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# Harnessing the Power of CAR-NK Cells: A Promising Off-the-Shelf Therapeutic Strategy for CD38-Positive Malignancies

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## ABSTRACT

**Background:** CD38 is highly expressed on multiple myeloma (MM) cells and has been successfully targeted by different target therapy methods. This molecule is a critical prognostic marker in both diffuse large B-cell lymphoma and chronic lymphocytic leukemia.

**Objective:** We have designed and generated an anti-CD38 CAR-NK cell applying NK 92 cell line. The approach has potential application as an off-the-shelf strategy for treatment of CD38 positive malignancies.

**Methods:** A second generation of anti-CD38 CAR-NK cell was designed and generated, and their efficacy against CD38-positive cell lines was assessed *in vitro*. The PE-Annexin V and 7-AAD methods were used to determine the percentage of apoptotic target cells. Flow cytometry was used to measure IFN-γ, Perforin, and Granzyme-B production following intracellular staining. Using *in silico* analyses, the binding capacity and interaction interface were evaluated.

**Results:** Using Lentivirus, cells were transduced with anti-CD38 construct and were expanded. The expression of anti-CD38 CAR on the surface of NK 92 cells was approximately 25%. As we expected from *in silico* analysis, our designed CD38-chimeric antigen receptor was bound appropriately to the CD38 protein. NK 92 cells that transduced with the CD38 chimeric antigen receptor, generated significantly more IFN-γ, perforin, and granzyme than Mock cells, and successfully lysed Daudi and Jurkat malignant cells in a CD38-dependent manner.

**Conclusion:** The *in vitro* findings indicated that the anti-CD38 CAR-NK cells have the potential to be used as an off-the-shelf therapeutic strategy against CD38-positive malignancies. It is recommended that the present engineered NK cells undergo additional preclinical investigations before they can be considered for subsequent clinical trial studies.

**Keywords:** CD38, CAR NK cell, Malignancy, Multiple Myeloma

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## INTRODUCTION

Cancer is a leading cause of death worldwide, demanding the development of more efficient therapeutic approaches. Multiple myeloma (MM), the well known plasma cell malignancy, is the second most frequent hematological cancer (1). The patients with MM still have a poor prognosis, despite high doses of chemotherapy and stem cell transplantation (2). Cancer immunotherapy has emerged as a revolutionary technique for treating various malignancies via the immune system's empowerment to target and eradicate cancer cells (3). Daratumumab, is a highly effective monoclonal antibody-based medication used to treat MM. In relapsed/refractory (R/R) patients, daratumumab monotherapy caused only a low remission rate (29.2%). Consequently, the majority of the patients with multiple myeloma still face the challenge of an incurable condition (4).

Immunotherapy, such as chimeric antigen receptor (CAR) T cells, indicated promising results in the treatment of B-cell malignancies (5). However, several challenges, such as the high cost and complexity of the manufacturing process, cytokine release syndrome (CRS), neurotoxicity, and graft-versus-host disease side effects, have prompted the investigation of alternative cell-based immunotherapies (6, 7). CARs are engineered receptors that are expressed on the surface of the immune cells to enhance T or NK cell activity against a specific antigen. The receptors are chimeric in both the signaling domain and antigen-binding parts. A CAR construct is composed of three fundamental components. First, the single chain variable fragment of the antibody (scFv) contains an extracellular ligand binding domain. This region detects specific target antigens. The second section of a CAR construct is a transmembrane domain and a hinge/extracellular spacer. This area connects the other two CAR components and increases the overall flexibility, stability, and dimerization of the construct. The third section consists of a cytoplasmic signaling domain and costimulatory molecules. This section is in charge of signal transmission, and it is where the majority of CAR construct modifications are made to increase a particular CAR's signal transmission capability (8). A chimeric antigen receptor is depicted in Fig. 1.

Therefore, the wise selection of extracellular sequences such as scFv and hinge, using *in silico* analysis, to ensure optimal structural performance leads to cost savings from laboratory tests and optimal resource management.

Natural killer (NK) cells, with their innate cytotoxicity and potential for allogeneic use, offer several advantages over T cells as a cellular platform for immunotherapy (9). NK cells can mediate the direct killing of tumor cells without needing prior sensitization or major histocompatibility complex (MHC) matching (10). Additionally, CAR-NK cells can traffic to solid tumor sites and exhibit greater persistence than CAR-T cells (11).

Although recently approved anti-B cell maturation antigen (BCMA) CAR-T is highly efficient in MM treatments (12), targeting CD38 will not allow MM cells to escape due to its high, stable, and homogenous expression, unlike CAR targeting BCMA (13). CD38, a transmembrane glycoprotein, is frequently expressed in a variety of malignancies, including MM and some B-cell lymphomas (14). Several investigations revealed that CD38 is an important prognostic marker in diffuse large B-cell lymphoma and chronic lymphocytic leukemia (15, 16). The CD38 expression on solid tumors has recently received much attention. It might be related to immune evasion and progression in solid tumors (17), and its overexpression has been related to poor survival of lung cancer patients (18). Since a transient increase in CD38 expression was observed in checkpoint inhibitor-treated cells, such as PD-1/PD-L1 blocking antibodies, it has been proposed that CD38 is a checkpoint inhibitor (19). Due to the importance of CD38 expression in the proliferation, migration, prognosis,

and tumorigenic properties of the tumor microenvironment (20, 21), this marker has been widely used as a therapeutic target in clinical trials to treat hematological and solid tumors, particularly MM (21).

Researchers evaluated the effectiveness of anti-CD38 CAR in KHYG-1 cells against CD38-positive MM cell lines (22). To overcome self-killing caused by the NK cell CD38 expression, they used CRISPR/Cas9 genome editing tools to destroy the NK cell CD38 gene (23). The number of studies investigating the anti-MM effects of various types of anti-CD38 CAR-NK cells has recently increased (22, 24).

In this study, we designed and generated anti-CD38 CAR-NK cells using the NK 92 cell line as an off-the-shelf approach that could be used for a wide range of patients. Before evaluating our CAR-NK cells *in vitro*, we generated a virtual model of our construct and checked its attachment using *in silico* analysis. By engineering NK cells to express the anti-CD38 CAR for CD38 targeting, we sought to enhance their targeting and cytotoxic potential against CD38-expressing cancer cells (25).

