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이학박사 학위논문

# BTLA와 PD-1 신호 전달 경로에 의한 인간 말초 혈액 γδ T 세포의 증식과 세포 독성의 개별적인 조절

BTLA and PD-1 signaling pathways regulate independently proliferation and cytotoxicity of human peripheral blood γδ T cells

울 산 대 학 교 대 학 원

의 학 과

황 현 주

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이 논문을 이학박사학위 논문으로 제출함

**2021 년 08 월**

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## Contents

**Abstract** ····· iv

**Introduction** ····· 1

**Materials and Methods** ····· 13

Human subjects ····· 13

Isolation of peripheral blood mononuclear cells (PBMCs) ····· 13

Generation of HVEM$^{low}$ tumor cell lines ····· 14

Flow cytometry ····· 16

Expansion of γδ T cells ····· 17

Cell proliferation assay ····· 18

Cell death assay ····· 19

Degranulation assay ····· 20

Intracellular staining ····· 21

Detection of phosphoprotein ····· 21

Statistics ····· 23

**Results** ····· 24

BTLA expression is sustained in peripheral blood γδ T cells of ALL and AML patients · 24

BTLA signaling plays a critical role in proliferation of γδ T cells ····· 27

The blockade of BTLA/HVEM and PD-1/PD-L1 signaling does not affect both BTLA and PD-1 expression in *in vitro* expanded γδ T cells ····· 35

Blockade of BTLA signaling reduces the more differentiated γδ T cell population ····· 39

The antitumor activity of γδ T cells is not affected by inhibition of BTLA signaling ····· 42

PD-L1 signaling regulates the degranulation of γδ T cells ····· 45

The IFN-γ production of γδ T cells is not affected by blockade of BTLA signaling ····· 50

BTLA and PD-1 signaling pathways are independent of each other in γδ T cells ····· 52

**Discussion** ····· 56

**Reference** ····· 66

국문요약 ····· 84

# 그림 목차

**Figure 1.** PD-1 and BTLA expression in γδ T cells from childhood leukemia patients ··25

**Figure 2.** Blocking BTLA/HVEM interaction increases γδ T cell proliferation ·····30

**Figure 3.** Co-culture with HVEM$^{low}$ Jurkat cells does not affect BTLA and PD-1 expression in γδ T cells ·····37

**Figure 4.** Blockade of BTLA/HVEM interaction represses the terminal differentiation of γδ T cells ·····40

**Figure 5.** The target cell death by *in vitro* expanded γδ T cells was not altered by the blockade of BTLA/HVEM signaling ·····43

**Figure 6.** Blockade of the BTLA/HVEM signaling does not affect the degranulation of in vitro expanded γδ T cells ·····47

**Figure 7.** Effects of the BTLA/HVEM and PD-1/PD-L1 signals on IFN-γ production in γδ T cells ·····51

**Figure 8.** Effects of blocking the BTLA/HVEM and PD-1/PD-L1 signals on the downstream signaling in resting γδ T cells ·····54

**Abstract**

**Background:** B- and T-lymphocyte attenuator (BTLA) and programmed cell death-1 (PD-1) inhibit gamma-delta (γδ) T cell homeostasis and activation. This study was set up to examine whether BTLA and PD-1 signaling pathways were convergent or independent in human peripheral blood γδ T cells, where Vγ9Vδ2 T cells are majority.

**Materials & methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from adult healthy volunteers (HV) and patients with pediatric B-cell acute lymphoblastic leukemia (B-ALL) and acute myeloid leukemia (AML). CRISPR-Cas9 system was used to generate the herpesvirus entry mediator (HVEM)-deleted Jurkat or HL-60 cell lines. PBMCs and inactivated WT or $HVEM^{low}$ tumor cells were co-cultured in the presence of interleukin (IL)-2 and zoledronate to induce BTLA/HVEM and PD-1/ PD-ligand 1 (PD-L1) signaling during expansion. The cell surface markers on those cells were analyzed by flow cytometry. The intracellular IFN-γ, and induction of phosphoproteins, such as protein kinase B (AKT), extracellular signal-regulated kinases (ERK) 1/2, and src homology region 2-containing protein tyrosine phosphatase 2 (SHP-2), were also assessed by flow cytometry. Additionally,

flow cytometry was used to evaluate the cell proliferation, degranulation, and cytotoxicity of.

**Results:** PD-1 was extensively expressed in γδ T cells from patients with B-ALL, but not AML. However, the BTLA was similarly expressed in γδ T cells of three groups, suggesting an essential role of BTLA on γδ T cells. Proliferation of γδ T cells was increased when PBMCs were co-cultured with inactivated $HVEM^{low}$ tumor cells compared with WT tumor cells. The cytotoxicity of the expanded γδ T cells was not affected by co-culture with inactivated $HVEM^{low}$ Jurkat cells and was further increased in the presence of anti-PD-L1 monoclonal antibody (mAb). The inhibition of BTLA or PD-1 signaling increased the phosphorylation of AKT, whereas repressed phosphorylation of SHP-2 in γδ T cells. However, there were no synergistic or additive effects by a combined inhibition.

**Conclusion:** Herein, I demonstrate that the interactions between BTLA/HVEM and between PD-1/PD-L1 regulated proliferation and cytotoxicity of human peripheral γδ T cells, respectively. The results suggest that inactivation of BTLA/HVEM signaling pathway during expansion could help produce more γδ T cells without compromising cytotoxicity.

## Introduction

Gamma-delta (γδ) T cells have been in the limelight, as potential immunological therapeutic targets against a dynamic array of diseases. γδ T cell is the unconventional T lymphocyte and accounts for 1–10% of circulating T cells (1). It has characteristics that distinguish it from conventional αβ T cells, which occupy most of T cells, in the context of cell biology and function. (2, 3). They act as a bridge between innate and adaptive immune responses and recognize pathogens and tumor cells (4). They could participate in the early phase of infection and tumorigenesis through T cell receptor (TCR), natural killer group 2 member D (NKG2D), CD16, and toll like receptor (TLR), and induce antibody-dependent cellular cytotoxicity (ADCC) (3). Furthermore, they kill target cells through cytolysis by granzyme/perforin and tumor necrosis factor related apoptosis-inducing ligand (TRAIL), Fas/Fas ligand pathways, and cytokine secretion, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (5, 6). Unlike αβ T cells, γδ T cells do not have antigen specificity as they do not require major histocompatibility complex (MHC) molecules for antigen recognition, suggesting they are potent candidates for immunotherapy targeting the tumor

with neoantigens and allogeneic tumor cells (7). Indeed, γδ T cells exert potent cytotoxicity against various tumor types, including hematopoietic malignancies (8).

There are main subsets in human γδ T cells, distinguished by TCR Vδ chain expression; Vδ1 and Vδ2. Vδ1 T cells, which are paired with Vγ2, 3, 4, 5, or 8 chains, account for 10-30% of peripheral blood γδ T cells, whereas Vδ2 T cells, which are almost associated with Vγ9 chains, occupy the majority of peripheral blood γδ T cells (9). The Vδ1 T cells are mainly residing in the tissues, such as skin and colon, and recognize the stress-related antigens. Most Vδ2 T cells are present in the peripheral blood and recognize the nonpeptidic phospho-antigens (PAgs) (10). Most studies focused on Vδ2 T cells rather than Vδ1 T cells because they are readily obtained from peripheral blood and can be expanded from PBMCs with high efficiency *ex vivo* using PAgs and interleukin (IL)-2 (11). The large amount of Vδ2 T cells can be expanded upon short-term culture and they have no alloreactivity (12). Several studies have demonstrated that expanded Vδ2 T cells by aminobisphosphonates (NBPs) have increased anti-tumor response *in vitro* and in preclinical

*in vivo* models, suggesting potent availability of it in clinical cancer immunotherapy (13, 14). The mechanism of activating Vδ2 T cells is better known than that of Vδ1 T cells.

Human Vγ9Vδ2 T cells are a unique subtype in human peripheral blood, which require cell-to-cell interaction in reactivity and are directly activated by recognizing PAgs, through their TCR (7). Through the mevalonate isoprenoid synthesis pathway, intracellular PAgs accumulate in infected or tumor transformed mammalian cells. NBPs, such as zoledronate, lead to the accumulation of isopentenyl pyrophosphates (IPP) by inhibiting farnesyl-pyrophosphate synthase, resulting in selective activation of Vγ9Vδ2 T cells [9, 10]. They are not activated under physiological conditions but activated by recognizing the increased level of IPP, which is the metabolites of the mevalonate pathway, resulting in exerted cytotoxicity against the tumor, microbiome, and viruses (15-17).

There are two strategies of Vδ2 T cell-based cancer immunotherapy (18). First, a systemic administration of IL-2 and either an IPP analog or NBPs that directly induces stimulation and expansion of Vδ2 T cells. Second, adoptive transfer of *ex vivo* expanded Vδ2 T cells by synthetic PAgs or NBPs. The two strategies have been applied in clinical trials,

which target not only hematopoietic malignancy but also solid tumors (19-23). Repeated and combined administration of NBP and human Vγ9Vδ2 T cells have been reported to effectively control tumor growth in a xenograft model, suggesting the potential availability of a combination of the two strategies (24).

Multiple methods have been established for the adoptive transfer of γδ T cells from PBMCs (25). Bromohydrin pyrophosphate (BrHPP) and zoledronate are synthetic drugs commonly used for *in vitro* and *in vivo* expansion of Vδ2 T cells in combination with exogenous IL-2. Zoledronate is a drug that has been used as a therapeutic agent for osteoporosis. Thus, its safety has been proven clinically. It has been reported that after zoledronate infusion into patients, differentiation, and cytotoxicity of γδ T-cells are enhanced by sensing increased IPP levels (9). Furthermore, following treatment of IL-2 and zoledronate, Vγ9Vδ2 T cells from patients with chronic myelogenous leukemia (CML) have autologous and allogeneic antileukemic activity, both *in vitro* and *in vivo* (26).

Allogeneic stem cell transplantation is one of the standard therapies for patients with hematologic malignancies, which evoke graft-versus-leukemia (GVL) effect by the new

donor-derived immune system (27). However, there is a possibility that αβ T cells from graft contribute to the development of graft-versus-host disease (GVHD) (28). It has been proved that the depletion of αβ T cells is safe and effective for haploidentical hematopoietic stem cell transplantation (HSCT) (29). In particular, one report revealed that the disease-free survival was higher in leukemia patients, who received αβ T-cell-depleted bone marrow transplants with increased frequency of γδ T cells (30). These reports provide a possibility that the remaining γδ T and NK cells, which are not alloreactive, are the main inducers of anti-infectious and anti-tumor responses to prevent life-threatening infectious disease and leukemia relapse. Human Vγ9Vδ2 T cells are a promising candidate for immunotherapy as they can directly kill leukemic cells (31) without causing GVHD (32). Clinical studies are underway to activate and expand γδ T cells by administering zoledronate after αβ T cell depleted HSCT to obtain optimal therapeutic effect (33-35). In addition, co-infusion of αβ T cell-depleted grafts and γδ T cells expanded from donors could be an attractive option (27). It is hence necessary to establish optimal methods which efficiently enhance the proliferation and activation of Vγ9Vδ2 T cells, and to understand underlying mechanisms thereof. The

activation and differentiation status of γδ T cells, that are regulated by surface receptors, including TCR, costimulatory receptors, and inhibitory receptors, and downstream intracellular signals, are related to the efficiency of γδ T cell-based immunotherapies (36). Among the surface receptors, inhibitory receptors negatively regulate the effector function and expansion of γδ T cells to control homeostasis. Co-inhibition signaling is one of the regulatory mechanisms that inhibit excessive T cell activation, which causes autoimmunity. (37). However, it also causes tumor immune escape in the tumor microenvironment by suppressing T-cell-mediated antitumor response. Consequently, immune checkpoint inhibitors have been developed to enhance the antitumor response by reinvigorating T cells from dysfunctional exhausted T cells (38). The blockade of inhibitory pathways by immune checkpoint inhibitors is an effective strategy to enhance the T cell responses against tumors. Thus, combinations of immune therapies, for example adoptive transfer of immune cells in combination with immune checkpoint inhibitors, may be useful in the treatment of malignancies. Several reports revealed that blockades of immune checkpoint, such as

programmed cell death-1 (PD-1; CD279) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; CD152) after adoptive cell transfer, can enhance anti-tumor response (39).

PD-1 is one of the well-known immune checkpoint molecules, which is expressed on T, B, NKT, and monocytes. Engagement with programmed death-ligand 1 (PD-L1; B7-H1; CD274) or PD-L2 (B7-DC) inhibits T cell activation and cytotoxicity (40, 41). PD-L1 is expressed on immune and nonimmune cells, indicating it could be expressed on various types of tumor cells, while PD-L2 is expressed on dendritic cells, macrophages, and germinal center B cells (42). PD-1 is highly expressed in exhausted T cells, which is associated with impaired effector function and increased apoptosis (43-45). Therefore, increasing the anti-tumor response of T cells by PD-1 blockade by anti-PD1 or PD-L1 monoclonal antibody (mAb) has emerged as a promising therapy and has been confirmed in pre-clinical and clinical trials (46-48).

