Immune Masking Strategies to Extend the Pharmacokinetics of Allogeneic Cell Therapies

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Introduction

Allogenic “universal” cell therapies address the challenges associated with autologous cell therapies such as lengthy production time, variability in starting material and final product, and high production costs. Allogenic cell therapies are engineered healthy donor cells expanded in large quantities to provide products of consistent quality and potency that are available “off the shelf”. Methods are under investigation to improve the pharmacokinetic properties of allogeneic cell products by engineering them to avoid host vs. graft disease, where allogeneic NK and T cells are rapidly targeted by the patient’s own immune system. A conventional method for preventing host T rejection of allogenic T cells, is to knockout (KO) β-2 microglobulin (β2M) to diminish expression of MHC class I proteins, combined with overexpression of nonclassical MHC class I protein, HLA-E, to evade host NK cell rejection. Here, we evaluated the effectiveness of HLA-E and other molecules in β2M deficient T cells for inhibiting NK cell cytotoxicity at different NK:T ratios and timepoints. Concurrently, a high throughput platform was developed to screen a wide variety of NK inhibitory peptides and synthetic ligands to identify novel immune masking strategies for extending allogeneic cell therapy persistence for broad patient populations.

Methods

Two approaches were used to test the effectiveness of immune evasion strategies:

a) Primary NK and T cell screen. NK and T cells from the same donors were expanded. NK cells were transduced with gamma-retrovirus encoding CD19 CAR. T cells were edited by knocking out β2M, using CRISPR/Cas9 and further modified by transduction via retrovirus encoding various hypoimmune constructs. CD19 CAR-NK cells and β2M KO hypoimmune T cells were co-cultured at various NK:T ratios in IncuCyte-based *in vitro* assays to assess NK killing of T cells.

b) High-throughput screen utilizes NKG2D CAR-NK and MHC I/ MHC II-negative K562 cell line labeled with nuclear red (K562NR). NK cells were expanded and transduced with NKG2D CAR retrovirus. K562NR cells were transduced with various retroviral hypoimmune constructs with GFP, as the reporter gene. Double positive K562 cells were sorted for NR$^+$ and GFP$^+$ cells and allowed to recover before co-culture with NKG2D CAR-NK cells at 1:1 E:T ratio in an IncuCyte *in vitro* assay. Flow cytometry was used to quantify hypoimmune construct expression and characterize NKG2A expression on NK cells.

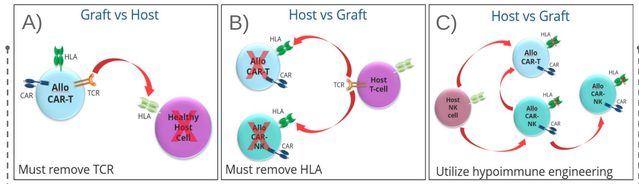


Figure 1: Schematic depicting various scenarios to combat challenges associated with allogenic CAR cell therapies. (A) T cell receptor (TCR) knockout prevents graft-versus-host disease (GvHD) of alloreactive CAR-T cells targeting against host tissue. **(B)** β2M (MHC I/HLA) knockout prevents T-cell mediated host-versus-graft disease (HvGD). **(C)** HLA deficient allogenic CAR cells are vulnerable to NK fratricide and host NK cells due to the missing self hypothesis.

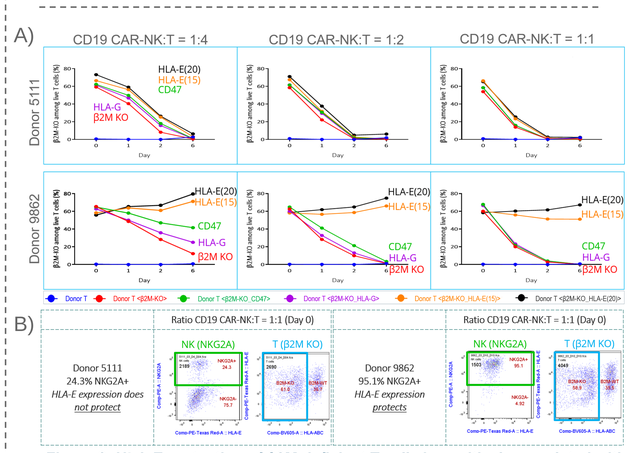


Figure 2: HLA-E protection of β2M deficient T-cells is positively correlated with NKG2A+ expression on NK cells. (A) Evaluation of immune masking with HLA-E (linker size, 20 and 15), HLA-G, and CD47 at various NK:T ratios over a course of 6 days. HLA-E protection of β2M deficient T cells observed at low NK:T ratios and shorter incubation time and positively correlated with NK NKG2A$^+$ expression. **(B)** NKG2A and β2M knockout efficiency was assessed by flow cytometry.

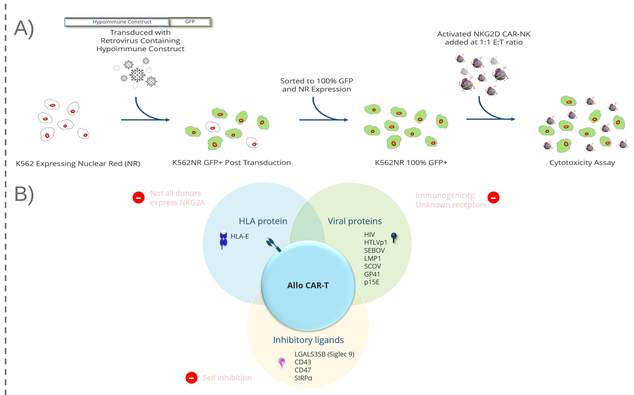


Figure 3: High throughput assay to evaluate immune evasion strategies. (A) K562 cell lines expressing nuclear red (NR) transduced with immune constructs were sorted and co-cultured with NKG2D CAR-NK cells to test for NK killing inhibition. **(B)** Categories of NK inhibitory molecules tested in the screening assay.