## MATERIAL AND METHODS

*CAR Construct Designing*

A second-generation CAR construct encoding a leader sequence, anti-CD38 single chain variable fragment (scFv) of daratumumab antibody, CD8α hinge, a transmembrane region, and 4-1BB-CD3 zeta signaling domains were assembled. Green fluorescent protein (GFP) was applied as a marker gene. Two enzymatic restriction sites were available on both sides of the GFP sequences, which allows them to be removed for subsequent clinical trial studies. According to binding energies and conformations, the sequences of the anti-CD38 scFv were selected after an *in silico* analysis of different combinations of daratumumab with juxtapositioning of light and heavy variable chains (Drug Bank) separated by a different length of G4S linker. The remaining sequences were obtained from NCBI and EMBL-EBI databases. The construct was synthesized after gene optimization (GeneScript: https://www.genscript.com). It was ordered in a pCDH vector (Biomatik, Canada). The construct was confirmed by sequencing (Biomatik, Canada). The empty vector was used as a control (Mock).

*In silico Analysis*

We conducted an *in silico* assessment, to evaluate and select the optimal scFv chain for CAR construct designing. The cell surface proteins scFv and hinge were modeled, and the final model was subsequently docked against the CD38 chain obtained from the RCSB server.

*Sequence Retrieval and 3D Structure Modeling*

All sequences were retrieved from the Gene Bank in the FASTA format. The three-dimensional (3D) structure of CD38 was obtained from PDB 1zvm chain A, and the 3D structure of the anti-CD38 scFv protein was modeled by the Swiss model server (https://swissmodel.expasy.org) using homology modeling. Besides, the hinge was modeled by Quark (https://zhanglab.ccmb.med.umich.edu/C-QUARK), the server for ab initio structural prediction. It is worth mentioning that we initially evaluated all possible daratumumab scFv models, which included juxtaposing light and heavy variable chains with multiple linkers, and finally docked with CD38 and selected the best model based on the lowest energy level. Models were refined structurally using Mod Refiner (https://zhanglab.ccmb.med.umich.edu/ModRefiner/). The stereochemical quality of the scFv model was evaluated using the PROCHECK server. In addition to PROCHECK software, other model evaluation algorithms such as RAMPAGE, verify 3D, ERRAT, RMSD, and TM-score, (Fig. S1) were applied. The hinge region and scFv structures were assembled by the AIDA server (http://ffas.sanfordburnham.

org/AIDA/). It was refined once more. The 3D structures of proteins were displayed using Chimera 1.10.2 software and modeler, version 9.11.

*Docking of the Hinge-scFv Fusion Protein against CD38 Receptor*

To evaluate the hinge-scFv fusion protein and CD38 interactions docking against CD38 protein was done using the ZDOCK server (http://zdock.umassmed.edu). The best docking result was selected according to the lowest energy level. Visualization was performed using Pymol software. The PIC server (http://pic.mbu.iisc.ernet.in/cgi/submit_job.cgi) was used to analyze the antigen-antibody interactions.

*Cell Culture*

The cell lines Daudi (Human Burkitt Lymphoma), Jurkat (Human Acute T Cell Leukemia), K562 (Human Chronic Myelogenous Leukemia) were cultured in RPMI 1640 medium (BI1031-500, Biosera, France) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% PenStrep (Penicillin, 10,000 U/mL; Streptomycin, 10,000 µg/mL). Hek293T (Human Embryonic Kidney cell line) was cultured in DMEM high glucose (Gibco, USA) containing 10% fetal bovine serum and 1% PenStrep and NK 92 cell line (Human non-Hodgkin's Lymphoma Natural Killer Cells, a kind gift from prof. Bruce Walcheck, University of Minnesota, USA) was cultured in α-MEM containing 12.5% fetal bovine serum and 12.5% horse serum (Gibco, USA). The NK 92 cells were supplemented with interleukin IL-2 at a final culture concentration of 200 International Units per mL.

*Retroviral Transduction*

The envelope, gag-pol packaging vectors, and anti-CD38 CAR constructs were transfected into HEK293T cells by the calcium phosphate method. After 16 hours, the entire medium (DMEM+10% FBS) was refreshed. Viral supernatant was harvested 24, 48, and 72 hours after transfection and concentrated with ultracentrifuge at 100,000 rpm for one hour before being used for transduction. The viral transduction of the NK 92 cell was facilitated with three µg/mL Polybrene (Sigma, Germany). The transduction was repeated after 18 hours. The cells' supernatant was replaced eight hours after the second transduction. Two days later, transduction success was visualized using a fluorescent microscope, and CAR expression was confirmed using flow cytometry using CD38-His tag recombinant protein conjugated to PE (SinoBiological, China). The transduced cells were sorted and expanded.

*Assessment of Anti-CD38-CAR-NK Cell Phenotype*

Expanded NK cells were collected and rinsed with phosphate-buffered saline (PBS) to prepare them for flow cytometry analysis. To identify transduction efficiency and anti-CD38 CAR-NK expression, flow cytometry was used to gate the population of NK cells expressing GFP. The gating strategy involved setting a gate on the GFP channel to include cells with GFP fluorescence above background levels, which indicated successful transduction of the anti-CD38 CAR. Then, the expression of CAR protein in the cell surfaces was measured using PE-conjugated recombinant human CD38-His tag protein, (NP_001766.2), extracellular domain, Val 43-Ile 300, (SinoBiological, 10818-H08H-P), according to the manufacturer's

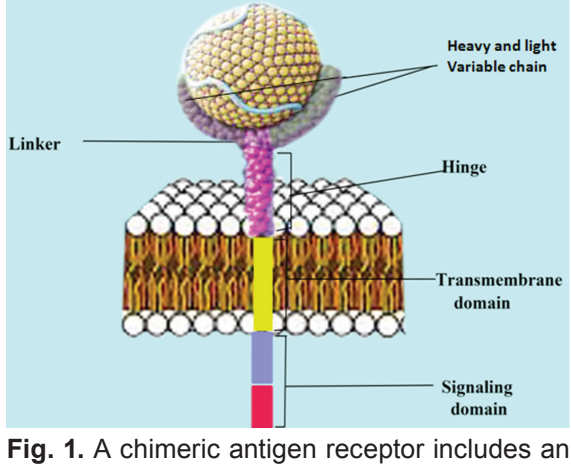


**Fig. 1.** A chimeric antigen receptor includes an extracellular target binding domain (scFv), a hinge domain, a transmembrane domain, and one or more intracellular signaling domains

manual. To further characterize the phenotype of anti-CD38 CAR-NK cells, cell surface staining was carried out for NK cell markers, FITC-conjugated anti-human CD16 (clone: B73.1), phycoerythrin (PE)-conjugated anti-human CD56 (5.1H11), Allophycocyanin (APC)-conjugated anti-human CD3 (all from Biolegend, USA). CAR-NK cells were incubated with the antibody panel according to the manufacturer's instructions. The proper antibody concentrations were determined based on titration experiments. An isotype control antibody was used to set the gating strategy and establish the background staining levels. CAR-NK cells were differentiated from other cell types using gating strategies based on their distinct GFP expression and marker expression patterns.

