CD19/CD22 bispecific chimeric antigen receptor-NK-92 cells are developed and evaluated

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Abstract. Anti-CD19 chimeric antigen receptor (CAR)-T cells have improved the outcomes of patients with B cell leukemia and lymphoma. However, their applications and positive outcomes remain limited. CAR-T cells are currently restricted to autologous blood as their source and their use can lead to downregulation of CD19 expression along with complications such as graft-versus-host disease and cytokine release syndrome. The present study aimed to develop anti-CD19/CD22 bispecific CAR structures using an anti-CD22 monoclonal antibody clone from chickens and analyze them in natural killer (NK)-92 cells, a human NK cell line, *in vitro* and *in vivo*. Anti-CD19/CD22 CAR-NK-92 cell cytotoxicity was assessed by the survival of target cells and counted using flow cytometry. Anti-CD22/CD19 and loop-structured anti-CD19/CD22 bi-specific CAR-NK-92 cells showed improved efficacy against OCI-Ly7 cells, a human B cell lymphoma cell line, compared with other CAR structures. These results demonstrate the potential of anti-CD19/CD22 bispecific CAR-NK cells and suggested that optimizing CAR structures in NK cells can improve the efficacy of CAR therapy.

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Introduction

Anti-CD19 chimeric antigen receptor (CAR)-T cells have demonstrated notable therapeutic effects on B cell leukemia and lymphoma in clinical trials following landmark studies (1,2). Anti-CD19 CAR-T cell therapy is effective for treating relapsed or refractory aggressive B cell lymphoma and second-generation anti-CD19 CARs have been approved by the US Food and Drug Administration and are currently being evaluated in clinical trials to expand their applications (3-5). In certain cases, persistent CD4+ CAR-T cells have achieved leukemia remission lasting a decade (6). However, CD19-expressing malignant B cells evade CAR-T cell therapy (7) and T cell exhaustion can occur due to continued antigen stimulation by cancer cells (8). Furthermore, there is a risk of cytokine release syndrome (9), and autologous blood is the only source of CAR-T cells in the clinic to prevent potential graft-versus-host disease (GVHD). CAR-T cells produce cytokines and exert cytotoxicity to control tumor cells; tumor cells can produce cytokines that interact with immune cells in the tumor microenvironment (10). Infusion of CAR-T cells increases circulating cell count, resulting in elevated serum inflammatory cytokine levels, which elicit cytokine release syndrome (9).

To overcome the limitations of CAR-T cell therapy, natural killer (NK) cells are suitable candidates for cancer immunotherapy. They are not major histocompatibility complex (MHC)-restricted and are thus less likely to elicit GVHD while retaining anti-tumor activity via degranulation of cytolytic molecules, including granzyme and perforin, and cytokine secretion (11). In addition, NK cells have short lifespans, which may prevent cytokine release syndrome. Infusion of human leukocyte antigen-mismatched anti-CD19 CAR-NK cells derived from umbilical cord blood does not cause cytokine release syndrome, neurotoxicity or GVHD in patients with CD19+ relapsed or refractory lymphoma or leukemia (12). Notably, 73% of patients exhibit a response to treatment with these cells, and there is no increase in inflammatory cytokine levels (12). According to phase I clinical trials, infusion of NK-92, a human NK cell line, is safe in patients with various types of solid tumor, refractory lymphoma, and multiple myeloma (MM) (13-15). NK-92 cells are easily

cultured and have a higher transfection efficiency than primary NK cells (16). NK-92 cells express high levels of molecules involved in the perforin-granzyme apoptosis pathway (17). To eliminate *in vivo* proliferation and tumorigenic potential of CAR-NK-92, irradiation is often performed before administration in patients, and phase I clinical trials demonstrating their stability profile have been performed (18,19). The safety of anti-CD33 CAR-NK-92 cells has also been previously examined in patients with relapsed and refractory acute myeloid leukemia (20).

CD22 is expressed during the pre-B cell stage and is maintained in mature B cells, making it a promising target for immunotherapy against malignant B cells (21,22). CD19 and CD22 are expressed in normal B cells and associated malignancies and CD22 expression is sustained in CD19^{low} or CD19^{-} malignant B cells (4,23). By targeting both CD19 and CD22, bispecific CARs may broaden the range of diseases treated by CAR-based immune cells. Anti-CD19 and anti-CD22 CAR-T cell cocktail therapy and anti-CD19/CD22 bispecific CAR-T cell therapy have shown promising results in phase I clinical trials (4,24,25). To the best of our knowledge, however, the efficacy of anti-CD19/CD22 bispecific CAR-NK cells has not been reported.

The present study aimed to overcome the limitations of anti-CD19 CAR-T cell therapy. To generate anti-CD19/CD22 bispecific CAR structures, anti-CD22 monoclonal antibodies (mAbs) were developed, and their antigen-binding sites were integrated into CAR backbone structures. The present study also aimed to evaluate the cytotoxicity of anti-CD19/CD22 bispecific CAR-NK-92 cells against OCI-Ly7 cells, a human diffuse large B cell lymphoma cell line, *in vitro* and *in vivo* and identify the anti-CD19/CD22 CAR structures that had the highest efficacy in NK cells.

Materials and methods

Generation of anti-CD22 single-chain variable fragment (scFv) Ab. Specific-pathogen-free (SPF) White Leghorn chickens (Gallus gallus) were hatched from SPF eggs (VALO BioMedia GmbH). All chickens were kept at 24-30°C and 9:15 light-dark cycle in 40-60% humidity, and had free access to food and water until the end of the experiment. A total of three 4-week-old male chickens were immunized with 20 μg recombinant human CD22-Fc protein (cat. no. 1968-SL; R&D Systems, Inc.) subcutaneously four times at two-week intervals by BIOPOA Co., Ltd. The spleen, bursa of Fabricius and bone marrow were harvested for total RNA isolation using TRI Reagent™ (Invitrogen, Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized using the Superscript IV First-Strand Synthesis system (Invitrogen, Thermo Fisher Scientific, Inc.), with oligo(dT) primers, according to the manufacturer's instructions. The genes encoding Vλ and VH were amplified from the oligo(dT)-synthesized cDNA using gene-specific PCR primers. Primer sequences for Vλ (CSCVK_forward: 5'GTGGCCCAGGCGGCCCTGACT CAGCCGTCCTCGGTGTC-3' and CKJo-B_reverse:5'-GGA AGATCTAGAGGACTGACCTAGGACGGTCAGG-3') and VH (CSCVHo-FL_forward: 5'-GGTCAGTCCTCTAGA TCTTCCGGCGGTGGTGGCAGCTCCGGTGGTGGCGGT TCCGCCGTGACGTTGGACGAG-3' and CSCG-B_reverse:

