이학석사 학위논문

졸레드론산으로 증식시킨 간 관류액 내 감마 델타 T 세포의 암 세포 표면 인 항원 및 자연 살상 세포 수용체 신호전달에 대한 세포독성 연구

Cytotoxicity of Human Hepatic Intrasinusoidal γδ T Cells Expanded with Zoledronate Depends on Phospho-antigen on Tumor Cells and NK Receptor Signalings

울산대학교대학원

의 과 학 과

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지도교수 정동환

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Master of Science

Cytotoxicity of Human Hepatic Intrasinusoidal γδ T Cells Expanded with Zoledronate Depends on Phospho-antigen on Tumor Cells and NK Receptor Signalings

The Graduate School

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Department of Medical science

Yoorha Kang

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Supervisor: Prof. Dong-Hwan Jung

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Contents

그림목차

Abstract

Background: Hepatocellular carcinoma (HCC) is most common primary liver cancer and the third leading cause of cancer mortality world-wide. Therefore, developing a new strategy to reduce the incidence of liver cancer is urgently needed. Cancer immunotherapy has been revolutionized by introducing an adoptive immune cell transfer (ACT) technique, using mostly T cells from patient or healthy individual to eliminate cancer cells. Recent ACT therapies begin to incorporate other immune cell types, like natural killer (NK) cells and γδ T cells. Many studies have demonstrated that *ex vivo* expanded γδ T cells exert cytotoxicity against HCC cells, making γδ T cells a feasible candidate for liver cancer. Herein hepatic intrasinusoidal (HI) γδ T cells were investigated as a novel candidate for immune cell therapy.

Methods: HI γδ T cells were isolated from liver perfusate and cultured for 2 weeks in the presence of IL-2, IL-15, and Zoledronate. Lactate dehydrogenase (LDH) assay and CD107a degranulation assay were performed to measure cytotoxicity of γδ T cells from liver perfusate and PBMC. Expression of activating receptors (NKG2D, NKp46, CD16, and DNAM-1), death ligand (TRAIL, and Fas-L), and inhibitory receptors (PD-1, BTLA, and

TIGIT) were assessed by flow cytometry. CFSE-labeled HCC cell lines (Huh7, and SNU398) and HI γδ T cells were intraperitoneally injected into NSG mouse. After 48 hours, mice were sacrificed and $CFSE^{+}/CFSE^{-}$ ratio was calculated. Pyrophosphate expression on HCC cell lines and human prostate cancer cell lines were measured. Flow cytometry was used to measure the expression of DNAM-1 ligands, CD112 and CD155.

Results: The results of degranulation assay and LDH assay showed that HI γδ T cells exhibited strong cytotoxicity against HCC cell lines, especially Huh7. HI γδ T cells expressed higher levels of NKG2D and DNAM-1 than PBMC γδ T cells. LDH assay with blocking antibodies revealed that HI γδ T cells relied on an activating receptor, NKG2D, in killing of SNU398. Huh7 cells might activate Vδ2 γδ T cells by expressing high level of pyrophosphate antigen. HI γδ T cells were shown to recognize pyrophosphate antigen on and kill human prostate cancer cells as well.

Conclusion: HI γδ T cells exhibited stronger cytotoxicity against HCC cells than PBMC γδ T cells. Huh7 cells, previously known as an NK-resistant cell line, were killed effectively by HI γδ T cells. HI γδ T cells utilized both pyrophosphate antigen

and activating receptor. These results suggest that HI γδ T cells are a promising candidate for cancer immune cell therapy.

Introduction

γδ T cells are unconventional T cells with T cell receptor (TCR) γ and δ chains, and constitute a minor subset both in mice and humans. Unlike conventional αβ T cells, they are derived from $CD4^{-}CD8^{-}$ double-negative stage and develop into functional γδ T cells depending on the intensity of TCR signaling [1]. γδ T cells play an important role in stress surveillance and homeostasis, acting at the interface of the innate and adaptive immune systems [2]. γδ T cells share many effector functions with αβ T cells but one distinct difference is that γδ T cells can recognize a various range of antigens without the presence of major histocompatibility complex (MHC) protein [3]. Several studies have shown that γδ T cells exhibit strong cytotoxicity without causing graft versus host disease (GvHD) [4] ,and can produce inflammatory cytokines such as TNF-α, IFN-γ, and IL-17 against tumor cells [5]. These combined features enable γδ T cells to gain attention as a promising candidate for tumor therapy over the past decade. The cytotoxicity of γδ T cells relies on the engagement of T cell receptors (TCR) and NK cell receptors, such as natural killer group 2D (NKG2D) [6] and DNAX accessory molecule-1 (DNAM-1) [7]. Notably, NKG2D has

been widely investigated in γδ T cells. This receptor contributes to the recognition and killing of tumor cells by NKG2D-NKG2D ligands binding. Their activation triggers γδ T cells to lysis tumor cells [8]. DNAM-1 is involved in γδ T cell-mediated killing of tumor cells. Interaction with their cognate ligands, such as nectin-2 (CD122) and necl-5 (CD155), is needed to lyse tumor cells since blockade with anti-DNAM-1 did not affect the cytotoxicity of γδ T cells against the Burkitt's lymphoma cell line Daudi which had no expression of its ligands [7]. Upon γδ TCRs activation, expression of Fas ligand (FasL), as well as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), is increased, which can promote killing of Fas^+ and TRAIL $receptor^+$ tumor cells [9]. CD16, an Fcγ receptor mediates antibody-dependent cellular cytotoxicity (ADCC) by binding to Fc region of monoclonal antibodies that target tumor antigens [10].

HCC cells expressed not only NKG2D ligands including MHC class I-chain-related antigens (MIC) A/B [11] and UL16-binding proteins (ULBPs) [12] but also DNAM-1 ligands [13], TRAIL receptor DR4, DR5 [14], NKp46 ligand [15], and Fas [16, 17], suggesting γδ T cells may have a beneficial role in HCC regression. Human γδ T cells are

grouped by δ chain rather than γ chain, and are classified into Vδ2 and non-Vδ2 γδ T cells. Vδ2 T cells are typically paired with Vγ9 chain and constitute most of the peripheral blood γδ T cells [18, 19], whereas Vδ1 γδ T cells are predominant in solid tissues and mucosal epithelial.

Lymphocytes in liver are different from those in peripheral blood (PB). Many studies have shown that innate lymphoid cells including NK cells, invariant NK T (iNKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ T cells are enriched in the liver [20]. In particular, hepatic γδ T cells occupy up to 6.6% of T lymphocytes in the liver while peripheral blood only contains 0.9% of them [21]. Intrahepatic γδ T cells display tissue-resident features. Compared with the equivalent subset in blood, $CD27^{lo}CD45RA^{lo}$ subset of $V\delta1^{+}$ in the liver was shown to express liver-resident associate marker CD69, CXCR3, and CXCR6 [22]. Secretion of cytokines such as interferon (IFN)- γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, and IL-4 were increased in malignancy, and strong cytotoxicity against K562 and Daudi cell lines by hepatic γδ T cells were observed, suggesting the anti-tumor role of liver-resident γδ T cells [21]. One major obstacle which slows the advance of

γδ T cells in cancer biology is the low number of its population (0.5-5% of all T-lymphocytes). Considering that liver perfusate acquired before liver transplantation is an abundant source of hepatic intrasinusoidal (HI) T cells [23], I utilized HI γδ T cells isolated from liver perfusate and cultured HI γδ T cells in the presence of IL-2, IL-15, and zoledronate to overcome this problem in this study. Zoledronate which inhibits farnesyl pyrophosphate (FPP) synthase in mevalonate pathway induces activation of Vγ9Vδ2 T cell subset [24].

I proposed NK receptor signaling and phospho-antigen on tumor cells as possible mechanism for tumor cell killing. Phospho-antigens (pAgs) containing pyrophosphate are intermediates made in a mevalonate pathway. The cytoplasmic domain of Butyrophilin 3A1 (BTN3A1) are involved in pAgs sensing, and binding of BTN3A1 and TCR directly leads to activation of Vγ9Vδ2 T cells [25]. For example, Isopentyl pyrophosphate (IPP) made during mevalonate pathway activates Vγ9Vδ2 T cells, which promotes the anti-tumor activity of γδT cells.

This study aimed to characterize HI γδ T cells and PB γδ T cells and to investigate the mechanism underlying the cytotoxicity of HI γδ T cells against human HCC cell lines, SNU398 and Huh7. Our result indicated that using HI γδ T cells may contribute to treating intractable HCC

Materials and Methods

Human HI or PB γδ T Cell Expansion

Healthy living donor right lobe grafts were washed with 1 L of histidine–tryptophan–ketoglutarate solution, and liver perfusate was collected. All participants provided written informed consent, and all procedures were approved by the Institutional Review Board (IRB), Asan Medical Center, Seoul, Republic of Korea (Approval No. 2019-1665). HI mononuclear cells were isolated by a Ficoll–Paque density gradient method (GE Healthcare Life Sciences, Waukesha, WI, USA). Isolated cells were frozen in 90% fetal bovine serum (FBS; Welgene, Gyeongsan-si, Gyeongsangbuk-do, Republic of Korea) and 10% DMSO (Sigma Aldrich, St.