*Cytotoxicity Assay*

To evaluate the functional activity of anti-CD38 CAR-NK cells and their ability to induce programmed cell death in CD38-expressing target cells, flow cytometry analysis was used to assess the proportion of apoptotic cells within the target cells. Daudi and Jurkat cell lines, which were positive for CD38 molecules, were considered positive controls. As a negative control, the K-562 cell line was selected. For functional analysis, anti-CD38 CAR-NK cells and empty vector-transduced (Mock) NK 92 cells were co-cultured ($0.5\times10^6$ cells/well) in 24-well plates for 18-20 hours with target cell lines at an Effector: Target cell ratio (E: T) 1:1, 1:3 and 3:1. After the incubation period, the cells were harvested for further analysis. A fluorescent apoptosis detection kit (Biolegend, USA) was used for staining the cells. The anti-CD38 CAR-NK cells were gated based on their distinct marker expression, allowing them to be distinguished from the target cells. The target cells (Daudi, Jurkat, and K-562) were selected as positive or negative controls based on CD38 expression.

*IFN-γ, Perforin and Granzyme-B Production Assay*

For the IFN-γ, perforin, and granzyme B production assay, target cells were seeded. After that, anti-CD38 CAR-NK cells and Mock were added to the wells in a 1:1 E:T ratio. Following the co-culture period, the cells were collected from the wells and centrifuged (1800 rpm, 5 min) to pellet them. For intracellular staining (ICS), using an appropriate fixative/permeabilization buffer, the pelleted cells were permeabilized and fixed (Cytofix/Cytoperm, BD Biosciences) according to the manufacturer's instructions.

The cells were treated with fluorescently labeled antibodies specific for the markers (PerCP Cy5.5-Perforin, PerCP Cy5.5-IFN-γ [both Biolegend, USA], Alexa flour 647-Granzym B [BD, USA]). The cells were rinsed after staining to remove any remaining unbound antibodies. The stained cells were then analyzed using a four-color FACS Calibur flow cytometer (BD Biosciences, USA).

*Flow Cytometry Data Analysis*

The obtained data from the functional assays were analyzed using appropriate flow cytometry analysis software (CellQuest Pro, BD Biosciences).

*Statistical Analysis*

Statistical analyses were carried out using the GraphPad Prism software package (version 8) and SPSS software (version 20). The two-tailed student's t-tests were used to compare the anti-CD38 CAR-NK cells with those of the Mock-NK 92 cell. P-values less than 0.05 were considered statistically significant.

## RESULTS

*In Silico Analysis*

*Sequence Retrieval and Analysis*

The daratumumab sequence was retrieved from the Drug Bank. All possible models of daratumumab scFv were considered and evaluated, which included juxtaposing light and heavy variable chains with multiple


**Fig. 2.** The sequences of scFv and scFvs from daratumumab deposited in the RCSB server

linkers. Finally, the best model (VH sequence is EVQLLESGGG (102aa) GQGTLVTVSS, and VL sequence is EIVLTQSPAT (87aa) FGQGTKVEIK) was applied for further evaluation (Fig. 2).

*Homology Modelling of the Structures*

The most homologous proteins of the scFv fusion protein were obtained and supplied as a template to the SWISS-MODEL server. The scFv was modeled based on 5wym.1A protein as a template with 82% sequence identity and 95% coverage. The GMQE and QMEAN were 0.81 and 0.30, respectively. Based on QUARK ab initio approaches, the hinge domain was modeled. AIDA server put together the hinge region and scFv structures. The CAR construct, modeled scFv and hinge are illustrated in Fig. 3. PROCHECK's Ramachandran plot assessment of simulated structures revealed that 88.2% and 89.5% of residues were in favorable regions before and after refinement, respectively. The average 3D-1D score for the assembled modeled, hinge-scFv, proteins was greater than 0.2.

*Docking of the Hinge-scFv with CD38 and Interaction Interface Analysis*

The best hinge-scFv fusion protein and CD38 docking result from the ZDOCK server based on the interaction binds and lowest energy score was chosen. Twenty residues from the hinge-scFv fusion protein and 19 residues

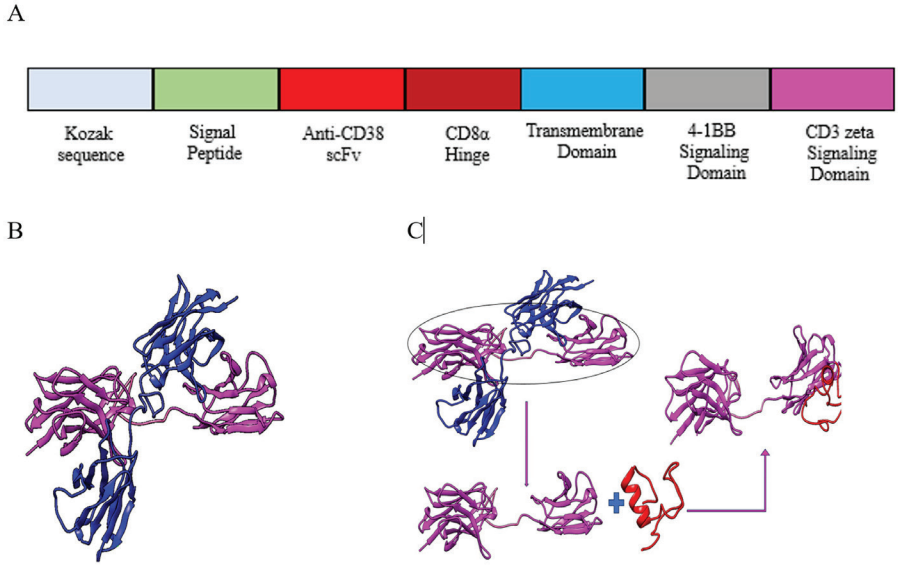


**Fig. 3.** CAR construct encoding a leader sequence, anti-CD38 scFv of daratumumab, CD8α hinge, transmembrane regions, and 4-1BB-CD3 zeta signaling domains (a). The daratumumab scFv visualized by Chimera version 1.16. The pink color represents one of the scFv and the blue color is another similar scFv. The linker is shown as the faded color of the structure (b). The 3D structure of scFv homology modeled based on 5wym.1A protein in Swiss-model server. The hinge region highlighted in red color was conjugated to scFv by the server (c). Chimera's "rounded ribbon" design displays the molecule's second and third configurations using various colors.

from CD38 took part in interactions, according to PyMol and PIC sever analyses of the two molecules' orientation to one another. Among the several forms of interacting forces between the antibody and CD38, hydrogen bonds, hydrophobic contacts, cation-pi interactions, and aromatic-aromatic interactions were observed. Hydrophobic contacts, hydrogen bonds, cation-pi interactions, and aromatic-aromatic interactions were among the different types of interaction forces between the antibody and CD38.

