Cytokine-Autonomous, CAR-Directed, Off-the-Shelf Natural Killer Cells Derived from a Clonal Engineered Master Pluripotent Cell Line

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ABSTRACT

Natural killer (NK) cells are potent anti-tumor effectors that play an important role in innate and adaptive immunity. Despite recent clinical advances in the therapeutic use of NK cells, significant opportunities remain to harness their full potential in adoptive immunotherapy. For example, achieving consistent manufacturing of cancer immunotherapies using patient- and donor-sourced cells remains a significant challenge to delivering therapies to all patients who may benefit. There is also the need to improve the efficacy and persistence of adoptively transferred lymphocytes to promote favorable patient outcomes.

We have previously demonstrated that induced pluripotent stem cells (iPSCs) can be genetically edited at the single-cell level and expanded to create clonal master engineered pluripotent cell lines, representing a renewable and reliable starting material for the creation of off-the-shelf (OTS) adoptive NK cell therapy. Using this unique platform, we evaluated the combination of NK cell-specific chimeric antigen receptors (CARs) with an autonomous protein to create a highly effective, persistent, and targeted NK cell therapy. The NK cell optimized CAR (NK-CAR) backbone contains the NKG2D transmembrane domain and the 2B4 co-stimulatory and the CD3ζ signaling domains to mediate a strong increase in NK cell signaling. To provide directed anti-tumor activity, anti-mesothelin (meso) and anti-CD19 scFvs were added to the NK-CAR backbone, and then engineered into the iPSC and subsequently differentiated to NK cells expressing NK-CARmeso or NK-CAR19.

Using an ovarian cancer xenograft model in the initial study, a single dose of NK-CARmeso NK cells markedly inhibited tumor growth and mediated enhanced survival (84 days) compared to various controls, including NK cells harboring a 3rd-generation T-cell CAR construct with CD28 and 4-1BB costimulatory domains ($p < 0.002$). We next engineered an IL-15RF fusion protein to provide self-stimulating signals to support NK cell function and persistence. The IL-15RF construct was created by fusing mature IL-15Rα to IL-15 at the C-terminus through a flexible linker. The design mimics the trans-presentation of IL-15 bound to IL-15Rα that is presented to IL-15Rβ/γC dimer to initiate signaling. While both iPSC-derived NK cells (iNKs) and iNKs bearing IL-15RF expanded *in vitro* in a similar manner in the presence of soluble IL-15 and IL-2 (2040- and 3615-fold expansion in 14 days, respectively), only the iNKs bearing IL-15RF significantly proliferated in the absence of cytokines (10- vs. 701-fold expansion in 14 days, respectively).

We are currently investigating the combination of NK-CAR and IL-15RF to determine the potential synergy between the two modalities to create cytokine-autonomous, OTS NK cell cancer immunotherapies, including those that are CAR-targeted against solid and liquid tumors.

CONCLUSION

- iPSCs can be used as an effective off-the-shelf platform to create engineered NK cells with enhanced function and efficacy.
- iPSCs derived NK cells expressing NK-CARs against solid (mesothelin) and liquid (CD19) tumors both displayed effective *in vitro* and *in vivo* anti-tumor activities.
- Expression of IL-15RF enhances NK cell expansion and persistence *in vitro* and *in vivo* in the absence of exogenous cytokines.

RESULTS

NK-CARmeso Expressing iNK Cells Display Improved *in vitro* and *in vivo* Anti-Tumor Activities Compared to T-CARmeso

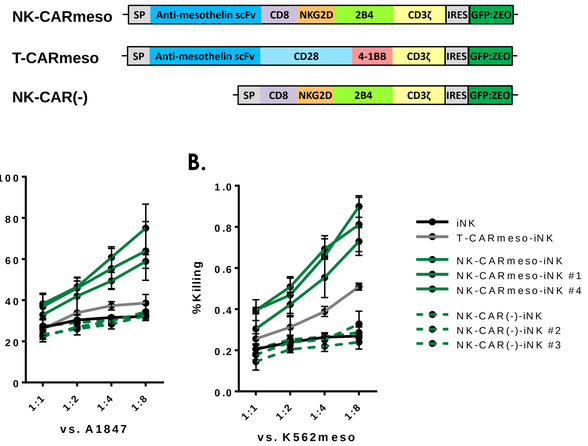

Figure 1. *in vitro* anti-tumor activity of NK-CARmeso expressing iNK cells. NK cells derived from pooled or clonal [NK-CARmeso-iPSC #1/4, CAR(-)-iPSC #2/3], and T-CARmeso were co-cultured with europium-loaded meso-high target cells of meso-high K562meso and A1847 cells at different effector to target ratios.

