A LAYERED CLOAKING STRATEGY TO GENERATE ALLOGENEIC IPSC-DERIVED CD8 T-CELLS THAT EVADE NK CLEARANCE

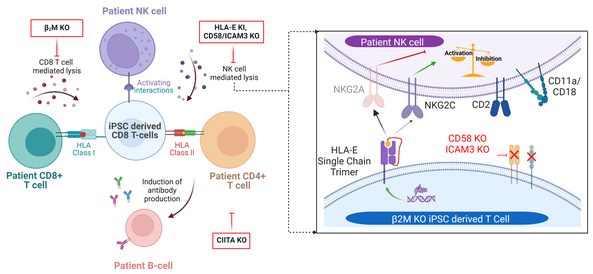
Notch THERAPEUTICS

notchtx.com

ASH 2024

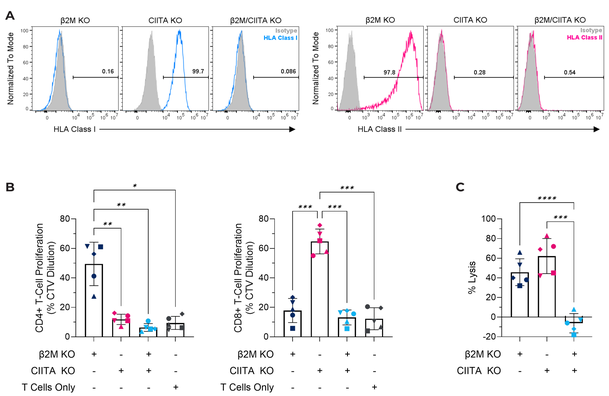
Siddarth Chandrasekaran, Vignesh Janardhanam, Ian Cardle, Justin Yoo, Yue Zhang-Wong, Julia Bershadsky, Elisa Martinez, April Marple, Laxsha Thaya, Sommer Apelu, Charlie Liu, Dhanya Vijayan, Leanne Jamieson, Lifu Sheng, Elizabeth Csaszar, Emily Titus and Chris Bond

Fig.1: Notch has developed a novel and potent approach to protect iPSC-derived T cells from the patient's immune system.



Allogeneic clearance limits the persistence of induced pluripotent stem-cell (iPSC) derived CD8 T-cell therapies. iPSC-derived CD8-T cells can be recognized and eliminated by patient CD8 and CD4 T-cells through Human Leukocyte Adhesion (HLA) class I and class II molecules. Every gene of HLA I forms heterodimers with the B2 microglobulin protein that is encoded by β2M gene. CIITA is a transcriptional coactivator essential for all HLA class II expression. Deletion of β2M and CIITA leads to the formation of cells that can completely mitigate patient T-cell mediated clearance. However, patient Natural Killer (NK) cells eliminate cells that do not express HLA class I receptors on their surface, since it is an important inhibitory ligand for NK cell receptors. To address NK clearance, we have designed a unique layered approach to knock-in HLA-E and knock-out adhesion ligands CD58 and ICAM3 to prevent the formation of a stable immune synapse between product cells and patient NK cells. We show that our layered approach outperforms HLA-E KI alone in a NKG2C+ human NK donor pool and has the potential to support immune evasion and product persistence in a broader population of patients than previous approaches.

Fig.2: Combined β2M and CIITA knockout mitigates T-cell mediated rejection of iPSC-derived T cells.

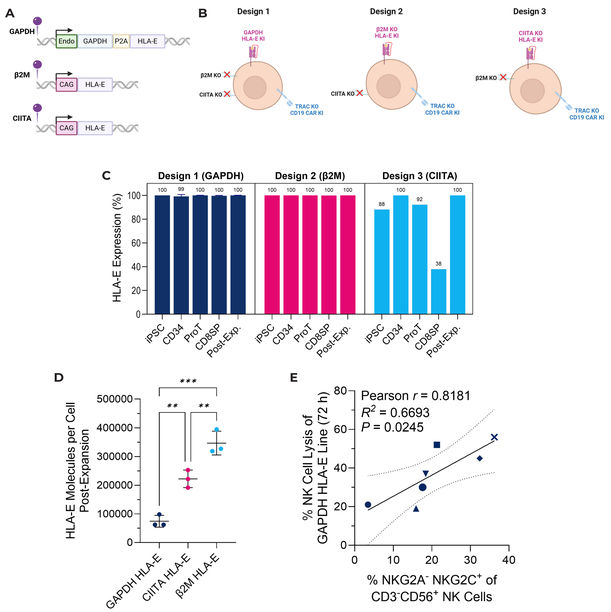


A) HLA-ABC (Class I) and HLA-DR/DP/DQ (Class II) expression on expanded iPSC-derived T cells with clonal β2M and CIITA knockouts (KOs), measured by flow cytometry. Double KO cells do not express any HLA class I and II.