The CDR3 from variable heavy of scFv participated in more than half of the hydrogenic interactions, with glutamic acid 106 from the fusion antibody. The hydrogen bonds, hydrophobic and ionic and aromatic-aromatic and cation-PI interactions between the antibody and its receptor are illustrated in supplementary Tables 1-3, and Fig. 4.

*Anti-CD38 CAR-NK Generation*

We generated the second-generation CAR construct to target the CD38 marker (Fig. 3A). Lentiviral vector particles were produced using HEK293T cells. Transduction efficiency in HEK293, which was measured by a fluorescent microscope, was greater than 90% (Fig. 5). Lentiviral vector particles were used for NK 92 cell transduction. To comprehensively assess the surface marker expression and activation status of anti-CD38 CAR-NK cells, a combination of fluorescent microscopy and flow cytometry methods was employed (Figs. 6a-c).

The expression of scFv at the surface of NK 92 was determined by flow cytometry using PE-conjugated recombinant human CD38-His tag, in the GFP-expressing population of NK cells, which was approximately 25% (Fig. 6a). FACS enrichment of anti-CD38 CAR-NK resulted in homogeneous anti-CD38 NK 92 cells that were more than 90% GFP-positive. Restriction of gene transfer to the NK cells led to lower expression of anti-CD38 scFv at the surface of NK 92 cells than HEK293.

*Fluorescent Microscopy*

Fluorescent microscopy revealed distinct morphological features indicative of NK cell lineage in anti-CD38 CAR-NK cells. These morphological features included a granular cytoplasm and well-defined nuclei. Utilizing the GFP marker, the entrance of the CD38-specific CAR construct was vividly

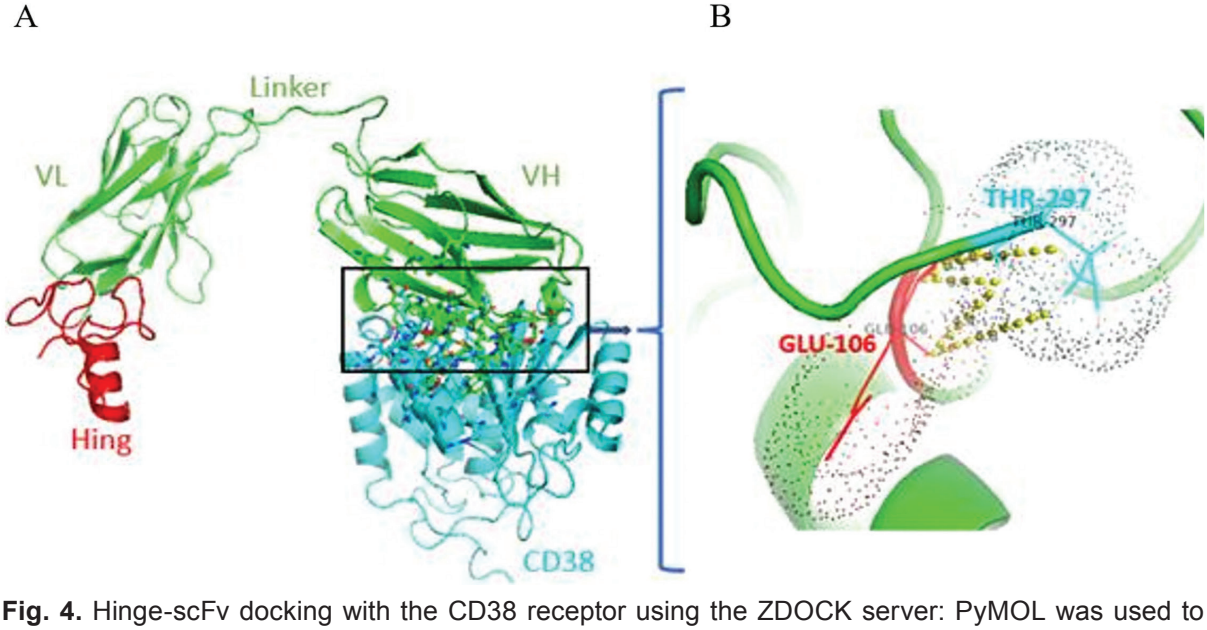


**Fig. 4.** Hinge-scFv docking with the CD38 receptor using the ZDOCK server: PyMOL was used to perform the visualizations. CD38 is shown in blue. VH, VL, and linker of anti-Cd38 scFv are indicated in green, and the hinge is displayed in red (a) presentation of anti-CD38 scFv, CD38 receptor, and key residues at the interaction interface (b). The interaction of GLU 106 from the scFv's VHCDR3 with THR 297 from the CD38 receptor forms a hydrogen bond.

