CRISPR/Cas9-gRNA RNP mediated gene knockout of TGFβR2 and CISH enhances CD19-CAR NK cell function and provides resistance to TGFβ

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Introduction

Natural killer (NK) cells are known for their ability to kill tumor cells and represent a promising 'off the shelf' therapeutic for cell-based cancer immunotherapy. One of the major challenges with NK cell-based therapies is their lack of persistence and expansion after adoptive transfer. In addition, factors within the tumor microenvironment may impede tumor infiltration and inhibit the activity of NK cells. To improve NK cell function, we developed an optimized CRISPR/Cas9 system utilizing electroporation of CRISPR-gRNA ribonucleoproteins (RNPs) to disrupt important regulatory genes to enhance and/or protect primary human NK cell function. This method allowed efficient knockout of the TGFβR2 and CISH genes in NK cells. FACS and Amplicon NGS Sequencing data confirmed both genes were successfully disrupted, and each knockout had measurable impacts on NK cell function. Furthermore, we were able to transduce and expand CRISPR/Cas9 edited NK cells to express a CD19-CAR construct and a membrane-bound form of IL-15 (mbIL15). Cytotoxicity assays demonstrated that TGFβR2 knockout CD19-CAR NK cells were resistant to TGFβ inhibition of cytotoxicity. We also showed that CISH gene-edited CD19-CAR NK cells have significantly improved proliferation, survival, cytokine production, and cancer cell cytotoxicity. In summary, we show here that efficient primary human NK cell gene knockouts can enhance CAR NK function and provide resistance to tumor microenvironment inhibition.

Methods

NK cells were generated by co-culture of peripheral blood mononuclear cells (PBMCs) with irradiated K562 stimulatory cells genetically modified to express mbIL-15 and 4-1BBL. NK cells were transduced at a MOI of 2-3 with a γ-retrovirus encoding a CD19-CAR and mbIL-15. NK expansion and CAR expression were evaluated by flow cytometry. *In vitro* cytotoxicity of transduced NK cells was measured using both flow cytometry and the IncuCyte S3 live cell analysis system. Primary human NK cells were electroporated under selected conditions with Cas9-RNP complexes targeting human TGFβR2 or CISH. Deletion efficiency was assessed 3-4 days post-electroporation by FACS or western blot.

Results

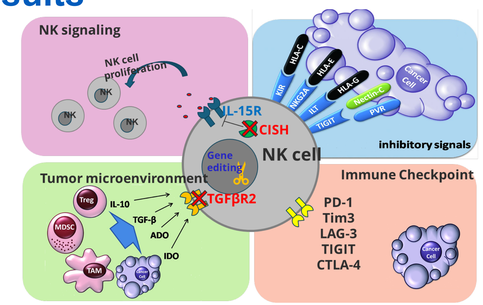


Figure 1. CISH and TGFβR2 were chosen as knockout targets to assess CAR NK cell function (image partly adapted from Li et al, 2018)

