



The BTLA and PD-1 signaling pathways independently regulate the proliferation and cytotoxicity of human peripheral blood $\gamma\delta$ T cells

Hyun J. Hwang^{1,2} | Jae J. Lee¹ | Sung H. Kang³ | Jin K. Suh³ |
Eun S. Choi³ | Seongsoo Jang⁴ | Sang-Hyun Hwang⁴ | Kyung-Nam Koh³ |
Ho J. Im³ | Nayoung Kim¹

¹Asan Institute for Life Sciences and Department of Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

²Asan Medical Institute for Convergence Science and Technology, Asan Medical Center, Seoul, Republic of Korea

³Department of Pediatrics, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

⁴Department of Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

Correspondence

Nayoung Kim, Asan Institute for Life Sciences and Department of Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, Republic of Korea.
Email: naykim@amc.seoul.kr

Funding information

National Research Foundation of Korea, Grant/Award Number: 2018R1C1B6008852; National R&D Program for Cancer Control, Republic of Korea, Grant/Award Number: 1520060; Asan Institute for Life Sciences, Asan Medical Center, Grant/Award Numbers: 2019IP0837, 2020IP0002

Abstract

Background: B- and T-lymphocyte attenuator (BTLA) and programmed cell death-1 (PD-1) inhibit $\gamma\delta$ T cell homeostasis and activation. This study aimed to determine whether BTLA and PD-1 signaling pathways were convergent or independent in human peripheral blood $\gamma\delta$ T cells. Herein we demonstrate that the signalings of BTLA and PD-1 regulated proliferation and cytotoxicity of human $\gamma\delta$ T cells, respectively.

Methods: Human peripheral blood $\gamma\delta$ T cells were cultured with inactivated Jurkat cells in the presence of interleukin-2 and zoledronate (Zol) for 14 days. Flow cytometry was performed to evaluate the phenotypes and functions of $\gamma\delta$ T cells.

Results: The proliferation of the $\gamma\delta$ T cells was increased when PBMCs were cocultured with inactivated herpes virus entry mediator (HVEM)^{low} Jurkat cells. The cytotoxicity of the expanded $\gamma\delta$ T cells was not affected by coculture with inactivated HVEM^{low} Jurkat cells and was further increased in the presence of anti-PD-L1 mAb. These results suggest that the inactivation of the

Abbreviations: AV, annexin V; BTLA, B- and T-lymphocyte attenuator; CFSE, carboxyfluorescein succinimidyl ester; FBS, fetal bovine serum; GVHD, graft versus host disease; HSCT, hematopoietic stem cell transplantation; HVEM, herpes virus entry mediator; IPP, isopentenyl pyrophosphates; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; mAb, monoclonal antibody; NBP, aminobisphosphonate; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PD-1, programmed cell death-1; PD-L1, PD-ligand 1; PI, propidium iodide; SHP, src homology region 2 domain-containing phosphatase; WT, wild-type; Zol, zoledronate.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. *Immunity, Inflammation and Disease* published by John Wiley & Sons Ltd

BTLA signaling pathway during expansion could help produce more $\gamma\delta$ T cells without compromising $\gamma\delta$ T cell function. The inhibition of BTLA or PD-1 signaling repressed phosphorylation of the src homology region 2-containing protein tyrosine phosphatase 2 and increased the phosphorylation of protein kinase B in $\gamma\delta$ T cells. However, there were no synergistic or additive effects by a combination of BTLA and PD-1 blockade.

Conclusion: These results suggest that BTLA signaling is crucial in regulating $\gamma\delta$ T cell proliferation and function and that the BTLA and PD-1 signaling pathways act independently on the proliferation and cytotoxicity of human peripheral $\gamma\delta$ T cells.

KEYWORDS

AKT, BTLA, cytotoxicity, human peripheral blood $\gamma\delta$ T cells, PD-1, proliferation, SHP2

1 | INTRODUCTION

$\gamma\delta$ T cells are innate-like immune cells that participate in the early phase of infection and they kill tumor cells directly by degranulation of granzymes and perforin.¹ $\gamma\delta$ T cells constitute 1%–10% of total T cells. They have unique characteristics that distinguish them from $\alpha\beta$ T cells, which make up the majority of T cells.² Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells do not require MHC molecules for antigen recognition.³ Human V γ 9V δ 2 T cells, which are the majority of circulating $\gamma\delta$ T cells, require cell-to-cell interactions to induce their reactivity. V γ 9V δ 2 T cells proliferate and are activated by recognizing phospho-antigen directly.¹

Through the mevalonate pathway, aminobisphosphonates (NBPs), such as zoledronate (Zol), lead to the accumulation of isopentenyl pyrophosphates (IPP) by inhibiting farnesyl-pyrophosphate synthase. IPP is then recognized by a $\gamma\delta$ T cell, which induces it to proliferate.⁴ The $\gamma\delta$ T cells expanded and activated in response to IPP exert antitumor, antimicrobial, and antiviral responses.^{5–7} It has been reported that repeated and combined administration of NBPs and human V γ 9V δ 2 T cells can effectively control tumor growth *in vivo*.⁸ Human V γ 9V δ 2 T cells can directly kill leukemia cells⁹ without causing graft versus host disease (GVHD).¹⁰ It has been proven that the depletion of $\alpha\beta$ T cells is a safe and effective tumor therapy.¹¹ Clinical studies are underway to activate $\gamma\delta$ T cells by administering Zol after $\alpha\beta$ T and B cell-depleted hematopoietic stem cell transplantation (HSCT) to obtain an optimal therapeutic effect based on the activity of $\gamma\delta$ T cells.^{12,13} Thus, $\gamma\delta$ T-cell adoptive transfer after HSCT could be an attractive therapeutic option.^{14,15} It is, however, necessary to optimize methods of efficiently triggering the proliferation and activation of V γ 9V δ 2 T cells.

Coinhibitory or immune checkpoint signaling is one of the regulatory mechanisms to inhibit excessive T cell activation.¹⁶ However, it also causes tumor immune escape. Programmed cell death-1 (PD-1), an immune checkpoint molecule, reacts with programmed death-ligand 1 (PD-L1) to inhibit T cell activation, resulting in reduced cytotoxicity.^{17,18} PD-1 is highly expressed in exhausted T cells and is associated with impaired effector function and increased apoptosis.^{19–21} Increasing the antitumor response of T cells by inhibiting PD-1 signaling has emerged as a promising therapy and its efficacy has been confirmed in preclinical and clinical trials.^{22,23} B- and T-lymphocyte attenuator (BTLA), a member of the CD28 family, negatively regulates T cell activation as does PD-1.²⁴ Both BTLA and PD-1 contain Ig-like domains that are expressed in T cells and B cells and present as monomers in T cells.²⁵ After TCR stimulation, the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) of PD-1 and BTLA are phosphorylated. Then, the src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 are recruited.²⁶ As a result, downstream molecules of TCR and CD28 are dephosphorylated.²⁷ Although PD-1 and BTLA both have ITIM and ITSM motifs, PD-1 essentially only requires ITSM for its inhibitory function,²⁸ whereas BTLA requires both ITIM and ITSM.²⁶ CD160, BTLA, and LIGHT share their common ligand, herpes virus entry mediator (HVEM), but BTLA is expressed almost exclusively in T cells.²⁹ Our previous results showed that BTLA is robustly expressed on $\gamma\delta$ T cells from leukemia patients.³⁰ Blocking the BTLA/HVEM inhibitory signal improves the proliferation of $\gamma\delta$ T cells.²⁹ BTLA regulates the proliferation of V γ 9V δ 2 T cells,^{29,31} whereas PD-1 regulates their cytotoxicity.³²

However, the interaction between the two inhibitory signaling pathways in $\gamma\delta$ T cells is poorly understood. Therefore, we examined whether the BTLA and PD-1 signaling pathways are convergent or independent in $\gamma\delta$ T cells by evaluating the proliferation, cytotoxicity, and expression of signaling molecules. The results showed that the BTLA and PD-1 signaling independently regulate the proliferation and activation of $\gamma\delta$ T cells, suggesting that inhibition of BTLA signaling could improve the expansion of $\gamma\delta$ T cells without compromising cytotoxicity.

