Efficient knockout of both CD33 and CLL-1 by multiplex genome editing of human hematopoietic stem cells enhances the potential of next-generation transplants for acute myeloid leukemia (AML) treatment

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P150

INTRODUCTION

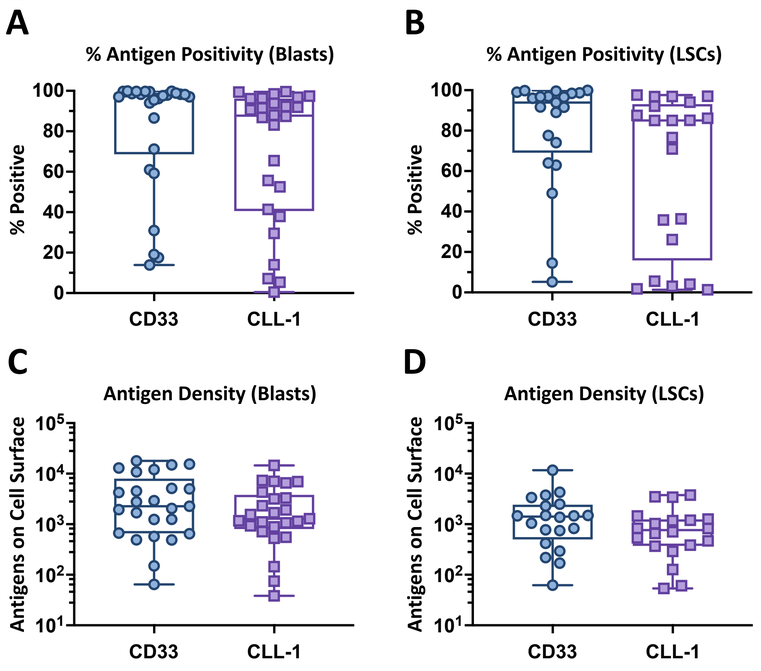
- Acute myeloid leukemia (AML) is a heterogeneous disease characterized by abnormal clonal expansion; it is the most common form of adult acute leukemia.
- Though hematopoietic stem cell transplantation (HCT) is the standard of care for patients with high-risk AML, post-HCT relapse occurs in 40% of these patients, highlighting the need for new therapeutic approaches such as immunotherapy.
- Cluster of differentiation 33 (CD33) and C-type lectin-like molecule-1 (CLL-1) are highly expressed in AML patient blasts/leukemic stem cells (LSCs), suggesting that immunotargeting both CD33 and CLL-1 can address AML heterogeneity and reduce chances of tumor resistance. Targeting these antigens, however, can lead to cytopenia due to shared expression on normal hematopoietic cells.
- Deleting both CD33 and CLL-1 from hematopoietic stem cell (HSC) grafts prior to HCT restricts these antigens to AML cells in the event that relapse occurs post-HCT, thereby enabling the potential for subsequent immunotherapy without risk of on-target, off-tumor toxicities.

OBJECTIVES

- Demonstrate that multiplex (MPX) deletion of CD33 and CLL-1 from CD34⁺ human hematopoietic stem and progenitor cells (hHSPCs) does not impact HSC function.
- Demonstrate that cells deleted for CD33 and CLL-1 are protected from targeted immunotherapies

