Characterization of Allogeneic Engineered Human Tissue-Tregs for Treatment of Acute Inflammatory Diseases

Maegan E Hoover#, Priya Saikumar#, Yash Agarwal#, Guoping Deng, Madison Milaszewski, Carlos Frias, Abigail Doherty, Nicole Reed, Scott Hussell, Rebecca Brunette, Marko Repic, Tiffany F Chen, Thomas J Wickham, Payam Zarin

GentiBio, Inc., Cambridge, MA, USA

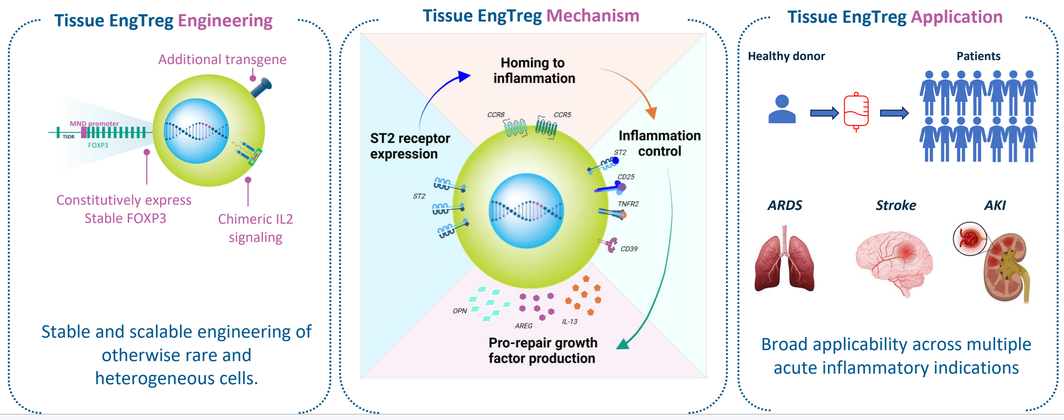
ABSTRACT

FOXP3+ Regulatory T cells (Tregs) orchestrate immune tolerance, thus preventing life-threatening autoimmunity, while maintaining tissue homeostasis and promoting repair following tissue damage. Tissue-resident Tregs appear to have a highly specialized gene expression program that enables the simultaneous activation of the anti-inflammatory and pro-repair functions. Adoptive transfer of allogeneic tissue Tregs could prove effective in patients with acute inflammatory conditions such as Acute Respiratory Distress Syndrome, acute kidney injury, or acute ischemic stroke where only a swift intervention could be useful, necessitating an "off-the-shelf " therapeutic that could ameliorate disease within hours. However, while the repair program has been well characterized in murine models, the understanding of tissue Tregs in humans is limited. Here, we engineer tissue Tregs from bulk CD4+ T cells with enhanced tissue-homing and suppression. Further, we demonstrate the effectiveness of allogeneic Tregs when transferred into a mouse model of acute lung injury.

Engineered human regulatory T cells (EngTregs) were generated from CD4+ T cells using CRISPR-Cas9 to knock-in a strong promoter at the *FOXP3* gene locus to stabilize FOXP3 expression and express a chemically inducible IL-2 signaling complex (CISC). This promotes rapamycin-activated IL-2-like signaling specific to the engineered cells, thus enabling enrichment of FOXP3+ cells in the culture process. To impart a tissue-Treg phenotype, we overexpressed a 'tissue-Treg' associated protein in EngTregs. We found that the final product (Tissue EngTregs) had a robust Treg phenotype as measured by high expression of key Treg-associated proteins such as FOXP3, CD25, CD39, HLA-DR, TNFRII and more, and increased suppression of proliferation of a polyclonal pool of effector T cells relative to control mock-engineered cells. Tissue EngTregs also upregulated several chemokine receptors (CCR4, CCR5, and CCR8) that would enable chemokine-dependent homing to inflamed tissues and demonstrated modulation of tissue repair. When exposed to an acute inflammatory cytokine milieu, tissue EngTregs also respond by rapid sequestration of several inflammatory molecules (e.g. TNF-α, IL-2, IL-4, IL-33) *in vitro*. Therefore, Tissue EngTregs have significant potential for the treatment of acute inflammatory diseases.