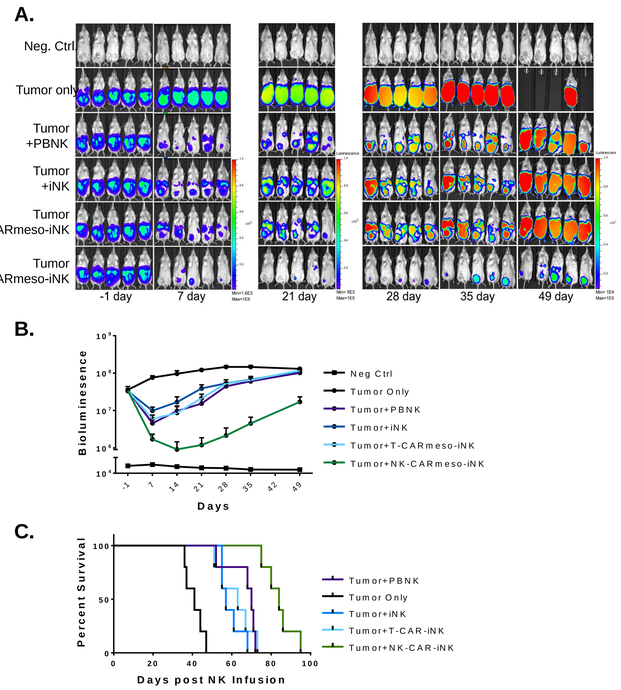

Figure 2. NK-CARmeso-expressing iNK cells demonstrate potent anti-ovarian cancer activity *in vivo*. Luciferase (luc)-expressing A1847 cells (meso^{high}) in a mouse xenograft model were treated with indicated NK cell. NSG mice were inoculated intraperitoneally with 2×10^5 luc$^+$ A1847 cells. 3 days later, mice received 225cGy radiation and 1 day later, one dose of 1.5×10^7 of NK population intraperitoneally. Cytokines hIL-2 and hIL-15 were administrated for the following 21 days. **A**. Tumor burden was determined by weekly bioluminescent imaging (BLI) until day 49. Images of representative time points were shown. **B**. Tumor burden of each group was monitored for 49 days post NK cell infusion. The BLI data is plotted, mean ± S.D are shown. **C**. Kaplan-Meier curve representing the percent survival of the experimental groups, n=5 for all groups: Tumor only, median survival (MS) 41 days; PBNK: MS 70 days; iNK: MS 57 days; T-CAR[28bbz]-iNK: MS 63 days; NK-CAR-iNK: MS 84 days. Statistics: iNK vs. NK-CAR-iNK, P=0.0017, HR=0.2236; T-CAR-iNK vs. NK-CAR-iNK, P=0.0018, HR=0.2153.

NK-CAR19 Expressing iNK Cells Display *in vitro* and *in vivo* Anti-Tumor Activities

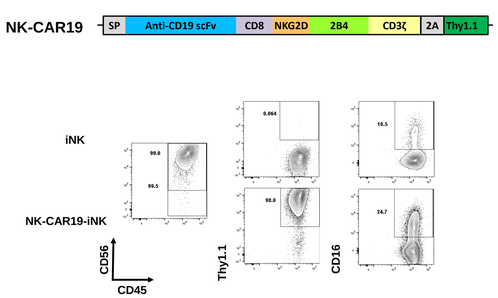

Figure 3. Phenotype of iPSC derived NK cells expressing NK-CAR19. iPSCs were engineered with NK-CAR19, and the Thy1.1+ population was sorted and differentiated into NK cells. NK surface markers CD45, CD56, and CD16, and transgene expression marker Thy1.1 were analyzed by flow cytometry.

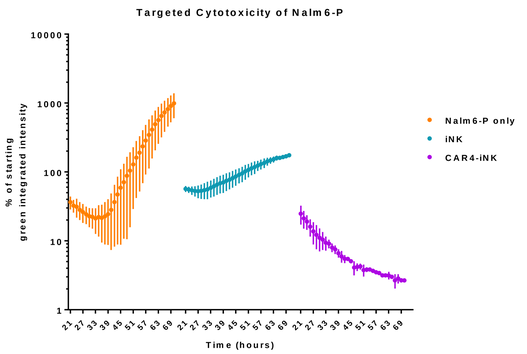

Figure 4. NK-CAR19 expressing iNK cells display improved *in vitro* cytotoxicity against CD-19 expressing tumor lines. NK-CAR19 and parental iNK cells were co-cultured with Nalm6-P cells in 96-well plate at a 1:1 effector-to-target ratio. Following incubation, survival of target cells was quantified by Incucyte Zoom Imaging at indicated time points.

