P#618

Gene Editing and Immunotherapeutic Targeting of ADGRE2/EMR2 to Enable Combinatorial Targeting in Acute Myeloid Leukemia (AML)

Julia Etchin, Yonina Keschner, Mariana Silva, Hillary Hoyt, Hannah Mager, Amanda Halfond, Julia DiFazio, Matthew Ung, Guy Mundelboim, Ciara Tucker, Jaime Sanchez, Julian Scherer, John Lydeard, Tirtha Chakraborty

Vor Biopharma, Cambridge, MA, USA 02140

INTRODUCTION

- Targeted immunotherapy of Acute Myeloid Leukemia (AML) has been limited due to lack of tumor-specific antigens resulting in "on-target, off-tumor" effects that can lead to severe cytopenia
- To unlock the full potential of targeted treatments, we engineer hematopoietic stem and progenitor cells (HSPCs) by genetically ablating target antigens from healthy, donor-derived HSPCs for hematopoietic stem cell (HSC) transplant. This allows compatible immuno-therapy to specifically kill leukemic cells bearing the AML target-antigen while sparing the antigen-null allogenic graft[1-4]
- Combinatorial targeting of AML antigens may be required to avoid antigen escape and address tissue heterogeneity, while multiplex genome editing of these antigens is necessary to protect the transplanted healthy graft (Figure 1)

OBJECTIVE

- Elucidate EMR2 antigen as a prospective AML target and advance novel EMR2-targeting CAR-T therapies
- Assess EMR2 expression in healthy heme tissues, evaluate its biological dispensability, and demonstrate its efficient multiplexing in healthy HSCs to enable combinatorial targeting of AML cells while sparing normal cells

Figure 1. Vor eHSC Multiplex Platform

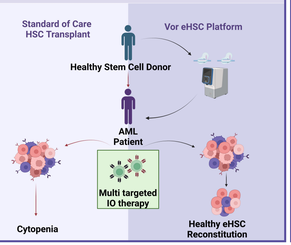
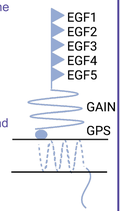


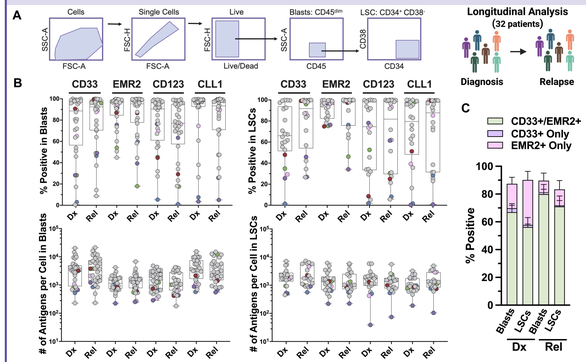
Figure 2. ADGRE2/EMR2 Structure and Background

- Member of the epidermal growth factor (EGF) – seven-transmembrane (TM7) sub-family (EMR1, 2, 3, 4, and CD97) of Adhesion G protein-coupled receptor (GPCR) family[5]
- Alternative splicing results in multiple isoforms that contain varying number of EGF domains
- Plays a role in adhesion, migration, invasion, angiogenesis, and inflammatory response[6-8]
- Expressed on healthy myeloid cells and the majority of AML blasts and leukemic stem cells (LSCs)[9]
- High occurrence of loss-of-function (LOF) alleles reported in the gnomAD database[10]
- A gain-of-function mutation within the GAIN domain of EMR2 causes familial vibratory urticaria, a rare autosomal dominant dermal allergic disorder resulting from vibration-induced mast cell activation[11-12]



Results: Novel Immunotherapeutic Targeting of EMR2

Figure 3. EMR2 is Expressed on AML Blasts and Leukemia Stem Cells

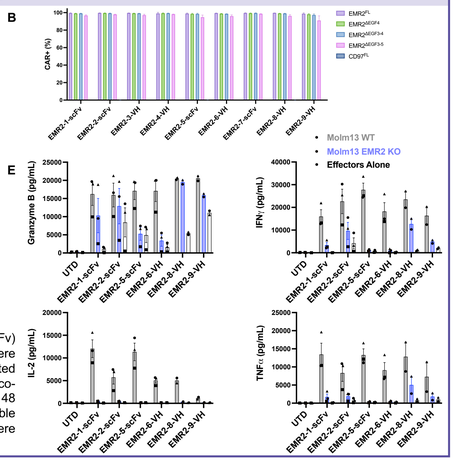


A. Gating schema for the flow cytometric surface expression longitudinal profiling of bone marrow samples from AML patients (N=32). **B**. Percent positivity and number of antigens per antigen$^+$ cell in blasts and LSCs. QuantiBrite beads were used for antigen quantitation. Colored dots trace expression data for select patients to demonstrate expression pattern for the four antigens. Data shown as box and whiskers. **C**. CD33 and EMR2 co-expression analysis for samples from **B**. Data shown as median ± standard error of mean. Leukemic stem cells (LSCs), Diagnosis (Dx), Relapse (Rel).

