Stable Expression of FOXP3 and Core Tolerogenic Genes by Engineered Human Tregs

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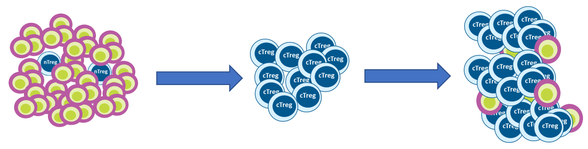
ABSTRACT

FOXP3+ Regulatory T cells (Tregs) play a critical role in the prevention of fatal autoimmunity and maintaining tissue homeostasis. The functional stability of Tregs remains critical in their contribution towards immune tolerance rather than runaway immunity, particularly for cell therapy whereby an inflammatory microenvironment may impact the expression of FOXP3 and associated tolerogenic genes. To address potential Treg instability, we generated human Engineered Tregs (EngTregs) through a gene editing approach of PBMC isolated CD4+ T cells, leading to stable FOXP3 expression and a rapamycin-activated signaling complex that provides tunable IL-2 signal, thereby effectively divorcing FOXP3 expression from existing regulatory elements known to promote Treg instability under inflammatory conditions. Using transcriptomic analysis, we evaluated the stability of cultured sorted Tregs (cTregs) compared to EngTregs. We found EngTregs expressed higher levels of key "Core Treg" and "FOXP3 Synergy" genes based on bulk and single cell RNA-seq analysis and as confirmed by flow cytometry. Additionally, a favorable expression pattern of key Treg stability markers, CD27, CD70 and IKZF4 (EOS) was observed in EngTregs compared to cTregs. Conversely, cTregs express higher levels of cytotoxic genes including GZMA and PERFORIN1 compared to EngTregs along with other inflammatory genes. Finally, we observed that these correlations have a functional implication as demonstrated by higher expression of key tolerogenic markers in EngTregs compared to cTregs. This work strongly supports EngTregs as stable, tolerogenic FOXP3+ T cells, thereby providing an invaluable asset for treating life-threatening autoimmune and autoinflammatory diseases.

PREMISE

Potential limitations of expanded natural regulatory T cells

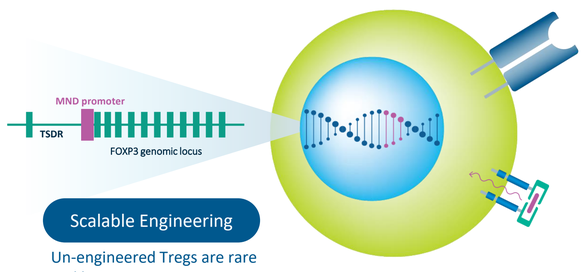
- Natural Tregs are an inherently rare and heterogenous subset.
- Downregulation of FOXP3 due to lack of epigenetic commitment



- Increased heterogeneity due to limited availability of stabilizing factors in host (e.g. low IL2)
- Possibility of inflammatory skewing as a result of exposure to de-stabilizing factors (e.g. inflammatory microenvironment)

Limitations of expanding sorted natural regulatory T cell. Natural Tregs are a rare subset within the PBMCs of most individuals. While the expansion of these rare cells in culture following cell sorting has been a common approach over the past decade, several well documented, key inherent limitations involving stability of natural Tregs have come to light (Zhou X., *Nat. Immunol.*, 2009; Junius S., *Sci. Immunol.*, 2020).

Engineered Tregs address key shortcomings of sorted Treg approach



Tissue Targeting

Additional transgene enables tissue specificity

- Modular TCRs or CARs for optimal efficacy and safety
- Antigen specificity confers tissue localization, engages bystander suppression and drives infectious tolerance
- Genti approach to TCR screening and qualification identifies the rare TCRs that are optimal for pairing with EngTregs

Scalable Engineering

Un-engineered Tregs are rare and heterogenous

- Tregs engineered from more abundant bulk T-cells are scalable, and achieve better phenotypes by stabilizing Treg differentiation factors (e.g. FOXP3) from either autologous and allogeneic sources.

