Engineering of regulatory T cells with hepatocyte-specific chimeric antigen receptor (CAR) enhances liver tissue trafficking

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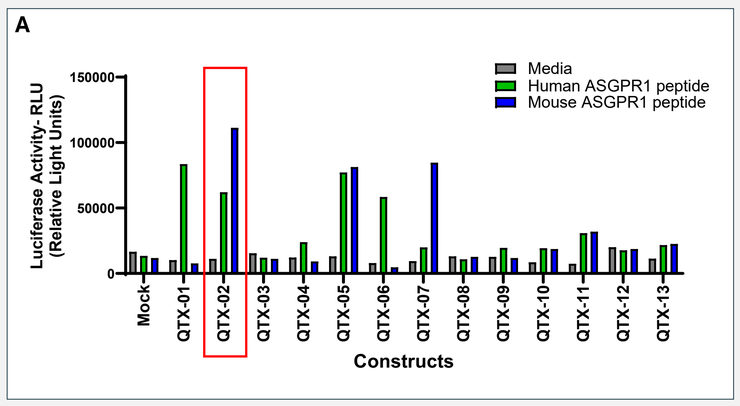
Autologous engineered liver-specific CAR-Treg therapy to modulate immune responses in transplantation and autoimmunity

Naturally occurring regulatory T cells (Tregs) play a critical role in maintaining immune homeostasis by suppressing excessive or autoreactive immune responses. They achieve this through multiple mechanisms, including the secretion of anti-inflammatory cytokines, direct inhibition of effector T cells via cell-cell interactions, and modulation of antigen-presenting cells to promote tolerance. Tregs require T-cell receptor (TCR)-mediated cognate antigen engagement for activation and pre-clinical studies demonstrated that antigen-specific Tregs preferentially migrate to inflammation sites, enabling targeted immunosuppression.

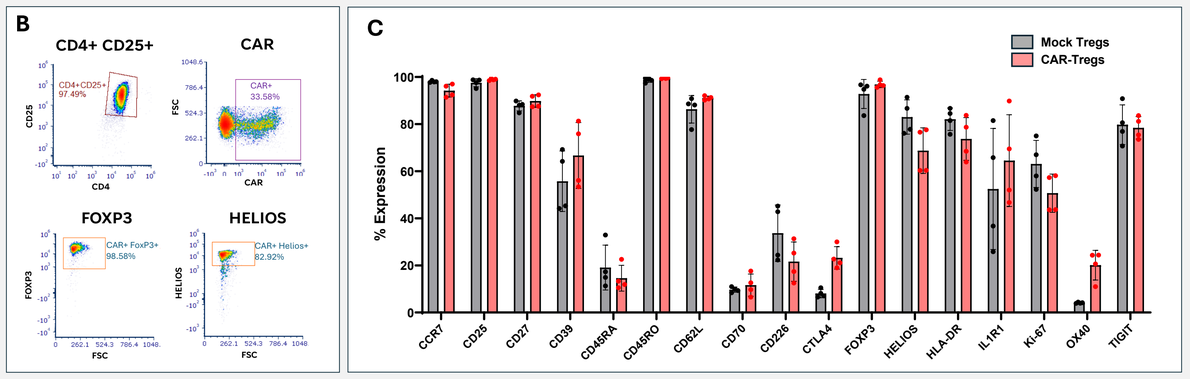
Chimeric antigen receptor (CAR)-engineered Tregs (CAR-Tregs) offer a transformative approach to restore immune tolerance in autoimmune and inflammatory liver diseases by leveraging the unique immunomodulatory capabilities of Tregs. By engineering Tregs with CARs targeting liver-specific antigens, we aim to enhance their specificity and suppressive function, directing them to the liver microenvironment to selectively dampen pathogenic immune responses. To test this, we designed and synthesized multiple second-generation CAR constructs incorporating single-chain antibody fragments specific for asialoglycoprotein receptor 1 (ASGPR1) and varying transmembrane and hinge domains. ASGPR1 is a universal antigen found highly and selectively expressed in hepatocytes, which is ~80% of the liver mass. ASGPR1 is well-characterized and functions by binding, internalizing and clearing of glycoproteins and asialoglycoproteins from the circulating serum.