5'-CTGGCCGGCCTGGCCACTAGTGGAGGAGACGAT GACTTCGGTCC-3') were synthesized by Integrated DNA Technologies, Inc. (26). scFv-displaying phage libraries were generated as described previously (27). The libraries were subjected to five rounds of bio-panning with CD22-conjugated magnetic beads (cat. no. 14302; Invitrogen, Thermo Fisher Scientific, Inc.). To select candidates able to bind CD22, reactivity of antibody-displaying phages was tested in phage enzyme immunoassay. The microtiter plates were coated with 100 ng/well human CD22-His protein (cat. No. 11958-H08H, Sino Biological, Inc.) and incubated at 4°C overnight. After blocking with 3% bovine serum albumin (BSA, cat. no. BSAS 0.1; Bovogen Biologicals Pty Ltd.) dissolved in PBS (w/v), the plate was then sequentially incubated with scFv-displaying phages in the culture supernatant, HRP-conjugated anti-M13 antibody (dilution fold 1:5,000, cat. no. 27-9421-01;Cytiva) and then finally with 2,2'-Azinobis [3-ethylbenzothiazoline-6-sul-fonic acid]-diammonium salt (ABTS) substrate solutions (cat. no.002024, Thermo Fisher Scientific, Inc.) with intermittent washing using 0.05% Tween-20 in PBS. The optical density was measured at 405 nm using a microplate spectrophotometer (Sunrise™, Tecan. Phage clones showing positive signals were selected (optical density at 405 nm ≥0.6), and their nucleotide sequences were determined by Sanger sequencing (Cosmo Genetech Co., Ltd., Korea).

Expression and purification of anti-CD22 scFv-Cκ-hemagglutinin (HA) fusion protein. The gene encoding anti-CD22 scFv was subcloned into modified pCEP4 mammalian expression vectors to express human constant κ region (Cκ) and HA-tagged proteins as described previously (28). The construct was transfected into FreeStyle™ 293-F cells (cat. no. R790-07, Thermo Fisher Scientific, Inc.) using 25-kDa linear polyethylenimine (PEI, cat. no. 23966-1, Polysciences). The mixture of 2 μg plasmid DNA and 4 μg linear PEI in 100 μl 150 mM NaCl solution was prepared per ml of cell culture volume. Following 15-min incubation at room temperature, the mixture was added to HEK293F cells ($2x10^6$ cells ml^{-1}) and the cells were grown in FreeStyle 293 Expression Medium (cat. no. 12338018, Thermo Fisher Sciences) for 5 days at 37°C in an atmosphere containing 7% CO_2 on an orbital shaking incubator (cat/no. NB-206CXL, N-Biotek) at 135 rpm. The scFv-Cκ-HA fusion proteins were purified from culture supernatant which was collected 48 h after transfection by affinity chromatography using KappaSelect Resin (cat. no. 17545801, Cytiva) according to the manufacturer's instructions.

Binding assay using anti-CD22 scFv-Cκ-HA fusion protein. Microtiter plates were coated with 100 ng/well human CD22-His protein (Sino Biological, Inc.) and incubated at 4°C overnight. Wells were blocked with 150 ml 3% BSA (Sigma Aldrich; Merck KGaA) in PBS at 37°C for 1 h. The anti-CD22 scFv-Cκ-HA fusion protein was diluted serially in 3% BSA in PBS starting from 800 nM with 4-fold dilutions, resulting in seven dilutions, and added to each well. After incubation at 37°C for 1.5 h, the plate was washed three times with 0.05% Tween-20 in PBS and incubated with HRP-conjugated anti-human Cκ antibody (dilution fold 1:5,000, cat. no. AP502P, MilliporeSigma) at 37°C for 1 h. The plate was washed three times with 0.05% Tween-20 in PBS and bound antibody was

determined by adding ABTS solution. Absorbance at 405 nm was measured using a microplate spectrophotometer.

OCI-Ly7 cells, donated by Professor Sung Ho Jeon (Hallym University, Chuncheon, Korea) or Raji cells (ATCC) were seeded into a 1.5 ml tube at a density of $3x10^5$ cells/tube. The anti-CD22 scFv-Cκ-HA fusion protein was added at 0.25 μM to each tube and incubated on ice for 30 min. After washing twice with MACS buffer (Miltenyi Biotec GmbH), Alexa Fluor 647-labeled mouse anti-HA antibody (Clone 912426; cat. no. IC6875R; R&D Systems) was added at 1:1,000 dilution and the sample was incubated on ice for 30 min. The cells were washed twice with MACS buffer, resuspended in 150 μl 1% paraformaldehyde in MACS buffer, and incubated at 37°C overnight. The cells were analyzed using flow cytometry with a FACS Canto II instrument (BD Biosciences) using a FACSDiva 6.0 software (BD Biosciences). For each sample, 10,000 cells were analyzed.

Anti-CD19/CD22 bispecific CAR construction. CD19 CAR-expressing vector (FMC63-CD8 hinge-BBz-pCL20C-MND) was provided by Dr Byoung Ryu (St Jude Hospital, Memphis, TN, USA). The pCAG vector backbone was derived from pCAGGS prepared by Dr Jun-ichi Miyazaki (Kumamoto University, Kumamoto, Japan) (29). CD19 CAR construct contained the FMC63 scFv, hinge and transmembrane domain of CD8, cytoplasmic domain of 4-1BB and CD3ζ signaling domain. The CD19 CAR vector was tagged with MYC. Representative structures of CARs are shown in Fig. 1.

Cell culture. NK-92 cells were obtained from American Type Culture Collection (cat. no. CRL-2407) and maintained in α-Minimum Essential Medium (Welgene, Inc.) containing 12.5% non-heat inactivated FBS (Welgene, Inc.), 12.5% heat-inactivated horse serum (Gibco; Thermo Fisher Scientific, Inc.) 10 U/ml penicillin, 10 μg/ml streptomycin, 20 mM folic acid, 20 mM myo-inositol (MilliporeSigma), 55 mM 2-mercaptoethanol and 100 U/ml recombinant human interleukin-2 (rhIL-2; PeproTech, Inc.); this was named NK92 medium. The human B cell lymphoma cell lines, OCI-Ly7 and Raji, and human embryonic kidney epithelial cell line 293(T) cells (Korean Cell Line Bank; Korean Cell Line Research Foundation) were maintained in DMEM (Welgene, Inc.) containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained in a fully humidified incubator at 37°C in 5% CO_2.

