이학석사 학위논문

B 세포 림프종 (BCL) 세포주에 대한 NK-92 세포, αβ T 세포 및 γδ T 세포에서 CD19/CD22 이중 특이적 키메라 항원 수용체 (CAR)의 효용성 연구

The Efficacy of CD19/CD22 Bispecific Chimeric Antigen Receptor (CAR) in NK-92 Cells, αβ T Cells, and γδ T Cells for B Cell Lymphoma (BCL) Cells

울산대학교 대학원

의 과 학 과

한 민 아

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지 도 교 수 고 경 남

이 논문을 이학석사학위 논문으로 제출함

2023 년 08 월

울 산 대 학 교 대 학 원

의 과 학 과

한 민 아

한 민 아의 이학석사학위 논문을 인준함

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2023년 08월

Master of Science

The Efficacy of CD19/CD22 Bispecific Chimeric Antigen Receptor (CAR) in NK-92 Cells, αβ T Cells, and γδ T Cells for B Cell Lymphoma (BCL) Cells

The Graduate School

of the University of Ulsan

Department of Medicine

Mina Han

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Supervisors: Prof. Kyung-Nam Koh

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Mina Han

Department of Medicine

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August, 2023

The Efficacy of CD19/CD22 Bispecific Chimeric Antigen Receptor (CAR) in NK-92 Cells, αβ T Cells, and γδ T Cells for B Cell Lymphoma (BCL) Cells

This certifies that the masters thesis
of Mina Han is approved.

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Ulsan, Korea

August, 2023

Contents

그림 목차

Abstract

Background: Anti-CD19 chimeric antigen receptor (CAR) T cell therapy is effective in the treatment of relapsed or refractory aggressive B-cell lymphomas, but it can induce antigen-escape such as $CD19^{low}$ or $CD19^{-}$ malignant B cells and relapse. By developing bispecific CARs for CD22 and CD19, it is intended to prevent relapse through antigen escape of tumor cells and to reduce complications such as GVHD by using an NK-92 cell line and γδ T cells as immune effector cells.

Methods: Seven CAR-expressing vectors were produced using anti-CD19 (clone FMC63) and a novel anti-CD22 monoclonal antibody by modifying the backbone structures. The efficacy of the CAR structures was evaluated in both *in vitro* and *in vivo* experiments, using OCI-Ly7 cells, a human B cell lymphoma cell line. OCI-Ly7 cells were labeled with CFSE to distinguish from CAR-NK-92, CAR-αβ T, and CAR-γδ T cells as effector cells. Cell death

was assessed with annexin V and 7-aminoactinomycin D *in vitro*. For *in vivo* analysis, OCI-Ly7 cells and effector cells were subsequently injected intraperitoneally in NSG mice and collected in the peritoneal lavage 48 hours later.

Results: The decrease of OCI-Ly7 cells was statistically significant in FMC63_CD8-NK-92 (CD19 CAR-NK-92) cells, CI4-47_FMC63-NK-92, and FMC63_2-3-14-NK-92 (CD19/CD22 CAR-NK-92) cells compared with NK-92 cells alone *in vitro*. CI4-47_FMC63-NK-92 and LOOPCAR6_FMC63_CI4-47-NK-92 (CD19/CD22 CAR-NK-92) cells had higher cytotoxicity *in vivo*. Several CD19/CD22 CAR-αβ T cells increased apoptosis of OCI-Ly7 cells although it was not statistically significant.

Conclusion: CD19/CD22 bispecific CAR-NK-92 cells had higher cytotoxicity against B cell lymphoma cells than CD19 CAR-NK-92 cells. The efficacy of the CARs could differ,

depending on the secondary structure of the protein, the sequence of the antibody domains, and the linker structures.

Introduction

Cytotoxic T- or natural killer cells have cytotoxicity to acute and chronic B-cell lymphoma. Nevertheless, T cell exhaustion occurs due to the continued pressure of antigen stimulation by cancer, and apoptosis is induced [1]. NK cells have been reported for resistance to exhaustion by the expression of HLA-G or the production of molecules by lymphoblasts such as MICA and MICB that bind to NKG2D [2]. γδ T cells are a subset of T cells, which account for 1-5% of peripheral blood, but they have abundant cytokine secretion capacity, suggesting that they possess a high antitumor and infection-defense capability [3, 4]. γδ T cells express T cell receptor (TCR) γ and δ chains, that can recognize broad range antigens without recognition of the peptide-MHC complexes. Therefore, unlike αβ T cells that are restricted to MHC-restricted antigen presentation, γδ T cells do not induce GVHD because they have allogenic characteristics, so they are useful for immunotherapy [5-7].

Chimeric antigen receptor therapy is a type of immunotherapy used to kill cancer cells with CAR-expressing immune cells [8]. Mainly T cells and NK cells are genetically engineered to

express CARs, then used to specifically target cancer cells via recognition of tumor-associated antigens [9]. Anti-CD19 CAR T cells therapy is effective in the treatment of relapsed or refractory aggressive B-cell lymphomas, and second-generation CD19 CARs have been approved by the FDA and are being used in clinical trials [10-12]. However, CD19-directed CAR therapies can induce antigen-escape relapse such as $CD19^{low}$ or $CD19^{-}$ malignant B cells [11].

CD22 is expressed at the pre-B cell stage and retained in mature B cells, exclusively to B lymphocytes, thus it is useful for targeted therapy of malignant B-cells [13, 14]. CD22 usually retains to $CD19^{low}$ or $CD19^{-}$ malignant B cells [11, 15]. Therefore, CD22 targeted therapy can overcome the problem of CD19 downregulation. The CD19/CD22 bispecific CARs have dual targets for CD19 and CD22, preventing antigen escape developed in malignant B cells, thereby increasing the cytotoxicity of CAR-expressing immune cells. I conducted the experiments with various immune cell types for effective CAR therapy using CD19/CD22 bispecific CARs of second-generation structures.

When using NK cells from peripheral blood for CAR-NK cells therapy, the number of expanded NK cells can be variable, and transfection efficiency of NK cells is also poorer compared with T cells. T cell depletion is performed on patients for clinical use to prevent graft-versus-host disease (GVHD) [2, 16]. In contrast, NK-92 cells, a human NK cell line, can easily be expanded in culture and has higher transfection efficiency than primary NK cells [17]. NK-92 cell line expresses high levels of molecules involved in the perforin-granzyme apoptosis pathway [18]. To eliminate the *in vivo* proliferation and tumorigenic potential of CAR-NK-92 cells, irradiation is performed before administration to patients, but phase 1 clinical trials showing a stability profile have been completed [19, 20].

I evaluated the cytotoxicity of CD19/CD22 bispecific CAR-NK-92, CAR-αβ T, and CAR-γδ T cells by targeting OCI-Ly7, a diffuse large B cell lymphoma (DLBCL) cell line, one of the types of non-Hodgkin lymphoma (NHL).

Materials and Methods

Plasmid construction

The CAR backbone vector and packaging vectors were from kind gifts from Dr. B. Ryn (St. Jude Hospital, Memphis, USA) and the CD22 scFv (CI4-47) was produced and provided by Dr. H. Kim (Asan Medical center, Seoul, Korea). In brief, bispecific CD19-CD22 CARs were constructed by DNA fragments encoding the CD19 single-chain variable fragment (scFv) derived from FMC63 mAb, the hinge and transmembrane domain of CD8, and the cytoplasmic domains of 4-1BB and CD3ζ. Sequences of extracellular spacers and linkers connecting scFv domains are listed in Figure 1. All CARs were tagged to the MYC gene to facilitate antibody staining.

Cells culture and medium

Natural killer 92 (NK-92) cells were maintained in α-MEM medium (Welgene, Gyeongsangbuk-do, Republic of Korea) containing 12.5% non-heat inactivated fetal bovine

serum (FBS) (Welgene), 12.5% heat-inactivated horse serum (Gibco, ThermoFisher Scientific, Waltham, MA, USA), 10 U/ml penicillin, 10 μg/ml streptomycin (Welgene), 55mM 2-mercaptoethanol (ThermoFisher Scientific) and 100 U/ml recombinant human interleukin-2 (rhIL-2) (PeproTech, Rocky Hill, NH, USA). A human diffuse large B cell lymphoma cell (DCBLC) cell line, OCI-Ly7, and HEK293T cells were maintained DMEM medium (Welgene) containing 10% heat-inactivated FBS (Welgene), 100 U/ml penicillin, and 100 μg/ml streptomycin (Welgene).

PBMCs or αβ T cell-depleted PBMCs from healthy adult volunteers were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Milwaukee, WI, USA). αβ T cells-depleted PBMCs were isolated using CliniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). All participants provided written informed consent, and all procedures were approved by the Institutional Review Board (IRB), Asan Medical Center, Seoul, Republic of Korea (IRB Approval No. 2018-0445). The study was performed ethically, in accordance with the Declaration of Helsinki. PBMCs were cryopreserved in heat-inactivated

FBS (Welgene) containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and stored in a liquid nitrogen tank until use.

PBMCs at 2×10^6 cells/ml in a 6-well plate were cultured with immobilized 1 μg/ml anti-CD3 and anti-CD28 mAbs (BD biosciences, Franklin Lakes, New Jersey, USA) in the presence of 100 U/ml rhIL-2 (PeproTech) in RPMI-1640 (Welgene) containing 10% inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM sodium pyruvate (Sigma-Aldrich), and 0.55 mM 2-mercaptoethanol (ThermoFisher Scientific) for 2 weeks. Every two days, half the medium was replaced and refreshed with the same concentrations of rhIL-2.