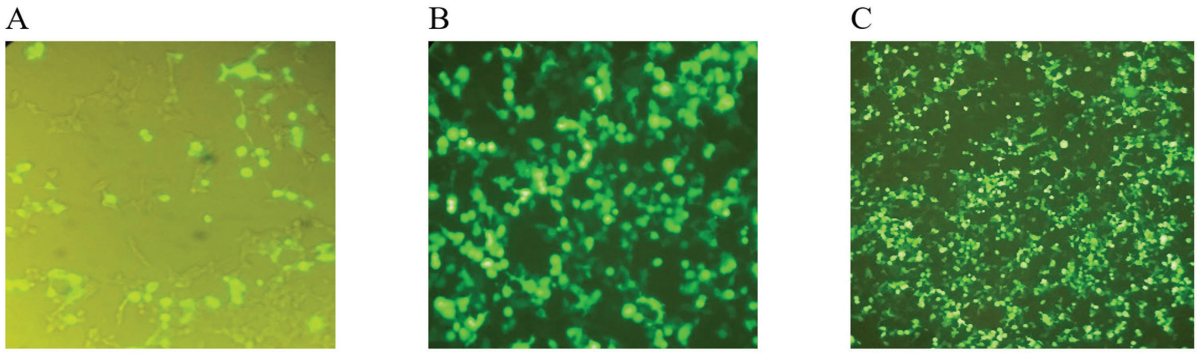


**Fig. 5.** Fluorescent microscopy of HEK293T transduced cells after 16h (a), 48h (b) and 72h (c)

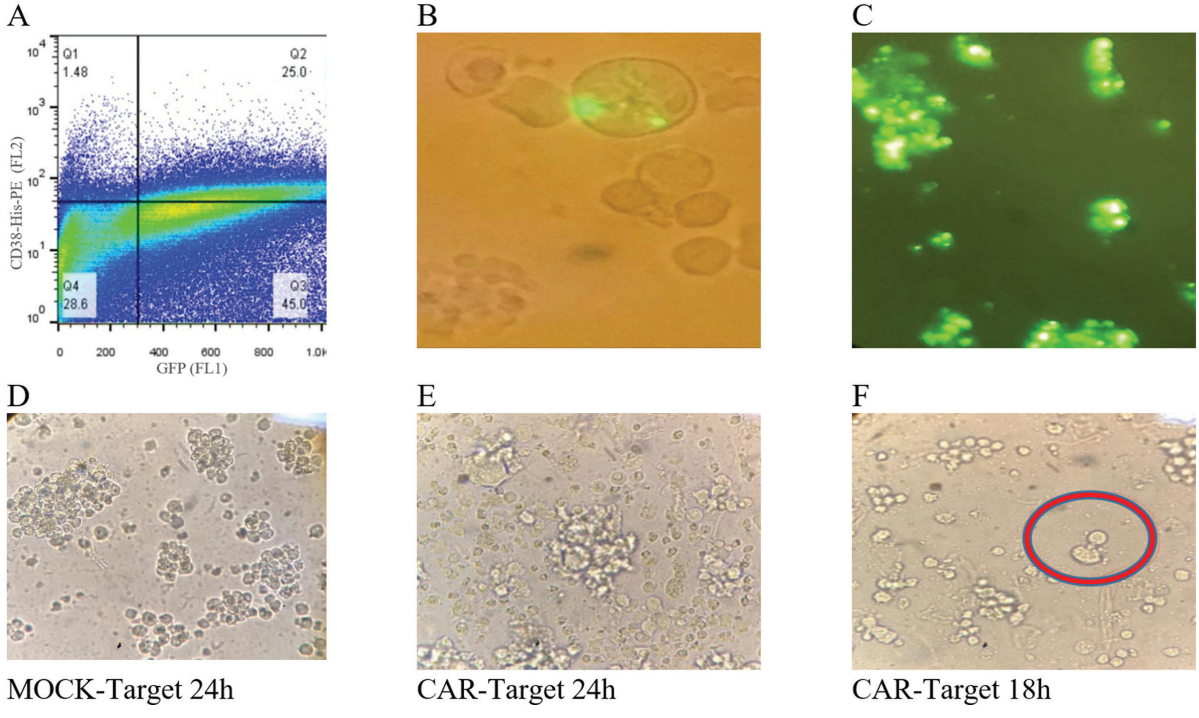


**Fig. 6.** Flow cytometry and fluorescence microscopy were used to detect anti-CD38 CAR expression on NK-92 cell. A representative flow cytometry plot illustrating the expression of anti-CD38 CAR on NK-92 cells following a 16-hour treatment. Q2: Depicts NK-92 cells that are both GFP and anti-CD38 CAR positive simultaneously; Q3: Represents NK-92 cells that are GFP positive; Q4: Corresponds to NK-92 cells with both GFP and anti-CD38 CAR negative, indicating cells that did not successfully express anti-CD38 CAR (a). GFP expression on NK 92 after 16h, detected by fluorescent microscope (b); GFP expression on NK 92 after 24h, detected by fluorescent microscope (C); Co-culture of Mock-Target 24h (d); Co-culture of anti-CD38 CAR-NK with Target 24h, using the light microscope (e); Co-culture of anti-CD38 CAR-NK with Target 18h, using the light microscope (f).

visualized in NK 92 cells. Upon exposure to CD38-positive target cells, they exhibited augmented cell spreading and membrane ruffling, indicative of an activated state (Figs. 6d-f). Staining with NK cell lineage markers, CD56 confirmed the NK cell identity of the engineered cells.

*Flow Cytometry*

Quantitative analysis of anti-CD38 CAR-NK cell phenotype was conducted using flow cytometry. The expression levels of CD38 on Daudi, Jurkat, and K562 were 88.7%, 64.8%, and 2.4%, respectively (Fig. S2). The GFP signal represents CAR vector entrance into cells (Fig. 6A).

*Anti-CD38 CAR-NK Cytotoxicity against Target Cells*

We conducted experiments to assess

the cytotoxic activity (anti-tumor activity) of anti-CD38 CAR-NK cells and Mock cells against various target cells at different Effector: Target ratios (1:1, 1:3, and 3:1). K562 cells were used as a negative control. There was no significant difference in K562 target cell lysis between transduced and non-transduced NK cells (1:3: 42.77±1.7 and 42.35±2.01, respectively; $p$=0.8; 1:1: 23.7±1.8 and 23.6±2.21, respectively; $p$=0.95; 3:1: 38.1±0.36 and 36.33±0.9, respectively; $p$=0.6). However, anti-CD38 CAR-NK cells exhibited a higher level of lysis towards CD38 expressing target cells such as Daudi (1:3: 58.31±7.4 and 35.42±3.9, respectively; $p$=0.009; 1:1: 76.56±0.51 and 36.93±0.94, respectively; $p$<0.0001; 3:1: 62.8±3.5 and 40.8±4.4, respectively; $p$=0.002) and Jurkat (1:3: 61.12±1.7 and 51.33±2.6, respectively; $p$=0.006; 1:1: 72.18±1.4 and 51.03±2.3, respectively; $p$<0.0001; 3:1: 51.07±1.9 and 68.2±1.04, respectively; $p$<0.0001) cells compared to non-transduced NK cells. These findings were statistically significant at all effector: target ratios (1:1, 1:3, and 3:1) in comparison with the Mock group (Fig. 7). The findings of the present study revealed that anti-CD38 CAR-NK cells induced the most cytotoxicity effect against CD38 expressing cells (Daudi and Jurkat) at an E:T ratio of 1:1. Therefore, we selected this ratio for IFN-γ, perforin, and granzyme production assay.