Louis, MO, USA) in liquid nitrogen until usage. PB mononuclear cells (MC) from healthy adult volunteers were also collected as describe above. All participants provided written informed consent, and all procedures were approved by the IRB, Asan Medical Center, Seoul, Republic of Korea (Approval No. 2018-0445). The study was performed ethically, in accordance with the Declaration of Helsinki. HIMC or PBMC were cultured at 2 x 10^6 cells/well in RPMI 1640 medium (WelGene) supplemented with 10% (v/v) heat-inactivated FBS (WelGene), 100 U/mL penicillin, 100 μg/mL streptomycin (Cellgro, Manassas, VA, USA), 5 mM sodium pyruvate (Pan Biotech, Aidenbach, Germany), and 55 μM 2-mercaptoethanol (Thermo Scientific, Waltham, MA, USA). HIMC or PBMC were cultured with 100 U/mL recombinant human IL-2 (rhIL-2) (PeproTech, Rocky Hill, NH, USA), 1 μM zoledronate (Selleckchem, Houston, TX, USA), and 50 ng/mL rhIL-15 (PeproTech) for 2 weeks. Zoledronate was treated once on day 0. RhL-2 and rhIL-15 were treated at same concentrations every 2 days. Anti-HVEM peptides were purchased from Peptron (Yuseong-gu, Daejeon, Republic of Korea). Anti-HVEM peptide (Ac-YRVKEACGELTGTVCEP-NH_2)

[26] was treated at 1 mg/ml in the culture media and refreshed every 3 days at same concentration.

Cell Culture

Human cell lines, Huh7, PC3, Du145, and LNCaP were purchased from ATCC (Manassas, VA, USA) and SNU398 cells were purchased from Korean Cell Line Bank (Seoul, Korea). Huh7, PC3 and Du145 cells were cultured in DMEM medium (WelGene) supplemented with 10% (v/v) heat-inactivated FBS (WelGene), 100 U/mL penicillin, and 100 μg/mL streptomycin (Cellgro). SNU398 and LNCaP cells were cultured in RPMI 1640 medium (WelGene) supplemented with 10% (v/v) heat-inactivated FBS (welgene), 100 U/mL penicillin, 100 μg/mL streptomycin (Cellgro), 5 mM sodium pyruvate (Pan Biotech).

Pyrophosphate Assay

For pyrophosphate assay, target cells were washed with cold PBS and lysed in RIPA buffer (Biosesang, Seongnam-si, Gyeonggi-do, Republic of Korea) supplemented with 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA, USA). The

cell lysates were incubated on ice for 30 min, and collected by centrifugation at 14,000 RPM 4 °C for 5 min. The assay was performed using a Pyrophosphate assay kit (Abcam, Cambridge, UK) and the result was calculated according to the manufacturer's instructions. Fluorescence was measured using an VICTOR X3 2030 multilabel reader at Ex/Em 316/456 nm absorbance (PerkinElmer, Waltham, MA, USA).

Flow Cytometry

HIMC and PBMC were incubated with the fluorescence-conjugated antibodies and washed twice with 1x PBS containing 2% FBS (Welgene). The following antibodies were used for surface staining; anti-human CD3 (Clone SK7 or UCHT1), CD16 (4G7), Vδ1 (TS8.2), Vδ2 (B6), NKp46 (9E2), NKG2D (1D11), TRAIL (RIK-2), CD178 (NOK-1), BTLA (MIH26), CD226 (11A8), PD-1 (EH12.2H7), CD8 (HIT8a), CD107a (H4A3), and TIGIT (A15153G) monoclonal antibodies (mAb). These antibodies and matched isotype controls were purchased from BD Biosciences (San Diego, CA, USA), BioLegend (San Diego, CA, USA), or eBioscience (San Diego, CA, USA). FcR blocking reagent (Miltenyi Biotec, Bergisch

Gladbach, Germany) was used for Fc receptor blocking. Flow cytometric data were acquired on a Cytoflex (Beckman Coulter, Brea, CA, USA) and analyzed with a FlowJo software (TreeStar, Ashland, OR, USA).

Three different human prostate cancer cell lines, Du145, PC3, and LNCaP, were incubated with anti-human CD112 (TX31), CD155 (SKIL4), HLA-E (3D12), DR4 (DJR1), DR5 (DJR2-4), MICA/B (GD4) , PD-L1(29E.2A3), Fas (DX2), and CD40 mAb (5C3) from Biolegend and HLA-ABC (G46-2.6), and HVEM (94081) mAb from BD at 4°C for 30 min. After staining, cells were washed twice with 1x PBS containing 2% FBS. The results were analyzed as describe above.

Degranulation Assay

HI γδ T cells as effector cells and tumor cells as target cells were co-incubated at a ratio of 1:2 at 37 °C in a humidified atmosphere of 5% CO_2 for 16 h. Positive control was stimulated for 15 hr with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) (Calbiochem, San Diego, CA, USA) and 1 μg/ml ionomycin (Sigma Aldrich). Monensin (eBioscience) was treated at

2 μM in the presence of fluorescence conjugated anti-CD107a mAb. After washing, cells were stained for cell surface markers at 4°C for 30 min. For some experiments, HIMC were pre-incubated with anti-human NKG2D (1D11) or DNAM-1 (11A8) (all from BioLegend) at 10 μg/mL at 4°C for 30 min, following 5 min incubation with human FcR blocking reagent (Miltenyi Biotec) at room temperature. Negative control was precoated with matched isotype control, mouse IgG1, κ. The results were analyzed by flow cytometry.

***In Vivo* Experiments**

Huh7 and SNU398 cells were harvested and labeled with 1.25 uM CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Waltham, MA, USA) in PBS at 37 °C for 6 min. Female NSG mice of 6-8 week-old were purchased from JA BIO (Suwon-si, Gyeonggi-do, Republic of Korea) and were injected intraperitoneally with Huh7 cells or SNU398 cells at 5 x 10^6 cells 30 min before injection with HI γδ T cells expanded with IL-2, IL-15, and zoledronate. Peritoneal lavages were collected by washing the peritoneal cavity with 10 ml of sterile PBS

48 hr later. The animal experiment was approved by IACUC, Asan Medical Center (Approval No. 2022-13-162).

Lactate Dehydrogenase (LDH) Assay

Target cells were seeded in a round-bottom 96-well plate at 7000 cells/well. HI γδ T cells were seeded at 140000 cells/well. The effector to target (E: T) ratios was 20:1. In experiments with blocking antibodies, HI γδ T cells were incubated with anti-human NKG2D (1D11), TRAIL (RIK-2), FASL (NOK-1), or DNAM-1 (11A8) (all from BioLegend) mAb at 10 μg/mL for 30 min at 4°C, following 5-min incubation with human FcR blocking reagent (Miltenyi Biotec) at room temperature. Negative control was precoated with matched isotype control, mouse IgG1, κ. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 6 hr. The assay was performed using CytoTox 96 Non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) and the result was calculated according to the manufacturer's instructions. Optical density was measured using a Sunrise ELISA reader at 490 nm absorbance (Tecan, Mannedorf, Switzerland).

Statistics

All data are presented as the means ± SEM. P values were calculated by one-way ANOVA for degranulation assay, LDH assay, *in vivo* experiment, and pyrophosphate assay, or by two-way ANOVA for degranulation assay with blocking antibodies and the expression of cell surface markers on HIMC and PBMC using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The sample numbers (n) represent the number of biological samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p<0.0001$.

Result

HI γδ T Cells Exhibited Strong Cytotoxicity against HCC cell lines.

HI γδ T cells were cultured in the presence of IL-2, IL-15, and Zoledronate to evaluate the cytotoxicity of γδ T cells from liver perfusate and PB, LDH assay was performed with Huh7 and SNU398 cell lines. I found that HI γδ T cells showed enhanced cytotoxicity against HCC cell lines *in vitro* compared with PBMCs (Fig. 1A). To validate the anti-tumor activity of HI γδ T cells in vivo, NSG mice were intraperitoneally (i.p.) injected with CFSE-labeled Huh7 or SNU398 cells. After 30 min, HI γδ T cells were i.p. injected, and the peritoneal lavage was collected 48 hours later. $CFSE^+$ cells were gated (Fig. 2) and the ratio of $CFSE^+/CFSE^-$ was calculated. HCC cells were effectively eradicated by injected HI γδ T cells. Huh7 cells, which are previously reported as NK cell-resistant HCC cells were killed 20 folds more than SNU398 (Fig. 1B). To understand stronger cytotoxicity of HI γδ T cells against Huh7 than SNU398 cells, I measured the level of pAg on HCC cells lines. SNU398 expressed significantly less pAg than Huh7 cells (Fig. 1C). These results suggest that increased pAg expression on tumor cells was correlated to anti-tumor activity of HI γδ T cells.

Figure 1.