αβ T cell-depleted PBMCs were cultured into a 6-well plate at a density of 3×10^6 cells/ml in complete RPMI1640 supplemented with 100 U/ml rhIL-2 (PeproTech) and 50 ng/ml recombinant human IL-15 (rhIL-15) (PeproTech) in the presence of 1 μM Zoledronic acid (Selleckchem, Houston, TX, USA) for 2 weeks. Every two days, half the medium was replaced and refreshed with the same concentrations of rhIL-2 and rhIL-15. All the cells were cultured in a fully humidified incubator with 5% CO_2 at 37℃

Generation of CAR transduced cells

Plasmid DNA was transformed by heat shock into competent E.coli strain Stbl3 prepared using a 0.1 M solution of $CaCl_2$, following standard protocols. The CAR vector was isolated using a Plasmid DNA Midi prep kit (Qiagen, Venlo, Netherlands). To produce lentivirus particles, CAR-expressing vector was transfected into HEK293T 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). Virus-containing medium was harvested 48 and 72 h after transfection and filtered through a 0.45 μM filter (Millipore, Burlington, MA, USA). Viral supernatant was used to transfect NK-92 cells, αβ T cells, and γδ T cells pre-treated with 8 μg/ml polybrene (Millipore, Burlington, Massachusetts, USA). For CAR-NK-92 cells, NK-92 cells were sorted about 80-100% CAR-expressing Myc^+ cells by BD FACSAria™III Cell sorter (BD Bioscience). CAR-NK-92 cells were cryopreserved in heat-inactivated FBS (Welgene) containing 10% dimethyl sulfoxide (Sigma-Aldrich) and stored in a liquid nitrogen tank until use.

***In vitro* cytotoxicity assay**

Cytotoxicity was assessed using a 7-amino-actinomycin D (7-AAD) and Annexin V (AV) assay. As target cells, OCI-Ly7 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using a Cell Trace Cell Proliferation Kit (ThermoFisher Scientific) according to the manufacturer's protocols. On day 2 post-transduction, CAR-αβ T and γδ T cells were harvested and resuspended in complete RPMI medium and CAR-NK-92 cells were used complete NK-92 medium. CAR transduced cells (effector cells) were co-cultured with CFSE-labeled target cells at an effector-to-target ratio of 2:1 (effector:target). After overnight incubation, cells were washed with Annexin V binding buffer (BD Biosciences) and incubated at 37°C for 30 min. For CAR-NK-92 cells, they were cultured for 4 hours. Cells were treated with human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 min and stained with 7-AAD Viability Staining Solution (BioLegend) and Pacific Blue™ -conjugated Annexin V antibody (BioLegend) for 15 min on ice. The $CFSE^+$ target cells were evaluated for early and late apoptosis by flow cytometry

using a CytoFLEX (Beckman Coulter Life Sciences). Data analysis was performed using FlowJo v10 (Treestar, Inc., Ashland, OR, USA).

***In vivo* studies in NOD/SCID/γ_c-/- (NSG) mice**

The NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice were purchased (JABio, Suwon, Republic of Korea). OCI-Ly7 cells were labeled with CFSE. On day 0, NSG mice were injected intraperitoneally (i.p) with 5×10^6 cells in 200 μl of 1×PBS (Biosesang, Gyeonggi-do, Republic of Korea). After 30 min, mice were injected i.p with 5×10^6 CAR-transduced cells. On day 2, 15 ml 1×PBS was injected into the peritoneal cavity under anesthesia. Peritoneal lavage was then harvested from mice, and the ratio and number of $CFSE^+$ cells were measured by flow cytometry. The data were analyzed by FlowJo v10 software. All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee at Asan Medical Center, Seoul, Korea (Approval No. 2020-12-251).

Flow cytometry

Flow cytometry was performed using the following antibodies : APC/Fire™750-conjugated anti-human CD3 (Clone SK7; Biolegend, San Diego, California, USA), APC-conjugated CD56 (NCAM) monoclonal antibody (Clone CMSSB; Invitrogen), PE-Cyanine7-conjugated TCR V delta 1 (Clone TS8.2; eBioscience™, Greater San Diego, West Coast, US), Brilliant Violet 510™-conjugated TCR V delta 2 (Biolegend). For detection of the CAR receptors, transduced cells were stained with AlexaFluor-647-conjugated Myc-Tag mouse monoclonal antibody (mAb) (Clone 9B11; Cell Signaling Technology, Beverly, MA, USA) or a matched AlexaFluor-647-conjugated mouse IgG2a, κ isotype control (Clone MOPC-173; BioLegend). For detection of *in vitro* cytotoxicity assay, cells were stained with 7-AAD Viability Staining Solution (Biolegend), Pacific Blue™-conjugated Annexin V (Biolegend). Before antibody treatment, cells were treated with human FcR blocking reagent (Miltenyi Biotec) at room temperature for 5 min. Cells were stained with the above antibodies at 4℃ for 30 min, and then washed with 1×PBS containing 2% FBS (Welgene). Flow cytometry

was performed using a CytoFLEX. The data were analyzed with a FlowJo v10 software.

Statistical analysis

Graphical and statistical analyses were performed with a GraphPad Prism v6 software (GraphPad Software Inc., San Diego, CA, USA). The p values were calculated by the one-way ANOVA with multiple comparisons between the groups. P values <0.05 were regarded as statistically significant. All data are depicted as mean±standard error of the mean (SEM).

Result

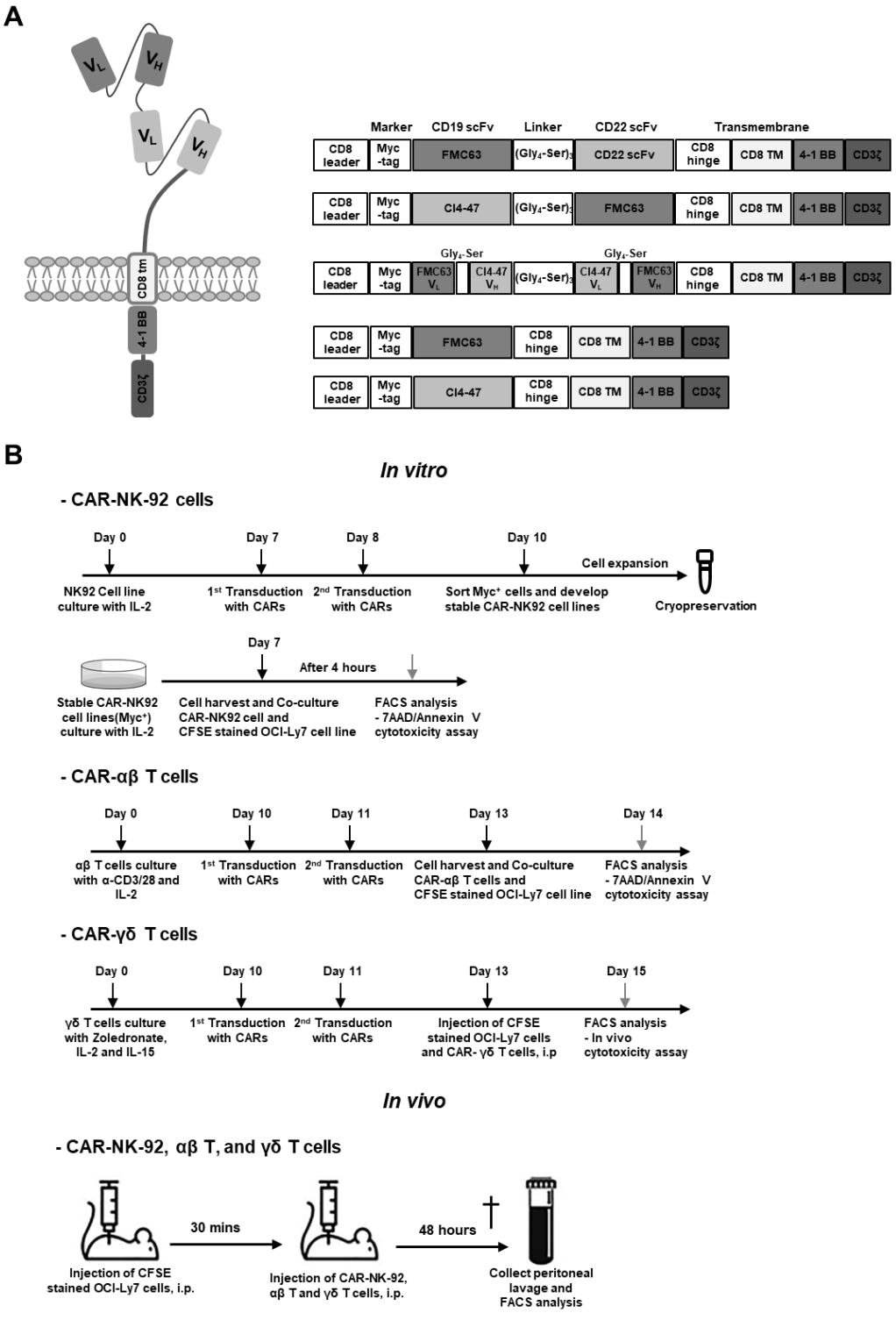
CD19/CD22 bispecific CAR structures are produced.

