Development of multi-omics approaches to evaluate NKG2D-ligand dynamics and anti-tumor immune responses during CAR-NK treatment

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Introduction

NK cells are highly potent, fast-acting cytolytic cells capable of eradicating cancer with limited risk of adverse effects such as cytokine release syndrome or GVHD. NKX101 is a healthy donor derived NK cell therapy product engineered to overexpress a chimeric receptor consisting of NKG2D ectodomain, costimulatory signaling motifs, and a membrane-bound form of IL-15. NKX101 is currently under clinical evaluation for treatment of relapsed/refractory AML and high risk MDS. To better understand patterns of response to NKX101, we describe the development of (i) a single-cell (sc) RNAseq approach to assess gene expression pattern changes in NKX101 and patient cells, (ii) a multiplex IHC panel to monitor NKG2D-ligand expression by cancer cells, and (iii) an ELISA method to detect shed NKG2D-ligand in serum.

Methods

To verify that our scRNAseq approach can distinguish NKX101 product cells from patient cells, healthy human PBMCs were pre-mixed with NKX101 cells at set ratios. 10x Genomics hybrid capture baits were prepared to enrich for exogene reads first via single-cell 5' gene expression assays and further processed for target enrichment using a human immunology gene set panel. Computational tools were developed to interrogate datasets from single-cell targeted gene expression. Multiplex IHC was carried out using the Vectra Polaris Phenoptics platform. FFPE biopsy samples were stained with an 8-marker multiplex panel. Digital image analysis was conducted using the inForm analysis software. For the detection of shed NKG2D-ligands, an in-house sandwich ELISA was developed using recombinant human NKG2D to capture followed by detection using ligand-specific antibodies and HRP-conjugated secondary antibodies.

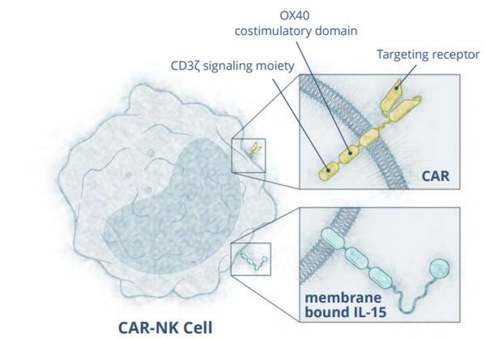
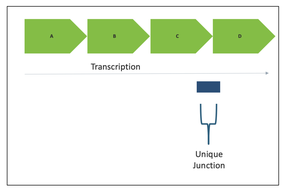


Figure 1. Diagram of a healthy donor derived CAR-NK cell.

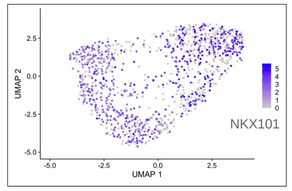
Results

NKX101 product cells were successfully separated from host PBMC cells using scRNAseq methods. NKX101 cell proportions in the transcriptome datasets correlated with the expected titration ratios against host PBMC, with samples titrated at 25% NKX101 cells yielding approximately 25% NKX101 exogene expressing cells by scRNAseq. Using multiplex IHC and digital image analysis, we quantified NKG2D-ligand expression levels in AML and hepatocellular carcinoma (HCC). MICA/MICB$^+$ and ULBP1/3$^+$ cells were found to be enriched in AML bone marrow compared to normal age-matched controls. In solid tumors, NKG2D-ligands were found to be upregulated on HCC biopsy samples and co-expressed with tumor marker PanCK. Finally, we determined that certain commercial kits for the detection of soluble NKG2D ligands potentially under-report NKG2D-ligand concentrations due to their propensity for aggregation. Using recombinant NKG2D as a capture reagent, we developed an in-house sandwich ELISA method to detect NKG2D-ligands shed in cell culture supernatant or blood serum.

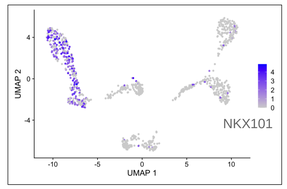
Hybrid-capture scRNAseq can quantitatively distinguish NKX101 cells from background PBMCs



A. CAR Transcription Module



B. NKX101 at 50% Titration



C. NKX101 at 25% Titration

% Expected Titration	% NKX101 Cells Identified in UMAP plot
50%	53%
25%	22%

D. NKX101 Quantification

Figure 2. A) Domains of the NKX101 chimeric construct are homologous to the human NKG2D endogenous sequence. To distinguish NKX101 cells from patient background cells, targeted baits across unique junctions of the chimeric receptor were designed and tested in two titration experiments. **B-C)** Dimensional-reduction representations of normalized chimeric receptor gene expression from samples with 50% (B) and 25% (C) NKX101 cells titrated into PBMCs. Relative expression levels of the NKX101 chimeric receptor is represented with purple/violet gradient **D)** Expected titration percentages of NKX101 cells compared to actual percentages recovered by hybrid-capture ssRNAseq.

