

Abstract #

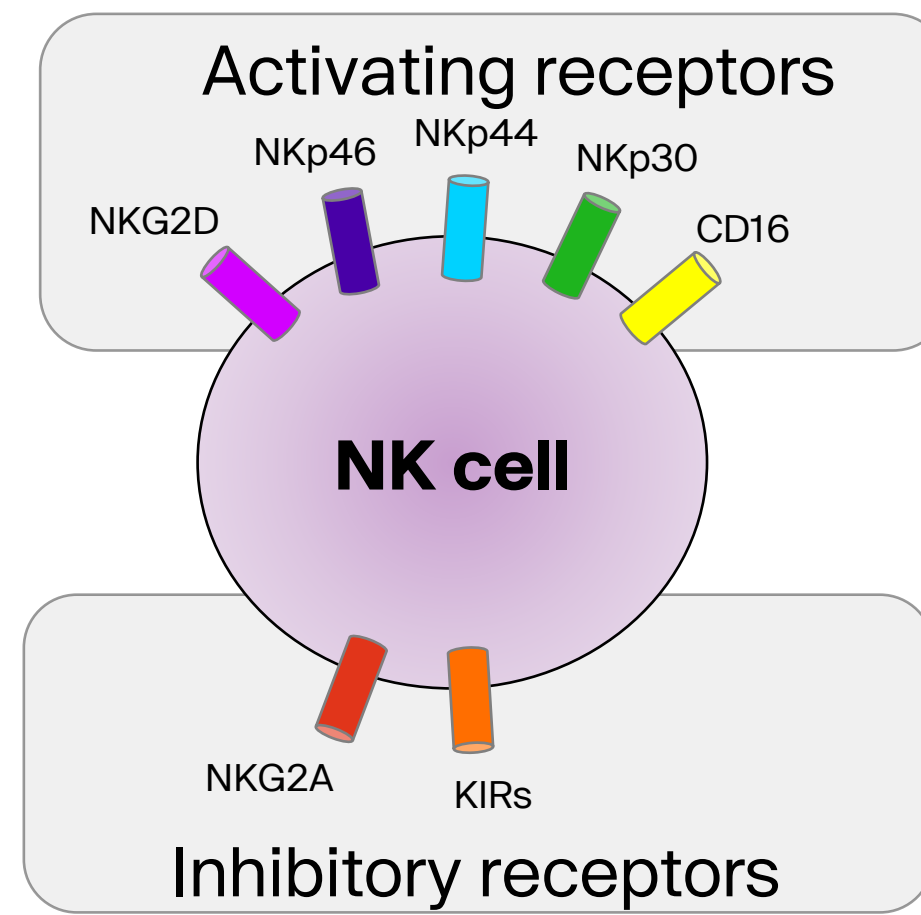
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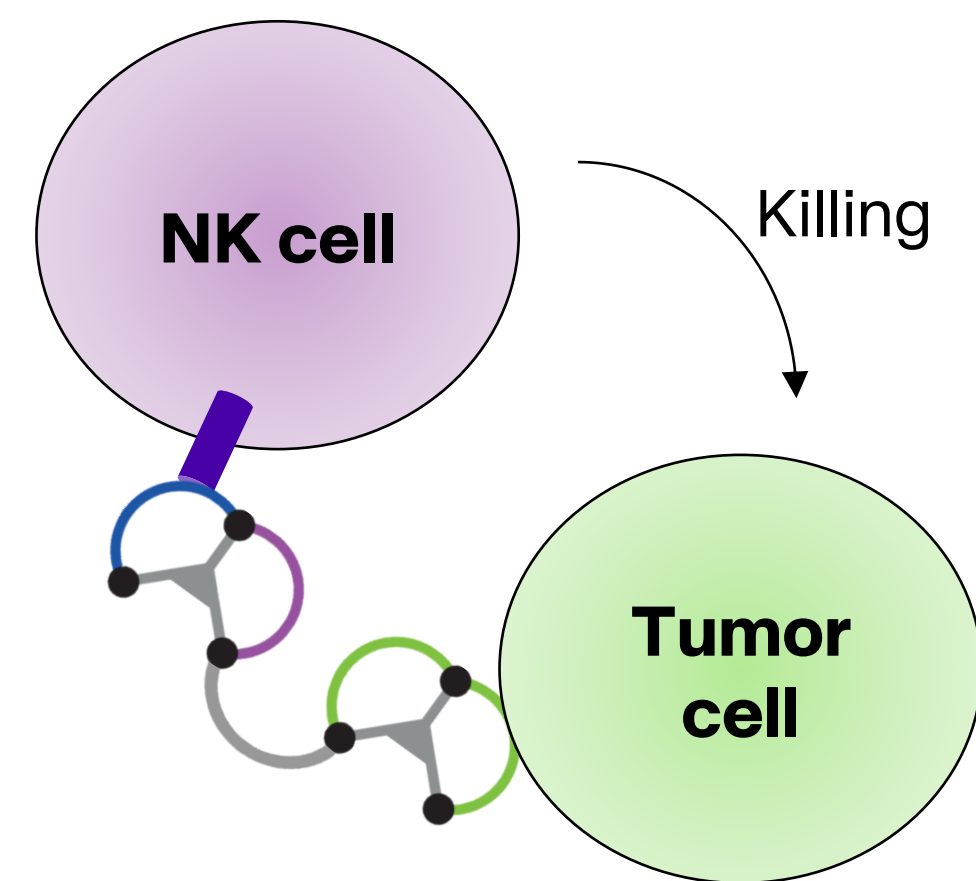
## INTRODUCTION