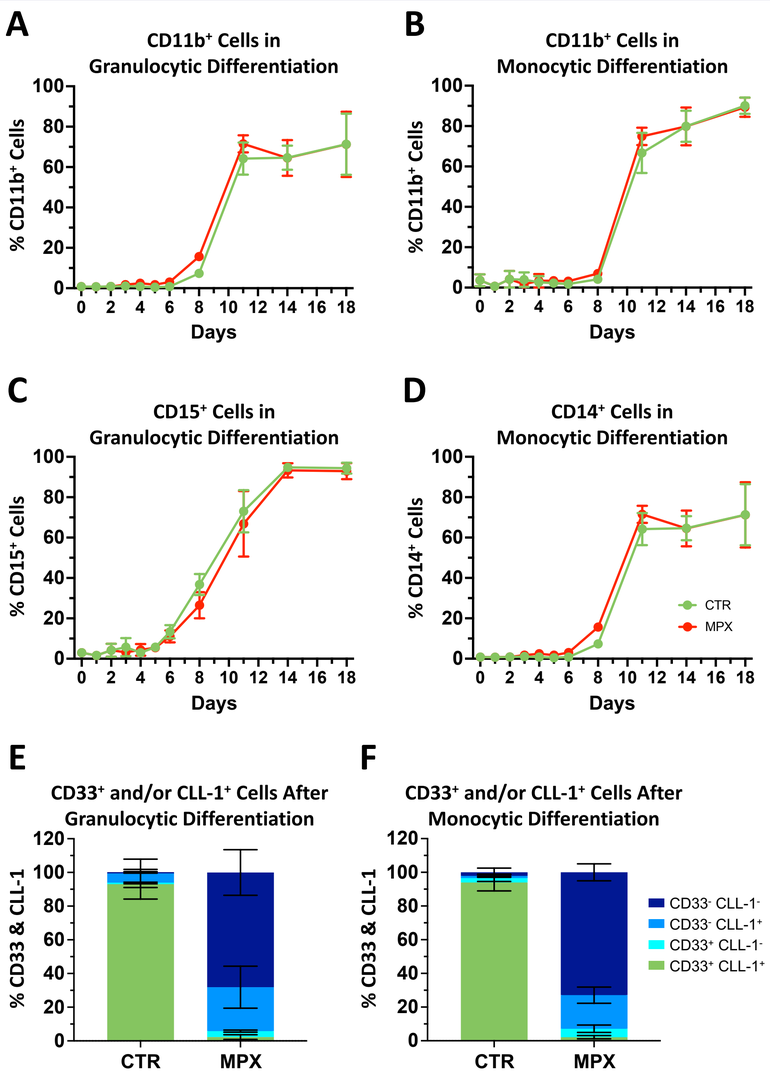
RESULTS

Figure 1. CD33 and CLL-1 are highly expressed in AML patient–derived blasts and LSCs



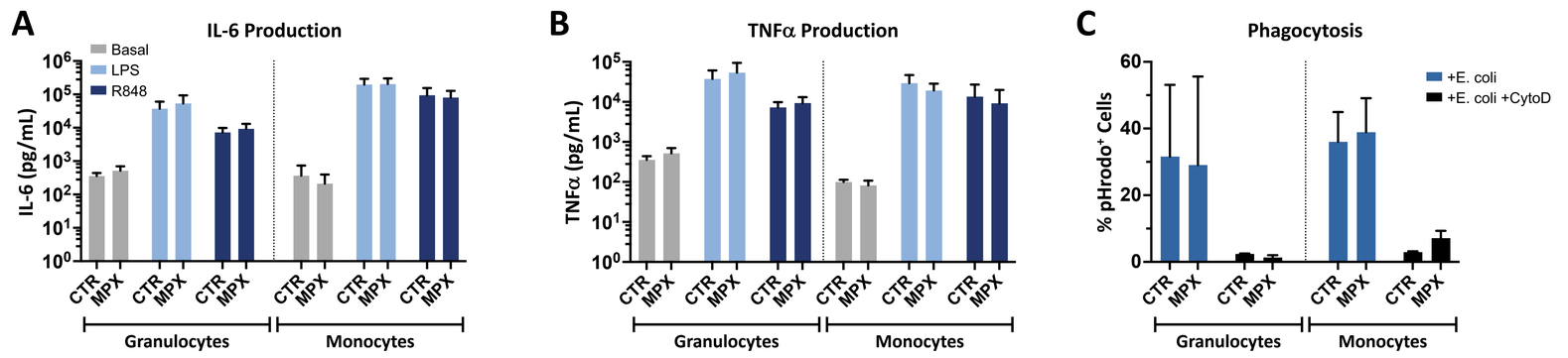
Percentage of CD33- and CLL-1–positive cells by flow cytometry in AML patient-derived **(A)** blasts (n=26) and **(B)** LSCs (n=21). CD33 and CLL-1 antigen density on the cell surface quantified by flow cytometry in AML **(C)** blasts (n=25) and **(D)** LSCs (n=20).

