# Generation and characterization of NK-92® (aNK™) cells with hypoimmunogenic modifications

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## ABSTRACT

NK-92® (also known as aNK™) cells are a natural killer (NK) cells line that have been studied in the treatment of cancer. The next generation of NK-92® cells will include inducible kill switches and enhanced cytotoxicity to make them safer and more effective, without the need to irradiate them before infusion into patients.

Because of their enhanced longevity *in vivo* and their allogeneic nature, aNK cells are susceptible to rejection by the patient's own immune system and need to be equipped with mechanisms to prevent such rejection. We have genetically engineered aNK cells by targeting a combination of molecules that allow them to be undetected by the patient's T-cells as well as resistant to lysis by peripheral blood NK cells (PBNKs).

We report here that abolishment of HLA class-I by *B2M* KO and re-expression of HLA-E on aNK cells effectively inhibited CD8⁺ T-cell recognition as well as provided significant protection against resting and IL-2 activated PBNKs. Additionally, expression of HLA-E on B2M-deficient aNK does not induce significant allogeneic response from resting and IL-2 activated NKG2C⁺ PBNKs.

Our results also suggest that these modifications are likely sufficient to allow the engineered aNK cells to evade immune alloreactivity in a majority of patients after infusion.

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## INTRODUCTION

- NK-92[1] (aNK) and its variants have been given to over 200 patients without serious side effects and potent anti-tumor efficacy.
- The next generation aNK will not require irradiation and thus need additional modifications to protect against allogeneic rejection.
- We have engineered HLA-I deficient aNK by knocking out the common $\beta_2$ microglobulin subunit (B2M)[2] gene to evade T-cell recognition.
- To protect HLA-I deficient aNK against lysis by PBNK via the 'missing-self' pathway[3], we further re-expressed the non-classical MHC class I molecule HLA-E to deliver an inhibitory signal through engagement with the NKG2A/CD94 heterodimer on PBNKs[4].
- We used mixed lymphocyte reaction (MLR) assays to assess T cell allogeneic response from healthy donor PBMC to modified aNK.
- Flow based cytotoxicity assays with labelling of CD107α and cell surface staining were used to assess HLA-E induced protection against both resting and IL-2 activated NKG2A⁺ and NKG2C⁺ PBNKs.

## METHODS AND MATERIALS

- **Cells and media:** K562 and K562-HLAE were cultured in RPMI 1640 medium + 10% FBS. aNK and its variants were grown in X-Vivo10™ medium + 5% human serum with 500 IU/mL of human recombinant IL-2. PBMCs were isolated from healthy donor buffy coats via Ficoll gradient and PBNKs were then isolated by bead-based enrichment. PBNKs were activated by 1000 IU/mL of IL-2 in NK-Xpander medium + 10% human serum.
- **Electroporation:** aNK cells were electroporated with Cas9-sgRNA ribonucleoprotein with sgRNA targeting *B2M* to generate HLA-I deficient aNK (aNK_B2M$^{KO}$). Electroporation was performed using the Neon™ system according to manufacturer's protocol. Bead-based column purification was subsequently performed.
- **Lentiviral transduction:** aNK_B2M$^{KO}$ and K562 cells were transduced with a pCDH-lentiviral vector with HLA-E single chain trimer gene. For aNK_B2M$^{KO}$_HLAE, bead-based column enrichment of HLA-E expressing cells was subsequently performed.
- **Mix Lymphocyte Reaction:** aNK and aNK_B2M$^{KO}$ as stimulator cells were treated with 3 µg/mL of mitomycin-C, labeled with CFSE and co-cultured at 1:1 ratio with donor PBMC labeled with the Cell Trace Violet proliferation dye. Cells were harvested after 7 and 10 days and labeled with anti-CD3e, CD4 and CD8 antibodies.
- **Flow-based Cytotoxicity assay:** Target cells were stained with the PKH67GL dye and co-cultured with resting or IL-2 activated PBNKs as effector cells for 4 hrs. In degranulation assays, anti-CD107α antibodies were added and after 1 hr of incubation, GolgiStop solution was added. Cell surface staining was performed after the cytotoxicity assay

