이학석사 학위논문

키메라 항원 수용체 (CAR)-γδ T 세포의 생산 및 평가

Production and Evaluation of Chimeric Antigen Receptor (CAR)-γδ T Cells

울 산 대 학 교 대 학 원

의 과 학 과

김 민 송

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지 도 교 수 고 경 남

이 논문을 이학석사학위 논문으로 제출함

2024 년 08 월

울 산 대 학 교 대 학 원

의 과 학 과

김 민 송

김민송의 이학석사학위 논문을 인준함

심사위원 김 혜 리 인

심사위원 고 경 남 인

심사위원 김 나 영 인

울 산 대 학 교 대 학 원

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Contents

List of Figures

Abstract

Background: Chimeric antigen receptor **(**CAR)-T cell therapy has been successful in clinics, but complications and side-effects hamper wide application. Gamma delta (γδ) T cells can recognize antigens without MHC restriction, reduceing the risk of graft-versus-host disease (GvHD). Thus, I set up the experiments to characterize expanded γδ T cells and produce CAR-γδ T cells.

Materials and Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers. Cell surface markers of γδ T cells were characterized using flow cytometry. γδ T cells were cultured for 2 weeks in three different conditions. Lentiviral vector expressing CD19-CAR (FMC63-CD8 hinge) was transduced at day 10 in culture. Cytotoxicity was analyzed using 7-amino actinomycin D (7-AAD) and Annexin V (AV) in OCI-Ly7 cells, a human B cell lymphoma cell line. NSG mice were injected intraperitoneally (i.p.) with SU-DHL6 cells labeled with Carboxyfluorescein succinimidyl ester (CFSE) followed by injection of CD19-CAR-transduced γδ T cells. After 48 hours, the peritoneal lavage was harvested and analyzed by flow cytometry to evaluate *in vivo* efficacy.

Results: Vδ1 γδ T cells were expanded in the presence of IL-2+IL-15, while Vδ2 γδ T significantly proliferated in the presence of IL-2+ZOL+IL-15. γδ T cells expressed the

activating receptors (NKG2D, CD16, and DNAM-1), cell death receptors (TRAIL and FasL) and, immune checkpoint receptors (PD-1 and BTLA) molecules sustainably. CAR-γδ T cells exhibited higher cytotoxicity than that of γδ T cells without CAR *in vitro*. Mice injected with *ex vivo* expanded CAR- γδ T cells showed lower numbers of SU-DHL6 cells than mice injected with γδ T cells without CAR.

Conclusion: This study shows *ex vivo* expanded γδ T cells expressed cell surface markers robustly, and γδ T cells transduced with CAR exhibited advanced tumor cell killing ability *in vitro* and *in vivo*.

Introduction

The chimeric antigen receptors (CARs) render the function of T cells focused on specific cell surface antigens on the tumor cells [1]. T cells with receptors that specifically bind to tumor cells have been developed, which are known as CAR-T cells [2]. CAR is produced by recombination of the single-chain variable fragment (scFv) sequence of a monoclonal antibody that recognizes a characteristic antigen expressed on the surface of cancer cells. CARs have specificity through scFv. CAR is composed of an extracellular domain including an scFv, a transmembrane domain, and a signaling domain [3-5]. The 1st generation CARs typically have a CD3-ζ signaling domain [6]. The 2nd and 3rd generation CARs have 1 and 2 co-stimulatory domains, respectively, and provide more activating signals than the 1st generation CARs [7]. CD28, 4-1BB, CD27, and OX40 co-stimulatory domains are commonly added to enhance T cell activation [8-11].

Genetically engineered CAR-T cells are used as cancer immunotherapy because they recognize antigens expressed by cancer cells [12]. In clinical trials, CD19-directed CAR-T cells have demonstrated efficacy in acute lymphoblastic leukemia (ALL) [13-16], chronic lymphocytic leukemia (CLL) [17-19], non-Hodgkin lymphoma (NHL) [20], and diffuse large B cell lymphoma (DLBCL) [21]. The two CD19 CAR-T cell therapy agents, Kymriah

(Tisagenlecleucel) [22] and Yescarta (Axicabtagene ciloleucel) [23] have been approved by the United States Food and Drug Administration. Compared with T cell receptor-modified T cells, CAR-T cells can recognize antigens without antigen-presenting cells, and regardless of MHC types, showing better clinical results [24, 25]. When CAR-T cell recognizes an antigen on the surface of a tumor cell, the signaling pathway is stimulated by an auxiliary stimulus signal that activates T cells, inducing the activation and proliferation of the CAR-T cell. Therefore, CAR-T therapy has the advantage of significantly higher clinical antitumor efficacy compared to monoclonal antibodies or other types of immunotherapy [26].

These treatments have been successful in hematologic malignancy but have yet to show any effect on solid tumors. In addition, side effect such as cytokine release syndrome has been reported for CAR-T cell due to the characteristics of T cells that divide rapidly when they meet target cells [27, 28]. Moreover, T cells that remain in the body for a long time as memory could have a problem that side effects may occur at any time if they recur. As an alternative to these disadvantages, CAR-NK and CAR-γδ T cells have begun to be developed by transducing CAR genes into NK and γδ T cells.

γδ T cells have distinctive features of combined adaptive and innate immunity to recognize and eliminate tumor cells over normal cells [29-32]. γδ T cells with T cell receptor γ and δ

chains are a small subset of T cells, accounting for 1-5% of peripheral blood mononuclear cells (PBMCs). Human γδ T cells are divided into subsets with Vδ1 and Vδ2 chains [33]. Most of the γδ T cells in human blood are Vγ9Vδ2 [34-36]. Vδ2 γδ T cells are a subset of γδ T cells that dominate about 50-75% of γδ T cells in PBMCs. As Vδ2 γδ T cells respond to phospho-antigens accumulated in cancer cells, they have been studied as a treatment for various malignancies. Vδ1 γδ T cells are present in less than about 30% of γδ T cells, and are the second most frequent subset in epithelial tissues including thymus, dermis, intestinal epithelium, and spleen [37]. Phospho-antigens are iso-prenoid synthesis products that specifically activate Vγ9Vδ2 T cells [38]. Iso-pentenyl pyrophosphate (IPP) is produced from the mevalonate pathway by cancer and HMBPP is produced in parasitic or bacterial infections [39, 40]. Vδ1 γδ T cells do not respond to phospho-antigen, but major histocompatibility complex (MHC) class I chain-related molecules A (MICA) and B (MICB) are known to bind [41]. It also responds to the stress signal by producing cytokines and chemokines. Vδ1 and Vδ2 γδ T cells have been applied in diverse cancer cell immunotherapy [42, 43].

Unlike αβ T cell, γδ T cells are not restricted to MHC and respond to phosphorylated non-peptide antigens called phospho-antigen produced by pathogens or cancer [44]. γδ T cells can recognize cancer antigens other than peptides, thus expanding the range of targets [45]. Herein

I set up the experiments to produce CAR-γδ T cells and evaluated them *in vitro* and *in vivo*.

Materials and Methods

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs from healthy volunteers (HV) were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Milwaukee, WI, USA). All the participants provided written informed consent (IRB Approval No. 2018-0445). PBMCs were cryopreserved in heat-inactivated fetal bovine serum (FBS) (Welgene, Gyeongsangbuk-do, Republic of Korea) containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and stored in a liquid nitrogen tank until use.