Generation of CAR-transduced cells. Plasmid DNA was transformed by heat shock into competent Escherichia coli Stbl3 cells in a 0.1 M solution CaCl2, following standard protocols (30). The CAR vector was isolated using a Plasmid DNA Midi prep kit (Qiagen GmbH). To produce lentivirus particles, the CAR-expressing vector was transfected into $1x10^5$ 293(T) cells with packaging plasmid vectors pCAG-KGP1-1R, pCAG4-RTR2, and pCAG-VSVG at a ratio of 6:3:1:1 μg using 100 μl of Lipofectamine® 3000 (Invitrogen, Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The virus-containing medium was harvested at 48 and 72 h after transfection and filtered through a 0.45 μM filter (MilliporeSigma). The viral supernatant was used to transfect NK92 cells pre-treated with 8 μg/ml polybrene (MilliporeSigma) for 5 min at room temperature. CAR-NK-92 cells were sorted to 80-99% purity CAR-expressing Myc+ cells using a BD FACSAria™ III cell sorter. Flow cytometry was performed using allophycocyanin anti-CD56 (clone NCAM, Cat. No. 17-0567-42; Invitrogen, Thermo Fisher Scientific, Inc.), AlexaFluor-647-conjugated anti-Myc-Tag mouse monoclonal antibody (Clone 9B11, Cat. No. 2276; Cell Signaling Technology, Inc.), and matched AlexaFluor-647-conjugated mouse IgG2a, κ isotype control (Clone MOPC-173, Cat. No. 400234; BioLegend, Inc.) by CytoFLEX (Beckman Coulter, Inc.) and FlowJo v10 software (Tree Star, Inc.) following 30 min incubation at 1:200 dilution on ice. The selected CAR-NK-92 cells were cryopreserved in heat-inactivated FBS containing 10% DMSO and stored at -196°C in liquid nitrogen until use. The purity of the Myc+ CAR-NK-92 cells was over 90% after thawing (Fig. S1). In *in vitro* experiments, naked lentiviral vector was transfected as NV control.

Apoptosis test. Cytotoxicity was analyzed by performing 7-amino-actinomycin D (7-AAD) and Annexin V assay. Target OCI-Ly7 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using a Cell Trace Cell Proliferation kit (Thermo Fisher Scientific, Inc.), as previously described (31). CAR-NK-92 cells were co-cultured with CFSE-labeled target cells at an effector-to-target ratio of 2:1. After incubation at 37°C overnight, the cells were washed with Annexin V binding buffer (BD Biosciences) and incubated at 37°C for 30 min. CAR-NK-92 cells were cultured at 37°C for 4 h, pre-treated with human FcR blocking reagent at 1:200 dilution (Miltenyi Biotec GmbH) for 5 min, and stained with 7-AAD Viability Staining Solution (BioLegend, Inc.) and Pacific Blue™ Annexin V (BioLegend, Inc.) for 15 min on ice. The CFSE⁺ target cells were evaluated for early and late apoptosis using flow cytometry using CytoFLEX (Beckman Coulter, Inc.). The data were analyzed using FlowJo v10 software (Tree Star, Inc.).

In vivo experiments. Female 8-12 week-old NSG (NOD. Cg-PrkdcscidIl2rgtm1Wjl/SzJ) mice were purchased (JABio,) and maintained in SPF facilities at 20-23°C and a 12/12 light/dark cycle with 40-60% humidity. They were free to access food and water. On day 0, mice were injected intraperitoneally with CFSE-labeled OCI-Ly7 cells at a density of $5x10^6$ cells/200 μl in 1X PBS. After 30 min, mice were injected intraperitoneally with $5x10^6$ CAR-transduced cells. On day 2, 15 ml 1X PBS was injected into the peritoneal cavity, and the peritoneal lavage was harvested (32). For euthanasia, 30-70% vol/min CO_2 was administered in a closed box, following injection of Tribromoethanol 200-400 mg/kg as anesthetics, intraperitoneally. For harvesting peritoneal lavage, anesthetics (Zoletil 20-40 mg/kg + Zylazine 5-10 mg/kg) were injected intraperitoneally. The ratio and number of CFSE⁺ cells were measured using flow cytometry, as described above. The data were analyzed with FlowJo v10 software. All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee of Asan Medical Center, Seoul, Korea (approval no. 2022-12-090) and performed ethically and in accordance with Animal Protection Act by Korean Government (33).
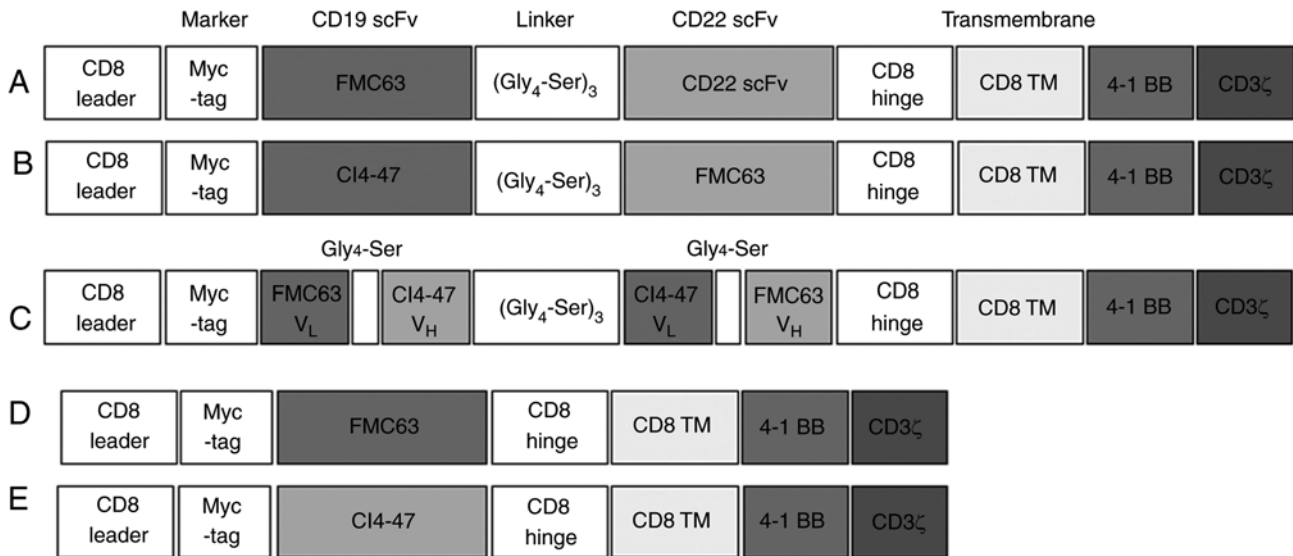
Figure 1. Representative structures of anti-CD19 and anti-CD22 specific and bispecific CAR structures. (A) A total of four bispecific CAR structures were produced using anti-CD22 antibody clones, CI4-47, CI3-23, 2-3-14, and 2-3-16. (B) Anti-CD19/CD22 bispecific CAR structures were produced using CI4-47 clone into the backbone structure. (C) Anti-CD19/CD22 bispecific CAR structures were produced using CI4-47 clone into the backbone structure with a loop to optimize the CAR structures for natural killer cells. (D) Anti-CD19 (FMC63) were integrated to the backbone structure. (E) Anti-CD22 antigen-binding domains were integrated as well. FMC63, anti-CD19 antigen-binding domain; CI4-47, anti-CD22 antigen-binding domain; CAR, chimeric antigen receptor; scFv, single chain variable fragment.

