iPSC-Derived Natural Killer Cells Expressing EGFR-CAR Against Glioblastoma

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Abstract

Chimeric antigen receptor (CAR) therapy has become a rising form of cancer treatment due to its ability to target cancer cells specifically and efficiently. Natural killer (NK) cells are a type of immune cells that belong to the innate immune system and executes a rapid immune reaction against cancer cells and viruses without prior activation. The huge success of CAR-T cells has motivated scientists to genetically modify human NK cells with CARs to improve their tumor killing capacity. NK cells can be derived from induced pluripotent stem cells (iPSCs) to produce a homogenous and standardized group of NK cells for the treatment of cancer. Here, we aim to engineer our EGFR-CAR construct into iPSC-derived hematopoietic stem cells (iPSC-HSC) to generate EGFR-CAR NK cells that will eradicate glioblastoma (GBM), a type of brain cancer with no cure. Our results demonstrated that iPSCs can be fully differentiated into NK cells, and that HSCs can be engineered with EGFR-CAR without affecting their differentiation into CAR-NK cells. Our engineered EGFR-CAR NK cells showed an enhanced antitumor activity against GBM compared to NK cells without the EGFR-CAR. Collectively, our study suggests that iPSCs can be utilized to generate an unlimited number of CAR-NK cells to treat cancer patients in the clinical setting.

Introduction

NK Cells are a type of lymphocytes that exist in the body and can kill target cells in a spontaneous fashion with little restriction unlike T cells. NK can be identified by unique markers expressed on their surface. In particular, they can be marked by the expression of CD56 and lack of CD3 (which is expressed on T or NKT cells). NK cells are a central component of the innate immune system and constitute the first line of defense against a variety of tumors and microbial pathogens (1,2). NK cells are dependent on the presence of cytokines such as interleukin (IL)-2, 15, and 21 that support their development and trigger their proliferation, motility, activation, and cytotoxic effector molecule expression (3,4). To be expanded and/or engineered in the laboratory for the treatment of cancer, NK cells may be superior to T cells because the former does not need to be collected from the same patient or a specific HLA-matched donor. Thus, NK cells has better potential to be used as a universal therapy.

CARs are chimeric receptor proteins that can be engineered to give T cells or NK cells, so called CAR-T or CAR-NK cells, a new ability to target a specific protein on the surface of tumor cells. The receptors are chimeric because they are hybrid molecules consisting of two parts. One part is outside of the cell membrane of CAR-T cells or CAR-NK cells that can bind to a protein (called an antigen) on tumor cells, while the other part is in the cytoplasm of the CAR cells that gives them the activating signal and enhances their function. The binding part makes the CAR cells specifically target tumor cells instead of normal cells, and the activating signal gives the CAR cells more power so that they can kill tumor cells better by secreting more "pore forming protein" called perforin, which makes pores on the targeted cell surfaces, and enzymes (for example, granzyme B) that degrade proteins inside of the tumor cells (5).

iPSCs have recently attracted much attention because new genetic editing technologies are advanced and these cells are capable of providing replensihable sources to generate NK cells without repetitive genome modifications for individual donors or patients. iPSC-derived NK cells can provide a homogeneous NK cell population which can be grown to a clinical scale for NK cell- or CAR-NK cell-based cancer immunotherapy. This approach allows that a single CAR cell product can be infused at multiple doses that may be needed for the same patient or among different patients, which is difficult to be achieved with the current CAR-T cell therapy. **Here, we aim to develop iPSC-derived CAR-NK cells targeting EGFR for the treatment of GBM via testing this feasibility with pre-clinical studies.**

Materials & Methods

Differentiation of iPSCs-derived HSCs into NK cells:

iPSC-derived HSCs were obtained from Dr. Yanhong Shi's lab at City of Hope (COH) and their phenotype was confirmed by flow cytometry to measure CD34 expression. The HSCs were placed in a 6-well plate containing OP9-DLL1 cells (a bone marrow stroma cell line) with NK differentiation media for 3 weeks. NK differentiation medium consists of 56% DMEM, 29% Ham's F-12 Nutrient Mixture, 15% heat-inactivated human AB serum, 1% Penicillin/Streptomycin (antibiotics), 2 mM L-glutamine, 25 µM β-mercaptoethanol, 5 ng/mL sodium selenite, 20 mg/L ascorbic acid, 5 ng/mL IL-3, 20 ng/mL SCF, 20 ng/mL IL-7, 10 ng/mL IL-15, etc. The medium was changed every 3-4 days and OP9-DLL1 cells were changed every 7 days.