Expansion of γδ T cells

PBMCs were seeded into a 6 well plate at a density of 3 X 10^6 cells/mL in RPMI-1640 (Welgene) supplemented with 10% inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM sodium pyruvate (Sigma-Aldrich), and 0.55 mM 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, USA). γδ T cells were cultured in the presence of 100 U/mL recombinant human IL-2 (rhIL-2) (PeproTech, Rocky Hill, NH, USA), 1 μM zoledronate (ZOL) (Selleckchem, Houston, TX, USA), and 50 ng/mL recombinant human IL-15 (rhIL-15) (PeproTech), in the presence of 100 U/mL rhIL-2, and 50 ng/mL rhIL-15, or in

the presence of 20 ng/mL recombinant human IL-7 (rhIL-7) (R&D systems, Minneapolis, MN, USA) and 1 μg/mL phytohemagglutinin-L (PHA) (eBioscience, San Diego, CA, USA) for 2 weeks. IL-2, IL-7, and IL-15 were refreshed at the same concentration every 2 days, and PHA every 4 days.

Cell lines

A human diffuse large B-cell lymphomas (DCBLC) cell line, OCI-Ly7, and a human embryonic kidney cell line, HEK293T, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). OCI-Ly7 and HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Corning, Manassas, VA, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Corning). All of the cells were cultured in 5% CO2 at 37℃.

Preparation of chimeric antigen receptor (CAR)-expressing lentiviral vector

CD19 CAR-expressing vector (FMC63-CD8 hinge-BBz-pCL20C-MND) was provided by Dr. Byoung Y. Ryu (St. Jude Hospital, Memphis, Tennessee, USA). CD19 CAR construct contained the FMC63 scFv fragment, the hinge and transmembrane domain of the CD8, the

cytoplasmic domain of 41-BB (CD137, TNFRSF9), and the CD3ζ signaling domain (Figure 1). To detect CAR expression, CD19 CAR was tagged with Myc. CAR vector was transformed into an *E.coli* strain, Stbl3. CD19 CAR vector was digested by AgeI (NEB, Ipswich, MA, USA) and NotI (ELPIS, Daejeon, Republic of Korea) restriction enzymes, and was confirmed by agarose gel electrophoresis. The competent Stbl3 cells were prepared using a 0.1 M solution of $CaCl_2$ following standard protocols. The transformation was performed using the heat shock method and the transformed bacteria were cultured on a LB (Duchefa biochemie B.V, Haarlem, Netherlands) Agar (BD Biosciences, San Diego, CA, USA) plate containing 100 μg/mL ampicillin (Duchefa biochemie B.V).

Figure 1

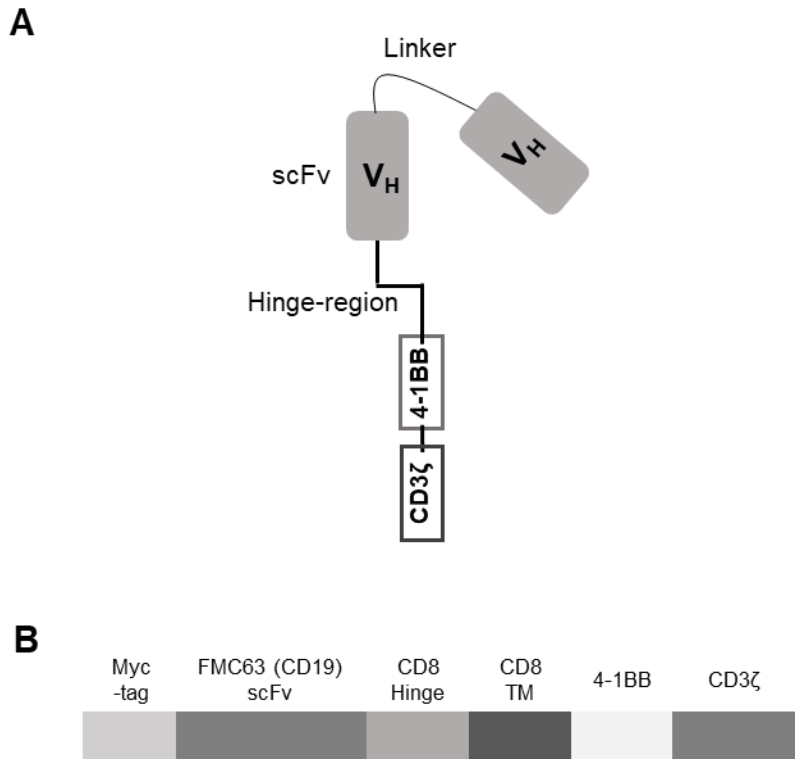

Figure 1. Chimeric antigen receptor (CAR) design. (A) The CARs consisted of a single-chain variable fragment (scFv) of an antibody, a non-signaling extracellular hinge domain, an intracellular CD3ζ domain, and a 4-1BB co-stimulatory domain. (B) Schematic diagram of CD19 CAR constructs.

Lentivirus production and transduction

All the vectors used for lentiviral transduction were purified using a Plasmid DNA Midi prep kit (Qiagen, Venlo, Netherlands). To produce lentivirus particles, 293T cells were seeded in a 100-mm culture dish at a density of 2 X 10^6 cells/mL one day before transfection. After 24 hours, the CAR-expressing vector construct was transfected into 293T cells with packaging plasmid vectors, pCAG-KGP1-1R, pCAG4-RTR2, and pCAG-VSVG, at a ratio of 6:3:1:1 using Lipofectamine 3000 (Invitrogen, Waltham, MA, USA). The transfected cells were incubated at 37℃ for 16-18 hours in serum-free DMEM medium without antibiotics. The medium was then replaced with fresh DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and the cells were incubated at 37℃ for another 24-48 hours. Virus-containing medium was harvested 48 and 72 hours after transfection and filtered through a 0.45 μM filter (Millipore, Burlington, MA, USA). Viral supernatant was used for infection of γδ T cells treated with 8 μg/ mL Polybrene (Millipore). Lentivirus was transduced twice on days 10 and 11 in culture.

Flow cytometry

The surface molecules of γδ T cells were recorded by staining with an APC mouse anti-human

CD314 (NKG2D) (clone 1D11, BD Biosciences), Brilliant Violet 605 anti-human CD226 (DNAM-1) (clone 11A8, BioLegend, San Diego, CA, USA), PE mouse anti-human CD16 (4G7, clone BD Biosciences), PE anti-human CD253 (TRAIL) (clone RIK-2, BioLegend), PE anti-human CD178 (Fas-L) (clone NOK-1, BioLegend), APC/Cyanine7 anti-human CD279 (PD-1) (clone EH12.2H7, BioLegend), and APC anti-human CD272 (BTLA) (clone MIH26, BioLegend). For detection of the CD19 CAR, transduced cells were stained with AlexaFluor-647-conjugated Myc-Tag mouse mAb (clone 9B11, Cell Signaling Technology, Beverly, MA, USA) and matched AlexaFluor-647-conjugated mouse IgG2a, κ isotype control (Clone MOPC-173, BioLegend). In order to analyze the phenotype of transduced cells, the following antibodies were used: APC/Fire75-conjugated anti-human CD3 antibody (Clone SK7, BioLegend), Pacific Blue-conjugated anti-human CD56 (NCAM) antibody (Clone 5.1H11, BioLegend), PE-Cy7-conjugated TCR Vδ1 monoclonal antibody (Clone TS8.2, Invitrogen), and Brilliant Violet-conjugated anti-human TCR Vδ2 antibody (Clone B6, BioLegend). Cells were treated with a human FcR blocking reagent at room temperature for 5 min before Ab treatment. Cells were stained with the above antibodies at 4℃ for 30 min, then washed. Flow cytometry was conducted with a CytoFlex (Beckman Coulter Life Sciences, Brea, CA, USA). The data was analyzed by a FlowJo software v10 (Treestar, Inc., Ashland, OR, USA).

