *ex vivo* expansion of γδ T cells. We tested 13 human PBMCs samples in G-Rex and the results are summarized in Table I. With nine PBMC samples from healthy donors and when the cell culture medium was supplemented with 1% plasma, the total cell expansion was 4616 ± 2632 (mean ± standard deviation [SD])–fold and the Vγ9Vδ2 T-cell purity achieved was 89.17 ± 4.36. Because the initial frequencies of Vγ9Vδ2 T cells varied from 0.2–7.9% in the nine samples, the expansion folds of Vγ9Vδ2 T cells ranged from 43 933 to 1,195,671 (median value, 187,040). It is well described that expansion of Vγ9Vδ2 T cells from the blood samples of patients with cancer is more difficult than from donors [31–34]. When we initially tested 1% plasma for three blood samples collected from patients with lymphoma, Vγ9Vδ2 T cells failed to expand effectively with our method. We then increased serum concentration to 5% and used AB serum. Vγ9Vδ2 T cells from all four tested blood samples collected from patients with lymphoma expanded massively (Table I). We obtained the total cell expansion fold of 10 995 ± 3078 for these cancer patient samples, yet the purity remained at 85.98% ± 10.28%. Thus, with our method and using G-Rex flasks, Vγ9Vδ2 T cells can easily be expanded to give clinically relevant numbers of cells (>1E9).

Phenotyping of the expanded Vγ9Vδ2 T cells

To examine the features of Vγ9Vδ2 T cells generated with our method in G-Rex, we conducted phenotyping analysis of Vγ9Vδ2 T cells collected during the expansion on day 0, 7 and 17. We observed that Vγ9Vδ2 T, with an initial frequency of approximately 2.3%, could be enriched to 87% after Zometa treatment for 7 days and the frequency increased slightly to 89% by day 17, after the 10-day co-culture in G-Rex (Figure 2A). On the contrary, the NK population decreased from an initial 20% in PBMCs to 4.6% by

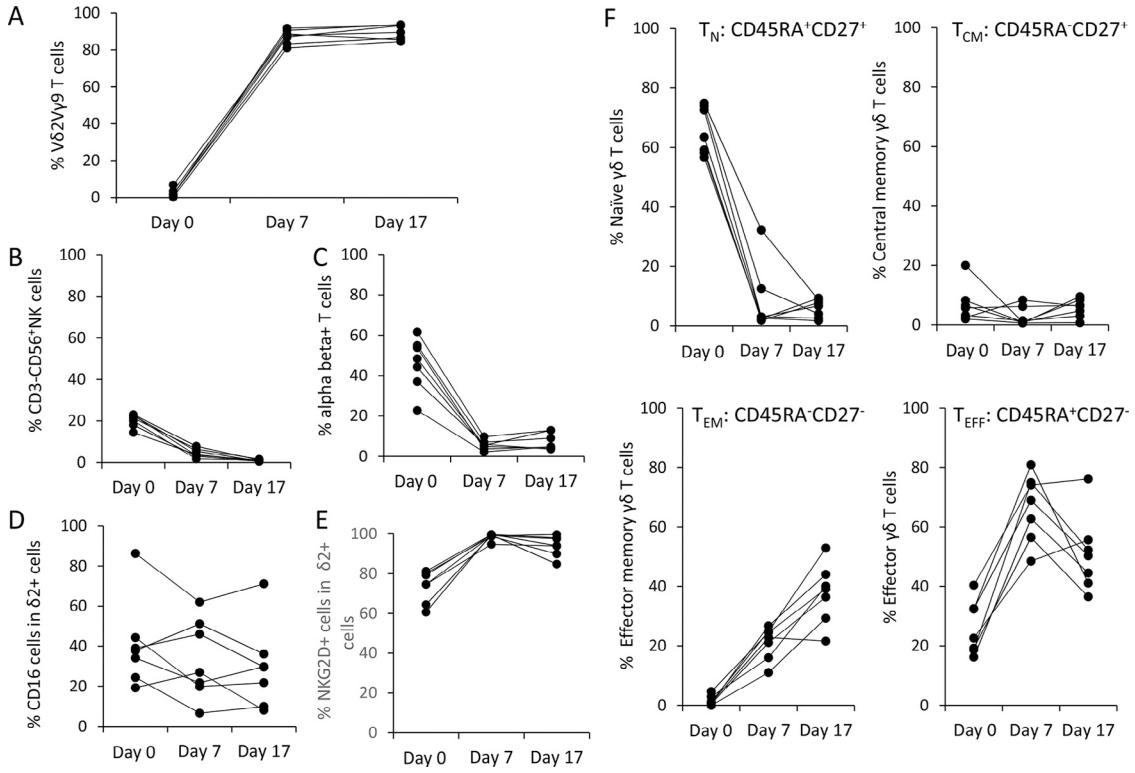

Figure 2. Phenotyping of Vγ9Vδ2 T cells expanded in G-Rex vessels. PBMCs (day 0) from 7 healthy donors listed in Table I and the derived cells on day 7 and day 17 of culture were analyzed. Each donor is represented by one line. (A) % Vγ9Vδ2 T cells. (B) % $CD3^-CD56^+$NK cells. (C) % αβ T cells. (D) % $CD16^+$ cells in $\delta2^+$ cells. (E) % $NKG2D^+$ cells in $\delta2^+$ cells. (F) Naïve, central memory, effector memory and terminally differentiated effector subsets during Vγ9Vδ2 T-cell expansion. T cells were gated for the presence of $CD45RA^+CD27^+$ naive, $CD45RA^-CD27^+$ central memory, $CD45RA^-CD27^-$ effector memory and $CD45RA^+CD27^-$ terminally differentiated effector subsets.

day 7 and to 1.0% by day 17, whereas the αβ T-cell population decreased from an initial 46% in PBMCs to 5.6% by day 7 and then slightly increased to 7.4% by day 17 (Figure 2B and 2C). Putting these results together with those in Table I, we concluded that Zometa treatment contributed to the significant increase of Vγ9Vδ2 T-cell frequency, while co-culturing with K562 aAPCs was responsible for massive expansion of Vγ9Vδ2 T cells.

CD16 is a low-affinity Fc receptor that is mainly expressed on NK cells and involved in mediating the antibody-dependent cell-mediated cytotoxicity (ADCC). Vγ9Vδ2 T cells were reported to be associated with a diverse expression pattern of CD16 [8]. Consistently, we observed that CD16 expression varied from 19–86% of δ2+ cells on day 0 across different donors. Zometa treatment and co-culturing with K562 aAPCs did not provide a consistent pattern of influencing CD16 expression (Figure 2D). NKG2D is another activating receptor expressed on NK cells and γδ T cells. On day 0, 74% of δ2+T cells were NKG2D-positive and the frequency increased to 98.6% by day 7 and 94% by day 17 (Figure 2E). Because Vγ9Vδ2 T cells can also function as antigen-presenting cells (APCs) [35], we examined the expression of APC markers (CD80, CD86, HLA-DR and CD40). As showed in Supplemental Figure S3, the percentages of the CD86- and HLA-DR-positive cells were 2.5% and 10% of δ2 T cells, respectively, on day 0. Their expression levels increased to 99% after Zometa treatment for 7 days and remained the same after co-culturing with K562 aAPCs.