2 | MATERIALS AND METHODS

2.1 | PBMC isolation and $\gamma\delta$ T cell proliferation

Peripheral blood mononuclear cells (PBMC) or $\alpha\beta$ T cell-depleted PBMCs from healthy adult volunteers were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (GE Healthcare). $\alpha\beta$ T cell-depleted PBMCs were isolated using CliniMACS (Miltenyi Biotec), as previously described.¹³ All of the participants provided written informed consent. All of the procedures were approved by the Institutional Review Board, Asan Medical Center, Seoul, Korea (Approval No. 2015-0307). The study was performed ethically, following the Declaration of Helsinki. Isolated cells were immediately frozen in heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) containing 10% dimethyl sulfoxide (Sigma-Aldrich) and were maintained in liquid nitrogen until use. The PBMCs were cultured at 6×10^5 cells/well in 24-well plates in Rosewell Park Memorial Institute (RPMI) 1640 (Corning) supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin plus 100 $\mu\text{g}/\text{ml}$ streptomycin (Corning), 1 mM sodium pyruvate (Sigma), and 55 μM 2-mercaptoethanol (Gibco) in the presence of 100 U/ml recombinant human interleukin-2 (IL-2) (PeproTech) and 1 μM Zol (Selleckchem). Every 2 days, fresh IL-2 was added.

2.2 | Generation of HVEM^{low} Jurkat cell lines

Jurkat cells (ATCC) were cotransfected with the HVEM CRISPR/Cas9 KO plasmid (Santa Cruz) and the HVEM HDR plasmid (Santa Cruz) according to the manufacturer's instructions. After 48 h, all transfected cells were selected with media containing 2 $\mu\text{g}/\text{ml}$ puromycin (TOCRIS Bioscience) for at least 2 weeks. Before use, the reduction of HVEM expression in the selected cells was confirmed by flow cytometry. The cells expressing lower

levels of HVEM were named HVEM^{low} Jurkat cells. Jurkat cell lines were maintained in RPMI 1640 supplemented with 10% inactivated FBS, 100 IU penicillin plus 100 $\mu\text{g}/\text{ml}$ streptomycin, and 1 mM sodium pyruvate. All cells were incubated in a humidified CO₂ incubator.

2.3 | Cell proliferation assay

PBMCs were washed and resuspended in phosphate-buffered saline (PBS) (Biosesang). Then, the cells were labeled with cell proliferation Dye eFluor™ 670 (EF670) (Thermo Fisher Scientific) according to the manufacturer's instructions.

For inactivation of the Jurkat cells, they were treated with 25 $\mu\text{g}/\text{ml}$ mitomycin C (Sigma) for 2 h and then it was washed away three times with 1 \times PBS. EF670-labeled PBMCs at 3×10^5 cells were cultured in 96-well plates with or without the indicated monoclonal antibody and mitomycin C-treated Jurkat cells at 1.5×10^5 cells in the presence of 100 U/ml rhIL-2 and 1 μM Zol. Anti-human PD-L1 blocking antibody (clone 29E.2A3; Biolegend) was added at 1 $\mu\text{g}/\text{ml}$ at the beginning of the culture. Fresh IL-2 was added every 2 days. In some experiments, BLTA/HVEM blocking peptides (Ac-YRVKEACGELTGTVCEP-NH₂; Peptron),³³ and anti-PD-1 mAb (clone EH12.2H7; Biolegend) were used. As control, scrambled peptide (Ac-ELCAGPVTRKVECTYGE-NH₂; Peptron) and isotype control (clone MOPC-21; Biolegend) were also used. The cultured cells were treated with FcR blocking reagent (Miltenyi Biotec) for 5 min and stained with phycoerythrin (PE)-conjugated anti-human TCR $\gamma\delta$ (clone MOPC-21; Biolegend) and APC-H7-conjugated anti-human CD3 antibody (clone SK7; BD Biosciences) for 30 min. The EF670 dilution was analyzed by FACS Canto II (BD Biosciences) or CytoFLEX (Beckman Coulter Life Sciences) flow cytometers and FlowJo (Tree Star, Inc.) software.

2.4 | Degranulation assay

In the presence of anti-human CD107a (clone eBioH4A3; eBioscience) monoclonal antibody, ex vivo expanded PBMC were incubated with tumor cells at a 1:1 ratio of effector:target. After 1 h, 50 μM monensin (eBioscience) was added. Following overnight incubation and washing, the cells were treated with FcR blocking reagent for 5 min, and stained with fluorescein isothiocyanate (FTIC)-conjugated anti-human TCR $\gamma\delta$ (clone B1.1; eBioscience) and APC-H7-conjugated anti-human CD3 antibody (clone SK7; BD Biosciences), then analyzed by FACS Canto II or CytoFLEX flow cytometers and FlowJo software (Tree Star).

2.5 | Cell death assay

Cytotoxicity was analyzed using annexin V (AV) and propidium iodide (PI) assays. Target cells were washed and resuspended in PBS. Then, the cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) by using Cell Trace Cell Proliferation Kits (Molecular Probes) according to the manufacturer's instructions. Expanded $\gamma\delta$ T cells were resuspended in media and incubated with CFSE-labeled target cells. After 1 day, the cells were washed with AV-binding buffer (BD Biosciences). The cells were treated with FcR blocking reagent for 5 min and stained with anti-AV-APC antibody (BD Biosciences) and PI (Biolegend). Early and late apoptosis was evaluated within the CFSE positive target cells by flow cytometry with FACS Canto II or CytoFLEX. The data were analyzed by FlowJo software.

2.6 | Flow cytometry

In vitro expanded $\gamma\delta$ T cells were harvested and stained with the following antibodies: PE-conjugated anti-human TCR $\gamma\delta$ (clone MOPC-21; Biolegend), APC-H7-conjugated anti-human CD3 antibody (clone SK7; BD Biosciences), FITC-conjugated anti-human CD279 antibody (clone EH12.2H7; Biolegend), and APC-conjugated anti-human CD272 antibody (clone MIH26; Biolegend). For analysis of the differentiation subsets, the following antibodies were used: FITC-conjugated anti-human TCR $\gamma\delta$ (clone B1.1; eBioscience), APC-H7-conjugated anti-human CD3 antibody (clone SK7; BD Biosciences), PE-conjugated anti-human CD45RA antibody (clone HI100; Biolegend), and APC-conjugated anti-human CD27 antibody (clone O323; eBioscience). Differentiation subsets were determined by CD45RA and CD27 expression within the $\gamma\delta$ T cells. In some experiments, interferon- γ (IFN- γ) (clone 4S.B3; Biolegend) was intracellularly stained using BD Cytofix/Cytoperm™ (BD Biosciences). Data were acquired by flow cytometry. Flow cytometry was conducted by FACS Canto II or CytoFLEX. The data were analyzed by FlowJo software.

2.7 | Detection of phosphoprotein by flow cytometry

Jurkat cells were preincubated with anti-human PD-L1 blocking antibody (Biolegend) for 30 min in the presence of FcR blocking Ab and then treated with 100 μ M pervanadate, an inhibitor of phosphatases, which was used as a phosphorylation inducer.³⁴ In the presence of IL-2 and Zol, PBMC and tumor cells were cocultured

for 20 min and immediately fixed in a 4% paraformaldehyde solution. After washing with PBS containing 2% FBS, the cells were permeabilized with BD™ Phosflow Perm Buffer III (BD Biosciences) for 30 min on ice. Then, they were incubated with anti-pSHP-2 (clone L99-921; BD Biosciences), anti-pAKT (Clone M89-61; BD Biosciences), anti-pERK1/2 (ab212153; Abcam), anti-pSHP-1 (ab192669; Abcam) antibody or IgG1 κ isotype control for 1 h, following FcR blocking. For pSHP-1 staining, PE-conjugated goat antirabbit IgG Ab (ab72465; Abcam) was treated. Thirty minutes before washing the cells, FTIC-conjugated TCR $\gamma\delta$ (clone B1.1, eBioscience) and APC-H7-conjugated CD3 antibody (clone SK7; BD Biosciences) were additionally added. Flow cytometry was conducted by CytoFLEX (Beckman Coulter Life Sciences). The data were analyzed by FlowJo (Tree Star, Inc.) software to understand the phosphorylation status of SHP-1/2, ERK1/2, and AKT.

2.8 | Statistical analyses

Growth curves were analyzed by Friedman test, non-parametric repeated-measures analysis of variance using InStat (GraphPad Software). Other results were analyzed using paired two-tailed *t* tests by GraphPad Prism Ver. 6.0 software (GraphPad Software). When the *p* value was less than .05, the difference was considered to be significant.

