Pooled Screening For CAR Signaling Optimizes Function In NK Cells

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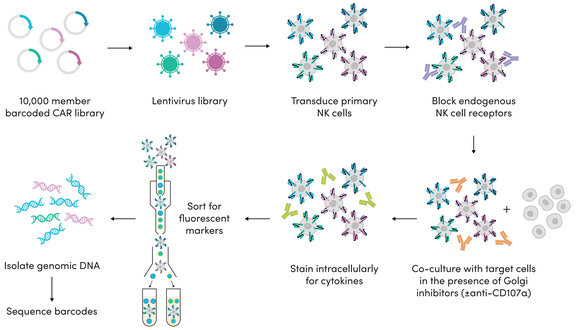
Introduction

NK cells are an attractive alternative to T cell-based therapies because they are capable of producing similar levels of anti-tumor cytotoxicity and are a potentially universal source for "off-the-shelf" CAR therapies with lower incidence of cytokine release syndrome. However, exploration of activating signals used for CAR-NK cell architectures has been fairly limited, with the majority of inputs being derived from T cell or NK cell receptors. **We hypothesized that a more systematic exploration of diversified CAR signaling in NK cells could enhance their antitumor function and expedite their utility beyond hematological malignancies.**

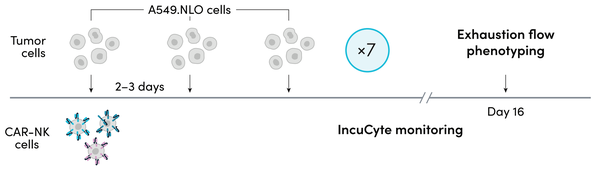
To address this, we evaluated 10,000 unique signaling domain combinations in CAR-NK cells. We expressed our previously reported library of anti-ROR1 2nd generation CARs with diversified signaling domains in human primary peripheral NK cells, which we then screened for cytotoxicity and cytokine release following endogenous NK cell receptor blockade and solid tumor challenge by sorting for degranulation markers and intracellular cytokines.

Screening workflow



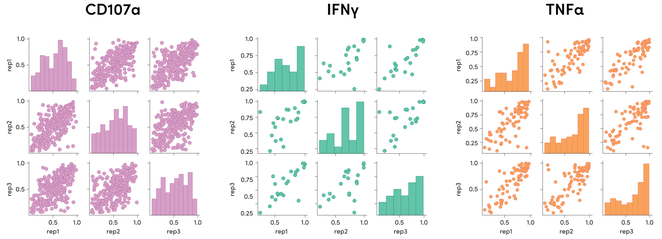
Validation methods

Same as screening, but flow cytometry analysis instead of sorting and sequencing
Additional tumor rechallenge validation workflow for assessing long-term tumor control:

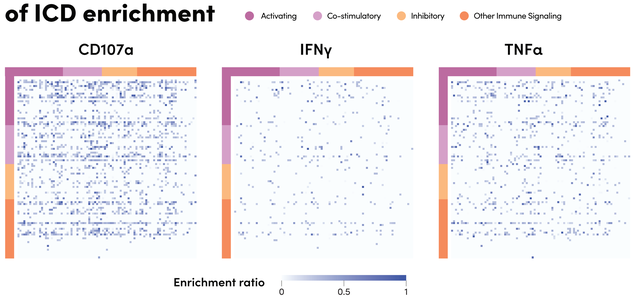


ICD Legend — **B**-cell associated; **CD3** variant; **M**yeloid-associated; **NK** cell-associated; **T** cell-associated; **TNFRSF** member

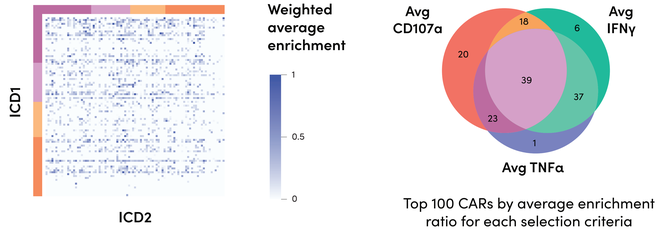
SortSeq produces reproducible enrichment