Cytotoxicity assay

Cytotoxicity was analyzed using 7-amino-actinomycin D (7-AAD) and Annexin V (AV) assay. For cytotoxicity assay, OCI-Ly7 cells as target cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using Cell Trace Cell Proliferation Kits (Thermo Fisher Scientific) according to the manufacturer's instructions. CAR-transduced γδ T cells as effector cells were harvested and resuspended in RPMI 1640 media. γδ T cells were co-cultured with CFSE labeled target cells at a 2:1 of E: T ratio. After 1 day, cells were washed with Annexin V binding buffer (BD Biosciences) and incubated at 37℃ for 30 min. Cells were treated with human FcR blocking reagent (Miltenyi Biotec B.V, Teterow, Germany) for 5 min and stained with 7-AAD Viability staining solution (BioLegend) and AV-Pacific Blue antibody (BioLegend) on ice for 15 min. $CFSE^+$ target cells were evaluated for early and late apoptosis by flow cytometry with a CytoFLEX (Beckman Coulter Life Sciences). Data analysis was performed using FlowJo v10.

***In vivo* evaluation of CAR-γδ T cells in response to B cell lymphoma cell line**

For *in vivo* evaluation, SU-DHL6 (SU) cells, a diffuse large B-cell lymphoma cell line, were labeled with CFSE. On day 0, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (JA Bio, Co.) were

injected intraperitoneally (i.p.) with 5 X 10^6 SU cells. After 30 minutes, Mice were subsequently injected i.p. with 5 X 10^6 γδ T cells transduced with CD19-CAR. On day2, 10 mL ice-cold 1X PBS (Biosesang, Gyeonggi-do, Republic of Korea) was injected into the peritoneal cavity, and the peritoneal lavage was harvested $CFSE^+$ cells reduction was analyzed by flow cytometry. The data was analyzed by a FlowJo software. All mouse experiments were approved by the Institutional Animal Care and Use Committee at Asan Medical Center (Approval No. 2020-12-251).

Statistical analysis

GraphPad Prism v6 (GraphPad Software Inc., San Diego, CA, USA) was used to generate graphs and statistical analyses. *P* values were determined by the paired two-tailed *t*-test or two-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant; ns, not significant; $*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$. All data are depicted as means±standard error of the mean (SEM)

Results

Stimulation with IL-2, ZOL and IL-15 enhances γδ T cell proliferation

γδ T cells were cultured using PBMCs from HV to optimize the expansion. It has been reported that Vδ2 γδ T cells proliferate upon stimulation of IL-2 and ZOL [46], and IL-7 and PHA enhances the proliferation of Vδ1 γδ T cells [47]. IL-15 enhances the proliferation of γδ T cells [48]. Thus, γδ T cells were cultured for 14 days under three conditions; The first condition was the treatment with IL-7 and PHA and the second was IL-2 and IL-15 treatment. Lastly, IL-2, ZOL, and IL-15 were cotreated. The subsets of expanded cells were analyzed by Vδ1, Vδ2, and CD56 expression (Figure 2A). IL-2+ZOL+IL-15 stimulation was most effective for γδ T cell proliferation (Figure 2B and 2C). Total lymphocytes were counted by FSC/SSC gating, and the number of γδ T cells were calculated using the percentages of $CD3^{+}V\delta1^{+}$ or $V\delta2^{+}$ cells. Vδ1 γδ T cells were expanded about 10-fold in the presence of IL-2 and IL-15 (Figure 2D). By IL-2, ZOL, and IL-15 cotreatment, Vδ2 γδ T cells and NK cells significantly proliferated 114 and 2-fold, respectively (Figure 2E and 2F). It was confirmed that Vδ1 γδ T cells were most expanded by IL-2 and IL-15 and Vδ2 γδ T cells by IL-2, ZOL, and IL-15.