Natural Killer (NK) cells are cytotoxic cells of the innate immune system with well characterized anti-tumor properties. Their ability to directly kill malignant cells and elicit an adaptive immune response makes them a promising candidate for a precision guided immunotherapy for cancer patients.

**RIGHT ▶**  
The major activating and inhibitory receptors expressed on the surface of NK cells. Abbreviations: CD, cluster of differentiation; KIRs, killer cell immunoglobulin-like receptors.



A target expressed on NK cells, NKp46 is a key activating receptor contributing to cytolytic function of NK cells. Bicycle® peptides are small (~1.5-2 kDa), chemically synthetic, structurally constrained bicyclic peptides discovered using phage display. NKp46-binding Bicycles conjugated to a tumor antigen-binding Bicycle® directed human NK cells to kill the tumor cells expressing the target antigen, and we term these molecules NK tumor-targeted immune cell agonists (NK-TICA™).



**LEFT**  
NK-TICA™ is composed of NKp46 targeting Bicycle® conjugated to a tumor antigen-binding Bicycle®. It is designed to cross-link NK cell and a tumor cell via binding to NKp46 on the NK cell surface and to an antigen on the tumor cell surface. This cross-linking triggers NK cell-mediated killing of the target cell.

The use of humanized animal models in preclinical evaluation of immunotherapeutics is often dictated by lack of homology between mouse and human target or divergence in the biology of studied immune cell subtypes. Due to low homology of NKp46 ectodomain between mouse and human (~63% by NCBI BLAST), we evaluated different approaches to animal humanization to establish the most suitable model for in vivo evaluation of NK-TICA™. The assessment of the models was mainly based on the number of circulating and tumor-infiltrating NKp46+ NKs as well as expression of activating and inhibitory receptors on these cells.

