Generation of human iPSC-derived Dual CAR-NK cells targeting EGFR and PD-L1 for glioblastoma

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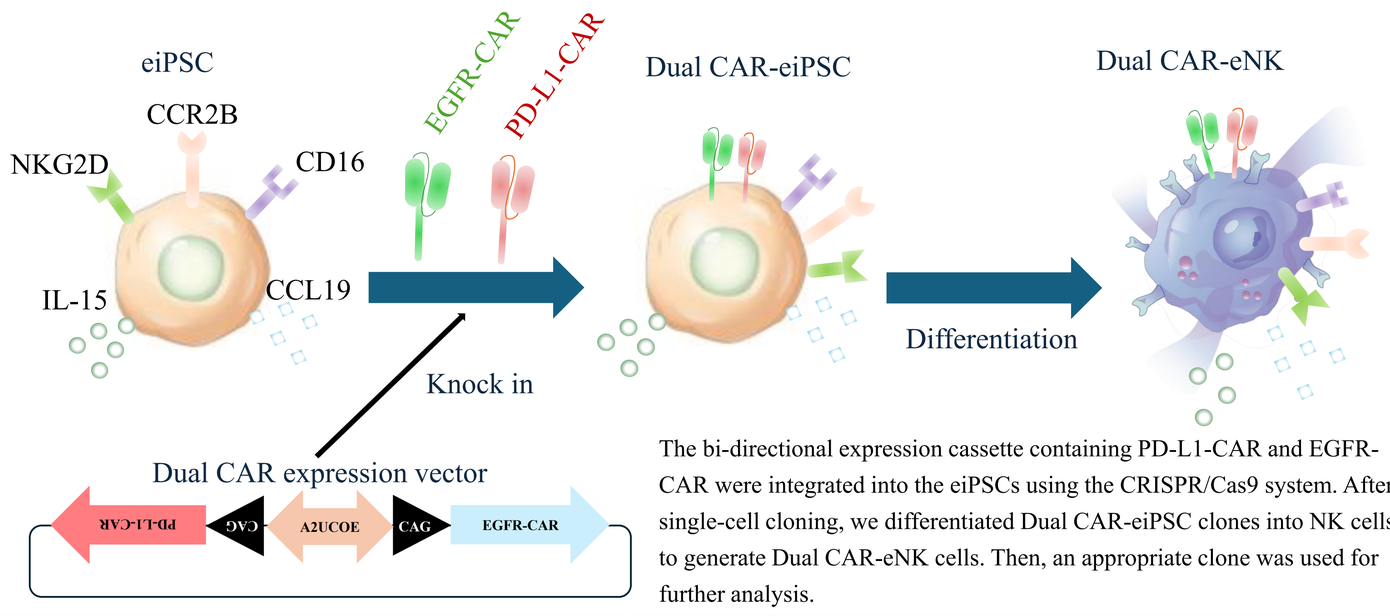
Abstruct

Glioblastoma is the most common malignant primary brain tumor in adults with a very poor prognosis. Conventional treatments have shown limited efficacy; therefore, it is necessary to develop novel therapeutic strategies for glioblastoma. We have previously generated engineered NK (eNK) cells derived from human iPSCs which are genetically engineered to express IL-15, CCR2B, CCL19, high-affinity CD16, and NKG2D/DAP10 complex (eiPSCs). The eNK cells demonstrated improvement of survival, persistence, infiltration of tumors, and recruitment of immune cells, and high cytotoxic activity against tumor spheroids, suggesting the eNK cells have potential to overcome challenges associated with immune cell therapies for solid tumors. In this study, we aimed to enhance eNK cell function by expressing CARs targeting EGFR and PD-L1. We made a CAR construct capable of stably co-expressing both CARs, introduced it into the eiPSCs, and subsequently differentiated them into NK cells (Dual CAR-eNK). We confirmed that the Dual CAR-eNK cells stably express both EGFR-CAR and PD-L1-CAR by flow cytometric analysis. Co-culture experiments of the Dual CAR-eNK cells with K562 cells ectopically expressing CAR target antigens (EGFR or PD-L1) revealed that the Dual CAR-eNK cells exhibited antigen-specific cytotoxicity and improvement of interferon-γ protein production. Live-imaging cytotoxicity assays showed that the Dual CAR-eNK cells exerted strong antigen-specific cytotoxicity against glioblastoma spheroids. These results indicate that our Dual CAR-eNK cells may offer a promising approach for treating glioblastoma.