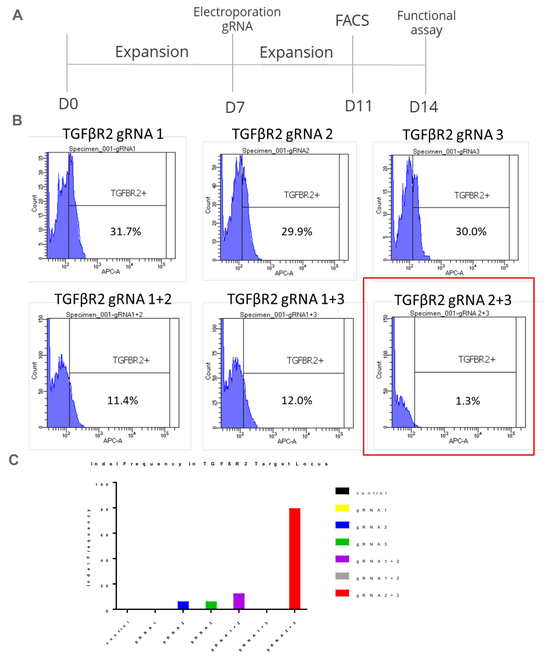
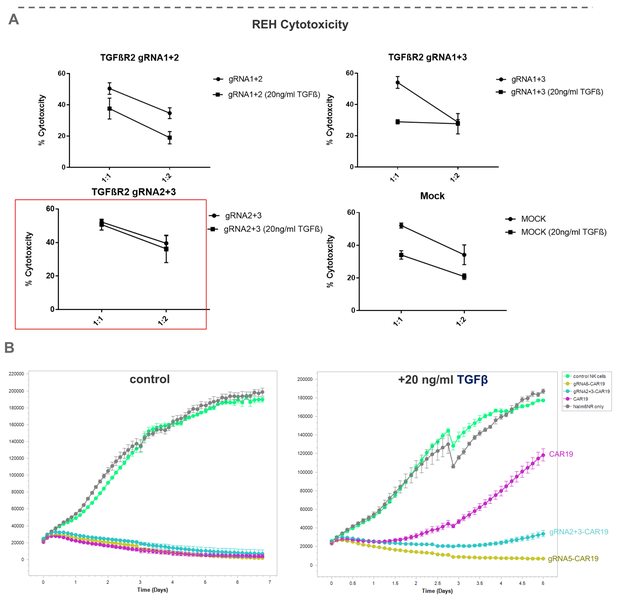


Figure 2. Cas9-RNP results in efficient TGFβR2 gene deletion in primary human NK cells. (A) Schematic timeline of TGFβR2 gene editing process for primary human NK cells. (B) Deletion efficiency of TGFβR2 on activated primary human NK cells using different gRNAs and combinations. TGFβR2 FACS staining was performed 3 days post-electroporation. (C) Indel frequency of different TGFβR2 gRNA/RNP based on targeted NGS of primary human NK cells. TGFβR2 gRNA2+3 shows best deletion efficiency (79.7%).



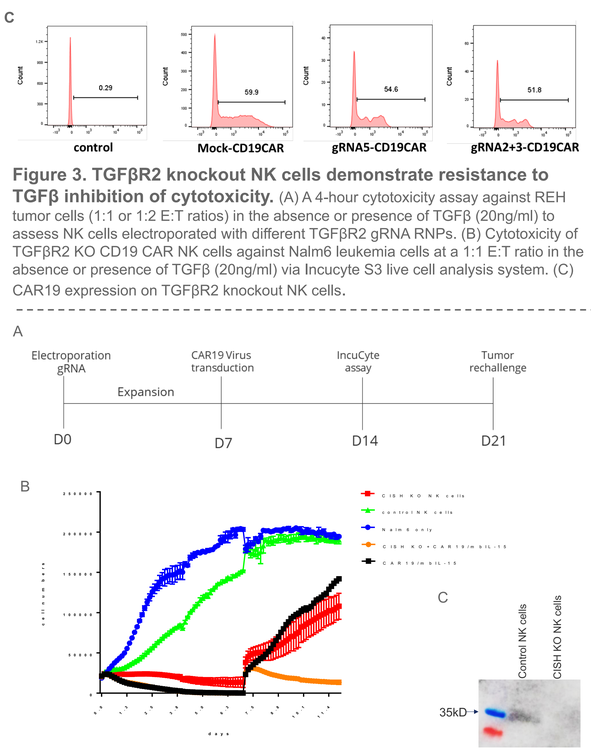


Figure 3. TGFβR2 knockout NK cells demonstrate resistance to TGFβ inhibition of cytotoxicity. (A) A 4-hour cytotoxicity assay against REH tumor cells (1:1 or 1:2 E:T ratios) in the absence or presence of TGFβ (20ng/ml) to assess NK cells electroporated with different TGFβR2 gRNA RNPs. (B) Cytotoxicity of TGFβR2 KO CD19 CAR NK cells against Nalm6 leukemia cells at a 1:1 E:T ratio in the absence or presence of TGFβ (20ng/ml) via Incucyte S3 live cell analysis system. (C) CAR19 expression on TGFβR2 knockout NK cells.