Figure 2

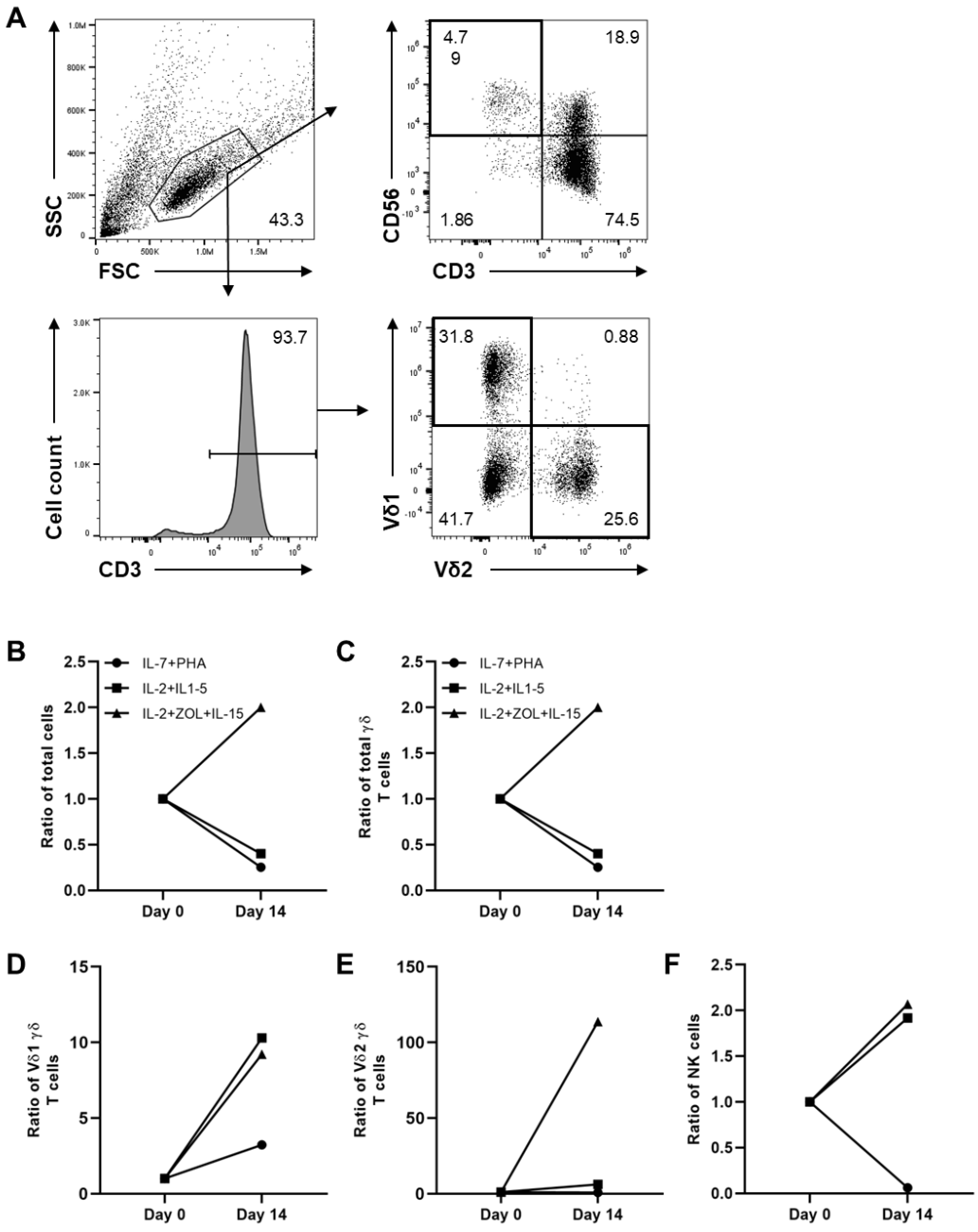

Figure 2. Expansion of γδ T cells *in vitro*. γδ T cells were cultured for 14 days under three different conditions: IL-7 and PHA; IL-2 and IL-15; IL-2, IL-15, and zoledronate (ZOL). Cells were stained with anti-human CD3, CD56, TCR Vδ1, and Vδ2 mAb for flow cytometry. (A) Representative flow cytometric plots of αβ T cell-depleted PBMCs from a healthy donor showing the proportion of $V\delta1^+$ and $V\delta2^+$ γδ T cells and NK cells ($CD3^-CD56^+$). The cell growth curves of total lymphocyte (B), total γδ T (C), Vδ1 γδ T (D), Vδ2 γδ T (E) and NK (F) cells are shown. The cell numbers was assessed at 0 and 14 days in culture (N=7). Average fold changes were displayed.

The phenotypes of Vδ1 and Vδ2 γδ T cells

To examine whether expanded γδ T cells maintained the phenotypes of cell surface receptors, I performed flow cytometry. The percentages of γδ T cell surface markers were calculated based on flow cytometric plots on days 0 and 14 of expansion (Figure 3A). I compared the phenotypes of Vδ1 and Vδ2 γδ T cells as well. γδ T cells expressed the activation, cell death, and immune checkpoint molecules which are predominantly expressed in cytotoxic immune cells. Almost all surface markers were expressed at similar levels between Vδ1 and Vδ2 γδ T cells before culture with an exception of CD226 (Figure 3B). CD226 was more expressed in Vδ2 γδ T cells than Vδ1 γδ T cells (Figure 3B). After IL-2+IL-15 and IL-2+ZOL+IL-15 stimulation, the NKG2D expression was lower in Vδ2 γδ T cells than Vδ1γδ T cells, as opposed to that of *ex vivo* (Figure 3C). The CD16 expression was increased in Vδ2 γδ T cells at day 14 (Figure 3C), whereas the percentages of NKG2D, CD16, and CD226 in Vδ1 γδ T cells were decreased in the presence of IL-7 and PHA (Figure 3C). Likewise, the expressions of TRAIL, FAS-L, PD-1, and BTLA were decreased in the presence of IL-7 and PHA after culture (Figures 3D and 3E). The $TRAIL^+$ and $FAS\text{-}L^+$ cells in Vδ1 and Vδ2 γδ T cells were increased by IL-2+IL-15 and IL-2+ZOL+IL-15 after culture, compared with those before culture (Figure 3D). More Vδ1 γδ T cells expressed PD-1 than Vδ2 γδ T cells (Figure

3E).

As shown in Figure 4, the mean fluorescence intensities (MFIs) of the cell surface molecules were measured before and after culture. The MFI of NKG2D was significantly higher in expanded Vδ1 γδ T cells than that of Vδ2 γδ T cells (Figure 4C). The MFI of PD-1 on expanded Vδ1 γδ T cells was higher than that of Vδ2 γδ T cells by IL-2+IL-15 (Figure 4E). These results suggest that the surface receptors of cultured γδ T cells are differentially expressed between Vδ1 and Vδ2 γδ T cells. Nonetheless, the surface markers on γδ T cells were robustly maintained after culture by IL-2+IL15 and IL-2+ZOL+IL-15.

