Multiplex Deletion of Myeloid Antigens CD33 and CLL-1 by CRISPR/Cas9 in Human Hematopoietic Stem Cells Highlights the Potential of Next-Generation Transplantation for AML Treatment

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INTRODUCTION

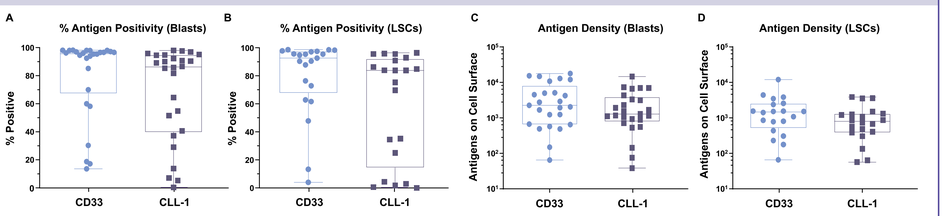
- Acute myeloid leukemia (AML) is a heterogeneous disease characterized by abnormal clonal expansion; it is the most common form of adult acute leukemia.
- Though hematopoietic stem cell transplantation (HCT) is the standard of care for patients with high-risk AML, post-HCT relapse occurs in 40% of these 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–derived 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.

OBJECTIVE

- 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

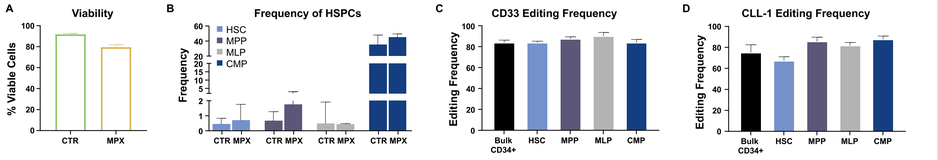
RESULTS

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



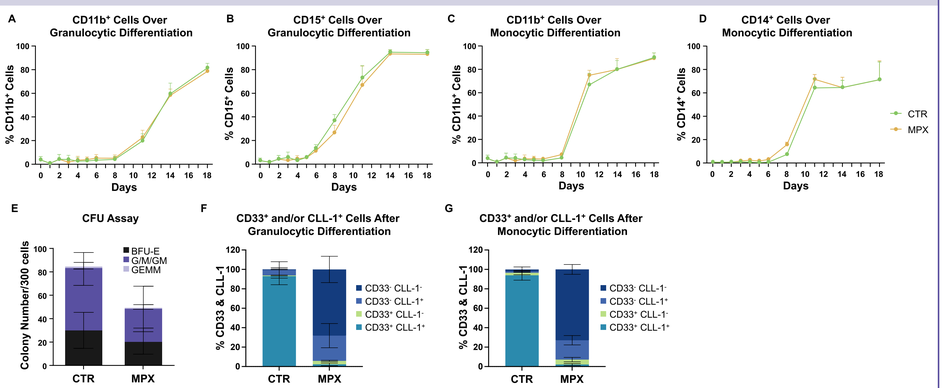
- Percentage of CD33- and CLL-1–positive cells by flow cytometry in AML **(A)** blasts (n=33) and **(B)** LSCs (n=27). CD33 and CLL-1 antigen density on the cell surface quantified by flow cytometry in **(C)** blasts (n=31) and **(D)** LSCs (n=25).

Fig. 2. MPX-edited hHSPCs for CD33 and CLL-1 retain high viability and normal distribution of hematopoietic progenitor subpopulations *in vitro*