Like αβ T cells, Vγ9Vδ2 T cells also exhibit different memory phenotypes. We used the cell surface memory phenotyping markers CD45RA and CD27 to determine naïve (CD45RA+CD27+, T_N), central memory (CD45RA-CD27+, T_{CM}), effector memory (CD45RA-CD27-, T_{EM}) and terminally differentiated effector (CD45RA+CD27-, T_{EFF}) Vγ9Vδ2 T cells before and after expansion (Figure 2F). On day 0, the majority (around 65.5%) of Vγ9Vδ2 T cells showed naïve phenotype, with only 6.8%, 1.5% and 26% of which being central memory, effector memory and terminally differentiated effector cells, respectively. Zometa treatment for 7 days dramatically decreased the proportion of naïve T cells; the percentage of T_N cells decreased to 8.2%. Meanwhile the percentage of effector memory and effector cells increased to 21% and 66.6%, respectively. Interestingly, co-culturing with K562 aAPCs in G-Rex promoted phenotypic changes: the T_{EFF} cell

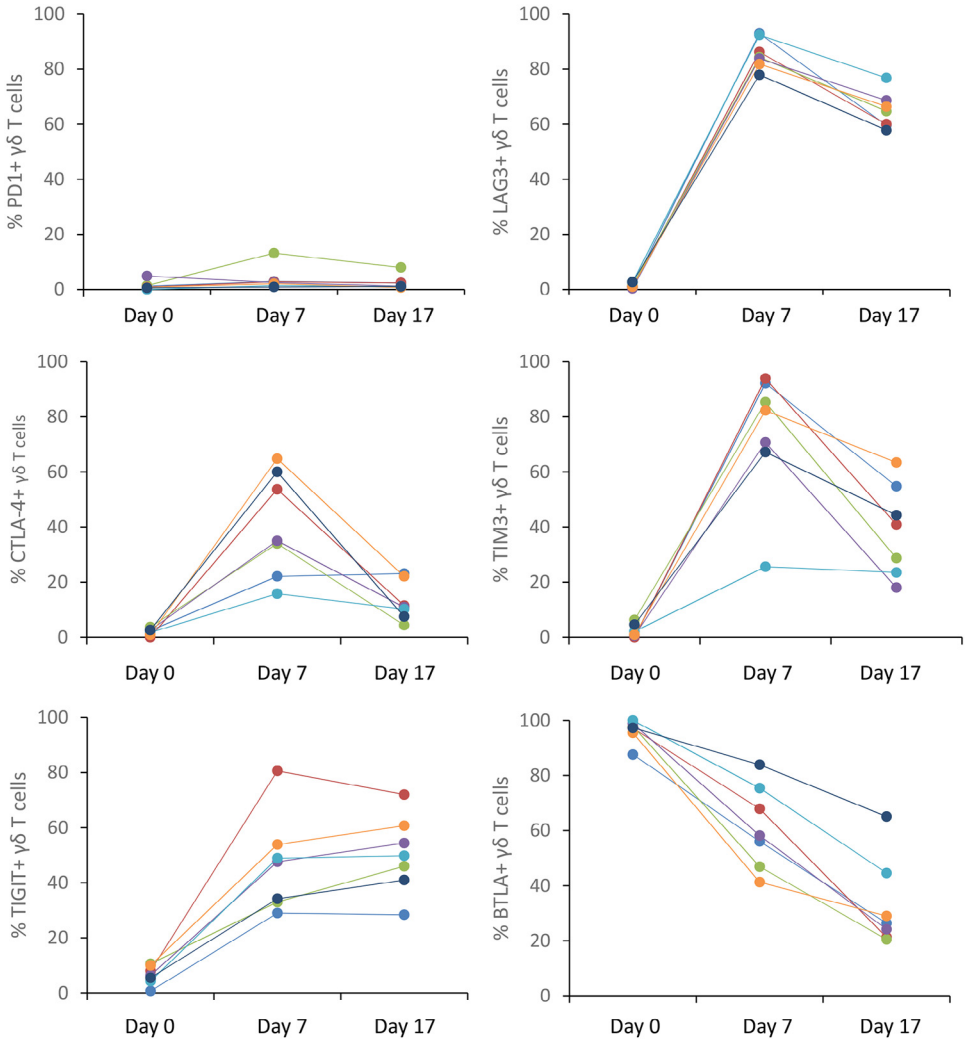

Figure 3. Checkpoint inhibitor phenotyping of Vγ9Vδ2 T cells expanded in G-Rex vessels. PBMCs (day 0) from seven donors listed in Table I and the derived cells on day 7 and day 17 of culture were analyzed. Each donor is represented by one line.

population decreased from 66.6–51% and the T_{EM} cell population increased from 21–37.6%. The T_{CM} cell population under either Zometa treatment or co-culturing with K562 aAPCs remained at a low level, being < 10% of Vγ9Vδ2 T cells. These results indicate that while Zometa promoted the differentiation of Vγ9Vδ2 T cells into effector cell status, K562 aAPCs can assist with the maintenance of the T_{EM} cell population.

The immune checkpoint receptors play an important role in regulating immune cell function. The immune checkpoint receptors expressed on γδ T cells include cytotoxic T lymphocyte (CTL)-associated antigen 4 (CTLA-4, CD152), programed cell death protein 1 (PD-1, CD279), lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin-containing protein 3 (TIM-3), T-cell immunoglobulin and ITIM domain (TIGIT) and B and T lymphocyte attenuator (BTLA). In the current study, the expression of the six checkpoint receptors on Vγ9Vδ2 T cells was examined before and after the expansion (Figure 3 and Supplemental Figure S4). Only very low levels (<5%) of PD-1 expression were detected on day 0 and in the expanded Vγ9Vδ2 T cells. The expression of LAG3, CTLA4, TIM3 and TIGIT, which were almost absent on day 0, was up-regulated significantly using Zometa treatment for 7 days to 85.6%, 40.8%, 73.8% and 46.7%, respectively. Co-culturing with aAPCs down-regulated the expressions of LAG3, CTLA4 and TIM3 to 64.8%, 12.8% and 39%, respectively, by day 17, but TIGIT expression was not obviously changed (50.3%). The expression of BTLA, a key regulator of Vγ9Vδ2 T-cell proliferation [36,37], was detected on almost all Vγ9Vδ2 T cells (96%) before the expansion, but decreased during the expansion, down to 61.4% and 33% on day 7 and day 17, respectively. Overall, the use of K562 aAPCs here promoted the down-regulation of immune checkpoint receptor expression on Vγ9Vδ2 T cells.