B) Mixed lymphocyte reaction of CTV-labeled allogeneic primary T cells co-cultured with iPSC-derived T cells containing single or double β2M and CIITA KOs at a 3:1 E:T ratio for 6 days. Proliferation of allogeneic CD4+ and CD8+ T cells was tracked by % CTV dilution. Allogeneic CD4+ T cells proliferate when cocultured with β2M KO iPSC-derived T cells that have intact HLA class II expression but not with CIITA KO iPSC-derived T cells, whereas allogeneic CD8+ T cells proliferate when cocultured with CIITA KO iPSC-derived T cells that have intact HLA class I expression but not with β2M KO iPSC-derived T cells. Data points and error bars represent the mean ± SD; $n = 5$ T cell donors. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (RM one-way ANOVA with Dunnett's test).

C) Resulting lysis of iPSC-derived T cells after co-culture with allogeneic T cells. While β2M and CIITA single KO iPSC-derived T cells are lysed after coculture, double KO cells are protected. Data points and error bars represent the mean ± SD; $n = 5$ T cell donors. ***$P < 0.001$ and ****$P < 0.0001$ (RM one-way ANOVA with Dunnett's test).

Fig.3: Site-specific integration of HLA-E at the GAPDH locus protects β2M KO iPSC-derived T cells against NKG2A+ but not NKG2C+ NK cells.



A) Schematic of *GAPDH*, *β2M*, and *CIITA* targeted integration sites explored for HLA-E knock-in. For integration at *GAPDH*, HLA-E was inserted directly downstream of the gene separated by a P2A ribosomal skip sequence to preserve GAPDH expression and co-opt the endogenous promoter for expression. For integration at *β2M* and *CIITA*, HLA-E was inserted with a CAG promoter in the gene to disrupt expression of HLA class I and II.

B) Schematic of the three resulting engineered iPSC clonal lines with targeted integration of HLA-E at *GAPDH* (Design 1), *β2M* (Design 2), and *CIITA* (Design 3). All three clonal lines have biallelic knockouts targeting *TRAC*, *β2M*, and *CIITA* loci, with integration of a CD19-CAR.

C) Percent HLA-E expression on iPSCs, CD34 hematopoietic progenitor cells (HPCs), progenitor T cells (ProTs), unexpanded CD8 single-positive T cells (CD8SPs), and expanded CD8SPs (Post-Exp) with targeted integration of HLA-E at *GAPDH*, *β2M*, and *CIITA* loci, measured by flow cytometry ($n = 6$ clones for GAPDH, $n = 1$ clone for *β2M* and CIITA). HLA-E expression at *GAPDH* and *β2M* loci is maintained throughout differentiation, whereas expression at *CIITA* locus is silenced at the end of differentiation but upregulated post-expansion.

D) HLA-E molecules per cell driven by *GAPDH*, *CIITA*, and *β2M* targeted integration on expanded iPSC-derived T cells, as determined by BD Quantibrite PE analysis. Targeted integration of HLA-E at *GAPDH* locus drives lower expression than integration at *CIITA* and *β2M* loci, which may be advantageous for mitigating NKG2C-driven NK clearance. Accordingly, targeted integration of HLA-E at *GAPDH* (Design 1) was selected for further testing. Lines and error bars represent mean ± SD; $n = 3$ independent experiments. **$P < 0.01$, ***$P < 0.0001$ (ordinary one-way ANOVA with Tukey correction).

E) Correlation of NKG2A⁻ NKG2C⁺ phenotype on CD3⁻CD56⁺ NK cells from $n = 7$ donors with NK lysis of expanded *β2M* KO iPSC-derived T cells containing targeted integration of HLA-E at *GAPDH* locus. NK lysis correlates positively with NKG2C expression, suggesting further engineering on top of HLA-E knock-in is needed to comprehensively mitigate NK clearance from different donors.

Fig.4: HLA-E knock-in combined with CD58 and ICAM3 knockouts results in more universal protection of β2M KO iPSC-derived T cells from NK cells.

