Human Engineered Tregs maintain stability in inflammatory environment

Martina Sassone-Corsi*, Payam Zarin*, Victoria De Vault* Adam Chicoine, Abigail Doherty, Jennifer Yam, Xiang Li, Fabien Depis, Adel Nada, Thomas J Wickham, Tiffany F Chen

GentiBio, Inc., Cambridge, MA, USA

**Equal contribution*

ABSTRACT

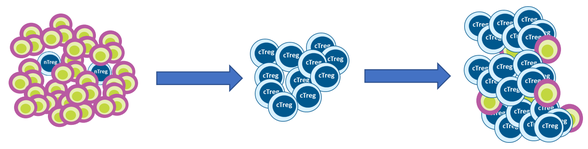
Regulatory T cells (Tregs) are essential for keeping the immune system in check, thus dysregulation of Tregs can lead to autoimmune disorders. Treg cell therapy has the potential to address this problem, however limitations of sorted and cultured Treg (cTregs) cell therapies include inherent plasticity and instability. Indeed, cTregs can lose FOXP3 expression and acquire the ability to express T effector (Teff) cytokines in non-favorable environments. To address these limitations, we have engineered human CD4 T cells into Tregs (EngTregs) endowed with stable FOXP3 expression and a rapamycin-activated, chemically induced IL-2 signaling complex (CISC).

To address the stability and functionality of EngTregs, we compared them to cTregs at both steady state and in inflammatory conditions. At steady state, EngTregs demonstrate enrichment of core T regulatory cell gene signatures (CTLA-4, IL2RB, TNFRSF1B, TNFRSF18) compared to cTregs. EngTregs but not cTregs, maintain stability as measured by expression of FOXP3, CD25 and other Treg stability markers (e.g. EOS, CD27+CD70-). Stable FOXP3 expression in EngTregs is reflected in their suppressive activity against Teff cells. Importantly, EngTregs maintain FOXP3 expression in inflammatory environments and secrete IL-10 similarly to cTreg. However, unlike cTregs, EngTregs express little or no key Th2 cytokines (e.g. IL-4 and IL-13) in such environments.

PREMISE

Potential limitations of expanded sorted and cultured legulatory T cells

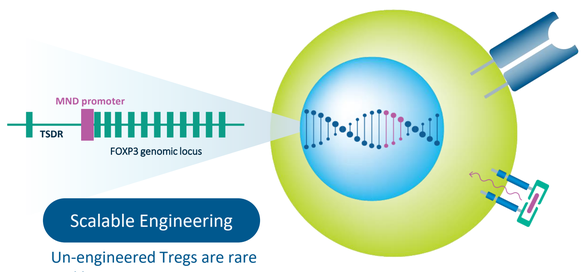
- Natural Tregs are an inherently rare and a heterogenous subset.
- Downregulation of FOXP3 due to lack of epigenetic commitment



- Increased heterogeneity due to limited availability of stabilizing factors in host (e.g. low IL-2)
- 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.