Statistical analysis. Graphical and statistical analysis was performed using GraphPad Prism v6 software (Dotmatics). P-values were calculated by one-way ANOVA followed by post hoc Tukey-Kramer and Bartlett's tests. $P < 0.05$ was considered to indicate a statistically significant difference. Data are shown as the mean ± standard error of the mean or SD of at least four independent experimental repeats.

Results

Production of anti-CD19/CD22 bispecific CAR constructs. Binding of mAb clones to CD22 protein was evaluated through enzyme immune assays (Figs. 2A and S1A) and flow cytometry (Figs. 2B and S1B). The antigen-binding domain of selected mAb clones, CI4-47, CI3-23, 2-3-14, and 2-3-16, was integrated into a CAR backbone structure, a second-generation molecule containing 4-1BB and CD3ζ signaling domains. To optimize the bispecific CAR structures for NK cells, the order of the anti-CD19 (clone FMC63) and anti-CD22 (clone CI4-47) was changed (Fig. 1A and B) and the loop structure was prepared (Fig. 1C). CI4-47 clone was selected by binding assay using flow cytometry (Fig. S1B). Anti-CD19- or anti-CD22 specific CAR-expressing plasmids were also produced (Fig. 1D and E) in same lentiviral vector.

Selection of anti-CD19/CD22 bispecific CAR-NK-92 cells in vitro. CAR-expressing lentivirus particles were used to infect NK-92 cells twice. After 48 h, CAR-expressing NK-92 cells were sorted using flow cytometry (Figs. 3A and S2). The CAR-NK-92 cells were cultured to obtain sufficient cell numbers, and CAR expression was confirmed before the experiments (Fig. S2). Following co-incubation with CFSE-labeled OCI-Ly7 cells, a B cell lymphoma cell line used as target cells, the death of target cells was assessed
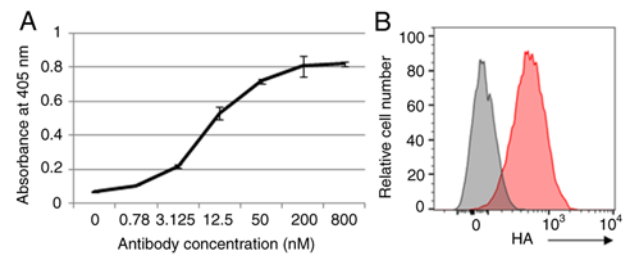

Figure 2. Binding assay of anti-CD22 antibody. Recombinant anti-CD22 scFv-Cκ-HA fusion protein was subjected to (A) ELISA and (B) flow cytometry. Grey, secondary antibody only; red, anti-CD22 scFv-Cκ-HA fusion protein tested. Enzyme immunoassay was performed in triplicate and data are presented as the mean ± SD. scFv, single chain variable fragment.

by calculating the percentages of CFSE+ cells using Annexin V/7-AAD staining (Figs. S3 and 3D). OCI-Ly7 cells expressed high levels of CD19 and CD22 (Fig. S4). The number of late apoptotic cells that were AV+7-AAD+ increased following 4-h co-incubation with anti-CD19/CD22 bispecific CAR-NK-92 cells, whereas the number of early apoptotic cells that were AV+7-AAD- did not increase due to the effect of CAR-NK-92 cells (Fig. 3B). Counting of CFSE+ target cells revealed that CAR-NK-72 cells exhibited higher cytotoxicity than NK-92 cells (Fig. 3C). Anti-CD19 single CAR, anti-CD22 (CI4-47)/CD19 (FMC63) bispecific CAR and anti-CD19 (FMC63)/CD22 (2-3-14) bispecific CAR significantly decreased target cell numbers compared with the effects of anti-CD19 specific CAR. The anti-CD19/CD22 (FMC62-CI4-47) and loop-structured anti-CD19/CD22 (LOOPCAR-FMC63-CI4-47) bispecific CARs decreased target cell numbers, but to a lesser extent. The NK-92 cells infected with naked lentiviral vector control did not change the viability of target cells compared with NK-92 cells
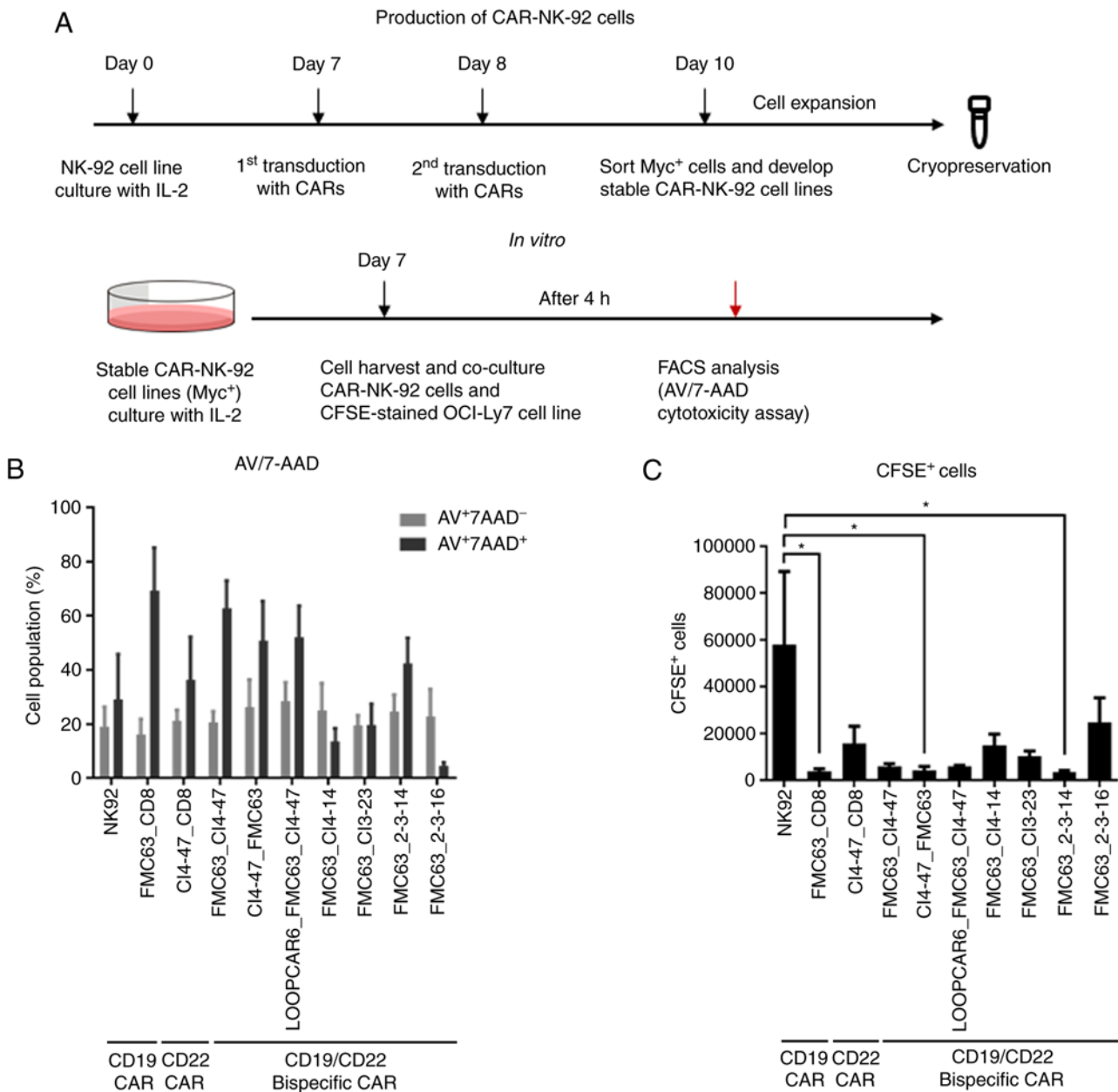
Figure 3. Continued.