This study presents the development and preclinical evaluation of CAR-Tregs designed to recognize a liver-specific antigen, demonstrating their potential to precisely modulate immune responses and promote durable, tissue-specific tolerance.

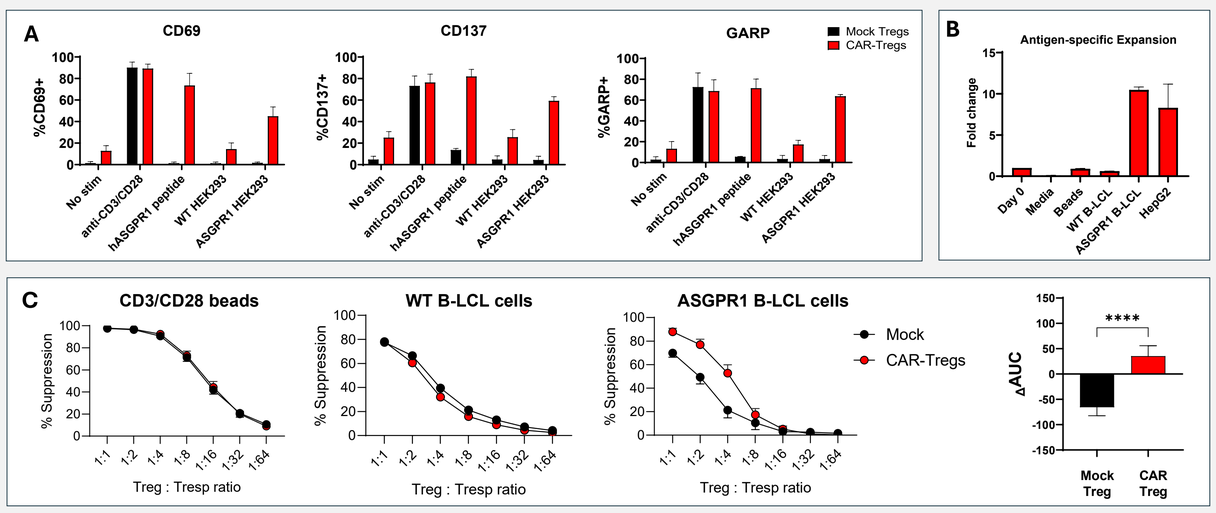
CAR Tregs exhibit a stable lineage phenotype and markers of suppressive functions



(A) Initial screening using Jurkat NFAT reporter cells which were transduced with various constructs that each had a different ASGPR1 scFV and CAR domain designs. Transduced Jurkat NFAT reporter cells were cultured with human or mouse ASGPR1 peptide for 24 hours before measuring luciferase activity. Data showed that QTX-02 scFV design performed the best. N=2 **(B)** Representative FACS plots showing that Tregs were successfully transduced with the CAR construct and had high FoxP3 expression levels. **(C)** Deep phenotype analysis of 4 healthy donors at day 13 post-transduction showing that CAR-Tregs have a similar cell marker expression profile as Mock Tregs and do not show phenotype instability. CAR-Tregs have high expression levels of suppressive markers FoxP3 and Helios. CAR-Tregs were pre-gated on CAR+ population. N=4