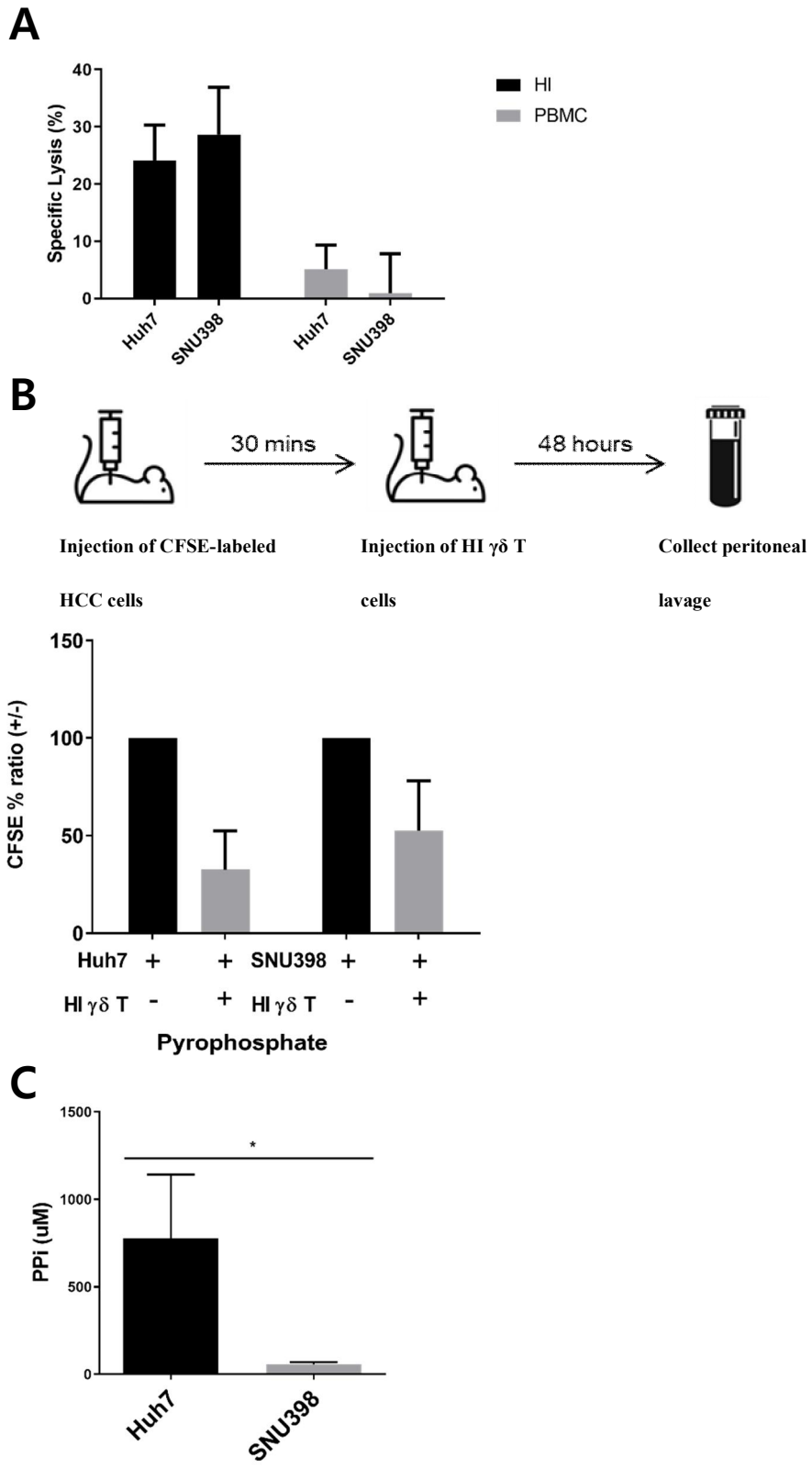

A
Specific Lysis (%)
40
30
20
10
0
HI
PBMC
Huh7
SNU398
Huh7
SNU398
B
30 mins
48 hours
Injection of CFSE-labeled HCC cells
Injection of HI γδ T cells
Collect peritoneal lavage
CFSE % ratio (+/-)
150
100
50
0
Huh7 + + SNU398 + +
HI γδ T - + HI γδ T - +
Pyrophosphate
C
PPi (uM)
1500
1000
500
0
*
Huh7
SNU398

Figure 1. HI γδ T Cells Effectively Eradicated HCC Cell Lines.

(A) LDH assay was performed with HI γδ T cells on HCC cell lines, Huh7 and SNU398. Effector to target (E:T) ratio was 20:1. N=11 for HI γδ T cells and N=3 for PBMC γδ T cells (Means ± SEM). Student's t test was performed. *$p<0.05$. (B) Scheme of *in vivo* experiment. CFSE labeled HCC cells were i.p. injected into NSG mouse at 5 x 10^6 cells/mouse. After 30 min of injection, HI γδ T cells were i.p. injected at 5 x 10^6 cells/mouse. After 48 hours, peritoneal lavage was collected and analyzed by flow cytometry. The results are representatives of two independent experiments (Means ± SEM). Student's t test was performed. *$p<0.05$; **$p<0.005$. (C) Pyrophosphate antigen expression on Huh7 and SNU398. The results are representatives of two independent experiments (Means ± SEM).

Figure 2.

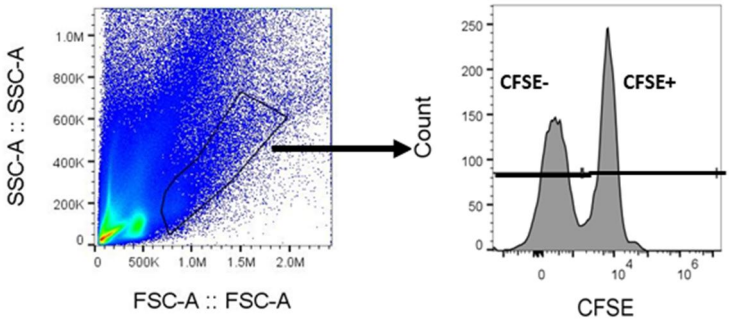


Figure 2. Gating Strategy for *In Vivo* Experiment. To calculate $CFSE^+/CFSE^-$cell ratio, lymphocytes were gated as shown in the left panel, and $CFSE^+$ cells and $CFSE^-$ cells were gated as shown in the right panel.

HI γδ T Cells and PB γδ T Cells Differentially Expressed an Array of Activating and Inhibitory Receptors

In Fig.1, I found that the phospho-antigen expression did not fully explain the cytotoxic effects of HI γδ T cells on SNU398 cells or the higher cytotoxicity of HI γδ T cells, compared with the PB γδ T cells. Therefore, I investigated the percentages (Fig. 3) and mean fluorescence indices (MFI) (Fig. 4) of activating receptors (NKG2D, NKp46, CD16, and DNAM-1), death ligands (TRAIL and Fas-L), and inhibitory receptors (PD-1, BTLA, and TIGIT) of HI and PB γδ T cells expanded with zoledronate. HI γδ T cells were gated into Vδ1 and Vδ2 (Fig. 5). None of the activating receptors was expressed at a higher level in the HI γδ T cells than in the PB γδ T cells. The percentages of HI Vδ2 T cells expressing Fas-L were significantly lower than those of PB γδ T cells, and PD-1 expression levels were lower in HI γδ T cells than in PB γδ T cells. The expression of BTLA was significantly higher in HI Vδ2 T cells than in HI Vδ1 T cells and the expansion rate of HI Vδ2 T cells was increased by blocking BTLA signaling [27] (Fig. 6). TIGIT was expressed significantly higher

in HI Vδ1 T cells than in HI Vδ2 T cells and PB γδ T cells, even though Vδ1 T cells were less than 5% of the total cells expanded by zoledronate. These results show that the lower expression of PD-1 may elicit greater cytotoxic effects from HI γδ T cells when compared with PB γδ T cells.

Figure 3.