IL2 Signaling

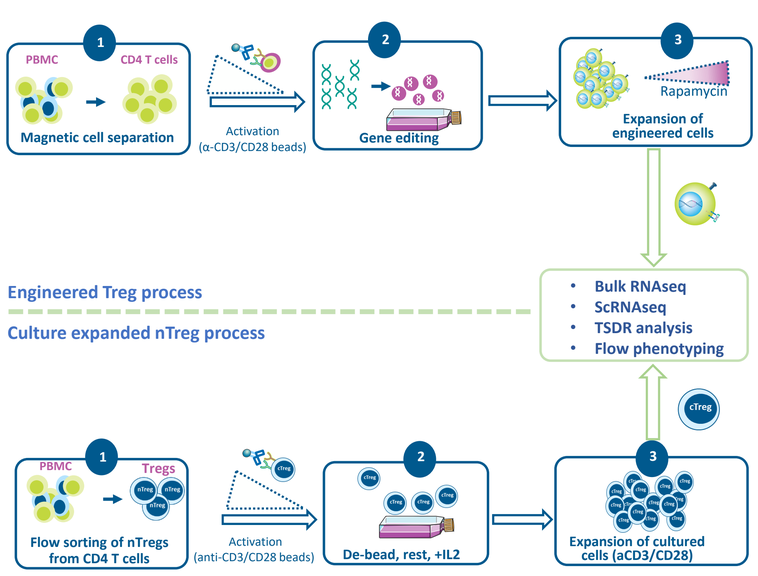
Tregs require IL2 but do not produce it

- Engineered Tregs incorporate Treg-selective and titratable IL2 signaling through a chemically-induced signaling complex (CISC)
- CISC enhances manufacturing and transforms Tregs into potent, long-lived drugs

Overview of human Engineered Tregs as a therapeutic approach. Briefly, the gene editing approach of PBMC isolated CD4+ T cells, leads to stable FOXP3 expression and expression of a rapamycin-activated signaling complex that provides tunable IL-2 signal, thereby effectively divorcing FOXP3 expression from existing regulatory elements known to promote Treg instability under inflammatory conditions. Additional key elements obtained through the manufacturing process and expression of additional transgenes would enable effective tissue homing/ specificity and mediation of Treg functional capabilities including enhanced proliferation / survival in response to signals from the inflammatory microenvironment.

Schematic: Research grade production of Engineered Tregs vs cultured, sorted Tregs

(A)



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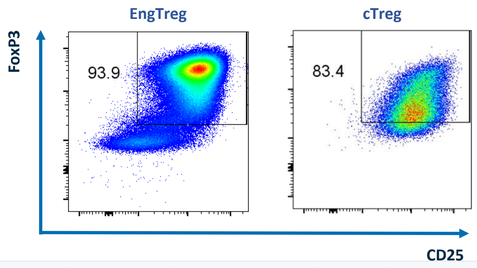
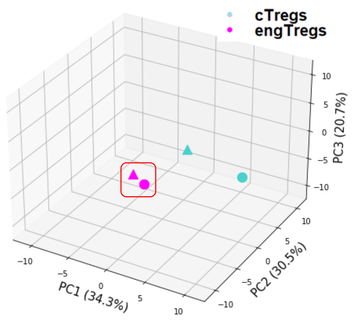


The process for generation of research grade Engineered Tregs (EngTregs) and culture expanded, sorted natural Tregs (cTregs). (A) For EngTregs, $CD4^+$ cells were isolated via magnetic enrichment from PBMCs. The cells were then genetically modified using CRISPR-Cas9 to knock-in transgenes delivered by AAV vectors. CISC receptor enables selective expansion and enrichment of engineered Tregs in the presence of rapamycin. For cTregs, $CD4^+$ $CD25^+$ $CD127^{lo}$ (further sorted as $CD45RA^+$ or "bulk") cells were isolated from PBMCs via flow cytometric cell sorting (BD A5 instrument). The cells were then activated using CD3/CD28 beads for an initial expansion, before rest to match EngTreg protocol timelines. IL2 (100ng/mL) was supplemented to cTregs throughout and these cells were stimulated again at D7 by CD3/CD28 beads. For cTregs and EngTregs, expanded cells were cryopreserved and used for further analyses and studies. (B) Flow cytometric analysis of CD25 and FoxP3 protein levels at process end timepoint by EngTregs vs cTregs.