B- and T-lymphocyte attenuator (BTLA; CD272), a member of the CD28 family, is expressed on T, B, and NK cells, like PD-1. It is another immune checkpoint molecule whose expression is transiently upregulated during T cell activation (49). Herpes Virus Entry

Mediator (HVEM; tumor necrosis factor receptor superfamily member 14; TNFRSF14), which expresses on cancer cells, antigen-presenting cells (APCs), and T cells, is a bidirectional switch capable of inducing co-stimulation or co-inhibition signals through their ligand binding (50). Its engagement with BTLA or CD160 induces a co-inhibitory signal, whereas that of LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) or lymphotoxin-α (LTα) induces co-stimulatory signal. HVEM is the most-studied ligand of BTLA, whose engagement with BTLA inhibits proliferation (51) and activation (52) of T cells. Notably, previous results have shown that the *ex-vivo* expansion of tumor-specific T cells from allogeneic stem cell transplant patients was increased by blocking BTLA-HVEM interaction (53), suggesting that BTLA-HVEM interaction is a candidate of novel therapeutic targets. Interestingly, exhausted tumor-infiltrating CD4 T cells show the $BTLA^{+}PD\text{-}1^{+}$ surface phenotype (54). Therefore, combined inhibition of BTLA and PD-1 may enhance the restoration of dysfunctional T cells.

PD-1 and BTLA have several structural and functional similarities and share a

common mechanism to attenuate the T cell activation (55, 56). First, both BTLA and PD-1 contain Ig-like ectodomain that are expressed on T and B cells and present as monomers in T cells. Second, PD-1 and BTLA negatively regulate the function of T cells via conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) and immune-receptor tyrosine-based switch motif (ITSM) signaling motif pathway. After TCR stimulation, ITIM and ITSM in intracellular tail of PD-1 and BTLA are phosphorylated, resulting in recruitment of Src homology 2 (SH2) domain-containing protein-tyrosine phosphatases, such as src homology region 2 domain-containing phosphatase (SHP)-1 or SHP-2. As results, the down-stream molecules of TCR and CD28 such as TCRξ, ξ-chain-associated protein kinase 70 kDa (ZAP70), phosphoinositide 3-kinase (PI3K), and AKT, which is a downstream target of PI3K are dephosphorylated, resulting in reduced proliferation and IL-2 secretion (57).

However, PD-1 and BTLA have distinct regulation pathways in T cells. PD-1 essentially requires ITSM for its inhibitory function (58), while BTLA requires both ITIM and ITSM (59), despite PD-1 and BTLA have both ITIM and ITSM. In addition, BTLA, not PD-1, has a growth factor receptor–bound protein 2 (Grb2) (YXN) signaling motif, which is

absent from other member of CD28 family, in cytoplasmic tail. Moreover, a recent report shows that both SHP-1 and SHP-2 are recruited by BTLA, whereas only SHP-2 is recruited by PD-1 in T cells (60). This observation agrees with earlier studies that SHP-1 is not recruited by PD-1 (61, 62). Other studies reveal that BTLA interaction with its ligand HVEM induces recruitment of both SHP-1 and SHP-2, using co-immunoprecipitation experiments (51, 63).

Since the cytotoxicity of Vγ9Vδ2 T cells is highly dependent on the interaction with other cells in tumor microenvironment, adoptive cell transfer of *ex vivo* expanded Vγ9Vδ2 T cells and combination therapy with immune checkpoint inhibitors, such as anti-PD-1, anti-BTLA or their ligand, might be a promising therapeutic strategy for Vγ9Vδ2 T cells. Repeated administration of BrHPP and low-dose IL-2 induce exhaustion of Vγ9Vδ2 T cells in a nonhuman primate model, implying the needs of immune checkpoint inhibitor in combination with Vγ9Vδ2 T cell-based therapy (64). Circulating γδ T cells express PD-1 at a low level, while they express BTLA constantly. However, during activation by IL-2 and BrHPP, BTLA is down-regulated in Vγ9Vδ2 T cells, whereas PD-1 is upregulated (65). In

addition, it is known that BTLA regulates the proliferation of Vγ9Vδ2 T cells (65, 66), and PD-1 regulates anti-tumor responses (67). These reports suggest that the two receptors play important roles in Vγ9Vδ2 T cells, in the context of homeostasis and function. Nevertheless, the effect of physiological interaction between these two signaling pathways in Vγ9Vδ2 T cells has not been explored. Thus, a better understanding of the combined role and mechanism of these inhibitory molecules in Vγ9Vδ2 T cells are important to improve the effect of Vγ9Vδ2 T cell-based immunotherapy against tumor cells.

In the current study, an *in vitro* cell culture system in which IL-2 and zoledronate drive peripheral blood Vδ2 T cell expansion was used. They were co-cultured with inactivated Jurkat cells in order to interact HVEM and PD-L1 on the surface of Jurkat cells to their respective partner BTLA and PD-1 inhibitory receptors of Vδ2 T cells. Taking advantage of this system, I examined whether BTLA and PD-1 signaling are convergent or independent in γδ T cells, by evaluating proliferation, cytotoxicity, and phosphorylation of downstream molecules, such as AKT, ERK, and SHP-2. BTLA/HVEM inhibitory signaling repressed the proliferation of γδ T cells, but not cytotoxicity, while PD-1/PD-L1 inhibitory

signaling down-regulated the anti-tumor activity of γδ T cells. The inhibition of BTLA signaling increased phosphorylation of AKT, whereas it reduced that of SHP-2, in a PD-1-independent manner. Collectively, these results revealed the role of BTLA and PD-1 signaling that the BTLA and PD-1 signaling pathways regulate proliferation and functionality of γδ T cells independently. Thus, co-blockade of PD-1 and BTLA might enhance the proliferation and functionality, providing a rationale for improving current γδ T-cell-based therapies.

## Materials and methods

### Human subjects

Whole blood samples of healthy adult volunteers (HV) and newly diagnosed patients with pediatric AML and B-cell ALL who visited the Asan Medical Center (Seoul, Republic of Korea) were collected. All the participants provided written informed consent, and the study protocols were approved by the Institutional Review Board (IRB) of the Asan Medical Center (IRB Approval No. 2015-0307 for HV and No. 2018-0445 for patients).

### Isolation of PBMCs

αβ T cell-depleted PBMCs were isolated using CliniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (68). Peripheral blood mononuclear cells (PBMCs) from HV, pediatric AML, and B-cell ALL (69) or αβ T cell-depleted PBMCs from HV were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Milwaukee, WI, USA). In brief, 10 ml of whole blood was layered on equivalent volume of Ficoll-Paque™ without disturbing the surface in 50 ml polypropylene tube

(Corning, NY, USA). It was then centrifugated at 400 × g without brake at 22 ± 2 ℃ for 20 min and the buffy coats were carefully transferred into a fresh tube and washed with phosphate-buffered saline (PBS) (Biosesang, Gyenggi-do, Korea) twice at 500 × g, at 22 ± 2 ℃ for 5 min. The cell number was counted by trypan blue (Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) exclusion method. Cells were immediately frozen in heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and stored in liquid nitrogen until use.

**Generation of HVEM$^{low}$ tumor cell lines**

To delete a HVEM gene from a tumor cell, CRISP/Cas9 technology was applied. In brief, wild-type (WT) Jurkat or HL-60 cells (ATCC, Manassas, VA, USA) were co-transfected with HVEM CRISPR/Cas9 KO plasmid (Santa Cruz Biotechnology, CA, USA) and HVEM HDR plasmid (Santa Cruz Biotechnology). One day prior to transfection, 2.0 × $10^5$ WT Jurkat or HL-60 cells were seeded in a 60 mm dish (Falcon, BD Biosciences) in 3 ml of

antibiotic-free RPMI 1640 growth medium to grow cells to a 40-80% confluency. HVEM CRISPR/Cas9 KO plasmid and HVEM HDR plasmid was mixed at a ratio of 3:1 and diluted with Opti-MEM (Gibco, Thermo Fisher Scientific). Lipofectamine 2000 (Thermo Fisher Scientific) was diluted with Opti-MEM following the manufacturer's instructions. After 5 min, diluted plasmid DNA was added to the diluted transfection reagent and incubated for further 25 min. Prior to transfection, the media of the cell culture was replaced with 3 ml of fresh serum-free growth medium, and the plasmid DNA/transfection reagent complex was added to well dropwise. After 3 hrs, 300 μl of FBS was added to well. Transfected cells were treated with media containing 2 μg/ml puromycin (TOCRIS Bioscience, Bristol, UK) for at least two weeks. The reduction of HVEM expression was confirmed by flow cytometry. The cells expressing lower levels of HVEM were named $HVEM^{low}$ Jurkat or HL-60 cells. Jurkat and HL-60 cell lines were maintained in RPMI 1640 media supplemented with 10% inactivated FBS, 100 IU penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate that is named complete RPMI. All cells were incubated in a humidified incubator in 5% $CO_2$ at 37 °C.

**Flow cytometry**

Frozen PBMCs were thawed and washed twice (500 × g, 22 ± 2 °C, 5 min) with complete RPMI 1640 (Corning) with 55 μM 2-Mercaptoethanol (Gibco). PBMCs or *in vitro* expanded γδ T cells were stained with antibodies as follow; PE-conjugated anti-human TCRγδ (clone MOPC-21, Biolegend, San Diego, CA, USA), APC-H7-conjugated anti-human CD3 (clone SK7, BD Biosciences, San Diego, CA, USA), FITC-conjugated anti-human CD279 (clone EH12.2H7, Biolegend), and APC-conjugated anti-human CD272 (clone MIH26, Biolegend) antibodies. To assess the HVEM and PD-L1 expression level, Jurkat, and HL-60 cells were stained with Alexa Fluor®647-conjugated anti-human HVEM (clone 94801, BD Biosciences) or APC-conjugated anti-human PD-L1 antibody (Ab) (clone 29E.2A3, Biolegend).

For analysis of T cell subsets, antibodies were used as follow; FITC-conjugated anti-human TCRγδ (clone B1.1, eBioscience), APC-H7-conjugated anti-human CD3 (clone SK7, BD Biosciences), PE-conjugated anti-human CD45RA (clone HI100, Biolegend), and APC-conjugated anti-human CD27 (clone O323, eBioscience) antibodies. T cell subsets

were determined by CD45RA and CD27 expression on γδ T cells. Flow cytometry was conducted by a FACS Canto II (BD Biosciences) or a CytoFLEX (Beckman Coulter Life Sciences, Brea, CA, USA). The data was analyzed by a FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Expansion of γδ T cells**

PBMCs were cultured at $6.0 \times 10^5$ cells/well in 24-well plates (Nunc, Naperville, IL, USA) in RPMI 1640 complete media in the presence of 100 U/ml recombinant human IL-2 (rhIL-2) (PeproTech, Rocky Hill, NJ, USA) and 1 μM zoledronate (Selleckchem, Houston, TX, USA). To inactivate Jurkat or HL-60 cells, mitomycin C (Sigma-Aldrich) was treated at 25 μg/ml for 2 hrs and washed away 3 times with 1x PBS. Mitomycin C-treated Jurkat or HL-60 cells were co-cultured at $3.0 \times 10^5$ cells with PBMCs in the presence of LEAF™ Purified Mouse IgG1, κ isotype control Ab (clone MOPC-21, Biolegend) or blocking anti-human PD-L1 mAb (clone 29E.2A3, Biolegend). Anti-human PD-L1 mAb or IgG1, κ isotype control Ab was added at 10 μg/ml every two days for the first 6 days. Fc receptor (FcR) blocking

reagent for human (Miltenyi Biotec, Auburn, CA, USA) was treated on the first day of the culture. Every two days, half of the medium was removed and replaced with fresh complete medium containing 100 U/ml rhIL-2.

**Cell proliferation assay**

For cell proliferation assay, αβ T cell depleted PBMCs were washed twice with 1X PBS. Then, cells were labeled with cell proliferation dye, eFluor™ 670 (EF670) (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. EF670-labeled PBMCs at $3.0 \times 10^5$ cells and mitomycin-treated Jurkat cells at $1.5 \times 10^5$ cells were co-cultured in 96-well plates (NUNC) with anti-human PD-L1 blocking mAb or IgG1, κ isotype control Ab at 10 μg/ml in the presence of 100 U/ml rhIL-2 and 1 μM zoledronate. Blocking anti-human PD-L1 mAb was treated every two days for the first 6 days, and rhIL-2 were added every two days.

In some experiments, EF670-labeled PBMCs at $3.0 \times 10^5$ cells were cultured in 96-well plates (NUNC) with scrambled peptide (Ac-ELCAGPVTRKVECTYGE-NH2, Peptron,

Daejeon, Korea) or HVEM-specific blocking peptide (Ac-YRVKEACGELTGTVCEP-NH2, Peptron) at 1 mg/ml (70). Anti-human PD-1 blocking mAb (clone EH12.2H7, Biolegend) or IgG1, κ isotype control Ab was also treated at 10 μg/ml in the presence of 100 U/ml rhIL-2 and 1 μM zoledronate. Blocking anti-human PD-1 mAb and rhIL-2 were added every other day. Scrambled or HVEM peptides were added every three days. Cultured cells were then treated with FcR blocking reagent for 5 min and stained with PE-conjugated anti-human TCRγδ (clone MOPC-21, Biolegend) and APC-H7-conjugated anti-human CD3 Ab (clone SK7, BD Biosciences) for 30 min. EF670 dilution was acquired by a FACS Canto II or a CytoFLEX flow cytometer and analyzed with a FlowJo software.