Figure 3

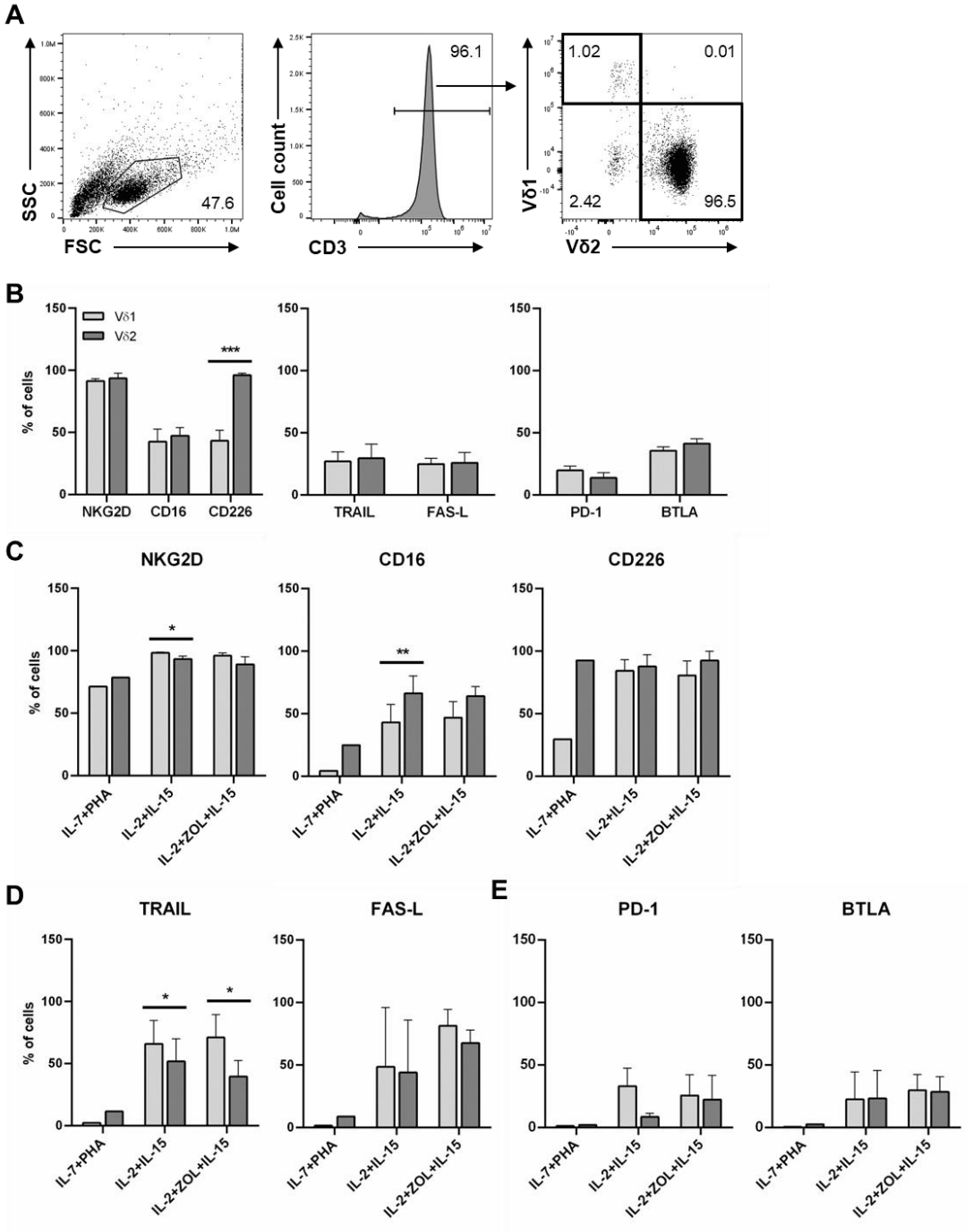

Figure 3. The percentages of γδ T cells expressing selected cell surface makers. (A) Gating strategy to define Vδ1 and Vδ2 γδ T cells. (B) Expression of activation (NKG2D, CD16, and CD226), cell death (TRAIL and FAS-L), and immune checkpoint (PD-1 and BTLA) receptors in Vδ1 and Vδ2 on day 0. (n=1-5, mean ± SEM; ***, $p<0.001$). (C) The percentages of NKG2D, CD16 and CD226-expressing Vδ1 and Vδ2 γδ T cells were shown in bar graphs (n=1-5, mean ± SEM; *, $p<0.05$; **, $p<0.01$). (D) The percentages of TRAIL and FAS-L-expressing Vδ1 and Vδ2 γδ T cells were shown in bar graphs (n=1 for IL-7+PHA: n=5 for IL-2+IL-15 and IL-2+ZOL+IL-15; mean ± SEM; *, $p<0.05$). (E) The percentages of PD-1 and BTLA expressing Vδ1 and Vδ2 γδ T cells were shown in bar graphs (n=1 for IL-7+PHA: n=5 for IL-2+IL-15 and IL-2+ZOL+IL-15; mean ± SEM).

Figure 4

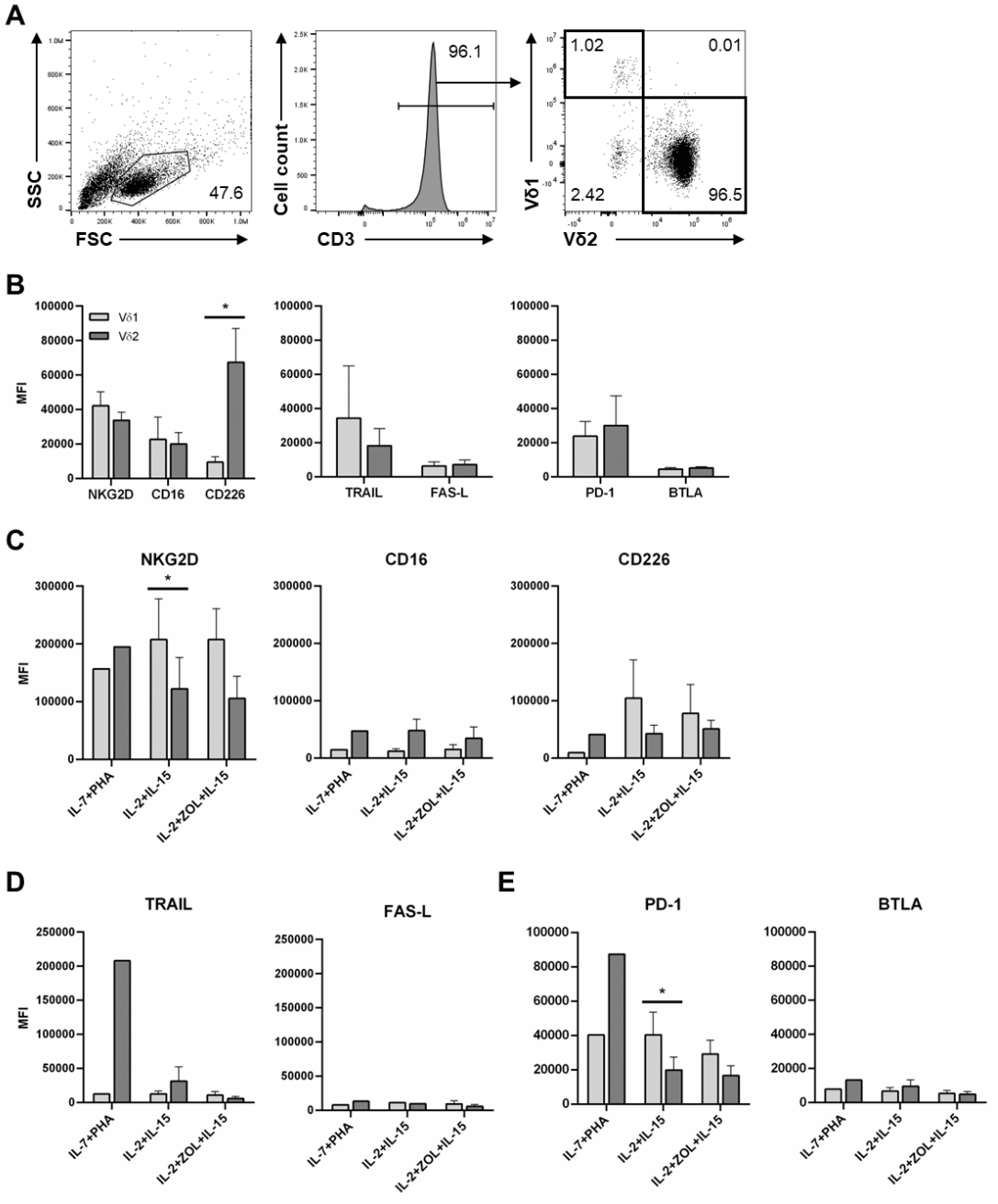

Figure 4. The mean fluorescence indices (MFIs) of γδ T cell surface receptors. (A) Gating strategy to define Vδ1 and Vδ2 γδ T cells. (B) Expression of activation (NKG2D, CD16, and CD226), cell death (TRAIL and FAS-L), and immune checkpoint (PD-1 and BTLA) surface receptors in Vδ1 and Vδ2 on day 0. (n=1-5, mean ± SEM; *, $p<0.05$). (C) The MFIs of NKG2D, CD16 and CD226-expressing Vδ1 and Vδ2 γδ T cells were shown in bar graphs (n=1 for IL-7+PHA: n=5 for IL-2+IL-15 and IL-2+ZOL+IL-15; mean ± SEM; *, $p<0.05$). (D) The MFIs of TRAIL and FAS-L-expressing Vδ1 and Vδ2 γδ T cells were shown in bar graphs (n=1 for IL-7+PHA: n=5 for IL-2+IL-15 and IL-2+ZOL+IL-15; mean ± SEM). (E) The MFIs of PD-1 and BTLA expressing Vδ1 and Vδ2 γδ T cells were shown in bar graphs (n=1 for IL-7+PHA: n=5 for IL-2+IL-15 and IL-2+ZOL+IL-15; mean ± SEM; *, $p<0.05$).