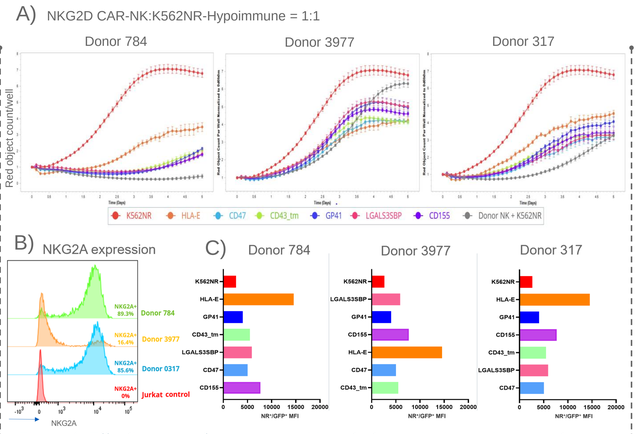


Figure 4: Effectiveness of immune masking strategies is donor dependent. **(A)** Evaluation of immune masking in three different NKG2D CAR-NK donors using high throughput screening with K562 NR cells at 1:1 E:T ratio. GP41 viral peptide inhibited NK killing, even with low expression; HLA-E protection dependent on NK cell NKG2A$^+$ expression **(B)** NKG2A expression between donors was assessed by flow cytometry **(C)** NR$^+$/GFP$^+$ MFI of hypoimmune expression of donors, arranged in order of immune masking capabilities; expression of peptides is not correlative to NK inhibition activities.

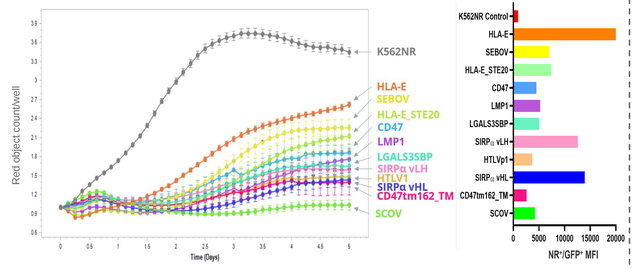
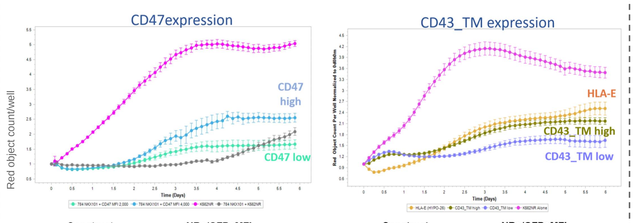


Figure 5: SEBOV viral protein comparable to HLA-E NK inhibition. (A) K562NR high throughput screening system showing broad range of immune masking capabilities with SEBOV comparable to HLA-E. **(B)** NR$^+$/GFP$^+$ MFI of hypoimmune expression arranged in order of immune masking capabilities; expression of peptides is not correlative to NK inhibition activities.



Construct	NR+/GFP+MFI
CD47 high	4,205
CD47 low	2,750

Construct	NR+/GFP+MFI
CD43_TM high	26,467
CD43_TM low	10,035

Figure 6: Increased expression of hypoimmune constructs can improve masking capabilities. K652NR cells expressing higher levels (~2X) of CD47 and CD43 decreased NK cytotoxicity.

Results

Two methods were implemented to investigate the effectiveness of the different immune evasion strategies. In both approaches, HLA-E protection from NK cell cytotoxicity in β2M deficient T cells and K562NR cells was highly correlated with positive expression of CD94/ NKG2A on host NK cells. In the primary cell screen, HLA-E, HLA-G and CD47 had greater NK evasion at lower NK:T ratios; with HLA-E being the most effective at all NK:T ratios tested (1:1, 1:2, 1:4). To mitigate the donor-to-donor variation on alloreactivity, we developed a high throughput screening method using β2M deficient- K562NR cells as a surrogate for T cells to identify candidate synthetic hypoimmune constructs that enhanced NK inhibitory function. From the screen we were able to identify several synthetic proteins that conferred NK cell evasion with similar efficiency as HLA-E. These included viral proteins SEBOV (glycoprotein from Sudan ebolavirus), and GP41 (a subunit of the envelope protein complex of retrovirus, such as HIV). The hypoimmune masking capabilities for the majority of the hypoimmune constructs screened were donor dependent. We also found that increasing the cell surface expression of CD47 and CD43 correlated with enhanced NK inhibitory function, suggesting that increasing expression of other NK inhibitory ligands could yield the same result.

Conclusion

Implementing hypoimmune strategies using gene editing of allogeneic cell therapies is necessary to increase their persistence by enabling them to evade host T cell and NK cell surveillance. Here we show different approaches to investigate the effectiveness of cell engineered immune evasion strategies. We found that the benefit of HLA-E expression in suppressing NK cytotoxicity is highly correlated with the expression of CD94/NKG2A on the host NK cells. Viral peptides were less dependent on donor NKG2A expression and potent even at low expression levels on K562NR cells. This suggests that viral peptide inhibition of NK cytotoxicity may be improved by increasing hypoimmune surface expression on primary T cells. While the implications of applying immune evasion strategies are broad with respect to allogeneic cell therapies, hypoimmune strategies are particularly advantageous for application of allogeneic products containing mixed NK and T cell populations to not only extend persistence of both cell types but to also minimize or eliminate potential cell fratricide. These data support further exploration of different immune masking strategies in order to extend the pharmacokinetics of allogeneic cell therapies.

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