Generation of Dual CAR-eNK cells

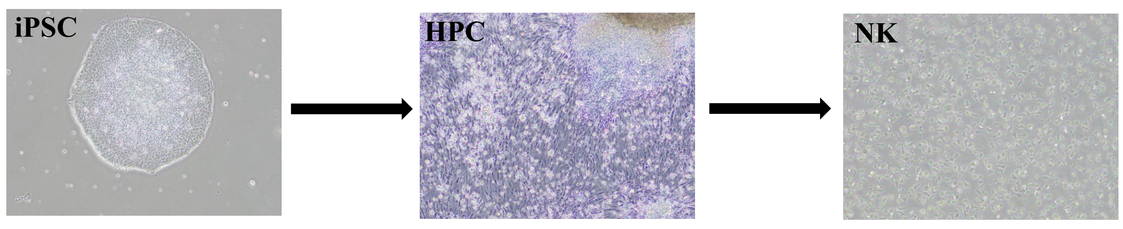


The bi-directional expression cassette containing PD-L1-CAR and EGFR-CAR were integrated into the eiPSCs using the CRISPR/Cas9 system. After single-cell cloning, we differentiated Dual CAR-eiPSC clones into NK cells to generate Dual CAR-eNK cells. Then, an appropriate clone was used for further analysis.

① Methods

(A) Differentiation of Dual CAR-eNK cells from iPSCs

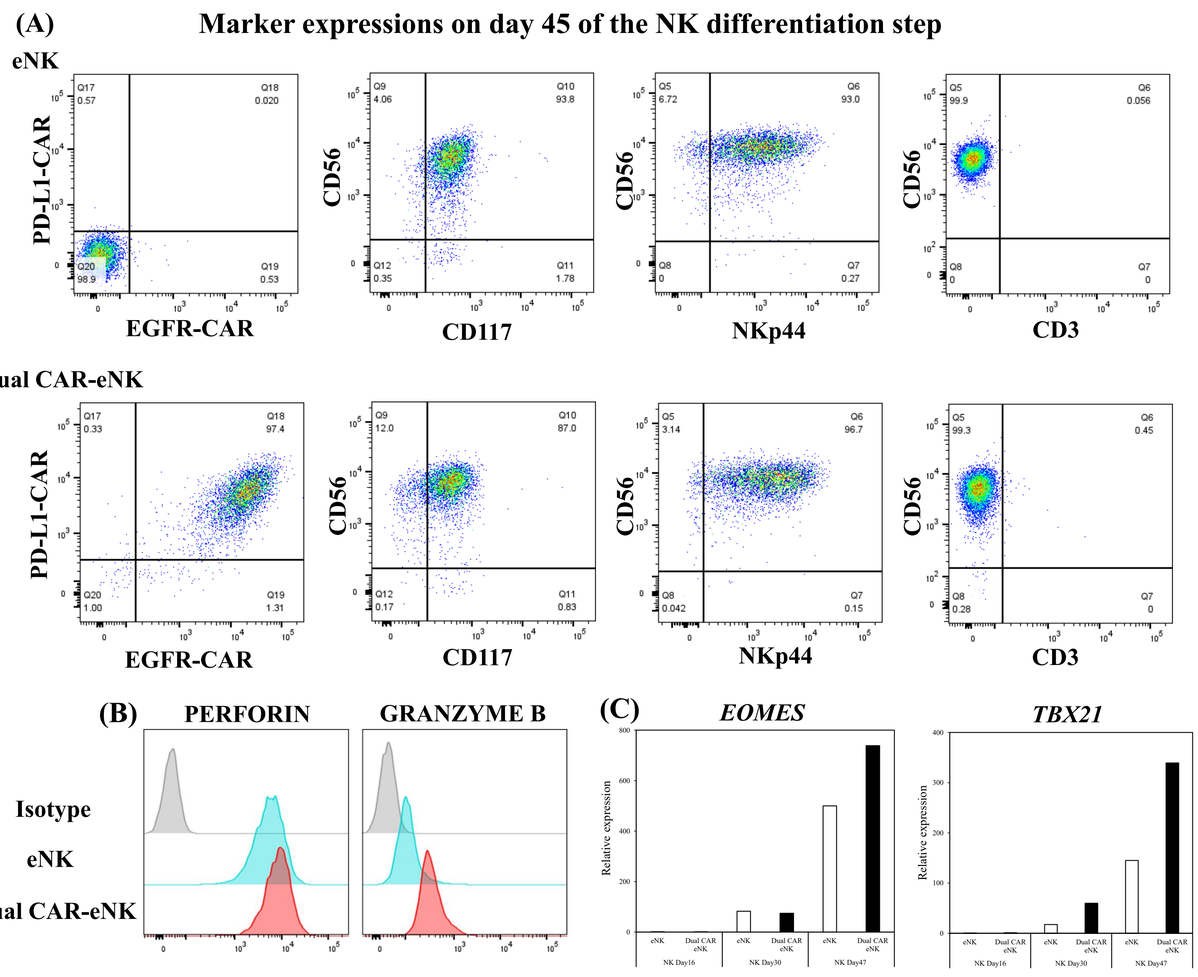
9 days	2 days	2 days	7 days	14 days	> 42 days
iPSC	ME	HE	HPC	NK	
AK03N	DMEM/F12 + CHIR99021 BMP4 VEGF165 bFGF Y-27632	Essential6 + VEGF165 SB431542 SCF bFGF Y-27632	HPCM0002 + SCF FLT3L BMP4 GSK-126	AIM-V + SCF FLT3L IL-7 IL-15 BMP4	AIM-V + SCF FLT3L IL-7 IL-15