Cytotoxicity of expanded Vγ9Vδ2 T cells

We moved on to examine the tumor cell–killing activity of the expanded Vγ9Vδ2 T cells using cytotoxicity and Enzyme-Linked ImmunoSpot (ELISpot) assays. Four different types of human tumor cell lines, colorectal cancer cell line SW480 and HRT-18G, ovarian cancer cell line CAOV3 and glioblastoma cell line U87, were tested as target cells. Because Zometa treatment can increase intracellular levels of IPP, thus sensitizing tumor cells to Vγ9Vδ2 T-cell–mediated cytolysis, we tested both tumor cells with or without Zometa pretreatment. As shown in Figure 4A, without Zometa pretreatment, the four tumor cell lines were resistant to anti-tumor cytolysis mediated by Vγ9Vδ2 T cells. Zometa treatment significantly increased tumor

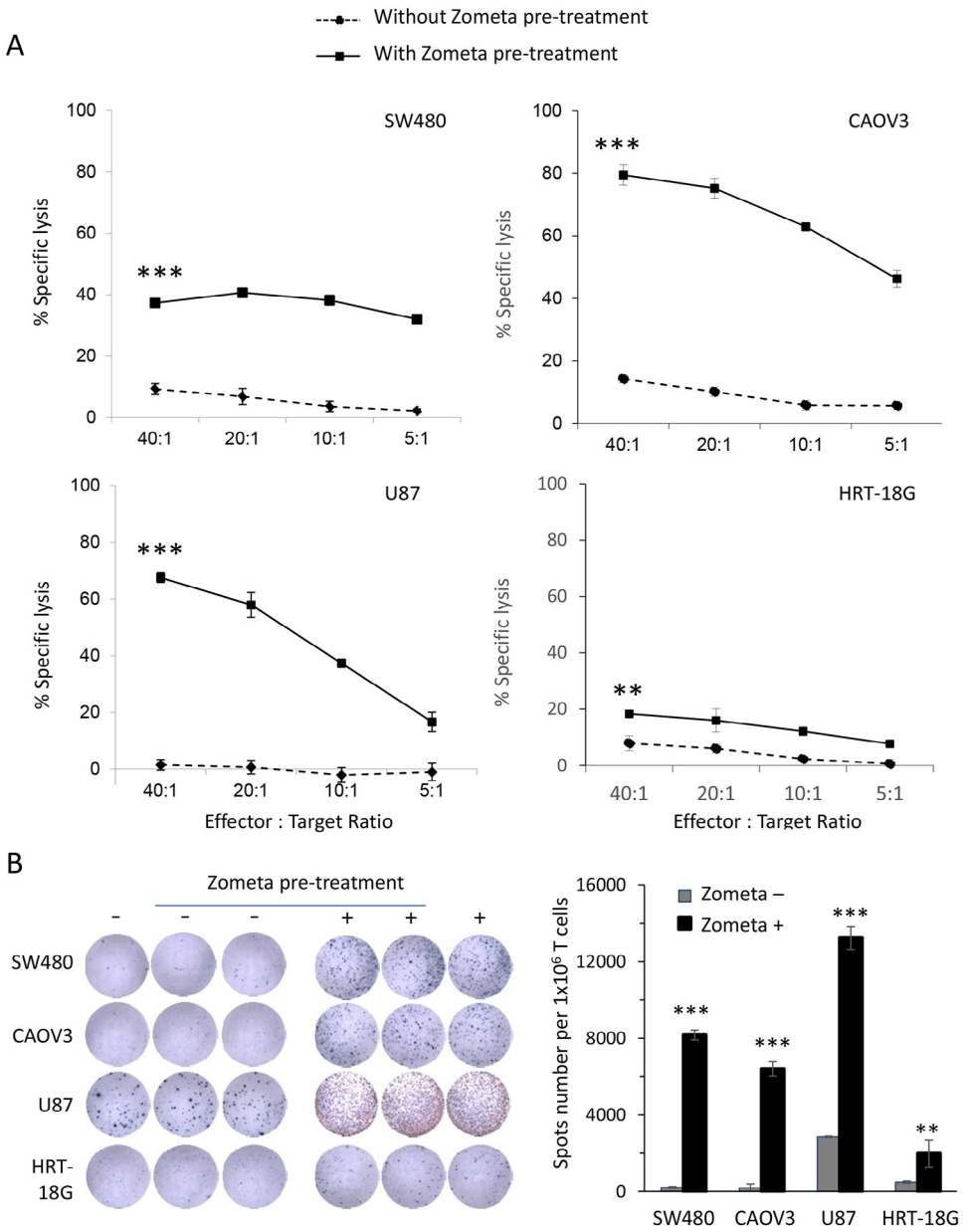

Figure 4. Cytotoxic immune responses induced by Vγ9Vδ2 T cells against tumor cells with or without Zometa pretreatment. Human colorectal cancer cell lines SW480 and HRT-18G, ovarian cancer cell line CAOV3 and glioblastoma cell line U87 were treated with or without 5 μmol/L Zometa overnight before co-culturing with day 17 γδ T cells for cytotoxicity and ELISpot assays. (A) Cytolytic effects. Delfia Europium thiodiglycolate (EuTDA) cytotoxicity assay (2 h of EuTDA culturing) was used to assess *in vitro* cell lysis activity. Mean ± SD of three validation runs is represented. $^{**}P < 0.01$, $^{***}P < 0.001$ between Zometa-treated tumor cells and those without the pretreatment. (B) The frequency of IFNγ-secreting γδ T cells as quantified with the IFNγ ELISpot assay. The images of the increased IFNγ secretion after co-culturing with the indicated cancer cells are shown on the left. Mean IFNγ spots per 1E6 immune cells ± SD from triplicate cultures are shown on the right. $^{**}P < 0.01$; $^{***}P < 0.001$.

cell sensitivity to Vγ9Vδ2 T-cell lysis, around 40–80% of SW480, CAOV3 and U87 tumor cells were killed at effector cell:target cell (E:T) ratio of 20:1. Only 20% of Zometa-treated HRT-18G tumor cells were killed at E:T ratio of 40:1, slightly higher than that for HRT-18G tumor cells without Zometa treatment. Consistently, human interferon (IFN)-γ ELISpot assay showed none or little IFN-γ secretion for the four tumor cell lines without Zometa pretreatment, but significantly increased IFN-γ release after Zometa treatment of the tumor cells (Figure 4B). Thus, Vγ9Vδ2 T cells plus Zometa pretreatment of tumor cells were effective in killing tumor cells.