*Flow Cytometry-Based IFN- γ, Perforin, and Granzyme Expression Detection*

We determined the IFN-γ, perforin, and granzyme production of anti-CD38 CAR-NK cells in comparison with Mock against target cells (Daudi, Jurkat, and K562 cell lines) at a1:1 E:T ratio. In the presence of K562, NK cells with or without CD38-CAR indicated no significant differences in IFN-γ, perforin, and granzyme production (IFN-γ: 12.1±0.1 and 11.9±0.5, respectively; $p$=0.52; perforin: 10.34±0.09 and 10.99±0.32, respectively; $p$=0.65; granzyme: 2.14±0.12 and 2.1±0.12, respectively; $p$=0.27). When exposed to CD38-positive cells, anti-CD38 CAR-NK cells showed a significantly higher expression of IFN-γ (Daudi: 21.36±1.3 and 13.9±0.61, respectively; $p$=0.004; Jurkat: 16.38±1.2 and 3.1±0.61, respectively; $p$<0.0001), perforin (Daudi: 18.05±1.4 and 2.4±0.32, respectively; $p$=0.002; Jurkat: 38.2±1.1 and 0.7±0.09, respectively; $p$<0.0001) and granzyme (Daudi: 16.3±1.5 and 8.1±0.3, respectively; $p$=0.009; Jurkat: 16.5±1.2 and 9.9±1.45, respectively; $p$=0.004) than non-transduced NK cells (Fig. 7)

## DISCUSSION

The CD38 was chosen as a target antigen because of its frequent expression on various types of hematopoietic malignant cells, making it an attractive candidate for targeted immunotherapy. Targeted therapy of overexpressed CD38 malignant cells requires the design of a best-fit project anti-CD38 CAR-NK cell (26). The proper design and appropriate selection of each fragment and sequence of the CAR-NK protein, including scFv, hinge, transmembrane, and signaling, have a significant impact on the affinity, specificity, and expression level of CAR-NK protein, making it the most important step of the project (Table S4).

CAR-T cells are classified into generations based on the organization of their intracellular signaling domain. Second-generation chimeric antigen receptors (CARs) with two signaling fragments are highly active against B cell malignancies. Meanwhile, third-generation CAR-T cells did not outperform second-generation CAR-T cells in terms of efficacy (27). As a result, we engineered a second-generation anti-CD38 CAR that harnessed the strength of 4-1BB-CD3 zeta dual signaling to induce significant NK cell proliferation. It is worth noting that according to certain previous findings, 4-1BB-CAR-T cells might remain longer in the body than CD28-CAR-T cells. Moreover, the CD28ζ-CAR was reported to be activated undesirably in the absence of the antigen (28).

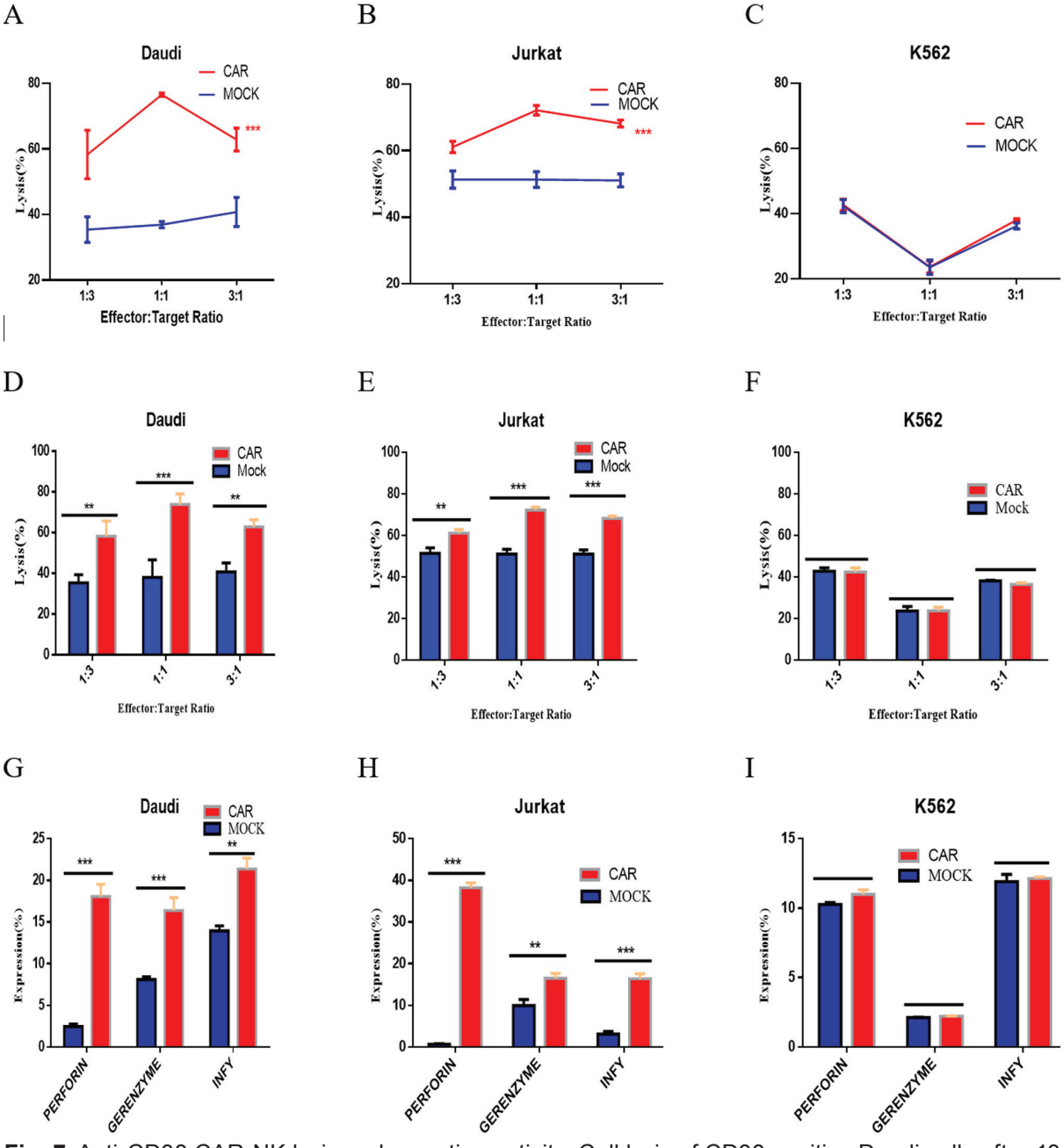


**Fig. 7.** Anti-CD38 CAR-NK lysis and secretion activity. Cell lysis of CD38 positive Daudi cells after 18 hours' co-culture with anti-CD38 CAR-NK 92 or Mock cells using flow cytometry (a, d); Cell lysis of CD38 positive Jurkat cells after 18 hours' co-culture with anti-CD38 CAR-NK 92 or Mock cells using flow cytometry (b, e); Flow cytometry analysis of CD38 negative K562 cells after 18 hours' co-culture with anti-CD38 CAR-NK 92 or Mock cells (c, f); INF gamma, perforin, and granzyme detection in CD38 positive Daudi cells after 18 hours' co-culture with anti-CD38 CAR-NK 92 or Mock cells using flow cytometry (g); INF gamma, perforin, and granzyme detection in CD38 positive Jurkat cells after 18 hours' co-culture with anti-CD38 CAR-NK 92 or Mock cells using flow cytometry (h); INF gamma, perforin, and granzyme detection in CD38 negative K562 cells after 18 hours' co-culture with anti-CD38 CAR-NK 92 or Mock cells using flow cytometry (i). $^{**}p<0.01$, $^{***}p<0.001$.

