A novel engineered Treg platform that addresses Treg stability, IL-2 signaling and targeting, for the treatment of autoimmune diseases

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OVERVIEW | GentBio Treg Engineering Designed to Overcome Limitations of Sorted Tregs

Introduction

Clinical studies aiming to restore the immune homeostasis via in vitro sorted cultured Treg cell therapy were shown to be safe, however, several hurdles such as lack of stability, specificity and IL-2 signaling support, have hindered translation of Tregs into an effective treatment for autoimmune and autoinflammatory diseases. Treg identity can be impacted by the inflammatory environment which can skew the immunosuppressive phenotype towards proinflammatory effector function. Therefore, a safe and efficacious Treg product must thus be endowed with a locked Treg phenotype resistant to destabilizing cytokine cues. To enhance retention at the site of inflammation, a Treg cell therapy product can be armed with a TCR or CAR specific for a disease relevant antigen. Lastly, defects in IL-2 availability associated with autoimmune conditions impair Treg homeostasis, indicating that providing IL-2 support will be critical for Treg stability, engraftment and function in patients.
Here, we report an Engineered Treg (EngTreg) cell therapy platform designed to address major limitations of sorted cultured Tregs.

Three Pillars of EngTreg Therapy Platform

1. Treg stability

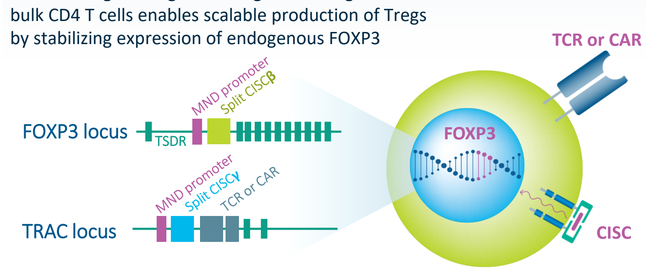
GentiBio engineering of autologous or allogeneic bulk CD4 T cells enables scalable production of Tregs by stabilizing expression of endogenous FOXP3

2. Specificity

Antigen specificity conferred by expression of TCR or CAR provides disease-tailored EngTreg cell retention at site of autoimmunity and engages bystander suppression

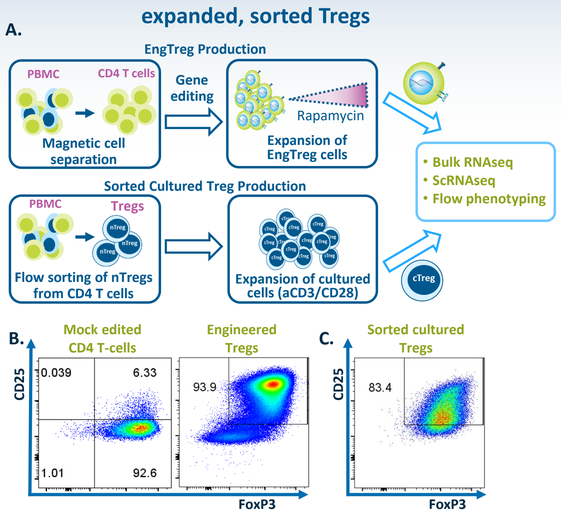
3. IL-2 signaling support

Chemically-inducible signaling complex (CISC) provides EngTreg -selective and tunable IL-2 signal to circumvent IL-2 scarcity associated with autoimmunity and enable long-lived restoration of immune homeostasis