**Cell death assay**

Cytotoxicity was analyzed using annexin V (AV) and propidium iodide (PI) assay. WT Jurkat cells were washed and resuspended in PBS. Cells were labeled with CFSE by using Cell Trace Cell Proliferation Kits (Molecular Probes, Eugene, OR, USA). according to the manufacturer's instructions. Expanded γδ T cells were resuspended in RPMI 1640 media and

incubated with CFSE-labeled target cells at a 1:1 of effector:target ratio. After 1 day, cells were washed with AV binding buffer (BD Biosciences) and incubated at 37℃ for 30 min. Cells were treated with FcR blocking reagent for 5 min and stained with anti-AV-APC Ab (BD Biosciences) and PI (Biolegend) on ice for 15 min. Cells gated as $CFSE^{+}$ were analyzed for early and late apoptosis by flow cytometry with a FACS Canto II or a CytoFLEX. The data was analyzed by a FlowJo software.

**Degranulation assay**

Expanded PBMCs were incubated with target cells at a 1:1 of effector:target ratio. Alternatively, PBMCs were incubated with target cells in the presence of anti-human PD-1 blocking mAb or IgG1, κ isotype control Ab. The PerCP/Cy5.5-conjugated anti-human CD107a (clone eBioH4A3, eBioscience, San Diego, CA, USA) mAb was added in the culture. After 1 hr, monensin (eBioscience) was added at 50 μM. Following overnight incubation and washing, cells were treated with FcR blocking reagent for 5 min and stained with FITC-conjugated anti-human TCRγδ (clone B1.1, eBioscience) and APC-H7-

conjugated anti-human CD3 Ab (clone SK7, BD Biosciences) and analyzed by a FACS Canto II or a CytoFLEX flow cytometer and a FlowJo software.

**Intracellular staining**

To detect intracellular IFN-γ, HVEM blocking peptide at 1mg/ml and anti-human PD-L1 mAb at 10 μg/ml were pretreated to WT HL-60 cells. Thirty min later, *in vitro* expanded PBMCs were stimulated with WT HL-60 cells at a 2:1 of the effector:target ratio and incubated for 4 hrs with 1X brefeldin A solution (Biolegend). Cells were treated with FcR blocking reagent for 5 min and stained with FITC-conjugated anti-human TCRγδ (clone B1.1, eBioscience) and APC-H7-conjugated anti-human CD3 Ab (clone SK7, BD Biosciences). After fixed and permeabilized Cytofix/Cytoperm kit (BD), cells were then stained with PE/Cy7-conjugated anti-human IFN-γ Ab (clone 4S.B3, Biolegend) and analyzed by a FACS Canto II or a CytoFLEX flow cytometer and a FlowJo software.

**Detection of phosphoprotein**

Jurkat cells were pre-incubated with anti-human PD-L1 blocking Ab (Biolegend) for 30 min following FcR blocking Ab treatment. Cells were further treated with 20 mM pervanadate at a 1:200 ratio. Pervanadate was made by adding 2 μl of 30 % (w/w) hydrogen peroxide (Sigma-Aldrich) and 50 μl of 200 mM sodium orthovanadate (Sigma-Aldrich) to 448 μl of PBS. Fresh PBMCs and Jurkat cell lines were co-incubated at a 2:1 of effector:target ratio in the presence of IL-2 and zoledronate for 20 min and immediately fixed in a 4% paraformaldehyde (Sigma-Aldrich) solution. Washing with PBS containing 2% FBS, cells were permeabilized with BD™ Phosflow Perm Buffer III (BD Biosciences) on ice for 30 min. They were pre-incubated with FcR blocking reagent for 5 min and stained with anti-phospho-AKT-Brilliant Violet 421 (pS473) (Clone M89-61, BD Biosciences), anti-phospho-ERK1/2-Alexa Fluor®647 (pThr202/Tyr204), anti-phospho-SHP-2-PE (pY542) (clone L99-921, BD Biosciences) Ab, or fluorescence-matched IgG1κ isotype control for 1 hr. Thirty minutes before washing, FITC-conjugated TCRγδ (clone B1.1, eBioscience) and APC-H7-conjugated CD3 Ab (clone SK7, BD Biosciences) were added. Flow cytometry was conducted by a FACS Canto II or a CytoFLEX. The data was analyzed by a FlowJo software.

**Statistics**

Data are shown as mean ± SEM, and the numbers and replicates from individual samples are indicated in the figure legends. All results were analyzed using student *t*-test by a GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Differences between mean values in HV or patients with AML and B-cell ALL in Figure 1A and B were evaluated using the Mann-Whitney *U*-test. When *p* value as less than 0.05, the differences were considered to be statistically significant. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

## Results

### BTLA expression is sustained in peripheral blood γδ T cells of ALL and AML patients.

The expression of PD-1 and BTLA was analyzed on PBMCs from HV and pediatric ALL and AML patients by flow cytometry. The gating strategies to assess the PD-1 expression on $CD3^{+}TCR\gamma\delta^{+}$ γδ T cells are shown in Figure 1A. Flow cytometric analysis revealed that PD-1 and BTLA were expressed in all circulating γδ T cells from not only HV but also leukemia patients (Figures 1A and 1B). Notably, the percentage of PD-$1^{+}$ γδ T cells in pediatric ALL patients was significantly higher than that of HV, as well as that of AML ($p$=0.0239 and 0.0028, respectively), suggesting that ALL patients may have a higher responsiveness to PD-1/PD-L1 blockade therapy than AML patients (Figures 1A and 1B). However, it was found that the expression of BTLA on γδ T cells was not significantly different in all three groups, unlike that of PD-1 (Figure 1C), suggesting an important role of BTLA in γδ T cell signaling pathway.

**Figure 1.**

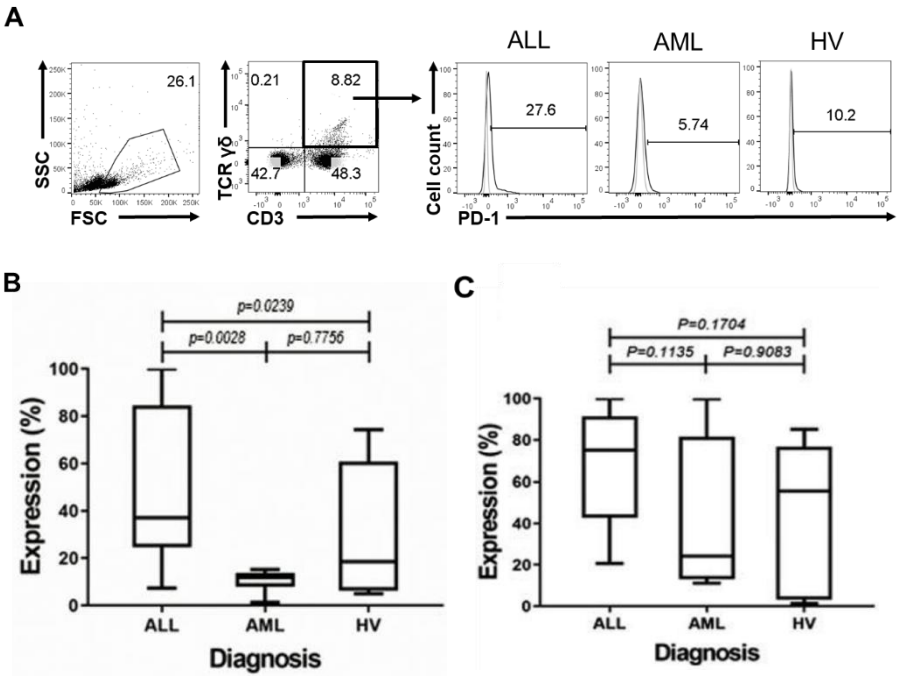

A
26.1
SSC
FSC
0.21
8.82
42.7
48.3
TCR γδ
CD3
ALL
AML
HV
27.6
5.74
10.2
Cell count
PD-1
B
p=0.0239
p=0.0028
p=0.7756
Expression (%)
ALL
AML
HV
Diagnosis
C
P=0.1704
P=0.1135
P=0.9083
Expression (%)
ALL
AML
HV
Diagnosis

**Figure 1. PD-1 and BTLA expression in γδ T cells from childhood leukemia patients.** Flow cytometric analysis was performed to assess PD-1 and BTLA expression in peripheral blood γδ T ($CD3^+TCR\gamma\delta^+$) cells from patients with ALL, AML, and HV. (A) Representative gating strategy was displayed. Representative data are shown. (B-C) The percentages of PD-$1^+$ (B) and $BTLA^+$ (C) γδ T cells are shown. FSC: forward scatter; SSC: side scatter; TCR: T-cell receptor. Data shown as mean ± SEM. N = 9 / group.

**BTLA signaling plays a critical role in proliferation of γδ T cells**

To study the roles of BTLA in expanding γδ T cells in the presence of $HVEM^{+}$ tumor cells, a human acute T cell leukemia cell line, Jurkat cells, that do not express BTLA and PD-1 were used (60). Thus, the unexpected response of BTLA and PD-1 of tumor cells to the blockades of BTLA and PD-1 could be avoided. I investigated whether the inhibition of the interaction of inhibitory receptors BTLA and PD-1 with their cognate ligands HVEM and PD-L1 during co-cultivation of γδ T cells and Jurkat cells could improve *in vitro* expansion of γδ T cells. Jurkat cells were co-transfected with HVEM CRISPR/Cas9 knockout and HVEM HDR plasmids to establish $HVEM^{low}$ Jurkat cell lines that *HVEM* gene was deleted. HVEM surface expression was reduced in $HVEM^{low}$ Jurkat cells than that of mock-transfected wild-type (WT) Jurkat cells from 22.3% to 0.28% (Figure 2A upper panel). It was determined whether down-regulated HVEM expression affects the endogenous expression of PD-L1 in $HVEM^{low}$ Jurkat cells. The expression of PD-L1 was not changed by the deletion of the *HVEM* gene in Jurkat cells (Figure 2A lower panel).

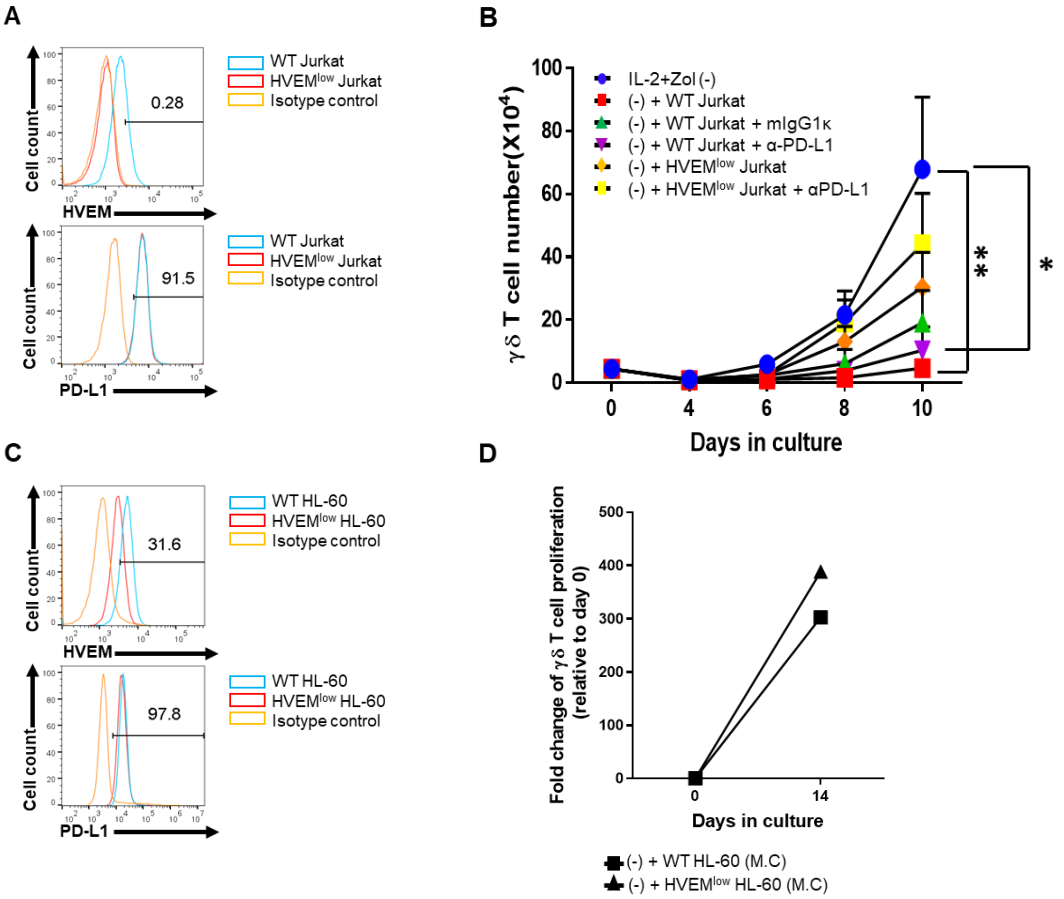
αβ T cell-depleted PBMCs were cultured with inactivated $HVEM^{low}$ or WT Jurkat

cells by mitomycin C (M.C.), in the presence or absence of anti-PD-L1 blocking mAb, in the medium containing 100 U/ml rhIL-2 and 1 μM zoledronate. Mitomycin C, which is an alternative method to irradiation, was treated on Jurkat cells prior to co-culture to induce inhibition of proliferation (71). The cell number of viable γδ T cells was significantly reduced by co-incubation with inactivated WT Jurkat cells, which express HVEM (Figure 2B). Notably, it was partially rescued by co-incubation with inactivated $HVEM^{low}$ Jurkat cells, suggesting that BTLA/HVEM interaction is responsible for the attenuated proliferation of γδ T cells when cultured with WT Jurkat cells (Figure 2B). The treatment with anti-PD-L1 mAb failed to increase γδ T cell expansion both in groups co-cultured with WT and $HVEM^{low}$ Jurkat cells at day 10 (Figure 2B).

