# A combination of CAR-NK and CAR-T cells results in rapid and persistent anti-tumor efficacy while reducing CAR-T cell mediated cytokine release and T-cell proliferation

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## Introduction

CAR-NK cells show rapid and potent anti-tumor cytotoxic activity even at very low effector-to-target ratios; however, they do not typically expand upon encounter with antigen and lack the long-term persistence commonly associated with autologous CAR-T cells. Conversely, CAR-T cells generally exhibit persistent killing activity, even though they are activated more slowly. In addition, the explosive expansion of CAR-T cells is frequently associated with a variety of toxicities (e.g. CRS, CRES, etc.). To address the potential shortcomings associated with either cell type, we have assessed a novel platform that combines CAR-NK and CAR-T cells as a novel cancer therapeutic.

## Methods

NK and T cell were expanded separately to allow evaluation of varying cell ratios. CAR19-NK and –T cells were made by transduction with bicistronic retroviral construct encoding CD19 CAR (αCD19 scFv-CD8αTM-OX40 - CD3ζ) and membrane-bound IL15.

To evaluate the overall outcomes of combining CAR-NK and CAR-T cells as an anti-tumor therapy, we have developed two novel *in vitro* assays:

(a) IncuCyte-based matrixed cytotoxicity assay, which allows direct assessment of tumor killing kinetically with different E:T and NK:T ratios;
(b) IncuCyte-based tumor re-challenge assay, which provides a convenient and quick assessment of anti-tumor persistence. Additionally, we measured a panel of cytokines from these two assays by LEGENDplex.

To further investigate the cellular mechanism(s) underlying the effects of combining CAR-NK and CAR-T cells, we performed flow cytometry-based phenotyping and a Violet Cell Trace-based proliferation assay.

Finally, we evaluated the anti-tumor efficacy of the NK plus T platform *in vivo* using a CDX tumor model in immunodeficient NSG mice.

## Results

### Combining of CAR19-NK and CAR19-T cells enhances cytotoxic outcome and kinetics

As shown in **Figure 1**, 20K/well Nalm6-NucRed target cells were seeded in a 96-well plate. Different ratios of CAR19-T and CAR19-NK effector cells to targets cells were established as indicated in (**A**) and (**B**). After 7 days of culture in an IncuCyte assay, combinations of NK and T cells that showed greater cytotoxicity than NK or T cells alone are indicated by the boxed green area (**A**). Additionally, the combination of the two cell types showed more rapid killing even at very low E:T ratios as shown in the red boxed areas (**B**).

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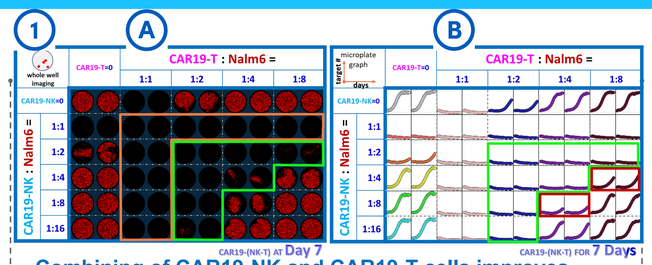


### Combining of CAR19-NK and CAR19-T cells improves cytotoxic persistence

As shown in **Figure 2**, during the first round of tumor challenge, CAR19-T cells alone at the indicated E:T ratio failed to arrest tumor growth, whereas CAR19-NK cells alone or in combination reduced tumor counts. However, following a second round of tumor challenge, CAR19-NK cells alone at these E:T ratios were no longer able to control tumor growth, whereas only the combination of both could arrest tumor growth after 14 days of culture.

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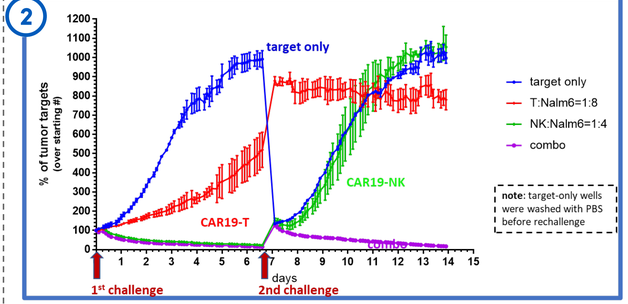


### Combining CAR19-NK with CAR19-T cells lowers the accumulation of cytokines associated with CRS

Different ratios of CAR19-T and CAR19-NK effector cells to Nalm6 targets were established as in Figure 1. At day 5, culture media were collected for cytokine measurement by LEGENDplex. Data from donors #103 (pink) and #452 (green) are shown in **Figure 3**. The levels of key mediators of CRS (e.g. GM-CSF, IL6) were donor-dependent, CAR19-T:target ratio dependent, and inversely correlated with CAR19-NK:target ratio.