## METHODS

### Summary of in vivo models assessed for NK-TICA™ preclinical studies

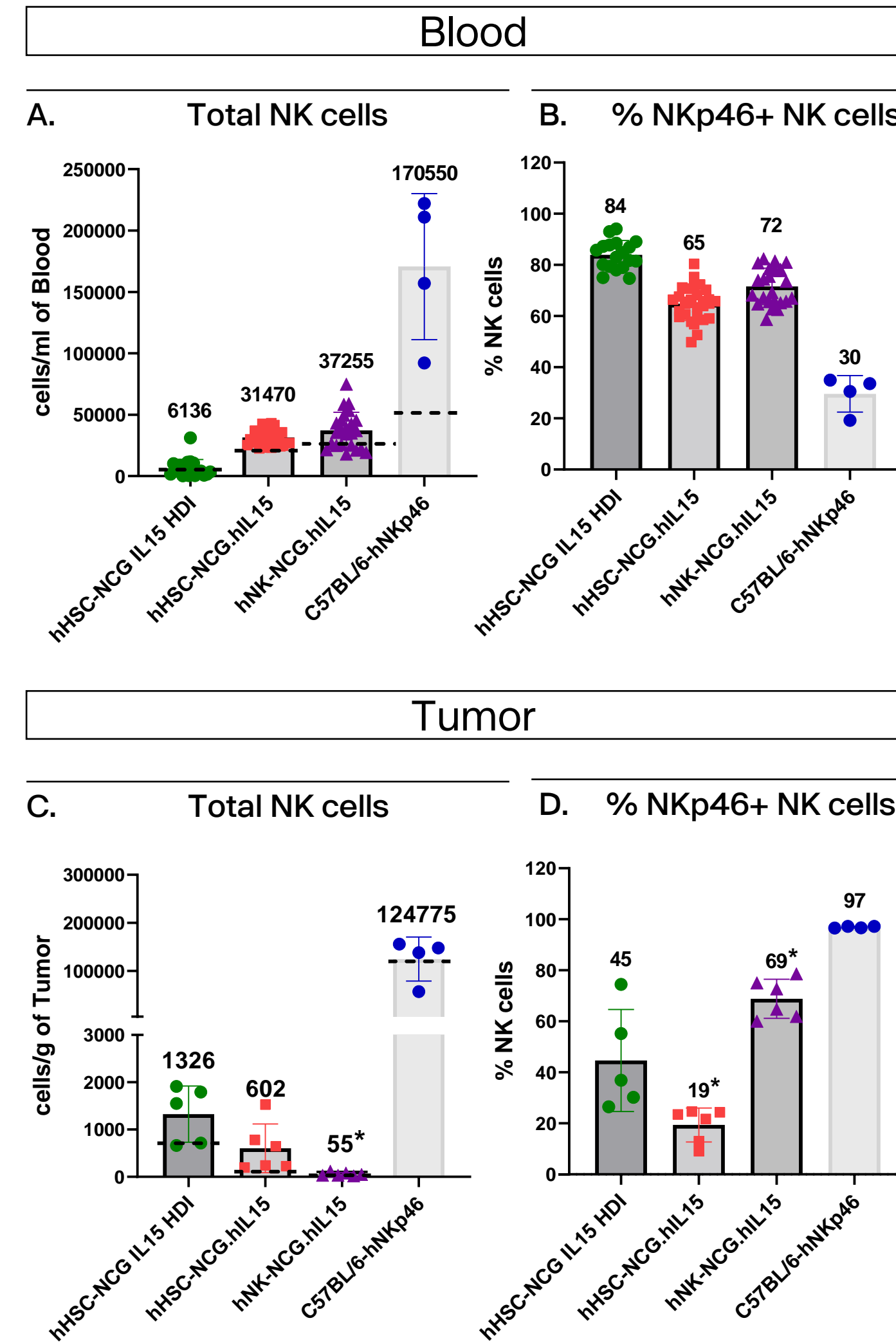
| No | Strain         | Tumor model type | NK source          | hIL-15 supplementation |
|----|----------------|------------------|--------------------|------------------------|
| 1  | NCG            | xenograft        | human CD34+ HSC    | Yes                    |
| 2  | NCG-hIL-15     | xenograft        | human CD34+ HSC    | Yes                    |
| 3  | NCG-hIL-15     | xenograft        | human PBMC         | Yes                    |
| 4  | C57BL/6-hNKp46 | syngeneic        | mouse (endogenous) | No                     |

Description of the humanized models:

- 1) hHSC-NCG IL15 HDI (1,2,3)** – 4 weeks-old females were engrafted with human CD34+ HSC derived from cord blood from 3 donors. 15 weeks post engraftment, mice received a single 10-second IV hydrodynamic injection (HDI) of 2ml solution containing plasmid DNA encoding hIL-15 that peaks at ~30 pg/ml 1 week post HDI and lasts for up to 2 weeks (unpublished data). FACS analysis of blood samples was performed 1 week post HDI (Day 0) on mice bearing A431 subcutaneous tumors (Avg. ~200mm<sup>3</sup>).
- 2) hHSC-NCG.hIL15 (3,4)** – 4 weeks-old females were engrafted with human CD34+ HSC derived from cord blood from 2 donors. These mice have constitutive transgenic expression of human IL-15 at plasma conc. ~100pg/ml. FACS analysis of blood samples was performed 13 weeks post HSC engraftment (Day 0) on mice bearing A431 subcutaneous tumors (Avg. ~100 mm<sup>3</sup>).
- 3) hNK-NCG.hIL15 (4,5)** – 6 weeks old females were IV infused with 2x10<sup>6</sup> NK cells purified using magnetic beads from human PBMC from 2 donors. These mice have constitutive transgenic expression of human IL-15 at plasma conc. ~100pg/ml. FACS analysis of blood samples was performed 2 weeks post NK cell infusion (Day 0) on mice bearing A431 subcutaneous tumors (Avg. ~50mm<sup>3</sup>).
- 4) C57BL/6-hNKp46 (6)** – 8-10 weeks old immunocompetent females with a knock-in of human NKp46 extracellular domain were subcutaneously engrafted with MC38 syngeneic tumors. FACS analysis of blood samples was performed 2 weeks later (Day 0) when the tumors reached (Avg. ~300mm<sup>3</sup>).

## RESULTS

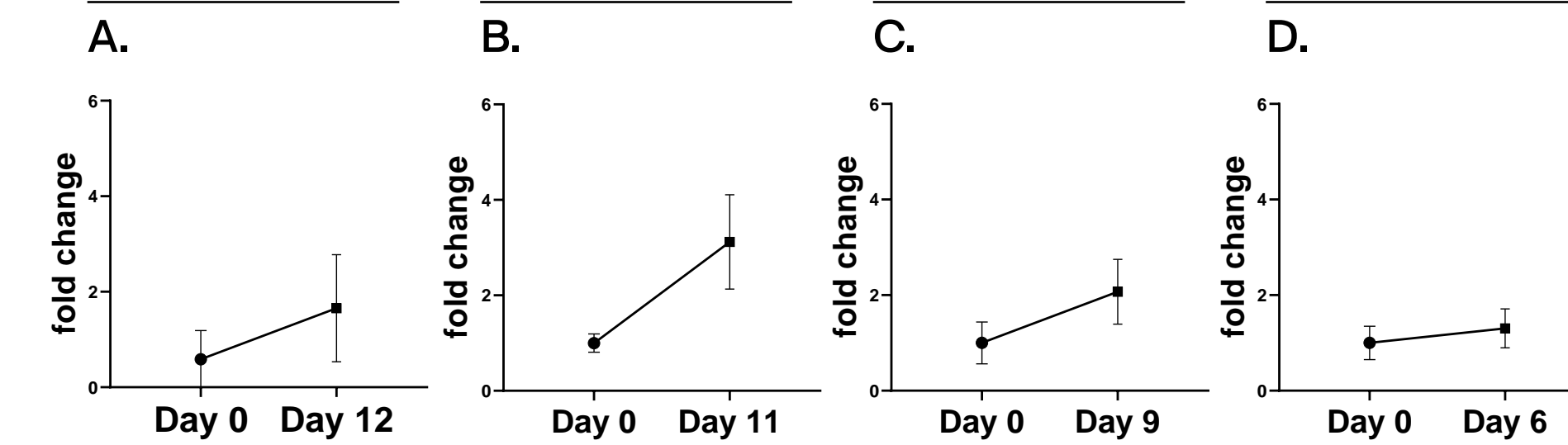
### C57BL/6-hNKp46 mouse model with MC38 syngeneic tumor shows the highest number of NKp46+ NK cells in blood and tumor tissue