(B) Expansion: NK cells were co-cultured with K562-mbIL-21 and 4-1BBL feeder cells.

② Characterization of iPSC-derived NK cells

(A) Marker expressions on day 45 of the NK differentiation step



(B) PERFORIN GRANZYME B

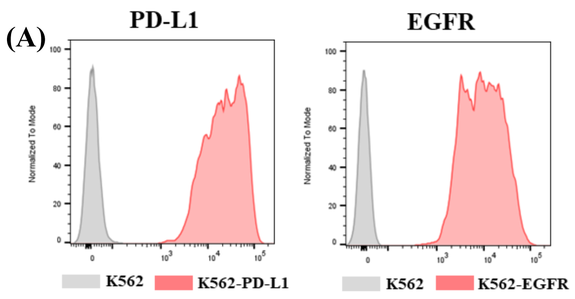
(C) *EOMES* *TBX21*

(A) The expression of PD-L1-CAR, EGFR-CAR, CD56, CD117, NKp44 and CD3 on iPSC-derived NK cells on day 45 of the NK differentiation step was determined by flow cytometry. (B) The expression of PERFORIN and GRANZYME B on iPSC-derived NK cells on day 46 of the NK differentiation step was determined by flow cytometry after intracellular protein staining. (C) The expression levels of *EOMES* and *TBX21* gene during the NK cell differentiation step were measured by RT-qPCR.

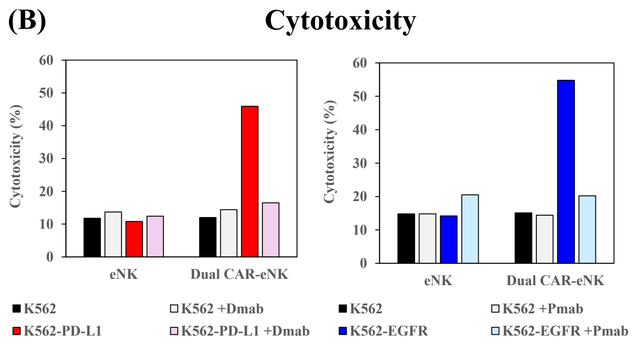
Dual CAR-eiPSCs can be differentiated into NK cells and stably express both PD-L1-CAR and EGFR-CAR.

③ Dual CAR response to target antigens

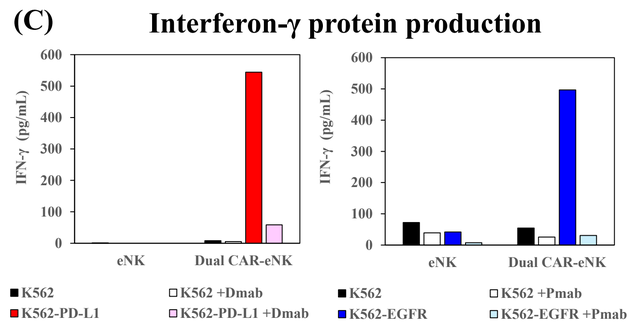
(A)