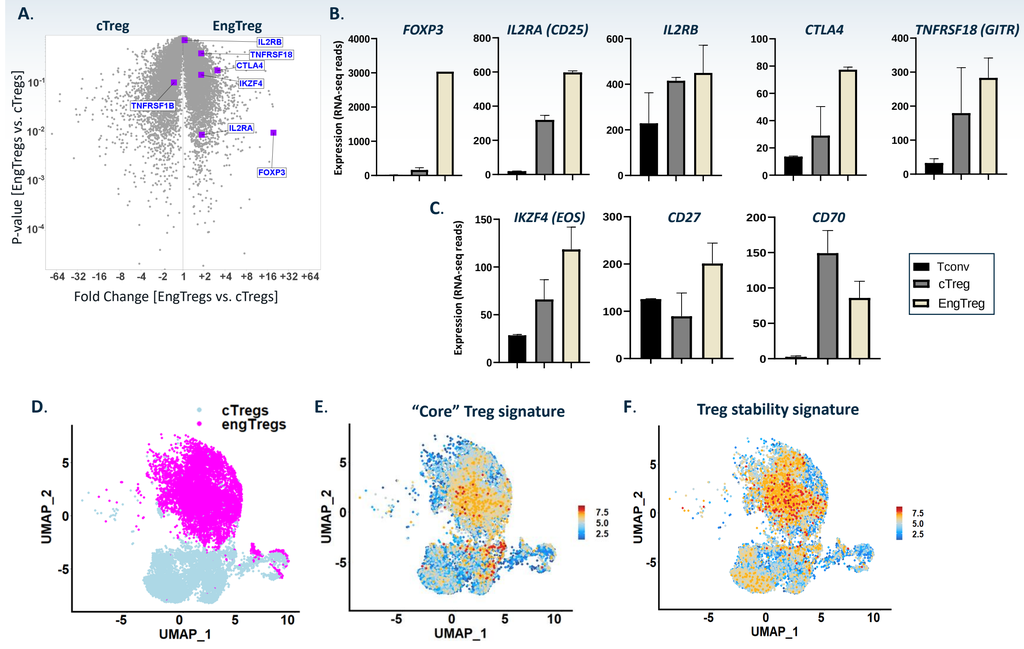
RESULTS | EngTregs Stably Express FOXP3 and Core Tolerogenic Genes

Generation of research grade EngTregs and culture expanded, sorted Tregs



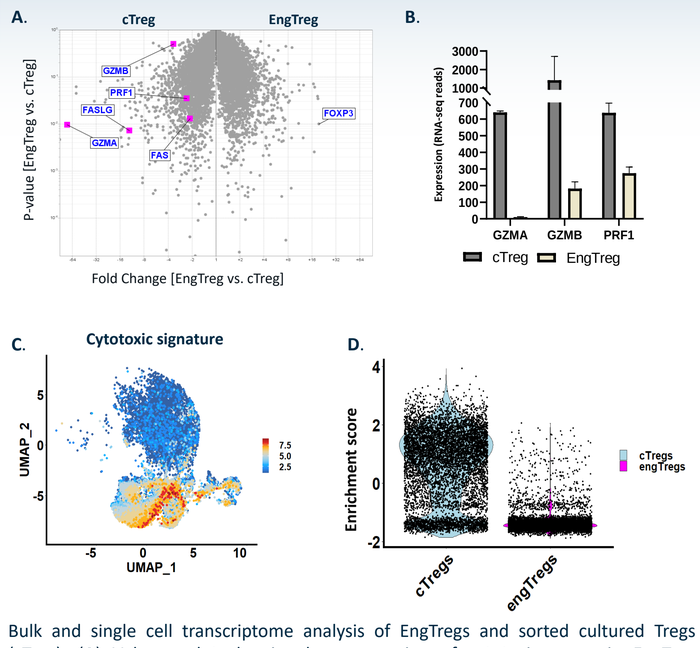
(A) For EngTregs, CD4⁺ T cells were isolated from PBMCs. The cells were genetically modified using an RNA-guided nuclease to insert transgenes delivered by AAV vectors. CISC enables selective expansion and enrichment of EngTregs in the presence of rapamycin. For cultured Tregs (cTreg), CD4⁺ CD25⁺ CD127^{lo} (further sorted as CD45RA⁺ or "bulk") cells were isolated from PBMCs via FACS. The cells were then activated using CD3/CD28 beads for an initial expansion, before rest to match EngTreg protocol timelines. IL-2 was supplemented to cTregs throughout and these cells were stimulated again at D7 by CD3/CD28 beads. (**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 Day 21.

EngTregs express higher levels of "Core" Treg and stability genes



Bulk and single cell transcriptome analysis of EngTregs and cultured Tregs (cTreg). (**A**) Volcano plot reveals higher expression of most "Core" Treg genes (Zemmour D., *Nat. Immunol.*, 2018) by EngTregs compared to cTregs. Absolute read counts of key (**B**) "Core" Treg and (**C**) Treg stability genes. (**D**) UMAP plot identifying EngTreg vs. cTregs. (**E**) UMAP plot of EngTregs vs. cTregs overlaid with Core Treg signature. (**F**) EngTregs appear to more uniformly express Treg stability genes.