**Figure 1:** Numbers of NK cells based on FACS analysis of cells isolated from blood (A-B) or tumor (C-D). Human NK cells were defined as: hCD45+, hCD3-, hCD56+ (hHSC.NCG and hNK.NCG models) and mouse NK cells defined as mCD45+, mCD3-, mCD49b+ (C57BL/6-hNKp46 model). Blood samples were collected from mice with small tumors (Avg. ~200mm<sup>3</sup>, ~100mm<sup>3</sup>, ~50mm<sup>3</sup> and ~300mm<sup>3</sup> respectively) and tumor tissue was collected when tumors were larger (Avg. ~600mm<sup>3</sup>, ~500mm<sup>3</sup>, ~1600mm<sup>3</sup> and ~2000mm<sup>3</sup> respectively). C57BL/6-hNKp46 model showed higher proportion of NKp46+ cells in blood which could be due to unspecific staining of pan NK marker CD49b. Dotted line in A and C represents the average number of NKp46+ NK cells. Numbers above columns represent mean; \* indicates cohorts where some of the samples had low event count (>20). Error bars indicate mean with SD;

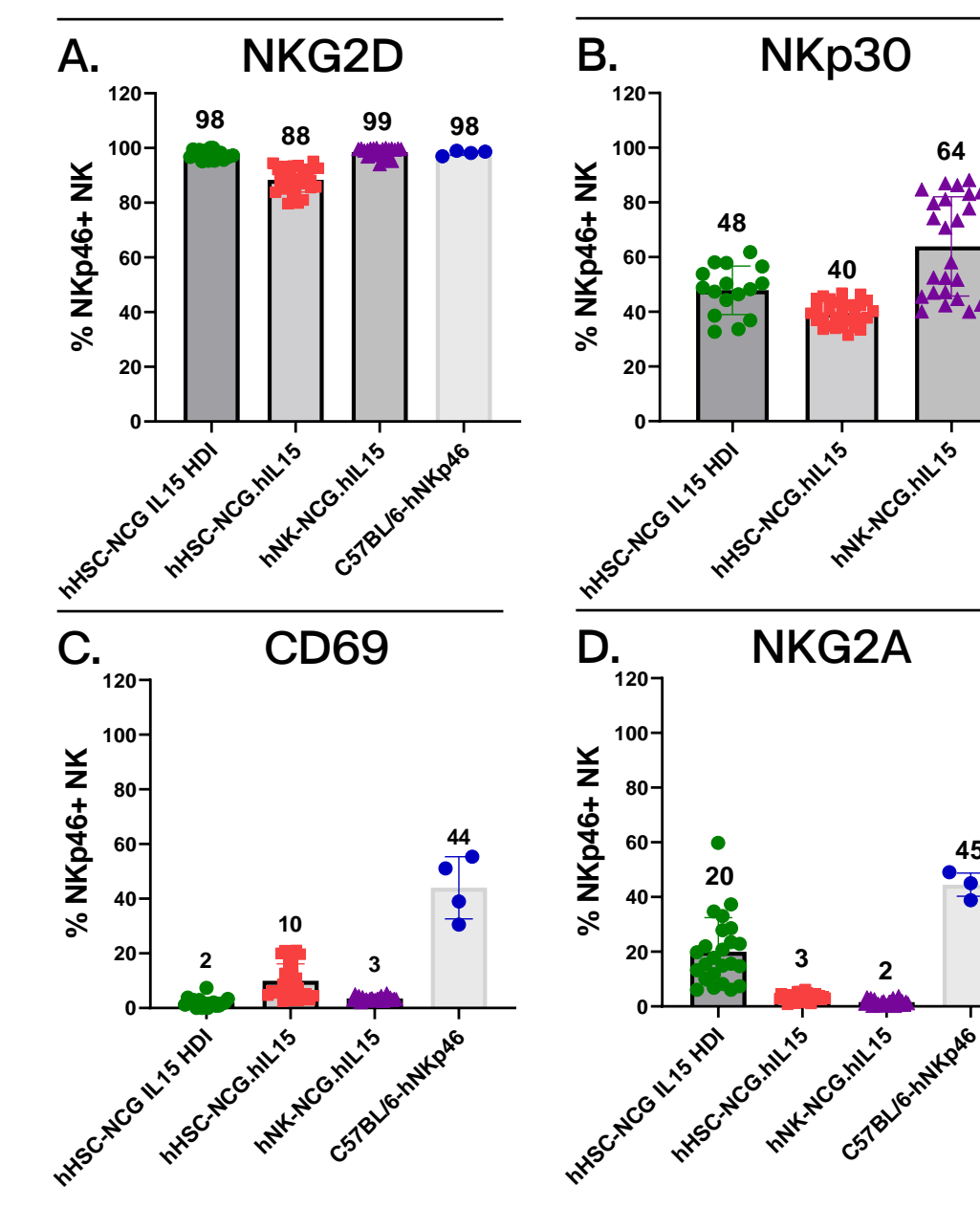
## RESULTS

### Transgenic expression of hIL-15 leads to expansion of NK cells in blood



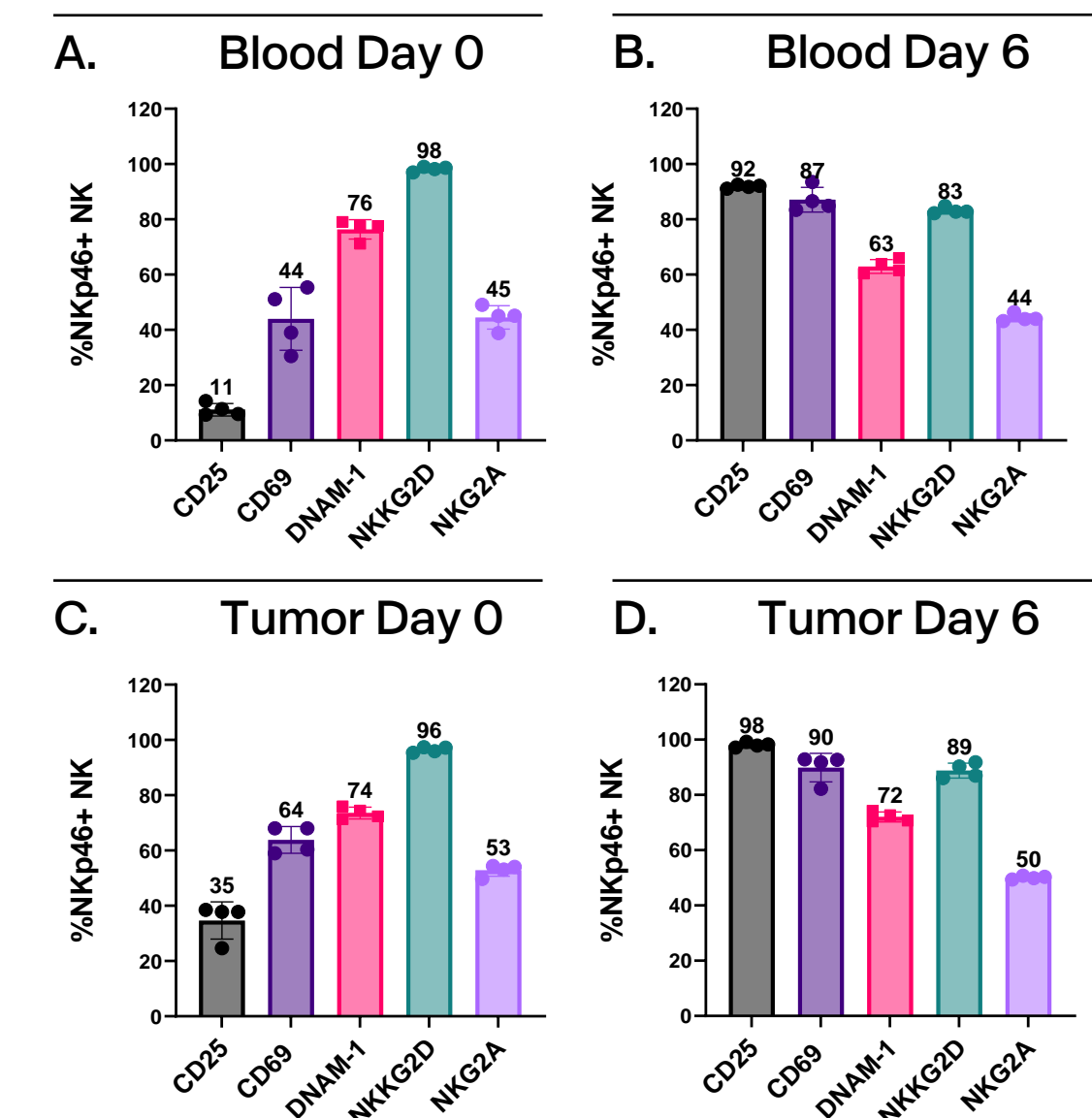
**Figure 2:** Changes in the number of circulating NKp46+ NK cells over time in the following models: A) hHSC-NCG IL15 HDI; B) hHSC-NCG.hIL15; C) hNK-NCG.hIL15; D) C57BL/6-hNKp46. Cells were enumerated in blood samples by FACS. Human NK cells were defined as: hCD45+, hCD3-, hCD56+, hNKp46+ (A-C) and mouse NK cells defined as mCD45+, mCD3-, mCD49b+, hNKp46+ (D). Error bars indicate mean with SD.