Given the low expression of HVEM on WT Jurkat cells, HL60 cells, an acute myeloid leukemia cell line was used to confirm the results. WT HL60 cells expressed much higher levels of HVEM than WT Jurkat cells, 82.2%, compared with 22.3% on WT Jurkat cells (Figures 2A and 2C upper panel). The $HVEM^{low}$ HL-60 cells were generated, and knockdown efficiency was assessed by flow cytometry as above (82.2% to 31.6%) (Figure

2C upper panel). The expression of PD-L1 was not altered by the HVEM knockdown in HL-60 cells that is consistent with the results of Jurkat cells (Figure 2C lower panel). The proliferation rate was increased in γδ T cells co-cultured with HVEM$^{low}$ HL-60 cells than that WT HL-60 cells at day 14 (Figure 2D). These results were confirmed by EF670 dilution, as seen in Figures 2E-I. αβ T depleted-PBMCs were labeled with EF670 and cultured as Figure 2B up to 8 days. As expected, more γδ T cells without co-incubation remained viable and proliferating than γδ T cells with co-incubation with inactivated WT Jurkat cells. By day 8, EF670$^{low/-}$ proliferating cells were 97.5% when only IL-2 and zoledronate were treated. Deletion of *HVEM* in Jurkat cells rescued proliferating cells up to 95.4% at day 8, compared with 57.3% when cultured with WT Jurkat cells. In line with Figure 2B, γδ T cells co-cultured with HVEM$^{low}$ Jurkat cells proliferated more rapidly than that with WT cells on day 6, and 8 (Figures 2F, H, and I). Taken together, the results suggest that the reduction of BTLA/HVEM signaling is a prerequisite for the optimal proliferation of Vδ2 γδ T cells, whereas PD-1/PD-L1 signaling is not.

**Figure 2.**


A
Cell count
HVEM
0.28
WT Jurkat
HVEMlow Jurkat
Isotype control
PD-L1
91.5
B
γδ T cell number(X10⁴)
IL-2+Zol (-)
(-) + WT Jurkat
(-) + WT Jurkat + mIgG1κ
(-) + WT Jurkat + α-PD-L1
(-) + HVEMlow Jurkat
(-) + HVEMlow Jurkat + αPD-L1
Days in culture
C
31.6
97.8
WT HL-60
HVEMlow HL-60
D
Fold change of γδ T cell proliferation (relative to day 0)
(-) + WT HL-60 (M.C)
(-) + HVEMlow HL-60 (M.C)

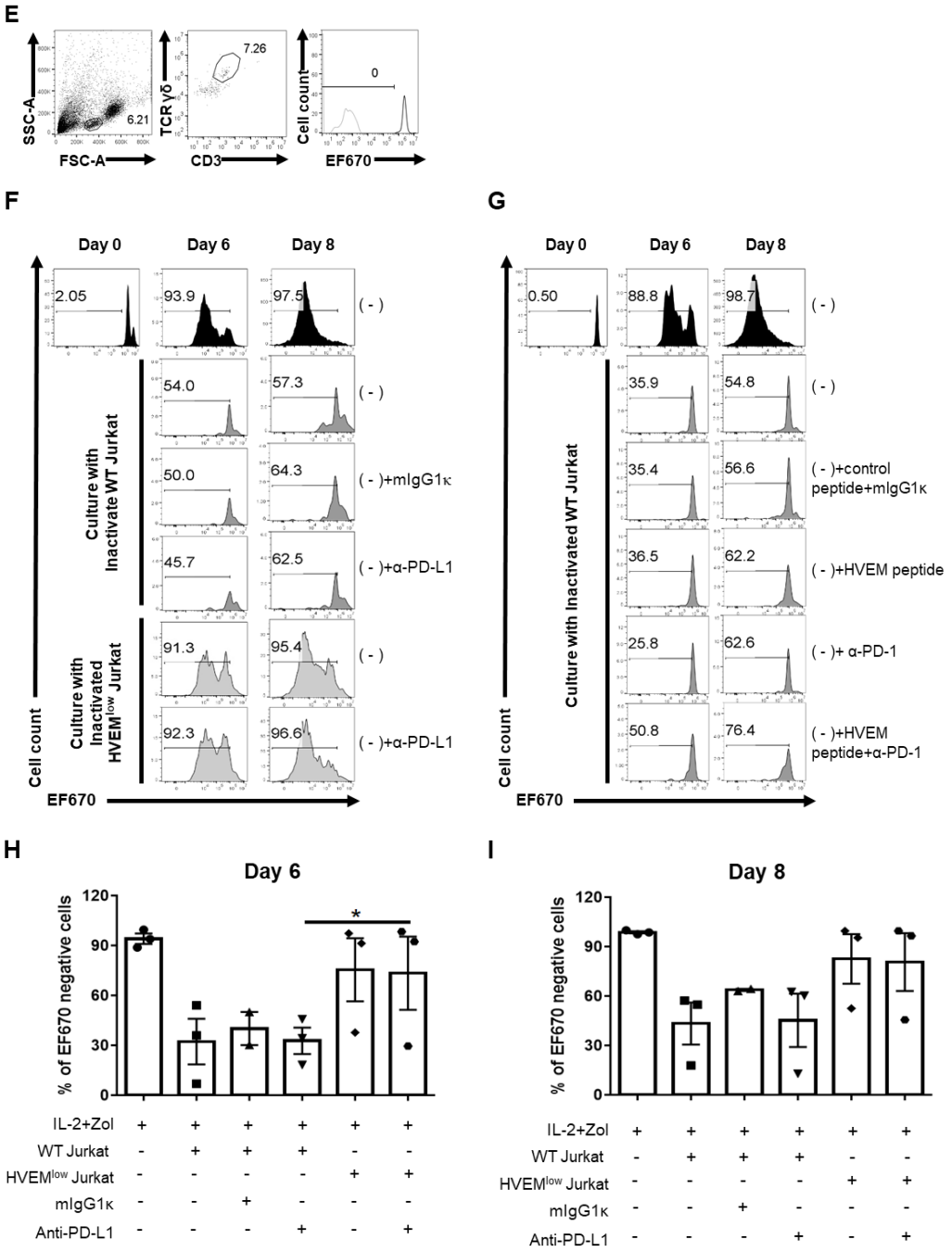

E
SSC-A
FSC-A
6.21
TCR γδ
CD3
7.26
Cell count
EF670
0
F
Day 0
Day 6
Day 8
2.05
93.9
97.5
( - )
54.0
57.3
( - )
50.0
64.3
( - )+mIgG1κ
45.7
62.5
( - )+α-PD-L1
91.3
95.4
( - )
92.3
96.6
( - )+α-PD-L1
Culture with Inactivate WT Jurkat
Culture with Inactivated HVEMlow Jurkat
Cell count
EF670
G
Day 0
Day 6
Day 8
0.50
88.8
98.7
( - )
35.9
54.8
( - )
35.4
56.6
( - )+control peptide+mIgG1κ
36.5
62.2
( - )+HVEM peptide
25.8
62.6
( - )+ α-PD-1
50.8
76.4
( - )+HVEM peptide+α-PD-1
Culture with Inactivated WT Jurkat
Cell count
EF670
H
Day 6
% of EF670 negative cells
120
90
60
30
0
*
IL-2+Zol + + + + + +
WT Jurkat - + + + - -
HVEMlow Jurkat - - - - + +
mIgG1κ - - + - - -
Anti-PD-L1 - - - + - +
I
Day 8
% of EF670 negative cells
120
90
60
30
0
IL-2+Zol + + + + + +
WT Jurkat - + + + - -
HVEMlow Jurkat - - - - + +
mIgG1κ - - + - - -
Anti-PD-L1 - - - + - +

**Figure 2. Blocking BTLA/HVEM interaction increases γδ T cell proliferation.** (A) The expression of HVEM (upper panel) and the PD-L1 (lower panel) on WT Jurkat (Blue line) and $HVEM^{low}$ Jurkat cells (Red line) was evaluated by flow cytometry. The yellow lines indicate isotype control. The representative histograms are shown. The numerical values indicate the percentages of $HVEM^{+}$ or $PD\text{-}L1^{+}$ cells among the $HVEM^{low}$ Jurkat cells. Representative data are shown out of 3 independent experiments. (B) αβ 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 (-) for the indicated days. mIgG1k was used as isotype control. The cell numbers of viable of γδ T cells ($CD3^{+}TCR\gamma\delta^{+}$) was assessed after culture. The frequency of γδ T cells was determined by flow cytometry. Results are representative of independent experiments using 3-6 distinct donors per group, and error bars represent mean ± SEM. (C) The expression of HVEM (upper panel) and the PD-L1 (lower panel) on WT HL-60 (Blue line) and $HVEM^{low}$ HL-60 cells (Red line) was assessed by flow cytometry. The yellow lines indicate the isotype control. The representative histograms are shown. The numerical values indicate the percentages of $HVEM^{+}$ or $PD\text{-}L1^{+}$

cells among the HVEM$^{low}$ HL-60 cells. (D) PBMCs were cultured with inactivated WT or HVEM$^{low}$ HL-60 cells in the presence of IL-2 and zoledronate (-). The frequency of γδ T cells (CD3$^{+}$TCRγδ$^{+}$) was determined by flow cytometry. The cell numbers of γδ T cells (CD3$^{+}$TCRγδ$^{+}$) at day 14 in culture was divided by that of day 0 to calculate fold changes. Results are representative of independent experiments using 2 distinct donors per group, and error bars represent mean ± SEM. αβ T cell depleted PBMCs were labeled with EF670, and EF670 dilution was determined as shown in Figure E. (F) After EF670-labeling, αβ T-cell-depleted PBMCs were stimulated as described above. Cells were harvested at day 6 or 8, and proliferation was evaluated as EF670 dilution in γδ T cells by flow cytometry. Representative histograms are shown out of 2-3 experiments using distinct donors. (G) After EF670-labeling, αβ T cell-depleted PBMCs were cultured with inactivated Jurkat cells with or without HVEM blocking peptide and anti-human PD-1 blocking antibodies in the presence of IL-2 and zoledronate (-). Representative histograms are shown out of 2-3 experiments using distinct donors. (H-I) The frequency of EF670$^{-}$ cells in cultured γδ T cells. Bar graphs are derived from FACS data in (F). (-), IL-2- and Zol-treated; M.C., mitomycin

C-treated. Paired *t*-test was used. *$p<0.05$.

**The blockade of BTLA/HVEM and PD-1/PD-L1 signaling does not affect both BTLA and PD-1 expression in *in vitro* expanded γδ T cells**

The BTLA and PD-1 expression on γδ T cells were assessed to understand the effect of BTLA/HVEM and/or PD-1/PD-L1 engagement during the *in vitro* expansion of γδ T cells (Figure 3A). αβ T cell-depleted PBMCs from HV were cultured as described in Figure 2B and the cell surface expression of BTLA and PD-1 in γδ T cells was evaluated by flow cytometry. The expression of these receptors in γδ T cells was similar at relatively low levels in the resting state, but it was significantly upregulated at least two-fold in 14 days ($p$=0.0404 in γδ T cells before stimulation versus after 10 d-stimulation of PBMCs with zoledronate and IL-2; $p$=0.0469 in γδ T cells before stimulation versus after 10 d-stimulation of PBMC, which is co-cultivated with $HVEM^{low}$ Jurkat cells in the presence of zoledronate and IL-2 in Fig. B; $p$=0.0417 in γδ T cells before stimulation versus after 10 d-stimulation of PBMCs with zoledronate and IL-2 in Figure. C.) (Figures 3B and 3C). Persistence inhibitory signaling by WT Jurkat cells did not induce further increment of those receptor expressions in activated γδ T cells, compared with only IL-2 and zoledronate treated group (Figures 3B

and 3C). The reduction of BTLA signaling by HVEM$^{low}$ Jurkat cells did not induce a significant change in those receptor expressions on γδ T cells, compared to that of WT Jurkat cells (Figures 3B and 3C). Those receptor expressions of γδ T cells did not alter in PD-L1 mAb-treated group, compared with that of the untreated group (Figures 3B and 3C). The simultaneous inhibition of those signaling did not result in increased or decreased expression of both BTLA and PD-1 in γδ T cells either. The expression of BTLA and PD-1 was upregulated by IL-2 and zoledronate in γδ T cells, which remained intact despite the reduction of inhibitory signaling.

