ENGINEERED SINGLE AMINO ACID SUBSTITUTIONS PROTECT HEMATOPOIETIC STEM AND PROGENITOR CELLS FROM CD123 TARGETED IMMUNOTHERAPY

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INTRODUCTION

- Targeted immunotherapy using monoclonal antibodies (mAb), T cell engager (TCE), or chimeric antigen receptor (CAR)) cells is standard of care in hematologic malignancies.
- CD123 (IL-3 receptor α-chain) is expressed in hematologic malignancies (e.g. acute myeloid leukaemia AML, blastic plasmacytoid dendritic cell neoplasm BPDCN), but also on healthy hematopoietic stem and progenitor cells (HSPCs)[1].
- Shared antigen expression leads to on-target off-tumor effect with risk of extensive myelotoxicity following targeted cell depletion[2].
- Proposed solution includes HSC transplantation with antigen-deleted HSPCs prior to immunotherapy[3-5]. This is, however, limited to dispensable targets and bears risk for antigen negative relapses.

AIM

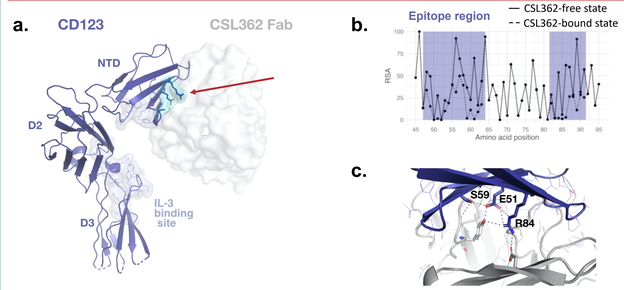
- We aimed to genetically engineer the CD123 epitope on HSPCs to shield the cells from targeted immunotherapy while preserving the antigen's structure and function.

METHODS

- HEK cell lines stably expressing wildtype CD123 (HEK-CD123) and 28 CD123 variants with individual amino acid substitutions.
- Targeted immunotherapy:
  - CSL362 biosimilar mAb MIRG123
  - CSL362/OKT3 TCE
  - Non-viral CRISPR/Cas9-based second generation CSL362-derived CAR T cells
- Non-viral engineering of HSPCs using CRISPR-Cas9 (High fidelity Cas9 RNPs and single strand oligodeoxynucleotides (ssODN) as homology directed repair template (HDRT).
- AAV6-mediated HDR editing of HSPCs (p-Stat5).
- *In vivo:* Injection of non-virally edited HSPCs into sublethally irradiated NSG mice.

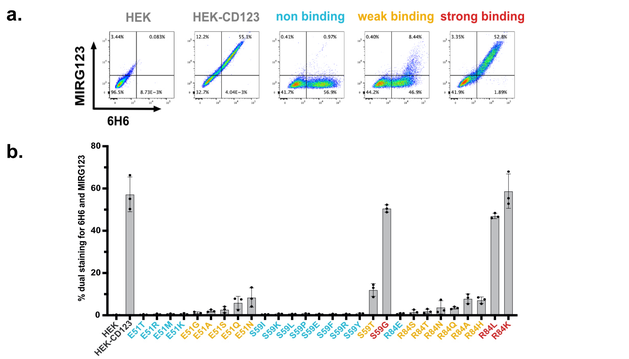
RESULTS

1. *In silico* design of human CD123 protein variants



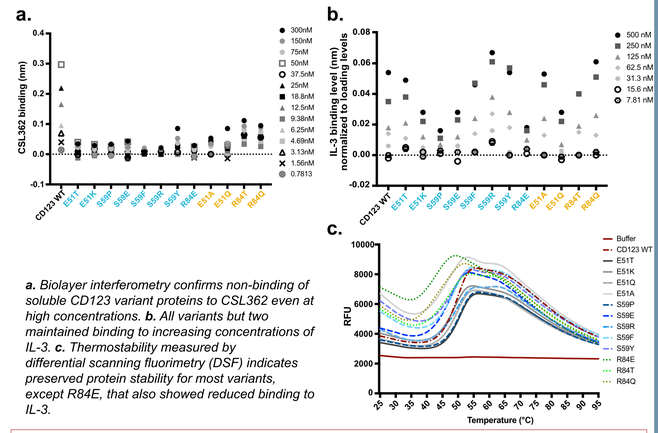
a. *Crystal structure of CD123 and the fragment antigen binding (Fab) of the CSL362 mAb (talacotuzumab). CSL362 epitope at the N-terminal domain (NTD) is indicated in light blue (red arrow).* ***b.*** *Per residue relative solvent accessibility computed on the CSL362-free (solid line) and -bound (dashed line) states based on the crystal structure.* ***c.*** *E51, S59 and R84 were identified as key amino acid (AA) residues for CSL362 binding and a total of 28 AA substitutions were chosen (not shown) that potentially disrupt binding but preserve function.*