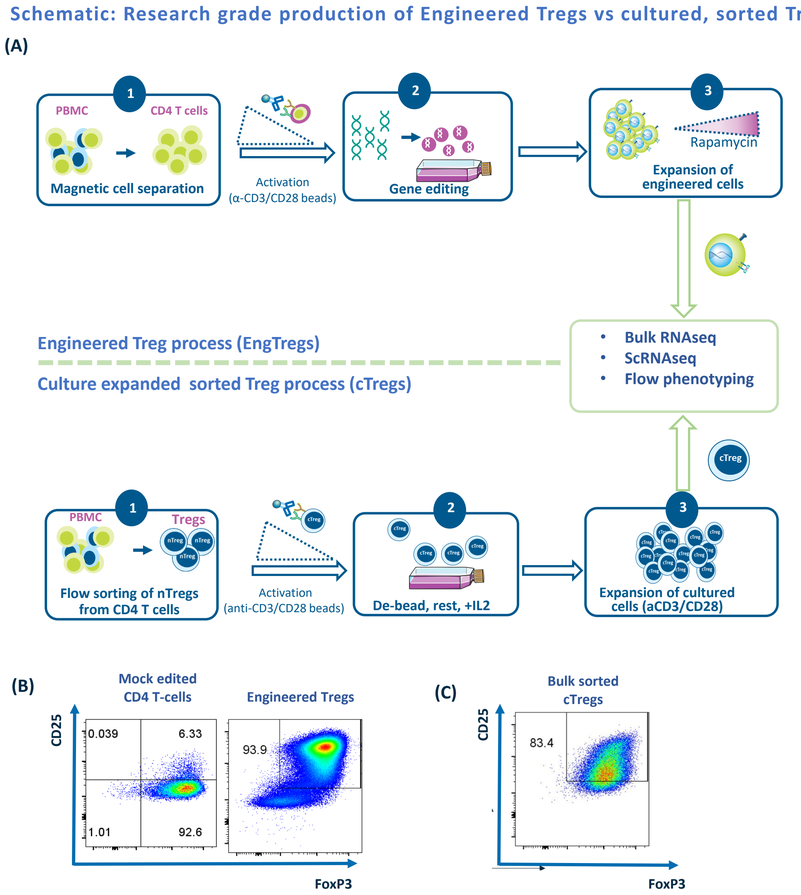
IL2 Signaling

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 enables enrichment of EngTregs during the cell production process.

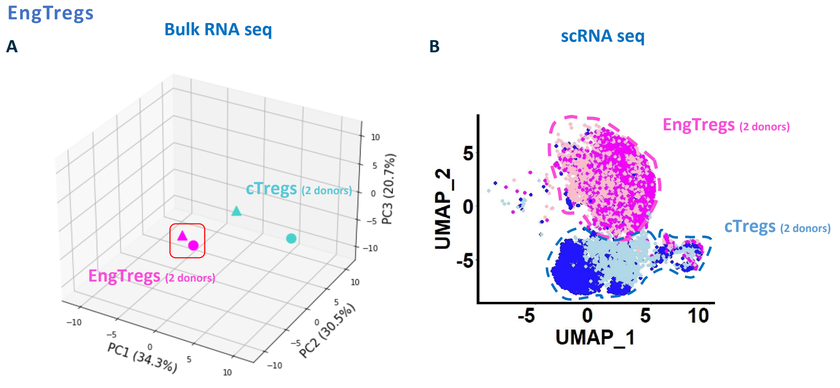
Overview of human Engineered Tregs as a therapeutic approach. Briefly, the gene editing approach of PBMC isolated CD4⁺ T cells, leads to stable FOXP3 expression and expression of a rapamycin-activated signaling complex that provides tunable IL-2 signal, thereby effectively divorcing FOXP3 expression 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 localization 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