Different selection criteria produce distinct patterns of ICD enrichment



CAR-NK screening can enrich CARs that produce polyfunctional phenotypes

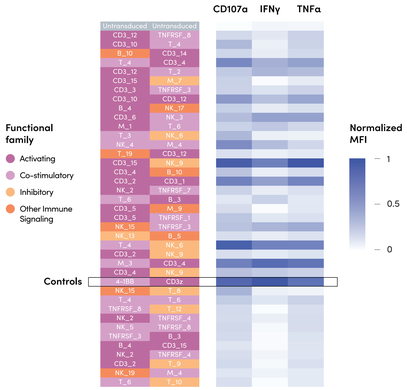


Top 100 CARs by average enrichment ratio for each selection criteria

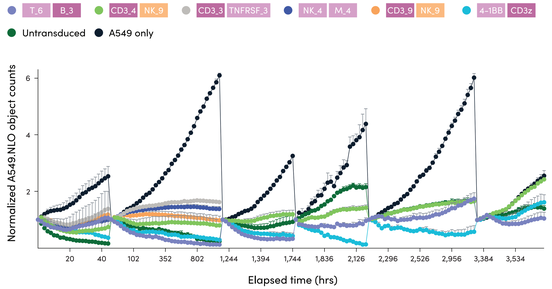
Validation set

Polyfunctionally enriched hits were selected for validation based on a weighted enrichment ratio metric, defined as the frequency within the selected population relative to the sum of the frequencies in the selected and unselected population for each CAR. Ratios were weighted 4:2:1 using the average CD107a, IFNγ, and TNFα enrichment ratios.

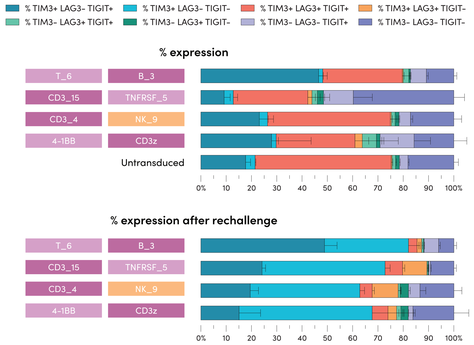
Novel CARs exhibit potent cytotoxicity and cytokine release upon tumor challenge



Novel CAR-NKs outperform BBz upon long-term rechallenge



Novel CARs show variable exhaustion profiles following rechallenge



Conclusion

Here, we demonstrate that selection for anti-tumor function in CAR-NK cells identifies active CARs that produce polyfunctional anti-tumor phenotypes. We found hits that were enriched across multiple selection criteria, with most containing activating and costimulatory domains from various immune cell types. Notably, the membrane proximal position drove stronger enrichment of these signaling domains, while the membrane distal position showed overall lower domain preference with the exception of 5 costimulatory domains. Furthermore, screening for IFNγ secretion was the most stringent selection criteria and produced the lowest levels of enrichment, while screening for CD107a was the least stringent.

Many CAR signaling domain combinations produced higher levels of CD107a, IFNγ, and TNFα expressing cells relative to untransduced (UTD) NK cells, which can respond to tumor detection through endogenous NK cell receptors, when subjected to tumor exposure for 6 hours. Several CARs were capable of eliciting comparable degranulation and cytokine secretion to that of an FDA-approved 4-1BB harboring second-generation CAR.

Additionally, several CARs were capable of long-term tumor control upon repeated co-culture, an elusive phenotype in NK cells given their inherently short lifespans, with one being on par with current clinical standards. Notably, we found that long KIR receptor signaling in combination with ITAM-harboring domains produced especially potent phenotypes in NK cells.