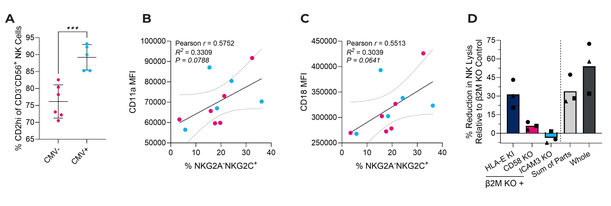
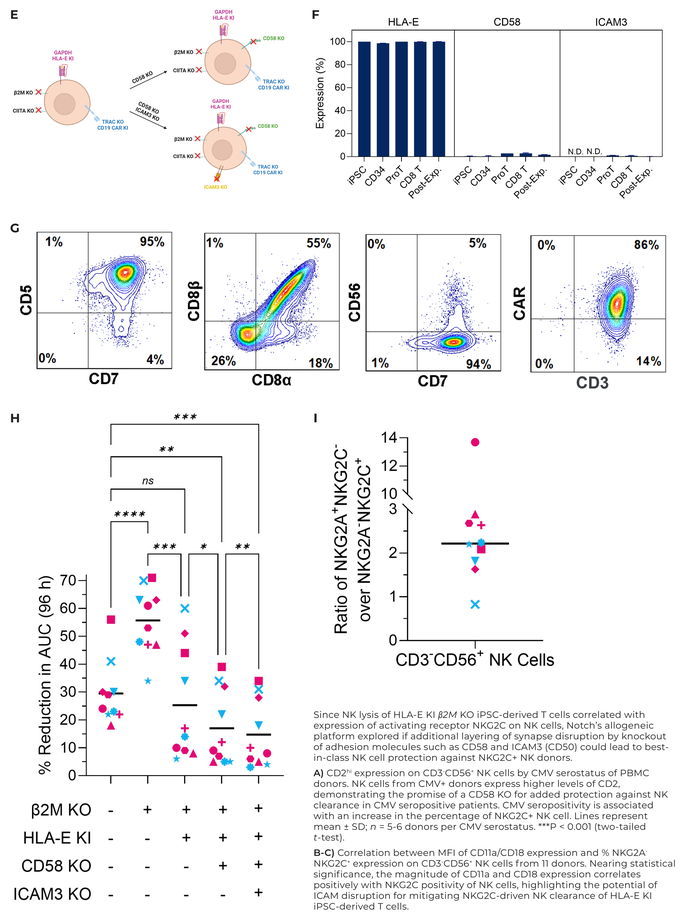


Fig.4 (CONT'D)



D) Whole versus sum of parts analysis of % reduction in NK cell lysis provided by HLA-E KI, CD58 KO, and ICAM3 KO in a β2M-deficient background. iPSC-derived T cells were expanded and engineered with a β2M KO plus HLA-E KI alone, CD58 KO alone, ICAM3 KO alone, or the combination of all three and cocultured with primary NK cells from $n = 3$ donors at a 1:1 ratio for 72h. Viability of iPSC-derived T cells in the presence and absence of NK cells was monitored daily by flow cytometry, analyzed as reduction in area under the curve, and normalized to a β2M KO only control to measure % reduction in NK lysis. While adhesion molecule KOs in the absence of HLA-E KI were not very protective, the combination of HLA-E KI with CD58 KO and ICAM3 KO provide greater protection than the sum of their parts, suggesting synergy.

E) Schematic representation of two iPSC clonal cell lines derived from Design 1 with targeted integration of HLA-E at the *GAPDH* locus. Each clonal line features bi-allelic knockouts, targeting either CD58 alone or both CD58 and ICAM3.

F) Percent HLA-E, CD58, and ICAM3 expression on iPSCs, CD34 HPCs, ProTs, unexpanded, and expanded T cells derived from Design 1 with CD58 and ICAM3 KOs, measured by flow cytometry ($n = 2$ clones). CD58 and ICAM3 KOs result in disruption of protein expression through all stages of differentiation.

G) Representative flow cytometry plots of key phenotypic attributes of iPSC-derived T cells. CD58 and ICAM3 KOs do not impact the differentiation of iPSC-derived T cells.

H) NK cell clearance of expanded clonal iPSC-derived T cells containing layering of β2M KO, HLA-E KI at *GAPDH* locus, CD58 KO, and ICAM3 KO. Clearance at a 1:1 NK to iPSC-derived T cell ratio was evaluated by tracking iPSC-derived T cell viability daily in the presence and absence of NK cells for up to 96h by flow cytometry and analyzing the difference in viability as % reduction in area under the curve (AUC). The combination of HLA-E KI, CD58 KO, and ICAM3 KO drives increasing protection from NK cell clearance. Lines represent the mean; $n = 10$ unique NK donors colored according to their CMV serostatus as in Figure 4A. *ns* > 0.05, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ (RM one-way ANOVA with Holm-Šídák correction).