**Figure 3.**

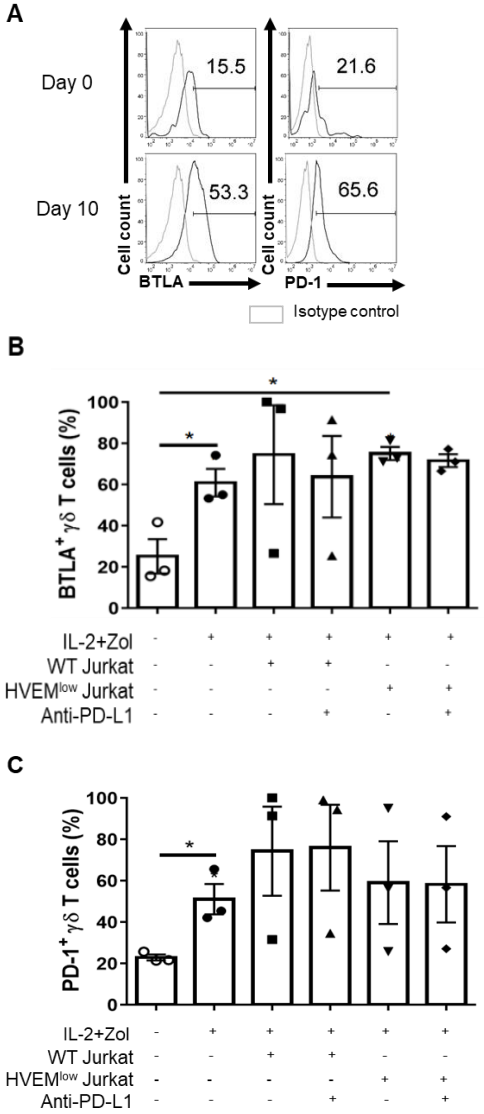

A
Day 0
Day 10
15.5
21.6
53.3
65.6
Cell count
BTLA
PD-1
Isotype control
B
BTLA+ γδ T cells (%)
IL-2+Zol
WT Jurkat
HVEMlow Jurkat
Anti-PD-L1
C
PD-1+ γδ T cells (%)
IL-2+Zol
WT Jurkat
HVEMlow Jurkat
Anti-PD-L1

**Figure 3. Co-culture with HVEM$^{low}$ Jurkat cells does not affect BTLA and PD-1 expression in γδ T cells.** (A) αβ T cell-depleted PBMCs were expanded by IL-2 and zoledronate for 10 days and stained with anti-human CD3, TCRγδ, BTLA, and PD-1 mAb. Representative histograms are shown BTLA and PD-1 expression in the γδ T cells. Gray lines indicate isotype control. Representative data are shown out of 3 independent experiments using distinct donors. (B-C) αβ T cell-depleted PBMCs were expanded as described above. The bar graph indicates the percentage of BTLA$^{+}$ (B) and PD-1$^{+}$ (C) cells out of γδ T cells following indicated stimulation. Data are shown as mean ± SEM of 3 independent experiments using distinct donors per group. Paired *t*-test was used. *$p<0.05$.

**Blockade of BTLA signaling reduces the more differentiated γδ T cell population**

Because the more differentiated T cells are, the less antitumor efficacy they have (72), the differentiation status of *in vitro* expanded γδ T cells was determined through flow cytometry. Upon incubation with IL-2 and zoledronate, γδ T cells were successfully enriched (Figure 4A). As seen in Figure 4B, coincubation with $HVEM^{low}$ Jurkat cells reduced terminally differentiated $CD27^{-}CD45RA^{+}$ γδ T ($T_{EMRA}$) cells significantly ($p = 0.0093$), compared with that of WT Jurkat cells. Coincubation with WT Jurkat cells slightly increased the proportion of naïve T cells, whereas change was not observed in other groups. PD-L1 blockade slightly increased $CD27^{+}CD45RA^{-}$ central memory T ($T_{CM}$) cells in γδ T cells which co-cultured with WT Jurkat cells, compared with the untreated group. The proportion of $T_{CM}$ cells, which was increased by PD-L1 blockade, was further increased by reduction of BTLA/HVEM signaling, although the differences were not statistically significant (Figure 4B). These results imply that inhibiting BTLA signaling pathway during expansion could be beneficial for cancer immunotherapy, by preventing further differentiation of γδ T cells from the central memory stage.

**Figure 4.**

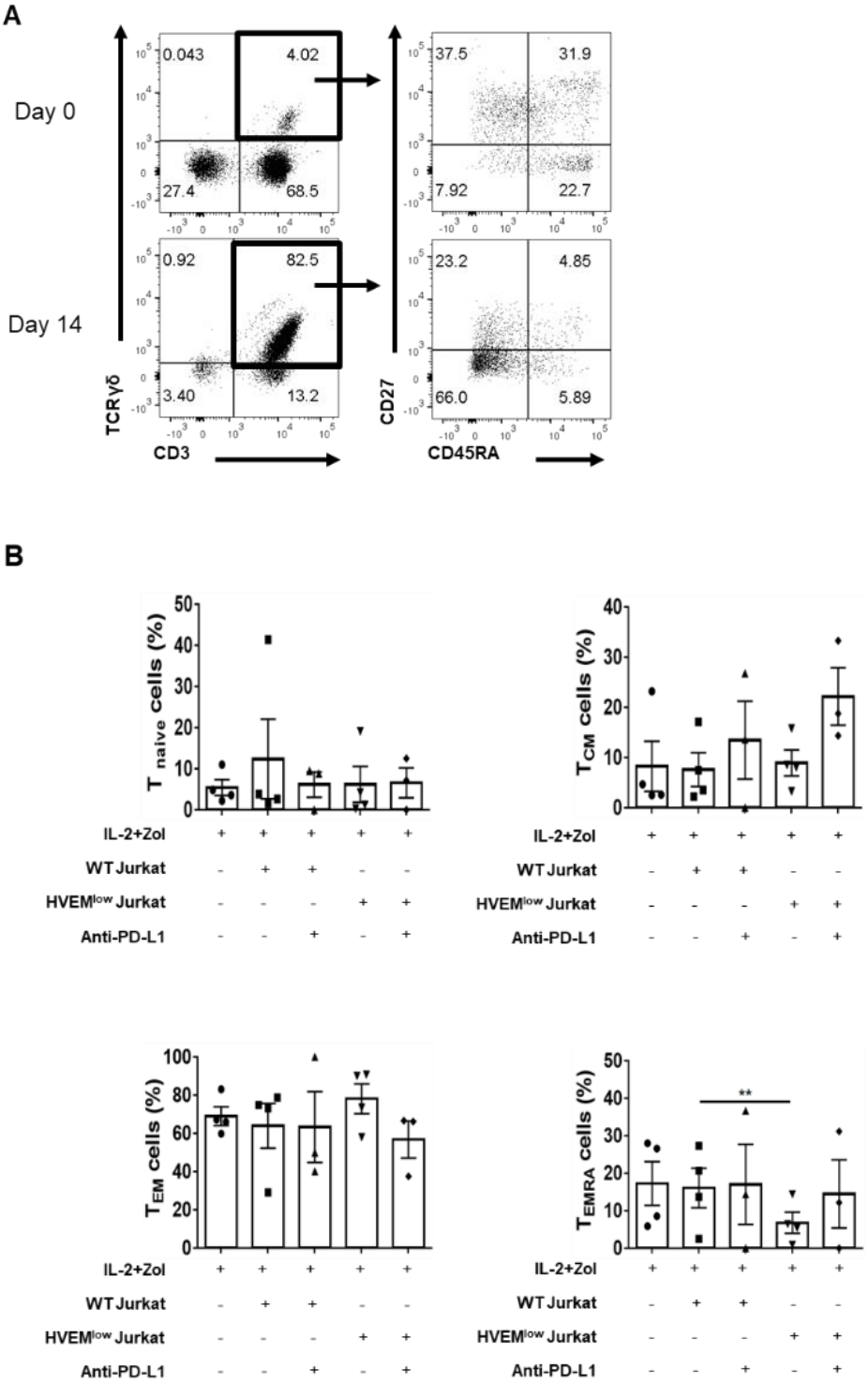

A
Day 0
Day 14
0.043
4.02
27.4
68.5
37.5
31.9
7.92
22.7
0.92
82.5
3.40
13.2
23.2
4.85
66.0
5.89
TCRγδ
CD3
CD27
CD45RA
B
T naive cells (%)
T CM cells (%)
T EM cells (%)
T EMRA cells (%)
**
IL-2+Zol
WT Jurkat
HVEMlow Jurkat
Anti-PD-L1

**Figure 4. Blockade of BTLA/HVEM interaction represses the terminal differentiation of γδ T cells.** (A) PBMCs from HV were expanded in the presence of IL-2 and zoledronate for 14 days. Representative dot plots showed that the phenotype and differentiation subsets of γδ T cells at Day 0 (upper panel) or following expansion (Day 14, lower panel). Representative data are shown out of 3-4 independent experiments using distinct donors. (B) PBMCs were activated in the indicated culture condition for 14 days. The frequency of differentiation subsets in the *in vitro* expanded γδ T cells is shown. Following subtypes were used for analysis: $T_{naive}$ ($CD27^{+}CD45RA^{+}$), $T_{CM}$ ($CD27^{+}CD45RA^{-}$), $T_{EM}$ ($CD27^{-}CD45RA^{-}$), and $T_{EMRA}$ ($CD27^{-}CD45RA^{+}$). Data are shown as mean ± SEM of 3-4 independent experiments using distinct donors. Paired *t*-test was used. **p*<0.05, ***p*<0.01, ****p*<0.001.

**The antitumor activity of γδ T cells is not affected by inhibition of BTLA signaling**

To evaluate the effect of BTLA/HVEM and PD-1/PD-L1 signaling on the cytotoxicity of γδ T cells, PBMCs were cocultured with WT Jurkat or HVEM$^{low}$ Jurkat cells with anti-human PD-L1 mAb in the presence of IL-2 and zoledronate. The expanded cells were incubated with same number of CFSE-labeled Jurkat cells for 1 day, and apoptosis was determined by PI and AV in CFSE$^{+}$ target cells (Figure 5A). Cells expanded by IL-2 and zoledronate treatment effectively induced apoptosis in WT Jurkat cells (Figures 5B and 5C). γδ T cells cultured with WT Jurkat cells or HVEM$^{low}$ Jurkat cells caused apoptosis comparable to the control group, indicating that BLTA/HVEM interaction did not regulate the cytotoxicity of γδ T cells. Anti-PD-L1 mAb treatment did not increase early and late apoptosis of target cells, despite increased expression of CD107a (Figure 6). These results revealed that inhibition of BTLA and PD-1 signaling did not increase or decrease the target cell death induced by γδ T cells, indicating that expanded γδ T cells have sufficient anti-leukemic property which was not affected by the blockade of BTLA and PD-1 inhibitory signalings.