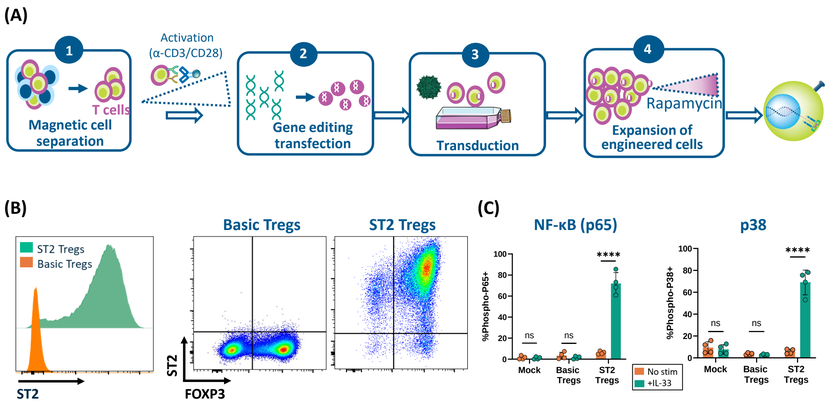
As proof-of-principle we also evaluated the efficacy of allogeneic surrogate murine Engineered Tregs (mEngTregs) in an LPS induced model of acute lung injury. mEngTreg treated animals showed significantly improved disease outcome and lower levels of inflammatory cytokines such as IFN-γ and TNF-α in the bronchoalveolar lavage (BAL). Also, mEngTregs were found to effectively home to the site of pulmonary inflammation and resemble endogenous Tregs in expression of key markers such as Foxp3, CD25, ST2 and CCR4. Overall, these data are supportive of the potential for developing a potent off-the-shelf engineered Treg cellular therapy for human acute onset inflammatory diseases.

PREMISE



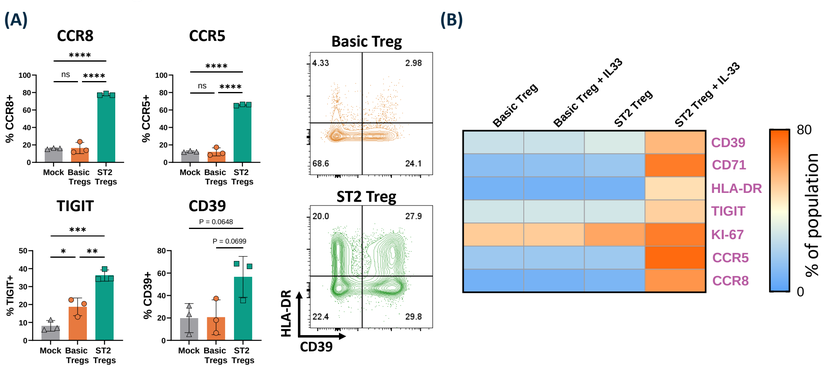
Overview of human Engineered Tissue Tregs as a therapeutic approach for acute inflammatory and ischemic diseases. Briefly, gene editing approach of PBMC isolated CD4+ T cells, leads to the expression of stable FOXP3 and a rapamycin-activated signaling complex that provides tunable IL-2 signal, effectively divorcing FOXP3 expression from existing regulatory elements known to promote Treg instability under inflammatory conditions (Honaker S, *Science Translational Medicine*, 2020, Cook P, *Molecular Therapy*, 2023). Additional key elements obtained through manufacturing process and expression of additional transgenes, such as the IL-33 receptor ST2, enable effective tissue homing and mediation of tissue Treg capabilities including enhanced proliferation / survival in response to signals from the inflammatory microenvironment as well as tissue repair. Scalable manufacturing of an allogeneic cell product from healthy donors would enable off-the-shelf treatment for acute inflammatory and ischemic diseases including Acute Respiratory Distress Syndrome (ARDS), ischemic stroke, and acute kidney injury (AKI).

1. Methods: Tissue EngTreg Generation and ST2 Signaling



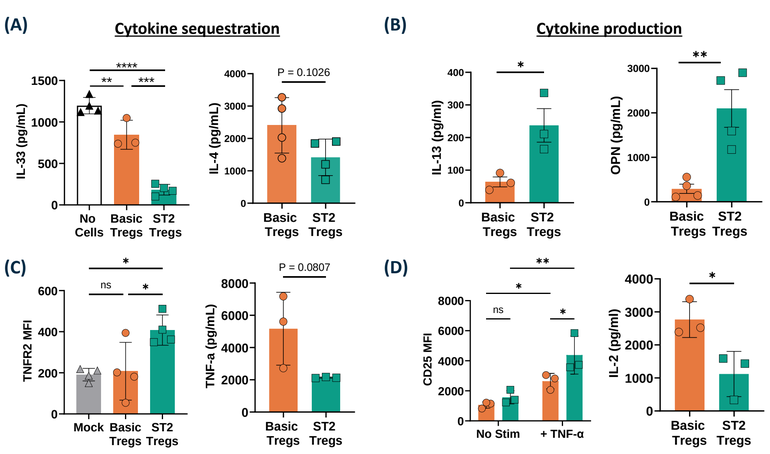
Tissue EngTregs overexpress the alarmin receptor, ST2, enabling P65 and P38 signaling in response to IL-33 provision. (A) Protocol outline detailing the generation of Engineered Tissue Tregs. (B) Flow cytometric analysis of ST2 and FOXP3 at process endpoint by basic EngTregs (Basic Tregs) and Tissue EngTregs (ST2 Tregs). (C) Phos-flow assay to measure P38 and P65 signaling downstream of the ST2 receptor with (green bars) or without (orange bars) 50ng/mL r-IL-33 in culture (statistics by two-way ANOVA).