(B) Cytotoxicity



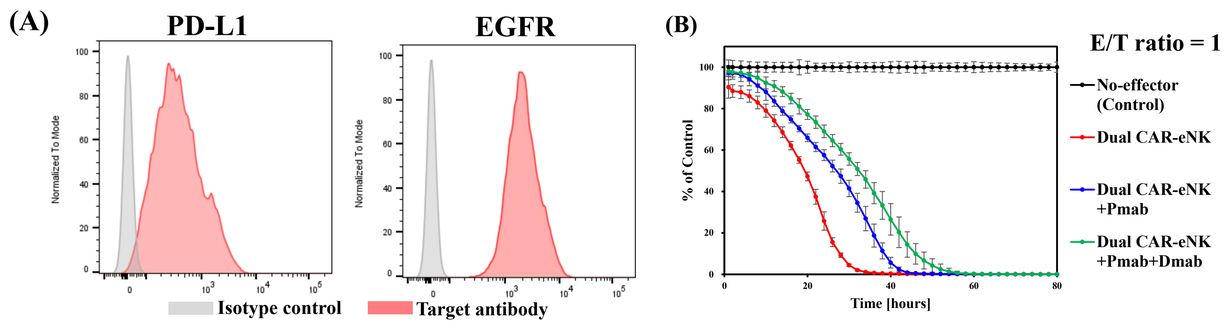
(C) Interferon-γ protein production



(A) Flow cytometry analysis of the expression of each CAR target antigen on K562 cells ectopically expressing EGFR or PD-L1. (B) The antitumor activity of eNK and Dual CAR-eNK cells against K562 cells. Calcein-labeled K562 cells were co-cultured with NK cells (E/T=2) for 4 hours in the presence or absence of anti-EGFR antibody (Pmab: Panitumumab) or anti-PD-L1 antibody (Dmab: Durvalumab). Dead cells stained with SYTOX were measured by flow cytometry. (C) The concentration of Interferon-γ in the culture supernatant after 4 hours in co-culture was measured by an Enzyme-Linked Immunosorbent Assay.

Dual CAR-eNK cells exhibited antigen-specific cytotoxicity and improvement of interferon-γ protein production.

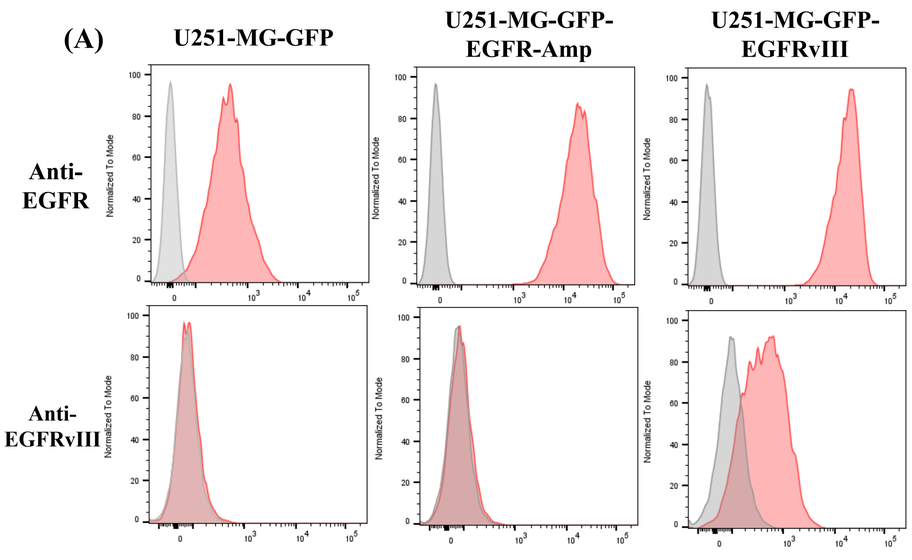
④ Antigen-specific cytotoxicity against glioblastoma