CD16 is an Fc receptor that can interact with the Fc portion of immunoglobulin G (IgG) to trigger the ADCC process. Because Vγ9Vδ2 T cells expanded with our method express variable levels of CD16, we selected two donors to test ADCC activity. Using Donor A's PBMCs 70.7% of the expanded Vγ9Vδ2 T cells were CD16-positive while in Donor B 8.7% of the expanded Vγ9Vδ2 T cells were CD16-positive (Figure 5A). Two human tumor cell lines, the head and neck cancer cell line SCC-25 and breast cancer cell line BT464 that overexpressed epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), respectively (Figure 5B), were used as target cells to test the anti-EGFR antibody Cetuximab and an anti-HER2 antibody Trastuzumab. ADCC activities were observed when Vγ9Vδ2 T cells from Donor A were tested together with Cetuximab and Trastuzumab against SCC-25 and BT464 tumor cells (Figure 5C). Using Vγ9Vδ2 T cells from Donor B, SCC-25 tumor cells could still been lyzed in the presence of Cetuximab, although we did

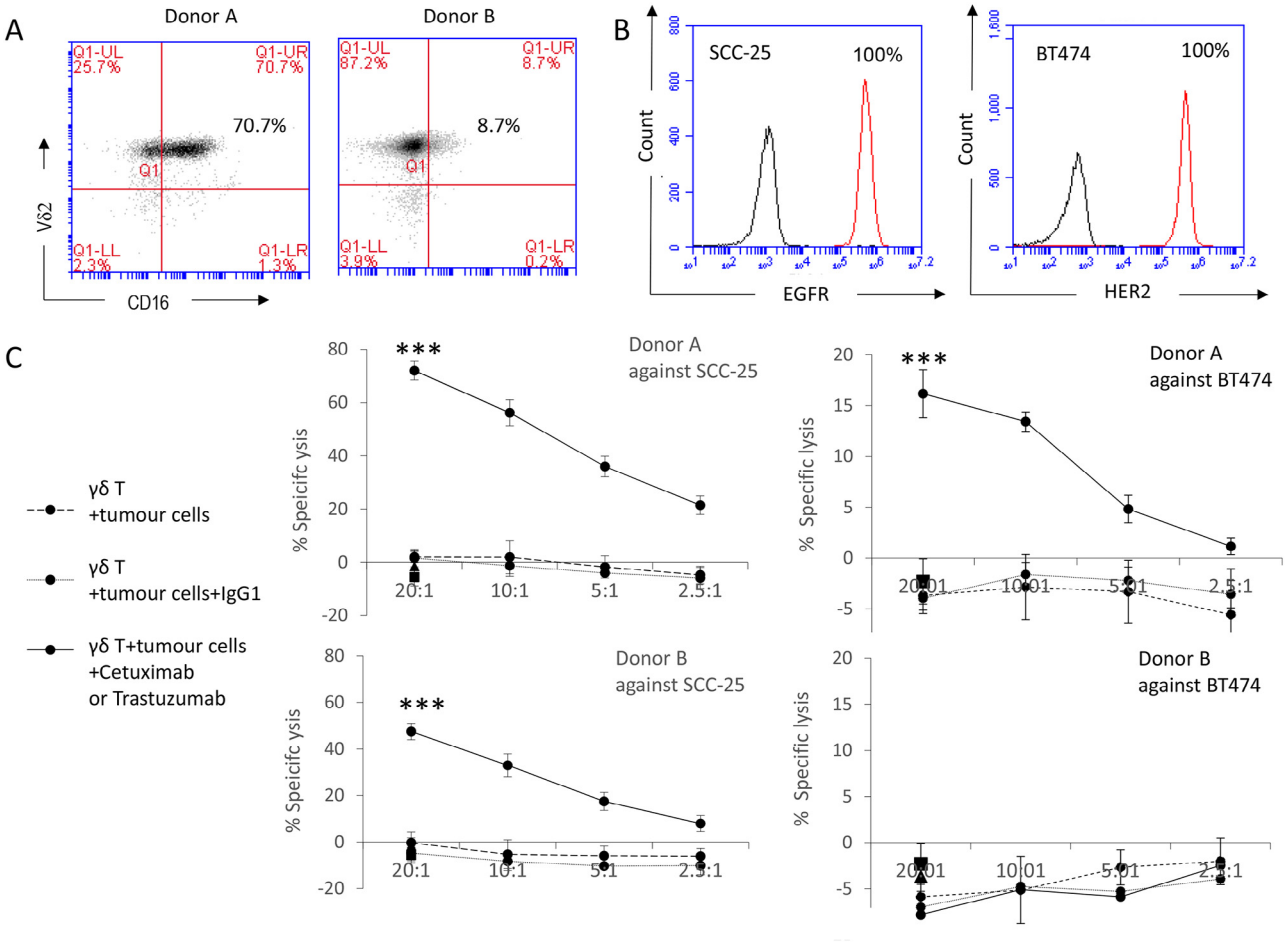

Figure 5. The ADCC activity of the expanded Vγ9Vδ2 T cells against tumor cells. (A) Flow cytometric analysis of CD16 expression on Vγ9Vδ2 T cells expanded from two selected donors with a high (Donor A) or low (Donor B) level expression. (B) EGFR and HER2 expression on the two cancer cell lines used to test Cetuximab- and Trastuzumab-mediated ADCC. SCC-25 is an enhanced green fluorescent protein-positive human head and neck cancer cell line and BT474 is a HER2-positive breast cancer cell line. Left curves: isotype controls; right curves: antibodies. (C) Anti-EGFR antibody Cetuximab (IgG1, 1 μg/mL)-mediated ADCC against SCC-25 cells (left) and anti-HER2 antibody Trastuzumab (IgG1, 1 μg/mL)-mediated ADCC against BT474 cells. Delfia EuTDA cytotoxicity assay (2 h EuTDA culturing) was used to assess ADCC activity. Mean ± SD of three validation runs is represented. ***$P < 0.001$. Cetuximab (1 μg/mL) or Trastuzumab (1 μg/mL) alone did not cause death of the tested cancer cells.

not detect ADCC activity against BT474 tumor cells in the presence of Trastuzumab (Figure 5C). Taken together, we demonstrated that Vγ9Vδ2 T cells generated with our method functioned well as ADCC effector cells and this effect was related to CD16 expression level on Vγ9Vδ2 T cells.

Expanded Vγ9Vδ2 T cells used as CAR effector cells

Genetic modification with a CAR gene is an effective way to enhance the specificity and tumor cell–killing activity of immune cells. CAOV3 and HRT-18G tumor cells are both epithelial cell adhesion molecule (EpCAM)-positive, but show different responses upon Zometa treatment. CAOV3 cells could be sensitized to Vγ9Vδ2 T-cell–mediated cytolysis using Zometa and HRT-18G cells were relatively resistant to Zometa sensitization (Figure 4). We tested the two tumor cell lines for their sensitivity to Vγ9Vδ2 T cells electroporated with an anti-EpCAM RNA CAR [38]. As shown in Figure 6, the CAR-modified Vγ9Vδ2 T cells were effective in lysing HRT-18G cells; all HRT-18G tumor cells, regardless of whether they were treated with (Figure 6A) or without (Figure 6C) Zometa, were killed at E:T ratio that ranged between 10:1 and 20:1. As observed above, Zometa treatment sensitizes CAOV3 cells to Vγ9Vδ2 T-cell–mediated cytolysis, resulting in 80% of cell death at E:T ratio of 20:1. CAR modification improved the killing activity to approximately 90% (Figure 6A).

