Development of Master Multiplexed-Engineered iPSC Bank for Off-the-Shelf Cell-Based Cancer Immunotherapy with Reduced Conditioning Chemotherapy

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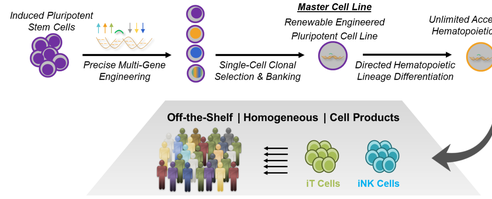
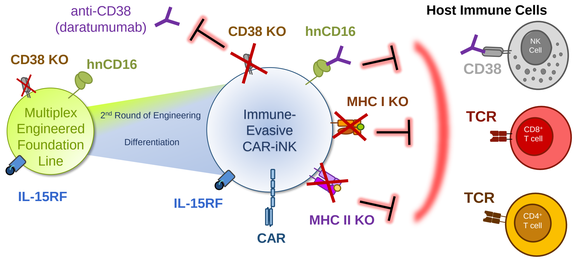
GRAPHICAL ABSTRACT & INTRODUCTION

A Novel Platform for Creating Off-the-shelf Multiplex-Engineered Hematopoietic Cell Products



- Autologous and allogeneic cell-based cancer immunotherapy requires administration of systemic conditioning chemotherapy to suppress the patient's immune system to potentiate the adoptively-transferred immune cells.
- Protracted immune suppression can lead to increased risk of complications such as severe infections and affects immune reconstitution
- New therapeutic strategies to prevent allorejection with minimal chemotherapy conditioning may significantly improve the application and efficacy of adoptively-transferred immune cells while preserving the patient's immune system

Multiplexed-Engineering of Off-the-Shelf Cell-Based Cancer Immunotherapy to Reduce the Need for Conditioning Chemotherapy



- iPSC derived NK cell (iNK cell) therapy is multiplex-engineered with a novel combination of immune-evasion modalities
  1. CD38 deletion for resistance to fratricide when combined with anti-CD38 monoclonal antibody to selectively eliminate host alloreactive lymphocytes
  2. Ablation of MHC class I molecules via knockout of Beta-2-Microglobulin (B2M) to prevent CD8+ T cell mediated rejection
  3. Ablation of MHC class II molecules via knockout of the Class II Transactivator (CIITA) to prevent CD4+ T cell mediated rejection
- In the first stage, a clonal foundation iPSC line was established incorporating IL-15/IL-15 receptor α fusion (IL-15RF) and a high-affinity, non-cleavable CD16 (hnCD16) at the CD38 locus for enhanced NK cell activity and antibody-dependent cellular cytotoxicity (ADCC), respectively
- In the second stage, iPSCs from the clonal master cell line were further engineered and subcloned to knockout B2M and CIITA and knock-in a tumor-targeting CAR simultaneously

RESULTS

Multi-Loci Engineering of Clonal iPSC Master Cell Lines with Six Edits to Drive Anti-Tumor Activity and Persistence