3 | RESULTS

3.1 | BTLA signaling plays a critical role in the proliferation of $\gamma\delta$ T cells

We found that the proliferation of $\gamma\delta$ T cells in the peripheral blood (PB) from healthy donors induced by IL-2 and Zol was significantly impaired by cocultivation with mitomycin C-inactivated wild-type (WT) Jurkat cells, which express HVEM (Figures 1A and S1). The differences between the control group, which was treated only with IL-2 and Zol (IL-2 + Zol(-)), and the groups cocultured with WT Jurkat in the absence or presence of anti-PD-L1 were statistically significant at Day 10. To determine whether blocking BTLA and/or PD-1 signaling could improve the proliferation of the $\gamma\delta$ T cells, PBMC were cultured with HVEM^{low} or WT Jurkat cells in the presence or absence of anti-PD-L1 blocking monoclonal antibody (mAb). The HVEM^{low} Jurkat cells were produced using a CRISPR/Cas9 system to delete the *HVEM* gene. As expected, HVEM expression was downregulated in HVEM^{low} Jurkat cells relative to mock-transfected

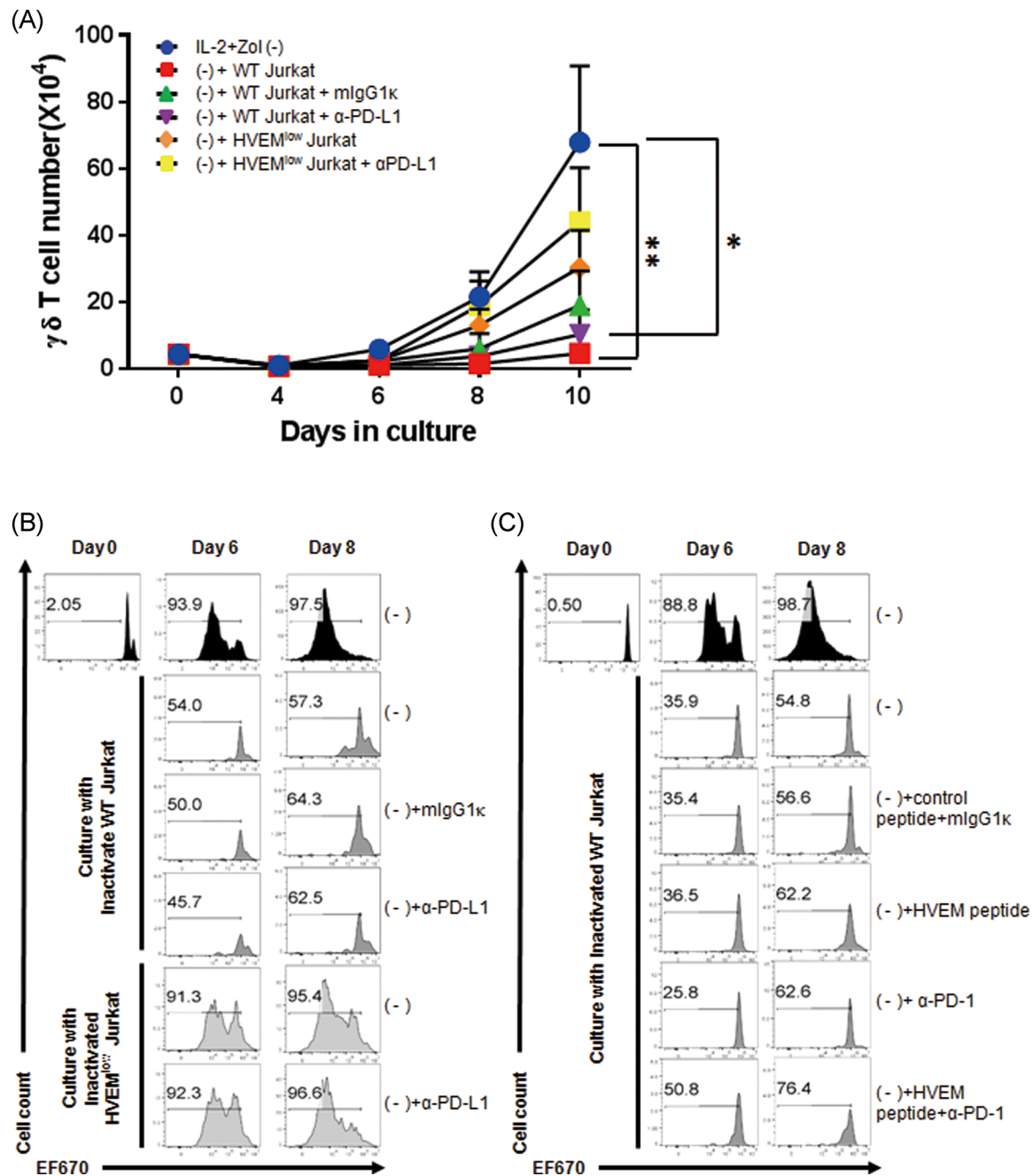


FIGURE 1 Blocking BTLA/HVEM interactions increase $\gamma\delta$ T cell proliferation. (A) $\alpha\beta$ T cell-depleted PBMCs were cultured with inactivated Jurkat cells with or without anti-human PD-L1 blocking antibodies in the presence of IL-2 and zoledronate(-). The gating strategy is shown in Figure S2. The frequency of $\gamma\delta$ T cells ($CD3^+TCR\gamma\delta^+$) was determined by flow cytometry. The absolute numbers of $\gamma\delta$ T cells ($CD3^+TCR\gamma\delta^+$) was calculated at the indicated days. Data were shown as mean \pm SEM of six independent experiments (donor no. $n = 6$, except $n = 3$ for isotype control group). Friedman test was performed ($*p < .05$). (B) After EF670-labeling, PBMCs were stimulated as described above. The top plots were only treated with IL-2 and zoledronate(-) (Zol) and the lower plots were cultured with inactivated leukemic cells as indicated on the left. Cells were harvested at 6 or 8 days after culture, and the proliferation was determined by EF670 dilution by flow cytometry. Representative histograms are shown out of three independent experiments. (C) Experiment was performed as above, except that BTLA/HVEM blocking peptides and anti-PD-1 mAb were used instead of HVEM^{low} Jurkat cells and anti-PD-L1 Ab(-), IL-2- and Zol-treated. BTLA, B- and T-lymphocyte attenuator; HVEM, herpes virus entry mediator; IL-2, interleukin-2; mAb, monoclonal antibody; mlgG1k, isotype control; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1

WT Jurkat cells (Figure S1). The expression of PD-L1 was not changed by the deletion of *HVEM* in Jurkat cells (Figure S1, lower panel).

The inhibited proliferation of the $\gamma\delta$ T cells in coculture with WT Jurkat cells was rescued by $HVEM^{low}$ Jurkat cells (Figure 1A; $p = .065$). The gating strategy to identify $\gamma\delta$ T cells was depicted in Figure S2. $HVEM^{low}$ HL-60 cells also increased $\gamma\delta$ T cell proliferation, compared with WT HL-60 cells (Figure S3A,B). Blocking PD-1/PD-L1 signaling increased the number of $\gamma\delta$ T cells only in the presence of $HVEM^{low}$ Jurkat cells. The treatment with anti-PD-L1 mAb alone failed to increase $\gamma\delta$ T cell proliferation when BTLA/HVEM signaling was intact. These results were confirmed by EF670 dilution, as seen in Figure 1B. By Day 8, EF670^{low/-} proliferating cells were 97.5% when treated only with IL-2 and Zol. Deletion of *HVEM* in Jurkat cells rescued the proliferating cells over 95% at Day 8, compared with 57.3% when cultured with WT Jurkat cells. The averages of the results were summarized in Figure S4. The treatment of BTLA/HVEM blocking peptides during culture increased $\gamma\delta$ T cell proliferation which was inhibited by WT Jurkat cells, and anti-PD-1 Ab alone did not increase it (Figure 1C). Taken together, these results suggest that reduction of BTLA/HVEM signaling is a prerequisite for the optimal proliferation of V δ 2 $\gamma\delta$ T cells, whereas PD-1/PD-L1 signaling has a limited role in their proliferation.

PD-1 expression on $\gamma\delta$ T cells was assessed to determine whether the reduction of BTLA/HVEM or PD-1/PD-L1 signaling during the ex vivo expansion altered PD-1 expression on the $\gamma\delta$ T cells. Stimulation with IL-2 and Zol for 10 days significantly upregulated BTLA and PD-1 expression on the $\gamma\delta$ T cells, and reduction of BTLA and PD-1 signaling did not change BTLA and PD-1 expression on the $\gamma\delta$ T cells (Figure S5).