without viral vector (Fig. S5). Therefore, five CAR structures were selected for further *in vivo* investigation.

Efficacy of anti-CD22/CD19 and loop-structured anti-CD19/CD22 CAR-NK-92 cells in vivo. To evaluate whether CAR-NK-92 cells induced cell death in B cell lymphoma cells *in vivo*, NSG immune-deficient mice were inoculated with CFSE-labeled OCI-Ly7 cells and with CAR-NK-92 cells (Fig. 4A). Among the CAR structures tested, anti-CD22/CD19 (CI4-47-FMC63) and loop-structured anti-CD19/CD22 (LOOPCAR-FMC63-CI4-47) bispecific CAR-NK-92 cells significantly eliminated target cells compared with anti-CD19 specific CAR-NK-92 cells after 48 h (Fig. 4B). The results suggested that anti-CD19/CD22 bispecific CARs were more cytotoxic than anti-CD19 CAR against B cell lymphoma cells *in vivo*. Additionally, the structures of bispecific CAR may modify the efficacy in NK-92 cells.

Discussion

Anti-CD19/CD22 bispecific CARs were developed and the efficacy of anti-CD19/CD22 CAR-NK-92 cells *in vitro* and *in vivo* was demonstrated. To the best of our knowledge, the present study is the first report of anti-CD19/CD22 bispecific CAR-NK-92 cells from a novel clone of chicken anti-CD22 Ab. The two distinct backbone structures of the bispecific CARs showed significant efficacy in a xenograft model of B cell lymphoma. Notably, anti-CD22/CD19 (CI4-47-FMC63) and loop-structured anti-CD19/CD22 (LOOPCAR-FMC63-CI4-47) bispecific CAR-NK-92 cells showed significantly higher cytotoxicity in mice after 48 h compared with anti-CD19 CAR-NK-92 cells. The CAR structures evaluated in the present study can be introduced into stem cell-derived CAR-NK cells. Notably, the two structures appeared to function more efficiently *in vivo* than *in vitro*;
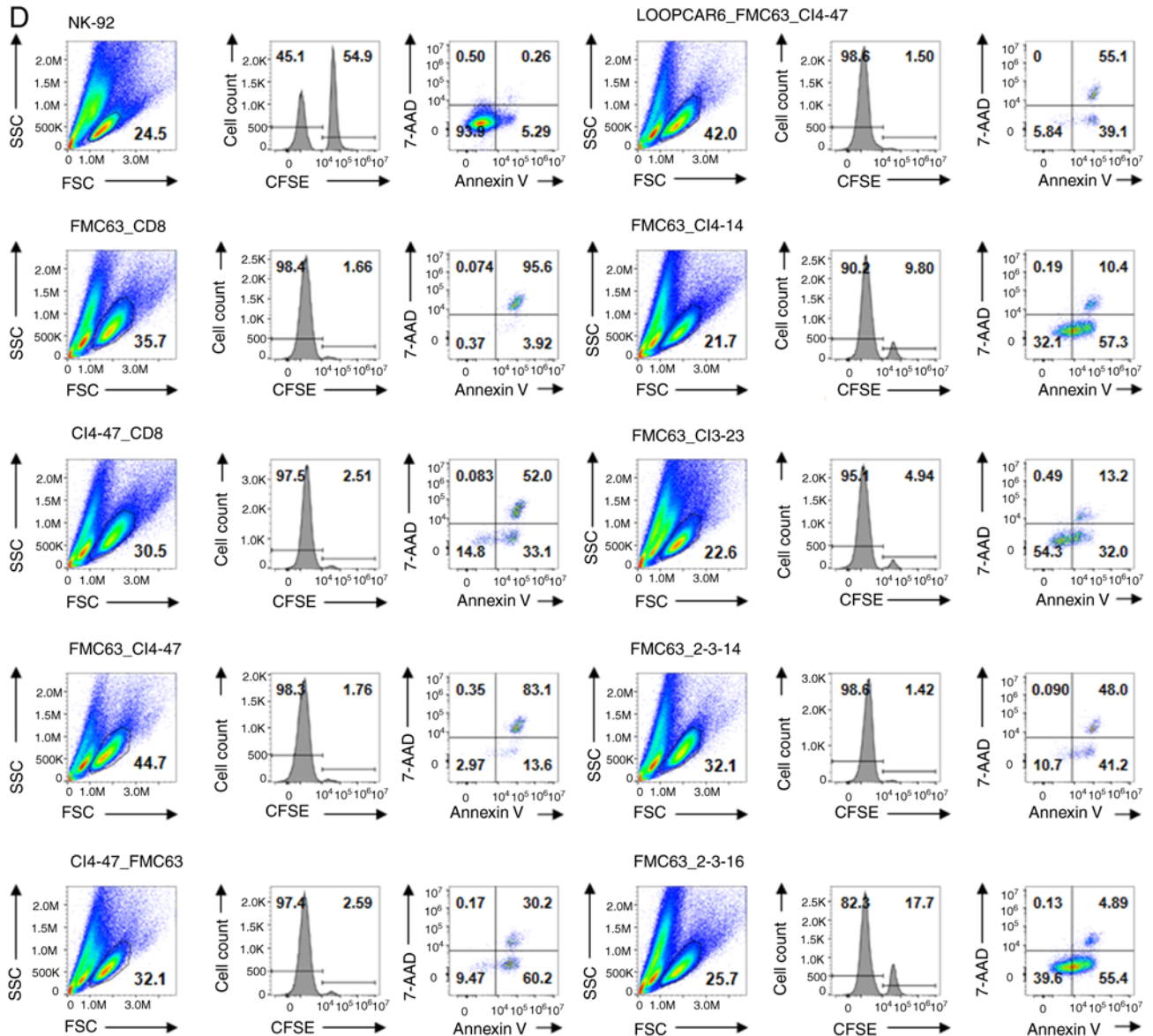
Figure 3. *In vitro* evaluation of anti-CD19/CD22 bispecific CAR-NK-92 cells. (A) *In vitro* experimental timeline. CAR-NK-92 cells were transduced with lentiviral vectors expressing CAR structures twice and Myc+ cells were sorted after 48 h. CAR-NK-92 cells were cryopreserved until use. For *in vitro* evaluation of anti-CD19/CD22 bispecific CAR-NK-92 cells, CAR-NK-92 cells were co-cultured with CFSE-labeled OCI-Ly7 cells for 4 h and measured for AV/7-AAD by flow cytometry. (B) Summary of AV+ 7-AAD- early and AV+ 7-AAD+ late apoptotic CFSE+ target cells. (C) Numbers of CFSE+ target cells (mean ± SEM; n=4). (D) Representative flow cytometry of AV/7-AAD cell death assay. *P<0.05. CAR, chimeric antigen receptor; NK, natural killer; CFSE, carboxyfluorescein diacetate succinimidyl ester; 7-AAD, 7-amino-actinomycin D; AV, Annexin V.