Figure 2. High biallelic editing achieved in CD33 and CLL-1 MPX-edited CD34⁺ hHSPCs with no impact to myeloid differentiation *in vitro*



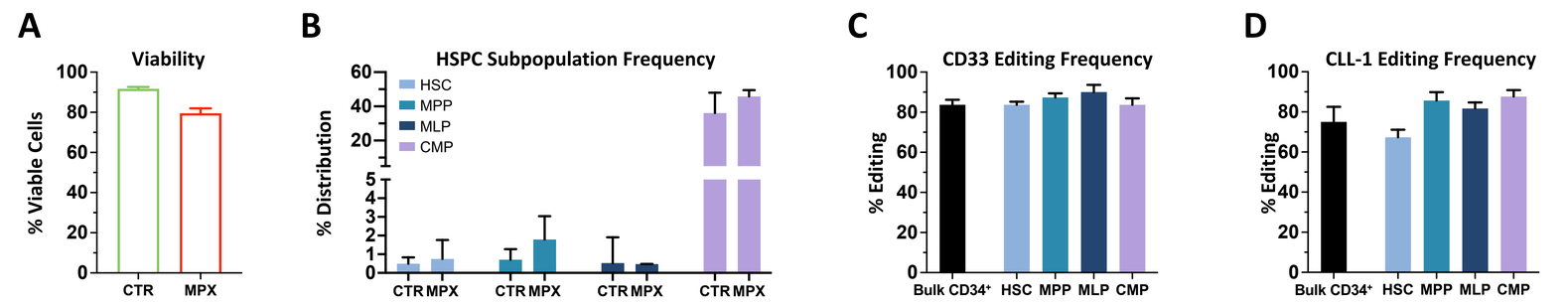
Mobilized peripheral blood CD34⁺ hHSPCs were sequentially edited using CRISPR/Cas9 with gRNAs against CD33 and CLL-1 and *in vitro* differentiated into **(A,C,E)** granulocytic or **(B,D,F)** monocytic lineages. Percentage of **(A,B)** CD11b⁺ cells, **(C)** CD15⁺ cells and **(D)** CD14⁺ cells differentiated from control (CTR) unedited or multiplex (MPX)-edited cells over differentiation time course. High levels of biallelic editing at both loci maintained throughout **(E)** granulocytic and **(F)** monocytic differentiation as assessed by flow cytometry analysis of CD33 and CLL-1 protein expression on Day 18. N=3 donors. Data shown as mean ± standard deviation.

Figure 3. *In vitro* myeloid differentiated cells derived from CD33 and CLL-1 MPX-edited hHSPCs maintain normal myeloid function with intact cytokine secretion and phagocytic capabilities



