P #3818

Knock Out of CD123 or CLL-1 by CRISPR-Cas9 Editing From Human Hematopoietic Stem Cell Transplantations Provide New Possibilities for Increasing Therapeutic Index and Safety for AML Treatment

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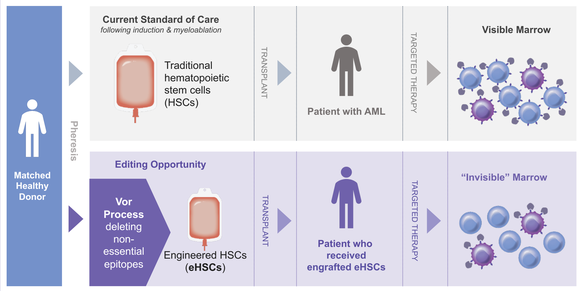
INTRODUCTION

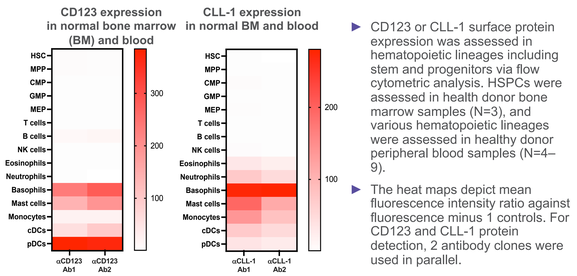
- Acute myeloid leukemia (AML) is a clonal disorder of hematopoiesis and the most common form of acute leukemia in adults that accounts for >11,000 deaths per year in the US.
- Most patients with AML relapse despite intensive chemotherapy. Allogeneic hematopoietic stem cell transplantation (HSCT) has become the standard of care for patients with intermediate or adverse genetics, with >3500 transplantations performed annually in the US.
- However, leukemia relapse after HSCT occurs in ~40% of these patients with a 2-year survival rate at <20%, necessitating new approaches to reduce relapse and improve overall outcomes.
- Targeted immunotherapies for the treatment of AML, while promising, are associated with myelosuppression caused by on-target off-tumor cytotoxicity owing to these targeted antigens such as cluster of differentiation 123 (CD123) or C-type lectin-like molecule-1 (CLL-1)[1] being present on both AML and normal myeloid cells.

OBJECTIVE

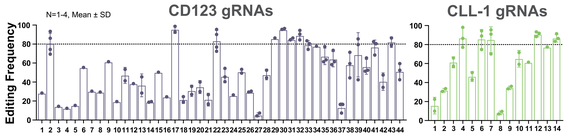
- To circumvent such myelotoxicity, CD123 or CLL-1 negative human hematopoietic stem and progenitor cells (hHSPCs) were created for HSCT to enable subsequent targeted therapy against these antigens to prevent post-HSCT relapse.
- Here, we present *in vitro* and *in vivo* preclinical evaluation to biologically de-risk CRISPR/Cas9 engineered CD123 or CLL-1 knock out (KO) hHSPC and to demonstrate as proof-of-concept, protection of CD123 or CLL-1 KO cells from targeted immunotherapies.

Fig. 1. Engineering the Patient to Make Treatment-Resistant Transplant





- CD123 or CLL-1 surface protein expression was assessed in hematopoietic lineages including stem and progenitors via flow cytometric analysis. HSPCs were assessed in health donor bone marrow samples (N=3), and various hematopoietic lineages were assessed in healthy donor peripheral blood samples (N=4–9).
- The heat maps depict mean fluorescence intensity ratio against fluorescence minus 1 controls. For CD123 and CLL-1 protein detection, 2 antibody clones were used in parallel.
- A saturated guide RNA (gRNA) screen was designed and produced 209 and 123 gRNAs for CD123 and CLL-1, respectively. On-target editing was assessed in THP-1 cells followed by validation in CD34⁺ HSPCs (44 CD123 gRNAs and 14 CLL-1 gRNAs). Lead gRNAs were identified as those with >80% mean on-target editing frequency, optimal insertion/deletion (Indel) distribution (primarily +/- 1, 2 bp) and minimal *in silico* predicted off-target editing.

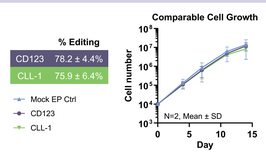


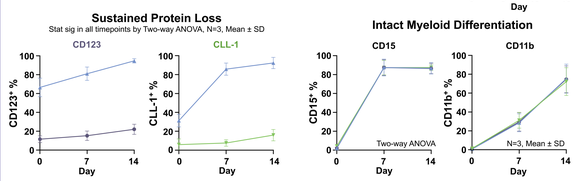
RESULTS

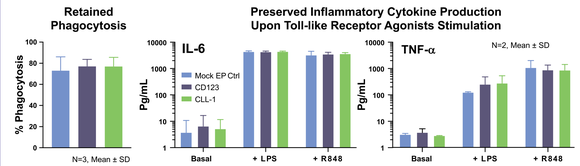
Fig. 2. Myeloid/Erythroid *In Vitro* Differentiation

Myeloid *In Vitro* Differentiation

- CD34⁺ hHSPCs were electroporated (EP) with CRISPR-Cas9 ribonucleoprotein complex using CD123 or CLL-1 gRNA and *in vitro* differentiated into granulocytic lineage.
- Both gRNAs attained high editing frequencies.