inconsistent results between *in vitro* and *in vivo* experiments may be associated with different experimental times (4 vs. 48 h) and the tumor environment *in vivo*.

To optimize the design of CARs, modules of CAR structures should be carefully considered, including the ligand-binding, spacer, transmembrane and cytoplastic domains (33). In addition to the affinity and avidity of antigen binding sites, structural factors such as antigen epitope proximity and accessibility, scFv aggregation, length of spacer domain and epitope proximity to the membrane affect the efficacy of CAR-T cells (33). The protein structures were varied, including the order of scFv sequences, to optimize the stability and efficacy of the molecules. Although the present study did not have the means to predict the actual tertiary structures of the CAR and antigens, it is hypothesized that

the order of the two antigen binding sites may determine the interaction and proximity between target cells and NK cells. CI4-47-FMC63 and LOOPCAR6-FMC63-CI4-47 may interact with the membrane proximal epitope of CD22 via CI4-47. Furthermore, changes in variable region heavy chain (VH)/variable region light chain (VL) combination, scFv order, length and flexibility/rigidity of the linkers and extracellular spacer length in the structures of bispecific CARs modify the expression and the activity of the CARs (34). The linker domain in LOOPCAR6-FMC63-CI4-47 is shorter than that of FMC63-CI4-47. This difference in length may facilitate optimal space between target cells and NK cells, allowing the formation of immune synapses. In a previous study, loop-structured, bi-specific CD19/CD22 CAR-T cells were developed and evaluated in patient-derived mouse xenograft
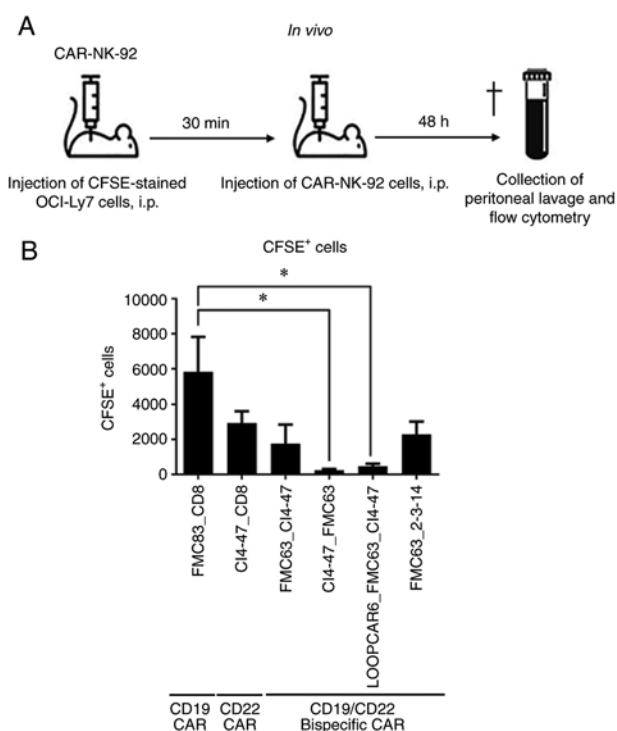
Figure 4. *In vivo* evaluation of anti-CD19/CD22 bispecific CAR-NK-92 cells. (A) *In vivo* experimental timeline. CFSE-labeled OCI-Ly7 cells were injected i.p. into NSG mice and after 30 min, CAR-NK-92 cells were injected i.p. After 48 h, peritoneal lavage was harvested and flow cytometry was performed. (B) Summary of numbers of CFSE+ target cells (mean ± SEM; n=4-7). *P<0.05. CAR, chimeric antigen receptor; NK, natural killer; CFSE, carboxyfluorescein diacetate succinimidyl ester; i.p., intraperitoneal.

models (35). Although underlying mechanisms of how structural changes in CARs improve their efficacy in NK-92 cells remains unclear, the present results suggested that optimizing CAR structures in NK and T cells may lead to improved clinical outcomes.