Isolation of HSCs from blood to be engineered with EGFR-CAR for generating CAR-NK cells:

CD34(+) stem cells were enriched from leukopacks obtained from the blood bank at COH under the institutional review board-approved protocols using a RossetteSep kit initially and then were purified with CD34 Miltenyi beads. Purity of the CD34(+) stem cells was confirmed with flow cytometry and the stem cells were grown in StemSpan II media supplemented with UM171 (a pyrimido-[4,5-b]-indole derivative) and StemSpan CD34+ expansion supplement (Flt3L, SCF, IL-3, IL-6, etc.) at day 0.

Materials & Methods (cont'd)

On day 2, HSCs were infected with retroviral supernatant (pCIR_GFP and pCIR_EGFR_CAR) in human retronectin-coated plates for 48 hr to generate EGFR-CAR HSCs. Two days post-infection the CAR-HSC cells were collected and transferred to a 6-well plate containing OP9-DLL1 cells in the NK differentiation media to obtain CAR-NK cells, as we performed for the iPSC-derived HSCs.

Expansion of NK cells to generate EGFR-CAR NK cells:

Blood luekopacks were obtained from the blood bank at COH. Peripheral blood mononuclear cells (PBMCs) from three donors were obtained by Ficol centrifugation. At day 0, two million PBMCs were stimulated with K562 feeder cells expressing 4-1BBL and IL-21 and recombinant human IL-2 (50 IU/ml) in X-Vivo 10 media. The K562 feeder cells were pre-treated with mitomycin C (10 µg/ml) to prevent their growth but were capable of promoting NK cell expansion. Purity of expanded NK cells at day 7 was confirmed by flow cytometry (> 95% purity) and the expanded cells were infected with retroviral supernatants (pCIR_GFP and pCIR_EGFR_CAR) in human retronectin-coated plates to generate EGFR-CAR NK cells and control cells marked with GFP. Four days post-infection these cells were collected for cytotoxicity assay to test the efficacy of CAR-NK cells.

Cytotoxicity assay:

To assess cytotoxicity, CAR and control NK cells were cocultured with ^{51}Cr-labeled LN229 cells (a glioblastoma cell line) at multiple Effector:Target (E:T) ratios, followed by measuring the levels of ^{51}Cr release.

Results

Differentiation of iPSC-derived HSCs into NK cells

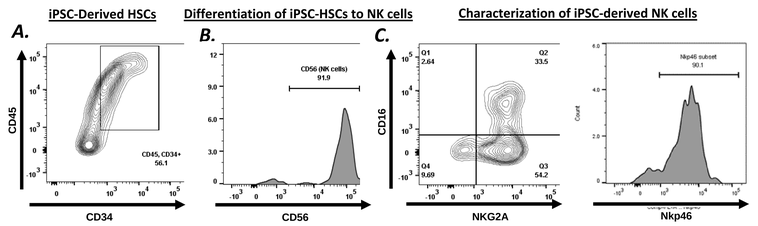


Figure 1 A. A representative FACS plot of iPSC-derived HSC cells. **B.** A representative FACS plot indicating differentiation of iPSC-derived HSCs into NK cells (CD45+, CD3-, CD56+) at 21 days post-incubation in NK differentiation media with OP9-DLL1 cells. **C.** Expression of CD16, NKG2A, and Nkp46, all of which are NK cell markers, on iPSC-derived NK cells.

Differentiation of EGFR-CAR-transduced HSCs into CAR-NK cells

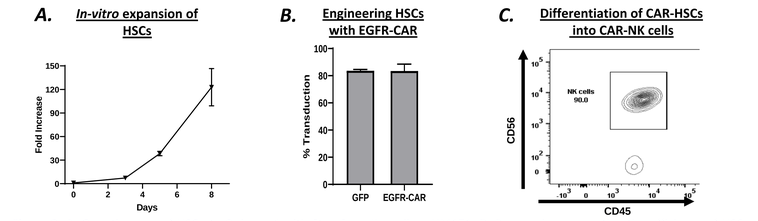


Figure 2. A. Growth curve of HSCs (n=3 donors) with StemSpan II media + UM171. **B.** Bar graph representing the efficiency of HSCs transduction with pCIR_GFP and pCIR_EGFR_CAR for 48 hrs. **C.** A representative FACS plot indicating differentiation of EGFR-CAR HSCs into CAR NK cells (CD45+, CD3-, CD56+) at 31 days post incubation in NK differentiation media with OP9-DLL1 cells.