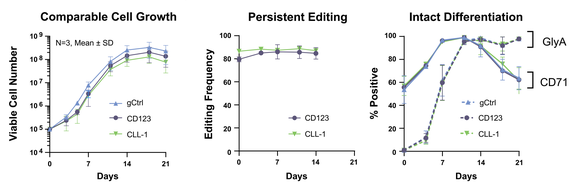






- Loss of CD123 or CLL-1 did not impair *in vitro* granulocyte cell expansion or differentiation as assessed by CD15, CD11b, CD33 (data not shown), and HLA-DR (data not shown) expression.
- High editing % was maintained throughout, resulting in sustained CD123 or CLL-1 protein loss.
- Myeloid functions, assessed by phagocytosis and secretion of inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β (data not shown), and macrophage inflammatory protein-1α (data not shown) were unaltered by CD123 or CLL-1 loss.
- Similar findings were observed when *in vitro* differentiated into the monocytic lineage.

Erythroid *In Vitro* Differentiation



- Loss of CD123 or CLL-1 did not impair *in vitro* erythroid cell expansion or differentiation as assessed by CD71, GlyA, α4-integrin (data not shown), and Band3 (data not shown) expression.
- High editing % was maintained throughout differentiation.
- Enucleation rates were similar to cells edited with control gRNA (gCtrl).

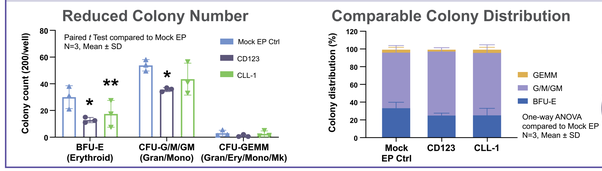
% Enucleation	
gCtrl edited	64.9 ± 4.4%
CD123 edited	63.1 ± 2.9%
CLL-1 edited	65.1 ± 3.8%

Fig. 3. Off-target Summary

Lead gRNA	# pOT sites assessed	# of sites w/ sig editing	Location of significant pOTs
CD123 g2	948	2	cds/splice site/ intron/3'UTR
CD123 g22	784	2	cds/intron/ intergenic
CD123 g32	650	1	splice site/gene upstream/intron
CLL-1 g4	954	1	intron
CLL-1 g6	388	0	
CLL-1 g12	776	1	gene upstream

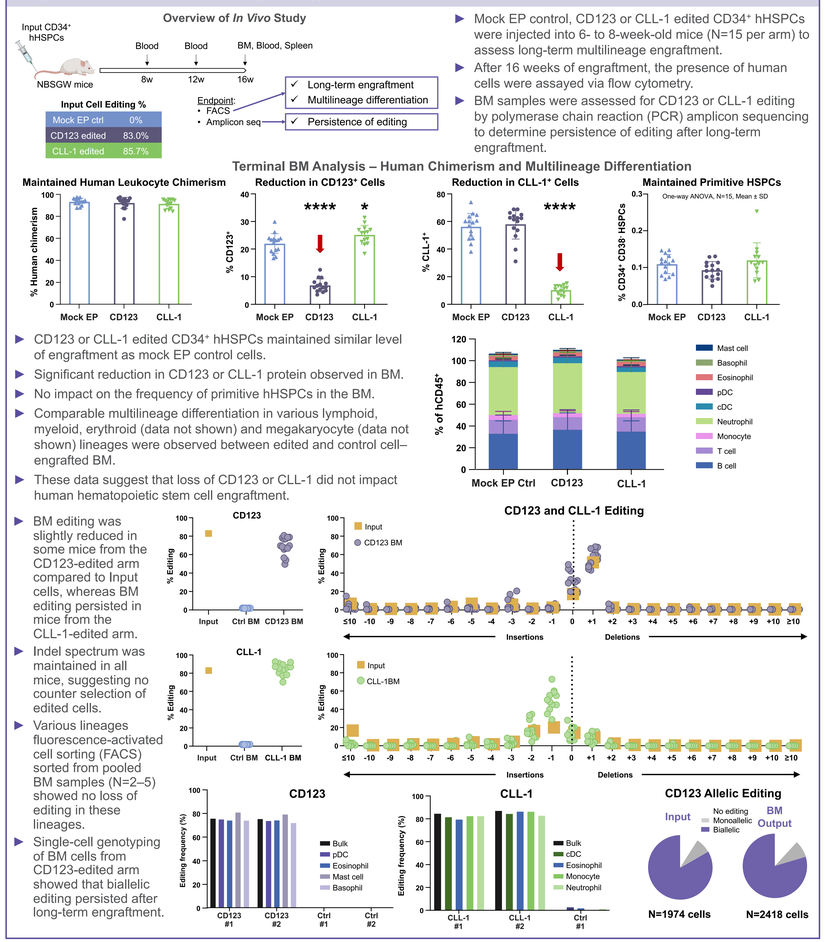
- The # of sites homologous ≤5 mismatches to the on-target/PAM sequence of candidate gRNAs were assessed (N=3) using hybrid capture followed by next-generation sequencing.
- The # of potential off-target (pOT) sites with observable editing % at least 0.2% above that of Mock EP were indicated as sites with significant (sig) editing.
- These sites were further risk stratified based their genomic location.