The intensity of CAR signaling in cells was influenced not only by CAR expression level but also by the hinge and transmembrane region (29). The hinge region, which provides the flexibility of the antigen-binding domain to access the targeted epitopes, was also important in modifying the CAR's cytotoxic response (30). Regardless of whether the signaling domain is CD28 or the 4-1BB, the CD28 hinge-transmembrane is associated

with a lower activation threshold and higher frequency of neurotoxicity and cytokine release syndrome than the comparable CD8 sequences (31). Considering the low expression of CD38 on normal blood cells and NK 92 itself, the low activation threshold of CAR NK is undesirable. Since toxicity and efficacy can be moderated by using the appropriate hinge length for a given scFv, *in silico* analysis can improve the design of an optimal CAR (30).

Performing the *in silico* docking procedures before starting the practical expression of the CAR construct on NK cells ensured the capability of the protein sequence to bind to its CD38 target. It also determined the critical parameters influencing protein-protein binding, such as particular residues and the corresponding bond (32). The affinity and specificity produced by residues in immunotherapeutic agents such as antibodies, scFv, and nanobodies are critical issues that influence the therapy's safety and effectiveness (33). Thus, the type of amino acids in the antigen-antibody binding site was particularly evaluated. Based on docking results, extracellular parts of anti-CD38 bind to CD38 efficiently and at a low energy level, which is attributable to stabilization by multiple types of interaction (34). The majority of the interactions consisted of hydrogen and hydrophobic bonds (Supplementary Table 1). The CDR3 from variable heavy of scFv participated in more than half of the hydrogenic interactions, while glutamic acid 106 from the fusion antibody participated in most of the interactions. Similarly, Faraji et al. showed that diversity in the HCDR3 plays a critical role in antigen binding, and a single residue change might seriously disrupt site structure and affect the antigen binding properties (35). Locating the most interactive amino acids in HCDR3 suggested using nanobodies in the construction of anti-CD38 CAR-NK construct to reduce cost and increase stability.

A previous study applied nanobodies, which were the variable domains (VHHs) of the heavy chain, and reported that nanobodies paired with 4-1BB and CD3ζ signaling domains effectively lysed CD38-positive MM cell lines, expanded efficiently, and generated more inflammatory cytokines. The efficiency of nanobody-based CAR expression was 30% higher than our anti-CD38 CAR expression. This could be due to the nanobody's small size (36).

HCDR3 located glutamic acid 106, which is the most interactive residue of anti-CD38 CAR, mostly interacts with CYS 296 and CYS287 in CD38. Tryptophan 103 in HCDR3 in anti-CD38 scFv interacts with isoleucine 278 and phenylalanine 284 in complementarity-determining regions, tryptophan exploited deeper concavity than any other amino acid residue, making it suitable for accessing concave antigen surfaces (33). The anti-CD38 scFv contains 12 interacted tyrosine, making it the most interactive residue, and three interacted lysine in CD38. Similar to the findings of the present study, Ramaraj et al. analyzed the 53 complexes of antibodies–antigen and reported that the most prevalent amino acids on paratope and epitope surfaces were tyrosine and lysine, respectively (37).

CD38 is a type II transmembrane protein that serves as a receptor for antibody-induced cell adhesion, differentiation, apoptosis, and cytokine production as well as a multi-functional enzyme that catalyzes a variety of processes. CD38-catalyzed reactions occur in a distinct active site pocket defined by the residues E226, S193, E146, W125, R127, W189, and D155. The human CD38 catalytic site was induced by calcium mentioned at residues 121-141 (38), and none of the mentioned active site amino acids indicated interaction with our anti-CD38 CAR residues. Therefore, it could be concluded that by connecting these CAR residues, the CD38 active site will not be stimulated or inhibited, and the effects of CD38 activation in the tumor cell will be avoided. Similarly, a previous study confirmed that daratumumab did not bind to the active site of CD38 (39).

Daratumumab binds to 233-246 and 267-280 residues of CD38 with a high affinity constant (KD) of 4.36 nM (40). Four residues of our CAR interactive to CD38 were similar to the daratumumab's CD38-interactive residues. This could imply that the present anti-CD38 CAR had a different binding affinity and interaction bond with CD38 in comparison to daratumumab. Drenet et al. examined three distinct human anti-CD38 CARs with different sequences and affinity to CD38-positive cells. They showed the side effects of CAR cells on normal cells could be regulated by modifying and adjusting the sequences of amino acids that lead to changes in the binding affinity (23).

The combined use of fluorescent microscopy and flow cytometry demonstrated the successful generation of anti-CD38 CAR-NK cells. The visualization of anti-CD38 CAR expression/ entrance through GFP tagging, along with the confirmation of NK cell lineage and CD38 markers, provided a comprehensive understanding of the engineered cells' phenotype.

Activation-induced morphological changes and upregulation of activation markers (Figs. 6 a, b, and f) further confirmed the responsiveness of anti-CD38 CAR-NK cells upon encountering CD38-expressing target cells. These findings provide valuable insights into the functional and phenotypic attributes of the engineered cells, which are crucial for their thorough characterization and potential clinical application.

The cytotoxic activity of anti-CD38 CAR NK cells against a spectrum of target cells (Daudi, Jurkat) was evaluated at spanning different effector: Target (E: T) ratios (1:1, 3:1, and 1:3). Utilizing K562 cells, devoid of CD38 expression, as a negative control facilitated the differentiation of CD38-CAR-mediated effects. When compared to their non-transduced counterparts (Mock), CD38-CAR NK cells significantly increased the lysis of CD38-expressing target cells, particularly Daudi cells. We found that Daudi cells had significantly higher levels of anti-CD38 CAR-NK dependent lysis than Mock cells or Jurkat cells. It could be due to a higher level of CD38 expression in Daudi cells, which is shown in Supplementary Figs. S2 (Based on the geometric mean of fluorescent intensity (GFI), Daudi cells were 51.8% CD38 positive compared to 40 % GFI in Jurkat cells). Daratumumab was also shown to increase lysis activity in Daudi compared to the other CD38-positive cells (41).

This increased cytotoxicity was not dose-dependent and remained significant across all E:T ratios (1:1, 1:3, and 3:1), highlighting the potent anti-tumor activity of anti-CD38 CAR-NK cells.