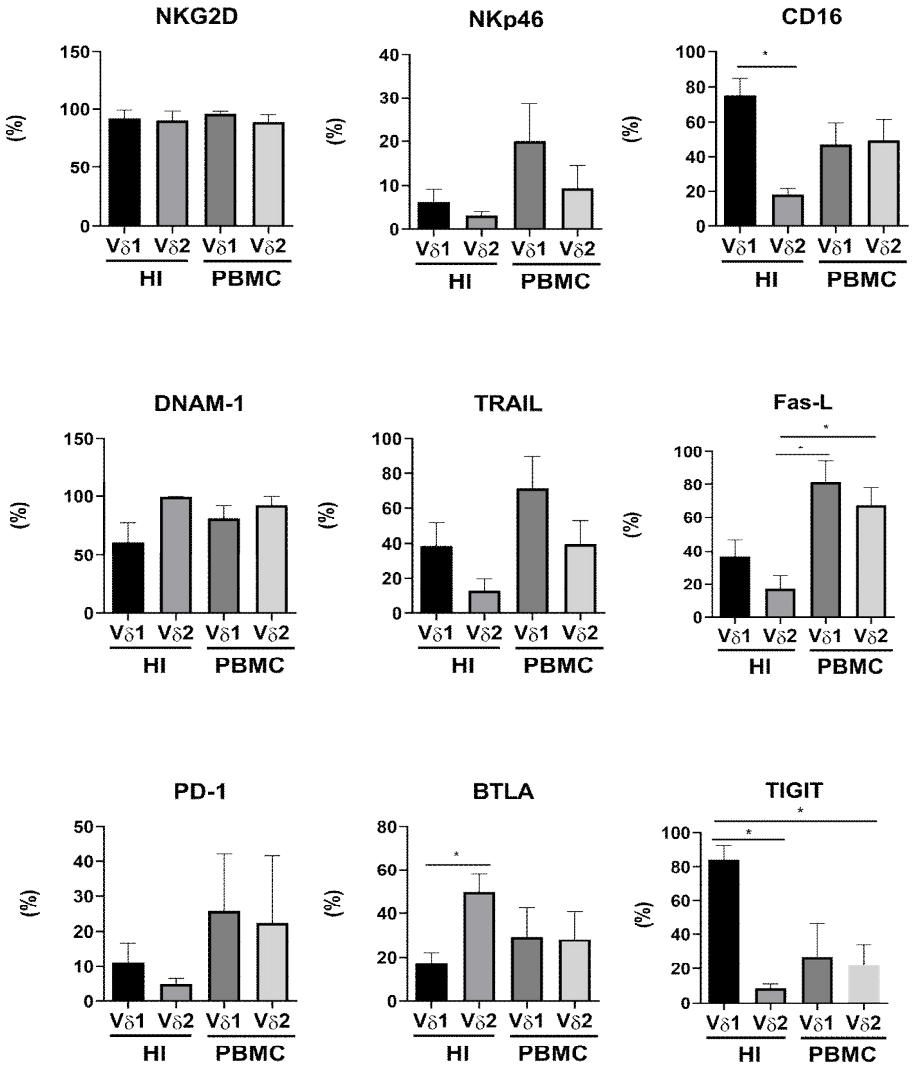

NKG2D
NKp46
CD16
DNAM-1
TRAIL
Fas-L
PD-1
BTLA
TIGIT
(%)
Vδ1 Vδ2 Vδ1 Vδ2
HI
PBMC

Figure 3. Percentages of Activating and Inhibitory Receptors, and Death Ligands Expressed in HI γδ T Cells and PB γδ T Cells.

Percentages of receptor expression were measured by flow cytometry. In HI γδ T cells, n = 11 for BTLA and PD-1 and N = 3 for others. In PB γδ T cells, n = 5. One-way ANOVA with Tukey's multiple comparison was performed. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.

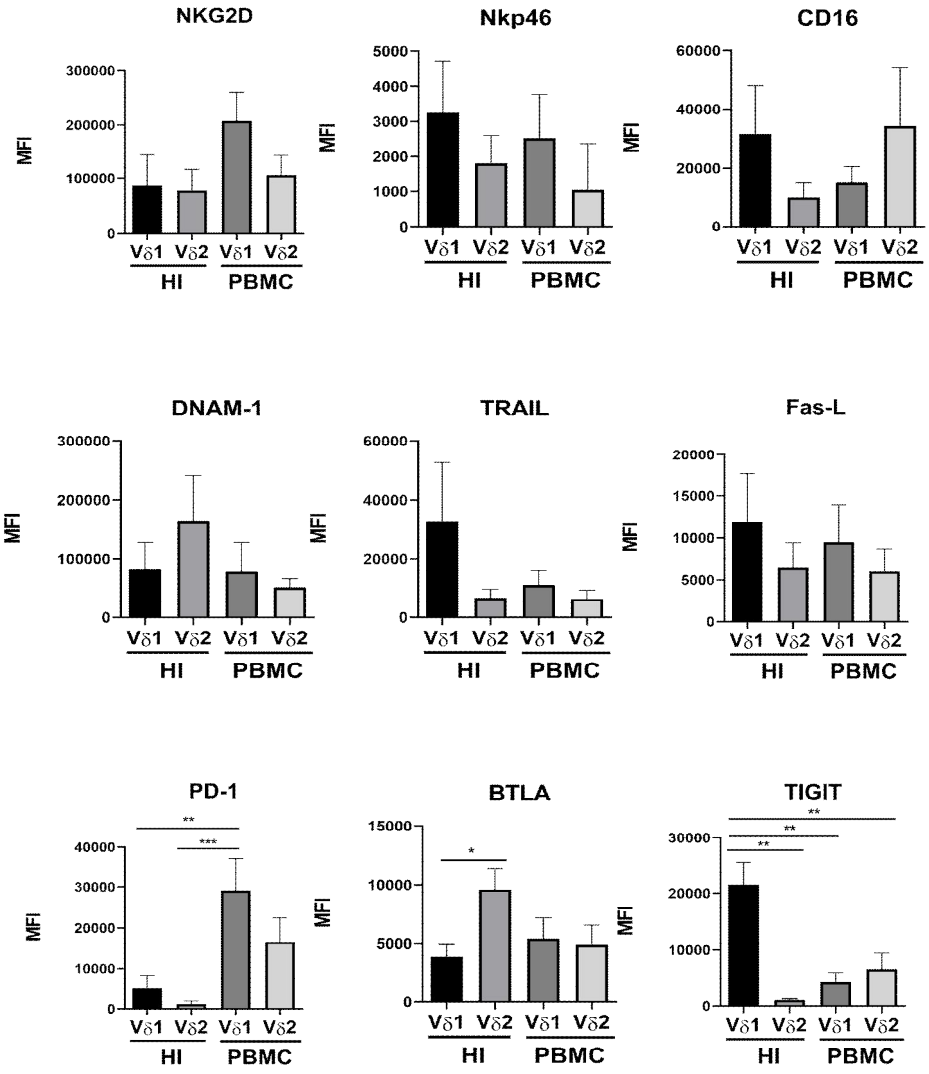

NKG2D
Nkp46
CD16
DNAM-1
TRAIL
Fas-L
PD-1
BTLA
TIGIT
MFI
Vδ1 Vδ2 Vδ1 Vδ2
HI
PBMC

Figure 4. MFI of Activating and Inhibitory Receptors, and Death Ligands Expressed in HI γδ T Cells and PB γδ T Cells.

MFI of receptor expression was measured by flow cytometry. In HI γδ T cells, n = 11 for BTLA and PD-1 and N = 3 for others. In PB γδ T cells, n = 5. One-way ANOVA with Tukey's multiple comparison was performed. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 5.

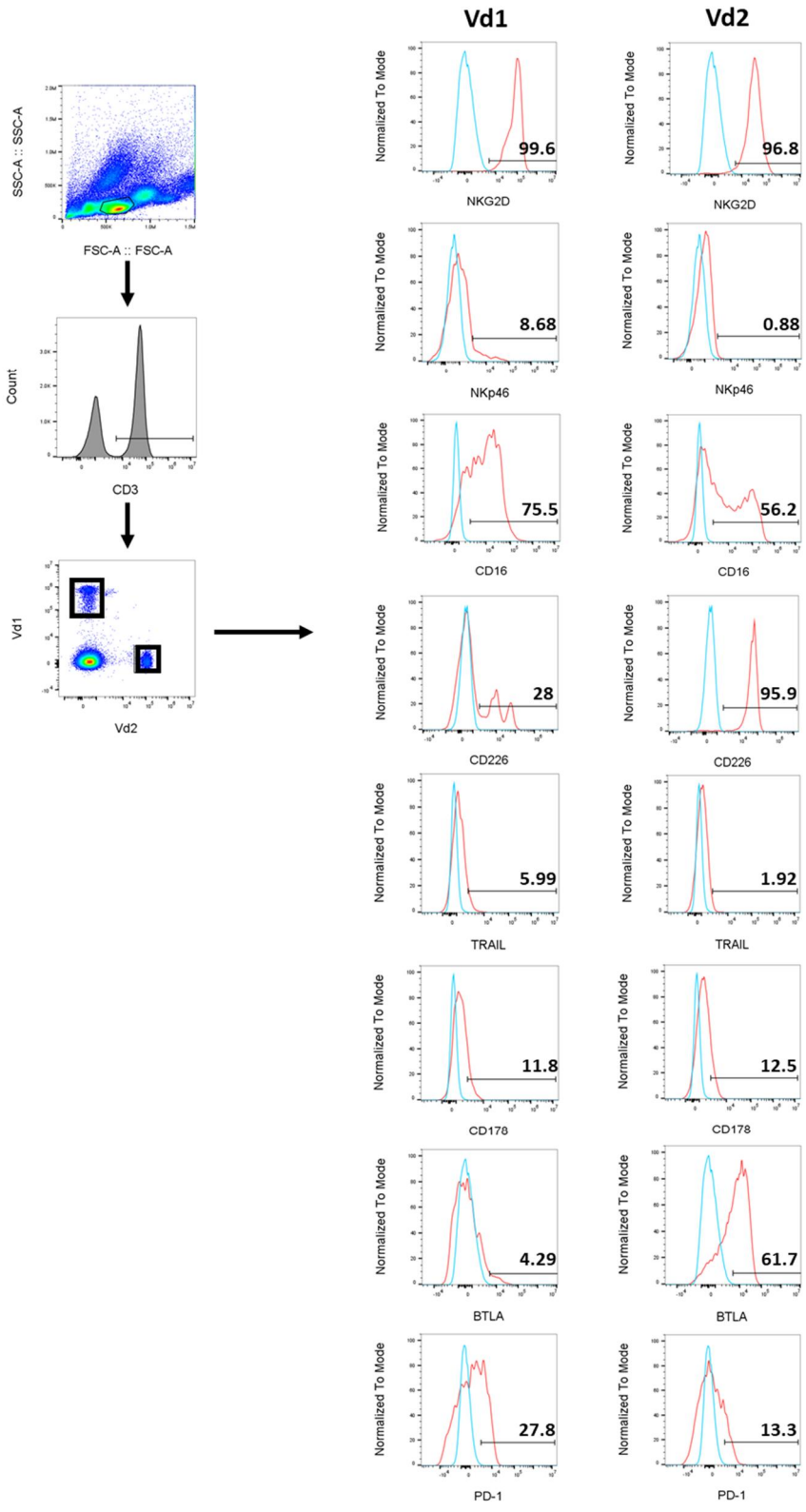

SSC-A :: SSC-A
FSC-A :: FSC-A
Count
CD3
Vd1
Vd2
Vd1
Vd2
Normalized To Mode
99.6
96.8
NKG2D
8.68
0.88
NKp46
75.5
56.2
CD16
28
95.9
CD226
5.99
1.92
TRAIL
11.8
12.5
CD178
4.29
61.7
BTLA
27.8
13.3
PD-1

Figure 5 Gating Strategies and Representative Flow Cytometric Plots of Cell Surface Markers on HI Vδ1 and Vδ2 γδ T Cells. Numeric values show % of cell surface marker^{+} cells. Blue lines, isotype control; red lines, cell surface markers.