Generation of CD19 CAR-expressing γδ T Cells

Among the three conditions described in Figure 2, IL-2+IL-15 and IL-2+ZOL+IL-15 were more effective in expanding γδ T cells than IL-7+PHA. Therefore, CD19 CAR was transduced into the γδ T cell cultured with IL-2+IL-15 and IL-2+ZOL+IL-15. γδ T cells were transduced with lentiviral particles expressing CD19 CAR on day 10 of culture, and second transduction was performed 24 hours after the transduction to increase CD19 CAR- γδ T cell production (Figure 5A). γδ T cells transduced with CD19 CAR were harvested to measure the CAR's transduction efficiency 48 hours after the second transduction. CD19 CAR-γδ T cells were identified by measuring Myc^+ cells (Figure 5B). The CAR transduction efficacy was low, but γδ T cells transduced with CD19 CAR had about 1.5 times higher $CD3^+Myc^+$ cells than the control (Figures 5C and 5D).

Figure 5

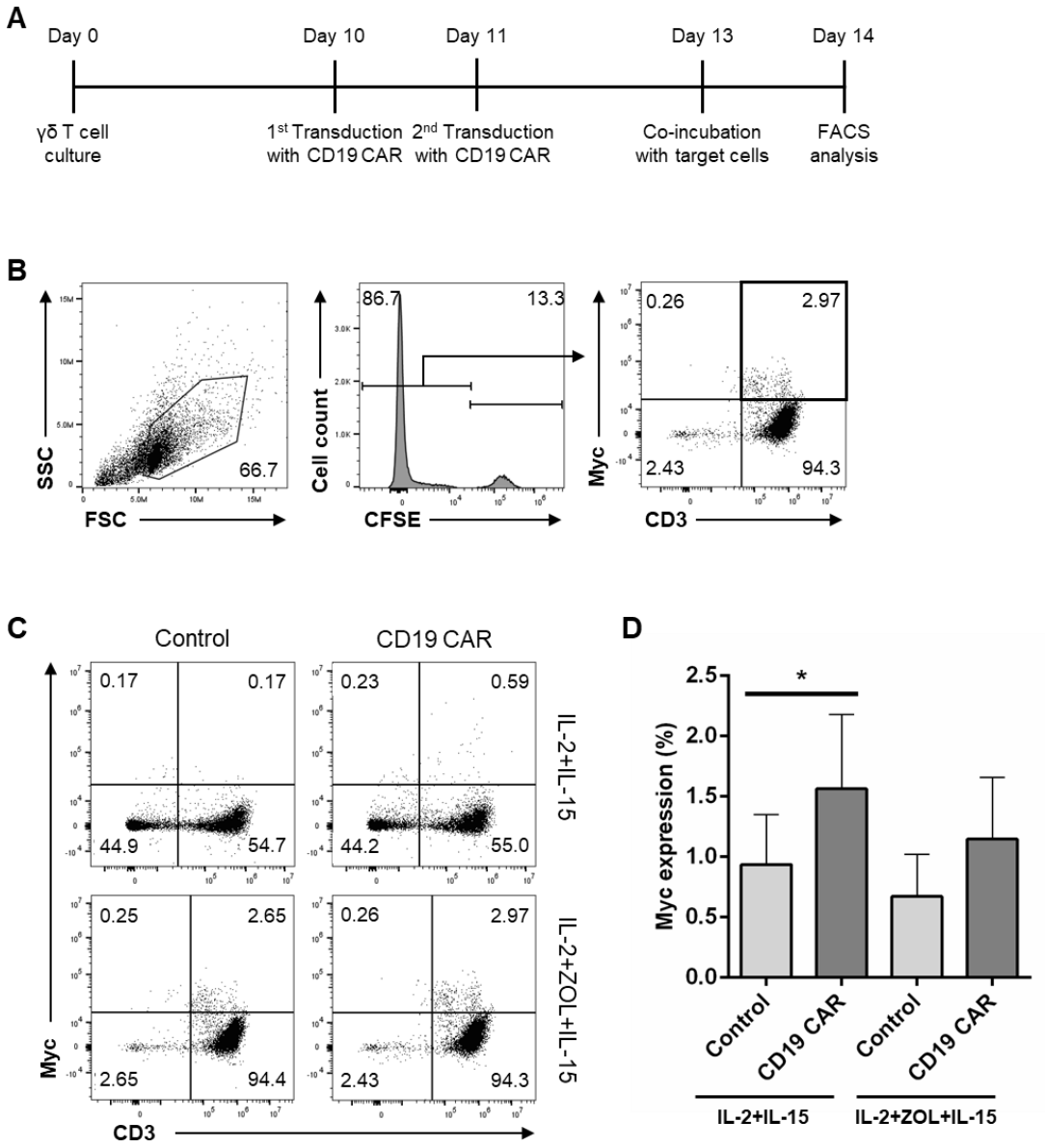

Figure 5. Production of CAR-γδ T cells. γδ T cells were cultured for 14 days under two conditions; IL-2 and IL-15; IL-2, IL-15, and ZOL. Lentiviral vectors expressing CD19 CAR were transduced on both day 10 and 11. (A) Schematic outline of the experimental procedure for CD19 CAR transduction into γδ T cells (B) Gating strategy for γδ T cells transduced with CD19 CAR lentiviral particle expressing Myc ($CD3^{+}Myc^{+}$). (C) Myc expression of γδ T cells with and without CD19 CAR was measured by flow cytometry in the presence of IL-2 and IL-15; IL-2, IL-15, and ZOL. (D) The summary of CAR-expressing cells which was measured by Myc expression were shown in bar graphs (n=5-6, mean ± SEM; *, $p<0.05$).

CD19 CAR-γδ T cells enhance the cytotoxicity against a $CD19^+$ B cell lymphoma cell line

The cytotoxicity of CD19 CAR-γδ T cells against $CD19^+$ tumor cells was evaluated. To test the efficacy of the CD19 CAR-γδ T cells *in vitro*, they were cocultured with CFSE-labeled OCI-Ly7 cells, $CD19^+$ B cell lymphoma cells (Figure 6A). The anti-tumor activity of CD19 CAR-γδ T cells was analyzed using Annexin V (AV) and 7-amino actinomycin D (7-AAD) in OCI-Ly7 cells (Figure 6B). The early apoptotic (AV^+7AAD^- cells) and the late apoptotic (AV^+7AAD^+) target cells were not different between control and CD19 CAR-γδ T cells expanded with IL-2+ZOL+IL-15 (Figures 6C and 6D). AV^+7AAD^- cells were significantly higher than the control in the presence of CD19 CAR-γδ T cells expanded with IL-2+ZOL+IL-15 (Figures 6C). The AV^+7AAD^+ cells were also slightly increased in the presence of ZOL (Figure 6D).