### Different strategies of humanization result in comparable biomarker expression profile on NKs



**Figure 3:** FACS analysis of blood samples from humanized tumor-bearing animal models at Day 0 showing proportion of NKp46+ NK cells expressing activating receptors (A-B), activation marker (C) and inhibitory receptor (D). Human NK cells defined as: hCD45+, hCD3-, hCD56+, hNKp46+ (hHSC.NCG and hNK.NCG models) and mouse NK cells defined as mCD45+, mCD3-, mCD49b+, hNKp46+ (C57BL/6-hNKp46 model). No NKp30 data available for C57BL/6-hNKp46 model due to lack of its expression on mouse NK cells. Number above each column represents mean. Error bars indicate mean with SD.

### NKp46+ NKs show similar biomarker expression profile between blood and tumor in C57BL/6-hNKp46 MC38 syngeneic model



**Figure 4:** FACS analysis of blood (A-B) and tumor (C-D) samples from C57BL/6-hNKp46 mice with syngeneic MC38 tumors at Day 0 (A and C, avg. tumor size ~300mm<sup>3</sup>) and Day 6 (C and D, avg. tumor size ~2000mm<sup>3</sup>). Graphs represent a proportion of NKp46+ NK cells expressing a panel of biomarkers. Mouse NK cells were defined as mCD45+, mCD3-, mCD49b+. Number above each column represents mean. Error bars indicate mean with SD.

## CONCLUSIONS

- ▶ C57BL/6-hNKp46 mice with MC38 syngeneic tumors are immunocompetent and show the highest number of circulating and tumor-infiltrating NKp46+ NK cells across models tested here. These mice, in contrast to NCG.hIL15 strain, show lack of non-physiological NK cell expansion and are free of donor-dependent variability. Across the models tested, C57BL/6-hNKp46 MC38 model is the most optimal experimental tool for in vivo evaluation of NK-TICA™ providing the tumor-targeting Bicycle® cross-reacts with the mouse ortholog expressed on MC38 cell line.
- ▶ hHSC- and hNK-NCG.hIL-15 models with A431 xenografts have utility in NK-TICA™ studies wherein the analysis of human NK cells is critical. However, the limitation of these models is poor NK tumor infiltration, which confines biomarker analysis to circulating NK cells.

## REFERENCES

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