Seven CAR-expressing vectors were produced using CD19 (clone FMC63) and novel anti-CD22 mAbs by modifying the backbone structures. Bispecific CD19/CD22 CARs were commonly constructed with the CD8 leader sequence, anti-CD19 scFv (FMC63), $(G4S)_3$ linker, anti-CD22 scFv (CI4-47), CD8 hinge region, CD8 transmembrane (TM) domain, and

4-1 BB costimulatory signaling domain and cytoplasmic domain of CD3ζ (Fig 1A). FMC63_CI4-47-CAR consists of anti-CD19 and anti-CD22 scFvs connected in tandem via a $(G4S)_3$ flexible linker, and CI4-47_FMC63-CAR consists of anti-CD22 and anti-CD19 scFvs sequences. LOOPCAR6_FMC63_CI4-47-CAR had structures composed of a short $(G4S)_1$ linker between anti-CD19 light chains (V_L) and anti-CD22 heavy chains (V_H) and structures composed of a $(G4S)_1$ linker between anti-CD22 V_L and anti-CD19 V_H. FMC63_CI4-14, FMC63_CI3-23, FMC63_2-3-14, and FMC63_2-3-16 CARs had same structures. Unispecific CARs with anti-CD19 (FMC63_CD8-CAR) or anti-CD22 (CI4-47_CD8-CAR) Ab domains were also used (Fig 1A). To evaluate the CAR expression, CAR vectors were incorporated a Myc gene next to the CD8 leader sequence of the N-terminal (Fig 1A). CAR-expressing lentiviral vectors were transduced into cultured immune effector cells, such as NK-92, αβ T, and γδ T cells. Myc^+ CAR-NK-92 cells were sorted by FACS sorter at 80-100% of purity, cryopreserved, and then used for experiments. Fluorescence was detected for CFSE-labeled OCI-Ly7 (Target) cells to identify the cell killing ability of CAR-

NK-92 cells, CAR-αβ T, and CAR-γδ T cells against human B-cell lymphoma cells. *In vitro* experiments were conducted AV/7-AAD apoptosis assay at a 2:1 effector:target (E:T) ratio. The NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice were injected i.p. at a ratio of a 1:1 effector:target (E:T) for *in vivo* experiments and CFSE fluorescence was analyzed in the peritoneal lavage after 48 hours (Fig 1B).

Figure 1.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Marker
CD19 scFv
Linker
CD22 scFv
Transmembrane
CD8 leader
Myc -tag
FMC63
(Gly4-Ser)3
CD22 scFv
CD8 hinge
CD8 TM
4-1 BB
CD3ζ
CI4-47
Gly4-Ser
CD8 tm
B
In vitro
- CAR-NK-92 cells
Day 0
Day 7
Day 8
Day 10
Cell expansion
NK92 Cell line culture with IL-2
1st Transduction with CARs
2nd Transduction with CARs
Sort Myc+ cells and develop stable CAR-NK92 cell lines
Cryopreservation
After 4 hours
Stable CAR-NK92 cell lines(Myc+) culture with IL-2
Cell harvest and Co-culture CAR-NK92 cell and CFSE stained OCI-Ly7 cell line
FACS analysis - 7AAD/Annexin V cytotoxicity assay
- CAR-αβ T cells
Day 11
Day 13
Day 14
αβ T cells culture with α-CD3/28 and IL-2
Cell harvest and Co-culture CAR-αβ T cells and CFSE stained OCI-Ly7 cell line
- CAR-γδ T cells
Day 15
γδ T cells culture with Zoledronate, IL-2 and IL-15
Injection of CFSE stained OCI-Ly7 cells and CAR- γδ T cells, i.p
FACS analysis - In vivo cytotoxicity assay
In vivo
- CAR-NK-92, αβ T, and γδ T cells
30 mins
48 hours
Injection of CFSE stained OCI-Ly7 cells, i.p.
Injection of CAR-NK-92, αβ T and γδ T cells, i.p.
Collect peritoneal lavage and FACS analysis

Figure 1. Chimeric antigen receptor (CAR) structure, vector designs, and experimental scheme. (A) Structure of an exemplary bispecific CAR and gene maps of CAR vectors. A total of five bispecific CAR structures were produced using anti-CD22 antibody clones, CI4-47, CI4-14, CI3-23, 2-3-14, and 2-3-16. Anti-CD19/CD22 bispecific CAR were produced CI4-47_FMC63, LOOPCAR6_FMC63_CI4-47. Anti-CD19 (FMC63) were integrated to the backbone structure. Anti-CD22 antigen-binding domains were integrated as well. (B) *In vitro* and *In vivo* experimental schemes. In CAR-NK-92 *in vitro* experiments, the NK-92 cell line was cultured with 100 U/ml IL-2. CAR transduction was performed at day 7 and 8 of culture. After sorting Myc^+ cell on day 10, cells were expanded and the stable CAR-NK-92 cells were cryopreserved. After thawing and culturing CAR-NK-92 cells for 7 days, the cells were harvested and co-cultured with CFSE-labeled OCI-Ly7 cells for 4 hours, and AV/7-AAD apoptosis assay was performed. αβ T cells were cultured with immobilized anti-CD3 and anti-CD28 mAbs at 1 μg/ml in the presence of 100 U/ml IL-2. On day 10 and 11 of culture, CAR transduction was performed, and CAR-αβ T cells were harvested on day 13 of

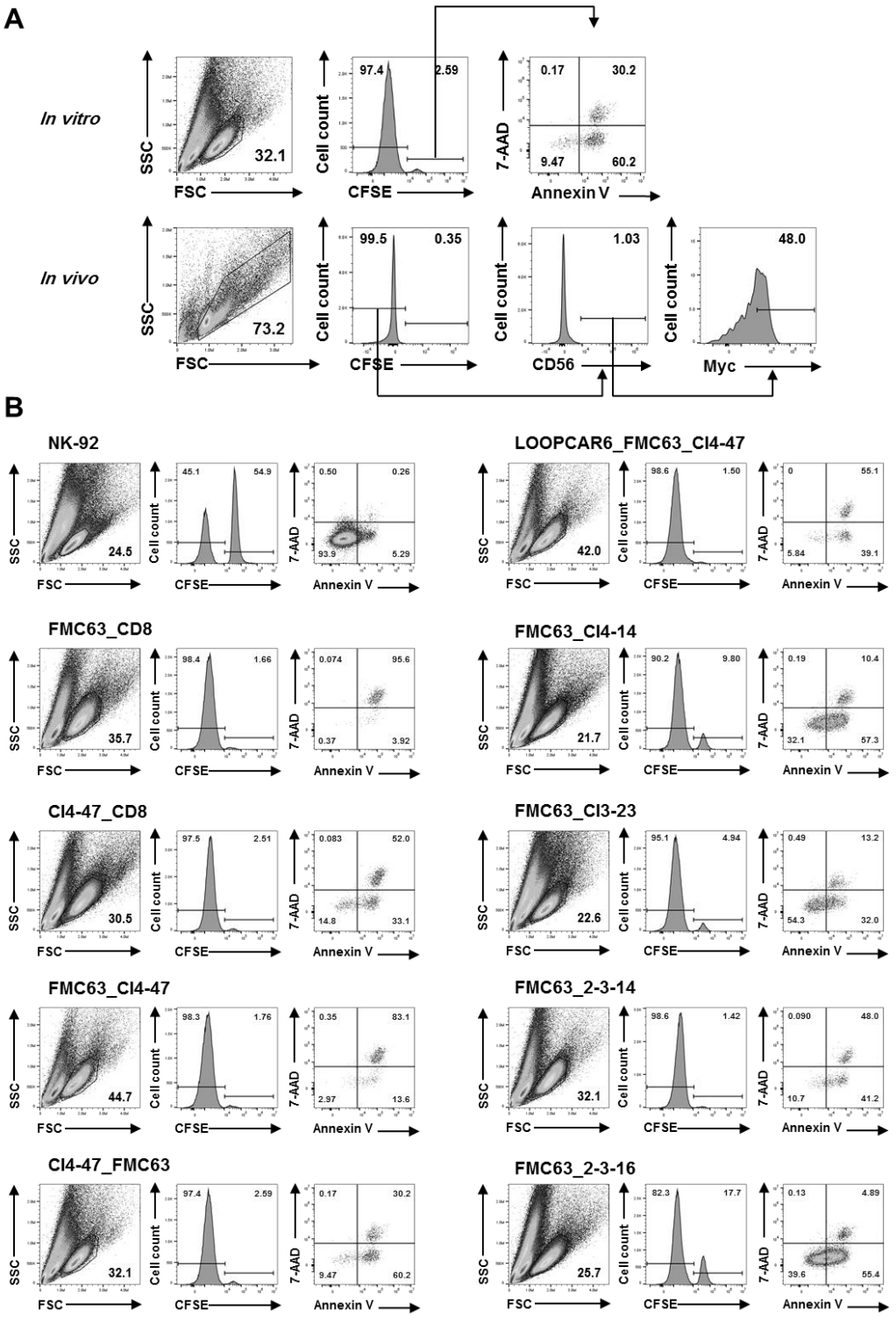
culture, CAR-αβ T cells were co-cultured with CFSE-labeled OCI-Ly7 cells for 24 hours, and AV/7-AAD apoptosis assay was performed. γδ T cells were cultured with 1 μM Zoledronate, 100 U/ml IL-2 and 50 ng/ml IL-15. On day 10 and 11 of culture, CAR transduction was performed. In the *in vivo* experiment, CFSE-labeled OCI-Ly7 cells were i.p. injected into NSG mice, and 30 minutes later, CAR-transduced cells were i.p. injected. After 48 hours, intraperitoneal cells were harvested and flow cytometry was performed.

CD19/CD22 bispecific CAR-NK-92 cells have high cytotoxicity against B cell lymphoma cells.