Figure 6

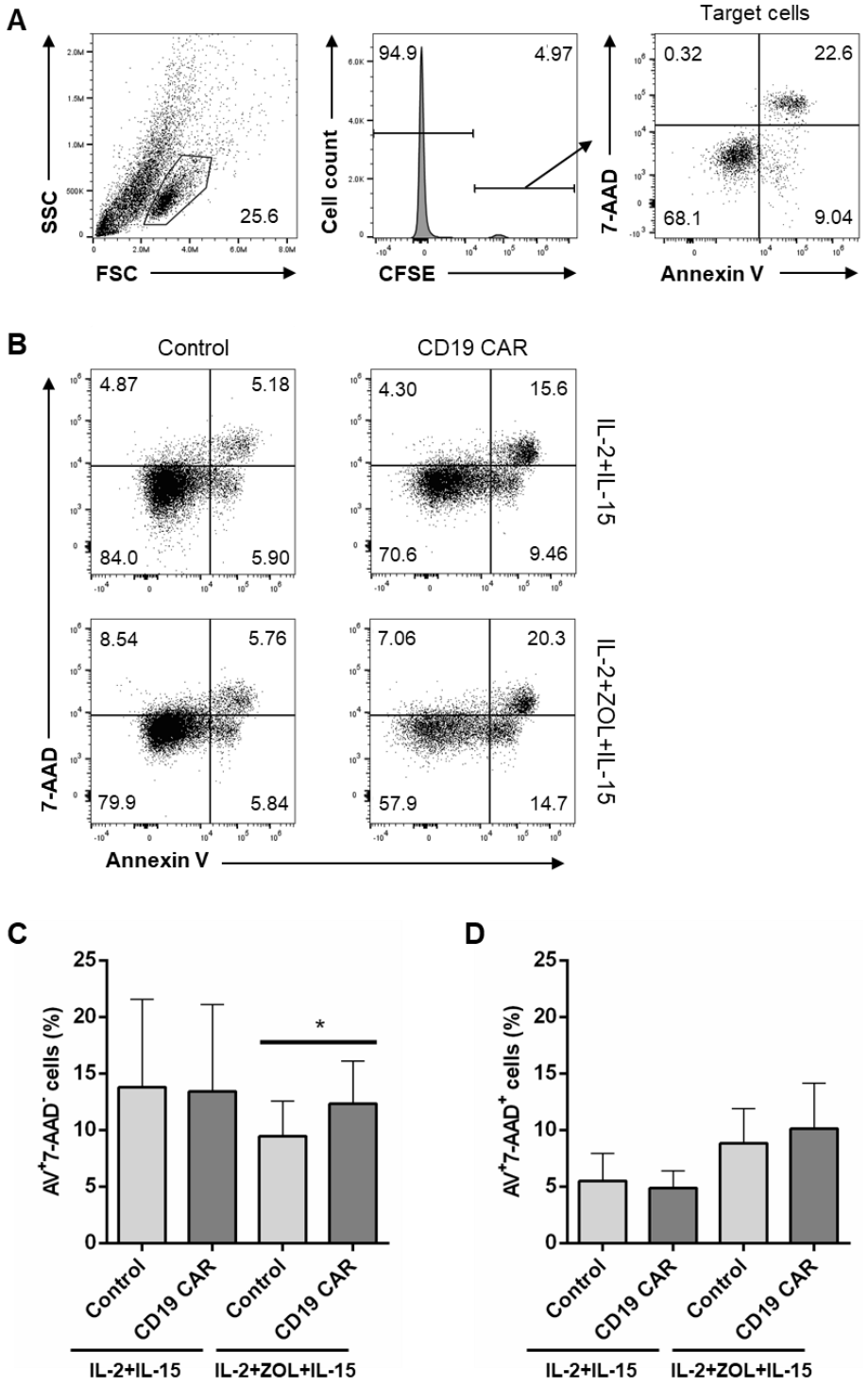

Figure 6. Cytotoxicity of CAR-γδ T cells against B cell lymphoma cells. OCI-Ly7 cells as target cells were labeled with CFSE and co-cultured with CAR-γδ T cells as effector cells at an effector to target cell ratio of 2:1 for 16-18 hours. Cells were then stained with AV and 7-AAD to detect apoptosis by flow cytometry. (A) Gating strategy to evaluate cell death. (B) AV and 7-AAD expression in $CFSE^+$ target cells was measured by flow cytometry. (C) The percentages of AV^+7AAD^- (early apoptosis) target cells were shown in bar graphs (n=7, mean ± SEM; *, $p<0.05$). (D) The percentages of AV^+7AAD^+ (late apoptosis) target cells were shown in bar graphs (n=7, mean ± SEM).

CD19 CAR-γδ T cells show effective cytotoxicity *in vivo* against a B cell lymphoma cell line

To investigate the *in vivo* efficacy of CD 19 CAR-γδ T cells for $CD19^+$ tumor cells, NSG mice were intraperitoneally injected with CFSE stained SU-DHL6 (SU) cells, and 30 minutes later, with CAR-γδ T cells or γδ T cells at an E:T ratio of 1:1 (Figure 7A). CD19 CAR-γδ T cells were produced under the condition of IL-2+ZOL+IL-15, as described above. Mice injected with B-cell lymphoma cells without immune cells were used as negative control. Two days after the injection, the peritoneal lavages were harvested to evaluate the quantities of B cell lymphoma cells (Figure 8A). The percentages of CFSE-labeled SU cells were lower in mice injected with CD19 CAR-γδ T cells, compared with those of control (Figure 7B). Approximately 80% of B cell lymphoma cells were eliminated by CD19 CAR-γδ T cells (Figure 7C). These data indicate that CD19 CAR-γδ T cells have effective anti-tumor activity against B cell lymphoma.

Figure 7

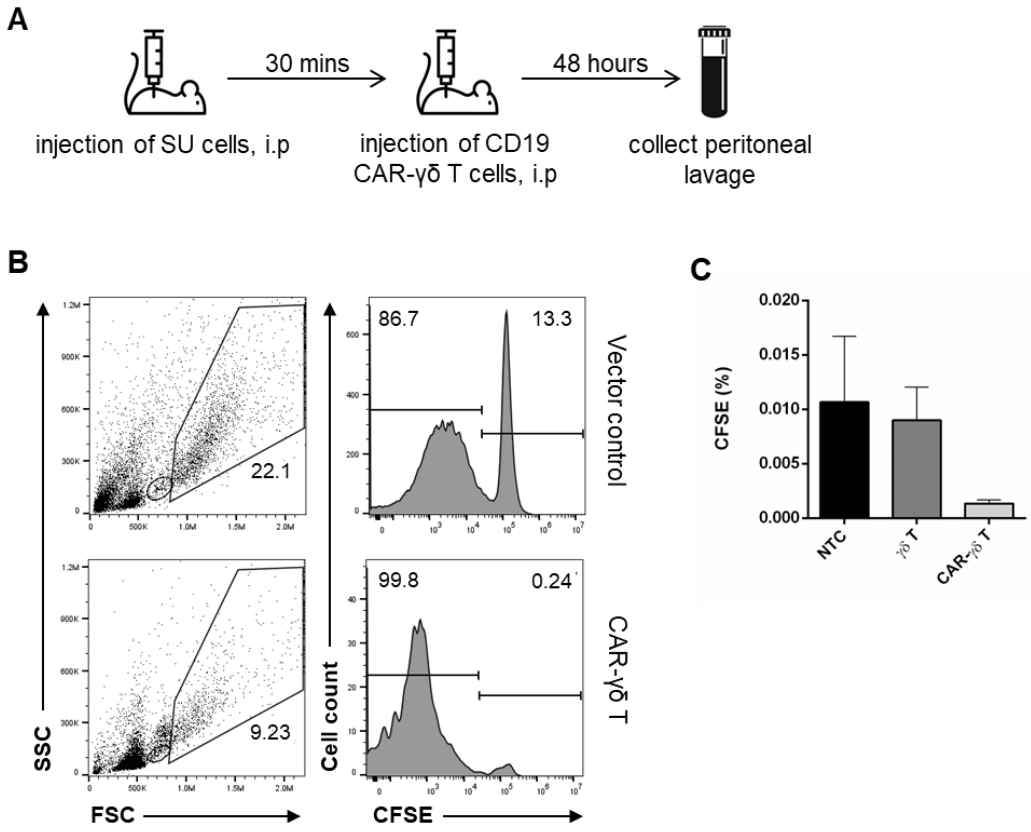

Figure 7. Evaluation of CD19 CAR- γδ T cells against CD19$^+$ B cell lymphoma cells *in vivo*. (A) Schematic outline of *in vivo* experiment. NSG mice were injected intraperitoneally with 5 X 10^6 CFSE labeled SU cells followed by a single intraperitoneal injection of 5 X 10^6 of CD19 CAR-T cells 30 minutes later. Peritoneal lavage was harvested and analyzed by flow cytometry on day 2. (B) Gating strategy for CFSE$^+$ tumor cells. (C) The percentages of CFSE$^+$ SU cells in the peritoneal lavages (n=3, mean ± SEM). **i.p.**, intraperitoneal injection; **NTC**, negative control