## RESULTS

### Generation of B2M-deficient aNK and re-expression of HLA-E on B2M-deficient aNK

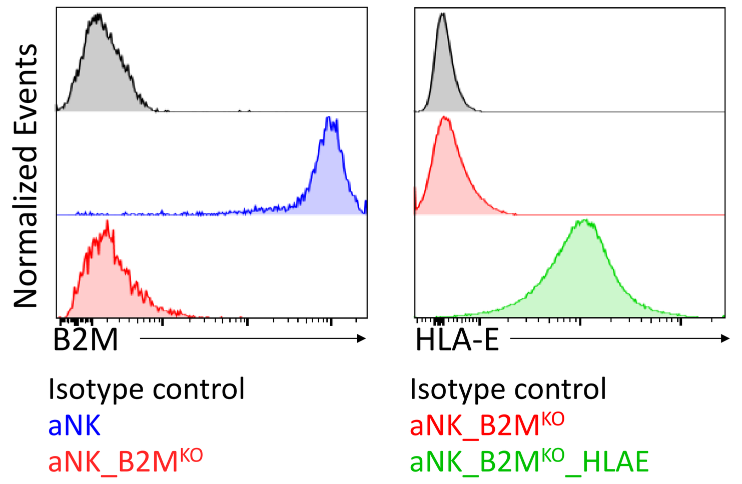


**Figure 1.** *B2M* was knocked out in aNK by electroporation of Cas9-sgRNA ribonucleoprotein complexes targeting *B2M*. B2M-deficient aNK cells were subsequently engineered to re-express HLA-E via lentiviral transduction.