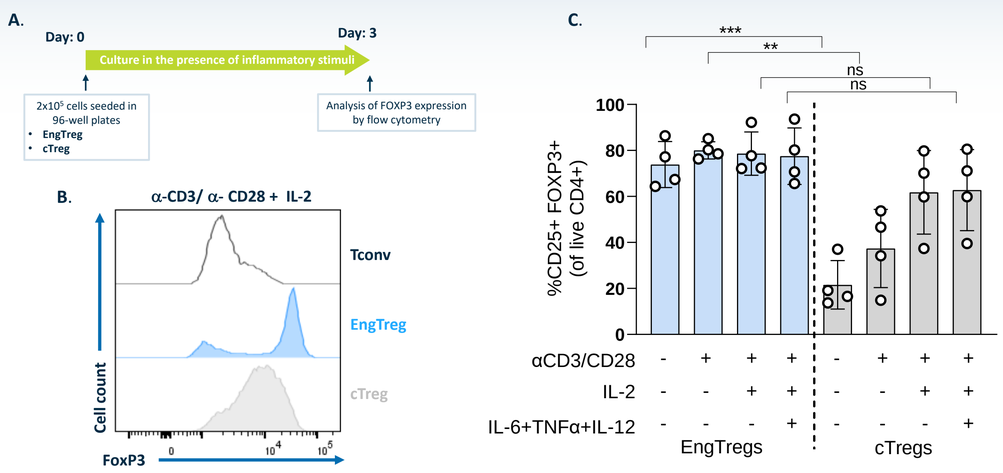
Cytotoxic skewing of cTregs but not EngTregs at steady state



Bulk and single cell transcriptome analysis of EngTregs and sorted cultured Tregs (cTreg). (**A**) Volcano plot showing low expression of cytotoxic genes in EngTreg compared to cTreg at steady state. 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**) Enrichment score of cytotoxic genes in cTregs compared to EngTreg.

RESULTS | EngTregs are Stable in an Inflammatory Environment

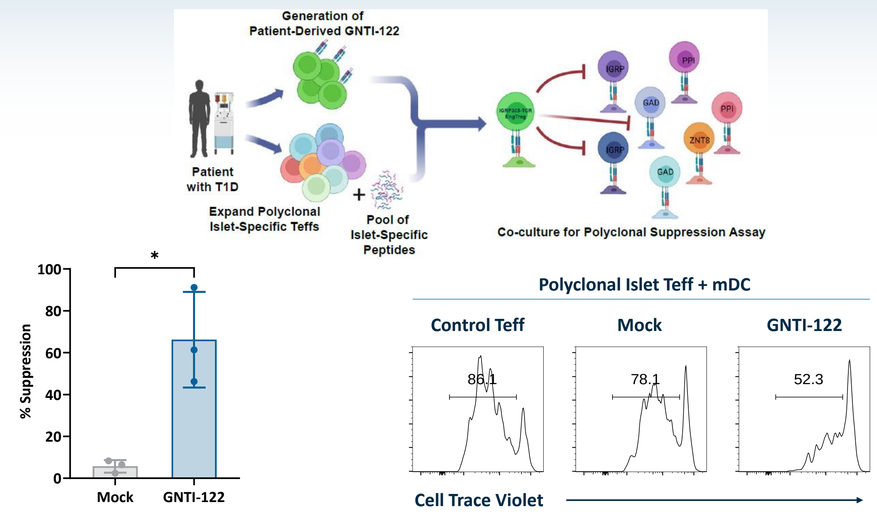
EngTregs maintain stable FOXP3 expression in the absence of TCR or IL-2 stimulation and post-inflammatory challenge



EngTregs and cTregs were cultured with and without αCD3/CD28 , IL-2 (50 ng/mL), a cocktail of IL-6 (50 ng/mL), TNFα (50 ng/mL), and IL-12 (10ng/mL) for 72 hours prior to flow cytometric analysis of FOXP3 expression. (**A**) Schematic of experimental setup. Representative histogram plots (**B**) and frequency of CD25+ FOXP3+ EngTregs vs cTregs (**C**) from 4 different human donors.

RESULTS | EngTregs Expressing Autoantigen-specific TCR Display Bystander Suppression

Islet antigen-specific EngTregs (GNTI-122) inhibit proliferation of T1D patient-derived polyclonal islet-reactive Teffs



