Molecular shielding of CD52 retains expression, anti-phagocytic ‘don’t eat me’ function and protects from Alemtuzumab-mediated depletion

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Introduction

Targeted immunotherapies such as monoclonal antibodies aim to **eliminate transformed or dysregulated cells** expressing a **specific target antigen** and are highly **effective** in the treatment of various hematologic diseases. E.g., **Alemtuzumab is approved** for treatment of CD52+ malignancies as well as multiple sclerosis and is clinically assessed in **reduced intensity conditioning** and for lymphodepletion prior to administration of CAR-T cells. These novel applications raised the **interest in engineered cell products** with resistance to Alemtuzumab, which is usually achieved by CD52 knock-out (KO).

CD52 is found on most cells of the **lymphoid lineage** as well as on monocytes and macrophages. Mature CD52 is a **highly glycosylated[1] 12-mer peptide**, which is GPI-anchored to the cell surface and acts as a regulator suppressing inflammatory responses. This implies that **CD52 KO on T cells will not be functionally inert** and thus influence the activity and exhaustion of CD52 KO lymphocytes.

Here, we **shield CD52 from Alemtuzumab binding** by introducing single amino acid (aa) substitutions. Careful selection of the substitutions preserved expression and function of the engineered protein.

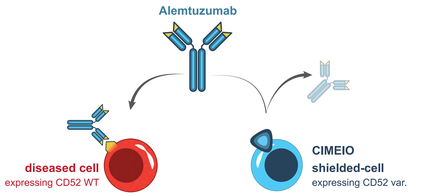


Figure 1: Concept of pairing shielded CD52 cell & Alemtuzumab immunotherapy. Alemtuzumab depletes specifically diseased cells, while CIMEIO's cell-shielding technology protects healthy cells expressing CD52 proteins with single amino acid substitutions.

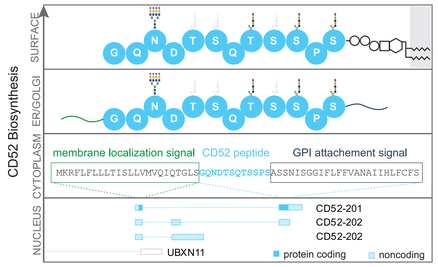
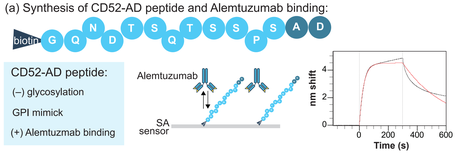


Figure 2: CD52 biosynthesis and GPI (glycosylphosphatidylinositol) anchoring starting in protein synthesis in the cell nucleus until cell surface representation.

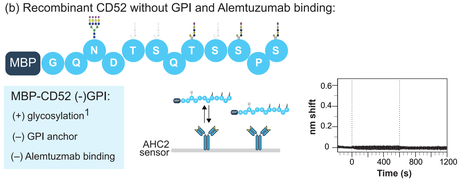
Results

1. Design of active CD52 construct

(a) Synthesis of CD52-AD peptide and Alemtuzumab binding:



(b) Recombinant CD52 without GPI and Alemtuzumab binding:



(c) Recombinant CD52 with partial GPI anchor and Alemtuzumab binding:

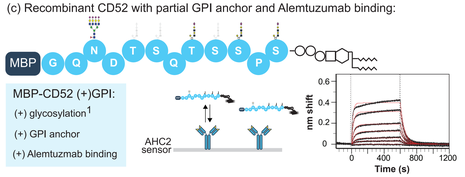


Figure 3: (a) Peptide synthesis of CD52 analogs containing a C-terminal AD dipeptide mimicking GPI anchor[2]. Alemtuzumab binding to the immobilized synthetic CD52 peptide was detected by BLI Octet. **(b)** Recombinant protein production of CD52 with a maltose-binding protein (MBP) tag using Expi293 PGAP2 KO[3] with an N-terminal signalling peptide (MBP-CD52 (-)GPI). Alemtuzumab was immobilized on anti-human capturing (AHC2) biosensors and no binding to MBP-CD52 (-)GPI was observed by BLI Octet. **(c)** Recombinant protein MBP-CD52 (+)GPI expressed in Expi293 PGAP2 KO[3] cells. Alemtuzumab binding was observed when part of the GPI anchor was present.

2. Engineered CD52 is shielded from Alemtuzumab binding

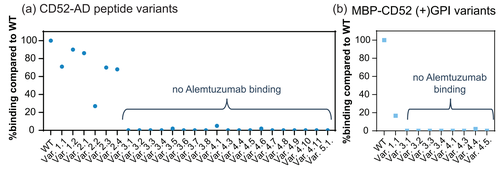
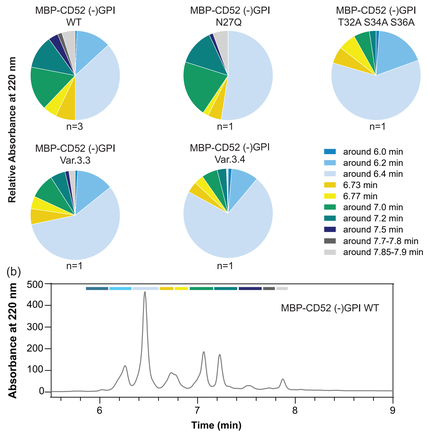


Figure 4: Alemtuzumab binding screen to **(a)** CD52-AD peptide variants and **(b)** selected MBP-CD52 (+)GPI variants containing a palette of different single amino acid substitutions. A large fraction of variants were fully shielded from Alemtuzumab binding compared to CD52 WT peptide/protein.