### Deletion of HLA-I by B2M$^{KO}$ on aNK eliminated allo-reaction from CD8⁺ T cells

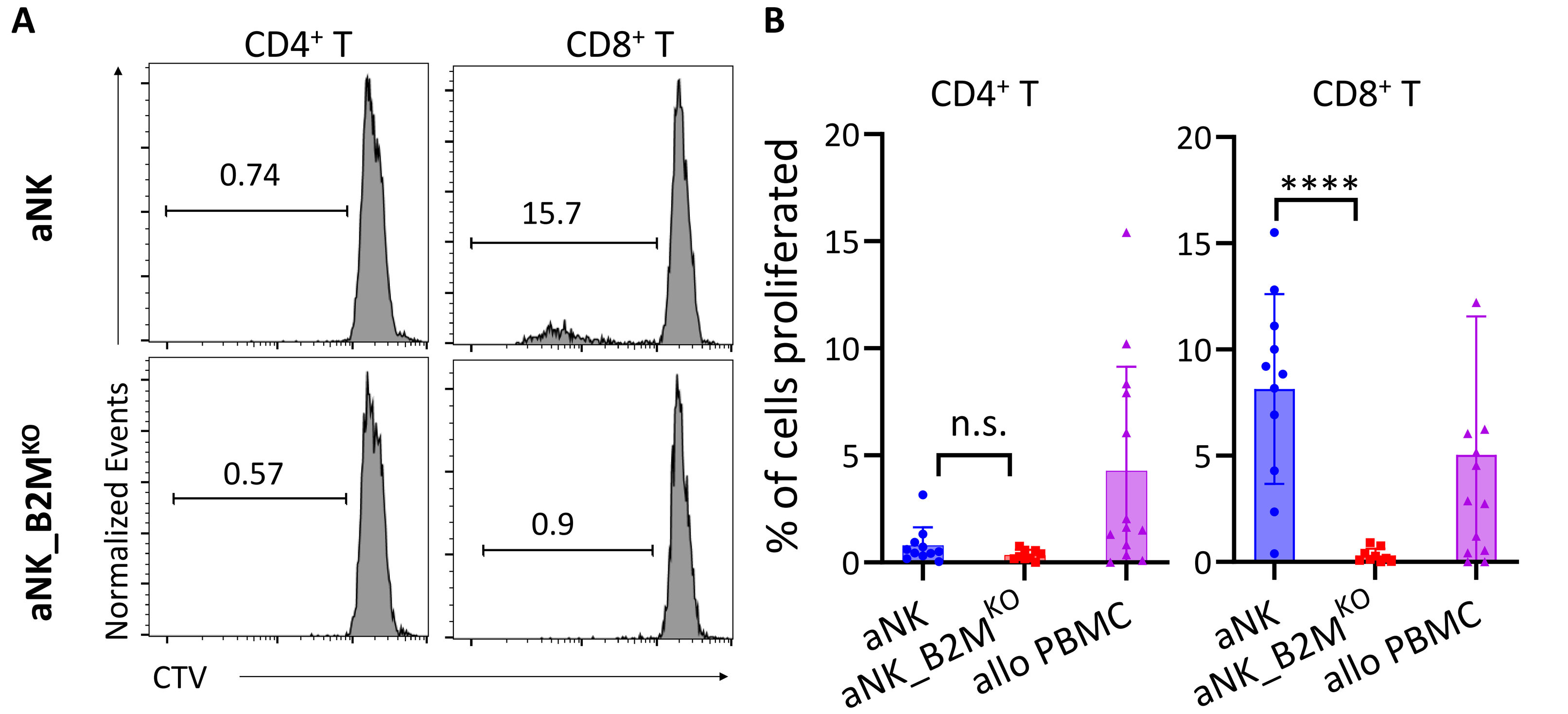


**Figure 2. A)** Representative flow cytometry profiles of percentages of CD4⁺ and CD8⁺ T cells from healthy donor PBMCs that have proliferated, as measured by the Cell Trace Violet (CTV) proliferation dye, at day 7 post-co-incubation with mitomycin-treated aNK or aNK_B2M$^{KO}$ cells. **B)** Quantification of CD4⁺ and CD8⁺ T cells proliferation (n=10-13, *student t-test).*

