**Abstract**

Acute myelogenous leukemia (AML) has a high mortality rate and remains difficult to treat, making new treatment approaches critical. Chimeric antigen receptor T (CAR) T cell therapies have shown impressive clinical responses in B-cell neoplasms. However, comparable success has not been reported to date in most malignancies, potentially due to difficulty in manufacturing efficacious CAR-T cells from AML patients and lack of a suitable tumor antigen for AML. To address these issues, we have developed allogeneic CAR T cells from healthy donors targeting the CD33/Siglec-3 antigen, a protein expressed on most AML cells and subpopulations in the majority of AML patients at presentation and relapse. Allogeneic anti-CD33 CAR T cells were produced from healthy donor-derived T cells using CRISPR/Cas9 gene editing. In these cells, the CD33 locus was disrupted to reduce the risk of graft versus host disease (GVHD). At the same time, a CAR construct targeting CD33 was inserted site-specifically into the TCR locus. In addition, the beta-2-microglobulin locus was disrupted to prevent clearance of the allogeneic CAR T cells by the host immune system. These allogeneic anti-CD33 CAR T cells showed potent effector activity in vitro against human AML-derived cell lines, as measured by both tumor cell lysis and effector cytokine secretion. The allogeneic anti-CD33 CAR T cell approach allows for the controlled manufacturing of product from healthy donor T cell populations. Benefits of the allogeneic approach include off-the-shelf administration, more potent starting material, a more consistent product, broader access, and flexible dosing (e.g., re-dosing).

**Conclusions**

- Allogeneic edited anti-CD33 CAR positive T cells can be generated from healthy donor T cells.
- Allogeneic anti-CD33 CAR T cells can potently and specifically kill CD33 expressing AML cells in vitro.
- Allogeneic CAR T cells outdated robust IFNγ and IL-2 secretion when exposed to target cells.

**Figure 2:** Healthy Donor-derived CAR-T Cells Offer Benefits Over Autologous Approaches

**Figure 3:** Development of CRISPR-Edited Allogeneic Anti-CD33 CAR-T Cells

**Figure 4:** CRISPR-Edited Anti-CD33 CAR-T Cells Exhibit Specific and Potent Cell Killing of AML Cells in Vitro

**Figure 5:** CRISPR-Edited Anti-CD33 CAR-T Cells Secretion Effector Cytokines in the Presence of AML Cells in Vitro

**Figure 6:** Allogeneic Anti-CD33 CAR-T Cells Eliminate AML Cells In Vivo

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**Figure 1:** CRISPR-Edited Allogeneic CAR-T Cells for CD33- Malignancies

- Improve persistence in the allogeneic setting with 2αC3 knock out to eliminate MHC I expression
- Prevent G418 via T2α disruption
- Improve safety and potency by genomic insertion of CAR constructs into TRAC locus

CRISPR-edited allogeneic CAR-T cells are produced by targeted disruption of the TRAC locus with consensuses insertions of an anti-CD3 CAR Cassette into the TRAC locus by homology-directed repair. TRAC gene disruption leads to loss of surface expression TCR complex and reduces the risk of graft versus host disease (GVHD). Targeted disruption of the β2M gene leads to loss of surface expressed MHC I and the reduced the risk of rejection of allogeneic CAR-T cells by the host immune system.

**Figure 2:** Healthy Donor-derived CAR-T Cells Offer Benefits Over Autologous Approaches

- CRISPR-Edited CAR-T cells are more potent and specific towards target cells than autologous T cells.
- CRISPR-Edited CAR-T cells show robust cytotoxic activity against all of the indicated AML cell lines.

**Figure 3:** Development of CRISPR-Edited Allogeneic Anti-CD33 CAR-T Cells

- Multiple anti-CD33 CAR constructs were screened in human primary T cells to identify ones with high surface staining. CAR surface expression is shown for three constructs selected for further evaluation: CRISPR-CAR33-1, CRISPR-CAR33-2, and CRISPR-CAR33-3. No RNP represents T cells mock transfected without CRISPR/RNA ribonucleoprotein (RNP).

**Figure 4:** CRISPR-Edited Anti-CD33 CAR-T Cells Exhibit Specific and Potent Cell Killing of AML Cells in Vitro

- CRISPR-CAR33-1 and CRISPR-CAR33-2 both secrete high levels of IFNγ when exposed to target cells. No relapse was observed.

**Figure 5:** CRISPR-Edited Anti-CD33 CAR-T Cells Secretion Effector Cytokines in the Presence of AML Cells in Vitro

- No RNP represents T cells mock transfected without CRISPR/Cas9.
- Effector cytokine secretion in response to target cell engagement was also examined. (A) CRISPR-CAR33-1 and CRISPR-CAR33-2 both secrete high levels of IFNγ when exposed to target cells. No relapse was observed.

**Figure 6:** Allogeneic Anti-CD33 CAR-T Cells Eliminate AML Cells In Vivo

- No RNP represents T cells mock transfected without CRISPR/Cas9.