Results 1: Donor dependent variability of Treg signature observed in cTregs but not EngTregs

(A)



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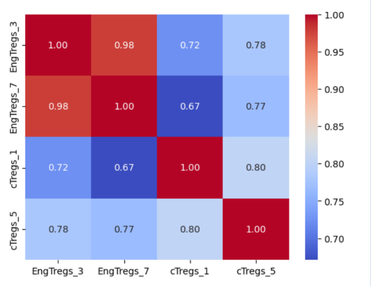
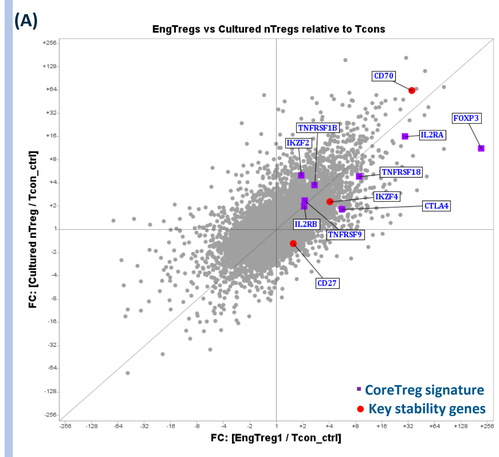


EngTregs cluster more closely to one another compared to cTregs. (A) (B) 3-dimensional principal component analysis and variance heat map of cultured Tregs vs EngTregs based on 70 hallmark genes upregulated in Tregs compared to Tcons (Kwon H., *Nat. Immunol.*, 2017). Data based on two donors used for cTreg and EngTreg production processes.

Results 2: EngTregs express higher levels of "Core" Treg function and stability genes compared to cTregs

(A)



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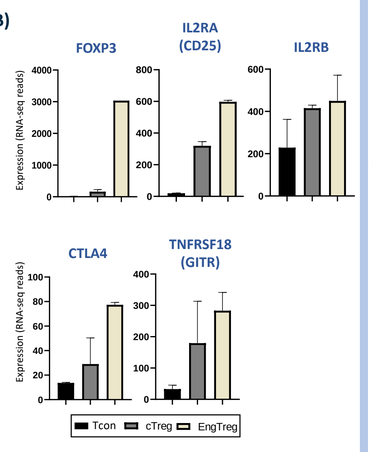

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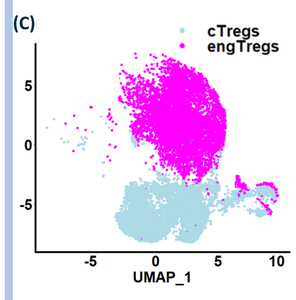

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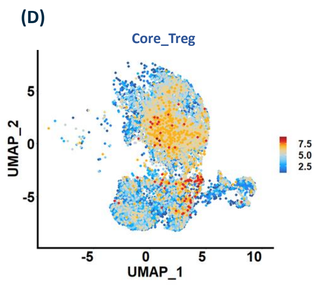

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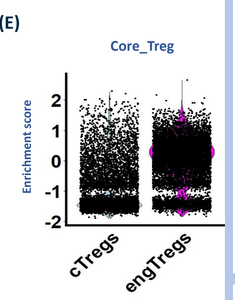
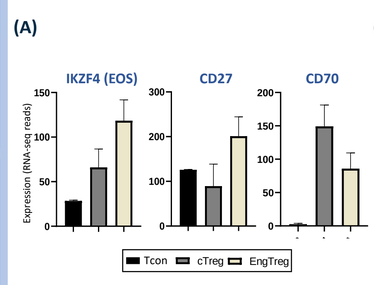


EngTregs cluster express higher levels of most Core Treg genes. (A) Fold change by fold change analysis of EngTreg and cTregs normalized to PBMC sorted T conventional cells reveals higher expression of most Core Treg genes (Zemmour D., *Nat. Immunol.*, 2018) by EngTregs compared to cultured Tregs. (B) Absolute read counts of key Core Treg genes. (C) UMAP plot identifying EngTreg vs. cTregs. (D) UMAP plot of EngTregs vs. cTregs overlaid with Core Treg signature. EngTregs appear to more uniformly express higher levels of Core Treg genes. (E) Enrichment score of Core Treg genes in EngTregs vs cTregs.