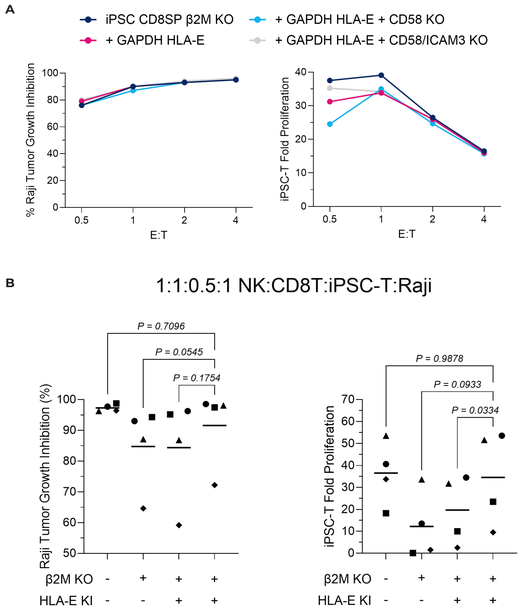
I) Ratio of % NKG2A⁺NKG2C⁺ expression over % NKG2A⁻NKG2C⁺ expression on CD3⁻CD56⁺ NK cells used in the NK clearance assay. NK cell donors comprised a wide range of NKG2A/C ratios. Line represents the mean; $n = 10$ unique NK donors colored according to their CMV serostatus as in Figure 4A.

Since NK lysis of HLA-E KI *β2M* KO iPSC-derived T cells correlated with expression of activating receptor NKG2C on NK cells, Notch's allogeneic platform explored if additional layering of synapse disruption by knockout of adhesion molecules such as CD58 and ICAM3 (CD50) could lead to best-in-class NK cell protection against NKG2C+ NK donors.

A) CD2^{hi} expression on CD3$^-$CD56$^+$ NK cells by CMV serostatus of PBMC donors. NK cells from CMV+ donors express higher levels of CD2, demonstrating the promise of a CD58 KO for added protection against NK clearance in CMV seropositive patients. CMV seropositivity is associated with an increase in the percentage of NKG2C+ NK cell. Lines represent mean ± SD; $n = 5$-6 donors per CMV serostatus. ***$P < 0.001$ (two-tailed *t*-test).

B-C) Correlation between MFI of CD11a/CD18 expression and % NKG2A$^-$ NKG2C$^+$ expression on CD3$^-$CD56$^+$ NK cells from 11 donors. Nearing statistical significance, the magnitude of CD11a and CD18 expression correlates positively with NKG2C positivity of NK cells, highlighting the potential of ICAM disruption for mitigating NKG2C-driven NK clearance of HLA-E KI iPSC-derived T cells.

Fig. 5: GAPDH-driven HLA-E and CD58/ICAM3 knockouts enhance CD19 CAR function of β2M KO iPSC-derived T cells in the presence of allogeneic NK and T cells.



A) Raji tumor growth inhibition (TGI) and iPSC-derived T cell proliferation after a 5-day coculture at multiple E:Ts. TGI was determined by Incucyte-based monitoring of Raji fluorescence, and iPSC CD8SP proliferation was quantified by normalizing manual counts at the end of the co-culture with % iPSC-derived T cells as determined by flow cytometry. HLA-E KI at the *GAPDH* locus with additional CD58/ICAM3 KOs does not impact the CAR function of iPSC-derived T cells in a single stim assay.

B) Raji TGI and iPSC-derived T cell proliferation after a 5-day coculture with allogeneic NK and CD8$^+$ T cells at a 1:1:0.5:1 NK:CD8T:iPSC:Raji ratio. TGI and iPSC-derived T cell proliferation were determined by manual counting and flow cytometry to distinguish the different cell types. HLA-E KI at the *GAPDH* locus and additional CD58/ICAM3 KOs drives improved tumor control and proliferation of β2M KO iPSC-derived T cells in the presence of allogeneic NK and CD8$^+$ T cells compared to HLA-E KI alone and was comparable to wild-type counterparts. Lines represent the mean; $n = 4$ unique NK donors. *ns* > 0.05, *$P < 0.05$ (RM one-way ANOVA with Dunnett correction).

SUMMARY AND CONCLUSIONS

- HLA-E knock-in combined with allogeneic synapse disruption provides comprehensive protection of β2M KO allogeneic iPSC-derived T-cells against a broad and diverse population of donor NK cells.
- Knock out of adhesion proteins that disrupt the allogeneic synapse does not decrease the potency of iPSC-derived CAR-T cells.
- Notch's novel combination of allogeneic edits improves the performance of iPSC-derived CAR-T cells in the presence of co-cultured allogeneic T and NK cells *in vitro*.
- This work supports the development of scalable, potent, and persistent off-the-shelf allogeneic CAR-T cell therapies for oncology and autoimmune patients.