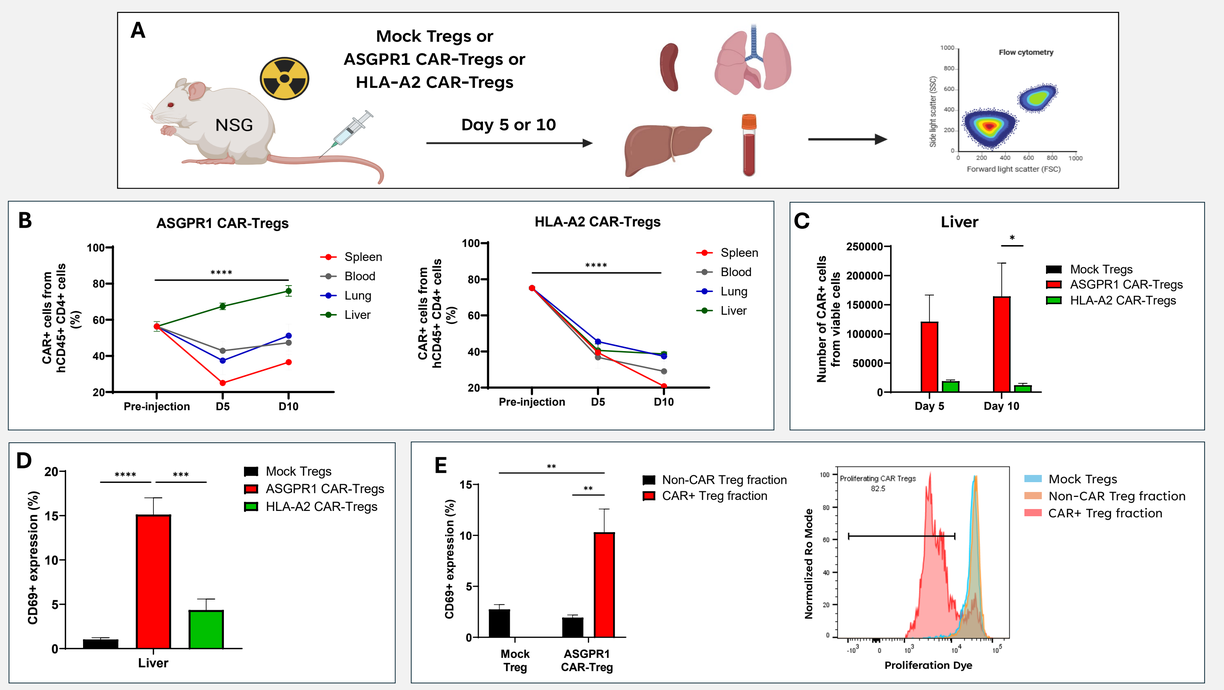


CAR Tregs activate and expand in response to ASGPR1 antigen and suppress T cells proliferation *in vitro*

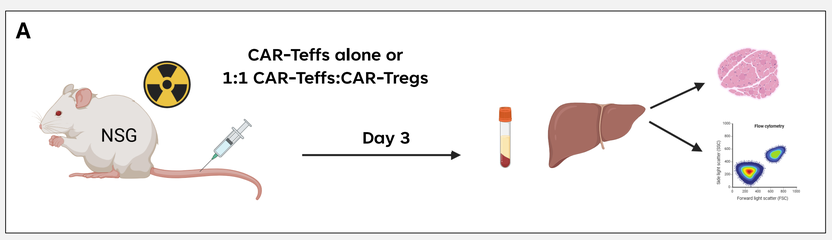


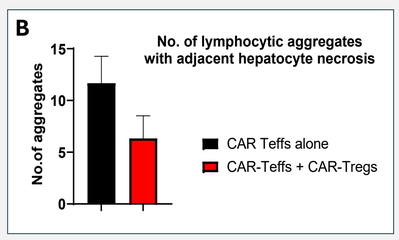
(A) Activation assay results showing that CAR-Tregs specifically recognise Human ASGPR1 peptide and ASGPR1+ HEK293 cells and upregulate activation markers of CD69, CD137 and GARP after 24 hours of co-culture. As expected, Mock Tregs did not recognise nor respond to ASGPR1 but did upregulate activation markers in response to anti-CD3/CD28 beads as a positive control. N= 6 **(B)** CAR-Tregs expand ~10 fold in the presence of ASGPR1 antigen (B-LCL expressing ASGPR1 and HepG2 cell lines). CAR-Tregs were pre-gated on the transduced fraction before assessing expanded cell numbers. N= 2 **(C)** Suppression assay results show that CAR Tregs suppress T cell proliferation more potently compared to Mock Tregs in the presence of ASGPR1 antigen. AUC=Area under the curve. Tresp=T cell responders. N=6

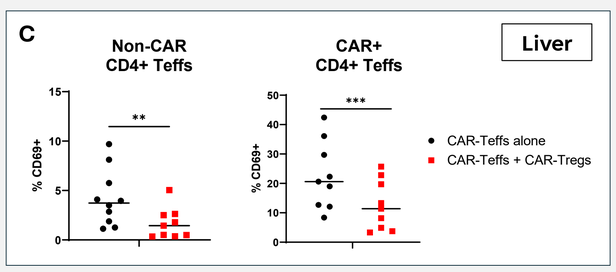
CAR Tregs traffic, engraft, activate and proliferate in the liver *in vivo*