Figure 6.

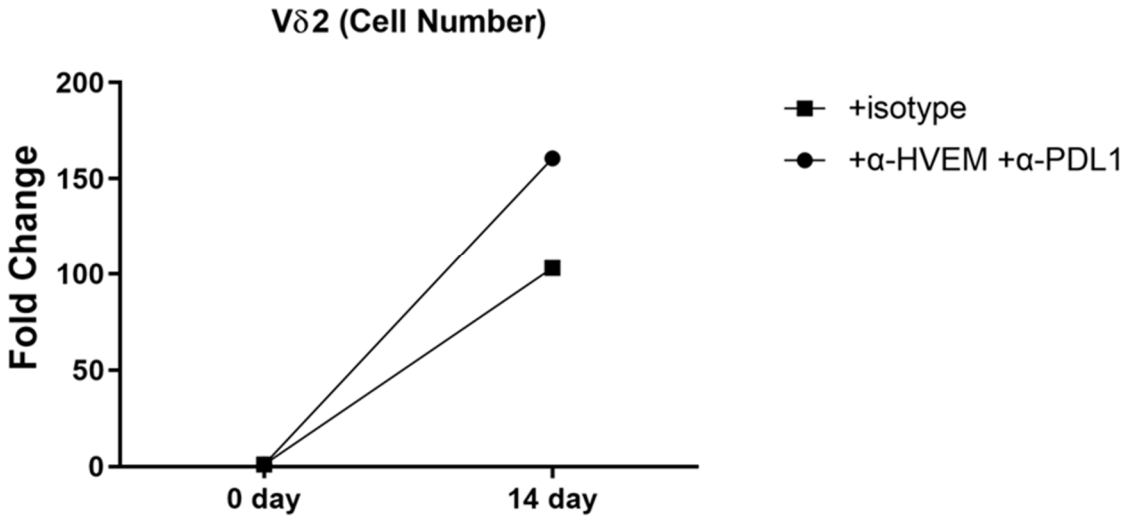


Figure 6. Improved Proliferation of HI γδ T Cells in the Presence of Anti-HVEM Peptide and Anti-PD-L1 mAb. HI γδ T cells were expanded in the presence of anti-HVEM peptide and anti-PD-L1 mAb in addition to zoledronate, IL-2, and IL-15, as described in the Materials and Methods. The cells were treated with the anti-HVEM peptide twice a week [28]. Anti-human PD-L1 at 1 μg/ml (clone 29E.2A3; *Biolegend,* San Diego, CA, USA) was added at the beginning of the culture [27].

Cytotoxicity of HI γδ T Cells Against SNU398 Cells Rely on NKG2D Expression

SNU398 cells express more Fas and MICA/B, while Huh7 cells express more TRAIL receptors, DR4 and DR5 [16]. The expression of CD112 and CD155, DNAM-1 ligands were assessed in SNU398 and Huh7 cells using flow cytometry. The results showed that both cells expressed CD112 and CD155 robustly (Fig 7). I thus performed a cytotoxicity assay with blocking antibodies. The cytotoxicity of HI γδ T cells against SNU398 cells was found to be dependent on NKG2D signaling, as blocking NKG2D significantly reduced the cytotoxicity of HI γδ T cells (Fig. 8). Degranulation of HI γδ T cells with blocking antibodies was also evaluated. Among $CD3^+$ T cells, Vδ1 and Vδ2 cells were gated (Fig. 9A) and the percentages of $CD107a^+$ cells were analyzed by flow cytometry. The results suggest that PB γδ T cells degranulated more against SNU398 cells, while HI Vδ2 T cells degranulated slightly more in response to Huh7 cells (Fig. 9B).

Figure 7.

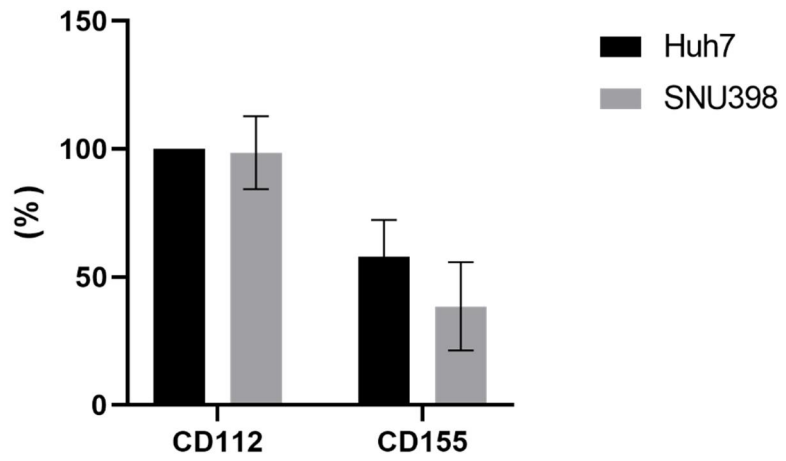


Figure 7. Expression of DNAM-1 Ligands in HCC cell lines. Flow cytometry was performed to assess CD112 and CD155 expression in Huh7 and SNU398 cells (means ± SEM). N = 3.

Figure 8.

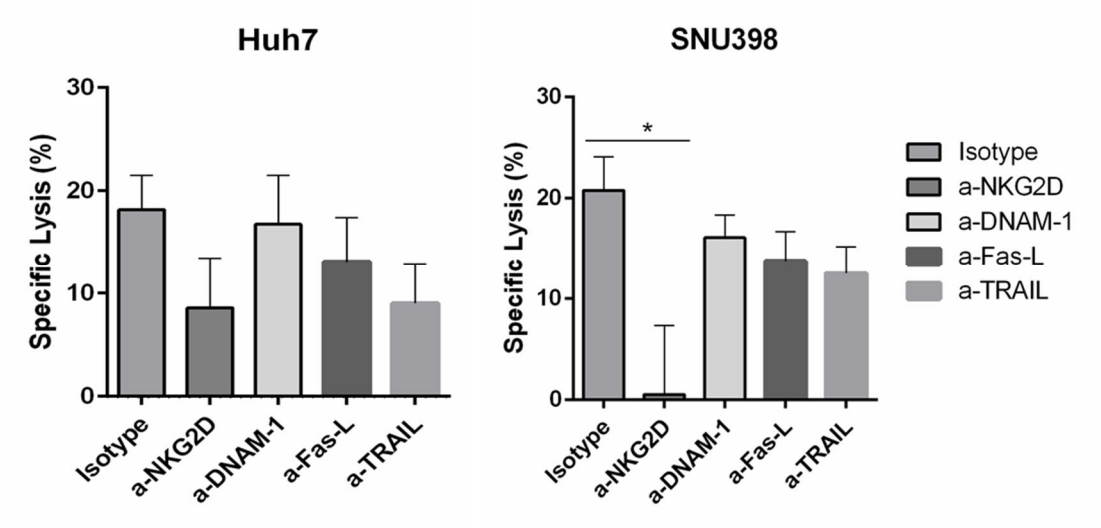
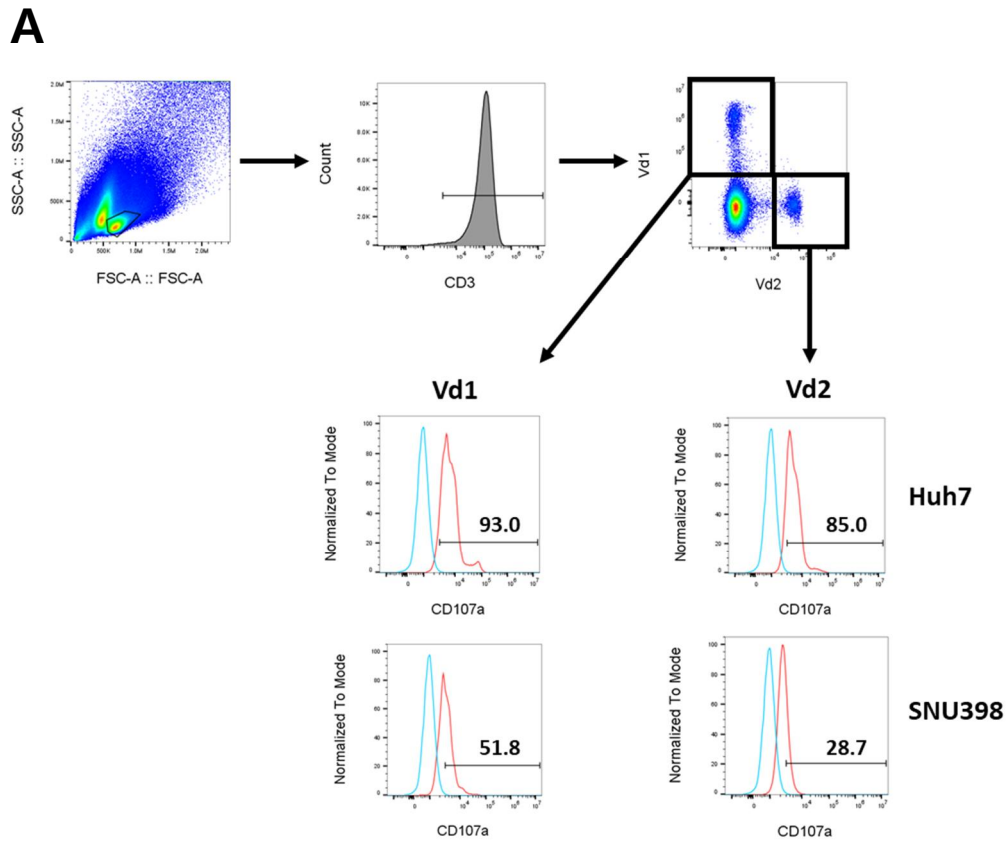