3.2 | Blocking BTLA signaling reduces the more differentiated $\gamma\delta$ T cell population

It has been suggested that the more differentiated T cells are, the less antitumor efficacy they have,³⁵ and thus the subsets of expanded $\gamma\delta$ T cells were analyzed by flow cytometry. As seen in Figure 2, coincubation with $HVEM^{low}$ Jurkat cells reduced the proportion of terminally differentiated CD27⁻CD45RA⁺ $\gamma\delta$ T cells significantly, compared with that incubated with WT Jurkat cells. Coincubation with WT Jurkat cells slightly increased the number of CD27⁺CD45RA⁺ naïve T cells, and coincubation with $HVEM^{low}$ Jurkat cells in the presence of PD-L1 blockade increased the

number of CD27⁺CD45RA⁻ central memory T cells, although the differences were not statistically significant. These results imply that inhibiting BTLA signaling pathway during expansion could be beneficial for cancer immunotherapy by preventing terminal differentiation of $\gamma\delta$ T cells.

3.3 | The antitumor activity of $\gamma\delta$ T cells is not affected by blocking BTLA signaling

To evaluate the effect of a blockade of BTLA/HVEM and PD-1/PD-L1 signaling on the effector function of $\gamma\delta$ T cells, PBMCs were treated with anti-human PD-L1 mAb while cocultured with $HVEM^{low}$ Jurkat or WT Jurkat cells in the presence of IL-2 and Zol. We then examined the antitumor activity of ex vivo expanded $\gamma\delta$ T cells using PI and AV in tumor cells. The cells expanded under the conditions shown in Figure 4 were incubated with the same number of CFSE-labeled Jurkat cells for 1 day (Figure 3A). PBMC proliferated by IL-2 and Zol treatment effectively induced apoptosis in all Jurkat cell lines (Figure 3B,C). Likewise, $\gamma\delta$ T cells cultured with WT Jurkat cells or $HVEM^{low}$ Jurkat cells caused apoptosis of the target cells comparable to the PBMC control group. Contrary to expectations, anti-PD-L1 mAb treatment did not increase both the early and late apoptosis of the target cells, despite increased expression of CD107a (Figure 4).

The expanded $\gamma\delta$ T cells were challenged by $HVEM^{low}$ Jurkat or WT Jurkat cells, and then, CD107a expression was measured to detect the degranulation of the ex vivo expanded $\gamma\delta$ T cells. Ex vivo expanded $\gamma\delta$ T cells in all five groups expressed similar levels of CD107a in response to $HVEM^{low}$ Jurkat and WT Jurkat cells. Inhibitory signaling by the WT and the $HVEM^{low}$ Jurkat cells did not affect the degranulation of $\gamma\delta$ T. However, the expression of CD107a on $\gamma\delta$ T cells cocultured with $HVEM^{low}$ Jurkat cells was significantly upregulated by the additional inhibition of PD-L1 signaling. Taken together, PD-L1 signaling, rather than HVEM signaling, regulated the degranulation of $\gamma\delta$ T cells. The experiments with $HVEM^{low}$ HL-60 cells also had no significant effect on degranulation (Figure S6). Collectively, the reduced HVEM signaling did not alter the target cell death by $\gamma\delta$ T cells during the culture as well as the reaction period.

$\gamma\delta$ T cells could produce cytokines, such as IFN- γ , upon stimulation. Thus we assessed intracellular IFN- γ by flow cytometry (Figure 4D,E). $\gamma\delta$ T cells were cultured with inactivated WT or $HVEM^{low}$ Jurkat cells in the presence or absence of anti-PD-L1 Ab for 2 weeks, and the cells were stimulated with WT Jurkat cells in the

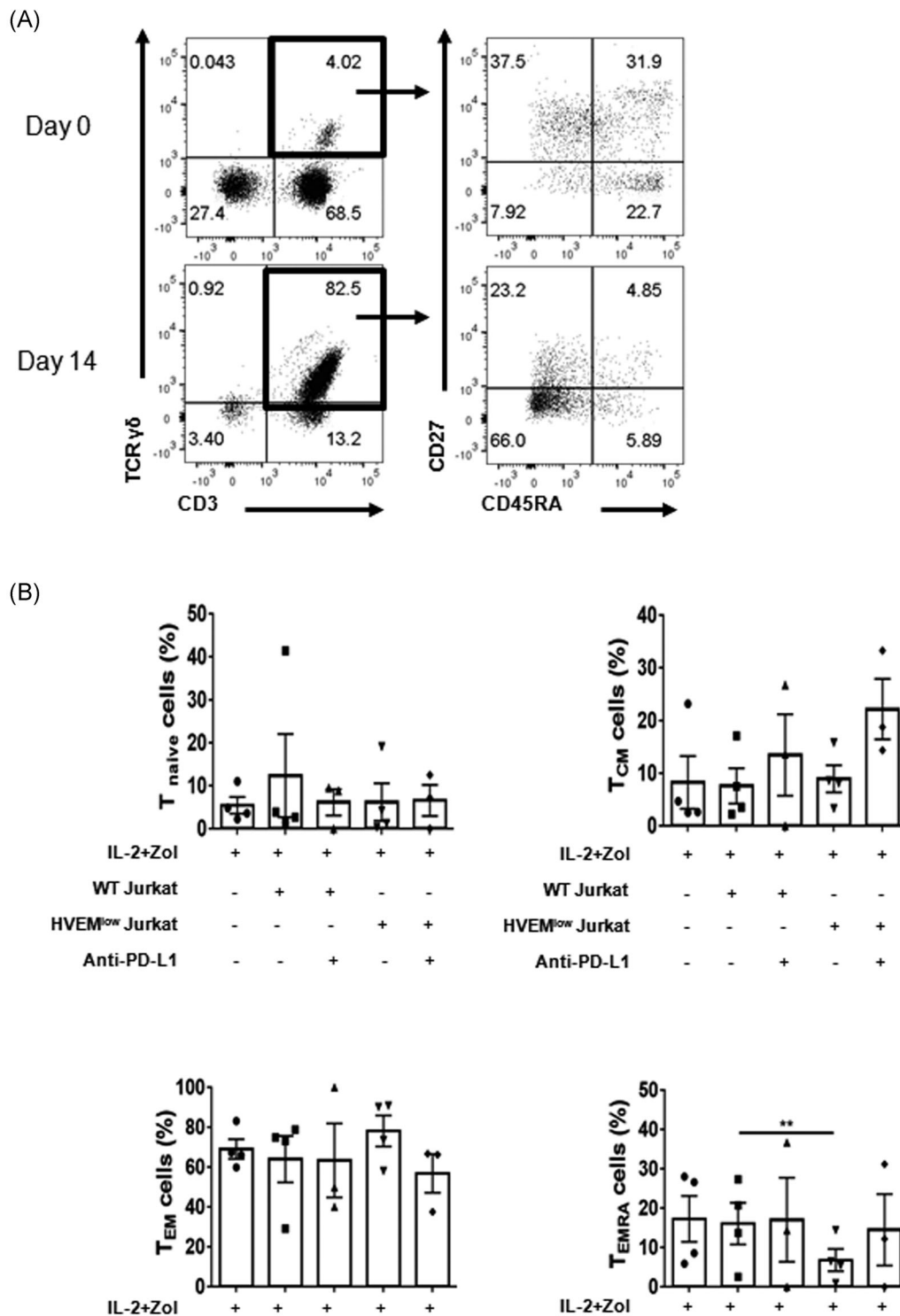


FIGURE 2 Blocking the BTLA/HVEM interaction reduces the terminally differentiated $\gamma\delta$ T cell subset. (A) PBMC from healthy donors expanded in the presence of IL-2 and zoledronate (Zol) for 14 days. Representative dot plots showing the phenotype of baseline or in vitro expanded cells (left panels) and the CD45RA and CD27 expression of the $\gamma\delta$ T (CD3⁺TCR $\gamma\delta$ ⁺) cells (right panels). Representative data are shown out of three to four independent experiments using distinct donors. (B) PBMC were activated in the indicated culture condition for 14 days. The frequency of differentiation subsets in the in vitro expanded $\gamma\delta$ T cells. The following subtypes were used for analysis: T naive (CD45RA⁺CD27⁺), T_{CM} (CD45RA⁻CD27⁺), T_{EM} (CD45RA⁻CD27⁻), and T_{EMRA} (CD45RA⁺CD27⁻). Data were shown as mean \pm SEM of three to four independent experiments using distinct donors per groups. BTLA, B- and T-lymphocyte attenuator; HVEM, herpes virus entry mediator; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cell; T_{CM}, central memory T; T_{EM}, effector memory T; T_{EMRA}, terminally differentiated T. Paired *t* test was used (***p* < .01 and *p* = .0093)