3. Glycosylation assessment of recombinant WT and engineered CD52

(a) Weak-anion exchange (WAX) chromatography to separate different N-and O-glycosylation:



(b)

Figure 5: O- and N-glycosylation analysis using weak-anion exchange chromatography. **(a)** Relative absorbance at 220 nm was integrated. Compared to control samples N27Q and T32A S34A S36A lacking almost all N- or O-glycosylation variant 3.3 retains the WT glycosylation pattern to a larger degree. **(b)** The integration strategy is depicted on one WT batch. These results were also confirmed by mass spectrometry of PNGase treated or untreated samples.

4. CD52 shielding protects from Alemtuzumab mediated antibody-dependent cellular cytotoxicity (ADCC)

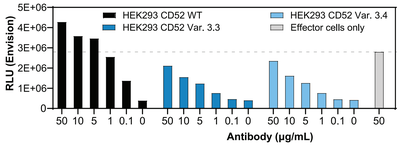
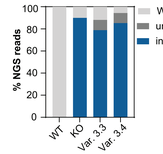


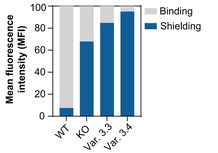
Figure 6: Antibody-dependent cellular cytotoxicity (ADCC) of Alemtuzumab on wildtype and variant CD52 expressing HEK293 cells was measured by an FcgammaRIIIa activation assay performed with the ADCC Reporter Bioassay, V Variant (Promega, ref G7015) determined by luminescence intensity (Y-axis; RLU). Alemtuzumab induced significant FcgammaRIIIa activation on the effector cells (Jurkat NFAT KO) when co-cultivated with WT CD52 HEK293 cells. CD52 variant cells show no FcgammaRIIIa activation above background (effector cells only with 50 µg/mL Alemtuzmab, dotted line), indicating that cells expressing CD52 variants are not susceptible to Alemtuzumab mediated ADCC.

5. Base editing on primary human T cells

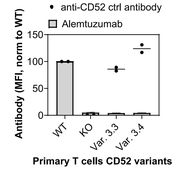
(a) Editing efficiency by NGS:



(b) Editing efficiency by flow cytometry:



(c) CD52 antibody binding:



(d) Alemtuzumab titration:

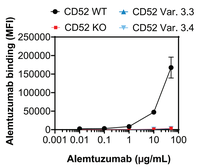


Figure 7: Primary human T cells were gene edited using base editing to engineer CD52 variants and CD52 knock-out cells (by RNP Cas9/sgRNA). Seven days post-editing, T cells were sorted by flow cytometry using Alemtuzumab to generate homogenously edited pools. **(a)** Editing efficiency was determined by next-generation sequencing (NGS) and **(b)** by flow cytometry. **(c)** Base edited, shielded CD52 variants expressed on primary T cells completely lose Alemtuzumab binding, while binding to the control anti-CD52 antibody 4C8 remains unchanged. This demonstrates expression levels of the shielding variants comparable to wildtype CD52. **(d)** To validate that engineered CD52 variants are fully shielded from Alemtuzumab binding, WT and edited primary T cells were stained with increasing concentrations of Alemtuzumab (0.01, 0.1, 1, 10, 50 µg/mL). Even at the highest concentration (50 µg/mL) shielding variants do not show any binding above the level of CD52-KO cells.

6. Shielding protects T cells from phagocytosis

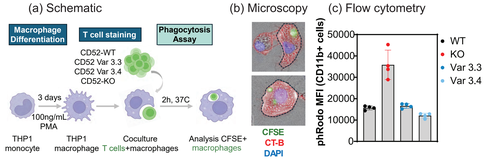


Figure 8: (a) THP-1 monocytes were differentiated to macrophages for 3 days with 100 ng/mL of PMA in vitro. Human primary T cells were edited to carry variant CD52 (var 3.3 or 3.4) and stained with CFSE or phRodo dye. CD52-WT or KO T cells were used as controls. Phagocytosis assay was performed at 1:2 effector (THP-1 derived macrophages) to target (T cells) cell ratio for 2 h at 37 °C. **(b)** Representative images of macrophages labelled with Cholera toxin subunit B-Alexa 647 (CT-B) containing phagocytosed CFSE-labelled T cells were taken by confocal microscopy using PerkinElmer Operetta high content analysis imaging system. Nuclei were stained with Dapi. **(c)** The median fluorescence intensity of phRodo dye in the macrophage (CD11b+ cells) population was measured by flow cytometry. CD52-KO T cells are phagocytosed at significantly increased levels when exposed to macrophages than WT T cells. Importantly, this was not observed with T cells expressing CD52 shielding variants, which also showed normal expression of phenotypic T cell markers and activation capabilities comparable to WT T cells.

Conclusion

In analogy to what we have shown for targeted immunotherapies paired with engineered CD123[4], CD117 and CD45, we **successfully shielded CD52 from Alemtuzumab binding** by single aa substitutions while maintaining cell surface expression, function and biochemical properties. Shielding variants could be precisely introduced in primary human T cells by base editing with high efficiency. Additionallly, our data is the first indication that CD52 can act as a novel **don’t eat me signal** and single aa substitutions in CD52 **retain this anti-phagocytic property**. Further studies need to verify increased in vivo persistence of engineered lymphocytes as compared to CD52 KO cells and thus being a superior alternative to approaches knocking out this cell surface receptor.

References

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Acknowledgements

- Lukas Jeker and the members of his research group at Department of Biomedicine, Basel University Hospital and University of Basel
- Anne Zeck and Sandra Maier at NMI Natural and Medical Science Institute Tuebingen
- Alessandro Sinopoli and Anna Haydn at Ridgeline Discovery