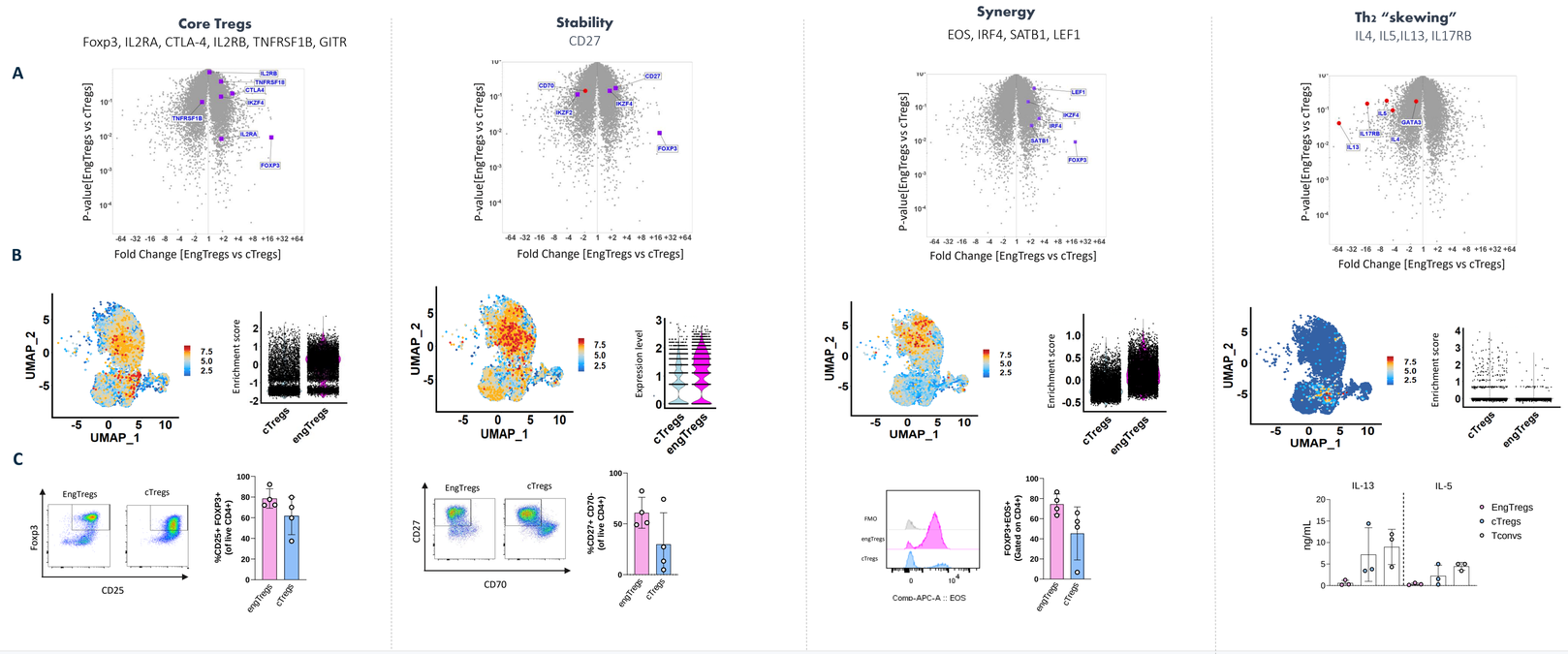
The process for generation of research grade Engineered Tregs (EngTregs) and culture expanded, sorted natural Tregs (cTregs). (A) For EngTregs, CD4⁺ cells were isolated via magnetic enrichment from PBMCs. The cells were then genetically modified using CRISPR-Cas9 to knock-in transgenes delivered by AAV 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) EngTregs but not mock edited PBMC derived CD4⁺ T cells express high levels of CD25 and FoxP3. (C) Expression of CD25 and FoxP3 by cultured Tregs at D21.

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



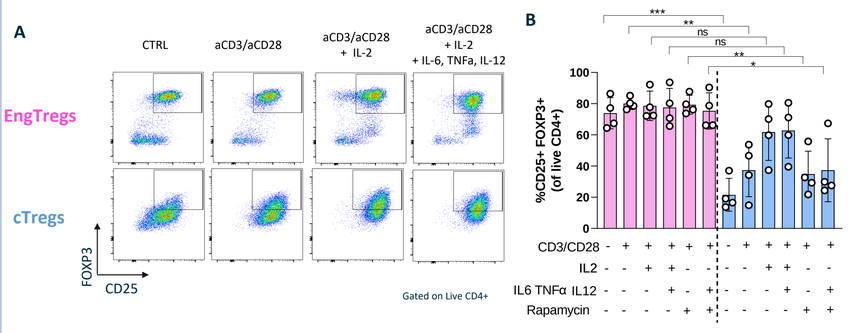
BulkRNA seq and scRNA seq in engTregs to culture Tregs (A) 3-dimensional principal component analysis and variance heat map of cultured Tregs vs EngTregs based on 70 hallmark genes upregulated in Tregs compared to Tconvs (Kwon H., *Nat. Immunol.*, 2017). Data based on two donors used for cTreg and EngTreg production processes. (B) UMAP projection of two donors used for transcriptomes from cTregs and EngTregs .