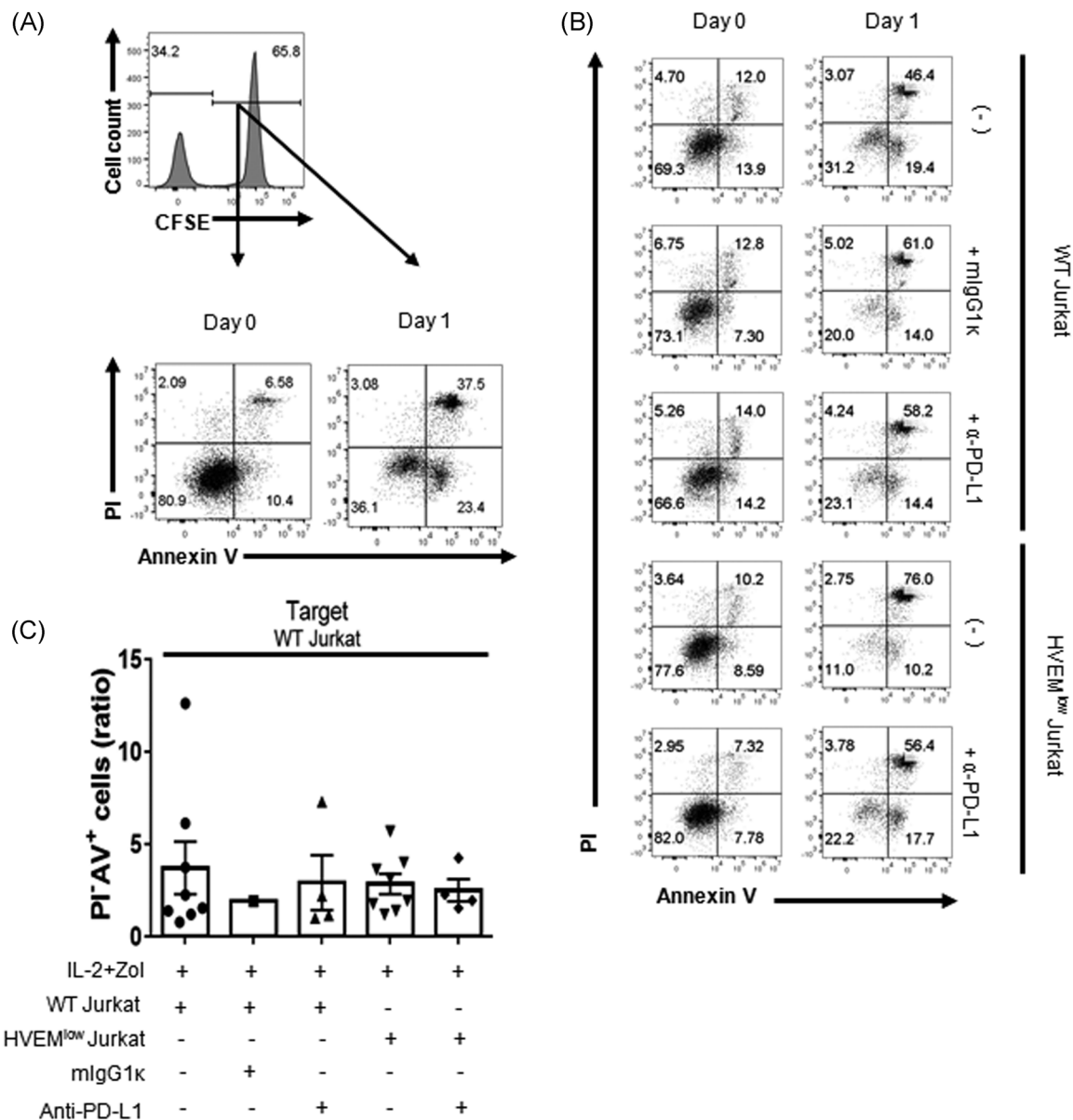


FIGURE 3 The targeted cell death by in vitro expanded $\gamma\delta$ T cells was not altered by the blockade of BTLA/HVEM interaction. WT Jurkat cells were stained with CFSE and incubated with in vitro expanded $\gamma\delta$ T cells at an effector to target cell ratio of 1:1 for 1 day. Cells were harvested and stained with PI and annexin V to detect apoptosis by flow cytometry. (A) Gating strategy to evaluate the PI and annexin V positive cells among the target cells. (B) Representative dot plots showing the proportion of early apoptosis ($PI^-Annexin V^+$) and late apoptosis ($PI^+Annexin V^+$) in the $CFSE^+$ target cells. Left panel indicates the baseline (Day 0) and the right panel indicates after 1 day of incubation. Representative data are shown out of three to seven independent experiments using distinct donors. (C) The percentage of $PI^-Annexin V^+$ apoptotic target cells in the experimental group were normalized by dividing by that of the control group. Data were shown as mean \pm SEM of three to seven independent experiments (donor no. $n = 3-7$). BTLA, B- and T-lymphocyte attenuator; CFSE, carboxyfluorescein succinimidyl ester; HVEM, herpes virus entry mediator; mIgG1k, isotype control; PI, propidium iodide; WT, wild-type. Paired t test was used ($*p < .05$ and $p = .0471$)

presence of BTLA/HVEM blocking peptides and/or anti-PD-L1 Ab to measure intracellular IFN- γ . Blocking PD-1 signaling clearly increased IFN- γ production, whereas that of BTLA had only minor effects. In summary, the reduction of BTLA signaling during expansion did not affect the function of $\gamma\delta$ T cells.

3.4 | BTLA and PD-1 signaling are independent of each other in $\gamma\delta$ T cells

BTLA and PD-1 control cell proliferation and apoptosis through downstream molecules, such as AKT, ERK, and SHP-2, which mediate T cell activating and