### aNK_B2M$^{KO}$ are lysed by PBNKs and re-expression of HLA-E confers significant protection

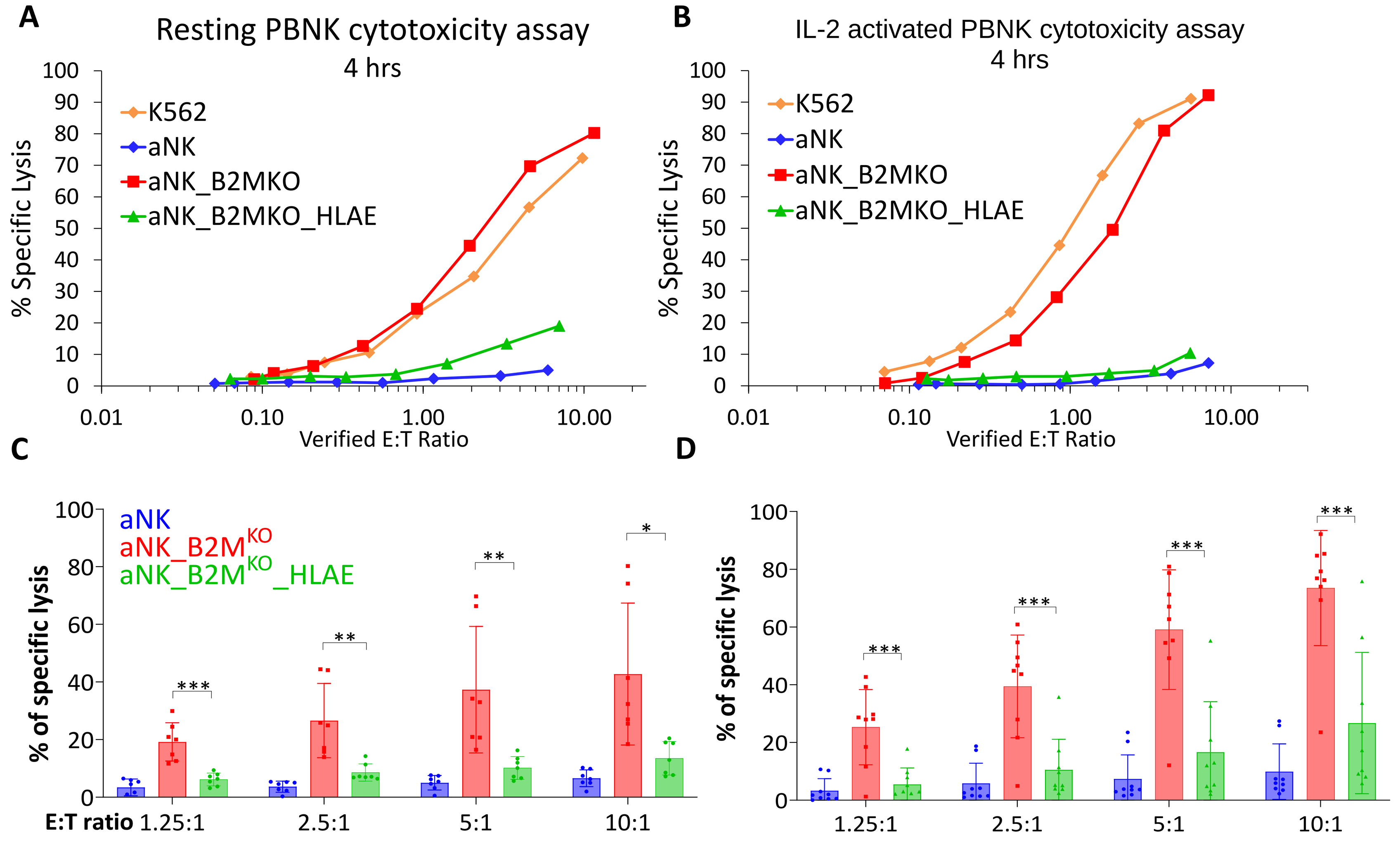


**Figure 3. A-B)** Representative 4-hrs cytotoxicity assay showing the percentages of specific lysis of indicated target cells by resting **(A)** and IL-2 activated **(B)** PBNKs isolated from healthy donors. **C-D)** Quantification of lysis by resting **(C)** and IL-2 activated PBNKs **(D)** against indicated target cells (n=7-9, *student t-test)*.