Discussion

This study shows γδ T cell expansion to be engineered with CAR under different conditions. CAR-γδ T cells revealed advanced tumor cell killing *in vitro* and *in vivo* without compromising the expression of activating receptors.

CAR-T cell therapy has emerged as an effective treatment for hematologic malignancy and is currently being developed to treat solid tumors [49]. CAR-T cell therapy has a crucial effect on targeting and killing malignant cells by recognizing cell surface antigens [50]. Several studies have shown that γδ T cells are effective against malignancies without causing GvHD [51]. Currently, CAR-T study is almost exclusively with CAR-αβ T cells, and there are very few studies on CAR-γδ T cells. However, the use of CAR-αβ T cells poses a risk of potential GvHD [52].

T cell survival is determined by the signals which T cells receive through the TCR and co-stimulatory molecules, adhesion, molecules and cytokine receptors [53]. Human γδ T cells are classified into Vδ1 γδ T cells present in the tissues and Vδ2 γδ T cells primarily present in the blood [37]. Studies have reported that stimulation of IL-2, ZOL and IL-15 effectively proliferates γδ T cells [54, 55]. This study demonstrated that stimulation of IL-2, ZOL and IL-15 promoted γδ T cell proliferation in particular, that of Vδ2 γδ T cells. Vδ2 γδ T cells

recognize the nonpeptidic phospho-antigens, whereas Vδ1 T cells do not respond to phospho-antigens. Human Vγ9Vδ2 T cells are a unique subtype of human peripheral blood that is activated directly by recognizing phospho-antigens through TCR. ZOL induced the activation of Vγ9Vδ2 T cells by increasing the intracellular level of IPP through the mevalonate pathway [56]. Therefore, the expansion of Vδ1 and Vδ2 γδ T cells was affected by ZOL, resulting in high yield.

In this study, the phenotypes of proliferated Vδ1 and Vδ2 γδ T cells were characterized in detail. γδ T cells expressed various activating and inhibitory receptors, and there are differences in the expression between Vδ1 and Vδ2 γδ T cells. Activating and cell death receptors, such as NKG2D, CD16, CD226, TRAIL, and FAS-L, of Vδ1 and Vδ2 γδ T cells were increased, and both PD-1 and BTLA did not increase. These results suggest that γδ T cells can expand while enhance cytotoxicity without interfering with immune checkpoint molecules. It also demonstrated the γδ T cells maintained functionality in culture.

To verify the production of CD19 CAR-γδ T cells, Myc-tag was used. The $CD3^{+}Myc^{+}$ cells were slightly more in the transduced CD19 CAR-γδ T cells, indicating that CAR-γδ T cells were generated. The production of CAR-γδ T cells was slightly more by the stimulation of IL-2, ZOL and IL-15. The stimulation of IL-2, ZOL and IL-15 was the only condition that I managed

to perform *in vivo* experiment in terms of the quantity of CAR-γδ T cells. The anti-tumor effect of CD19 CAR-γδ T cells *in vivo* was investigated in NSG mice injected with a B cell lymphoma cell line and CD19 CAR-γδ T cells. There are a few reports describing CAR-γδ T cells [57, 58]. Rozenbaume's group demonstrated that CD19 CAR-γδ T cells were effective in leukemia *in vitro* and *in vivo*. Retroviral particles expressing CD19 CAR were transduced into T cells on day 5 of culture, and αβ T cells were depleted 4 days of transduction. In this study, a lentiviral vector to express CD19 CAR was transduced twice into ab T depleted PBMCs on days 10 and 11 of culture. This study demonstrated the presence of the CD19 CAR-γδ T cells *in vivo*, despite a low proportion of CAR transduction efficacy

In conclusion, to overcome the limitations of CAR-T, γδ T cells are a good candidate as a novel CAR-immune cell. CAR-γδ T cells will increase the potential to be applied in developing a broad spectrum of cellular therapy.

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국문요약

배경: CAR-T 세포 요법은 임상에서 성공적이었지만 합병증과 부작용으로 인해 광범위한 적용이 제한된다. 감마 델타 T(γδ T) 세포는 MHC 제한 없이 항원을 인식하는 능력이 있어 이식편대숙주질환(GvHD)의 위험을 줄인다. 따라서 CAR-γδ T 세포를 생산하기 위한 실험을 설정하였다.

재료 및 방법: 건강한 지원자로부터 말초 혈액 단핵 세포(PBMC)를 분리하였다. γδ T 세포의 세포 표면 마커는 유세포 분석을 사용하여 특성화하였다. γδ T 세포를 3가지 다른 조건에서 2주 동안 배양하였다. CD19-CAR(FMC63-CD8 Hinge)를 발현하는 렌티바이러스 벡터를 배양 10일째에 형질도입하였다. 인간 B세포 림프종 세포주인 OCI-Ly7 세포에서 7-AAD와 AV를 사용하여 세포독성 능력을 분석하였다. NSG 마우스에 CFSE로 표지된 SU 세포를 복강내(i.p.) 주사한 후 CD19-CAR-형질도입된 γδ T 세포를 주사했다. 48시간 후, NSG 마우스로부터 복막 세척액을 얻어 생체 내 효능을 확인하기 위해 유세포 분석으로 분석하였다.

결과: Vδ1 γδ T 세포는 IL-2+IL-15의 존재 하에 확장된 반면, Vδ2 γδ T는 IL-2+ZOL+IL-15의 존재 하에 유의하게 증식되었다. γδ T 세포는 활성화 수용체 (NKG2D, CD16, DNAM-1), 세포 사멸 수용체 (TRAIL, FasL) 및 면역 체크포인트 수용체 (PD-1, BTLA) 분자를 발현했다. CAR-γδ T 세포는 시험관 내에서 CAR가

없는 γδ T 세포보다 더 높은 세포독성을 나타냈다. 생체 외 확장된 CAR-γδ T 세포가 주사된 마우스는 CAR 없이 γδ T 세포가 주사된 마우스보다 더 적은 수의 SU 세포를 나타냈다.

결론: 이 연구는 생체 외에서 확장된 γδ T 세포가 세포 표면 마커를 강력하게 발현했으며 CAR로 형질도입된 γδ T 세포가 시험관 내 생체 내에서 진행된 종양 세포 사멸 능력을 나타냈다는 것을 보여준다.