The anti-CD22 mAb clones used in the present study were produced in chickens. Due to the phylogenetic and evolutionary divergence of avians from mammals, raising Abs against mammalian antigens with better chances due to less homology (36). Furthermore, the use of chicken VH and VL genes simplifies mining of rearranged genes and preserves library diversity by avoiding the loss of rare transcripts during PCR amplification. Chicken scFv is flexible in structure and tolerate a range of mutations without compromising binding activity and solubility (37). Therefore, chicken scFv was used in the present study. ELISA and flow cytometry suggested that strong binding affinity may not guarantee improved efficacy to target cells as CAR, as the binding affinity of CI4-47 was not the best among the clones tested. However, the anti-CD19/CD22 bispecific CAR with CI4-14 showed improved efficacy *in vivo* compared with the 2-3-14 clone. The present results suggested the importance of optimizing CAR structures in addition to binding affinity.

NK-92 cells, although immortalized, have been shown to be safe in phase I clinical trials (13-15) and irradiation of NK-92 cells can further improve their safety (18-20). These results support the use of CAR-NK-92 cells as off-the-shelf therapeutics (38). However, NK cell activity is decreased in patients with MM, particularly in advanced stages, and patients with aggressive extramedullary cutaneous plasmacytoma (39,40). Furthermore, patients with MM who exhibit higher NK cell activity tend to show improved survival (40). As irradiated anti-CD33 CAR-NK-92 cells are safe for use in humans, infusion of NK-92 cells may be beneficial for patients with MM. In one study, the serum levels of IL-6 and IL-10 in one patient increased 5-6 days post-infusion, but returned to normal within a further 48 h (20). The other two patients did not show increased cytokine levels. However, IL-6 and IL-10 suppress NK cell activity (41), so it would be beneficial to control inflammatory cytokines to improve NK cell activity. Although cytokine production was not measured in mice injected with CAR-NK-92 cells, no mice showed any visible signs of sickness or weakness during the experiments. Hematopoietic and induced pluripotent stem cell-derived CAR-NK cells can be used in the clinic, instead of NK-92 cells (42). In phase I/II clinical trials, human leukocyte antigen-mismatched anti-CD19 CAR-NK cells are effective in treating patients with relapsed or refractory CD19+ lymphoma, with a 73% response rate (12,43). The infused CAR-NK cells proliferate and persist at low levels for at least 12 months, without increases in inflammatory cytokine levels. NK cells are unlikely to cause cytokine release syndrome and GVHD due to their relatively short lifespan and lack of MHC restriction, respectively. Irradiated NK-92 cells are safe, have a short lifespan, and are cost-effective, making repeated administration feasible in the clinic. The *in vivo* experimental model in the present study was a tumor rejection assay in the peritoneal cavity, rather than orthotropic xenograft model. This *in vivo* model was chosen because blood-origin tumors, which are often circulating or metastatic tumors, as well as residual tumor cells, are more likely to be targeted by CAR-NK-92 cells than primary solid tumors in the clinic. In addition, CAR expression remained stable after cells were cultured, frozen, and thawed.