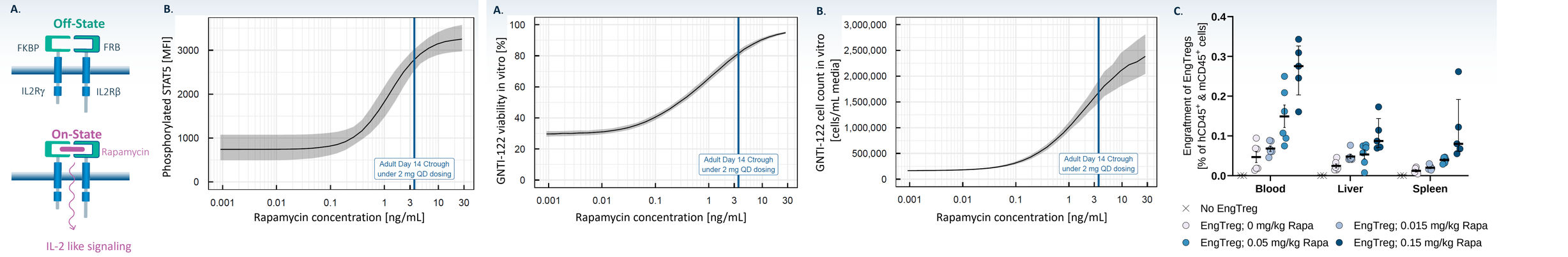
EngTreg cells (GNTI-122) expressing islet angtien-specific TCR are cocultured with autologous T effector cells (Teffs) expanded from patient donors with type 1 diabetes, and monocyte-derived dendritic cells (DC) as antigen-presenting cells. The Teffs specific to 9 different cognate peptides were isolated and DCs were loaded with their cognate peptides. Suppression was calculated as follows: % suppression = ((a-b)/a)x100, where "a" is the percentage of Teff proliferation in the absence of Tregs and "b" is the percentage of Teff proliferation in the presence of Tregs. Welch t-test (* p<0.05).

RESULTS | CISC Activation with Rapamycin Emulates IL-2 Signaling and Promotes EngTreg Expansion in the Absence of IL-2

CISC delivers tunable IL-2-like signal to EngTreg cells

CISC promotes survival and expansion of EngTregs cells in vitro

CISC supports in vivo engraftment of EngTregs



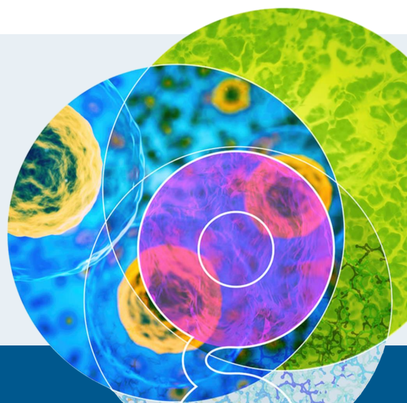
Phosphorylated STAT5 (pSTAT5) MFI show rapamycin concentration-dependent CISC activation in GNTI-122 cells in vitro. (**A**) Schematic depiction of CISC. (**B**) GNTI-122 cells were exposed to rapamycin and mean fluorescence intensity (MFI) of pSTAT5 at each concentration of rapamycin was quantified (N=3 donors). Simulation of rapamycin concentration-response relationship (Emax model) of in vitro CISC activation alongside rapamycin predicted trough exposures in adult humans at the 2 mg rapamycin dose. Median and 90% CI were derived from 500 population mean parameters sampled from the uncertainty distribution of the parameter estimates.

GNTI-122 expands in vitro and in vivo in the presence of TCR stimulation at clinically achievable rapamycin exposures. GNTI-122 cells were cultured for 10 days in the presence TCR stimulation via anti-CD3/CD28 beads, and increasing concentrations of rapamycin (in the absence of exogenously added IL-2). Cell viability and proliferation were analyzed at the end of the culture on Day 10. The relationships between rapamycin concentrations, and GNTI-122 viability (**A**) and expansion (**B**) on Day 10 of culture were characterized via a non-linear mixed effect (NLME) model trained on longitudinal in vitro GNTI-122 cell count and viability data. Rapamycin predicted trough exposures in adult humans at the 2 mg rapamycin dose and median and 90% CI derived from 500 population mean parameters sampled from the uncertainty distribution of the parameter estimates are shown. (**C**) Irradiated NSG mice given EngTregs at a dose of 5x10^6 cells/mouse were administered with increasing doses of rapamycin every other day intraperitoneally for 17 days post cell injection. On Day 19, mice were sacrificed and engraftment levels were assessed in each specified organ.

CONCLUSIONS

- EngTregs express higher levels of Core Treg and Treg stability genes, and maintain low expression of cytotoxic genes compared to cTregs
- EngTregs maintain stability in the absence of TCR and IL-2 stimulation and in the presence of pro-inflammatory cytokines
- EngTregs armed with pancreatic antigen-specific TCR elicit potent polyclonal suppression of islet antigen-reactive Teff cells isolated from patients with type 1 diabetes
- CISC provides on-demand IL-2-like signaling controllable by subtherapeutic rapamycin exposure levels to promote EngTreg viability and expansion in the absence of exogenous IL-2
- EngTregs represent a novel therapeutic modality with potential to restore immune tolerance in autoimmune diseases

We make Tregs. *Better.*



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