Results 2: EngTregs display a gene expression and marker profile consistent with a more stable and robust phenotypic profile compared to cultured Tregs



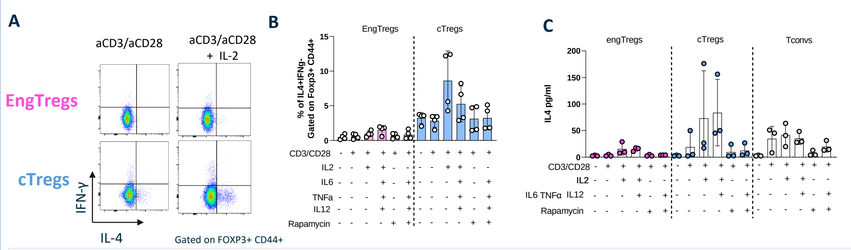
Comparison of Human EngTregs to cTregs gene expression and marker profile key differences (A) Volcano Plots (B) scRNA seq Feature Plots and Violin Plots (C) Flow Cytometry analysis or cytokine bead array (D2 post CD3CD28+ IL2) of EngTreg and cTregs reveal higher expression of most Core Treg genes (Zemmour D., *Nat. Immunol.*, 2018); stability gene CD27, FOXP3 Synergy genes (Fu W., *Nat. Immunol.*, 2012) IKZF4, LEF1, SATB1, IRF4 in EngTregs compared to cTregs, while higher expression of key Th2 genes signature (IL4, IL5, IL13, IL17RB) is observed in cTregs compared to EngTregs.

Results 3: EngTregs maintain stable FOXP3 and high CD25 expression post-inflammatory challenge and in the absence of TCR or IL-2 stimulation



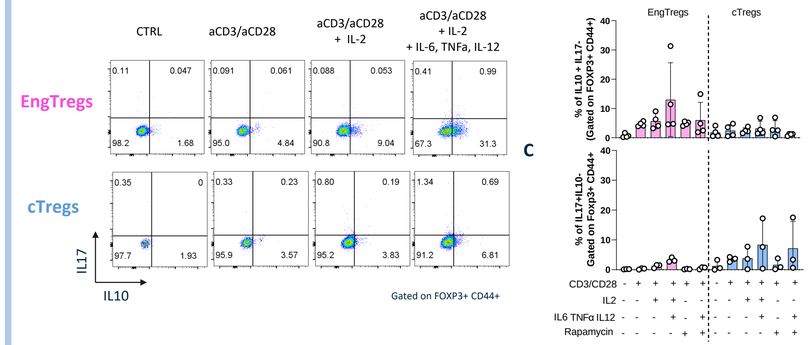
Genti EngTregs maintain stable FOXP3 and CD25 expression in the absence of TCR and IL2 stimulation and post–inflammatory cytokines challenge. 2x10^5 EngTregs and cTregs were cultured with and without αCD3/CD28 , IL2 (50ng/mL), a cocktail of IL6 (50ng/mL), TNFα (50ng/mL), and IL12 (10ng/mL) with or without Rapamycin (10nM) for 72hrs prior to flow cytometric analysis. (A) Representative FACS plots and (B) frequency of CD25+ FOXP3+ EngTregs vs cTregs in 4 different human donors.

Results 4: EngTregs express lower levels of Th2 cytokines in inflammatory environment



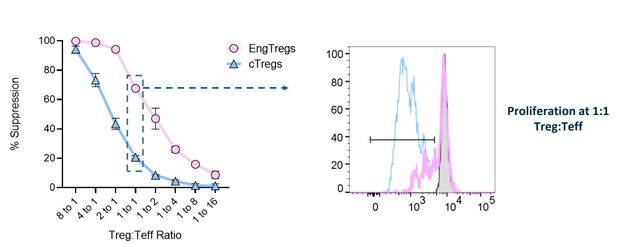
Inflammatory skewing of cTregs but not EngTregs. 2x10^5 EngTregs and cTregs were cultured with and without αCD3/CD28, IL-2 (50ng/mL), a cocktail of IL-6 (50ng/mL), TNFα (50ng/mL), and IL-12 (10ng/mL) with or without Rapamycin (10nM) for 72hrs prior to flow cytometric analysis. (A) Representative FACS plots and (B) frequency of IL-4⁺ IFNg⁻ in EngTregs and cTregs (C) secreted IL4 measured by cytokine bead array in EngTregs vs cTregs in 4 different human donors.