In conclusion, the present results suggest that a novel CD19/CD22 bispecific CAR, originating from a novel antibody clone from chickens, was stably expressed in NK-92 cells and was effective in eliminating a B cell lymphoma cell line *in vitro* and *in vivo*. The present results supported the potential use of anti-CD19/CD22 bispecific CAR-NK-92 cells for the treatment of B cell leukemia/lymphoma as well as the need for further experiments to improve CAR structures.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HyoK, HyeK, HJI, KNK and NK designed the research and wrote the manuscript. HyoK, MH, MK and NK performed the research and analyzed the results. HyeK, HJI and KNK provided samples. KNK obtained the grant. HyoK and NK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Asan Medical Center, Seoul, Korea (approval no. 2022-12-090).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, *et al*: Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368: 1509-1518, 2013.
2. Porter DL, Levine BL, Kalos M, Bagg A and June CH: Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365: 725-733, 2011.
3. Ying Z, Huang XF, Xiang X, Liu Y, Kang X, Song Y, Guo X, Liu H, Ding N, Zhang T, *et al*: A safe and potent anti-CD19 CAR T cell therapy. Nat Med 25: 947-953, 2019.
4. Wang N, Hu X, Cao W, Li C, Xiao Y, Cao Y, Gu C, Zhang S, Chen L, Cheng J, *et al*: Efficacy and safety of CAR19/22 T-cell cocktail therapy in patients with refractory/relapsed B-cell malignancies. Blood 135: 17-27, 2020.
5. Bouchkouj N, Kasamon YL, de Claro RA, George B, Lin X, Lee S, Blumenthal GM, Bryan W, McKee AE and Pazdur R: FDA approval summary: Axicabtagene ciloleucel for relapsed or refractory large B-cell lymphoma. Clin Cancer Res 25: 1702-1708, 2019.
6. Melenhorst JJ, Chen GM, Wang M, Porter DL, Chen C, Collins MA, Gao P, Bandyopadhyay S, Sun H, Zhao Z, *et al*: Decade-long leukaemia remissions with persistence of CD4+ CAR T cells. Nature 602: 503-509, 2022.
7. Plaks V, Rossi JM, Chou J, Wang L, Poddar S, Han G, Wang Z, Kuang SQ, Chu F, Davis RE, *et al*: CD19 target evasion as a mechanism of relapse in large B-cell lymphoma treated with axicabtagene ciloleucel. Blood 138: 1081-1085, 2021.
8. Wherry EJ: T cell exhaustion. Nat Immunol 12: 492-499, 2011.
9. Fajgenbaum DC and June CH: Cytokine storm. N Engl J Med 383: 2255-2273, 2020.
10. Jurisic V: Multiomic analysis of cytokines in immuno-oncology. Expert Rev Proteomics 17: 663-674, 2020.
11. Kim N, Lee DH, Choi WS, Yi E, Kim H, Kim JM, Jin HS and Kim HS: Harnessing NK cells for cancer immunotherapy: Immune checkpoint receptors and chimeric antigen receptors. BMB Rep 54: 44-58, 2021.
12. Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, Nassif Kerbauy L, Overman B, Thall P, Kaplan M, *et al*: Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. N Engl J Med 382: 545-553, 2020.
13. Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J and Klingemann H: Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: A phase I trial. Cytotherapy 10: 625-632, 2008.
14. Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, Suttorp M, Seifried E, Ottmann OG and Bug G: Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy 15: 1563-1570, 2013.
15. Williams BA, Law AD, Routy B, denHollander N, Gupta V, Wang XH, Chaboureau A, Viswanathan S and Keating A: A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. Oncotarget 8: 89256-89268, 2017.
16. Grund EM and Muise-Helmericks RC: Cost efficient and effective gene transfer into the human natural killer cell line, NK92. J Immunol Methods 296: 31-36, 2005.
17. Lu H, Zhao X, Li Z, Hu Y and Wang H: From CAR-T cells to CAR-NK cells: A developing immunotherapy method for hematological malignancies. Front Oncol 11: 720501, 2021.
18. Klingemann HG, Wong E and Maki G: A cytotoxic NK-cell line (NK-92) for ex vivo purging of leukemia from blood. Biol Blood Marrow Transplant 2: 68-75, 1996.
19. Fabian KP and Hodge JW: The emerging role of off-the-shelf engineered natural killer cells in targeted cancer immunotherapy. Mol Ther Oncolytics 23: 266-276, 2021.
20. Tang X, Yang L, Li Z, Nalin AP, Dai H, Xu T, Yin J, You F, Zhu M, Shen W, *et al*: First-in-man clinical trial of CAR NK-92 cells: Safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. Am J Cancer Res 8: 1083-1089, 2018.
21. Lanza F, Maffini E, Rondoni M, Massari E, Faini AC and Malavasi F: CD22 expression in B-cell acute lymphoblastic leukemia: Biological significance and implications for inotuzumab therapy in adults. Cancers (Basel) 12: 303, 2020.
22. Haso W, Lee DW, Shah NN, Stetler-Stevenson M, Yuan CM, Pastan IH, Dimitrov DS, Morgan RA, FitzGerald DJ, Barrett DM, *et al*: Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. Blood 121: 1165-1174, 2013.
23. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, Wolters P, Martin S, Delbrook C, Yates B, *et al*: CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat Med 24: 20-28, 2018.
24. Spiegel JY, Patel S, Muffly L, Hossain NM, Oak J, Baird JH, Frank MJ, Shiraz P, Sahaf B, Craig J, *et al*: CAR T cells with dual targeting of CD19 and CD22 in adult patients with recurrent or refractory B cell malignancies: A phase 1 trial. Nat Med 27: 1419-1431, 2021.
25. Cordoba S, Onuoha S, Thomas S, Pignataro DS, Hough R, Ghorashian S, Vora A, Bonney D, Veys P, Rao K, *et al*: CAR T cells with dual targeting of CD19 and CD22 in pediatric and young adult patients with relapsed or refractory B cell acute lymphoblastic leukemia: A phase 1 trial. Nat Med 27: 1797-1805, 2021.
26. Andris-Widhopf J, Rader C, Steinberger P, Fuller R and Barbas CF III: Methods for the generation of chicken monoclonal antibody fragments by phage display. J Immunol Methods 242: 159-181, 2000.
27. Rader C and Barbas CF III: Phage display of combinatorial antibody libraries. Curr Opin Biotechnol 8: 503-508, 1997.
28. Lee Y, Kim H and Chung J: An antibody reactive to the Gly63-Lys68 epitope of NT-proBNP exhibits O-glycosylation-independent binding. Exp Mol Med 46: e114, 2014.
29. Niwa H, Yamamura K and Miyazaki J: Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108: 193-199, 1991.
30. Jurisic V, Srdic-Rajic T, Konjevic G, Bogdanovic G and Colic M: TNF-α induced apoptosis is accompanied with rapid CD30 and slower CD45 shedding from K-562 cells. J Membr Biol 239: 115-122, 2011.
31. Sambrook J, Fritsche, EF and Maniatis T: Molecular cloning: A laboratory manual. Vol. 3. Cold Spring Harbor Laboratory Press, 1989.
32. Saudemont A, Garçon F, Yadi H, Roche-Molina M, Kim N, Segonds-Pichon A, Martín-Fontecha A, Okkenhaug K and Colucci F: p110gamma and p110delta isoforms of phosphoinositide 3-kinase differentially regulate natural killer cell migration in health and disease. Proc Natl Acad Sci USA 106: 5795-5800, 2009.
33. https://www.law.go.kr.
34. Jayaraman J, Mellody MP, Hou AJ, Desai RP, Fung AW, Pham AHT, Chen YY and Zhao W: CAR-T design: Elements and their synergistic function. EBioMedicine 58: 102931, 2020.
35. Zanetti SR, Velasco-Hernandez T, Gutierrez-Agüera F, Díaz VM, Romecín PA, Roca-Ho H, Sánchez-Martínez D, Tirado N, Baroni ML, Petazzi P, *et al*: A novel and efficient tandem CD19- and CD22-directed CAR for B cell ALL. Mol Ther 30: 550-563, 2022.

36. Qin H, Ramakrishna S, Nguyen S, Fountaine TJ, Ponduri A, Stetler-Stevenson M, Yuan CM, Haso W, Shern JF, Shah NN and Fry TJ: Preclinical development of bivalent chimeric antigen receptors targeting both CD19 and CD22. Mol Ther Oncolytics 11: 127-137, 2018.

37. Lee W, Syed Atif A, Tan SC and Leow CH: Insights into the chicken IgY with emphasis on the generation and applications of chicken recombinant monoclonal antibodies. J Immunol Methods 447: 71-85, 2017.

38. Yoon A, Shin JW, Kim S, Kim H and Chung J: Chicken scFvs with an artificial cysteine for site-directed conjugation. PLoS One 11: e0146907, 2016.

39. Zhang C, Oberoi P, Oelsner S, Waldmann A, Lindner A, Tonn T and Wels WS: Chimeric antigen receptor-engineered NK-92 cells: An off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity. Front Immunol 8: 533, 2017.

40. Jurisic V, Colovic N, Konjevic G, Minic I and Colovic M: An aggressive extramedullary cutaneous plasmacytoma associated with extreme alterations in the innate immune system. Onkologie 33: 113-115, 2010.

41. Jurisic V, Srdic T, Konjevic G, Markovic O and Colovic M: Clinical stage-depending decrease of NK cell activity in multiple myeloma patients. Med Oncol 24: 312-317, 2007.

42. Konjević GM, Vuletić AM, Mirjačić Martinović KM, Larsen AK and Jurišić VB: The role of cytokines in the regulation of NK cells in the tumor environment. Cytokine 117: 30-40, 2019.

43. Arias J, Yu J, Varshney M, Inzunza J and Nalvarte I: Hematopoietic stem cell- and induced pluripotent stem cell-derived CAR-NK cells as reliable cell-based therapy solutions. Stem Cells Transl Med 10: 987-995, 2021.

44. Gambella M, Carlomagno S, Raiola AM, Giannoni L, Ghiggi C, Setti C, Giordano C, Luchetti S, Serio A, Bo A, et al: CD19-targeted immunotherapies for diffuse large B-cell lymphoma. Front Immunol 13: 837457, 2022.