CAR-T-cell therapy often requires cryopreservation of the modified cells. We then tested effects of cryopreservation on the tumor cell–killing activity of RNA CAR-modified Vγ9Vδ2 T cells. After electroporation of the EpCAM-specific RNA CAR into expanded Vγ9Vδ2 T cells, the cells were frozen and stored in liquid nitrogen for 1 month. The cells were then thawed and allowed to rest for 24 h before being used for flow cytometric analysis and cytotoxicity assay. The post-thaw cell viability was 32.0% ± 3.45%. Using a control membrane-bound green fluorescent protein CAR [38], we observed the same levels of GFP expression on RNA CAR-modified Vγ9Vδ2 T cells before cryopreservation (fresh) and after freezing/thawing (Figure 6B). The cytolytic activity of the frozen/thawed CAR-Vγ9Vδ2 T cells was also comparable with that provided by fresh CAR-Vγ9Vδ2 T cells, suggesting the feasibility of using cryopreserved RNA CAR-modified Vγ9Vδ2 T cells for CAR-T-cell therapy.

Vγ9Vδ2 T cells function in an HLA-independent manner, offering opportunities for allogeneic CAR-T-cell therapy. However, in our Vγ9Vδ2 T-cell preparations, there were still residual αβ T cells (7.4% by day 17), which may increase the risk of graft-versus-host disease (GVHD). To minimize the risk, we have established a protocol for αβ T-cell depletion during Vγ9Vδ2 T-cell expansion (Figure 7A). αβ T-cell depletion was performed using an anti-TCRα/β antibody after PBMCs were activated using Zometa for 7 days. The depletion performed on day 7, not at the end of cell expansion, reduces the cost for the materials used for αβ T-cell depletion. Initially we tested magnetic-activated cell sorting (MACS) buffer during the depletion and obtained a Vγ9Vδ2 T-cell recovery rate of 50–60%, which was mainly because of heavy cell death in MACS buffer. To maintain cell viability, we tested Dulbecco's phosphate-buffered saline (DPBS) supplemented with 2% fetal bovine serum (FBS) and improved the Vγ9Vδ2 T-cell recovery rate to 60–80%. After the depletion, Vγ9Vδ2 T cells were co-cultured with K562-aAPCs for another 10 days. Cell expansion fold for Vγ9Vδ2 T-cell preparations that were depleted of αβ T cells in MACS buffer were expanded 689-fold, whereas those cells undergoing depletion in DPBS supplemented with 2% FBS were expanded 1349-fold ($P < 0.05$; Table II). Table II and the representative results shown in Figure 7B demonstrated that up to 21.3% of αβ T cells could be detected in day 7 Vγ9Vδ2 T-cell preparations and were almost totally depleted by the anti-TCRα/β antibody. While co-culturing with K562-aAPCs led to massive expansion of Vγ9Vδ2 T cells after αβ T-cell depletion, the percentage of αβ T cells remained very low (0.18% with MACS buffer to 0.15% with DPBS; Table II). *In vitro* cytotoxicity assays demonstrated that EpCAM-specific CAR-modified Vγ9Vδ2 T cells, either with or without αβ T-cell depletion, exhibited comparable cytotoxic activities to human ovarian cancer cell line SKOV3 and squamous cell carcinoma cell line FaDu, resulting in 100% tumor cell killing at E:T ratio of 20:1 (Figure 7C), indicating that αβ T-cell depletion did not affect immune effector cell functions of Vγ9Vδ2 T cells.

Discussion

Without the use of Zometa, heterogeneous γδ T-cell subsets have been expanded with K562 aAPCs expressing mIL-15, CD86 and CD137L after NK cell depletion and γδ T-cell isolation from PBMCs [19]. The method provides a 4.9E3 ± 1.7E3 (mean ± SD)–folds expansion of the isolated γδ T cells after co-culturing with K562 aAPCs for 22 days [19]. While the activation and propagation of the populations of γδ T cells expressing polyclonal repertoire of γ and δ TCR chains may be useful in facilitating γδ T-cell cancer immunotherapies, the importance of the polyclonal repertoire has yet to be thoroughly investigated because very little is known about human γδ T cells expressing TCR γδ alleles other than Vδ1 and Vδ2 [39,40]. Emerging pathogenic roles of γδ T cells in cancer progression have been reported, which are mainly