Results 3: EngTregs express correct pattern of key stability and FOXP3 Synergy genes

(A)



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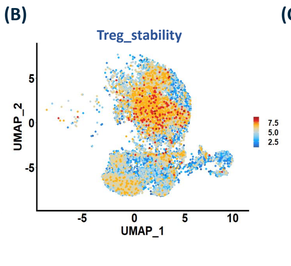

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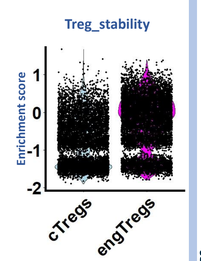

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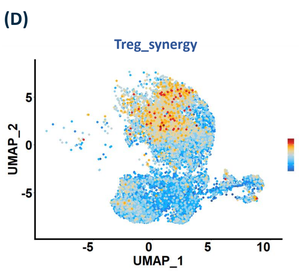

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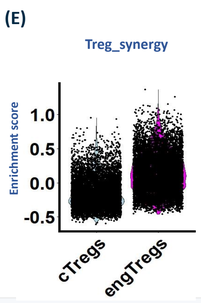

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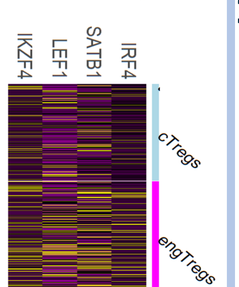
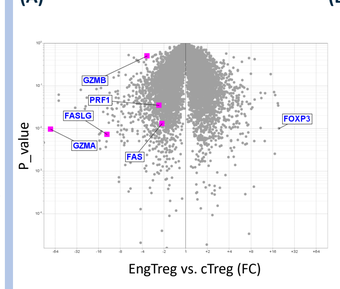


Genti EngTregs express correct pattern of key stability genes. (A) Higher expression of key stability genes CD27 and IKZF4 (EOS) in EngTregs compared to cTregs which conversely express higher levels of instability marker CD70 (RNA read counts). (B,C) UMAP (B) and enrichment score (C) of stability genes (CD27, IKZF4, FOXP3) shows higher expression in EngTregs compared to cTregs. (D, E, F) Projection on UMAP (D) with corresponding violin plot (E) and HeatMap of genes per cells (F), reveals higher expression of FOXP3 Synergy gene signature (Fu W., *Nat. Immunol.*, 2012) in EngTregs compared to cTregs. FOXP3 Synergy signature: IKZF4, LEF1, SATB1, IRF4.

Results 4: Inflammatory skewing of cTregs but not EngTregs at steady state

(A)



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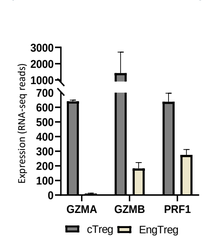

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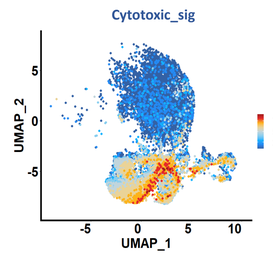

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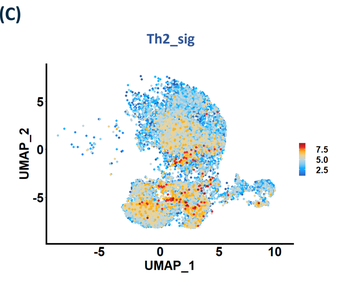

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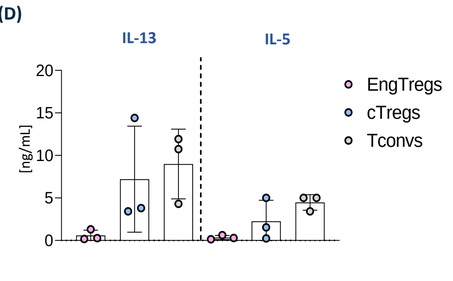
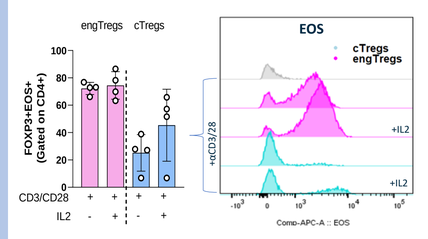


Inflammatory skewing of cTregs but not EngTregs at steady state. (A, B, C) Volcano plot (A) RNA read counts (B) and single cell analysis (C) comparing EngTregs and cTregs reveals upregulation of cytotoxic genes (GZMA, GZMB, PRF1, FAS, FASLG) in cTregs but not EngTregs. (D) single cell RNAseq analysis reveals higher expression of TH2 genes module (GATA3, IL4, IL5, IL13, IL17RB) in cTregs compared to EngTregs. (E) $2x10^5$ EngTregs and cTregs were cultured with αCD3/CD28 and IL2 (100ng/mL) for 72hrs prior to cytokine bead array analysis for indicated cytokines.