Figure 8. **NKG2D Expression Contributes to Cytotoxicity of HI γδ T Cells against SNU398 Cells.** LDH assay was performed with blocking anti-NKG2D, anti-DNAM-1, anti-FasL, and anti-TRAIL mAbs. The effector to target (E:T) ratio was 20:1. N = 11 for isotype control, n = 6 for anti-NKG2D and anti-DNAM-1, and n = 5 for anti-FasL and anti-TRAIL groups. Means ± SEM are displayed. One-way ANOVA with Tukey's multiple comparison was performed. *$p < 0.05$

Figure 9.


A
SSC-A :: SSC-A
FSC-A :: FSC-A
Count
CD3
Vd1
Vd2
Vd1
Vd2
Normalized To Mode
93.0
CD107a
85.0
Huh7
51.8
28.7
SNU398

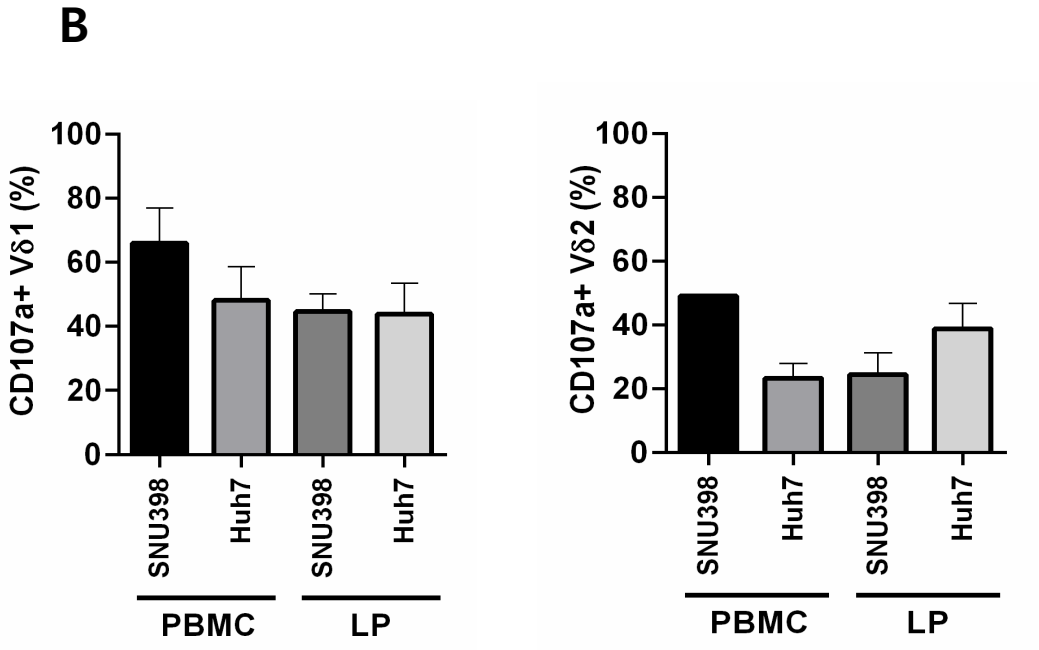

B
CD107a+ Vδ1 (%)
CD107a+ Vδ2 (%)
100
80
60
40
20
0
SNU398
Huh7
SNU398
Huh7
PBMC
LP

Figure 9. HI γδ T Cells Degranulates More in Response to Huh7 Cells Than to SNU398 Cells.

(A) A CD107a degranulation assay was conducted. Numeric values show % of $CD107a^+$ cells. Blue lines, isotype control; red lines, CD107a. **(B)** HI Vδ2 T cells showed higher degranulation against huh7 cells than SNU398 cells. Percentage of CD107a expression on PB γδ T cells and HI γδ T cells. PBMC, N=2; HI γδ T cells, N=7 for Huh7; N=8 for SNU398 (Means ± SEM). One-way ANOVA with Tukey's multiple comparison.

Activating and Death Ligand Signaling Are Associated with Cytotoxicity of HI γδ T Cells against Prostate Cancer Cells

To expand findings, I evaluated the quantities of pyrophosphate in prostate cancer cell lines, PC3, Du145, and LNCaP, and the cytotoxicity of HI γδ T cells expanded with zoledronate. Du145 cells appeared to express higher phospho-antigen than PC3 cells and LNCaP cells, but cytotoxicity of HI γδ T cells against Du145 was comparable to other prostate cancer cell lines (Fig. 10A). The expression of activating and inhibitory ligands, and death receptors of three cell lines was assessed by flow cytometry. The gating strategy was shown in Fig. 11. The expression of most of the cell surface receptors was lower in LNCaP cells than those of PC3 and Du145 cells, in percentages and MFI (Fig. 10B). The cytotoxicity assay with blocking antibodies showed that the cytotoxicity of HI γδ T cells against LNCaP depended on the Fas-Fas L pathway, although the expression of Fas on LNCaP cells was relatively low. The cytotoxicity of HI γδ T cells against PC3 and Du145 cells appeared to be dependent on NKG2D (Fig. 10C). Blockade of DNAM-1 also reduced the cytotoxicity of

HI γδ T cells against PC3. Taken together, cytotoxicity of HI γδ T cells was dependent on activating and death ligand signaling.

Figure 10.