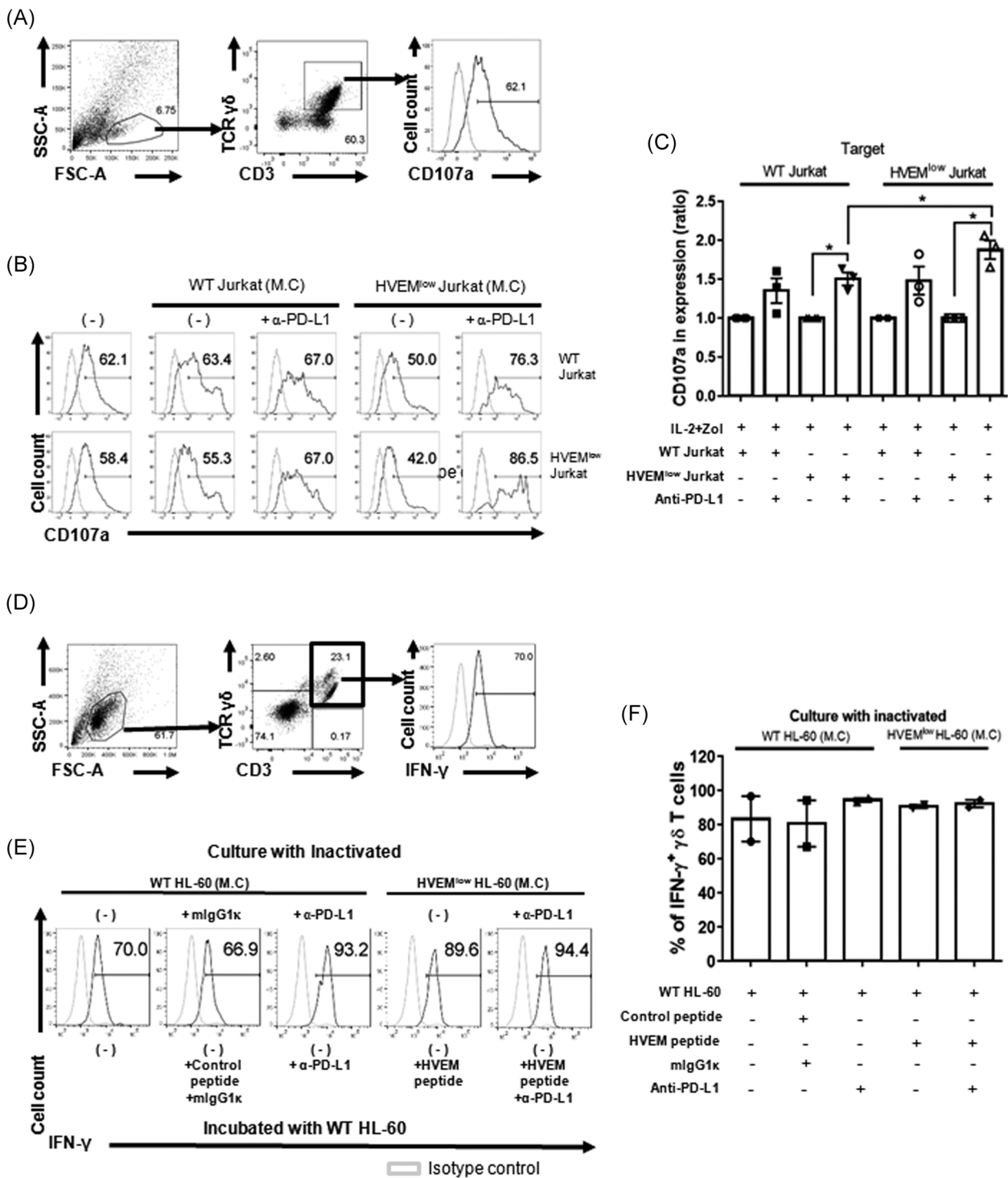


FIGURE 4 Blockade of the BTLA/HVEM signaling does not affect the degranulation of in vitro expanded $\gamma\delta$ T cells. (A) Representative histograms showing CD107a expression in the $\gamma\delta$ T cells. Gray lines indicate the isotype control. (B) Cells were expanded in the indicated culture condition for 14 days. In the presence of anti-human CD107a mAb, in vitro expanded $\gamma\delta$ T cells with the indicated inactivated cells (on top) were incubated overnight with WT Jurkat (upper panel) or HVEM^{low} Jurkat (lower panel) cells. Representative data are shown out of three to four independent experiments using distinct donors. (C) The percentages of CD107a⁺ $\gamma\delta$ T cells in the anti-PD-L1 treated group were normalized by dividing by that of the untreated group. Data were shown as mean \pm SEM of three to four independent experiments using distinct donors. Paired *t* test was used (**p* < .05). Each *P* value is .0190, .0345, and .0103 from left to right. (D) The gating strategy to identify intracellular IFN- γ is depicted. (E) Expanded $\gamma\delta$ T cells with the indicated inactivated cells (on top) were incubated overnight with WT Jurkat cells in the presence of BTLA/HVEM blocking peptides and anti-PD-L1 Ab or adequate controls for IFN- γ intracellular staining. (F) The percentages of IFN- γ ⁺ $\gamma\delta$ T cells were summarized. *N* = 2. BTLA, B- and T-lymphocyte attenuator; HVEM, herpes virus entry mediator; IFN- γ , interferon; mAb, monoclonal antibody; PD-L1, programmed death-ligand 1; WT, wild-type

inhibitory signalings, respectively. Thus, we next investigated the effects of immediate BTLA/HVEM and PD-1/PD-L1 signaling on the phosphorylation of AKT, ERK, and SHP-2 in resting $\gamma\delta$ T cells. PBMCs were

incubated with WT Jurkat or HVEM^{low} Jurkat cells and/or anti-PD-L1 mAb in the presence of IL-2 and Zol for 20 min, and the expression of the phosphorylated protein was evaluated in $\gamma\delta$ T cells by flow cytometry.

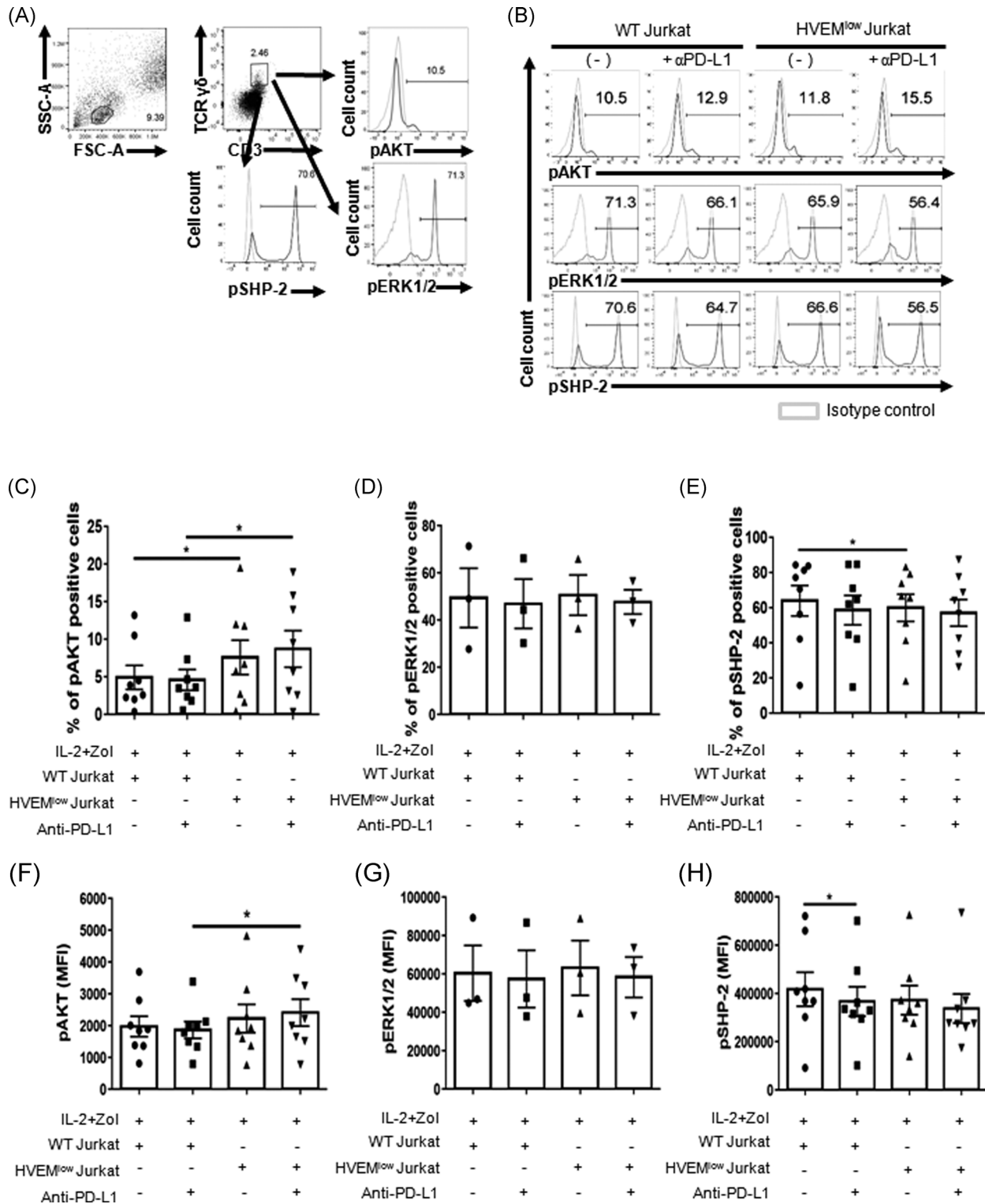


FIGURE 5 (See caption on next page)

We observed that the phosphorylation of AKT was increased slightly by blockade of the PD-1/PD-L1 interaction, and a reduction of BTLA/HVEM signaling in addition to it further increased pAKT, which was statistically significant in the percentages as well as MFI (Figures 5C and 5F). Likewise, cocubation with HVEM^{low} Jurkat cells also increased pAKT, compared with that of WT Jurkat cells. The activation of ERK was not changed in the presence of IL-2 and Zol (Figures 5D and 5G). The induction of pSHP-2 was slightly repressed by anti-human PD-L1 treatment in MFI, and inhibition of the BTLA/HVEM interaction reduced the percentage of pSHP-2⁺ $\gamma\delta$ T cells. However, BTLA/HVEM and PD-1/PD-L1 signaling had no synergistic or additive effects on the phosphorylation of SHP-2 (Figures 5E and 5H).