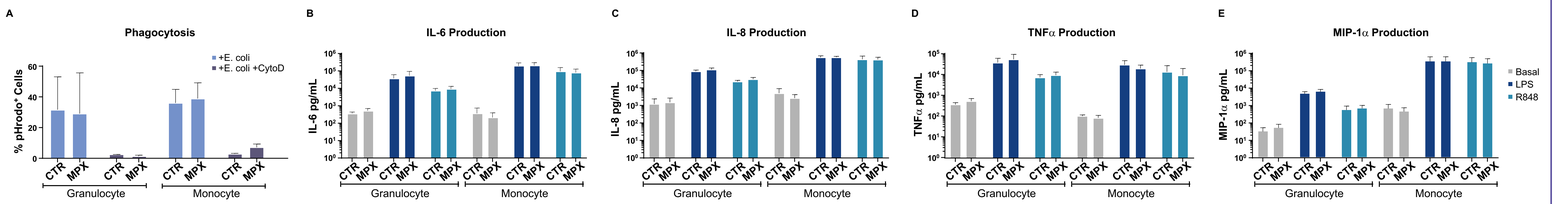
- Mobilized peripheral blood CD34$^+$ hHSPCs were sequentially gene edited using CRISPR/Cas9 with gRNAs against CD33 and CLL-1. **(A)** The viability of MPX-edited hHSPCs was maintained compared to non-electroporated cells (CTR) with **(B)** no impact on the distribution of hematopoietic stem cells, multipotent progenitors, or myeloid or lymphoid progenitors. The high editing efficiency in bulk CD34$^+$ cells was maintained for both **(C)** CD33 and **(D)** CLL-1. CMP, common myeloid progenitor; MLP, multi-lymphoid progenitor; MPP, multipotent progenitor. N=3 donors. Data shown as mean ± standard deviation.

Fig. 3. CD33 and CLL-1 MPX-edited hHSPCs maintain myeloid differentiation *in vitro*



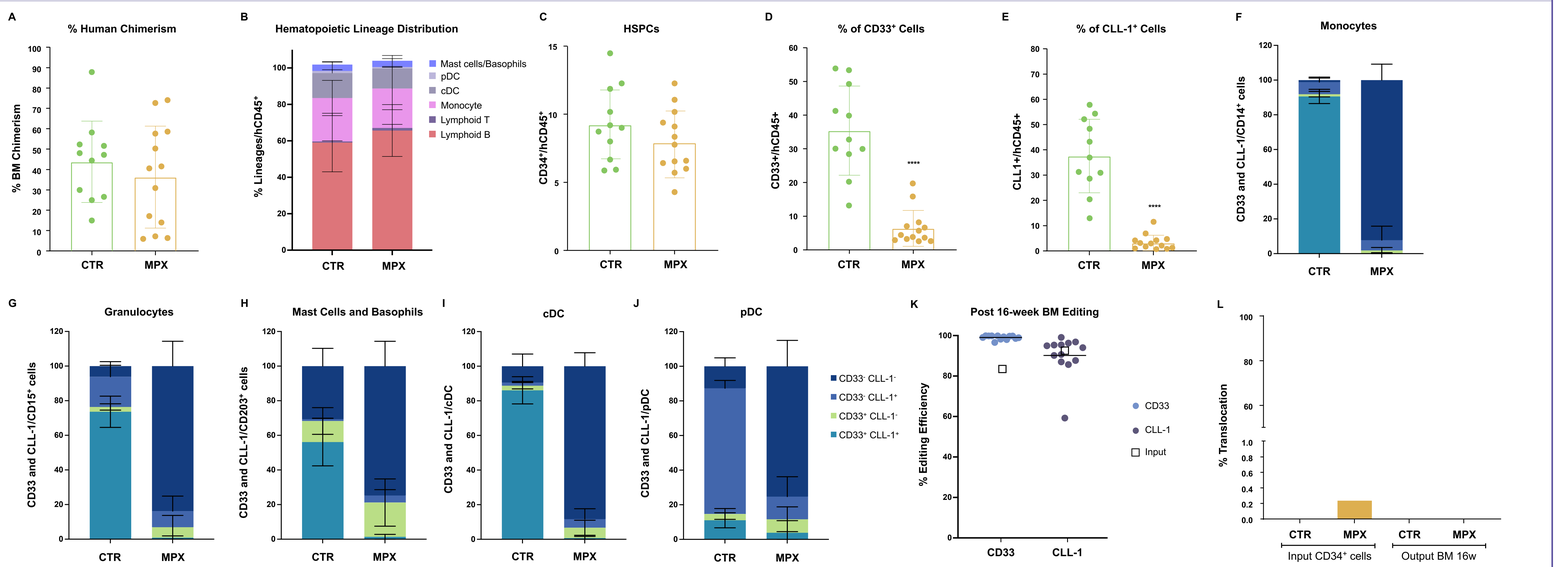
- **(A-D)** Effects of CD33 and CLL-1 MPX editing on granulocytic and monocytic differentiation in HSPCs, respectively. Percentage of **(A)** CD11b$^+$ cells and **(B)** CD15$^+$ cells at the indicated time points. Percentage of **(C)** CD11b$^+$ cells and **(D)** CD14$^+$ cells at the indicated time points. **(E)** Colony forming unit (CFU) number of CD33 and CLL-1 MPX-edited cells. **(F, G)** High levels of biallelic editing at both loci maintained throughout **(F)** granulocytic and **(G)** monocytic differentiation as assessed by flow cytometry analysis of CD33 and CLL-1 at the end of the *in vitro* differentiation. N=3 donors. BFU-E, burst-forming unit–erythroid; GEMM, multipotential myeloid progenitor cells that generate granulocyte, erythroid, macrophage, and megakaryocytes; G/M/GM, granulocyte, macrophage. Data shown as mean ± standard deviation.