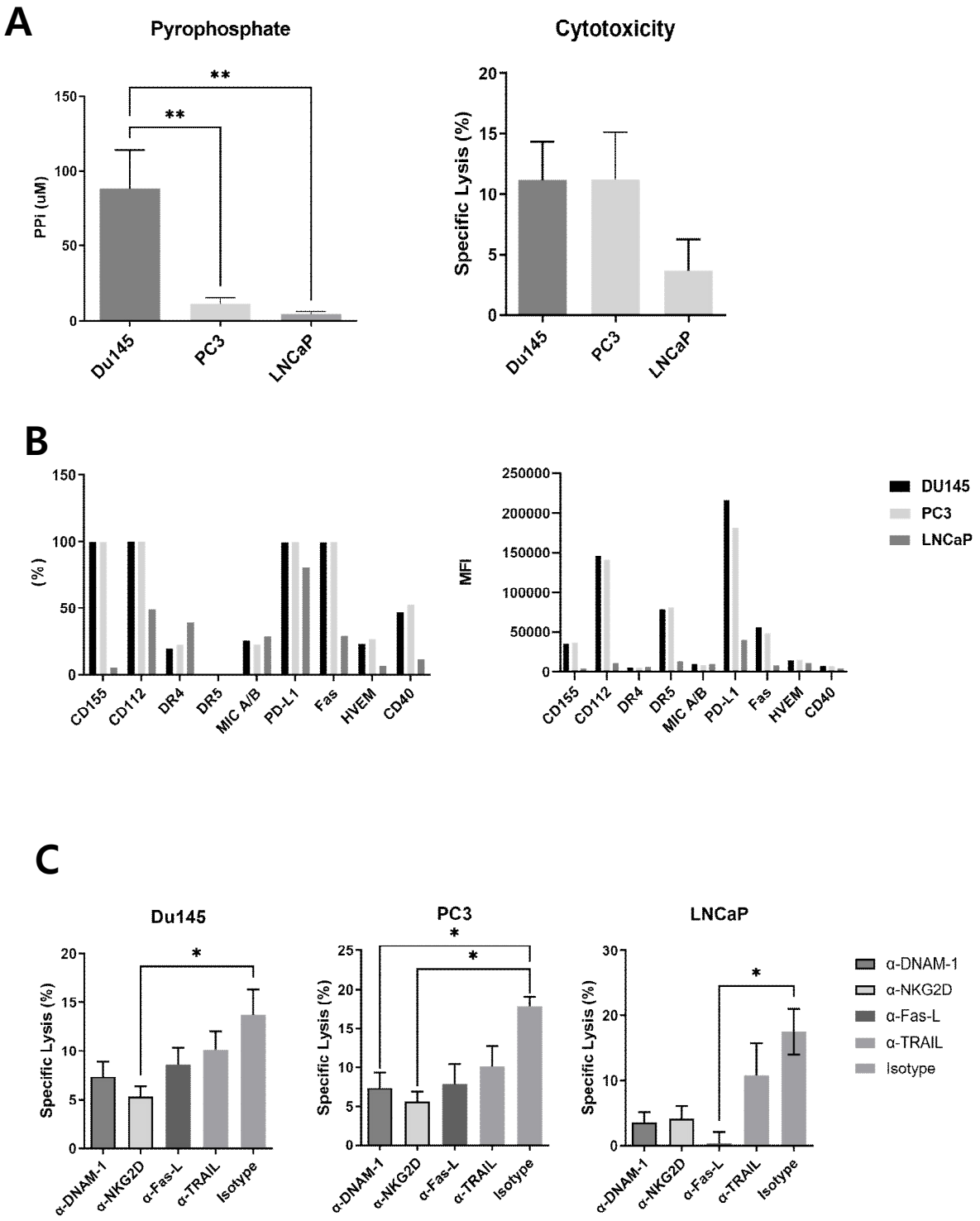
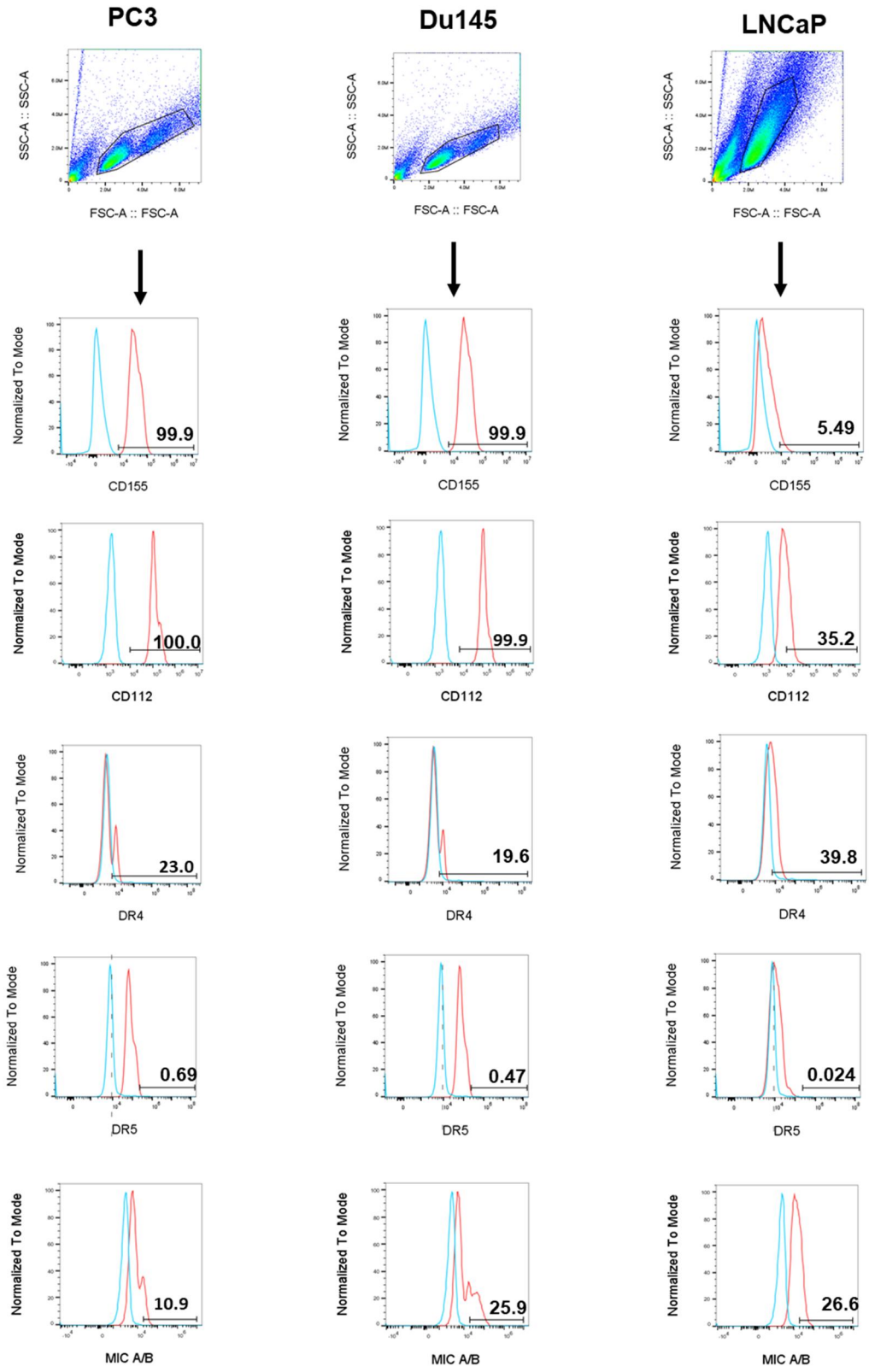

A
Pyrophosphate
PPi (uM)
150
100
50
0
**
**
Du145
PC3
LNCaP
Cytotoxicity
Specific Lysis (%)
20
15
10
5
0
Du145
PC3
LNCaP
B
(%)
150
100
50
0
CD155
CD112
DR4
DR5
MIC A/B
PD-L1
Fas
HVEM
CD40
MFI
250000
200000
150000
100000
50000
0
CD155
CD112
DR4
DR5
MIC A/B
PD-L1
Fas
HVEM
CD40
DU145
PC3
LNCaP
C
Du145
PC3
LNCaP
Specific Lysis (%)
20
15
10
5
0
25
30
*
*
*
α-DNAM-1
α-NKG2D
α-Fas-L
α-TRAIL
Isotype

Figure 10. **Cytotoxicity of HI γδ T cells Correlates to Activating, and Death Ligand Signaling in Prostate Cancer Cell Lines. (A)** Pyrophosphate expression on human prostate cancer cell lines (left panel) and cytotoxicity of HI γδ T cells (right panel) are shown (means ± SEM). N = 4 for pyrophosphate assay and N=9 for LDH assay. **(B)** Percentages (left panel) and MFI (right panel) of activating and inhibitory ligands, and death receptors expression in prostate cancer cell lines. The results are representatives of two independent experiments. **(C)** LDH assay was performed with anti-NKG2D, anti-DNAM-1, anti-FasL, and anti-TRAIL mAbs (means ± SEM). The effector to target (E:T) ratio was 20:1. One-way ANOVA with Tukey's multiple comparison was performed. N = 5 for Du145. In PC3 experiment; n=3 for isotype, n=4 for anti-NKG2D, n=5 for anti-DNAM-1, anti-Fas L and anti-TRAIL. In LNCaP experiment; n=8 for anti-DNAM-1, n=7 for anti-NKG2D and anti-TRAIL, and n=4 for anti-FasL and isotype mAbs.

Figure 11.


PC3
Du145
LNCaP
SSC-A :: SSC-A
FSC-A :: FSC-A
Normalized To Mode
99.9
CD155
99.9
CD155
5.49
CD155
100.0
CD112
99.9
CD112
35.2
CD112
23.0
DR4
19.6
DR4
39.8
DR4
0.69
DR5
0.47
DR5
0.024
DR5
10.9
MIC A/B
25.9
MIC A/B
26.6
MIC A/B