4 | DISCUSSION

Previous research on the individual signals of BTLA and PD-1 in circulating $\gamma\delta$ T cells has been conducted,^{29,32} but there are few reports on the interactions of these signals. In this study, the effects of the BTLA/HVEM and PD-1/PD-L1 inhibitory signaling pathways were evaluated on the proliferation and effector functions of human peripheral $\gamma\delta$ T cells, in particular V γ 9V δ 2 T cells. We found that BTLA/HVEM blockade could improve the efficient expansion of human peripheral $\gamma\delta$ T cells without compromising $\gamma\delta$ T cell function. These results could be utilized for the development of $\gamma\delta$ T cell therapy for leukemia patients. In addition, the results suggest that the BTLA and PD-1 signaling pathways independently act on the proliferation and cytotoxicity of $\gamma\delta$ T cells.

First, we found that coculture with inactivated leukemia cells hampered the expansion of human peripheral $\gamma\delta$ T cells severely. This may explain the insufficient elimination of tumor cells by $\gamma\delta$ T cells in

vivo in patients with leukemia, where BTLA on PB $\gamma\delta$ T cells can come into contact with HVEM on leukemic cells. Cocubation with WT Jurkat cells slightly increased the proportion of CD27⁺CD45RA⁺ naïve T cells, which are known to be associated with GVHD.³⁶ The proliferation capacity of $\gamma\delta$ T cells was upregulated by blocking the HVEM/BTLA interaction without reducing their cytotoxicity, whereas inhibition of PD-1/PD-L1 signaling induced degranulation of $\gamma\delta$ T cells. We chose to inhibit the BTLA and PD-1 ligands on the target cells, rather than BTLA and PD-1 directly because we were concerned that the direct blockade of BTLA and PD-1 might unexpectedly affect the proliferation and function of $\gamma\delta$ T cells during expansion. However, BTLA also binds to the HCMV UL144 protein to limit lymphocyte-induced inflammation.³⁷ Potential interactions of BTLA with other ligands might explain the partial inhibition of downstream molecules in this study. In addition, we cannot exclude the possibility that Fc receptors on the $\gamma\delta$ T cells might play a stimulating role in response to anti-PD-L1 mAb. Thus, we confirmed the results with BTLA/HVEM blocking peptides and anti-PD-1 mAb and with HVEM^{low} HL-60 cells, which also improved $\gamma\delta$ T cell expansion.

In previous reports, both PD-1 and BTLA inhibit the proliferation of T cells.^{24,38} Moreover, BTLA negatively controls the homeostasis of $\gamma\delta$ T cells³⁹ and inhibits phospho-antigen-induced $\gamma\delta$ T cell proliferation.²⁹ Consistent with previous reports, HVEM-deleted Jurkat cells enhanced the proliferation capacity of $\gamma\delta$ T cells more than WT Jurkat cells. However, an anti-PD-L1 blockade alone does not have a proliferative effect on $\gamma\delta$ T cells. Taken together, the expansion of $\gamma\delta$ T cells is tightly regulated by BTLA rather than PD-1.

It should be mentioned that the deletion of the *HVEM* gene did not affect endogenous PD-L1 expression on Jurkat cells and HL-60 cells and that blocking the BTLA and PD-1 signaling pathways did not affect each other's expression on $\gamma\delta$ T cells either. Although $\gamma\delta$ T cells can

FIGURE 5 BTLA/HVEM and PD-1/PD-L1 signals do not additively activate the downstream molecules. Phosphorylation of AKT, ERK1/2, and SHP-2 in $\gamma\delta$ T cells was measured by flow cytometry. WT or HVEM^{low} Jurkat cells were pretreated with anti-human PD-L1 antibody for 30 min. In the presence of pervanadate, PBMCs were stimulated with IL-2 and zoledronate and cocubated with indicated cells for 20 min. After fixation/permeabilization by PFA and methanol, the cells were stained with anti-human CD3, TCR $\gamma\delta$, phospho-AKT, ERK, and SHP-2 Abs. (A) The gating strategy was depicted. (B) Representative histograms are shown. (C, F) The percentages and mean fluorescence indices of pAKT. Data were shown as mean \pm SEM of eight to nine independent experiments. (D, G). The percentages and mean fluorescence indices of pERK1/2. Data were shown as mean \pm SEM of three independent experiments. (E, H) The percentages and mean fluorescence indices of pSHP-2. Data were shown as mean \pm SEM of eight to nine independent experiments. BTLA, B- and T-lymphocyte attenuator; HVEM, herpes virus entry mediator; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; SHP, src homology region 2 domain-containing phosphatase; WT, wild-type. Paired *t* test was used (**p* < .05). Individual *p* values as below: (C) *p* = .0384 and 0.0311 from left to right; (E) *p* = .0480; (F) *p* = .0307; (H) *p* = .0377

transiently upregulate PD-L1 expression,⁴⁰ PD-L1 do not have signaling domains in its short cytoplasmic tail.⁴¹ Thus, it is unlikely blocking PD-L1 on $\gamma\delta$ T cells could affect the results in this study. In one study, the expression of BTLA was downregulated by IL-2 and bromohydrin pyrophosphate after 4 days, whereas the expression of PD-1 was upregulated.²⁹ However, we observed a significant increase in the expression of BTLA and PD-1 in $\gamma\delta$ T cells expanded with IL-2 and Zol 10 days. It has previously been reported that the degranulation and cytotoxicity of PD-1⁺ $\gamma\delta$ T cells were reduced in response to PD-L1-expressing target cells, but they were recovered by anti-PD-L1 treatment.³² Similarly, we observed that inhibition of sustained PD-1 signaling increased CD107a degranulation of $\gamma\delta$ T cells. However, it was insufficient to cause enhanced cell death in the target cells.

PD-1 upregulates the expression of SHP-2 in tumor-infiltrating lymphocytes,⁴² and inhibition of PD-1 signaling suppresses the phosphorylation of SHP-2.⁴³ Likewise, pSHP-2 was slightly less induced by inhibition of PD-1 signaling against WT Jurkat cells in our system. In addition, SHP-2 is associated with the phosphorylation of BTLA by TCR engagement.²⁴ We observed that the specific inhibition of the BTLA/HVEM and PD-1/PD-L1 pathways in $\gamma\delta$ T cells reduced phosphorylation of SHP-2 in percentages or MFI, but the combined inhibition did not show any additional effect. Reduction of the BTLA/HVEM or PD-1/PD-L1 signal in $\gamma\delta$ T cells inhibited the induction of pSHP-2 in response to Jurkat cells, whereas phosphorylation of AKT was increased by the reduction of both BTLA/HVEM and PD-1/PD-L1 signals. As immune checkpoint receptors, BTLA and PD-1 signaling appear to play independent roles in inhibiting $\gamma\delta$ T cell proliferation and cytotoxicity, respectively, and they had no additive effect on each other. In a recent report, BTLA and PD-1 regulate T cell signaling differentially and only partially through SHP-1 and SHP-2,⁴⁴ that supports our study. In Terms of activating signaling, dual inhibition of BTLA and PD-1 increased pAKT, compared with that of BTLA single blockade, implying the importance of BTLA signaling in $\gamma\delta$ T cells.

In conclusion, BTLA/HVEM inhibitory signaling is a crucial factor in proliferation in ex vivo expanded V δ 2 $\gamma\delta$ T cells. On the contrary, PD-1/PD-L1 signaling could provide partial enhancement in proliferation and cytotoxicity. These results indicate the importance of BTLA signaling in $\gamma\delta$ T cells and suggest that simultaneous suppression of both signals could effectively improve immunotherapy with $\gamma\delta$ T cells. Furthermore, the results of this study could lead to a better understanding of the interactions between BTLA/HVEM and PD-1/PD-L1 signaling in tumor microenvironments.