Fig. 4. *In vitro* myeloid differentiated cells derived from CD33 and CLL-1 MPX-edited hHSPCs maintain function



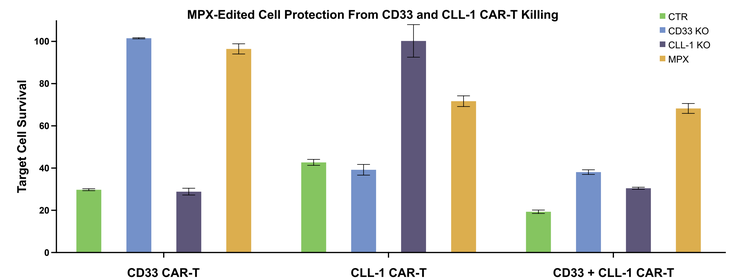
- **(A)** Phagocytic ability of differentiated granulocytes and monocytes following MPX editing. Phagocytic ability was analyzed as the percent pHrodo$^+$ cells that were subjected to pHrodo$^+$ *Escherichia coli* cells or pHrodo$^+$ *E coli* and CytoD. **(B-E)** Cytokine production from differentiated myeloid cells following MPX editing at basal levels and 24 hours after LPS or R848 stimulus. Cytokine release assay was performed by Luminex for **(B)** IL-6, **(C)** IL-8, **(D)**TNFα and **(E)** MIP-1α production. N=3 donors. Data shown as mean ± standard deviation.

Fig. 5. CD33 and CLL-1 MPX-edited hHSPCs maintain long-term engraftment and multilineage differentiation *in vivo*



- BM analysis of NSG mice at 16 weeks after xeno-transplantation with MPX-edited hHSPCs. Flow cytometry analysis of BM **(A)** human chimerism, **(B)** human hematopoietic lineages including lymphoid B and T cells, monocytes, cDC, pDCs and mast cells/basophils, and **(C)** CD34$^+$ HSPCs. Significant reduction of **(D)** CD33 and **(E)** CLL-1 protein in the total human cells in the BM. **(F–J)** CD33 and CLL-1 protein expression levels in myeloid cells showing long-term persistence of high levels of biallelic editing at both genes with no impact in reconstituted **(F)** monocytes, **(G)** granulocytes, **(H)** mast/basophils, **(I)** cDCs and **(J)** pDCs. **(K)** Quantification of on-target editing by NGS of PCR amplicons showing no loss of total editing frequencies after engraftment. The square data point indicates the editing frequency of input samples used to engraft the mice. **(L)** Total translocation events in input cells (CD34$^+$ hHSPCs) and in output xenograft BM cells. N=12 mice per group. BM, bone marrow; NSG, NOD-scid IL2Rγ null. Data shown as mean ± standard deviation.

Fig. 6. Protection of MPX-edited cells from CAR-Ts targeting CD33 and/or CLL-1



- 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. CTR, Control unedited cells, CAR-T, chimeric antigen receptor T.

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 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.
- 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.

Disclosures

All authors listed here are current employees and equity holders of Vor Biopharma, except for asterisked authors*, who are no longer employees at Vor Biopharma.

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