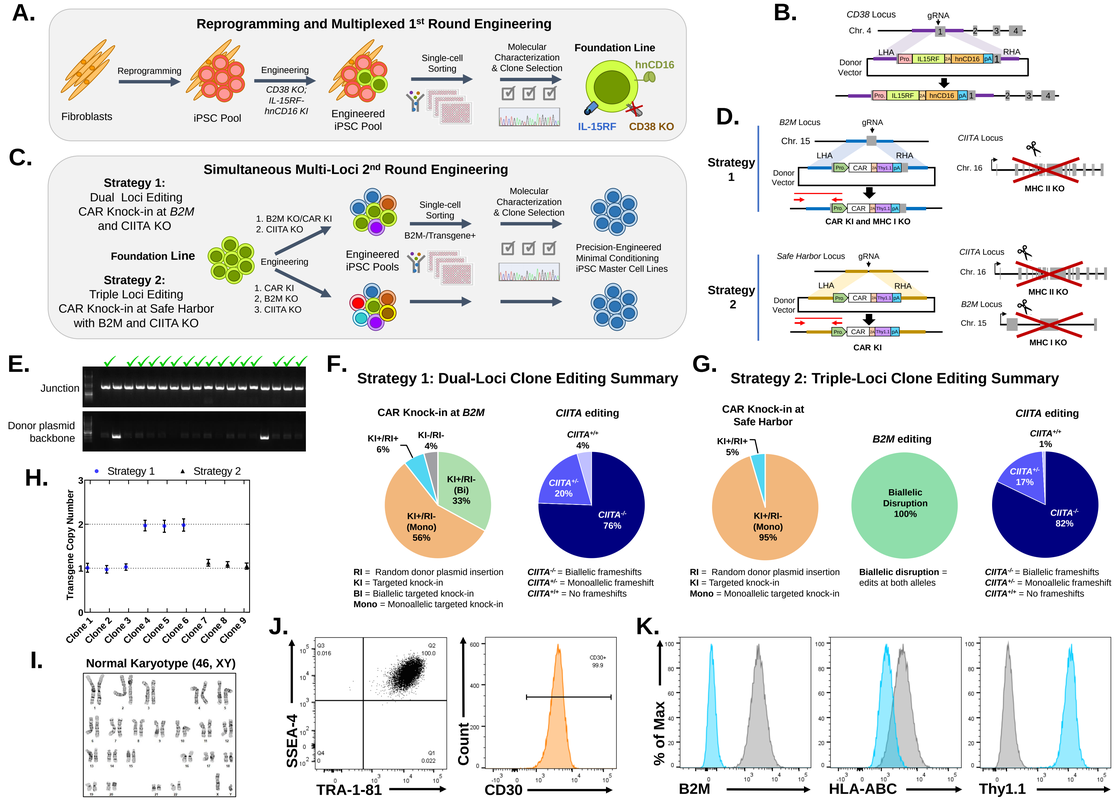


Figure 1. High precision, simultaneous multi-loci engineering of minimal conditioning iPSC clones from a multiplexed-engineered foundation line. (A) A multiplexed-engineered iPSC foundation line was generated from reprogrammed healthy donor fibroblasts that were engineered for simultaneous knock-in of the IL-15RF and hnCD16 at the CD38 locus. **(B)** Design of donor construct for simultaneous transgene knock-in into the CD38 locus to facilitate CD38 gene disruption. **(C)** Two multi-loci 2nd round engineering strategies for simultaneous knockout of B2M and CIITA and CAR knock-in were conducted in parallel to generate iPSC master cell lines with additional immune-evasive edits from the multiplexed-engineered foundation line. Strategy 1 consists of dual-loci engineering in which CAR knock-in was directed to the *B2M* locus for synchronized knockout of B2M with concurrent CIITA knockout via a *CIITA* targeted gRNA. Strategy 2 consists of triple-loci engineering in which CAR knock-in was directed to a safe harbor locus while gRNAs targeting *B2M* and *CIITA* were simultaneously administered for knockout. Engineered iPSCs were single cell sorted for transgene marker expression and loss of B2M surface expression in both strategies. **(D)** Multi-loci targeting layouts for strategies 1 and 2 with designs of donor constructs for simultaneous CAR knock-in and B2M knockout (strategy 1) and CAR knock-in at a safe harbor locus (strategy 2). **(E)** PCR assay to screen for clones with targeted integration of the transgene cassette into the *B2M* locus (Junction) and without the random integration of the donor plasmid (Donor plasmid backbone). A similar assay was used to screen for integration at the safe harbor locus. **(F, G)** Molecular characterization of sorted clones show highly efficient and precise transgene targeting (>90% of clones with on-target integration without random donor plasmid integration in both strategies) and high efficiency, *B2M* and *CIITA* editing. **(H)** Transgene copy numbers in isolated clones were determined using ddPCR. Both mono- and biallelic clones were identified. **(I)** Representative cytogenic analyses of engineered iPSC clones demonstrate normal, diploid karyotypes post multi-loci engineering for both strategies. **(J)** Representative flow cytometry profile of clonal iPSCs demonstrates uniform expression of pluripotency markers SSEA-4, TRA-1-81, and CD30 for all isolated clones. **(K)** Representative flow cytometry profile of engineered iPSCs demonstrates loss of B2M and MHC class I expression and uniform expression of the transgene marker (blue) compared to non-engineered iPSCs (grey).