CAR-NK-92 cells were expanded as described above and *in vitro* and *in vivo* studies were performed to identify CFSE-labeled OCI-Ly7 ($CFSE^+$) cells and CAR-NK-92 cells (Fig 2A). In *in vitro* results, FMC63_2-3-16 CAR-NK-92 cells, CD19/CD22 CAR-NK-92 cells, had lower anti-tumor effect against OCI-Ly7 cells than CD19 CAR-NK-92 cells in late-apoptotic cell population, which was defined as AV^+7AAD^+. FMC63_2-3-16 CAR-NK-92 cells had lower cytotoxicity than FMC63_CI4-47 CAR-NK-92 cells (Fig 2B-C). The number of $CFSE^+$ cells was reduced by FMC63_CD8 (CD19), CI4-47_FMC63, and FMC63-2-3-14 CAR-NK-92 cells (Fig 2B-C). I evaluated selected CD19/CD22 CAR-NK-92 cells from *in vitro* study to perform *in vivo* experiments. The frequency of $CFSE^+$ cells was slightly decreased by CD19/CD22 CAR-NK-92 cells, and the number of $CFSE^+$ cells were significantly reduced by the presence of CI4-47_FMC63 and LOOPCAR6_FMC63_CI4-47 CAR-NK-92 cells, compared with that of CD19 CAR-NK-92 cells (Fig 2D-E). Collectively,

the therapeutic effects of CD19/CD22 CARs differed depending on the CAR structures *in vitro* and *in vivo*, and CI4-47_FMC63 CAR-NK-92 cells were validated to have high cytotoxic activity against OCI-Ly7 cells than CD19 unispecific CAR-NK-92 cells *in vivo*.

Figure 2.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Cell count
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2.59
7-AAD
Annexin V
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9.47
60.2
In vivo
73.2
99.5
0.35
1.03
CD56
48.0
Myc
B
NK-92
24.5
45.1
54.9
0.50
0.26
93.9
5.29
LOOPCAR6_FMC63_CI4-47
42.0
98.6
1.50
0
55.1
5.84
39.1
FMC63_CD8
35.7
98.4
1.66
0.074
95.6
0.37
3.92
FMC63_CI4-14
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90.2
9.80
0.19
10.4
32.1
57.3
CI4-47_CD8
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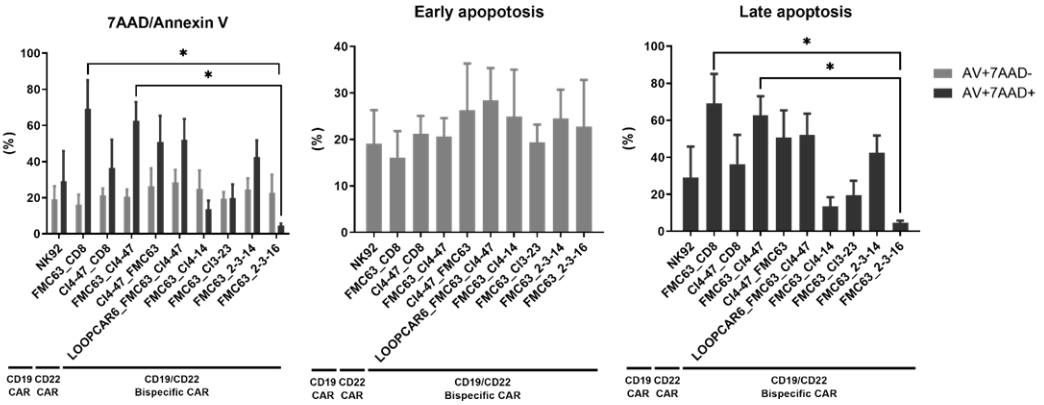
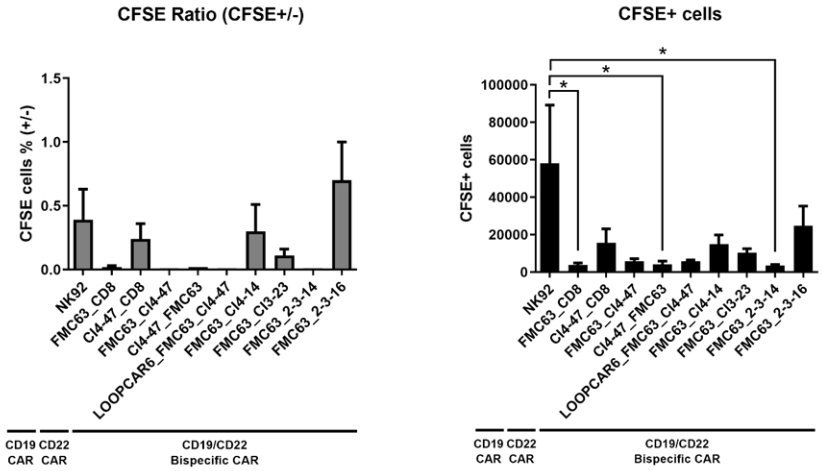
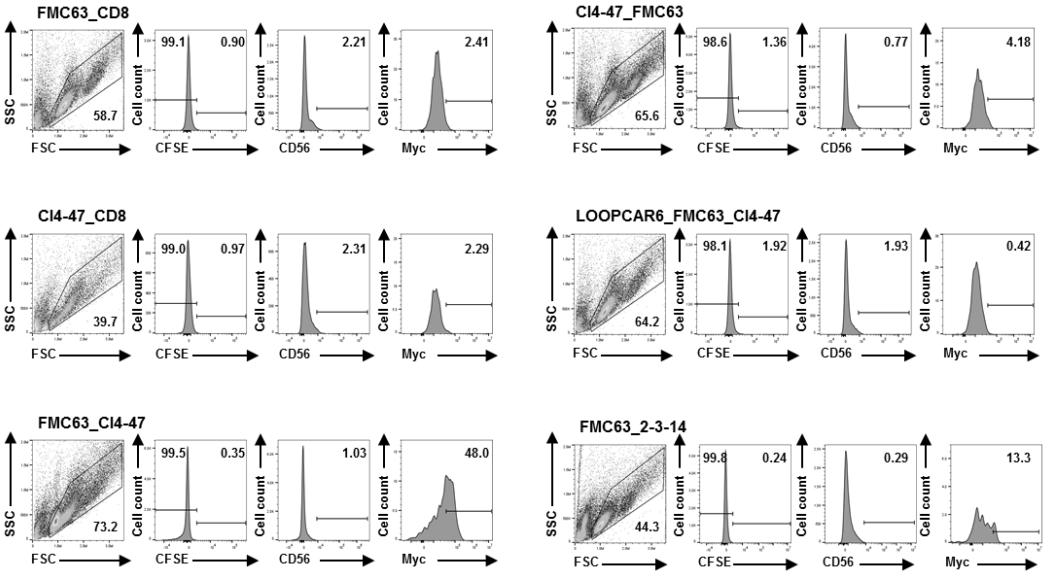
C


7AAD/Annexin V
Early apopotosis
Late apoptosis
(%)
AV+7AAD-
AV+7AAD+
NK92
FMC63_CD8
CI4-47_CD8
FMC63_CI4-47
CI4-47_FMC63
LOOPCAR6_FMC63_CI4-47
FMC63_CI4-14
FMC63_CI3-23
FMC63_2-3-14
FMC63_2-3-16
CD19 CAR
CD22 CAR
CD19/CD22 Bispecific CAR


CFSE Ratio (CFSE+/-)
CFSE+ cells
CFSE cells % (+/-)
CFSE+ cells
NK92
FMC63_CD8
CI4-47_CD8
FMC63_CI4-47
CI4-47_FMC63
LOOPCAR6_FMC63_CI4-47
FMC63_CI4-14
FMC63_CI3-23
FMC63_2-3-14
FMC63_2-3-16
CD19 CAR
CD22 CAR
CD19/CD22 Bispecific CAR

D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CFSE
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0.77
4.18
CI4-47_CD8
39.7
99.0
0.97
2.31
2.29
LOOPCAR6_FMC63_CI4-47
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1.93
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0.35
1.03
48.0
FMC63_2-3-14
44.3
99.8
0.24
0.29
13.3

E

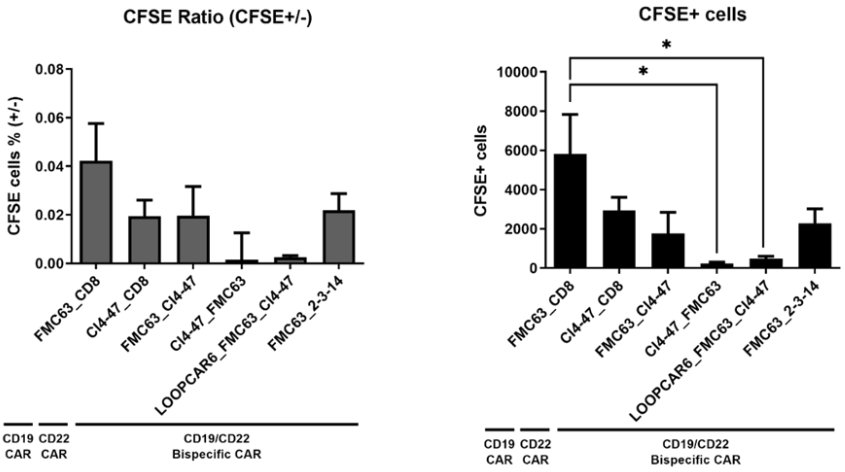

CFSE Ratio (CFSE+/-)
CFSE cells % (+/-)
0.08
0.06
0.04
0.02
0.00
FMC63_CD8
CI4-47_CD8
FMC63_CI4-47
CI4-47_FMC63
LOOPCAR6_FMC63_CI4-47
FMC63_2-3-14
CD19 CAR
CD22 CAR
CD19/CD22 Bispecific CAR
CFSE+ cells
CFSE+ cells
10000
8000
6000
4000
2000
0
*
*