**Figure 5.**

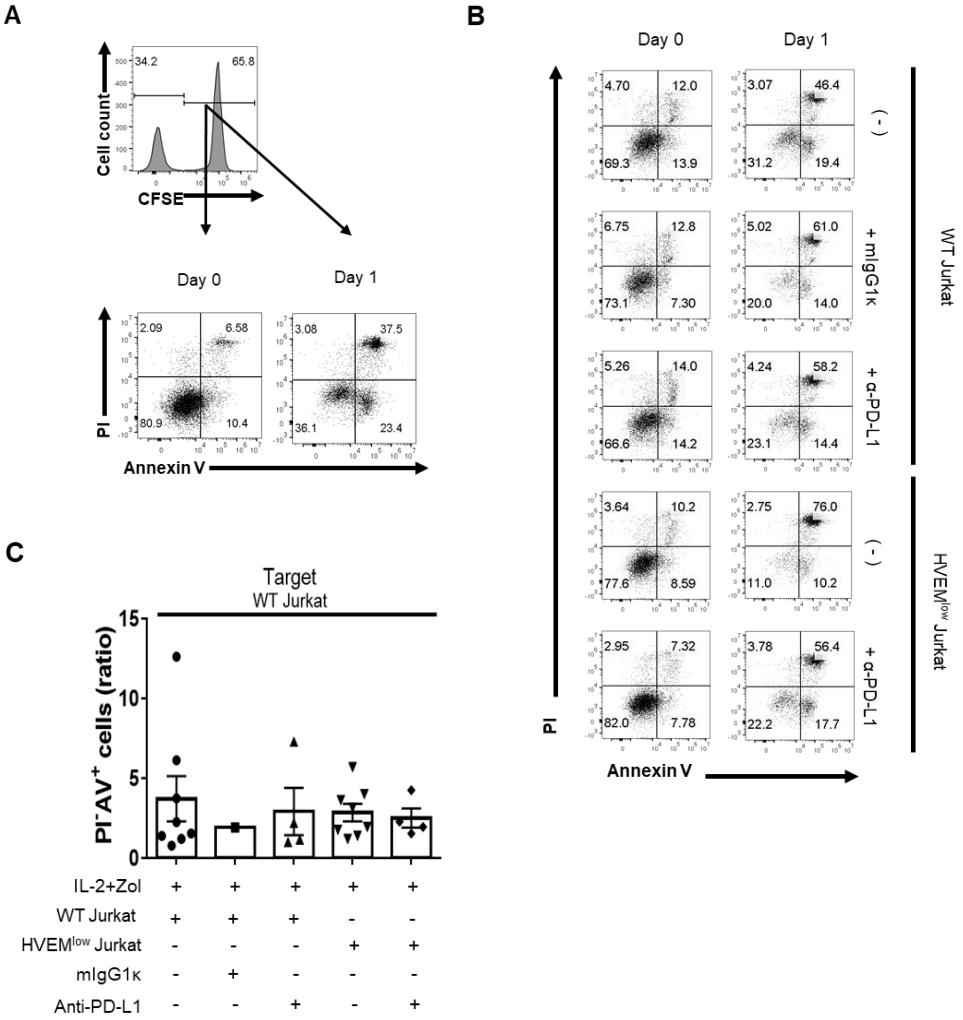

A
Cell count
CFSE
34.2
65.8
Day 0
Day 1
PI
Annexin V
2.09
6.58
80.9
10.4
3.08
37.5
36.1
23.4
B
Day 0
Day 1
PI
Annexin V
4.70
12.0
69.3
13.9
3.07
46.4
31.2
19.4
( - )
6.75
12.8
73.1
7.30
5.02
61.0
20.0
14.0
+ mIgG1κ
5.26
14.0
66.6
14.2
4.24
58.2
23.1
14.4
+ α-PD-L1
WT Jurkat
3.64
10.2
77.6
8.59
2.75
76.0
11.0
10.2
( - )
2.95
7.32
82.0
7.78
3.78
56.4
22.2
17.7
+ α-PD-L1
HVEMlow Jurkat
C
Target
WT Jurkat
PI-AV+ cells (ratio)
15
10
5
0
IL-2+Zol + + + + +
WT Jurkat + + + - -
HVEMlow Jurkat - - - + +
mIgG1κ - + - - -
Anti-PD-L1 - - + - +

**Figure 5. The target cell death by *in vitro* expanded γδ T cells was not altered by the blockade of BTLA/HVEM signaling.** WT Jurkat cells were stained with CFSE and incubated with *in vitro* expanded γδ T cells at an effector to target cell ratio of 1:1 for one day. Cells were harvested and stained with PI and AV to detect apoptosis by flow cytometry. (A) A gating strategy to evaluate the $PI^+$ and $AV^+$ cells among the target cells was displayed. (B) Representative dot plots are shown that the proportion of early apoptosis ($PI^-AV^+$) and late apoptosis ($PI^+AV^+$) in $CFSE^+$ target cells. The left panel indicates cell death rates at day 0, and the right panel indicates them after 1 day of incubation. Representative data are shown out of 1-8 independent experiments using distinct donors. (C) The percentages of $PI^-AV^+$ target cells in the experimental groups were normalized by dividing by those of day 0 control group. Data are shown as mean ± SEM of 1-8 independent experiments using distinct donors.

**PD-L1 signaling regulates the degranulation of γδ T cells.**

Vγ9Vδ2 T cell cytotoxicity against zoledronate-sensitized chronic myelogenous leukemia cells is mainly mediated by granule exocytosis pathway (26). To evaluate the effect of BTLA/HVEM and PD-1/PD-L1 signaling on the degranulation of γδ T cells, PBMCs were expanded under the conditions which was shown in Figure 5 (Figure 6A). The expanded γδ T cells were challenged by WT Jurkat or HVEM$^{low}$ Jurkat cells, and CD107a expression was measured to detect degranulation (Figure 6B). Inhibitory signaling by WT and the HVEM$^{low}$ Jurkat cells during the assay did not affect the degranulation of γδ T cells. However, the degranulation of γδ T cells co-cultured with HVEM$^{low}$ Jurkat cells was significantly increased by additional inhibition of PD-L1 signaling in response to both WT Jurkat cells ($p$=0.0190) and HVEM$^{low}$ Jurkat cells ($p$=0.0103). The CD107a expression was further increased in γδ T cells that challenged by HVEM$^{low}$ Jurkat cells than WT cells ($p$ = 0.0345) (Figure 6C). However, resting γδ T cells in all five groups expressed similar levels of CD107a in response to immediate signaling by HL-60 cells, suggesting that the proportion of CD107$^{+}$ γδ T cells was not changed by short-term BTLA and PD-1 signaling (Figure 6E and

6F). Taken together, these results indicate that PD-1 signaling regulates the cytolytic activity of γδ T cells, rather than HVEM signaling.

**Figure 6.**

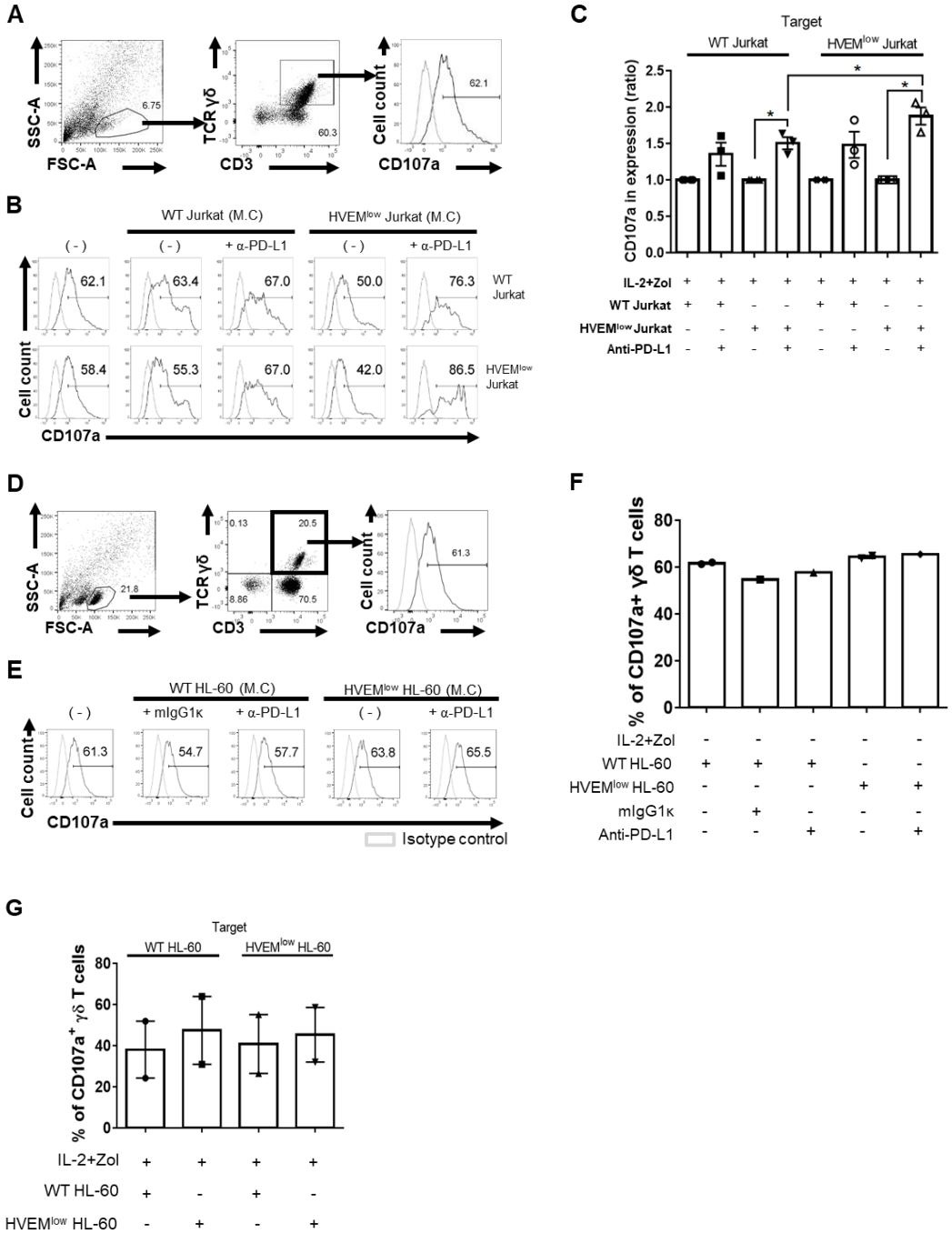

A
SSC-A
FSC-A
6.75
TCR γδ
CD3
60.3
Cell count
CD107a
62.1
B
WT Jurkat (M.C)
HVEMlow Jurkat (M.C)
( - )
( - )
+ α-PD-L1
( - )
+ α-PD-L1
62.1
63.4
67.0
50.0
76.3
WT Jurkat
58.4
55.3
67.0
42.0
86.5
HVEMlow Jurkat
Cell count
CD107a
C
Target
WT Jurkat
HVEMlow Jurkat
CD107a in expression (ratio)
2.5
2.0
1.5
1.0
0.5
0.0
IL-2+Zol + + + + + + + +
WT Jurkat + + - - + + - -
HVEMlow Jurkat - - + + - - + +
Anti-PD-L1 - + - + - + - +
D
SSC-A
FSC-A
21.8
TCR γδ
CD3
0.13
20.5
8.86
70.5
Cell count
CD107a
61.3
E
WT HL-60 (M.C)
HVEMlow HL-60 (M.C)
( - )
+ mIgG1κ
+ α-PD-L1
( - )
+ α-PD-L1
61.3
54.7
57.7
63.8
65.5
Cell count
CD107a
Isotype control
F
% of CD107a+ γδ T cells
80
60
40
20
0
IL-2+Zol - - - - -
WT HL-60 + + + - -
HVEMlow HL-60 - - - + +
mIgG1κ - + - - -
Anti-PD-L1 - - + - +
G
Target
WT HL-60
HVEMlow HL-60
% of CD107a+ γδ T cells
80
60
40
20
0
IL-2+Zol + + + +
WT HL-60 + - + -
HVEMlow HL-60 - + - +

**Figure 6. Blockade of BTLA/HVEM signaling does not affect degranulation of *in vitro* expanded γδ T cells.** (A) A gating strategy to evaluate the degranulation of γδ T cells was displayed. Representative histograms are shown CD107a expression in the γδ T cells in response to Jurkat cells. Gray lines indicate the isotype control. (B) γδ T cells were expanded in the indicated culture condition for 14 days. In the presence of anti-human CD107a mAb, *in vitro* expanded γδ T cells were incubated overnight with WT Jurkat (upper panel) or $HVEM^{low}$ Jurkat (Lower panel) cells. Representative data are shown out of 3-4 independent experiments using distinct donors. (C) The percentages of $CD107a^{+}$ γδ T cells were normalized to that of the untreated group. Data are shown as mean ± SEM of 3-4 independent experiments using distinct donors. (D) Degranulation during cytolysis of fresh γδ T cells was analyzed in response to HL-60 cells. In the presence of anti-human CD107a mAb, PBMCs were incubated overnight as indicated condition. (E) Representative histograms are shown CD107a expression in γδ T cells. Representative data are shown out of 1-2 independent experiments using distinct donors. (F) The results are summarized. (G) PBMCs were expanded in the indicated culture condition for 14 days. In the presence of

anti-human CD107a mAb, *in vitro* expanded γδ T cells were incubated overnight with WT HL-60 or HVEM$^{low}$ HL-60 cells. The graphs are showing the percentages of CD107a$^{+}$ γδ T cells. Data are shown as mean ± SEM of 1-2 independent experiments using distinct donors. Paired *t*-test was used. *$p<0.05$, **$p<0.01$

**The IFN-γ production of γδ T cells is not affected by blockade of BTLA signaling.**

Intracellular IFN-γ was assessed in γδ T cells. γδ T cells were cultured with inactivated WT or HVEM$^{low}$ HL-60 cells in the presence or absence of anti-PD-L1 mAb for 2 weeks, and the stimulated with WT HL-60 cells in the presence of BTLA/HVEM blocking peptides and/or anti-PD-L1 mAb to measure intracellular IFN-γ (Figure 7B). The expanded γδ T cells in all groups exhibited strong IFN-γ production in response to WT HL-60 cells. Blockades of PD-1 signaling increased IFN-γ production, whereas that of BTLA had no effect. (Figures 7B and 7C).