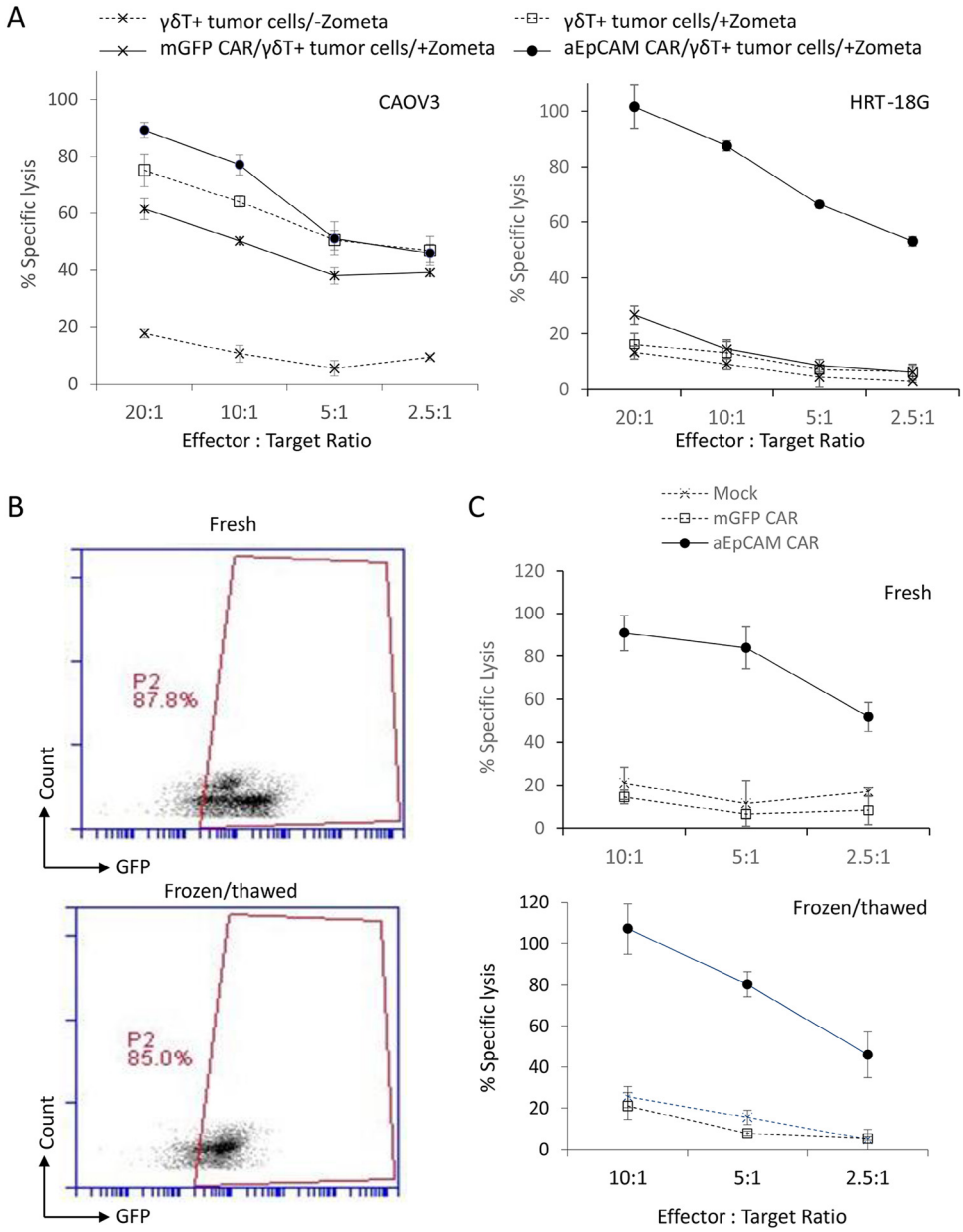

Figure 6. Vγ9Vδ2 T cells electroporated with mRNA encoding an anti-EpCAM CAR effectively lyse EpCAM-positive tumor cells. (A) Cell lysis of CAOV3 cancer cells that can be sensitized using Zometa and HRT-18G cancer cells that are resistant to Zometa sensitization. Delfia EuTDA cytotoxicity assay (2 h EuTDA culturing) was used to assess *in vitro* cell lysis activity. (B and C) Effects of freezing and thawing on the cell lytic activity of EpCAM CAR-modified Vγ9Vδ2 T cells. (B) Flow cytometric analysis of GFP expression on γδ T cells. Fresh: CAR mRNA electroporated cells were analysed before freezing (24 h post-electroporation). Frozen and thawed: The electroporated cells were cryopreserved, stored in a liquid nitrogen tank for 1 mo and analyzed 24 h after thawing. (C) Delfia EuTDA cytotoxicity assay (2 h EuTDA culturing) to assess tumor cell lysis efficiency of fresh and frozen/thawed EpCAM CAR-modified Vγ9Vδ2 T cells. HRT-18G cells were used as target cells.

attributed to IL-17 secreting γδ T-cell subset [41,42]. Given that adult human peripheral blood Vγ9Vδ2 T cells distinctively express Th1 signature with 50–80% of them producing IFNγ and only <5% secreting IL-17 [43], we have focused in the current study on the massive expansion of Vγ9Vδ2 T cells, a subset of γδ T cells that have been broadly tested in clinical trials for tolerability, safety and efficacy [5–10].

We report here a simple and straightforward process for Vγ9Vδ2 T-cell expansion using K562 aAPCs that express CD64, CD86 and CD137L. The features of the method include the following: starting directly with PBMCs without running γδ T-cell separation and purification at the initial stage, expansion in bulk performed in GMP-compliant G-Rex devices together with the K562 aAPCs and anti-CD3 antibody

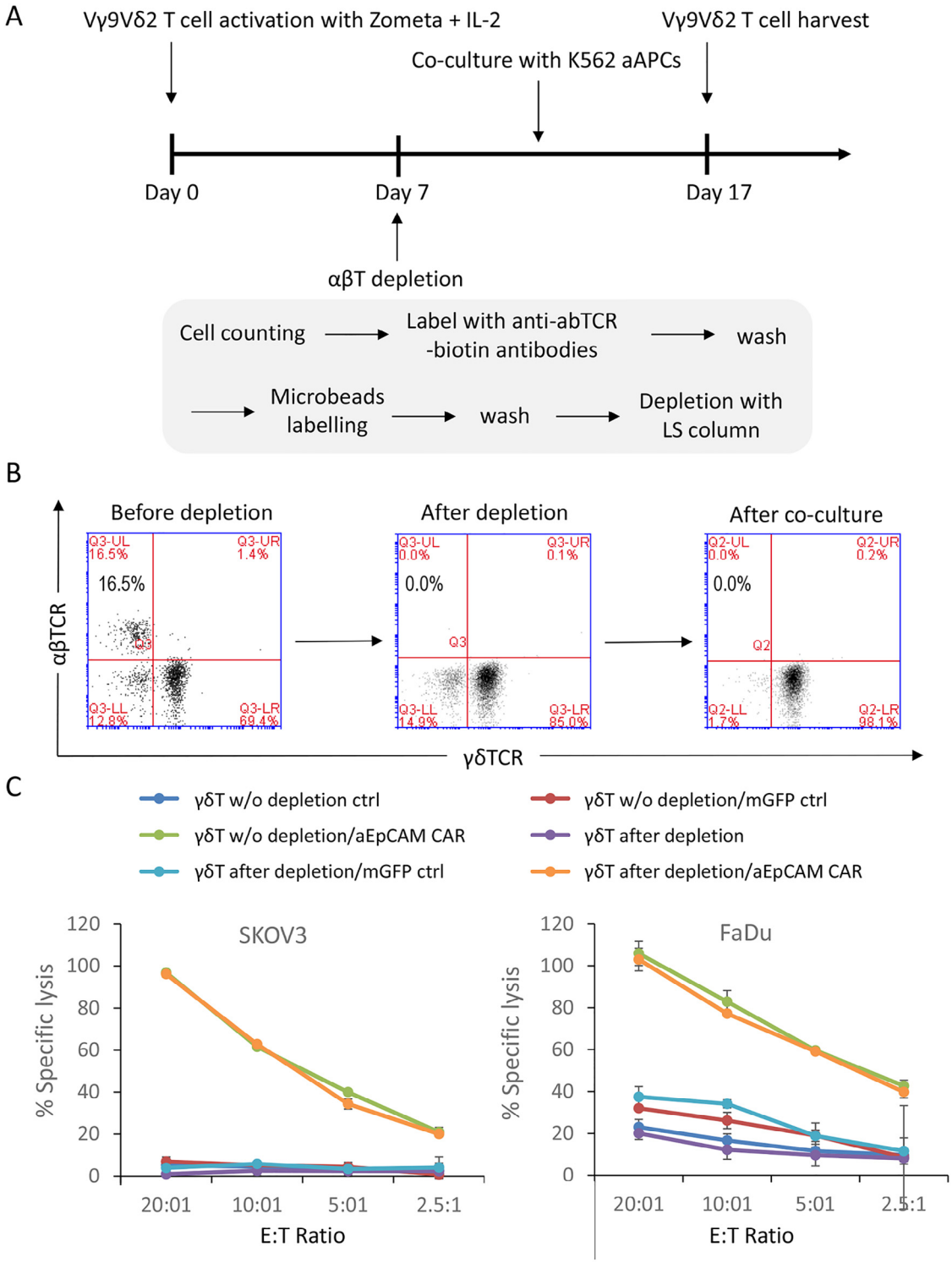

Figure 7. The use of αβ T-cell–depleted Vγ9Vδ2 T cells for CAR-T cell therapy. (A) Schematic of αβ T-cell depletion procedure. αβ T-cell depletion was conducted after PBMC activation using Zometa for 7 days. After the depletion, γδ T cells were co-cultured with γ-irradiated K562 aAPCs for another 10 days until harvest. (B) Representative flow charts of αβ T-cell percentage before depletion (left), immediately after depletion (middle) and after co-culture (right). (C) *In vitro* killing of human ovarian cancer cell line SKOV3 and squamous cell carcinoma cell line FaDu by EpCAM-specific RNA CAR modified Vγ9Vδ2 T cells prepared with or without αβ T-cell depletion.