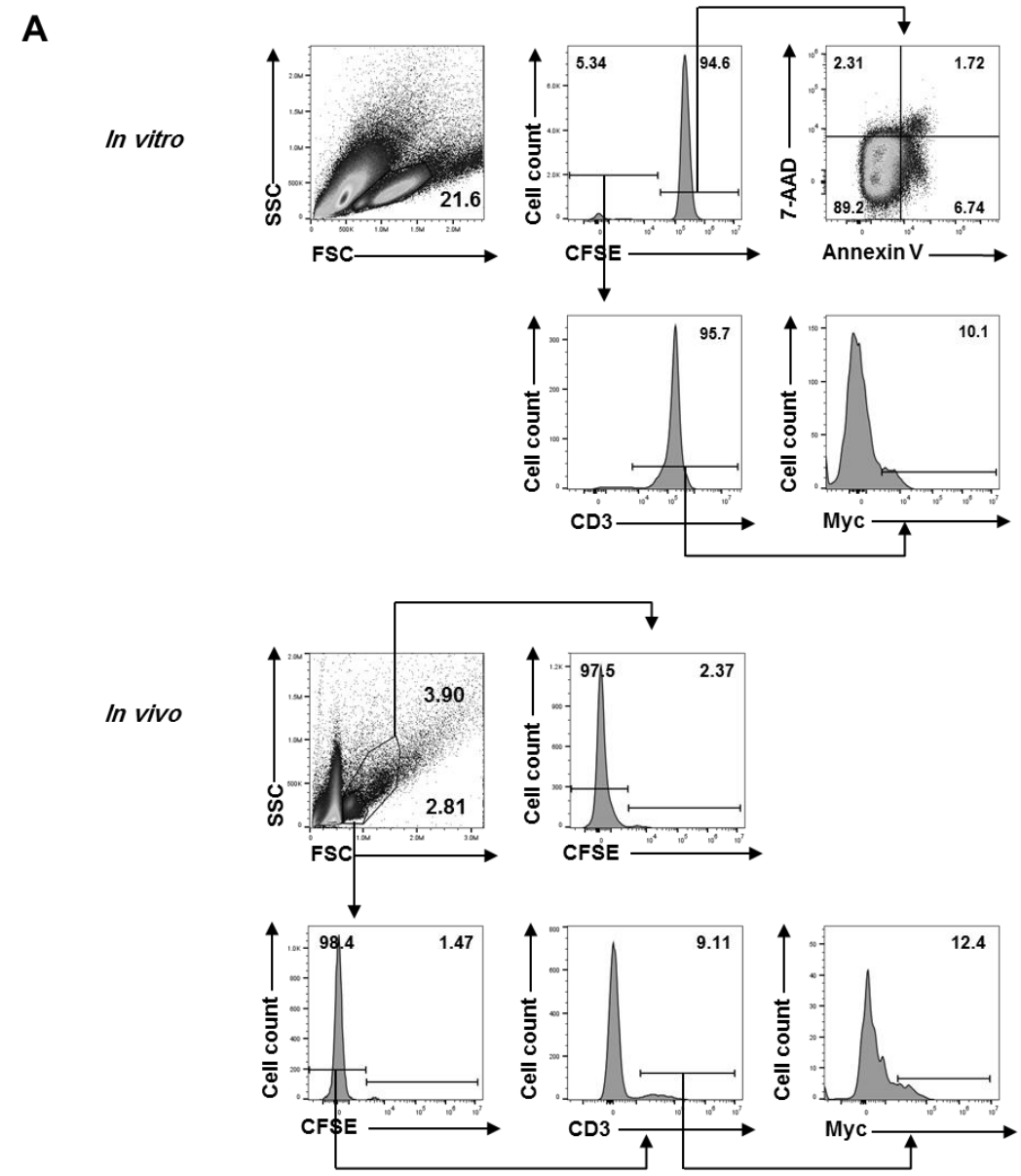
Figure 2. *In vitro* and *In vivo* CAR-NK-92 cell studies. (A) Gating strategy of *in vitro* and *in vivo* experiments. *In vitro* experiments (upper panel) were performed using AV/7-AAD to identify apoptotic CFSE-labeled OCI-Ly7 cells. *In vivo* experiments (lower panel) performed FACS analysis to identify CFSE-labeled OCI-Ly7 and CAR-NK-92 cells. (B) Representative FACS plots of AV/7-AAD apoptosis assay. Results are shown for FMC63_CD8 (CD19 CAR), CI4-47_CD8 (CD22 CAR), FMC63_CI4-47, CI4-47_FMC63, LOOPCAR6_FMC63_CI4-47, FMC63_CI4-14, FMC63_CI3-23, FMC63_2-3-14 and FMC63_2-3-16 bispecific CAR-NK-92 cells. (C) Graphs of AV^+ $7\text{-}AAD^-$ early apoptotic cells and AV^+ $7\text{-}AAD^+$ late apoptotic cells (upper panel). Graph of $CFSE^+$ cells, and the number of $CFSE^+$ cells (lower panel). CFSE-labeled OCI-Ly7 and CAR-NK-92 cells co-cultured for 4 hours, and flow cytometry was performed. NK-92 cells without any treatment were used as control. CAR-NK-92 cells were selected as Myc^+ with 80-100% of purity OCI-Ly7 cells were used as target cells. n=4, All values present as means ± SEM. *$p<0.05$. (D) Representative FACS plots of *in vivo* studies in NSG mice. Results of cytotoxicity assay

were shown for FMC63_CD8 (CD19 CAR), CI4-47_CD8 (CD22 CAR), FMC63_CI4-47, CI4-47_FMC63, LOOPCAR6_FMC63_CI4-47, and FMC63_2-3-14 bispecific CAR-NK-92 cells. (E) Graph of ratio and number of $CFSE^+$ cells. CFSE-labeled OCI-Ly7 and CAR-NK-92 cells were injected i.p. into NSG mice. After 48 hours, intraperitoneal cells were harvested and flow cytometry was performed. CAR-NK-92 cells were selected as Myc^+ with 80-100% of purity. OCI-Ly7 cells were used as target cells. n=7 for FMC63_CD8. n=6 for CI4-47_CD8 and LOOPCAR6_FMC63_CI4-47. n=5 for FMC63_CI4-47, FMC63_2-3-14. n=4 for CI4-47_FMC63. All values present as means ± SEM. *$p<0.05$.

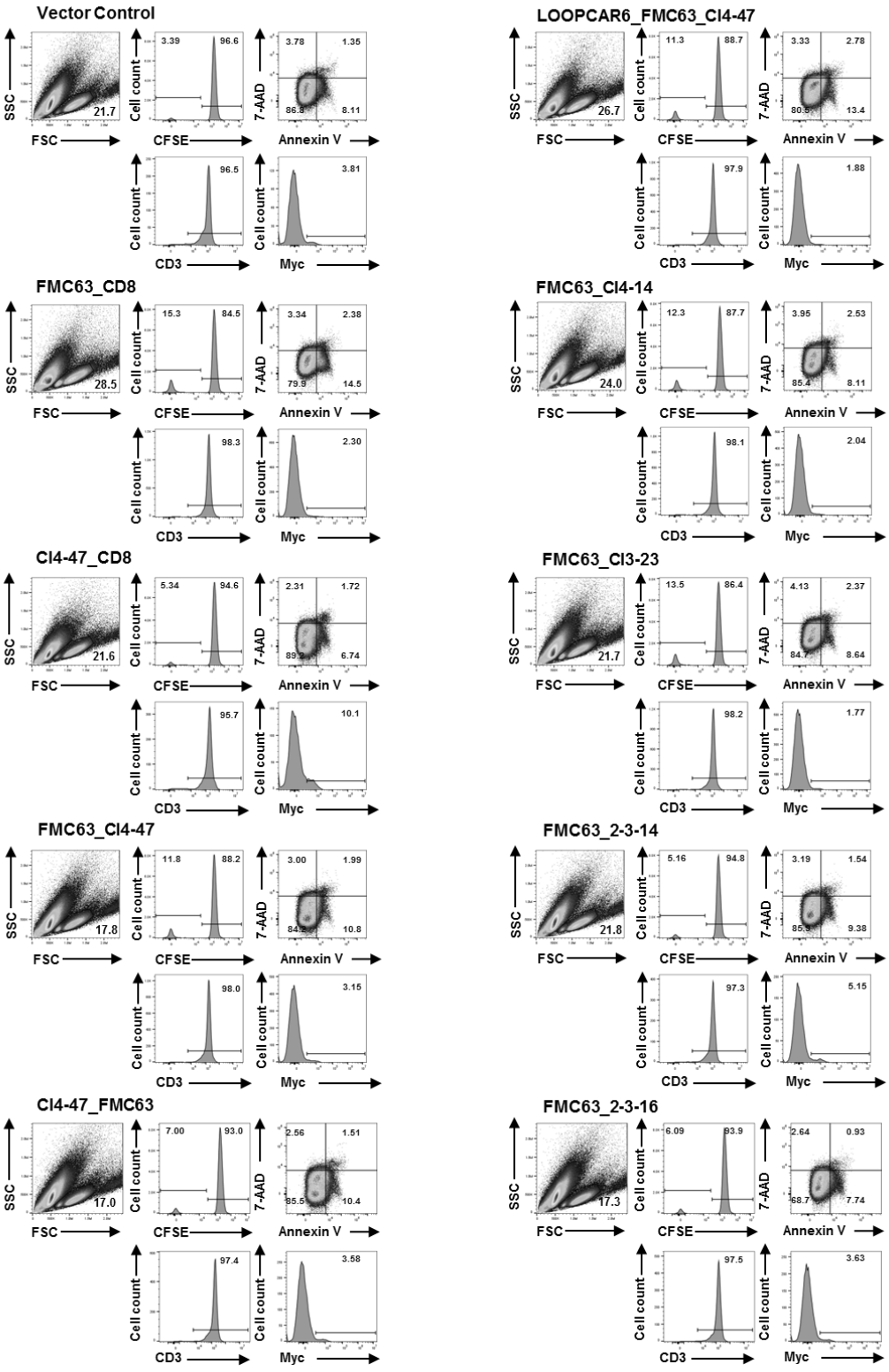
CD19 CAR-αβ T cells and CD19/CD22 bispecific CAR-αβ T cells eliminate B cell lymphoma cells *in vivo*.