### Re-Expression of HLA-E on aNK_B2M$^{KO}$ cells does not elicit increased response from NKG2C⁺ PBNKs

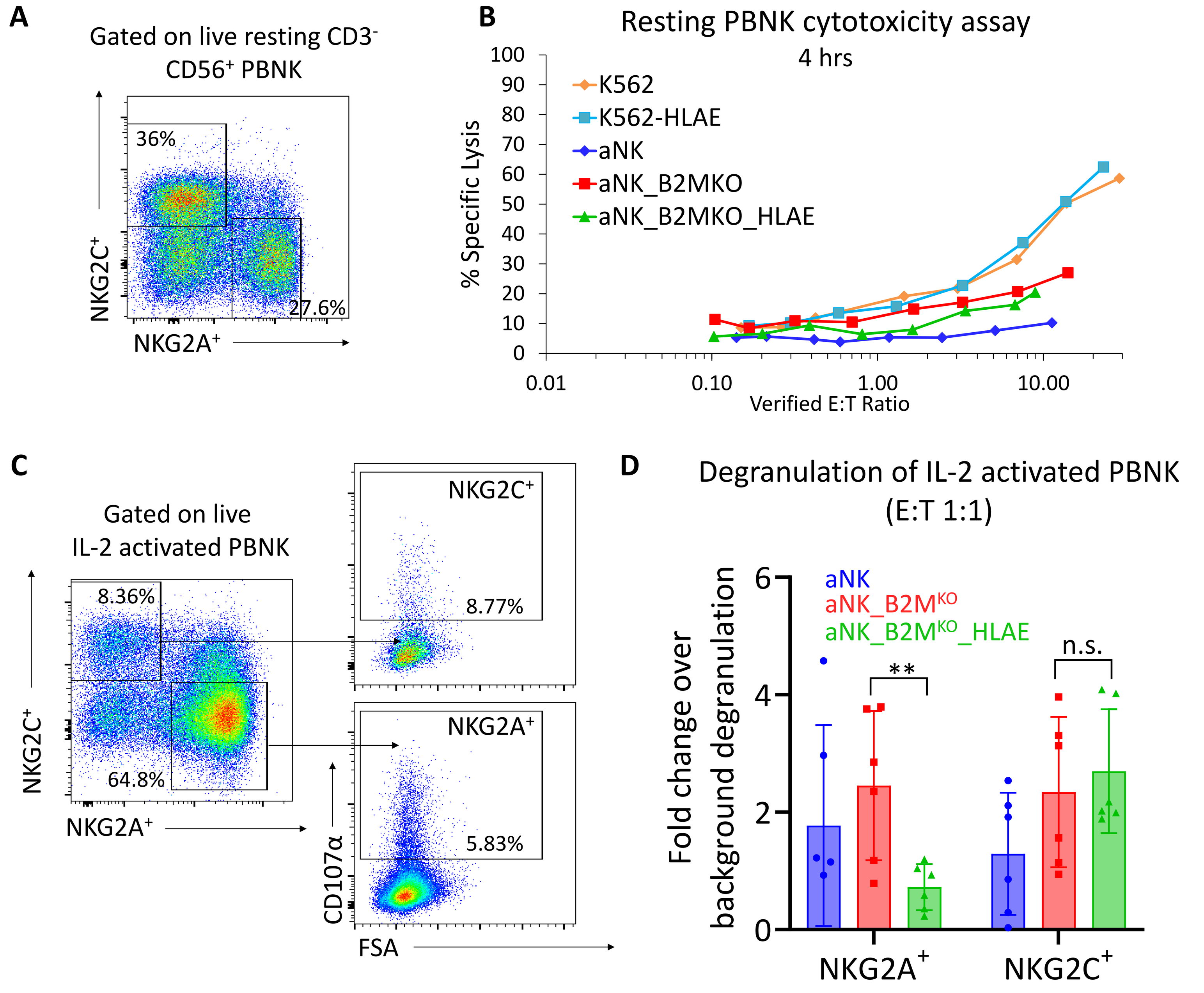


**Figure 4. A)** Example of a flow cytometric profile indicating the frequencies of NKG2A⁺ and NKG2C⁺ PBNKs in total live CD3⁻ CD56⁺ compartment from an NKG2C-rich PBMC donor. **B)** Representative 4-hrs cytotoxicity assay of resting donor PBNK **(A)** against indicated target cells. **C)** Representative flow cytometric profiles of percentages of degranulation by anti-CD107α staining in IL-2 activated NKG2C⁺ and NKG2A⁺ PBNKs after a 4-hrs cytotoxicity assay. **D)** Quantification showing the fold changes over background degranulation at 1:1 E:T ratio of indicated IL-2 activated PBNK subsets from 6 random donors (n=5 experiments, *student t-test*)

## CONCLUSIONS

We report here that the abolishment of HLA class-I and re-expression of HLA-E on aNK cells effectively inhibited CD4⁺ and CD8⁺ T-cell recognition as well as provided significant protection against resting and IL-2 activated PBNKs from healthy donors. Our results also show that the re-expression of HLA-E in aNK cells does not induce significant allo-response from NKG2C⁺ PBNKs. Together, our data suggests these modifications are sufficient to allow the engineered aNK cells to evade immune alloreactivity in a majority of patients after infusion.

## REFERENCES

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