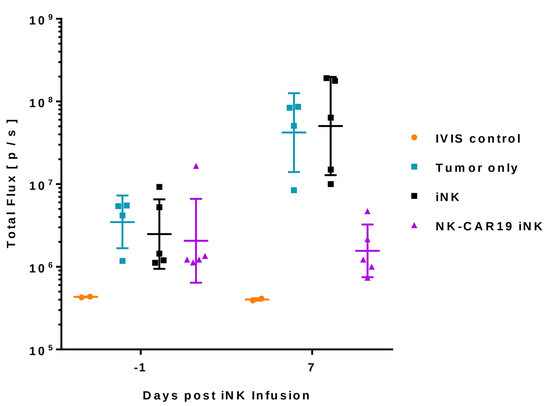

Figure 5. NK-CAR19 expressing iNK cells display effective anti-tumor activity xenograft mouse model (preliminary). NSG mice were inoculated intraperitoneally with $1x10^5$ Nalm6/ffLuc cells. Three days later, mice received one dose of $1x10^7$ of iNK cells intraperitoneally. hIL-2 was administrated twice a week. Tumor burden was determined at day -1 and day 7 by bioluminescent imaging (BLI). Geometric mean of BLI with geometric SD is shown.

IL-15RF Expression Promotes Cytokine-Independence and Improves *in vivo* Persistence of iNK Cells

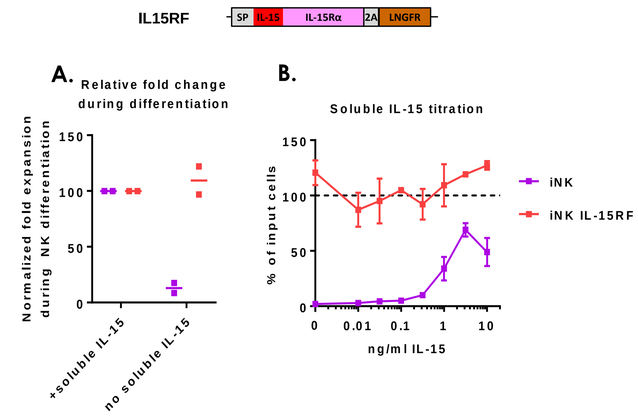

Figure 6. IL-15RF expression promotes differentiation and survival of iNK cells *in vitro* independent of addition of soluble, exogenous IL-15. A. iNK cells of the indicated genotypes were differentiated with or without the addition of of soluble IL-15. IL-15RF expressing cells differentiated equally well in both conditions. **B.** iNK cells were extensively washed and placed back into culture in concentrations of soluble IL-15 ranging from 10ng/ml to 0 ng/ml for 7 days. Expression of the IL-15RF rendered the cells independent of soluble IL-15.

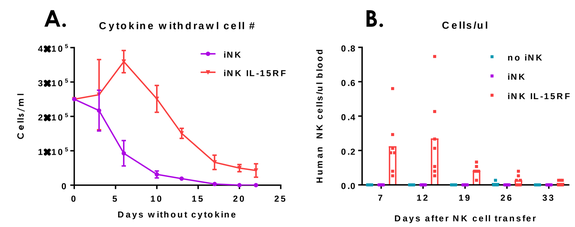

Figure 7. Expression of IL-15RF enhances iNK cells persistence *in vitro* and *in vivo* in the absence of soluble IL-15. **A**. 2.5×10^5 iNK or IL15-RF iNK cells were extensively washed and placed back into culture in the absence of soluble IL-15. Cells were counted at indicated time points. **B**. Eight million iNK or IL15-RF iNK cells were adoptively transferred to immunocompromised NOG mice. Only cells expressing the IL-15RF construct persisted in the absence of soluble IL-15.

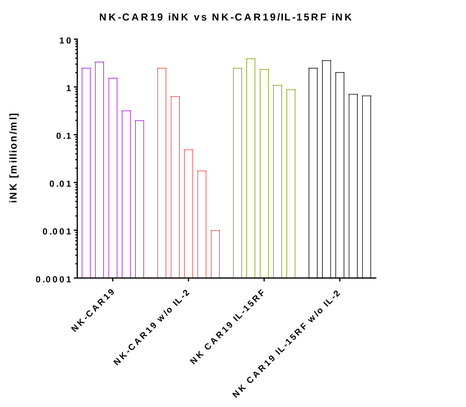

Figure 8. Co-expression of IL-15RF enhances NK-CAR19 expressing iNK cells *in vitro* persistence in the absence of soluble IL-2. NK-CAR19 expressing iNK cells with and without co-expression of IL-15-RF were cultured for 9 days in the presence and absence of 250 U/ml exogenous hIL-2. Expression of IL-15RF eliminated cytokine dependence of the NK-CAR19 iNK cells.