I evaluated the efficacy of CD19 CAR-αβ T cells and CD19/CD22 CAR-αβ T cells. PBMCs from healthy donors were expanded with anti-CD3/CD28 antibodies and hIL-2 to produce αβ T cells and transduced with CD19/CD22 CAR vectors. CAR expressions were assessed as $CFSE^{-}CD3^{+}Myc^{+}$ cells *in vitro* and *in vivo* (Fig 3A). In AV/7-AAD apoptosis assay, apoptotic cells slightly increased in the presence of CD19/CD22 CAR-αβ T cells such as FMC63_CI4-47, LOOPCAR6_FMC63_CI4-47 and FMC63_CI3-23 bispecific CAR-αβ T cells. The frequency of $CFSE^{+}$ cells decreased by CD19/CD22 CAR-αβ T cells such as FMC63_CI4-47 and FMC63_2-3-16 bispecific CAR-αβ T cells, but the number of $CFSE^{+}$ cells was not significantly changed by CD19/CD22 CAR-αβ T cells (Fig 3B-C). *In vivo* studies, $CFSE^{+}$ cells were decreased by CAR-expressing αβ T cells, especially CD19 and CD19/CD22 FMC63_CI4-47, LOOPCAR6_FMC63_CI4-47 and FMC63_CI3-23 CAR-αβ T cells, compared with vector control (Fig 3D-E).

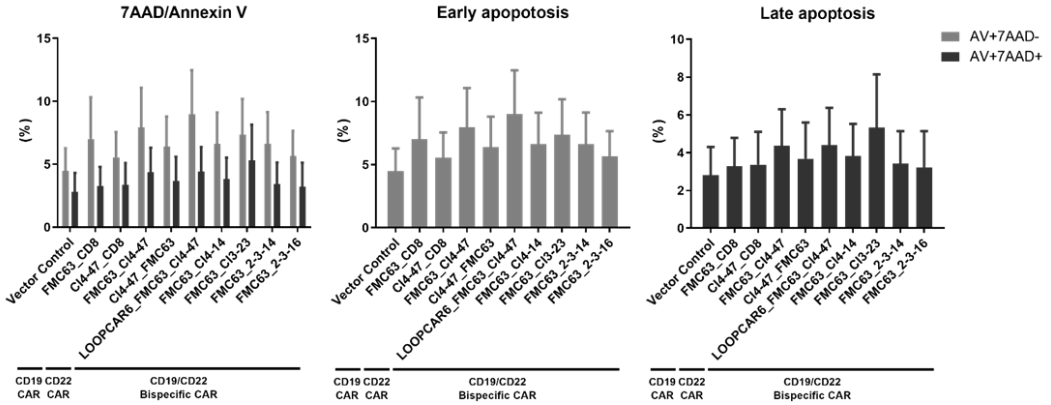
Figure 3.

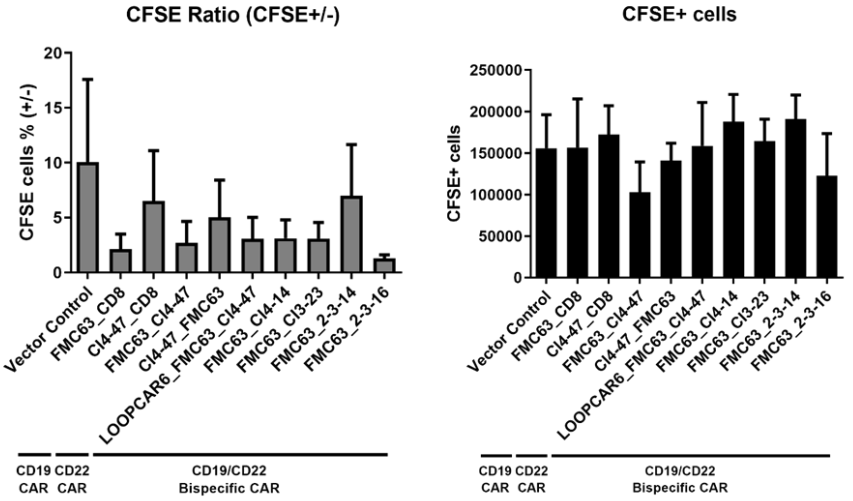

A
In vitro
SSC
FSC
21.6
Cell count
CFSE
5.34
94.6
7-AAD
Annexin V
2.31
1.72
89.2
6.74
CD3
95.7
Myc
10.1
In vivo
3.90
2.81
97.5
2.37
98.4
1.47
9.11
12.4

B

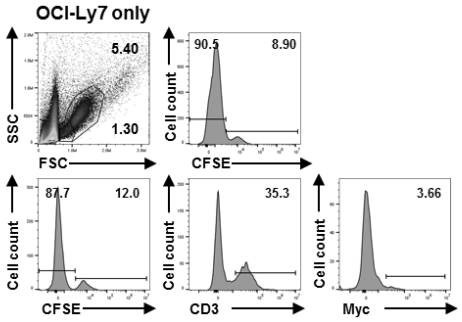

Vector Control
SSC
FSC
21.7
Cell count
CFSE
3.39
96.6
7-AAD
Annexin V
3.78
1.35
86.8
8.11
CD3
96.5
Myc
3.81
LOOPCAR6_FMC63_CI4-47
26.7
11.3
88.7
3.33
2.78
80.5
13.4
97.9
1.88
FMC63_CD8
28.5
15.3
84.5
3.34
2.38
79.5
14.5
98.3
2.30
FMC63_CI4-14
24.0
12.3
87.7
3.95
2.53
85.4
8.11
98.1
2.04
CI4-47_CD8
21.6
5.34
94.6
2.31
1.72
89.3
6.74
95.7
10.1
FMC63_CI3-23
21.7
13.5
86.4
4.13
2.37
84.7
8.64
98.2
1.77
FMC63_CI4-47
17.8
11.8
88.2
3.00
1.99
84.2
10.8
98.0
3.15
FMC63_2-3-14
21.8
5.16
94.8
3.19
1.54
85.9
9.38
97.3
5.15
CI4-47_FMC63
17.0
7.00
93.0
2.56
1.51
85.5
10.4
97.4
3.58
FMC63_2-3-16
17.3
6.09
93.9
2.64
0.93
68.7
7.74
97.5
3.63

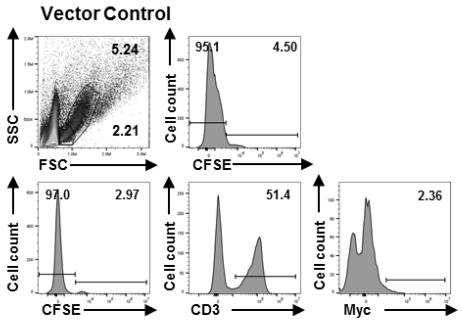
C

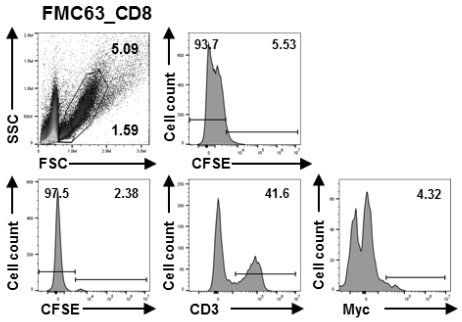

7AAD/Annexin V
Early apopotosis
Late apoptosis
AV+7AAD-
AV+7AAD+
(%)
Vector Control
FMC63_CD8
CI4-47_CD8
FMC63_CI4-47
CI4-47_FMC63
LOOPCAR6_FMC63_CI4-47
FMC63_CI4-14
FMC63_CI3-23
FMC63_2-3-14
FMC63_2-3-16
CD19 CAR
CD22 CAR
CD19/CD22 Bispecific CAR

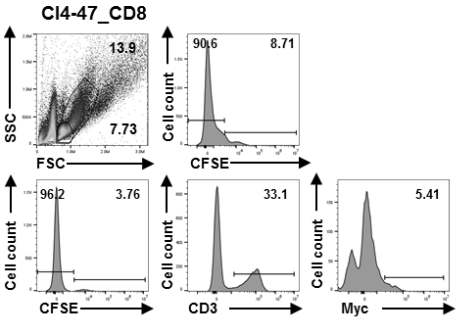

CFSE Ratio (CFSE+/-)
CFSE cells % (+/-)
CFSE+ cells
Vector Control
FMC63_CD8
CI4-47_CD8
FMC63_CI4-47
CI4-47_FMC63
LOOPCAR6_FMC63_CI4-47
FMC63_CI4-14
FMC63_CI3-23
FMC63_2-3-14
FMC63_2-3-16
CD19 CAR
CD22 CAR
CD19/CD22 Bispecific CAR