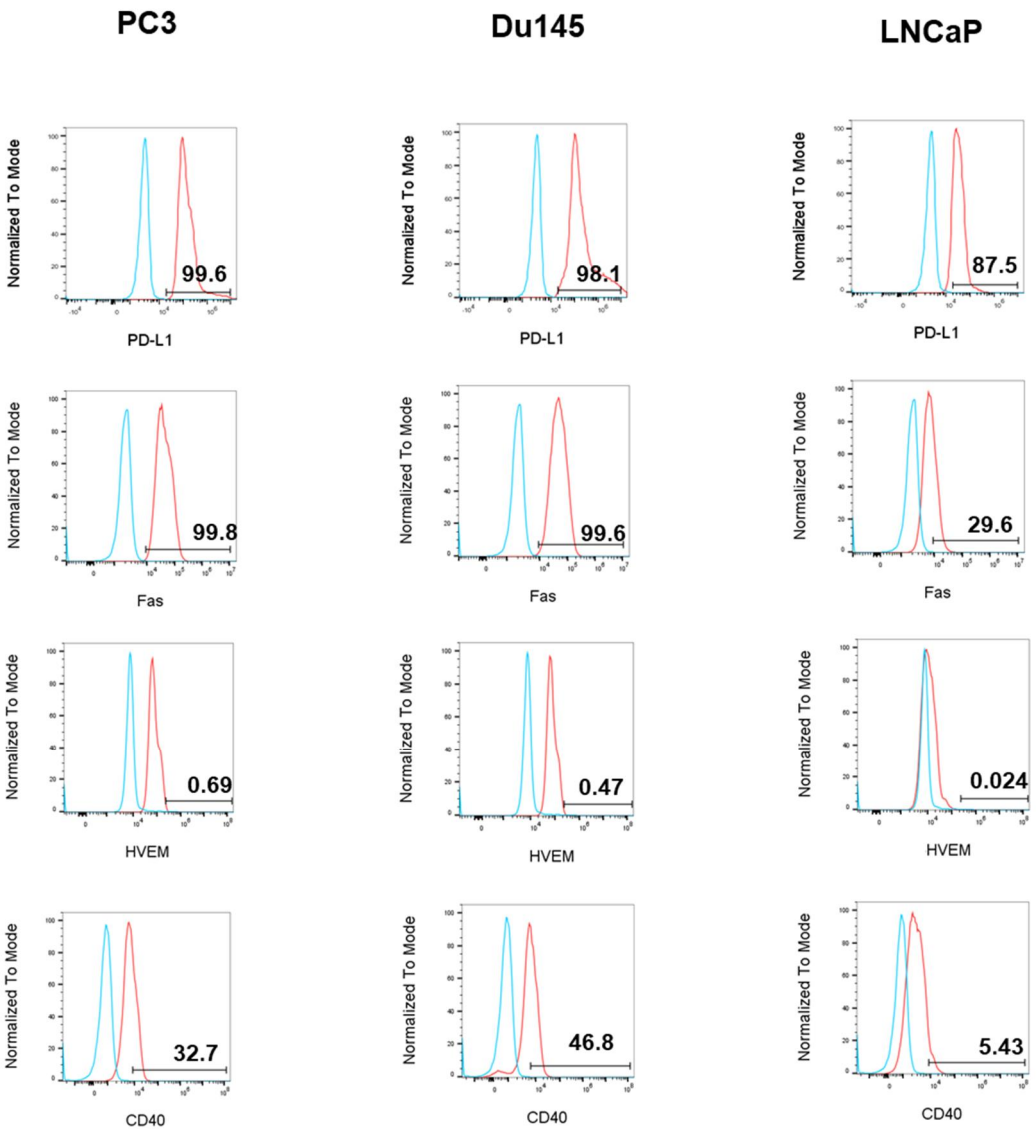

PC3
Du145
LNCaP
Normalized To Mode
99.6
PD-L1
98.1
PD-L1
87.5
PD-L1
99.8
Fas
99.6
Fas
29.6
Fas
0.69
HVEM
0.47
HVEM
0.024
HVEM
32.7
CD40
46.8
CD40
5.43
CD40

Figure 11. Representative Flow Cytometric Plots of Cell Surface Markers on the Prostate Cancer Cells, PC3, Du145 and LNCaP. The results are representative of 2 independent experiments. Numeric values show % of cell surface marker^{+} cells. Blue lines, isotype control; red lines, cell surface markers.

Discussion

In this study, I studied the phenotypes and cytotoxicity of HI γδ T cells from living donor liver transplantation and showed that HI γδ T cells that were expanded with zoledronate had potent cytotoxicity against HI NK-resistant Huh7 cells. The results of the pyrophosphate assay of HCC cells and prostate cancer cells showed that the strong cytotoxic effects of γδ T cells might be related to the expression of phospho-antigen. The expression levels of pyrophosphate were lower in prostate cancer cells than in HCC cells. Accordingly, the cytotoxicity of HI γδ T cells was lower against prostate cancer cells than against HCC cells. However, phospho-antigen expression did not fully explain the cytotoxic effects of HI γδ T cells on SNU398 cells or the higher cytotoxicity of HI γδ T cells than PB γδ T cells. Therefore, I evaluated the expression pattern of activating receptor on HI γδ T cells and found that NKG2D was an alternative modality for killing SNU398 HCC cells.

Given that γδ T cells are not MHC-restricted, and shared many activating receptors with NK cells, cancer treatment using γδ T cells has gained much

importance in recent years [29]. However, there are two major reasons leading to the limited clinical efficacy of γδ T cells. The first reason is that it remains a challenge to potentiate the anti-tumor cytotoxicity of non-modified immune cells. The second issue is difficulty of expansion. Therefore, a number of clinical trials had been conducted using γδ T cells, but only limited clinical trials were reported [30, 31]. To overcome these issues, the beneficial roles of zoledronate in promoting anti-cancer efficacy of Vγ9Vδ2 T cells has been reported. Zoledronate increases the recognition of HCC and colorectal carcinoma cells by Vδ2 T cells [32]. Furthermore, PB γδ T cells and HI Vδ2 T cells expanded sufficiently by zoledronate in this study. The percentages of Vδ2 T cells were over 90% on day 14 of culture, and the Vδ2 expression levels based on MFI were also comparable.

Along with anti-tumor role of γδ T cells, there also have been inconsistent views on the role of γδ T cells in tumor immunity. For example, IL-17 produced by γδ T17 cells can promote cancer metastasis by supporting angiogenesis in the tumor microenvironment of various cancer [33, 34]. γδ T17 cells promoted the accumulation of myeloid-derived suppressor cells in human colorectal cancer [35]. Studies claiming that γδ T cells had

protumor role also have been reported [36]. Although the role of γδ T cells in cancer is still controversial, this study aimed to characterize the anti-tumor feature of HI γδ T cells.

In summary, this study suggests that γδ T cells can be a candidate for cell-mediated cancer therapy since it showed strong cytotoxicity against various cancer cell lines including liver cancer and prostate cancer cell lines. Minority and dual roles of γδ T cells are challenging and slow the advance of γδ T cells biology. Nontheless understanding of its role may be an important key for promoting pre-existing cancer therapies.

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국문요약

배경: 간세포암은 가장 흔한 primary liver cancer로 전세계 암 사망율의 3 위를 차지하고 있는 난치성 질환으로서 새로운 치료 방법의 개발이 매우 시급한 상황이다. 본 연구는 암 세포 치료제의 새로운 후보로서 생체 간이식 시 얻어지는 간 관류액 내 감마 델타 T세포를 제시하였다. 간 관류액 내 림프구는 말초 혈액 림프구와 비교하였을 때 간에 존재하는 면역 세포에 가까운 특성을 가지고 있어서 간 면역 세포를 연구할 수 있는 매우 좋은 수단을 제공한다. 생체 간이식이 매우 활발한 한국에서는 간 관류액 내 림프구 세포를 얻기가 상대적으로 용이하여 큰 장점이 될 수 있다. 또한, 연구에 사용된 감마 델타 T 세포의 경우 알파 베타 T세포와 달리 MHC 분자의 제약을 받지 않음으로 graft versus host disease (GvHD)를 일으킬 가능성이 매우 낮고 제 3 자의 공여자 세포를 이용할 수 있어 기존 세포 치료제의 훌륭한 대안이 될 것으로 생각되어진다.

방법: 간 관류액 내 감마 델타 T세포와 말초 혈액 감마 델타 T세포의 간암 세포주에 대한 세포 살상능력을 비교하기 위하여 실험관 내 lactate dehydrogenase (LDH) assay를 수행하였다. 또한 생체 내 실험으로 면역 결핍 생쥐에 CFSE 표지된 간암

세포주와 간 관류액 내 감마 델타 T세포를 복강 내 주사하여 세포 독성능을 평가하였다. 그 다음으로 Zoledronate, IL-2, 그리고 IL-15 사이토카인의 조합으로 14 일동안 배양 후 유세포 분석을 통하여 세포 살상에 관여하는 세포 표면 수용체의 발현을 비교하였다. 선별된 세포 표면 수용체에 대한 차단 항체를 처리 후 실험관 내 LDH assay를 수행하여 각 수용체의 세포 살상에 대한 기여도를 측정하였다.

세포 표면 수용체 외에 또다른 세포 살상 기전으로 암세포의 표면에 발현하는 인항원에 의한 Vδ2 감마 델타 T세포의 활성화를 제시하였고 이를 입증하기 위하여 간암 세포주 및 전립선암 세포주에서 인 항원의 발현을 pyrophosphate assay를 통하여 측정하였다.

결과: 말초 혈액 단핵구 내 감마 델타 T세포에 비해 간 관류액 내 감마 델타 T세포의 간암 세포주에 대한 높은 세포독성능을 실험관 내, 생체 내 실험을 통하여 확인하였다. 특히 자연 살상 세포 민감성 세포주인 SNU398 보다 자연 살상 세포 저항성 세포주인 Huh7 에 대하여 더욱 높은 세포독성을 보이는 것을 확인하였다. 세포살상 기전으로는 자연 살상 세포와 발현을 함께하는 다양한 세포 표면 수용체 및 암세포 표면에 발현하는 인 항원에 의한 감마 델타 T세포의 활성화를 제시하였고 실험관 내 실험을

통해 Huh7 에 대한 높은 세포 독성의 경우 Huh7 표면의 높은 인항원의 발현에 의한 것임을 확인하였으며 SNU398 은 세포 표면 수용체 NKG2D와 해당하는 리간드 간의 결합에 의한 것임을 LDH assay 통해 확인하였다. 간암 세포주 뿐만 아니라 전립선암 세포주에 대해서도 간 관류액 내 감마 델타 T세포의 세포 독성 및 살상 기전을 조사하였으며 전립선 암의 경우 인항원의 발현뿐만 아니라 세포 표면 수용체 및 리간드의 결합이 세포살상에 기여하는 것임을 확인할 수 있었다.

결론: 간 관류액 내 감마 델타 T 세포가 말초 혈액 단핵구 감마 델타 T 세포보다 간암 세포주에 대해 높은 세포 독성을 보이는 것을 확인하였다. 특히 자연 살상세포 저항성 세포주인 Huh7 이 효과적으로 간 관류액 내 감마 델타 T 세포에 의해 제거됨을 확인하였다. 암세포 살상 기전으로 간 관류액 감마 델타 T 세포는 인 항원에 의한 활성화 그리고 세포표면 수용체를 활용함을 알 수 있었으며 이는 암세포 종류에 따라 다른 것을 실험을 통해 확인하였다.