Engineering Multi-modular Tregs with CARs, a Foxp3 Phenotype Lock™ and Synthetic Cytokine Receptors enhances their therapeutic potential for a broad range of immune conditions

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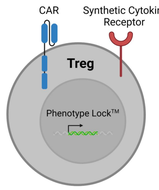
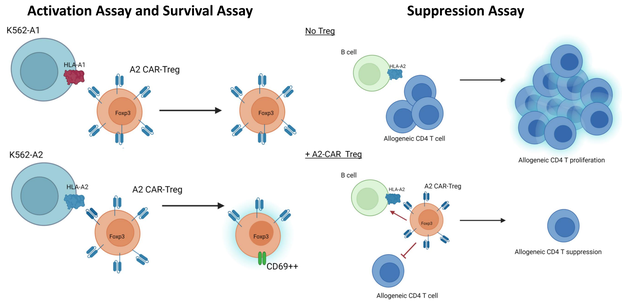
INTRODUCTION

- Adoptive transfer of Regulatory T (Treg) cells is a promising therapeutic approach to induce durable immune tolerance across organ transplantation and autoimmunity/inflammation.
- Limitations of polyclonal, non-engineered Treg cell therapy include lack of antigen-specificity, loss of Treg stability and limited durability.
- Chimeric antigen receptors (CARs) are recombinant fusion receptors that can be utilized to redirect the specificity of Tregs to recognize relevant target antigens.
- Foxp3 is the master transcription factor of Tregs which controls a transcriptional program driving Treg phenotype, stability and function.
- IL-2 cytokine signaling via pSTAT5 is necessary for Treg survival, expansion and stability.



We have designed several novel modules to generate multi-modular engineered CAR-Tregs with antigen-specificity via chimeric antigen receptors, our Foxp3 Phenotype Lock™ to drive Treg stability and a novel Synthetic Cytokine Receptor (SCR) module to provide pSTAT5 survival signaling independently of exogenous IL-2.

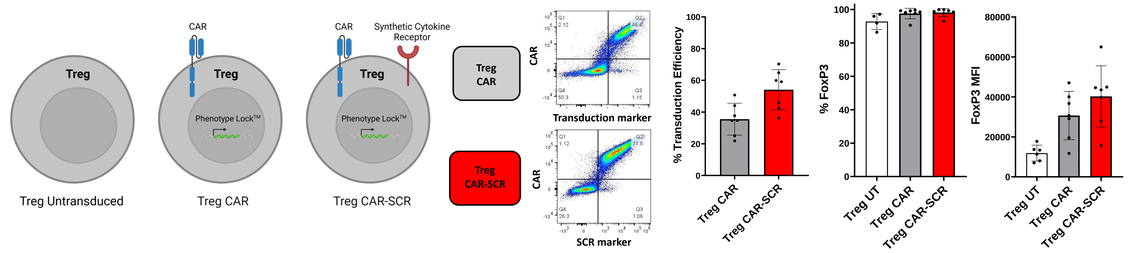
METHODS



- **Activation Assay-** CAR-Tregs were co-cultured with target or non-target cell lines, polyclonal activation beads or media for 18-22 hours before CD69 and CD137 detection via flow cytometry.
- **Survival Assay-** CAR Tregs were co-cultured with target cell lines for 0 to 14 days in the absence of IL-2. At certain time points, target cell lines were replenished to re-stimulate the CAR Tregs.
- **Suppression Assay**- CAR-Tregs were co-cultured with 3rd party Teffs at various indicated ratios with HLA A2+ B cells or HLA A2- B cells or polyclonal beads. Readout at day 5 via flow cytometry.

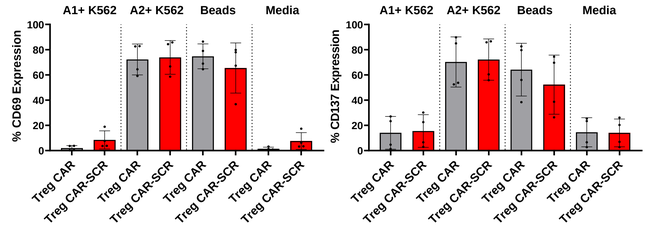
RESULTS

Multi-modular CAR-Tregs generated with 3 modules: CAR, Foxp3 Phenotype Lock™, Synthetic Cytokine Receptor



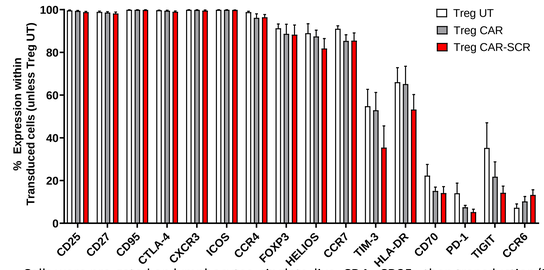
Cells were pre-gated on lymphocytes, singlets, live, CD4+ CD25+, transduction/SCR/CAR marker (except Treg UT) then Foxp3.