Results 5: EngTregs secrete IL-10 but no IL-17 post-inflammatory challenge



Production of IL-10 and IL-17 by EngTregs and cTregs. 2x10^5 EngTregs and cTregs were cultured with and without αCD3/CD28 , IL2 (50ng/mL), a cocktail of IL6 (50ng/mL) TNFα (50ng/mL), and IL12 (10ng/mL), with or without Rapamycin (10nM) for 72hrs prior to flow cytometric analysis. (A) Representative FACS plots and (B) frequency of IL10+ IL17- and (C) IL17+ IL10- EngTregs vs cTregs in 4 different human donors.

Results 6: EngTregs display superior suppressive activity compared to cTregs



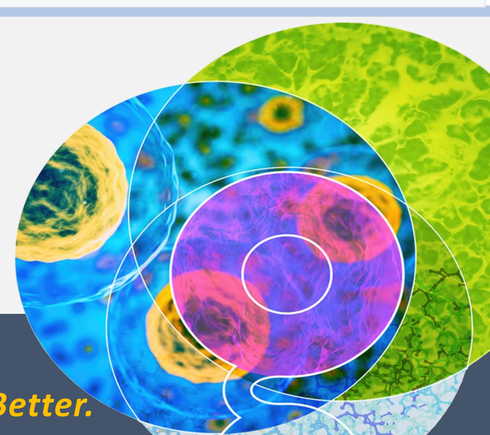
Enhanced suppressive activity observed with EngTregs compared to cTregs at steady state. Superior suppressive capability of EngTregs compared to cTregs based on representative donor suppression assay. Assay was set up by co-culturing various numbers of Tregs starting at 8:1 (200k→25k) with 25k autologous CD4⁺ T effector cells for 90hrs. Suppression is calculated as 100x ((Avg T resp max - % Treg+Tresp)/(Avg T resp max)) where T resp max = T responders with activation beads and no Tregs. 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 Treg cells by starting with more abundant T cell sources and enriching edited cells with a chemically induced IL-2 signaling complex.
- Principal component and scRNA seq analysis 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, stability gene CD27 and as well as FOXP3 Synergy genes, such as IRF4 and EOS compared to cTregs at both the bulk and single cell RNA transcript and protein level which correlate with higher functional capacity based on polyclonal Treg:Teff suppression assay.
- EngTregs show minimal enrichment of inflammatory gene signatures associated with Th2 cell phenotype, as opposed to cTregs.
- EngTregs maintain stability in the absence of TCR and IL2 stimulation and in the presence of pro-inflammatory cytokines.
- Overall, this work strongly supports EngTregs as more stable, tolerogenic FOXP3+ T cells, thereby providing an invaluable asset for treating 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.

References:
1)Honaker Y, Hubbard N, Gene editing to induce FOXP3 expression in human CD4+ T cells leads to a stable regulatory phenotype and function. Sci Transl Med. 12, 6422, 2020.
2)Zhou X., Bailey-Bucktrout S.L., ..., Bluestone J.A., Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat. Immunol. 10, 1000-1007 (2009).
3) Junius S., Mavrogiannis A., ..., Schlenner S. M., Unstable regulatory T cells, enriched for naive and Nrp1neg cells, are purged after fate challenge. Sci. Immunol. 6, 4723 (2021).
4) H. K. Kwon, H. M. Chen, D. Mathis, C. Benoist, Different molecular complexes that mediate transcriptional induction and repression by FoxP3. Nat. Immunol. 18, 1238–1248 (2017).
5) Zemmour D., Zilions R., Kiner E., Klein A. M., Mathis D., Benoist C., Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. Nat. Immunol., 19, 291-301 (2018).
6) Fu W., Ergun A., ..., Benoist C., A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells. Nat. Immunol. 13, 972-980 (2012).



We make Tregs. *Better.*