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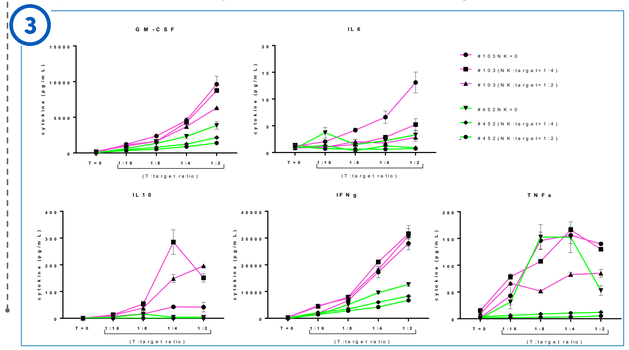


### Combining CAR19-NK and CAR19-T cells reduces CAR19-T cell proliferation and promotes CAR19-NK cell expansion

CAR19-NK and CAR19-T cells were labeled with Violet Cell Trace and then incubated for 6 days with Nalm6 at various ratios. As shown in **Figure 4,** in the absence of CAR19-NK cells, the majority of CAR19-T cells proliferated regardless of E:T ratio. In the absence of CAR19-T cells, CAR19-NK cells exhibited little to no proliferation. However, when CAR19-T cells and CAR19-NK cells were co-cultured with Nalm6, CAR19-T cell proliferation decreased with increased presence of CAR19-NK cells (**A**), and CAR19-NK cell proliferation was induced with an increased presence of CAR19-T cells (**B**). A cellular model is proposed for the mutual impact of CAR19-T and CAR19-NK cells upon encounter with CD19+ Nalm6 tumor cells (**C**).

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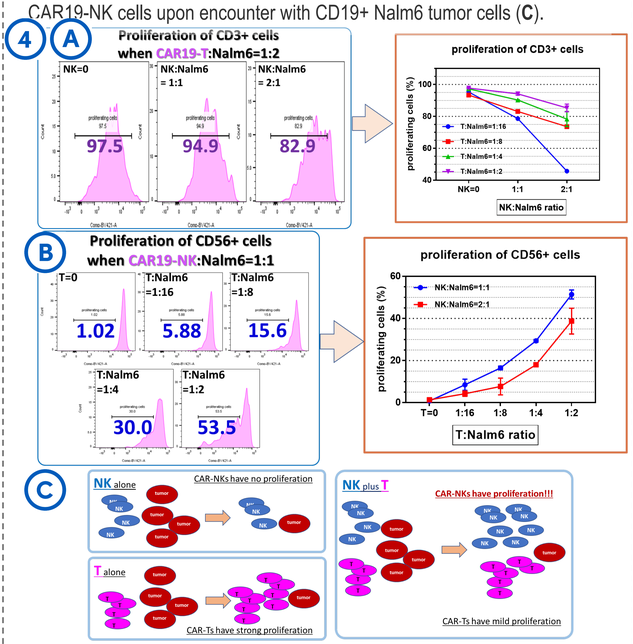


### Combining CAR19-NK and CAR19-T cells in an *in vivo model* improves anti-tumor efficacy and survival

As shown in **Figure 5A**, a a xenograft tumor model was established in immunodeficient NSG mice by tail vein injection of Nalm6.ffluc cells ($2x10^5$/animal). Treatment was performed at day 3 post tumor injection with CAR19-NK cells ($2.5x10^6$/animal) alone, or in combination with either a low dose ($5x10^5$/animal) or a high dose of CAR19-T cells ($2.5x10^6$/animal). Additional control arms were also included (**Figure 5B**). At day 121, 4/5 animals in CAR19-NK and high CAR19-T combo group survived and stayed tumor-free, as compared to only 1/5 in the high CAR19-T alone group (**Figure 5B-D**).

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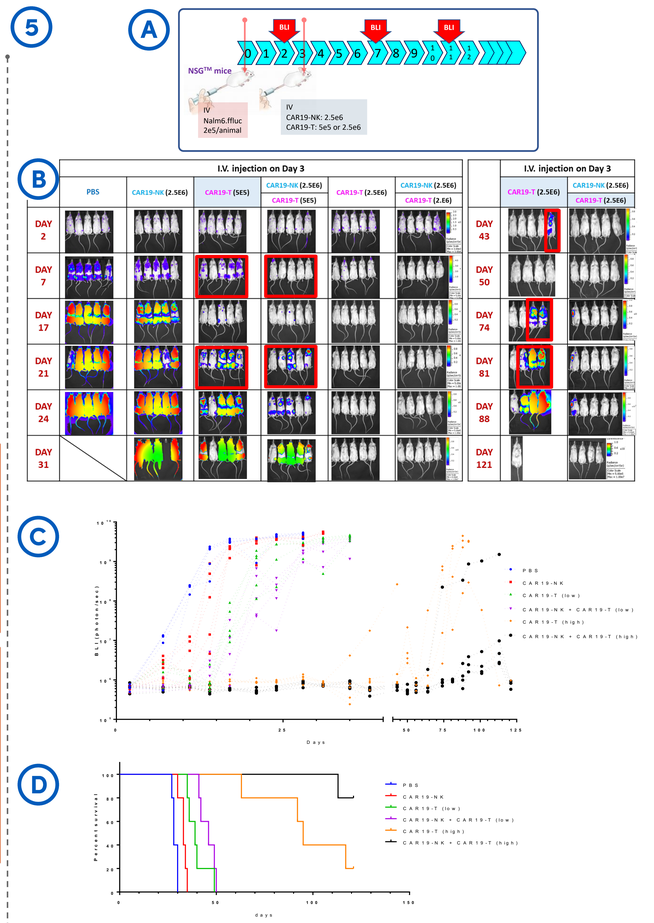


## Conclusion

Proof-of-concept studies using both innate and adaptive immunity with CAR19-NK and CAR19-T cells demonstrate that the combination of both can:

- Enhance anti-tumor cytotoxicity (kinetically and cumulatively) and persistence.
- Improve the general safety profile of cytokine release.
- Promote NK cell expansion but reduce antigen dependent proliferation of T cells
- Prevent tumor relapse in a Nalm6 CDX model for at least four months.

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