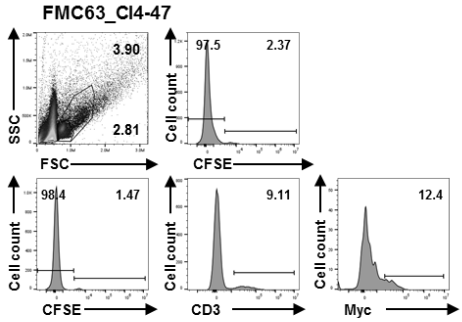
D

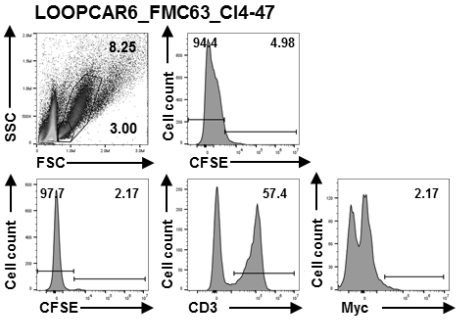

OCI-Ly7 only
5.40
1.30
90.5
8.90
87.7
12.0
35.3
3.66
SSC
FSC
Cell count
CFSE
CD3
Myc

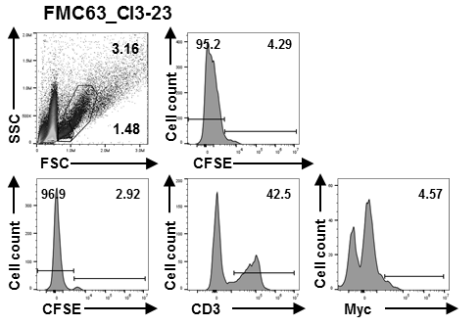

Vector Control
5.24
2.21
95.1
4.50
97.0
2.97
51.4
2.36
SSC
FSC
Cell count
CFSE
CD3
Myc


FMC63_CD8
5.09
1.59
93.7
5.53
97.5
2.38
41.6
4.32
SSC
FSC
Cell count
CFSE
CD3
Myc


Cl4-47_CD8
13.9
7.73
90.6
8.71
96.2
3.76
33.1
5.41
SSC
FSC
Cell count
CFSE
CD3
Myc


FMC63_Cl4-47
3.90
2.81
97.5
2.37
98.4
1.47
9.11
12.4
SSC
FSC
Cell count
CFSE
CD3
Myc


LOOPCAR6_FMC63_Cl4-47
8.25
3.00
94.4
4.98
97.7
2.17
57.4
2.17
SSC
FSC
Cell count
CFSE
CD3
Myc


FMC63_Cl3-23
3.16
1.48
95.2
4.29
96.9
2.92
42.5
4.57
SSC
FSC
Cell count
CFSE
CD3
Myc

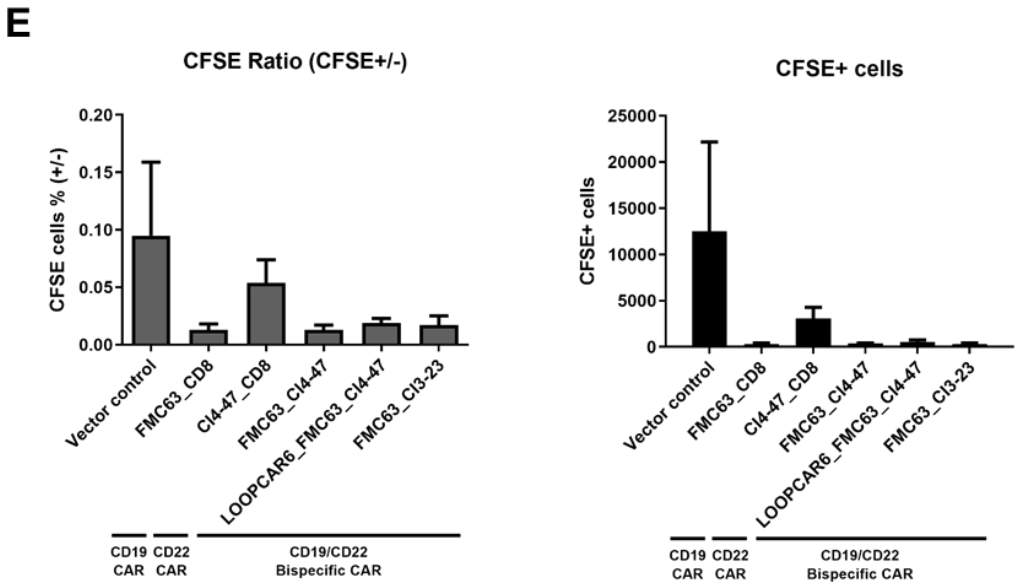


Figure 3. *In vitro* and *In vivo* experiments of CAR-αβ T cells. (A) Gating strategy of *in vitro* and *in vivo* experiments. AV/7-AAD apoptosis assay were performed to identify CFSE-labeled OCI-Ly7 and CAR-αβ T cells *in vitro*. *In vivo* experiments (lower panel) were performed to identify CFSE-labeled OCI-Ly7 by flow cytometry. (B) Representative FACS plots of AV/7-AAD apoptosis assay. Results are displayed for FMC63_CD8 (CD19 CAR), CI4-47_CD8 (CD22 CAR), FMC63_CI4-47, CI4-47_FMC63, LOOPCAR6_FMC63_CI4-47, FMC63_CI4-14, FMC63_CI3-23, FMC63_2-3-14 and FMC63_2-3-16 bispecific CAR-

αβ T cells. (C) Graphs of AV^+ 7-AAD^- early apoptotic cells and AV^+ 7-AAD^+ late apoptotic cells (upper panel). Graphs of $CFSE^+$ cells and the number of $CFSE^+$ cells (lower panel). CFSE-labeled OCI-Ly7 and CAR-αβ T cells were co-cultured for 24 hours and flow cytometry was performed. OCI-Ly7 cells were used as target cells. Vector control group was transduced with empty lentiviral vectors. n=3. All values present as means ± SEM. (D) Representative flow cytometric plots of *in vivo* studies in NSG mice. The results of cytotoxicity are displayed for FMC63_CD8 (CD19 CAR), CI4-47_CD8 (CD22 CAR), FMC63_CI4-47, LOOPCAR6_FMC63_CI4-47, and FMC63_CI3-23 bispecific CAR-αβ T cells. (E) Graphs of $CFSE^+$ cells and the number of $CFSE^+$ cells. CFSE-labeled OCI-Ly7 and CAR-αβ T cells were injected i.p. into NSG mice. After 48 hours, intraperitoneal cells were harvested, and flow cytometry was performed. OCI-Ly7 cells were used as target cells. n=2 for vector control, CI4-47_FMC63, LOOPCAR6_FMC63_CI4-47. n=3. All values present as means ± SEM.

Result of CD19/CD22 bispecific CAR-γδ T cells against B cell lymphoma cells *in vivo*.

γδ T cells, which are innate immune cells like NK cells, were transduced with CD19/CD22 CAR expression vectors. γδ T cells were expanded with hIL-2, hIL-15 and Zoledronic acid. CAR expression was analyzed as $CFSE^{-}CD3^{+}Myc^{+}V\delta1^{+}$cells and $CFSE^{-}CD3^{+}Myc^{+}$ $V\delta2^{+}$cells *in vivo* (Fig 4A). CAR expression was mainly showed in the Vδ2 T cell subset stimulated by zoledronic acid, but cytotoxicity of CAR-expressing γδ T cells were not significantly changed (Fig 4B-C).

Figure 4.