Table II. αβ T-cell depletion during Vγ9Vδ2 T-cell preparation[a].

ID no.	Depletion buffer	% αβTCR+ cell before depletion	% αβTCR+ cell after depletion and co-culture	Vγ9Vδ2 T recovery rate[b]	Fold change during the co-culture	Vγ9Vδ2 T cell: purity after co-culture
ID473	MACS	6.1%	0.1%	52%	775	86.3%
ID480		5.7%	0.2%	58%	620	92.5%
ID578		6.7%	0.3%	68%	594	95.8%
ID610		5.9%	0.1%	61%	930	91.5%
ID677		3.7%	0.2%	60%	525	92.4%
Mean	DPBS + 2% FBS	5.62%	0.18%	59.80%	689	91.70%
± SD		± 1.14%	± 0.08%	± 5.76%	± 163	± 3.43%
ID654		5.6%	0.1%	60%	938	89.9%
ID677		3.7%	0.1%	80%	1880	93.6%
ID699		6.8%	0.2%	68%	988	99%
ID743		9.1%	0.2%	78%	1490	95.3%
ID795		16.9%	0.0%	61%	1980	98.5%
ID799		21.3%	0.3%	58%	820	82%
Mean		10.57%	0.15%	67.50%	1349	93.05%
± SD		± 6.98%	± 0.10%	± 8.66%	± 506	± 6.36%

[a]Vγ9Vδ2 T cells were expanded with the standard protocol as illustrated in Figure 1A. After the first step (Zometa treatment for 7 days), the cell preparations were subjected to αβ T-cell depletion and the results are shown in the table.
[b]The number of Vγ9Vδ2 T cells after depletion divided by the number before depletion.

OKT3 and an extremely high expansion rate that can be achieved without the use of specialized culture media and reagents. With our standard cell expansion protocol, the total cell population can be expanded by more than 6500-fold and Vγ9Vδ2 T lymphocytes undergo a specific 530,000-fold expansion, corresponding to a Vγ9Vδ2 T-lymphocyte enrichment of >80%. When starting with 2 million PBMCs for Zometa activation and 1 million of the activated cells in G-Rex vessels, we were able to generate 2–15 billion Vγ9Vδ2 T cells with a mean purity of 88% (Tables I and II). The robust expansion efficiency offered by our method can potentially eliminate the need for costly and risky apheresis procedures and enable large-scale mass generation of Vγ9Vδ2 T cells. This will allow the preparation and single lot–release testing of multiple aliquots of cryopreserved cells for repeated infusions.

As demonstrated in the present study, the high expansion efficiency of the Vγ9Vδ2 T-cell population was achieved mainly during the step of co-culturing with our K562 aAPCs, where the anti-CD3 antibody OKT3 was included to promote interactions between K562 cells and CD3+ immune cells. During the activation of the αβ TCR by its cognate antigen, a conformational change of CD3 induced by the antigen is necessary for αβ TCR activation. However, Dopfer *et al.* [44] have shown that OKT3, which binds to the CD3 epsilon chain [45], can trigger great proliferation, as well as cytokine production, of Vγ9Vδ2 T cells without the induction of obvious CD3 conformational change. Furthermore, Vγ9Vδ2 TCR can be activated by phosphoantigens without obvious CD3 conformational change [44]. Thus, the authors have suggested that phosphoantigen stimulation followed by OKT3 stimulation should ensure the generation of a sufficient number of Vγ9Vδ2 T cells, which is demonstrated to be true by the current experimental data. Used together with K562 aAPCs expressing the high-affinity human IgG receptor FcγRI CD64, the Fc-portion of OKT3 can further attach to CD64 to promote the cell-cell interaction between CD3+ Vγ9Vδ2 T cells and CD64+ K562 aAPCs, which will in turn promote CD86 and CD137L on K562 cells to interact, respectively, with CD28 and CD137 on Vγ9Vδ2 T cells, providing co-stimulatory signals to stimulate the cells to proliferate numerically without triggering anergy or apoptosis. Thus, co-culturing of Vγ9Vδ2 T cells with the K562 aAPCs expressing CD64, CD86 and CD137L in the presence of OKT3 is a key attribute of our method that contributes to an increase of the number of Vγ9Vδ2 T cells by six orders of magnitude within 17 days. Whether including mIL-15, CD32 or CD83, as tested previously [19,20], into our K562 aAPCs could improve the efficiency further is worthy of investigation in future studies.

As an interesting observation, we found that the expression of several immune checkpoint inhibitors, including LAG3, CTLA4, TIM3 and TIGIT, increased upon Zometa treatment and decreased to some extent after co-culturing with K562-aAPCs (Figure 4). The co-inhibitory receptor BTLA is expressed by resting Vγ9Vδ2 T cells, and TCR activation by antigen stimulation down-regulates its expression on the cell surface [36,37]. In the current study, most of Vγ9Vδ2 T cells (96%) in PBMCs, which are possibly resting Vγ9Vδ2 T cells, expressed BTLA on their surface, but the expression can be quickly and significantly down-regulated with our cell expansion method. Hence, consistent with antigen stimulation, treating Vγ9Vδ2

T cells with OKT3 and K562 aAPCs expressing co-stimulatory factor inhibited the expression of BTLA. Immune checkpoint receptors play important roles in down-regulating immune responses to avoid excessive immune activation, providing a critical role in the maintenance of immune homeostasis. Inhibition and/or down-regulation of these receptors have proven to be an effective way to promote immune cell responses in cancer therapy. The down-regulation of LAG3, CTLA4, TIM3, TIGIT and BTLA expression by co-culturing with K562-aAPCs in this study might contribute to the observed cytolytic activity of the expanded Vγ9Vδ2 T cells against several types of tumor cells (Figure 5). However, when *in vitro* cytolytic activities of Vγ9Vδ2 T cells at day 7 and day 17 were compared, we did not observe obvious improvement in cytolysis; Vγ9Vδ2 T cells obtained after the K562 co-culture displayed similar tumor cell–killing effects as compared with Vγ9Vδ2 T cells collected after Zometa treatment for 7 days only (data not shown). Nevertheless, it is still possible that the down-regulated expression of the checkpoint receptors on day 17 Vγ9Vδ2 T cells may provide beneficial effects in the context of an *in vivo* tumor microenvironment, which could be highly immunosuppressive. This warrants further evaluation in future studies.