2. Abolished binding to the CSL362 biosimilar despite preserved expression of CD123



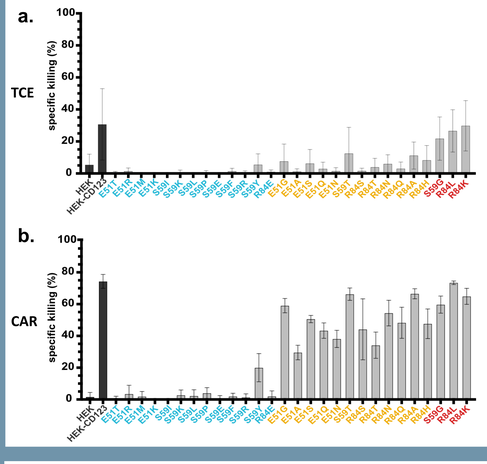
Stable expression of CD123 wildtype (HEK-CD123) and all 28 variants in HEK cells. ***a.*** *Variants were categorised as non binding (blue), weak binding (orange) and strong binding (red) using flow cytometry staining with the CSL362 biosimilar MIRG123 and the expression control mAb 6H6.* ***b.*** *13 non-binding, 12 weak-binding and 3 strong-binding variants were identified.*

3. Biophysical characterization of selected CD123 variants



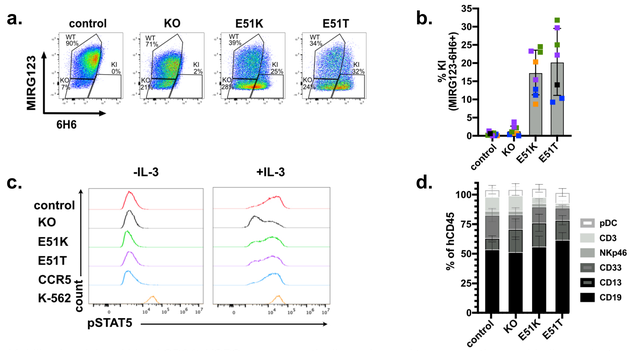
a. *Biolayer interferometry confirms non-binding of soluble CD123 variant proteins to CSL362 even at high concentrations.* ***b.*** *All variants but two maintained binding to increasing concentrations of IL-3.* ***c.*** *Thermostability measured by differential scanning fluorimetry (DSF) indicates preserved protein stability for most variants, except R84E, that also showed reduced binding to IL-3.*

4. Engineered CD123 variants are shielded from TCE- and CAR-mediated cytotoxicity in vitro



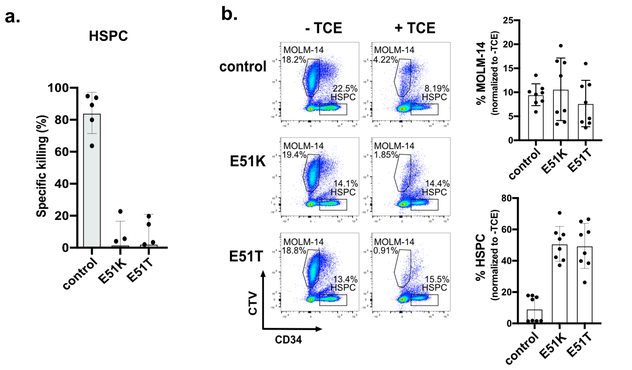
HEK cells stably expressing non-binding CD123 variants were protected from MIRG123-mediated cytotoxicity induced by ***a.*** *CSL362/OKT3 TCE and* ***b.*** *second generation CSL362-derived CD123 specific CAR T cells in vitro.*

5. Engineered human HSPCs expressing CD123 variants are functional, engraft and differentiate in vivo



Mobilised peripheral blood CD34⁺ HSPCs were genetically engineered to express the non-binding variants E51K and E51T using a non-viral and close to GMP-grade CRISPR/Cas9 protocol. ***a.*** *Representative flow cytometry dot plots and* ***b.*** *summary of KI (MIRG123⁻6H6⁺) frequency for multiple donors 5 days post-electroporation with RNPs and ssODN HDRT.* ***c.*** *Representative histogram indicating regular pSTAT5 signaling upon IL-3 exposure in engineered HSPCs.* ***d.*** *Multi-lineage differentiation of engineered HSPCs in blood 15 weeks after injection in NSG mice.*

6. Engineered HSPCs enable tumor-selective CD123 immunotherapy



a. *Selective CSL362/OKT3 TCE-mediated killing of non-edited HSPCs in vitro. E51K/E51T expressing HSPCs are spared.* ***b.*** *CD123 expressing MOLM-14 (CTV labelled AML cell line) cells are selectively killed in co-culture with engineered CD34⁺ HSPCs and the CSL362/OKT3 TCE.*

CONCLUSIONS

- Single amino acid substitutions abolish CSL362 binding but preserve function
- In vitro shielding of epitope engineered CD123 variants from TCE- and CAR-mediated killing
- Edited HSPCs are functional, engraft and differentiate regularly in vivo
- Tumor-selective killing of edited HSPCs in vitro
- Epitope engineering to shield cells may enable targeting of essential proteins

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