ACKNOWLEDGMENTS

We thank Dr. D.S. Choi at the Flow Cytometry Core Facility, Research Development Support Center, Asan Medical Center. This study was supported by grants from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Korea (Grant No. 1520060) and from the National Research Foundation of Korea, Ministry of Science and ICT, Korea (Grant No. 2018R1C1B6008852). Sang-Hyun Hwang and Nayoung Kim were supported by intramural grants from the Asan Institute for Life Sciences, Asan Medical Center (Grant Nos. 2019IP0837 and 2020IP0002).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Ho J. Im and Nayoung Kim conceived, designed, and discussed the research. Eun S. Choi, Sung H. Kang, and Kyung-Nam Koh provided samples and analyzed the results. Hyun J. Hwang, Jae J. Lee, Seongsoo Jang, Sang-Hyun Hwang, and Nayoung Kim conducted the research and analyzed the results. Kyung-Nam Koh, Sang-Hyun Hwang, and Nayoung Kim obtained the grants. Hyun J. Hwang and Nayoung Kim wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Nayoung Kim  <http://orcid.org/0000-0001-8598-0068>

REFERENCES

- Chien YH, Meyer C, Bonneville M. gammadelta T cells: first line of defense and beyond. *Annu Rev Immunol*. 2014;32:121-155.
- Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol*. 2013;13:88-100.
- Kabelitz D, Glatzel A, Wesch D. Antigen recognition by human gammadelta T lymphocytes. *Int Arch Allergy Immunol*. 2000;122:1-7.
- Thurnher M, Nussbaumer O, Gruenbacher G. Novel aspects of mevalonate pathway inhibitors as antitumor agents. *Clin Cancer Res*. 2012;18:3524-3531.
- Bonneville M, Scotet E. Human Vgamma9Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. *Curr Opin Immunol*. 2006;18:539-546.
- Qin G, Mao H, Zheng J, et al. Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. *J Infect Dis*. 2009;200:858-865.

7. Xiang Z, Liu Y, Zheng J, et al. Targeted activation of human Vgamma9Vdelta2-T cells controls epstein-barr virus-induced B cell lymphoproliferative disease. *Cancer Cell*. 2014;26:565-576.
8. Santolaria T, Robard M, Leger A, Catros V, Bonneville M, Scotet E. Repeated systemic administrations of both amino-bisphosphonates and human Vgamma9Vdelta2 T cells efficiently control tumor development in vivo. *J Immunol*. 2013;191:1993-2000.
9. Gertner-Dardenne J, Castellano R, Mamessier E, et al. Human Vgamma9Vdelta2 T cells specifically recognize and kill acute myeloid leukemic blasts. *J Immunol*. 2012;188:4701-4708.
10. Lamb LS, Jr., Lopez RD. gammadelta T cells: a new frontier for immunotherapy? *Biol Blood Marrow Transplant*. 2005;11:161-168.
11. Choi ES, Im HJ, Kim H, et al. Depletion of alphabeta(+) T cells for a haploidentical hematopoietic stem cell transplantation in children. *J Clin Apher*. 2018;33:521-528.
12. Bertaina A, Zorzoli A, Petretto A, et al. Zoledronic acid boosts gammadelta T-cell activity in children receiving alphabeta(+) T and CD19(+) cell-depleted grafts from an HLA-haploidentical donor. *Oncoimmunology*. 2017;6:e1216291.
13. Koh KN, Im HJ, Kim H, et al. alphabeta T-cell-depleted haploidentical hematopoietic cell transplantation and zoledronate/interleukin-2 therapy in children with relapsed, high-risk neuroblastoma. *Bone Marrow Transplant*. 2019;54:348-352.
14. Handgretinger R, Schilbach K. The potential role of gammadelta T cells after allogeneic HCT for leukemia. *Blood*. 2018;131:1063-1072.
15. Sebestyen Z, Prinz I, Dechanet-Merville J, Silva-Santos B, Kuball J. Translating gammadelta (gammadelta) T cells and their receptors into cancer cell therapies. *Nat Rev Drug Discovery*. 2020;19:169-184.
16. Zhang Q, Vignali DA. Co-stimulatory and co-inhibitory pathways in autoimmunity. *Immunity*. 2016;44:1034-1051.
17. Dong Y, Sun Q, Zhang X. PD-1 and its ligands are important immune checkpoints in cancer. *Oncotarget*. 2017;8:2171-2186.
18. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 2000;192:1027-1034.
19. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol*. 2015;15:486-499.
20. Jiang Y, Li Y, Zhu B. T-cell exhaustion in the tumor micro-environment. *Cell Death Dis*. 2015;6:e1792.
21. Pauken KE, Wherry EJ. SnapShot: T cell exhaustion. *Cell*. 2015;163:1038.
22. Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin Cancer Res*. 2013;19:1021-1034.
23. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3—potential mechanisms of action. *Nat Rev Immunol*. 2015;15:45-56.
24. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol*. 2003;4:670-679.
25. Chemnitz JM, Lanfranco AR, Braunstein I, Riley JL. B and T lymphocyte attenuator-mediated signal transduction provides a potent inhibitory signal to primary human CD4 T cells that can be initiated by multiple phosphotyrosine motifs. *J Immunol*. 2006;176:6603-6614.
26. Gavrieli M, Watanabe N, Loftin SK, Murphy TL, Murphy KM. Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of B and T lymphocyte attenuator required for association with protein tyrosine phosphatases SHP-1 and SHP-2. *Biochem Biophys Res Commun*. 2003;312:1236-1243.
27. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. 2013;13:227-242.
28. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol*. 2004;173:945-954.
29. Gertner-Dardenne J, Fauriat C, Orlanducci F, et al. The co-receptor BTLA negatively regulates human Vgamma9Vdelta2 T-cell proliferation: a potential way of immune escape for lymphoma cells. *Blood*. 2013;122:922-931.
30. Kang SH, Hwang HJ, Yoo JW, et al. Expression of immune checkpoint receptors on T-cells and their ligands on leukemia blasts in childhood acute leukemia. *Anticancer Res*. 2019;39:5531-5539.
31. Gertner-Dardenne J, Fauriat C, Olive D. BTLA, a key regulator of Vgamma9Vdelta2 T-cell proliferation. *Oncoimmunology*. 2013;2:e25853.
32. Iwasaki M, Tanaka Y, Kobayashi H, et al. Expression and function of PD-1 in human gammadelta T cells that recognize phosphoantigens. *Eur J Immunol*. 2011;41:345-355.
33. Spodzieja M, Kuncewicz K, Sieradzian A, et al. Disulfide-linked peptides for blocking BTLA/HVEM-binding. *Int J Mol Sci*. 2020;21:8876.
34. Imbert V, Peyron JF, Farahi Far D, Mari B, Auberger P, Rossi B. Induction of tyrosine phosphorylation and T-cell activation by vanadate peroxide, an inhibitor of protein tyrosine phosphatases. *Biochem J*. 1994;297(pt 1):163-173.
35. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol*. 2012;12:269-281.
36. Triplett BM, Shook DR, Eldridge P, et al. Rapid memory T-cell reconstitution recapitulating CD45RA-depleted haploidentical transplant graft content in patients with hematologic malignancies. *Bone Marrow Transplant*. 2015;50:968-977.
37. Sedy JR, Bjordahl RL, Bekiaris V, et al. CD160 activation by herpesvirus entry mediator augments inflammatory cytokine production and cytolytic function by NK cells. *J Immunol*. 2013;191:828-836.
38. Sheppard KA, Fitz LJ, Lee JM, et al. PD-1 inhibits T-cell receptor-induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett*. 2004;574:37-41.
39. Bekiaris V, Sedy JR, Macauley MG, Rhode-Kurnow A, Ware CF. The inhibitory receptor BTLA controls gammadelta T cell homeostasis and inflammatory responses. *Immunity*. 2013;39:1082-1094.

40. Peters C, Oberg HH, Kabelitz D, Wesch D. Phenotype and regulation of immunosuppressive Vdelta2-expressing gamma-delta T cells. *Cell Mol Life Sci.* 2014;71:1943-1960.
41. Akinleye A, Rasool Z. Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *J Hematol Oncol.* 2019;12:92.
42. Li J, Jie HB, Lei Y, et al. PD-1/SHP-2 inhibits Tc1/Th1 phenotypic responses and the activation of T cells in the tumor microenvironment. *Cancer Res.* 2015;75:508-518.
43. Yamamoto R, Nishikori M, Kitawaki T, et al. PD-1-PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. *Blood.* 2008;111:3220-3224.
44. Xu X, Hou B, Fulzele A, et al. PD-1 and BTLA regulate T cell signaling differentially and only partially through SHP1 and SHP2. *J Cell Biol.* 2020;219:e201905085.

SUPPORTING INFORMATION

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

How to cite this article: Hwang HJ, Lee JJ, Kang SH, et al. The BTLA and PD-1 signaling pathways independently regulate the proliferation and cytotoxicity of human peripheral blood $\gamma\delta$ T cells. *Immun Inflamm Dis.* 2020;1-14.
<https://doi.org/10.1002/iid3.390>