Figure 4. EMR2-directed CARs mediate potent EMR2-specific cytolytic activity against AML cells *in vitro*

A Table 1: Binder affinity and kinetic characterization

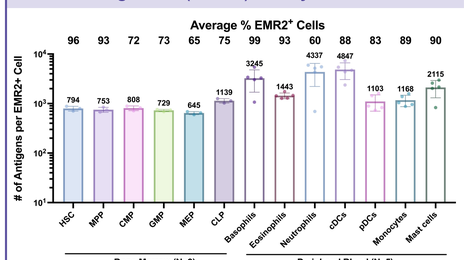
Property	Target	EMR2-1-scFv	EMR2-2-scFv	EMR2-3-scFv	EMR2-4-scFv	EMR2-5-scFv	EMR2-6-VH	EMR2-7-VH	EMR2-8-VH	EMR2-9-VH
EC50 (nM)	NAviHis-EMR2 ECD	1.0	0.6	0.3	1.9	1.59	31.7	2.59	NC	NC
	MOLM13	15.1	238.6	7.1	51.4	4736	96.8	47.4	NC	NC
	293s	NC	NC	NC	NC	NC	NC	NC	NC	NC
KD (nM)	EMR2 ECD-Biotin	59.2	6.3	53.4	10.1	29.7	31.5	1.97	10.9	16.5



A. EMR2-directed binders were identified by phage display panning against single-chain variable fragment (scFv) and heavy chain variable region (V_H) libraries. Binder kinetic and affinity characterization (EC_{50} and KD) were performed by multipoint flow cytometric, ELISA and Blitz analyses. **B.** Flow cytometric analysis of EMR2-directed binder reactivity to different EMR2 isoforms and CD97 in 293T cells. **C-E.** EMR2-directed CAR T cells were co-cultured with CFSE labeled MOLM13 WT and EMR2KO cell lines as AML target cells at 1:1 ratio for 24 and 48 hours (n=3). **C-D.** Percentage of viable target cells was determined by the absence of Annexin V and fixable viability dye reactivity by flow cytometry at 24 **(C)** and 48 **(D)** hours. **E.** The supernatants of co-cultures were analyzed for cytokine secretion using a Luminex 17-Plex kit. Data shown as median ± standard error of mean.

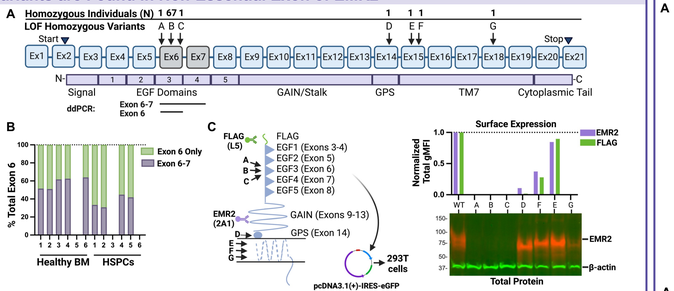
Genome Engineering and Characterization of Edited HSPCs

Figure 5. EMR2 is Expressed on Healthy Hematopoietic Stem and Progenitors (HSPCs) and Myeloid Cells



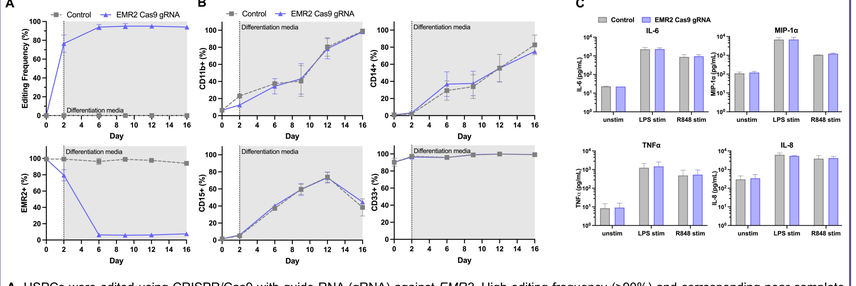
Flow cytometric analysis of EMR2 expression on healthy donor bone marrow and peripheral blood. QuantiBrite beads were used for antigen quantitation. Data shown as mean ± standard deviation. Hematopoietic stem cell (HSC), multipotent progenitor (MPP), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), megakaryocytic-erythroid progenitor (MEP), common lymphoid progenitor (CLP), classical and plasmacytoid dendritic cells (cDCs and pDCs).