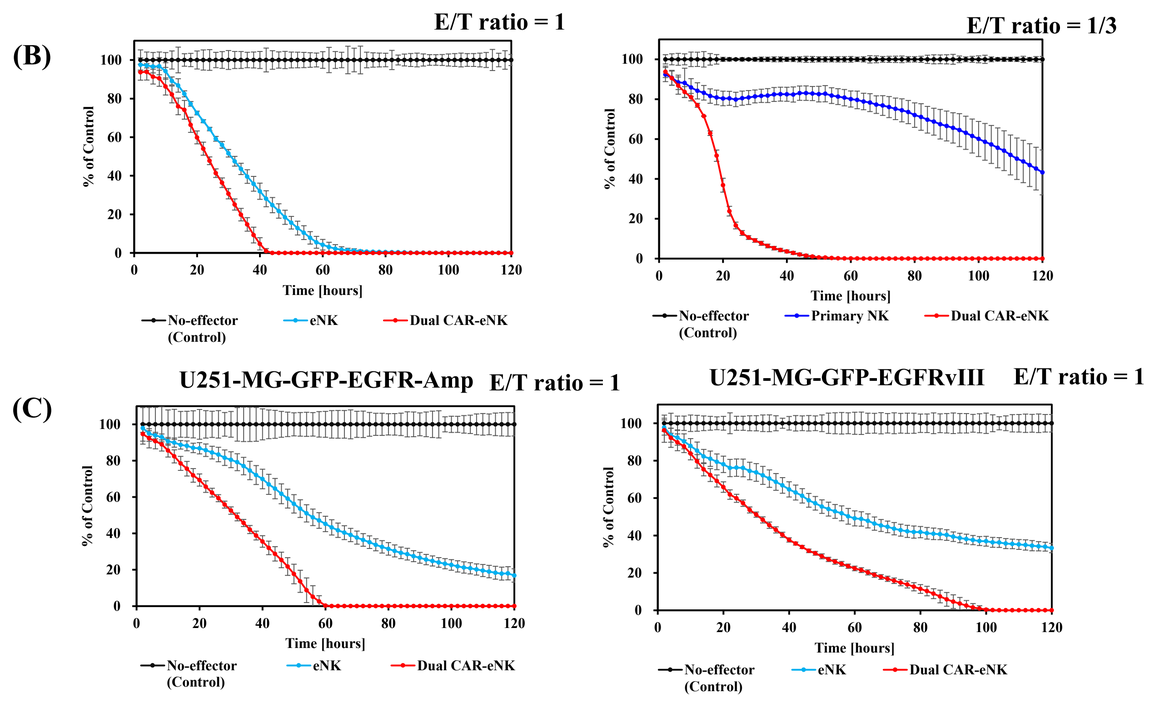
(A) The expression of PD-L1 and EGFR on U251-MG cells was determined by flow cytometry. (B) The antitumor activity of Dual CAR-eNK cells against U251-MG-GFP spheroids was determined by GFP fluorescence intensity. U251-MG-GFP spheroids were co-cultured with Dual CAR-eNK cells in the presence or absence of anti-EGFR antibody (Pmab: Panitumumab) and anti-PD-L1 antibody (Dmab: Durvalumab). The total GFP fluorescence intensity of each sample was recorded every two hours by the Incucyte SX5 system (Sartorius). GFP fluorescence intensity was normalized to that of control. All data are plotted as mean ± SD (n = 4)

Dual CAR-eNK cells exhibited antigen-specific cytotoxicity against U251-MG spheroids.

⑤ Cytotoxicity against EGFRvIII expressing glioblastoma



Amplification of EGFR genes and expression of mutant receptor **EGFR variant III (EGFRvIII)** are closely associated with tumor malignancy and poor clinical outcomes in glioblastoma.



(A) The expression of EGFR on U251-MG-GFP cells overexpressing EGFR (U251-MG-GFP-EGFR-Amp) and U251-MG-GFP cells ectopically expressing EGFR variant III (U251-MG-GFP-EGFRvIII) was determined by flowcytometry. (B, C) The antitumor activity of NK cells against U251-MG-GFP spheroids was determined by GFP fluorescence intensity. The total GFP fluorescence intensity of each sample was recorded every two hours by the Incucyte SX5 system. GFP fluorescence intensity was normalized to that of control. All data are plotted as mean ± SD (n = 4). (B) U251-MG-GFP spheroids were co-cultured with eNK cells, Dual CAR-eNK cells and primary NK cells. (C) U251-MG-GFP-EGFR and U251-MG-GFP-EGFRvIII spheroids were, respectively, co-cultured with eNK cells and Dual CAR-eNK cells.

Dual CAR-eNK cells exerted strong antigen-specific cytotoxicity against EGFR-overexpressing and EGFRvIII-expressing glioblastoma spheroids.

Conclusion

- **We established Dual CAR-eNK cells which stably co-express both PD-L1-CAR and EGFR-CAR.**
- **Dual CAR-eNK cells exhibited antigen-specific cytotoxicity and improvement of interferon-γ protein production.**
- **Dual CAR-eNK cells exerted strong antigen-specific cytotoxicity against EGFR-overexpressing and EGFRvIII-expressing glioblastoma spheroids.**

Dual CAR-eNK cells may offer a promising approach for treating glioblastoma.

【COI Disclosure】 First Author: Yuma Fukutani
I have no conflicts of interest to disclose in relation to this presentation.