**Figure 7.**

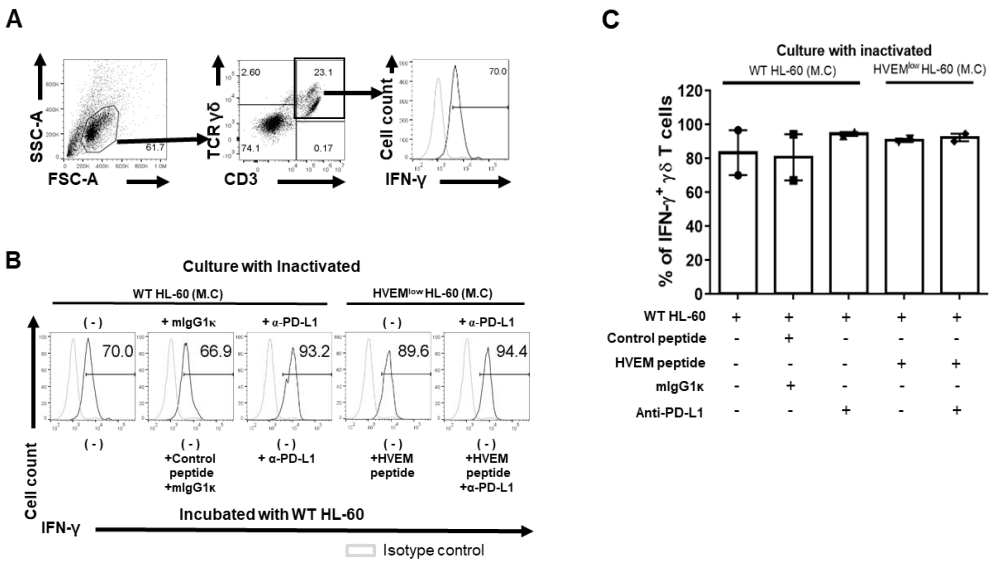



**Figure 7. Effects of the BTLA/HVEM and PD-1/PD-L1 signals on IFN-γ production in γδ T cells.** (A) Cells were expanded in the presence of IL-2 and zoledronate for 12 days. A gating strategy to evaluate the percentage of IFN-γ-producing γδ T cells in response to HL-60 cells was displayed. (B) *In vitro* expanded γδ T cells were incubated under indicated conditions for 4 hrs. Representative histograms are shown out of 2 independent experiments using distinct 2 donors. Gray lines indicate the isotype control. (C) The percentages of IFN-$\gamma^+$ cells in γδ T cells. Data are shown as mean ± SEM of 2 independent experiments. N=2.

**BTLA and PD-1 signaling pathways are independent of each other in γδ T cells**

BTLA and PD-1 control cell proliferation and apoptosis through their downstream molecules, such as AKT, ERK, and SHP-2, which mediate T cell activating and inhibitory signaling, respectively. Thus, the effects of immediate BTLA/HVEM and PD-1/PD-L1 signaling on the phosphorylation of AKT, ERK1/2, and SHP-2 in resting γδ T cells were investigated. PBMCs were incubated with WT Jurkat or HVEM$^{low}$ Jurkat cells and/or anti-PD-L1 mAb in the presence of IL-2 and zoledronate for 20 min, and the expression of phosphorylated proteins was evaluated in γδ T cells by flow cytometry (Figure 8A). Pervanadate, which is an inhibitor of phosphatases, was used as a phosphorylation inducer of PD-1 and BTLA cytoplasmic tail (73). The induction of pAKT was significantly increased in γδ T cells that stimulated with HVEM$^{low}$ Jurkat cells than WT cells ($p$=0.0384) (Figure 8C). Additional treatment of anti-PD-L1 mAb slightly increased the pAKT, which was significantly increased by reduction of BTLA/HVEM signaling in the percentages as well as mean fluorescence intensity (MFI) ($p$=0.0311, and $p$=0.0307, respectively) (Figures 8C and 8F). The induction of pERK1/2 by Jurkat cells was not influenced by blockade of BTLA and/or

PD-1 signaling (Figures 8D and 8G). The proportion of pSHP-2$^{+}$ cells was significantly reduced by inhibition of BTLA/HVEM signaling, whereas MFI of pSHP-2 was significantly repressed by anti-human PD-L1 mAb treatment ($p$=0.0480, and $p$=0.0377, respectively.) (Figures 8E and 8H). Together, these data suggest that BTLA/HVEM and PD-1/PD-L1 signaling have no additive or synergistic effects on the phosphorylation of AKT and SHP-2.

**Figure 8.**

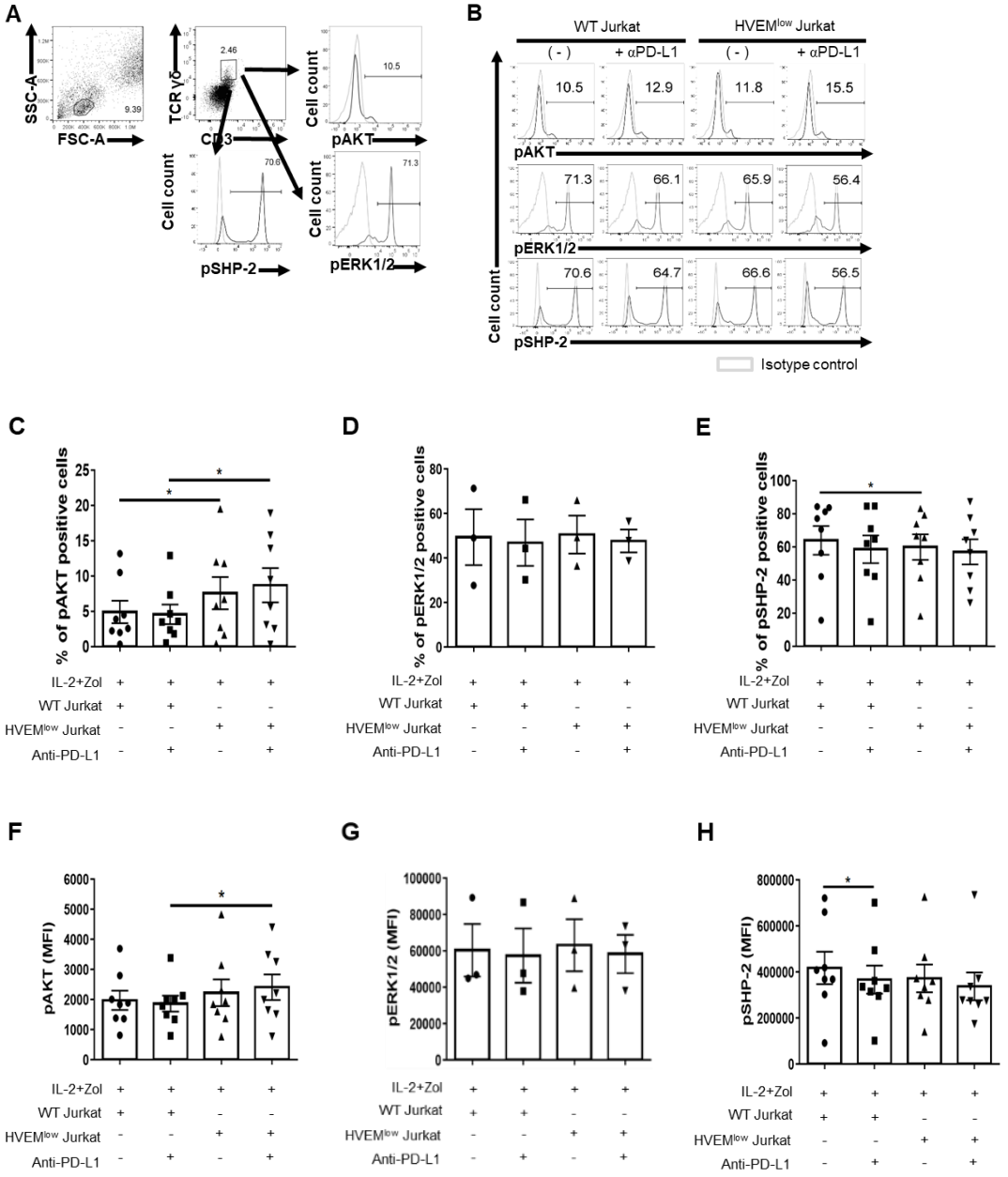

A
SSC-A
FSC-A
9.39
TCR γδ
CD3
2.46
Cell count
pAKT
10.5
pSHP-2
70.6
pERK1/2
71.3
B
WT Jurkat
HVEMlow Jurkat
( - )
+ αPD-L1
10.5
12.9
11.8
15.5
71.3
66.1
65.9
56.4
70.6
64.7
66.6
56.5
Isotype control
C
% of pAKT positive cells
D
% of pERK1/2 positive cells
E
% of pSHP-2 positive cells
F
pAKT (MFI)
G
pERK1/2 (MFI)
H
pSHP-2 (MFI)
IL-2+Zol
WT Jurkat
HVEMlow Jurkat
Anti-PD-L1

**Figure 8. Effects of blocking the BTLA/HVEM and PD-1/PD-L1 signals on the downstream signaling in resting γδ T cells.** Phosphorylation of AKT, ERK1/2, and SHP-2 in γδ T cells was measured by flow cytometry. WT or HVEM$^{low}$ Jurkat cells were pre-treated with anti-human PD-L1 Ab for 30 minutes. In the presence of pervanadate, αβ T cell-depleted PBMCs were stimulated with IL-2 and zoledronate, and co-incubated with WT or HVEM$^{low}$ Jurkat cells for 20 minutes. After fixing/permeabilization by PFA and methanol, the cells were stained with anti-human CD3, TCRγδ, phospho-AKT, phospho-ERK1/2, and phospho-SHP-2 antibodies. (A) Gating strategy to evaluate the phosphorylation status of AKT, ERK1/2 and SHP-2 in γδ T cells was displayed. (B) Representative histograms of pAKT, pERK1/2, and pSHP-2 induction in γδ T cells are shown. Representative data are shown out of 3-9 independent experiments using distinct donors. (C-H) The percentages or MFI of pAKT (C, F), pERK1/2 (D, G), and pSHP-2 (E, H) in γδ T cells are shown. Data are shown as mean ± SEM of 3-9 independent experiments using distinct donors. Paired *t*-test was used. *$p<0.05$, **$p<0.01$.

**Discussion**

BTLA is one of the inhibitory receptors, and its function and mechanism of action in T cells are less well known than PD-1 and CTLA-4. Although studies on the function of PD-1 in mouse and human αβ T cells have been conducted much more than BTLA, the knowledge of the PD-1 pathway in γδ T cells is still less elucidated. Recent studies have shown that the signals of BTLA and PD-1 are involved in proliferation and cytotoxicity of circulating γδ T cells, respectively (65, 67), whereas there are a few reports on the interaction of these signals.

In this study, the effect of combined BTLA/HVEM and PD-1/PD-L1 inhibitory signaling on the proliferation and effector function of peripheral blood γδ T cells were investigated to enhance the efficiency of Vγ9Vδ2 T cell-based immunotherapy. First, HVEM expression did not regulate endogenous PD-L1 expression in Jurkat cells, as well as in HL-60 cells. Second, the proliferation capacity of γδ T cells was suppressed by BTLA/HVEM ligation. Third, inhibition of PD-1/PD-L1 signaling induced degranulation of γδ T cells, that was enhanced by reduction of BTLA/HVEM signaling. Lastly, reduction of the BTLA/HVEM or PD-1/PD-L1 signal in γδ T cells inhibited the induction of pSHP-2 in

response to Jurkat cells, a human T-cell acute lymphoblastic leukemia cell line. In summary, BTLA and PD-1 signaling play an important role in inhibiting γδ T cell proliferation and cytotoxicity, respectively. The co-inhibition of these pathways could be helpful in eliciting anti-tumor responses of Vγ9Vδ2 T cells that are promising in immunotherapy, targeting hematologic malignancies (74).

Since the multiple inhibitory receptors are upregulated in tumor-infiltrating T cells, the expression profile of inhibitory receptors could be used as crucial biomarkers to predict prognosis and responses to immune checkpoint blockades (75). PD-$1^{hi}$TIM-$3^{+}$ T cells, which are associated with defective cytokine production, have been proposed as poor prognostic markers in leukemia relapse post-transplantation, indicating that PD-1 and TIM-3 co-blockade might be clinically effective (76). Subklewe and colleagues show that the peripheral blood T cells from patients with newly diagnosed AML and HV exhibit similar expression patterns in inhibitory receptors, including PD-1 and CD160 (77). The expression of PD-1 and BTLA on γδ T cells of patients with AML was comparable to those of HV, in agreement with that observation.

Previous studies have shown that engagement of PD-1/PD-L1 and BTLA/HVEM could interfere with the *trans*-interaction of T and APCs by interacting in *cis* on the same cell membrane of APCs or tumor cells. (78, 79). Concerning the *cis* interaction of BTLA/HVEM and PD-1/PD-L1 might affect the *trans* interaction unexpectedly, Jurkat cells, which do not express surface BTLA and PD-1, were chosen. Herein, HVEM and PD-L1 on the target cells were inhibited, rather than BTLA and PD-1 of γδ T cells directly. Although the UL144 protein of cytomegalovirus (CMV) could bind to BTLA, it has a lower affinity for BTLA than HVEM (80, 81). PD-L2 also binds to PD-1 with high affinity than PD-L1, but its expression is restricted compared with PD-L1 on immune cells (82). Nevertheless, it could not be ruled out that the blocking antibody might cause unexpected effects in this co-culture system. To overcome this limitation, a peptide-based specific inhibitor of BTLA/HVEM complexes (70) was used in this system, and similar results were observed.

