P #1716

G-CSF/Plerixafor Dual-Mobilized Donor Derived CD33CAR T-Cells as Potent and Effective AML Therapy in Pre-Clinical Models

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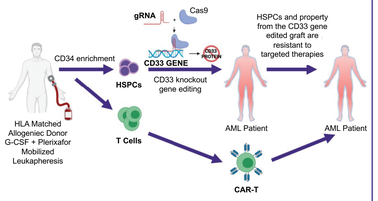
INTRODUCTION

- There are currently no known acute myeloid leukemia (AML) specific antigens. Genetic ablation of CD33 using CRISPR/Cas9 engineering of the hematopoietic stem and progenitor cells (HSPCs) transplant (VOR33) represents a synthetic biology approach to generating a leukemia-specific antigen in the transplant recipient.
- Mobilized leukapheresis product represents an attractive starting material for the generation of both a CD33 null HSC transplant and a complementary CD33 CAR T-cell product.

Fig. 1. VOR's approach to AML Treatment



VOR's approach to treat AML patients consists of two cell therapies: an initial transplant with CRISPR/Cas9-edited hematopoietic stem and progenitor cells (HSPCs) lacking CD33 protein expression (VOR33, top), followed by CD33-targeted CAR T-cells (VCAR33, bottom). Both cell therapies are manufactured from the apheresis product of the same dual-mobilized (G-CSF and plerixafor) human leukocyte antigen (HLA)-matched donor. VOR's approach allows selective killing of CD33+ AML cells by CAR T-cells while sparing donor HSPC-derived CD33- progenitors during hematopoietic reconstitution and function.

OBJECTIVE

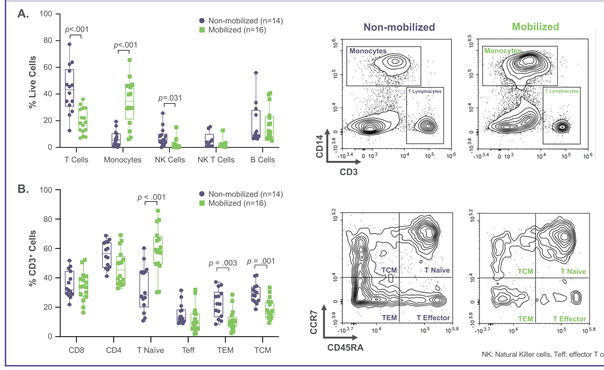
- In this study, we sought to determine the impact of dual mobilization with Granulocyte-Colony Stimulating Factor (G-CSF) and plerixafor (mozobil) on immune cell composition, T cell phenotype, and the functionality of these T cells to control AML tumor growth upon chimeric antigen receptor (CAR) transduction.

METHODS

- Mobilized (mob) peripheral blood mononuclear cells (PBMCs) were collected from healthy donors injected with G-CSF (10µg/kg/day, 5 consecutive days) and plerixafor (240µg/kg, on day 4 and 5). Non-mobilized (non-mob) PBMCs collected from the same donors were used as controls.
- Cells were analyzed by flow cytometry for immunophenotyping and T cell characterization, including differentiation and bone marrow homing markers, as well as responses to T cell activation with anti-CD3 (OKT3) and IL-2.
- Non-/mob PBMC populations were also analyzed by single-cell next generation sequencing (CITEseq) using ~130 immune cell phenotypic markers in combination with extensive transcriptome and T cell receptor repertoire analysis.
- In addition, lentiviral transduction of anti-CD33 CAR constructs enabled functional comparisons of mob- and non-mob-CAR T-cells in AML cell co-cultures as well as AML mouse models.

RESULTS

Fig. 2. *Ex-vivo* Immunophenotyping of Mob and Non-Mob PBMCs from Healthy Donors



PBMCs were isolated from dual-mobilized healthy donors. Non-mobilized PBMCs, collected from the same donors, were used as controls.

PBMCs immunophenotype was analyzed by flow cytometry.

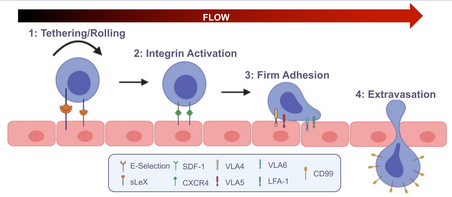
A. Mobilization decreases the overall percentage of CD3+ T cells and increases the percentages of monocytes (CD14+).

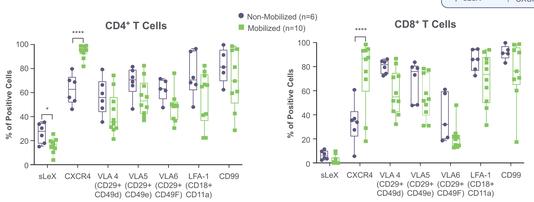
B. Donor mobilization increases the overall percentage of naïve T cells (CD45RA+/CCR7+). The opposite effect was seen for T effector-memory (CD45RA-/CCR7-) and T central-memory (CD45RA-/CCR7+) populations.