Figure 6. The majority of Loss-of-Function (LOF) Homozygous EMR2 Genetic Variants are Found in Non-Essential Exon of EMR2



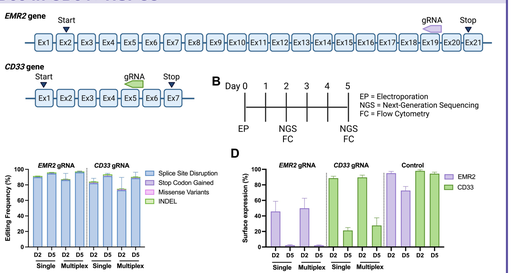
A. Gene structure of *EMR2* indicating the locations of LOF homozygous variants. The number of individuals identified for homozygous LOF variants[10] (A, B, C, D, E, F, and G) is shown. **B**. Presence and distribution of exon 6 only and exon 6-7 containing isoforms in healthy bone marrow (BM) and HSPCs as analyzed by ddPCR (amplified regions indicated in **A**). **C**. Surface and total protein expression of EMR2 variants. Plasmid constructs with *EMR2* variant mutations were transfected into 293T cells. EMR2 (2A1) and Flag antibodies were used for surface and total protein detection by flow cytometry and Western blotting, respectively.

Figure 7. EMR2-deleted HSPCs Maintain Normal Myeloid Differentiation and Cytokine Secretion *In Vitro*



A. HSPCs were edited using CRISPR/Cas9 with guide RNA (gRNA) against *EMR2*. High editing frequency (>90%) and corresponding near complete loss of EMR2 protein was achieved by day 6 post-electroporation (post-EP) and persisted throughout differentiation, as analyzed by inference of CRISPR edits (ICE) and flow cytometry, respectively. **B.** Two days post-EP, guide-control (Control) and *EMR2*-edited HSPCs were *in vitro* differentiated into monocytic lineage. There was no observable impact of *EMR2* editing on the ability of HSPCs to differentiate into monocytic lineage, as indicated by the expression of myeloid surface markers (CD11b, CD14, CD15, and CD33). Similar results were seen for granulocytic lineage (data not shown). **C**. Cytokine release was measured by Luminex for IL-6, IL-8, MIP-1α and TNFα. N=2 donors. Data shown as mean ± standard deviation.

Figure 8. Efficient Multiplex Adenosine Base Editing of *EMR2* and *CD33* in CD34+ HSPCs



A. Gene structures of *EMR2* and *CD33* indicating the adenosine base editing (ABE) gRNA locations. **B.** Experimental schema. **C.** Frequency of editing types at day 2 (D2) and day 5 (D5) after electroporation of HSPCs with ABE guides. **D**. Percentage of the EMR2 and CD33 surface protein expression of *EMR2* and *CD33* edited cells, in single and multiplex settings, at day 2 (D2) and day 5 (D5). N=2 donors. Data shown as mean ± standard deviation.

CONCLUSIONS

- EMR2 is expressed on a high percent of patient AML blasts and LSCs at similar expression intensities at both diagnosis and relapse timepoints
- Novel EMR2-directed CAR-Ts, engineered with phage display - derived scFv and V_H binders, mediate potent cytolytic activity against EMR2-expressing AML cells *in vitro*
- Surface protein expression analysis of EMR2 in healthy bone marrow HSPCs and peripheral blood lineages suggests that genetic editing may be necessary to protect healthy hematopoietic lineages from subsequent immunotherapeutic targeting
- Highly efficient multiplex base editing of EMR2 supports the feasibility of multi-target edited HSC transplant enabling selective eradication of AML blast and leukemic stem cells by combinatorial immunotherapy while sparing healthy grafts
- Evaluation of EMR2-edited HSPCs on long-term engraftment, multilineage differentiation, and persistence of editing *in vivo* is ongoing
- Further validation studies of the EMR2 human genetics data are ongoing

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Certain illustrations created with BioRender.com

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