CARs can activate Tregs upon recognition of cognate antigen

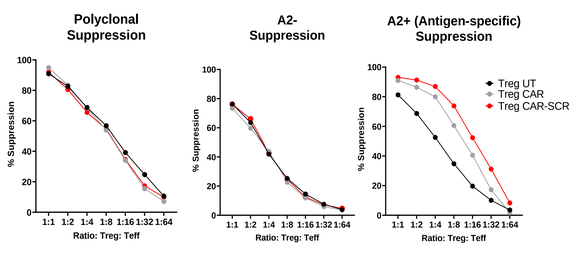


Cells were pre-gated on lymphocytes, singlets, live, CD4+ CD25+ and transduction/SCR marker

Stable Treg phenotype and suppressive function is observed in engineered CAR-Tregs



Cells were pre-gated on lymphocytes, singlets, live, CD4+ CD25+ then transduction/SCR marker (except Treg UT)



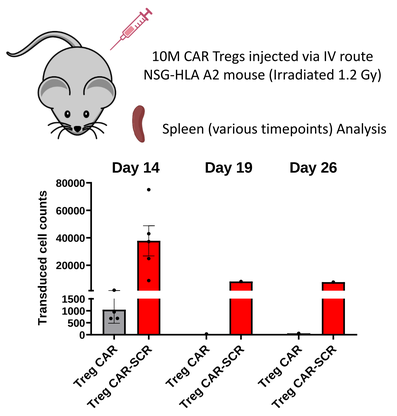
Treg CAR and Treg CAR-SCR maintain their antigen specific suppressive capacity driven through an A2 CAR

Synthetic Cytokine Receptor extend survival of Tregs independent of exogenous IL-2 in vitro

48 hours rested Tregs in the absence of IL-2 show that Treg CAR-SCR have higher pSTAT5 MFI levels compared to Treg CAR

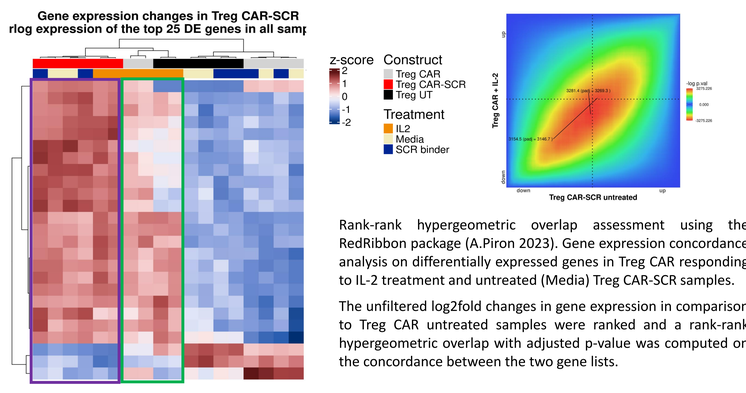
Treg CAR-SCR survive and persist longer than Treg CAR in the absence of IL-2 over several days

Synthetic Cytokine Receptor improves Treg survival in vivo



Mouse models are empty with no IL-2 source but with HLA A2 antigen on all cells. Treg CAR-SCR persisted longer than Treg CAR *in vivo*

RNASeq data reveals a pathway similar to IL-2 signalling in the Synthetic Cytokine Receptor engineered Tregs



When Treg CAR and Treg UT are treated with IL-2, a certain gene expression profile is upregulated (green box). A similar profile is seen in Treg CAR-SCR in untreated state (Media), IL-2 or SCR-binder treatments (purple box).

Rank-rank hypergeometric overlap assessment using the RedRibbon package (A.Piron 2023). Gene expression concordance analysis on differentially expressed genes in Treg CAR responding to IL-2 treatment and untreated (Media) Treg CAR-SCR samples.

The unfiltered log2fold changes in gene expression in comparison to Treg CAR untreated samples were ranked and a rank-rank hypergeometric overlap with adjusted p-value was computed on the concordance between the two gene lists.

CONCLUSIONS

- Engineering multi-modular Tregs with 3 proprietary modules including a CAR, the Foxp3 Phenotype Lock™ and our Synthetic Cytokine Receptor significantly improved Treg antigen-specificity, stability and durability.
- Our Foxp3 Phenotype Lock™ module increased the Foxp3 MFI levels within transduced Tregs, driving a stable Treg phenotype and maintained their suppressive ability.
- Our Synthetic Cytokine Receptor significantly increased pSTAT5 MFI levels (mechanism of action) and extended the survival of Tregs in the absence of exogenous IL-2 *in vitro* and *in vivo* (functional outputs).
- Our Synthetic Cytokine Receptor drives a gene expression profile similar to exogenous IL-2 signaling via IL-2 treatment.
- Thus, we have created a novel multi-modular CAR-Tregs with enhanced stability via the Foxp3 Phenotype Lock™ and with a novel Synthetic Cytokine Receptor module that confers IL-2 signaling, even when exogenous IL-2 may be limiting.