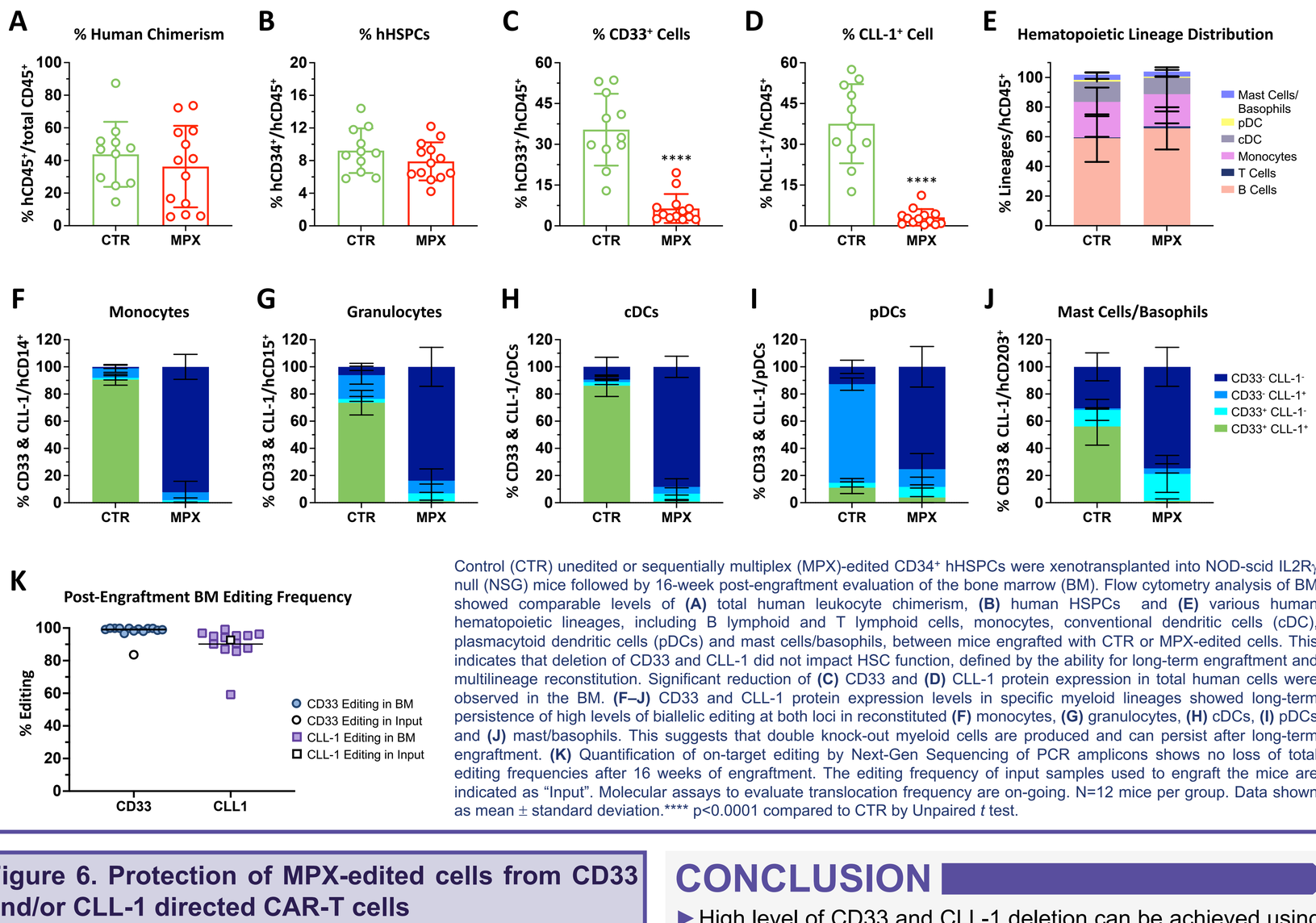
(A,B) Cytokine production from differentiated myeloid cells following MPX editing at basal levels and 24 hours after LPS or R848 stimulus. Cytokine release was measured by Luminex for **(A)** IL-6 and **(B)** TNFα production. Similar results observed for IL-8 and MIP-1α (data not shown). **(C)** Phagocytic ability of differentiated cells was analyzed by flow cytometry as the percent pHrodo⁺ cells after incubation with pHrodo-conjugated *E coli* alone or pHrodo-conjugated *E coli* and CytoD (as negative control). N=3 donors. Data shown as mean ± standard deviation.

Figure 4. MPX-edited hHSPCs for CD33 and CLL-1 retain high viability and normal distribution of hematopoietic stem and progenitor subpopulations *in vitro*



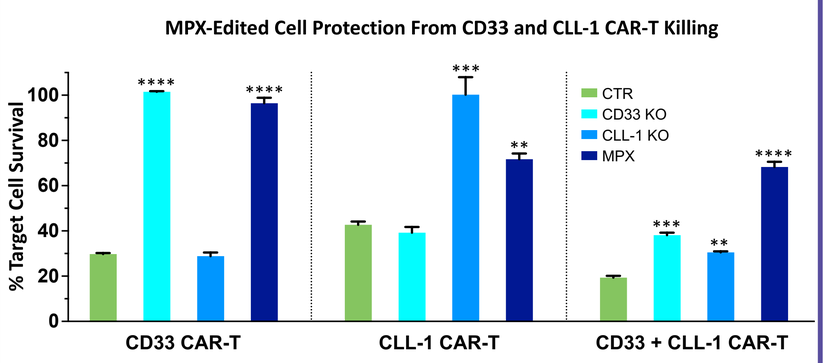
MPX-edited CD34⁺ hHSPCs maintained high viability **(A)** compared control cells (CTR) with no impact to hematopoietic stem cell (HSC), multipotent progenitor (MPP), multi-lymphoid progenitor (MLP) or common myeloid progenitor (CMP) frequencies **(B).** The high editing efficiency in bulk CD34⁺ cells was maintained for both **(C)** CD33 and **(D)** CLL-1 across all subpopulations. N=3 donors. Data shown as mean ± standard deviation.