Results 5: EngTregs express higher levels of tolerogenic markers and display superior suppressive capability compared to cTregs

(A)



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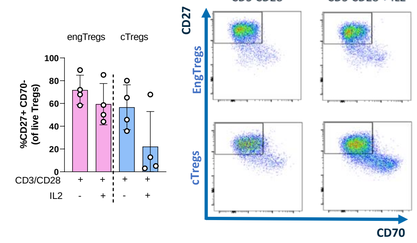

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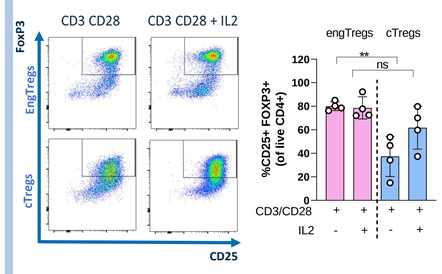

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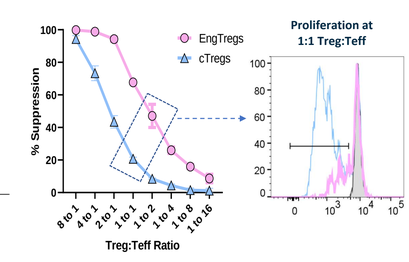
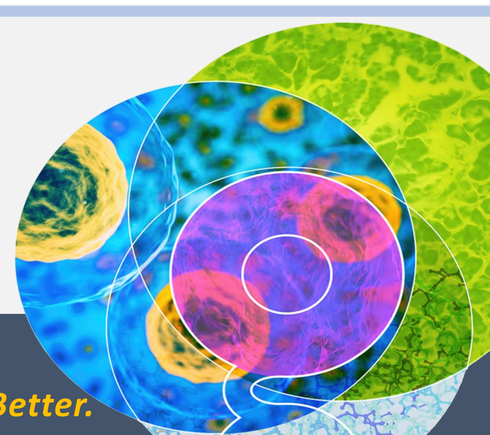


Higher expression of key Treg markers and superior suppressive capacity observed with EngTregs compared to cTregs at steady state. (A) Average frequency and representative flow cytometry histograms of EOS expressing EngTregs and cTregs cultured with αCD3/CD28, +/- IL2 (100ng/mL) as indicated for 72h. (B) Average frequency and representative FACS plots of CD27+ CD70- EngTregs vs cTregs cultured with aCD3/CD28 +/- IL2 (100ng/mL). (C) Representative FACS plots and frequency of CD25+ FOXP3+ EngTregs vs cTregs. (D) Superior suppressive capability of EngTregs compared to cTregs based on representative donor suppression assay. Histogram snapshot of proliferation at 1:1 Treg:Teff ratio. Data representative of EngTreg and cTregs produced from four donors.

CONCLUSIONS

- Engineered Treg platform overcomes the scaling and stability limitations of sorted patient Treg cells by starting with more abundant T cell sources and enriching edited cells with a chemically induced IL-2 signaling complex.
- Principal component analysis of top 70 genes upregulated in thymic derived Tregs reveals less variance between EngTregs produced from different donors compared to cTregs.
- Engineered Tregs express higher levels of Core Treg genes such as FOXP3, IL2RA and CTLA4 as well as FOXP3 Synergy genes, such as IRF4 and EOS compared to cTregs at both the bulk and single cell RNA transcript levels.
- These gene expression patterns correlate with higher levels of protein expression detected by flow cytometry and functional capacity based on polyclonal Treg:Teff suppression assay.
- EngTregs show minimal enrichment of inflammatory gene signatures associated with T cell mediated cytotoxicity or Th2 cell phenotype, as opposed to cTregs.
- Overall, this work strongly supports EngTregs as more stable, tolerogenic FOXP3+ T cells, thereby providing an invaluable asset for treating life-threatening autoimmune and autoinflammatory diseases.



ACKNOWLEDGEMENT: The laboratory of Dr. David Rawlings at Seattle Children's Hospital pioneered the gene editing approach to produce engineered Tregs.

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