NK: Natural Killer cells, Teff: effector T cells, TCM: central memory T cells, TEM: effector memory T cells

Fig. 3. *Ex-vivo* Immunophenotyping of Bone Homing Receptors in Mob and Non-Mob PBMCs from Healthy Donors

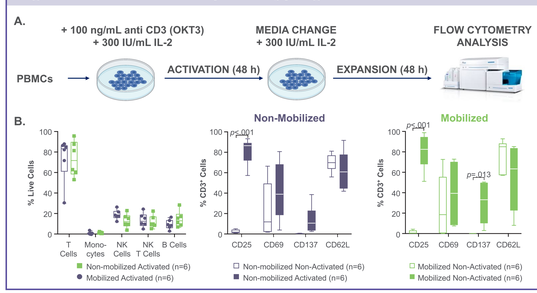
A. Schematic representation of leukocyte migration to sites of injury, inflammation, or bone marrow infiltration. This process involves multi-step adhesion interactions between circulating leukocytes and endothelial cells. Initially, weak and reversible interactions between vascular E-selectin and leukocyte Sialyl LewisX (sLeX) slow the leukocyte rolling velocity (**tethering/rolling**). Then, vascular SDF-1 interacts with its leukocyte receptor CXCR4 (**integrin activation**), which leads to tight leukocyte binding to the endothelium via VLA4, VLA5, VLA6 and LFA-1 (**firm adhesion**). Finally, leukocyte migration through the vessel wall is mediated by the expression of CD99 (**extravasation**).





B. and **C.** Flow cytometry analysis of PBMCs from healthy donor samples shows that mobilized CD4+ and CD8+ cells express lower levels of sLeX and higher levels of CXCR4 compared to Non-mobilized CD4+ and CD8+ cells. The overall percentage of VLA4, VLA5, VLA6, LFA-1 and CD99 is not significantly different between the two groups.

Fig. 4. *Ex-vivo* Immunophenotyping of Mob and Non-Mob activated T Cells

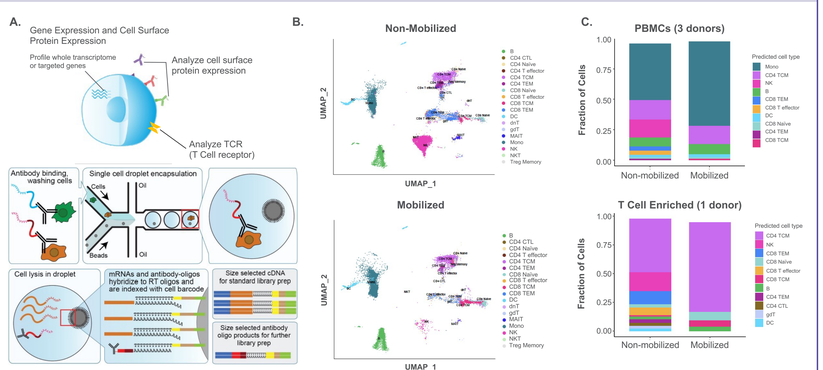


A. Schematic representation of the activation protocol: PBMCs are thawed and resuspended in medium containing OKT3 and IL-2. Cells are incubated for 48h (**T cell activation**), washed, and incubated in fresh medium containing IL-2 only for additional 48h (**T cell expansion**). Final cell populations are analyzed by flow cytometry.

B. Flow cytometry analysis shows high percentage of T cells in both Mobilized and Non-mobilized activated PBMCs. Low percentage of monocytes, NK cells and B cells are detected in both groups.

C. Flow cytometry analysis shows that activation/expansion leads to similar increases of the expression of T cell activation markers CD25, CD69 and CD137 in mobilized and non-mobilized T cells.

Fig. 5. Single Cell Immune Profiling of Mob and Non-Mob PBMCs and T Cells

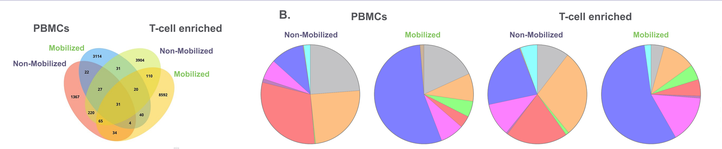


A. Schematic representation of the Chromium Single Cell Immune Profiling approach (CITEseq). PBMCs or enriched T cells from matched mobilized and non-mobilized donors were labelled with barcoded antibodies for 130 unique cell surface antigens. In order to index each cell's transcriptome and cell surface protein expression profile, cells were partitioned into nanoliter-scale Gel Beads-in-emulsion (GEMs), where all generated cDNA share a common 10x barcode. Sequence libraries for single cell transcriptome, protein expression and TCR profiling were generated and sequenced using the NextSeq 500 platform.