Figure 5. MPX editing of CD33 and CLL-1 in CD34⁺ hHSPCs does not impact HSC function. Biallelic MPX-edited cells can engraft long-term, differentiate into multilineages and persist *in vivo*



Control (CTR) unedited or sequentially multiplex (MPX)-edited CD34⁺ hHSPCs were xenotransplanted into NOD-scid IL2Rγ null (NSG) mice followed by 16-week post-engraftment evaluation of the bone marrow (BM). Flow cytometry analysis of BM showed comparable levels of **(A)** total human leukocyte chimerism, **(B)** human HSPCs and **(E)** various human hematopoietic lineages, including B lymphoid and T lymphoid cells, monocytes, conventional dendritic cells (cDC), plasmacytoid dendritic cells (pDCs) and mast cells/basophils, between mice engrafted with CTR or MPX-edited cells. This indicates that deletion of CD33 and CLL-1 did not impact HSC function, defined by the ability for long-term engraftment and multilineage reconstitution. Significant reduction of **(C)** CD33 and **(D)** CLL-1 protein expression in total human cells were observed in the BM. **(F–J)** CD33 and CLL-1 protein expression levels in specific myeloid lineages showed long-term persistence of high levels of biallelic editing at both loci in reconstituted **(F)** monocytes, **(G)** granulocytes, **(H)** cDCs, **(I)** pDCs and **(J)** mast/basophils. This suggests that double knock-out myeloid cells are produced and can persist after long-term engraftment. **(K)** Quantification of on-target editing by Next-Gen Sequencing of PCR amplicons shows no loss of total editing frequencies after 16 weeks of engraftment. The editing frequency of input samples used to engraft the mice are indicated as "Input". Molecular assays to evaluate translocation frequency are on-going. N=12 mice per group. Data shown as mean ± standard deviation.**** p<0.0001 compared to CTR by Unpaired *t* test.

Figure 6. Protection of MPX-edited cells from CD33 and/or CLL-1 directed CAR-T cells



HL-60 cells were either edited for CD33 or CLL-1, or MPX-edited for both, and knock-out (KO) cells were purified by FACS sorting prior to *in vitro* cytotoxicity assay with CD33 CAR-T, CLL-1 CAR-T or CD33 CAR-T pooled with CLL-1 CAR-T cells. The percentage of live cells were plotted. Single and double KO cells were significantly protected from their respective CAR-T cells. CTR, Control unedited cells, CAR-T, chimeric antigen receptor T. N=2. Data shown as mean ± standard deviation. ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to CTR by One-way ANOVA.

CONCLUSION

- High level of CD33 and CLL-1 deletion can be achieved using sequential Cas9 editing approach in CD34⁺ hHSPCs without affecting cell viability, HSPC distribution and *in vitro* myeloid differentiation/function.
- CD33 and CLL-1 multiplex-edited hHSPCs maintain robust hematopoiesis and multilineage reconstitution with high levels of biallelic editing at both loci *in vivo*.
- Gene modifications in dual-engineered cells can persist long-term after engraftment indicating no counterselection for these cells.
- CD33 and CLL-1 multiplex-edited cells also showed significant protection from CD33 and/or CLL-1 CAR-T cells.
- Pairing multiplex-edited hHSPCs with subsequent multi-specific immunotherapy can obviate concerns around tumor heterogeneity and escape mechanisms related to single antigen downregulation, transforming the current treatment approach for AML.

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Disclosures
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