Although it cannot be certainly stated, this non-dose dependence cytotoxicity could be attributed to the low expression of CD38 on NK 92 cells and its inconsiderable fratricide. As Esther Drent et al. reported, their CD38 blockage CAR-NK cells lysed target cells in a dose-dependent manner (42). When faced with K562 cells, there was no significant difference in target cell lysis between transduced and non-transduced NK cells. This observation suggests a potential specificity of CD38-CAR NK cells toward CD38-positive cells while retaining comparable cytotoxicity against CD38-negative targets. Although there was no significant cell lysis in K562 cells when co-cultured with both anti-CD38 CAR-NK cells and Mock, it could be due to the high expression of several NK activator markers such as beta 2-microglobulin, NKG2D ligands, on K562 cells which make them susceptible to NK cells lysis (43). Similarly, anti-CD38 CAR-T cells had negligible cytotoxicity on CD38 knocked out LP-1 cells or K562 cells (36).

In the next step, flow cytometry was used to examine the production of IFN-γ, perforin, and granzyme by ani-CD38 CAR-NK cells in comparison to non-transduced NK cells (Mock). Surprisingly, our findings revealed a significant upregulation of IFN-γ, perforin, and granzyme expression in anti-CD38 CAR-NK cells upon engagement with CD38-positive cells. This increased cytokine production

reinforces the multifaceted mechanisms that underscore the superior functional properties of CD38-CAR NK cells. The absence of significant cytokine variation between CD38-CAR and non-transduced NK cells, when co-cultured with K562 cells, underscored the specificity of CD38-CAR's impact.

The robust cytokine response induced by CD38-CAR NK cells had significant therapeutic implications. IFN-γ, with its multifaceted roles in orchestrating immune responses, enhancing NK cell cytotoxicity, and influencing the adaptive immune system, reinforces the potential of CD38-CAR NK cells to mount effective antitumor defenses (44).

Target cell death is brought on by the release of lytic granules containing the proteins perforin and granzyme B into the synaptic cleft when a target cell activates NK cell cytotoxicity (45). Elevated perforin and granzyme levels, indicative of enhanced target cell apoptosis, highlighted the potential of CD38-CAR NK cells to drive durable antitumor effects. Perforin/granzyme and the death-receptor pathway are the two main mechanisms by which immune effector cells, such as NK 92 cells, mediate cytotoxicity. A comprehensive understanding of the cytotoxic mechanisms, used by CAR-effector cells, is essential for discovering resistance mechanisms that may develop from resistance to apoptosis-inducing signals. Since cancer frequently harbors a mutation in apoptotic pathways, the effect on CAR-mediated cytotoxicity is of therapeutic interest. In 2023, Althaus et al. established CD19-CAR-NK 92 cell deletion models to investigate cytotoxic pathways *in vitro*. The cytotoxicity of D19-CAR-NK 92 cells was significantly reduced by the abrogation of the perforin/granzyme pathway and subsequent knockout of perforin 1 (Prf1) (46). The functional competence and endurance of CAR-modified cells were pivotal for the sustained success of adoptive cell therapies. Our findings suggested that the remarkable expansion potential of CD38 CAR-NK cells during prolonged *in vitro* culture showcases their replicative capacity, which was critical for prolonged therapeutic efficacy. The preservation of cytotoxic and cytokine-secreting capacities even after extended culture underscored the potential longevity and robustness of CD38-CAR NK cells in therapeutic contexts. Still, major improvements, such as CD38 knockout in NK 92, may be necessary to enhance the longevity and robustness of anti-CD38 CAR-NK. Gurney et al. used CRISPR/Cas9 genome editing to break the CD38 gene during expansion in primary expanded NK cells, with a knockdown efficacy of 84%. This inhibition prevented anticipated fratricide produced by NK cell CD38 expression. The results displayed decreased NK-cell fratricide and improved capacity to target primary AML blasts. They also reported that anti-CD38 CAR-T cells lysed CD38-positive hematopoietic progenitor cells but had no effect on the differentiation of progenitor cells into myeloid lineages. They also controlled anti-CD38 CAR-T cells' side effects with caspase-9-based suicide genes (23).

Drent et al. investigated the potential benefits of a doxycycline (DOX) inducible anti-CD38 CAR design for controlling CAR T cell off-target toxicity. This method might be more beneficial than suicide genes for controlling the potential toxicity of CAR T cells without being required to permanently eliminate them (47). Despite all attempts to reduce anti-CD38 CAR safety for normal cells, the safety and efficacy of CD38 targeting antibodies such as daratumumab and isatuximab have mostly eliminated the CD38 off-target concern (48-51). Besides, by lysing CD38+Tregs, which are known to have more potent T cell suppressive abilities than CD38- Tregs, anti-CD38 CAR-KHYG-1 cells may potentially have some positive immunomodulatory effects (52).

Therefore, all of these previous studies showed that it is extremely improbable that these fusion proteins will bind unintentionally to the normal or non-target cell surface and may also have certain beneficial effects. In comparison to previous research, our findings were consistent with recent studies on CAR-based immunotherapies that demonstrated

similar cytokine-mediated enhancement of T or NK cell cytotoxicity against CD38-expressing cells through CAR technology (47, 53). Similarly, researchers reported heightened cytokine production and cytotoxicity by CAR-modified cells targeting cancer cells (54-57). These analogies highlighted the broader applicability of CAR-based cell therapies across distinct cancer types.

The fact that NK cells are short-lived is a significant benefit of engineering anti-CD38 CARs into NK cells as opposed to T cells when taking into account the potential unforeseen safety issues. Thus, both their anti-tumor activities and any negative consequences will be restricted. Therefore, even if anti-CD38 CAR-NK cells have any negative consequences, they will likely be considerably less severe and last less time than anti-CD38 CAR-T cells, which can form memories and stay for longer (58, 59).

## CONCLUSION

In conclusion, our findings indicated that anti-CD38 CAR-NK cells has potential as a robust therapeutic strategy. Enhanced cytotoxicity against CD38-expressing cells, combined with potent IFN-γ, perforin, and granzyme production, exemplifies their capability to effectively target and eradicate CD38-positive malignant cells. The findings of the present study could contribute to the expanding body of evidence supporting the clinical utility of anti-CD38 CAR-NK cells as a promising treatment option for CD38-positive malignancies.

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## AUTHORS' CONTRIBUTION

MA performed lab experiments, provided manuscript draft and was involved in study design. RK was involved in experimental activities and data analysis. VR, AA, JF and AR were involved in study design and parts of experimental analyses. SNF contributed to *in silico* analysis. NE was the principal investigator involved in study design, supervision of lab experiments and data analysis. All authors took part in manuscript drafting and final proofreading.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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