B. UMAP projection and cell annotation of single cell data. Cell annotation was performed by projecting our datasets onto Azimuth's reference PBMC dataset using Seurat. CD4 and CD8 T cells were further divided into subpopulations based on surface protein expression of SELL, CD45RA, and CD45RO measured by CITEseq. UMAP coordinates of projected data are used to visualize cells in low dimensional space.

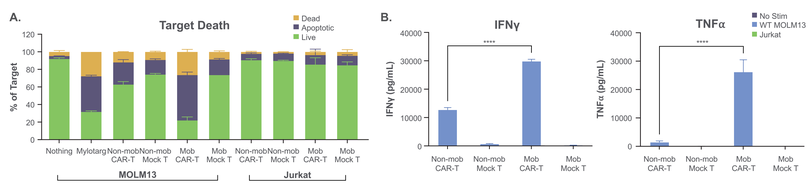
C. Comparison of cell state composition in bulk and T cell enriched PBMCs from non-mobilized and mobilized donor samples. Cell states present at low fractions (<1% of total cells) were excluded from bar plots.

Fig. 6. TCR Clonotype Analysis of Mob and Non-Mob PBMCs and T Cells



A. Venn diagram to indicate shared TCR clonotypes from the same donor across states (mob and non-mob) and sample preparations (PBMCs and T cell enriched). **B.** In depth phenotypic analysis of TCR clonotypes shared between states in PBMCs and T cell enriched samples reveals increased percentages of CD4+ and CD8+ T Central-Memory and decreased percentages of T Effector cells after mobilization.

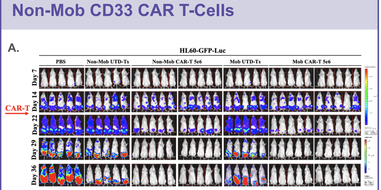
Fig. 7. *In Vitro* Cytotoxicity Activity of Mob and Non-Mob CD33 CAR T-Cells



A. Mobilized and non-mobilized CD33 CAR T-cells show comparable CD33-specific *in vitro* cytotoxicity of AML cells. CD33+ MOLM13 cells and CD33 null Jurkat target cells were co-cultured with CD33 CAR T-cells or untransduced T-cells (MOCK T) for 24 hours. Mylotarg™ (CD33 targeting antibody drug conjugate) was used as positive control for target cell killing. Target cell viability was assessed according to the gating strategy: Dead (DAPI+), Apoptotic (DAPIneg, Annexin V+), and Live (DAPIneg, Annexin Vneg), and expressed as a frequency of total target cells. MOLM13 showed a reduced proportion of live cells when cultured with CD33 CAR T-cells or Mylotarg™ compared to Jurkat target cells.

B. Cytokine profiling indicates that the secretion of IFN-gamma (IFNγ) and TNF-alpha (TNFα) is specific to CD33 CAR-expressing cells and is increased in CD33-directed mobilized compared to non-mobilized CAR T cells.

Fig. 8. *In Vivo* Killing Activity of Mob and Non-Mob CD33 CAR T-Cells



Weekly IVIS imaging analysis of animals injected with HL60-GFP-Lucifease cells. Both mobilized and non-mobilized CD33 CAR T-cell injections result in tumor burden decreases to a similar extent. Control animals received either no T cell (PBS), or injection of untransduced (UTD) T cells and exhibited continued tumor burden increase until the study endpoint (day 36). Importantly, all CAR-T cells injected animals show no signal of tumor above background at day 36.

Quantification of tumor burden signal indicates reduction of tumor signal to background levels only in animals injected with either mobilized or non-mobilized CD33 CAR T-cells.

Percentages of CAR positive cells of the input were compared with percentages in mouse samples at study endpoint. Mobilized (Mob) and non-mobilized (Non-Mob) CD33 CAR T-cells persisted over the course of the study and were equally detectable in blood and bone marrow at study endpoint.

CONCLUSION

- Our analysis showed phenotypical *ex vivo* differences between mob and non-mob PBMCs, which disappeared upon activation, indicating similar responses to T cell-specific stimulation.
- These findings are corroborated by similar *in vitro* cytotoxicity profiles of non-/mob-CAR T-cells. Non-transduced T cells in the mob-CAR T-cell population showed limited 'bystander' activation, indicating a potentially favorable clinical toxicity profile.
- Additional *in vivo* assessment of mob-CAR T-cell function shows effective tumor clearance, which supports further efforts towards their clinical use in combination with engineered HSPCs for the treatment of AML patients.

Acknowledgments

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Figures 1, 3 and 4 were in part generated by BioRender.

Disclosures

All authors: Vor Biopharma: Current Employment, current equity holder in publicly-traded company.

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