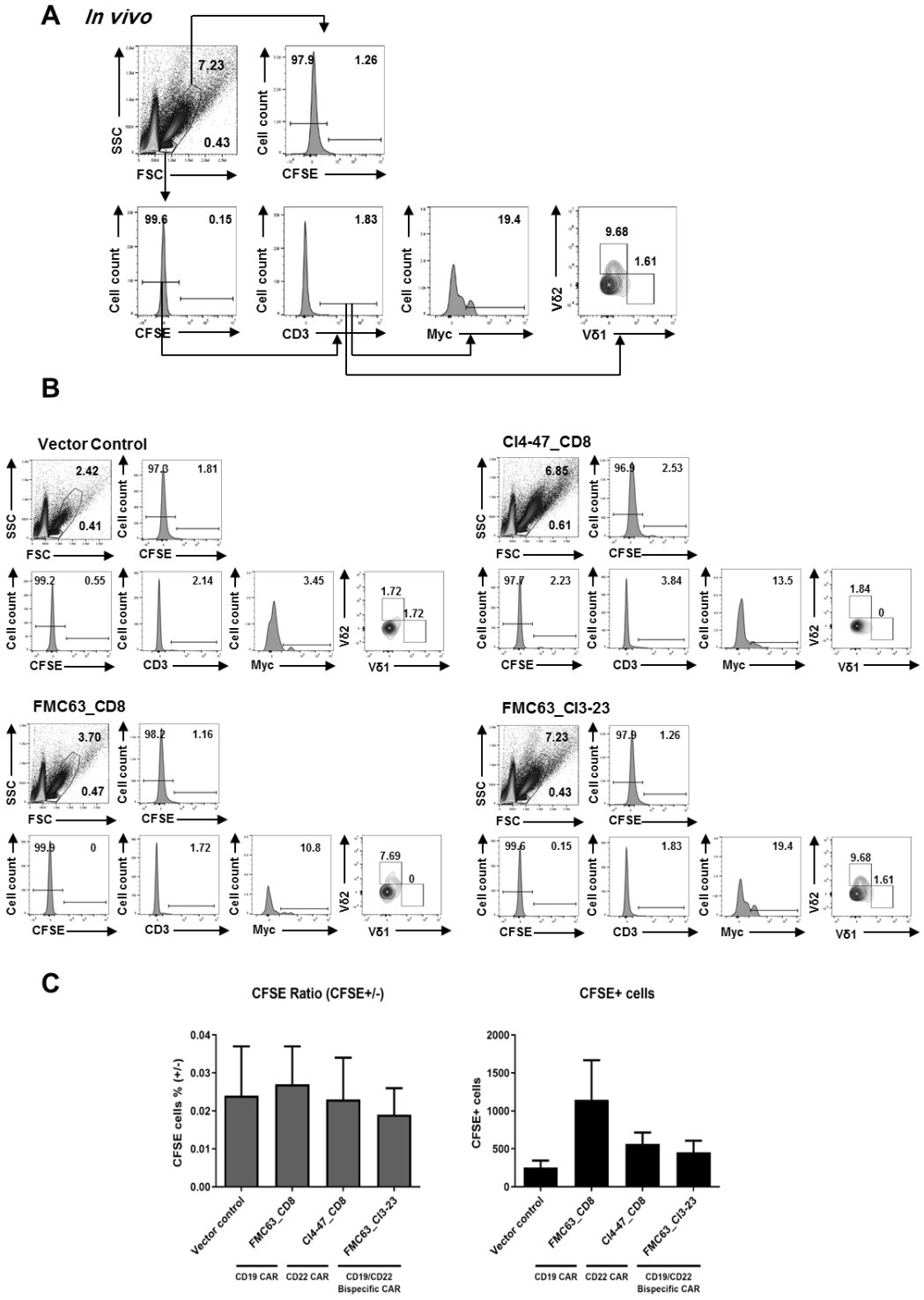

A
In vivo
7.23
0.43
SSC
FSC
97.9
1.26
Cell count
CFSE
99.6
0.15
1.83
19.4
9.68
1.61
CD3
Myc
Vδ2
Vδ1
B
Vector Control
2.42
0.41
97.8
1.81
99.2
0.55
2.14
3.45
1.72
1.72
CI4-47_CD8
6.85
0.61
96.9
2.53
97.7
2.23
3.84
13.5
1.84
0
FMC63_CD8
3.70
0.47
98.2
1.16
99.9
0
1.72
10.8
7.69
0
FMC63_CI3-23
7.23
0.43
97.9
1.26
99.6
0.15
1.83
19.4
9.68
1.61
C
CFSE Ratio (CFSE+/-)
CFSE cells % (+/-)
0.04
0.03
0.02
0.01
0.00
CFSE+ cells
2000
1500
1000
500
0
Vector control
FMC63_CD8
CI4-47_CD8
FMC63_CI3-23
CD19 CAR
CD22 CAR
CD19/CD22 Bispecific CAR

Figure 4. *In vivo* CAR-γδ T cells experiments. (A) Gating strategy of *in vivo* experiments. Flow cytometry was performed to identify CFSE-labeled OCI-Ly7 and CAR-γδ T cells. (B) Representative FACS plots of *in vivo* studies in NSG mice. Cytotoxicity assay results for FMC63_CD8 (CD19 CAR), CI4-47_CD8 (CD22 CAR), FMC63_CI4-47, and FMC63_CI3-23 bispecific CAR- γδ T cells. (C) Graphs of $CFSE^+$ cells and number of $CFSE^+$ cells. CFSE-labeled OCI-Ly7 and CAR-γδ T cells were injected i.p. into NSG mice. After 48 hours, intraperitoneal cells were harvested and flow cytometry was performed. OCI-Ly7 cells were used as target cells. Vector control was transduced with empty lentiviral vectors. n=3 for FMC63_CI3-23. n=4. All values present as means ± SEM.

Discussion

Cytotoxic T cells are MHC-restricted and specifically recognize epitopes presented by HLA class I molecules to exert cytotoxicity. But αβ T cells can induce GVHD as results of MHC restriction, thus they are not suitable for third-party immune cell therapy [5, 21]. NK cells and γδ T cells, which are non-MHC-restricted cytotoxic lymphocytes, derive from a common lymphoid precursor cells [7, 22]. These lymphocytes currently receive attention as CAR-expressing immune cells but have limitations their variability in functional competence and expansion potential [23]. CD19-targeted CAR T cell therapy has been effective in the treatment of relapsed or refractory B cell malignancies [10], but 40~60% of patients relapsed after CD19 CAR T cell therapy, due to CD19 loss [24]. Therefore, CD22, an activation marker of mature B lymphocytes, could be an effective target for immunotherapy of relapsed or refractory B cell malignancies [13]. Many studies have already been conducted on CD19/CD22 (2 single-specific) CAR-T cocktail therapy and on CAR T cells with dual targets of CD19 and CD22 [11, 25, 26]. Studies of the CD19/CD22 bispecific CAR T cells

were conducted in phase 1 clinical trials in relapsed or refractory children and adults [27, 28]. In this study, CD19/CD22 bispecific CAR structures were developed using a novel anti-CD22 monoclonal antibody domain. The structure of CARs, such as antibody domains (scFvs), structural components, and signaling domains, and the type of effector cells have different cytotoxicity outcome because they affect the accessibility of the target antigen by the CARs [29]. To evaluate the efficacy of CD19/CD22 bispecific CARs, the seven CARs were designed with different CAR antibody domains or hinge/spacer domains and transduced them to various immune effector cells. Anti-cancer effects were demonstrated by CI4-47_FMC63, FMC63_2-3-14 bispecific CAR-NK-92 cells *in vitro* and CI4-47_FMC63, LOOPCAR6_FMC63_CI4-47 bispecific CAR-NK-92 cells *in vivo*. Compared with CAR T cells therapy, CAR-NK cells do not cause cytokine release syndromes (CRS) or GVHD for hematological malignancies [30]. The NK-92 cell line resembles NK cells which are involved in the killing of tumor cells without prior exposure to antigen [31]. This cell line has the characteristics of activated NK cells *in vivo*, has been demonstrated safety in various

applications, and is currently been performed in clinical trials in a variety of tumors [32]. Therefore, CI4-47_FMC63 CAR-NK-92 cells were considered to have high cytotoxicity against B cell lymphoma (BCL) and were validated in this study. In CD19/CD22 CAR-αβ T cells studies, although not significant, it was observed that BCL cells were decreased by FMC63_CI4-47, LOOPCAR6_FMC63_CI4-47 and FMC63_CI3-23 bispecific CARs *in vivo*. The study of CD19/CD22 CAR-γδ T cells was expected to have resemblance results to those of CD19/CD22 CAR-NK-92 study, but there was no cell killing ability by the CARs. However, a low frequency of FMC-63_CI3-23 bispecific CAR-γδ T cells was observed in *in vivo* studies, and an increased frequency of FMC-63_CI3-23 bispecific CAR-γδ T cells could have cell killing effect on BCL. CD22 mostly retains to $CD19^{low}$ or $CD19^{-}$ malignant B cells [11, 15], but recently there are some studies that downregulation of CD19 also down-regulates CD22 [33], so further studies are required to validate whether the cytotoxicity of CD19/CD22 CARs is maintained to $CD19^{-}CD22^{-}$ malignant B cells.

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human natural killer cell line, NK92. J Immunol Methods. 2005;296:31-6.

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국문요약

배경: 항-CD19 키메라 항원 수용체 (CAR) T 세포 치료법은 재발성 또는 불응성 공격성 B 세포 림프종의 치료에 효과적이지만 $CD19^{low}$ 또는 $CD19^{-}$악성 B 세포와 같은 항원 소실 (antigen escape) 재발을 유발할 수 있다. CD22 와 CD19 에 대한 이중 특이성 CAR를 개발함으로써, 암세포의 항원 소실을 통한 재발을 방지하고 NK-92 세포주와 γδ T 세포를 면역효과세포로 사용함으로써 Graft-versus host disease (GVHD)와 같은 합병증을 줄이고자 한다.

방법: CD19 (클론 FMC63)와 backbone 구조를 변형하여 새로운 항-CD22 단일 클론 항체를 사용하여 7 개의 CAR-발현 벡터를 생산하였다. CAR 구조의 효능은 사람 B 세포 림프종 세포주인 OCI-Ly7 세포를 사용하여 시험관 내 및 생체 내 실험 모두에서 평가되었다. OCI-Ly7 세포를 CFSE로 표지하여 CAR-NK-92, CAR-αβ T 및 CAR-γδ T 세포(면역효과세포)와 구별했다. 세포 사멸은 시험관 내에서 Annexin V 및 7-

aminoactinomycin D로 평가되었다. 생체 내 분석을 위해 OCI-Ly7 세포와 면역효과 세포를 NSG 마우스에 복강 내 주사하고 48 시간 후 복막 세척액을 수집하였다.

결과: OCI-Ly7 세포는 시험관 내 실험에서 NK-92 세포에 비해 FMC63_CD8-NK-92 (CD19 CAR-NK-92) 세포, CI4-47_FMC63-NK-92 및 FMC63_2-3-14-NK-92 (CD19/CD22 CAR-NK-92) 세포에서 통계적으로 유의하게 감소했다. CI4-47_FMC63-NK-92 및 LOOPCAR6_FMC63_CI4-47-NK-92 (CD19/CD22 CAR-NK-92) 세포는 생체 내에서 더 높은 세포독성을 가졌다. 또한, 몇몇 CD19/CD22 CAR-αβ T 세포는 OCI-Ly7 세포의 세포사멸을 증가시키는 경향이 있었다.

결론: CD19/CD22 이중 특이성 CAR-NK-92 세포는 CD19 CAR-NK-92 세포보다 B 세포 림프종 세포에 대해 더 높은 세포독성을 가졌다. CAR의 효능은 단백질의 2 차 구조, 항체 도메인의 서열, 링커 구조에 따라 다를 수 있다.