It has been well known that both BTLA and PD-1 inhibit the proliferation of T cells (49, 62). A previous study reported that blockade of the BTLA/HVEM pathway enhances proliferation of tumor-reactive $CD8^+$ T cells by HVEM-expressing DCs, and an inhibitory

effect by a soluble form of HVEM is abolished by anti-BTLA mAb treatment (52). Inhibition of BTLA or PD-1 pathway augments *ex vivo* proliferation of minor histocompatibility antigens (MiHA)-specific $CD8^+$ T cells in allo-SCT patients (53). The proliferation capacity of peripheral blood PD-$1^+$ CD8 T cells from patients with advanced-stage non–small cell lung cancer (NSCLC) are increased following PD-1 therapy (83). The co-blockade of BTLA and PD-1 signals have a synergistic effect on the proliferation capacity of tumor-specific CD8 T cells (84). Additional blockade of BTLA further increases allogeneic DC-mediated proliferation of CD4 and CD8 T cells than PD-1 mono-blockade (85).

BTLA negatively controls the homeostasis of γδ T cells (86) and inhibits phospho-antigen-induced γδ T cell proliferation through partial cell cycle arrest in the S phase (65). Consistent with previous reports, this study revealed that disruption of BTLA/HVEM interaction enhances the proliferation capacity of γδ T cells. Anti-PD-L1 blockade alone did not have a proliferative effect on γδ T cells, but it slightly enhanced the proliferation capacity of γδ T cells that was induced by reduction of BTLA/HVEM signaling. These observations are supported by data from Massaia et al who show that PD-1 blockade only partially

recovers the proliferation of bone marrow-derived Vγ9Vδ2 T cells, which were stimulated by IL-2 and zoledronate (87). Taken together, the expansion of γδ T cells is regulated mainly by BTLA rather than PD-1, suggesting that co-blockade of BTLA and PD-1 is required for optimal proliferation capacity of γδ T cells.

This study revealed that BTLA was highly expressed, but PD-1 was less expressed in resting γδ T cells of healthy donors (65). Previous report has shown that the expression of BTLA is down-regulated by IL-2 and BrHPP treatment for 4 days, whereas the expression of PD-1 is upregulated in Vγ9Vδ2 T cells (65). In contrast to the previous findings, the expression of BTLA and PD-1 was significantly increased in γδ T cells that expanded by IL-2 and zoledronate for 10 days, compared with resting γδ T cells. These data might be associated with the previous report that BTLA is prominently expressed on human tumor antigen-specific $CD8^+$ T cells, while it is downregulated in human naive $CD8^+$ T cells during differentiation into effector cells (88). The expression of these receptors was similar in γδ T cells co-cultured with WT Jurkat or $HVEM^{low}$ Jurkat cells despite persistent antigen exposure, compared with the only IL-2 and zoledronate treated group.

The previous report suggests that anti-PD-1 mAb therapy could induce compensatory upregulation of other inhibitory receptors, such as BTLA and CTLA-4 (89). However, blockade of BTLA and PD-1 signaling pathways did not affect each other's expression on γδ T cells, suggesting that the enhanced proliferation capacity by blockade of co-inhibitory signaling is not resulted from reduced of expression of the inhibitory receptor. A similar proportion of each inhibitory receptor-positive γδ T cells on resting, as well as activating state was observed. Furthermore, the deletion of the *HVEM* gene did not affect endogenous PD-L1 expression on Jurkat and HL-60 cells either.

To define the differentiation status of γδ T cells, the CD27 and CD45RA surface marker expression has been measured on γδ T cells. The circulating naïve ($CD27^{+}CD45RA^{+}$) and $T_{CM}$ ($CD27^{+}CD45RA^{-}$) Vγ9Vδ2 T cells, which exist in the blood and secondary lymphoid organs, have no effector functions, but rapidly respond to PAgs (90). Circulating $T_{EM}$ ($CD27^{-}CD45RA^{-}$) and $T_{EMRA}$ ($CD27^{-}CD45RA^{+}$) Vγ9Vδ2 T cells, which are in the blood and peripheral tissues, have effector functions such as cytokine production and cytotoxicity (90). Naïve Vγ9Vδ2 T cells stimulated by isopentenyl pyrophosphate produce

low amounts of IFN-γ, and have low cytotoxicity, whereas a $T_{EMRA}$ Vγ9Vδ2 T cells produce high amounts of IFN-γ and exert cytotoxicity (91). $T_{EM}$ cells were the main subset of γδ T cells following *in vitro* expansion, indicating that γδ T-cells expanded by zoledronate have potent cytotoxicity. This observation is supported by the data from Anderson et al., showing that $V\delta2^{+}$ cells show a predominantly $T_{EM}$ phenotype, which is highly cytotoxic, at day 13 following activation with IL-2 and zoledronate (92). Blockade of the PD-1 pathway promotes naive-to-effector CD8 T cell transition in the model of acute lymphocytic choriomeningitis virus (LCMV) infection (93). The proportion of memory CD8 T cells are increased in BTLA or HVEM deficient mice, resulting in a hyperproliferative response (94). In line with this, co-incubation with WT Jurkat cells slightly increased the proportion of naïve γδ T cells, which are associated with GVHD (95), while the inhibition of BTLA/HVEM interaction reduced the proportion of $T_{EMRA}$ γδ T cells that have low proliferative capacity, compared with other subsets. Taken together, engagement of BTLA/HVEM, but not PD-1/PD-L1 might prevent with the differentiation of circulating γδ T cells.

Anti-PD-1 or PD-L1 mAb treatment could increase the degranulation of stimulated Vγ9Vδ2 T cells. Iwasaki and colleagues reported that the degranulation of PD-$1^+$ γδ T cells are reduced in response to PD-$L1^+$ target cells, which is recovered by anti-PD-L1 mAb treatment (67). Massaia and colleagues found that CD107a expression is increased by PD-1 blockade in BM Vγ9Vδ2 T cells that stimulated with IL-2 and zoledronate (87). The inhibition of sustained PD-1 signaling increased degranulation of γδ T cells in this study as well. Reduction of BTLA signaling more enhanced it, indicating that the co-blockade of PD-1 and BTLA pathway could enhance the anti-tumor function of γδ T cells.

Iwasaki and colleagues also revealed that impaired cytotoxic potential of PD-$1^+$γδ T cells is overcome by zoledronate treatment to have potent anti-tumor activity against zoledronate-sensitized tumor cells (96). γδ T cells expanded from PBMCs by IL-2 and zoledronate have potent cytotoxicity even under inhibitory signals, consistent with previous finding. Attenuation of PD-1 and BTLA signaling during expansion did not increase the cytotoxicity of γδ T-cells. These findings suggest that activated γδ T-cells by zoledronate are capable of mediating effective anti-tumor response.

PD-1 upregulates SHP-2, which binds to the ITSM, in tumor-infiltrating lymphocytes (3, 97). Upon engagement of PD-1 with its ligand, phosphorylation of SHP-2 is rapidly induced in T cells, whereas the inhibition of the PD-1 signaling suppresses phosphorylation of SHP-2 (98, 99). Furthermore, SHP-2 is associated with phosphorylation of BTLA by TCR engagement (49). Inhibition of the BTLA/HVEM or PD-1/PD-L1 pathways in γδ T cells reduced phosphorylation of SHP-2, but the combined inhibition did not show any synergistic and additional effects. PI3K–Akt and Ras-mitogen activated protein kinase kinase (MEK)-ERK-mediated signalings are indispensable to activation, proliferation, and cytotoxicity of human $V\gamma9^{+}$ γδ T cells by (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (100). The recruitment of SHP-2 could inhibit the PI3K–AKT and Ras signaling pathways, indicating that PD-1 and BTLA signaling negatively regulates the expansion and anti-tumor function of γδ T cells through SHP-2 recruitment (101). Reduction of the BTLA/HVEM or PD-1/PD-L1 signals in γδ T cells inhibited the induction of pSHP-2 in response to Jurkat cells, whereas phosphorylation of AKT was increased by the reduction of BTLA/HVEM signals. The PD-L1 blockade did not induce phosphorylation of AKT in γδ

T cells in response to Jurkat cells. BTLA-HVEM ligation suppresses phosphorylation in several pathways, which is related to T cell activation, including the AKT pathway in $CD8^{+}BTLA^{+}$ human tumor-infiltrating lymphocytes (102). In line with this, pAKT was significantly more induced by inhibition of HVEM in response to Jurkat cells. It might explain the more enhanced proliferation capacity of γδ T cells, which are co-cultured with HVEM-deleted Jurkat cells than with WT Jurkat cells.

In conclusion, BTLA/HVEM inhibitory signaling is a crucial factor in the proliferation of *ex vivo* expanded Vδ2 γδ T cells, while PD-1/PD-L1 signaling is important for the anti-tumor response. These results may establish preclinical rationality, suggesting that a combination blockade of BTLA and PD-1 could improve the efficiency of γδ T cell-based immunotherapy for the patients with hematopoietic malignancy, such as T cell leukemia/lymphoma and myeloid leukemia. Finally, this study might provide a benefit to better understanding the interaction of BTLA/HVEM and PD-1/PD-L1 signaling on γδ T cells in the immunosuppressive micro-tumor environment.

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국문요약

**배경:** B- and T-lymphocyte attenuator (BTLA) 그리고 programmed cell death-1 (PD-1)은 gamma-delta (γδ) T 세포의 항상성 및 활성화를 억제한다. 이 연구는 Vγ9Vδ2 T 세포가 대부분인 인간 말초 혈액 γδ T 세포에서 BTLA 및 PD-1 신호 전달 경로가 동일한 작용을 하는지 혹은 개별적으로 작용하는지를 조사하기 위해 설정되었다.

**방법:** 말초 혈액 단핵 세포 (PBMC)를 건강한 성인과 소아 B 세포 급성 림프모구 백혈병 (B-ALL) 및 급성 골수성 백혈병 (AML) 환자에서 분리하였다. CRISPR-Cas9 시스템을 사용하여 herpesvirus entry mediator (HVEM)이 결실된 Jurkat 또는 HL-60 세포주를 생성하였다. IL-2 및 졸레드로네이트의 존재 하에 PBMC 를 불활성화된 야생형 또는 HVEM 발현이 낮은 암세포와 공동 배양하여 배양 동안 BTLA/HVEM 및 PD-1/PD-Ligand 1 (PD-L1) 신호를 유도하였다. PBMC 및 Jurkat 세포 표면에서의 TCRγδ, CD3, BTLA (CD272), PD-1 (CD279), CD45RA, CD27, HVEM (CD270), 그리고 PD-L1 (CD274)의 발현은 유세포 분석에 의해 검출되었다. 또한 세포 내 IFN-γ, 그리고 AKT, ERK1/2 및 SHP-2 와 같은 인산화

단백질의 유도도 유세포 분석에 의해 평가되었다. 또한, CFSE 로 표지된 또는 표지되지 않은 γδ T 세포의 증식, 탈과립화 및 세포 독성 능력을 유세포 분석법을 사용하여 평가하였다.

결과: PD-1 은 AML 이 아닌 B-ALL 환자의 γδ T 세포에서만 광범위하게 발현되었지만 BTLA 는 세 그룹의 γδ T 세포에서 유사하게 발현되었다. 불활성화된 야생형보다 HVEM 발현이 낮은 암세포와 PBMC 를 공동 배양했을 때 γδ T 세포의 증식이 증가되었다. 확장된 γδ T 세포의 탈과립화는 BTLA/HVEM 신호 전달의 감소에 의해 영향을 받지 않았으며, 이는 PD-1/PD-L1 신호 전달의 억제에 의해 추가적으로 증가되었다. 그러나, 확장된 γδ T 세포의 세포 독성은 불활성화된 HVEM 발현이 낮은 Jurkat 세포와의 공동 배양 및 항-PD-L1 항체의 처리에 의해 영향을 받지 않았다. BTLA 또는 PD-1 신호 전달의 억제는 γδ T 세포에서 AKT 의 인산화를 증가시키는 반면, SHP-2 의 인산화를 억제하였다. 그러나 이들 단백질 인산화에 PD-1, BTLA 의 차단에 의한 상승 또는 부가적인 효과는 없었다.

결론: 이 논문은 BTLA/HVEM 과 PD-1/PD-L1 사이의 상호 작용은 각각 인간 γδ T 세포의 증식 및 세포 독성을 조절한다는 것을 입증했다. 이러한 결과는 γδ T 세포가 확장하는 동안 BTLA/HVEM 신호 경로의 비활성화가 세포 독성을 손상하지 않고 더 많은 γδ T 세포를 생산하는 데 도움이 될 수 있다는 것을 시사한다.