iPSCs with Engineered Immune-Evasion Modalities Robustly Differentiate into NK cells

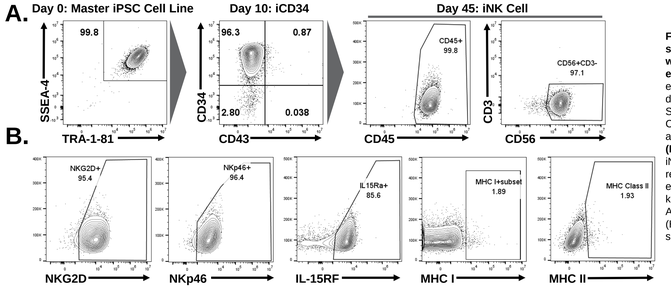


Figure 2. Phenotype of off-the-shelf NK product engineered with anti-tumor and immune-evasive elements. (A) Immune-evasive NK cells are produced by directed differentiation of SSEA4+TRA-1-81+ iPSCs to CD34+ hematopoietic progenitors and then to CD45+CD56+ NK cells. **(B)** CD45+/CD56+ immune-evasive iNK cells express the activating receptors NKG2D and NKp46 with expression of IL-15RF and knockout of all MHC class I (HLA-A, B, and C) and MHC class II (HLA-DR, DP, and DQ) receptor subtypes.

Engineered Immune-Evasion Modalities Prevent the Emergence of Alloreactive NK and T Cells

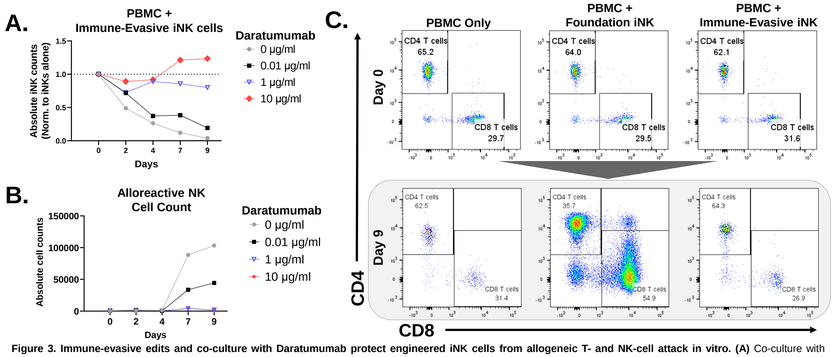


Figure 3. Immune-evasive edits and co-culture with Daratumumab protect engineered iNK cells from allogeneic T- and NK-cell attack in vitro. (A) Co-culture with Daratumumab protects immune-evasive iNK cells co-cultured with HLA-mismatched peripheral blood mononuclear cells (PBMCs) in a dose dependent manner. **(B)** Daratumumab decreases the number of alloreactive NK cells in a dose dependent manner when immune-evasive iNK cells are co-cultured with HLA-mismatched PBMCs. **(C)** iNK cells engineered with B2M and CIITA knockout edits do not stimulate the expansion of CD4+ and CD8+ T cells when co-cultured with HLA-mismatched PBMCs.

Summary

- As a first-of-kind demonstration, we successfully generated precision-engineered iPSC master cell lines incorporating six unique functional elements (including 3 bi-allelic disruptions) at clonal level that drive anti-tumor activity and functional persistence through multiple rounds of highly efficient, on-target engineering with simultaneous editing at multiple loci
- CD38 knockout combined with anti-CD38 Daratumumab treatment protects iNK cells from allorejection in vitro by selectively depleting alloreactive CD38+ lymphocytes
- Ablation of MHC class I and II via simultaneous knockout of B2M and CIITA prevents the activation of alloreactive CD4+ and CD8+ T cells by multiplex-engineered iNK cells
- These data validate the robustness of our proprietary iPSC product platform to support high-precision, multi-loci engineering of iPSCs to create clonally derived, multiplexed-engineered iPSC master cell lines to produce next-generation, off-the-shelf immune-evasive CAR-iNK cell therapies for use in patients with minimal requirements for immune suppression chemotherapy conditioning