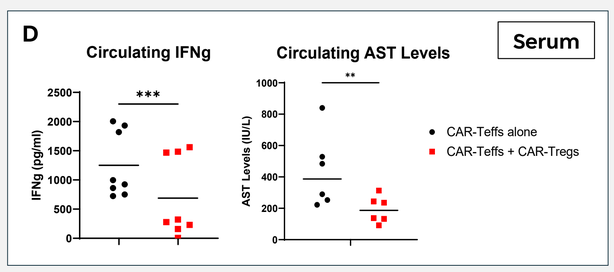


CAR Tregs suppress Teffs activity *in vivo*









(A) Diagram showing that 6-8 week old NSG mice were irradiated with 1.5 Gys 1 day prior to injecting mice with CAR T-effectors (CAR-Teffs) alone or CAR-Teffs with CAR-Tregs at a 1:1 ratio of CAR+ cells. CAR-Teffs were autologous Teffs transduced with the same ASGPR1-CAR construct as the CAR-Tregs and therefore inducing specific liver damage. After 3 days, mice were sacrificed, and liver tissue and blood serum were collected and assessed. **(B)** Graph of histology results showing the number of aggregates with adjacent hepatocyte necrosis. Results demonstrate a trend that the presence of CAR-Tregs reduces hepatocyte damage caused by CAR-Teffs. N=3 **(C)** Within the liver tissue, Non-CAR Teffs and CAR+ Teffs were assessed for CD69 expression levels. CAR-Teffs treated with CAR-Tregs had a reduced activation status as demonstrated by a lower CD69 expression level compared to without CAR Treg treatment. N= 9-10 **(D)** Within the blood serum, CAR Teffs treated with QTX-02 CAR Tregs had reduced IFNγ and AST levels compared to without CAR-Treg treatment. This demonstrates that CAR-Tregs suppressed the inflammatory response that would otherwise be observed from CAR-Teffs alone and that CAR-Tregs protected against liver damage as a lower AST level is observed, which is an indicator of liver damage. AST= aspartate transferase. N=6-8

(A) Diagram showing 8-12 weeks old NSG mice infused with 10M Mock, ASGPR1 or HLA-A2 CAR-Tregs. After 5 or 10 days, tissues were harvested and analysed **(B)** ASGPR1 or HLA-A2 CAR-Treg counts found in mouse spleen, blood, lung and liver after 5 or 10 days post-injection into the mice. The transduction efficiency pre-injection was recorded. Results show that there is enrichment of ASGPR1 CAR-Tregs in the mouse liver compared to the other tissues tested and compared to HLA-A2 CAR-Tregs, demonstrating that ASGPR1 CAR-Tregs specifically traffic to the liver where there is ASGPR1 antigen and engraft there. N=6 **(C)** Graph showing the absolute counts of CAR-Tregs within the liver tissue. N=6 **(D)** Within the liver tissue, ASGPR1 CAR-Tregs upregulated significantly higher levels of CD69 activation marker compared to HLA-A2 CAR Tregs. N=6 **(E)** (Left graph) Upregulation of CD69 activation marker in the liver tissue was driven by the transduced CAR-Treg fraction as opposed to the non-CAR Treg fraction of cells. N=9 (Right graph) Representative histogram plot of specific proliferation of the CAR+ fraction of QTX-02 CAR Tregs as opposed to the non-CAR fraction of Tregs within the liver tissue.

Conclusions and Acknowledgments

Conclusions

- We have designed a pan liver-specific CAR that recognizes ASGPR1 antigen, an extracellular protein with high tissue specificity
- The ASGPR1 CAR-Tregs showed efficient activation, proliferation and T cell suppression in vitro in an antigen-specific manner
- The development of a cross-reactive CAR with mouse ASGPR1 protein allowed the assessment of CAR-Treg trafficking and function in liver tissue of NSG humanized mouse models
- ASGPR1 CAR-Tregs showed enhanced activation and proliferation in the liver tissue compared to non-transduced Tregs or control CAR-Tregs
- Infusion of ASGPR1 CAR-Tregs protected against liver damaged and suppressed the activity of CAR-Teffs in a *in vivo* model of liver inflammation

Acknowledgements

Courtney Grant, Gabriele Indriuskaite, Stephen Orr, Andy Ko, Jelena Ruscic, Ines Meleiro, Stephen Perry, KCL members, Quell Tx members