Fig. 4. *In Vitro* Multilineage Potential and Allelic Editing



- CD123 edited (77.9±4.5%) or CLL-1 edited (79.2±7.5%) CD34⁺ hHSPCs were plated for *in vitro* colony-forming unit (CFU) assay to assess multilineage potential.
- While reduced number of bust-forming unit–erythroid (BFU-E) colonies was observed, loss of CD123 or CLL-1 retained distribution of colony types.
- Single cell-derived colonies genotyped for CD123 or CLL-1 on-target editing showed that the vast majority of cells was biallelically edited.

Fig. 5. *In Vivo* Multilineage Engraftment



- Mock EP control, CD123 or CLL-1 edited CD34⁺ hHSPCs were injected into 6- to 8-week-old mice (N=15 per arm) to assess long-term multilineage engraftment.
- After 16 weeks of engraftment, the presence of human cells were assayed via flow cytometry.
- BM samples were assessed for CD123 or CLL-1 editing by polymerase chain reaction (PCR) amplicon sequencing to determine persistence of editing after long-term engraftment.
- CD123 or CLL-1 edited CD34⁺ hHSPCs maintained similar level of engraftment as mock EP control cells.
- Significant reduction in CD123 or CLL-1 protein observed in BM.
- No impact on the frequency of primitive hHSPCs in the BM.
- Comparable multilineage differentiation in various lymphoid, myeloid, erythroid (data not shown) and megakaryocyte (data not shown) lineages were observed between edited and control cell–engrafted BM.
- These data suggest that loss of CD123 or CLL-1 did not impact human hematopoietic stem cell engraftment.
- BM editing was slightly reduced in some mice from the CD123-edited arm compared to Input cells, whereas BM editing persisted in mice from the CLL-1-edited arm.
- Indel spectrum was maintained in all mice, suggesting no counter selection of edited cells.
- Various lineages fluorescence-activated cell sorting (FACS) sorted from pooled BM samples (N=2–5) showed no loss of editing in these lineages.
- Single-cell genotyping of BM cells from CD123-edited arm showed that biallelic editing persisted after long-term engraftment.

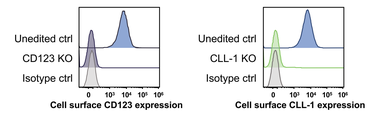
Fig. 6. Protection From Chimeric Antigen Receptor (CAR)-T Cells

CD123 KO MOLM-13

CD123 KO generated by CRISPR/Cas9
⇩
CD123 KO fraction purified by flow cytometry sorting

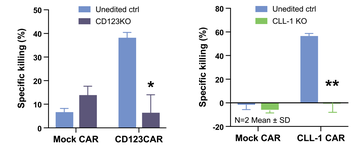
CLL-1 KO HL-60

CLL-1 KO generated by CRISPR/Cas9
⇩
CLL-1 KO fraction purified by flow cytometry sorting



CAR-T Cytotoxicity

Effector : Target = 1:1
Unpaired student *t* Test



- CD123-edited MOLM-13 KO and CLL-1-edited HL-60 KO AML cell lines were purified via FACS sorting.
- These cells were subjected to *in vitro* cytotoxicity assay when incubated with either CD123 CAR-T cells, CLL-1 CAR-T cells or untransduced control T cells (Mock CAR).
- % killing was significantly reduced when the CAR-T cells were incubated with the respective KO cell line.
- These data demonstrate proof-of-concept protection of CD123 or CLL-1 KO cells from targeted immunotherapy.

CONCLUSION

- In conclusion, we demonstrate that CD123 or CLL-1 negative hHSPCs can successfully carry out functional hematopoiesis and that the KO cells are resistant to CD123 or CLL-1 targeted therapies.
- Our findings provide a next-generation HSCT strategy that supports the safe and effective use of antigen-directed immunotherapy treatments for patients with AML.

Reference

1. Perna F, et al. *Cancer Cell.* 2017;32:506-519.

Acknowledgments

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Disclosures

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

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