Antitumor activity of EGFR-CAR NK cells

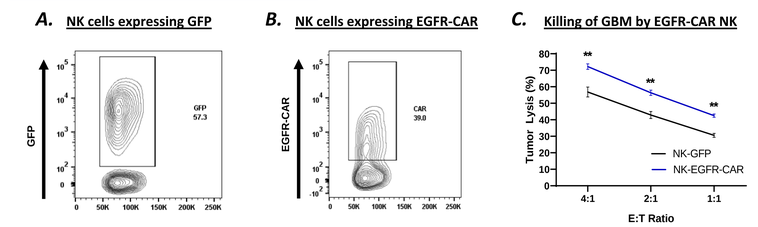
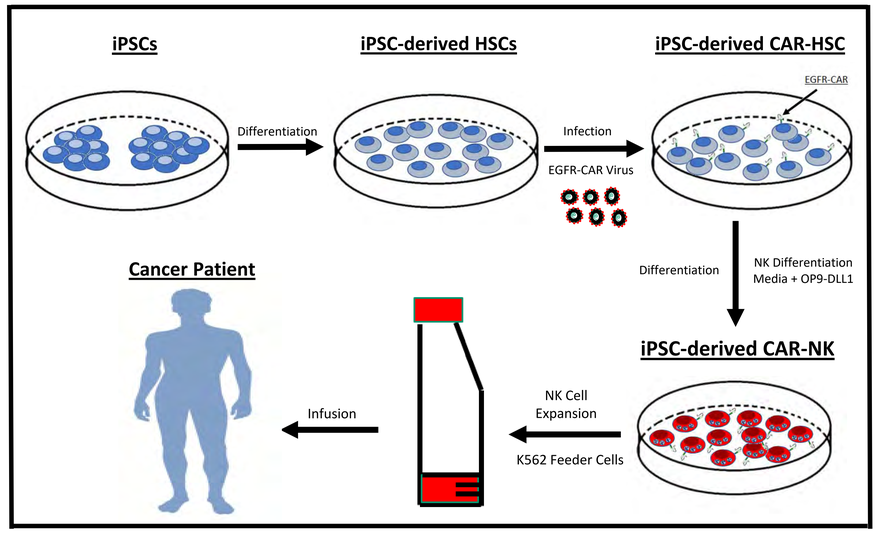


Figure 3. A and B . A representative FACS plot indicating the efficiency of NK cell infection with virus expressing GFP or CAR for 48 hrs. **C.** The cytotoxic activity of EGFR-CAR vector-transduced NK cells (blue line) vs GFP vector-transduced NK cells (black line) as measured by ^{51}Cr release assay. Target cells are the LN299 GBM cell line. Data are summarized from the results of three donors.

Project Summary



Conclusion

- iPSC-derived HSCs are capable of differentiating into NK cells expressing typical NK cell surface markers including CD56, NKp46, CD16, and NKG2A, etc.
- HSCs engineered with CAR can be fully differentiated into CAR NK cells and the expression of CAR on HSCs does not affect the differentiation process.
- EGFR-CAR NK cells show a significant improvement of cytotoxicity against GBM *in vitro* compared to control NK cells.
- Our approach potentially will allow for clinical scale production of iPSC-derived, off-the-shelf CAR NK cells to treat patients with cancer such as GBM.

Future Directions

- Alternatively, iPSCs can be genetically modified via CRISPR/Cas9, a genome editing technology, to remove some inhibitory signals such NKG2A, followed by a differentiation process to generate more potent CAR-NK cells.
- The efficacy and safety of iPSC-derived CAR-NK cells will be tested in animal models.
- If iPSC-derived EGFR-CAR-NK cells show a beneficial effect without substantial toxicities, the cells will be manufactured in a GMP facility for future treatment of GBM patients.

References

1. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nature Reviews Immunology*. 2006 Oct;6(10):715.
2. Bozzano F, Marras F, De Maria A. Natural Killer Cell Development and Maturation Revisited: Possible Implications of a Novel Distinct Lin− CD34+ DNAM-1brightCXCR4+ Cell Progenitor. *Frontiers in immunology*. 2017 Mar 9;8:268.
3. Carson WE, Giri JG, Lindemann M, Linett ML, Ahdieh M, Paxton R, Anderson D, Eisenmann J, Grabstein K, Caligiuri MA. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *Journal of Experimental Medicine*. 1994 Oct 1;180(4):1395-403.
4. Vosshenrich CA, Ranson T, Samson SI, Corcuff E, Colucci F, Rosmaraki EE, Di Santo JP. Roles for common cytokine receptor γ-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *The Journal of Immunology*. 2005 Feb 1;174(3):1213-21.
5. Gill S, June CH. Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies. *Immunological reviews*. 2015 Jan;263(1):68-89.