The specificity and strength of cytotoxicity of Vγ9Vδ2 T cells can be enhanced through ADCC mechanism or genetic modification with CARs specific for tumor antigens as demonstrated with EpCAM-specific RNA CARs in this study. EpCAM is up-regulated in a variety of epithelial-derived carcinomas, including adenocarcinomas of colon, stomach, pancreas, lung, ovarian and breast [46]. Especially, during the process of tumorigenesis, the subcellular distribution of EpCAM is changed from the basolateral location in normal tissues to a homogeneously dispersed pattern on the cancer cell surface in cancer tissues, making the molecule easily accessible to cellular immunotherapy [47,48] Vγ9Vδ2 T cells armed with EpCAM-specific RNA CAR elicited an extremely strong cytotoxic effect against not only Zometa-sensitive CAOV3 tumor cells, but also Zometa-resistant HRT-18G tumor cells (Figure 6A). Furthermore, we have developed two other clinically appropriate procedures related CAR-T-cell preparation: (i) cryopreservation of CAR-modified Vγ9Vδ2 T cells for long-term storage without compromising their cytolytic function and (ii) αβ T-cell depletion to generate highly pure Vγ9Vδ2 T-cell preparations suitable for allogeneic CAR-T-cell therapy. Our findings largely support the applications of Vγ9Vδ2 T cells expanded with our method for adoptive CAR-T-cell immunotherapy.

In conclusion, we report here an efficient approach for large-scale expansion of Vγ9Vδ2 T cells that possess a highly functional and less exhausted phenotype. Although the expanded cells are inherently cytotoxic to tumor cells, their tumor specificity and functionality as immune effector cells can be further enhanced by genetic modification to introduce a CAR transgene. Attractively, the lack of HLA restriction in Vγ9Vδ2 T-cell therapy should facilitate therapeutic applications of the cells in allogeneic settings. This is clinically important given the low frequencies of Vγ9Vδ2 T cells observed among cancer patients [49].

Materials and methods

Vγ9Vδ2 T-cell expansion

Buffy coats of healthy donors were collected from National University Hospital Singapore, Department of Laboratory Medicine Blood Transfusion Service, as approved by the institutional review board of National University of Singapore (NUS-IRB Reference Code B-14-133E). Human PBMCs were isolated from fresh buffy coats by density gradient centrifugation using Ficoll-Paque (GE Healthcare). To expand Vγ9Vδ2 T cells, 2E6 PBMCs were seeded in a 24 well plate at day 0 and activated using 5 μmol/L Zometa (Sigma-Aldrich) in 1 mL AIM-V (Life Technologies) supplemented with 1% human plasma (Valley Biomedical) or 5% human AB serum (Valley Biomedical), and 300 IU/mL human recombinant IL-2 (PeproTech). After 7 days of Zometa treatment, cells were mixed with γ-irradiated K562 Clone A aAPCs at an immune cell:K562 ratio indicated for co-culturing. K562 aAPCs were prepared as described before [17,30]. On the first day of co-culture, 5 μmol/L Zometa, 60 ng/mL OKT3 (eBioscience) and 300 IU/mL IL-2 were added. During the following co-culture, 300 IU/mL IL-2 were replenished every 2–3 days. After 10 days of co-culture, the cells were harvested for further analysis. After the initial optimization of co-culturing conditions in ordinary cell culture flasks, co-culturing was performed in G-Rex vessels (Wilson Wolf Manufacturing).

Tumor cell lines

Six different human solid tumor cell lines (ATCC) were used in the current study. They are human HRT-18G and SW480 colorectal cancer cell lines, SKOV3 and CAOV3 ovarian cancer cell lines, U87 glioblastoma cell line and FaDu hypopharyngeal carcinoma cell line. HRT-18G, SW480, CAOV3 and U87 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Biotech) supplemented with 10% FBS (Hyclone). SKOV3 cells were cultured in McCoy's 5A (Lonza) supplemented with 10% FBS. FaDu cells were cultured in RPMI (Lonza) supplemented with 10% FBS.

Flow cytometric analysis

The antibodies used in the current study are listed in Supplemental Table S1. For staining, 5E5 to 1E6 cells were suspended in 100 µL cold MACS buffer (Miltenyi Biotech). The antibodies were added according to the manufacturer's recommendations and incubated at 4°C for 15 min. After washing with MACS buffer, the cells were resuspended in 500 µL MACS buffer. Flow cytometry analysis after single-color staining or two-color staining was performed with Accuri C6 cytometer (BD Biosciences), while analysis after multi-color staining was performed with LSRII (BD Biosciences). Appropriate isotype controls that are matched to the primary antibody host species and class were used in flow cytometry analysis.

Anti-EpCAM messenger RNA CAR and electroporation of Vγ9Vδ2 T cells

The construction of anti-EpCAM messenger RNA (mRNA) CAR vector was described in our previous publication [30]. Polymerase chain reaction (PCR) was performed using the CAR vector as the DNA template, a forward primer CMV-F (5'-atccgctcgagtagttattaatagtaatcaattacggggtc-3'), and reverse primer T150-R. Capped mRNA was generated through *in vitro* transcription of the PCR DNA templates using the mMESSAGE mMACHINE T7 ULTRA transcription kit (Invitrogen) or the mScript RNA system (Epicentre). For mRNA electroporation, 0.2 mL of the expanded Vγ9Vδ2 T cells was mixed with 20 µg mRNA and electroporated in a 2-mm cuvette (Bio-Rad) using a NEPA21 electroporator (Nepagene) with the following parameters: voltage 240 V, pulse length 4 ms, pulse once. The electroporated T cells were rested for 3 h before testing.

Cytotoxicity assay and ELISPOT assay

The cytolytic activity of Vγ9Vδ2 T cells, ADCC activities by Vγ9Vδ2 T cells and tumor cell–specific lysis by CAR-modified Vγ9Vδ2 T cells were examined with a nonradioactive method (DELFIA EuTDA Cytotoxicity Reagents kit, PerkinElmer). Time-resolved fluorescence was measured in Victor3 multilabel plate counter (Perkin Elmer). The E:T ratios used ranged from 40:1 to 1:1. Control groups were set up to measure spontaneous release (only target cells added), maximum release (target cells added with 10 µL lysis buffer) and medium background (no cell added). Killing efficacy was calculated by using the following formula:

$$\%\ \text{Specific release} = \frac{\text{experimental release (counts)} - \text{spontaneous release (counts)}}{\text{maximum release (counts)} - \text{spontaneous release (counts)}} \times 100$$

IFNγ secretion triggered by tumor antigen recognition of Vγ9Vδ2 T cells was determined using IFNγ ELISPOT assays according to the protocols of ELISPOT kits (Mabtech). The plates were analyzed using an ELISPOT scanner (CTL, Ltd.).

αβ T-cell depletion

αβ T-cell depletion was performed after PBMCs were activated using Zometa for 7 days. Briefly, the cells were labelled with anti-TCR α/β-biotin antibody (Miltenyi) for 15 min at 4°C, followed by incubation with anti-biotin microbeads (Miltenyi) for 10 min at 4°C. After washing with MACS buffer or PBS supplemented with 2% FBS (Hyclone), the cells were resuspended in 500 µL of the corresponding buffer and passed through LS column (Miltenyi). After depletion, γδ T cells were co-cultured with γ-irradiated K562-aAPCs at 1:100 T:K562 ratio for another 10 days.

Statistical analysis

Data are presented as mean ± standard deviation (SD). All statistics were performed with GraphPad Prism 7. P values < 0.05 were considered significant.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2017.12.014.