Expression of NKG2D-ligands in AML and normal bone marrow can be quantified by IHC

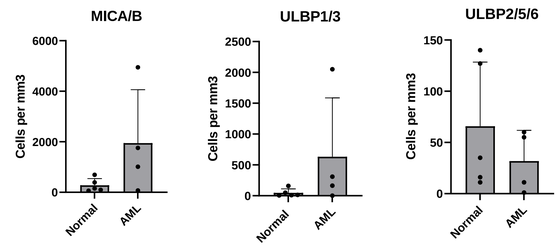


Figure 3. Cell densities of NKG2D-ligand positive cells in AML and normal bone marrow. Image data was analyzed using inForm digital image analysis software.

NKG2D-ligands are expressed at higher levels in HCC compared to matched normal liver samples

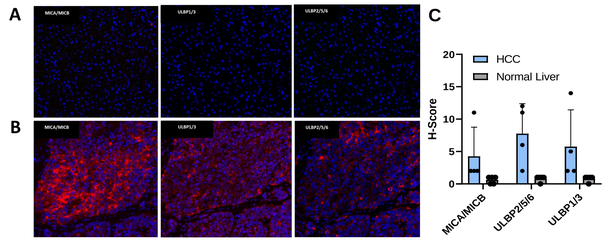


Figure 4. A-B) Representative IHC images of NKG2D-ligand expression (red) and DAPI (blue) in normal liver (A) and HCC samples (B). **C)** Histology scores (H-scores) from four HCC samples (light blue) and five normal liver samples (gray). Error bars represent standard deviation. **D)** Representative images of HCC samples stained with antibodies for NKG2D-ligands (red), PanCK (green), and DAPI dye (blue). Merged images are overlays of NKG2D-ligand, PanCK, and DAPI staining.

Commercial NKG2D-ligand immunoassays likely under-report shed NKG2D-ligands in their natural form

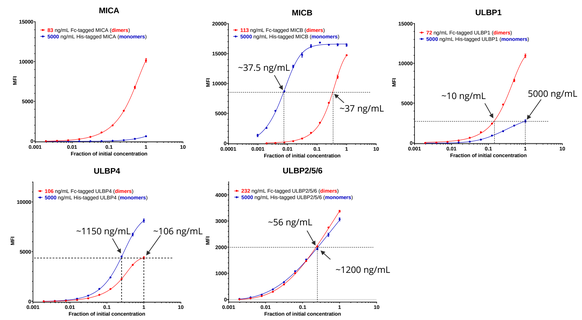


Figure 5. Accuracy assessment of a commercial Luminex assay. Known concentrations of His-tagged NKG2D-ligands relative to those of the Fc-tagged protein standards included in the kit were used for the assessment.

NKG2D-ligand ELISAs developed in-house can detect shed NKG2D-ligands in MDS sera

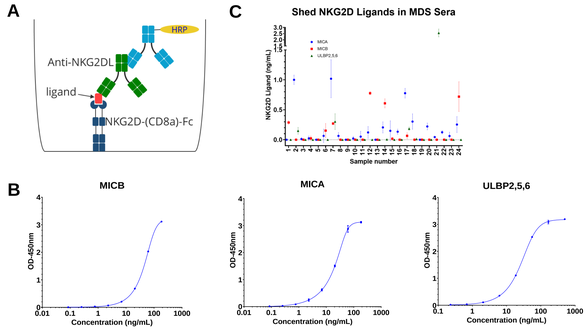


Figure 6. A) Depiction of the ELISA method used to detect shed NKG2D-ligands. **B)** Standard curves generated using His-tagged recombinant proteins. **C**) Shed NKG2D-ligand levels in commercially purchased MDS sera from patients with unknown medical histories.

Conclusion

In summary, we have developed several methods to better understand patient responses to CAR-NK cell therapy. First, we developed a scRNAseq approach capable of identifying NKX101 cells in a mixture of PBMCs. Employing multiplex IHC and digital image analysis, we found that membrane bound NKG2D-ligands are upregulated in AML and HCC compared to age-matched normal tissue controls. Lastly, using in-house developed ELISAs, we determined that shed NKG2D-ligands can be successfully detected in serum isolated from patients with MDS. Taken together, these assays provide methods for evaluation of NKG2D-ligand dynamics as well as the detection and phenotypic analysis of CAR-NK and immune cell populations in clinical samples.

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