Figure 4. CISH knockout increases cytotoxicity of CAR19-NK cells. (A) Schematic timeline for combining CD19 CAR expression and CISH gene KO for primary human NK cells. (B) Cytotoxicity of engineered or control NK cells measured against Nalm6 leukemia cells at a 1:1 E:T ratio. At day 7, a second bolus of Nalm6 was added. (C) Western blot for CISH protein from control or CISH KO NK cells.

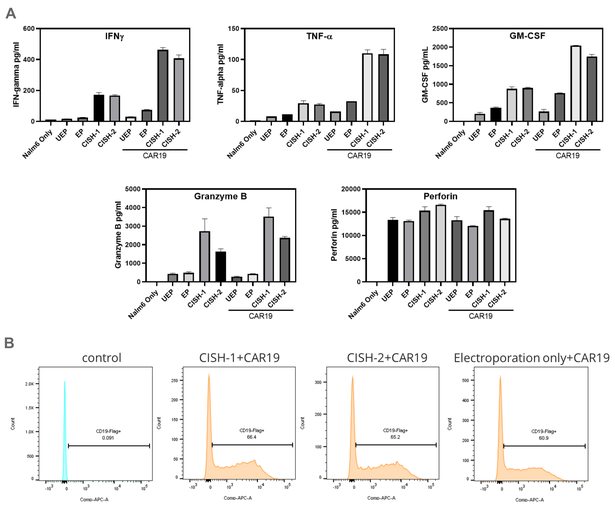


Figure 5. CISH knockout increases secretion of cytokines and cytotoxic proteins from CAR19-NK cells. (A) Luminex analysis of CISH KO CD19-CAR NK pro-inflammatory cytokine release in response to 72h co-culture with Nalm6 leukemia cell line at a 1:1 E:T ratio. (B) CAR19 expression on CISH knockout NK cells.

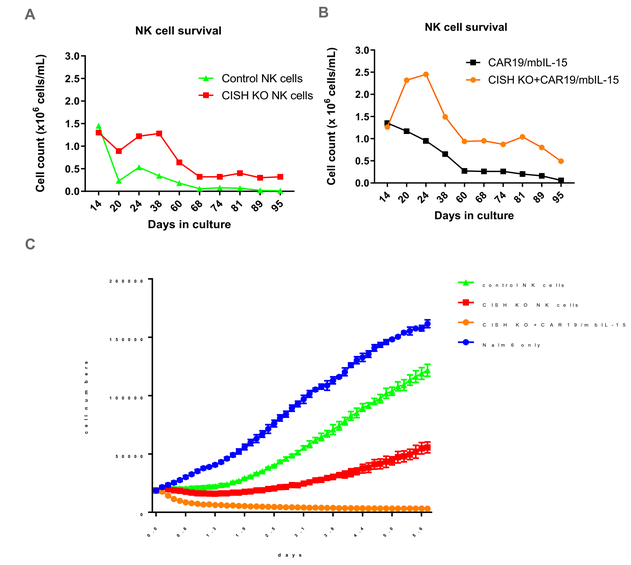


Figure 6. CISH knockout dramatically enhances NK cell persistence and activity. (A) CISH knock out allows NK cell persistence and maintained activity during prolonged culture in low IL-2 (40 IU/mL). (B) Effects are more pronounced in the presence of CAR19/mbIL-15. (C) Cytotoxicity of engineered or control NK cells against Nalm6 leukemia cells at a 1:1 E:T ratio. NK cell activity was measured after 100 days culture (control NK cells prepared fresh from same donor).

Conclusion

In summary, we have shown that efficient gene knockouts in primary human NK cells can be performed at multiple points in the culture and expansion of the cells, and can enhance NK function and provide resistance to tumor microenvironment inhibition.

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