2. Results: Tissue EngTregs express higher levels of homing, activation and tolerogenic markers



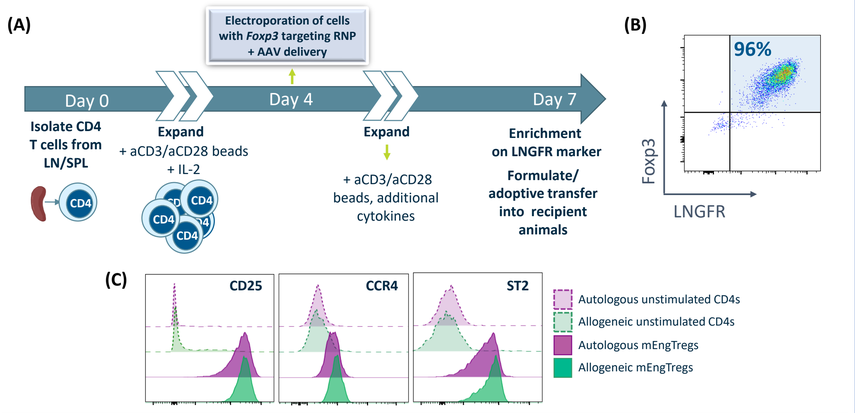
Tissue EngTregs are better suited for response to acute inflammation at the site of injury. (A)(B) Flow cytometric analysis of tissue homing, activation and tolerogenic markers comparing Tissue EngTregs (ST2 Tregs), basic EngTregs (Basic Tregs) and Mock engineered CD4 T cells (Mock). (B) Heat map of average frequency (n=3) of indicated tissue Treg markers determined by flow cytometric analysis. Statistics by One-way ANOVA.

3. Results: Tissue EngTregs show enhanced response to inflammatory cytokines and express repair mediating factors



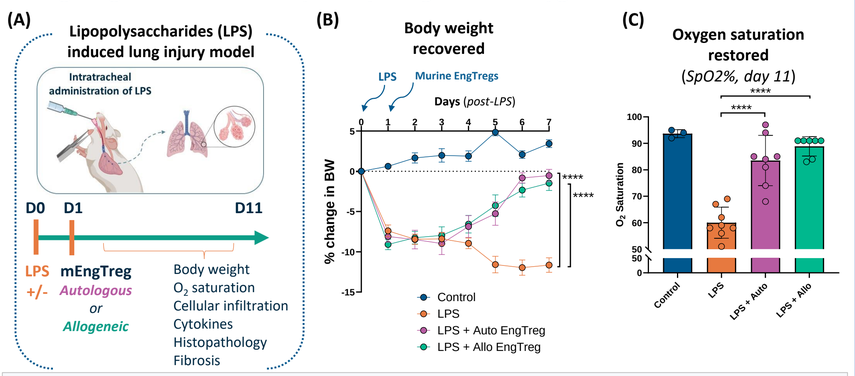
Tissue EngTregs show enhanced response to acute inflammatory cytokines. (A) Higher clearance of IL-33 and IL-4 by Tissue EngTregs in co-culture experiment as detected by ELISA. (B) Higher expression of IL-13 and Osteopontin (OPN) by Tissue EngTregs vs Basic EngTregs co-cultured with IL-33 (ELISA). (C) Higher expression of TNFR2 by Tissue EngTregs at baseline is associated with higher sequestration of TNF-α in co-culture assays (ELISA). (D) TNF-α co-culture of Tissue EngTregs results in greater relative upregulation of CD25 and high IL-2 uptake by Tissue EngTregs. Statistics by One-way ANOVA or unpaired T test except for CD25 MFI analysis which is by two-way ANOVA.

4. Methods: Murine polyclonal Engineered Treg (mEngTreg) surrogate process and cell characterization



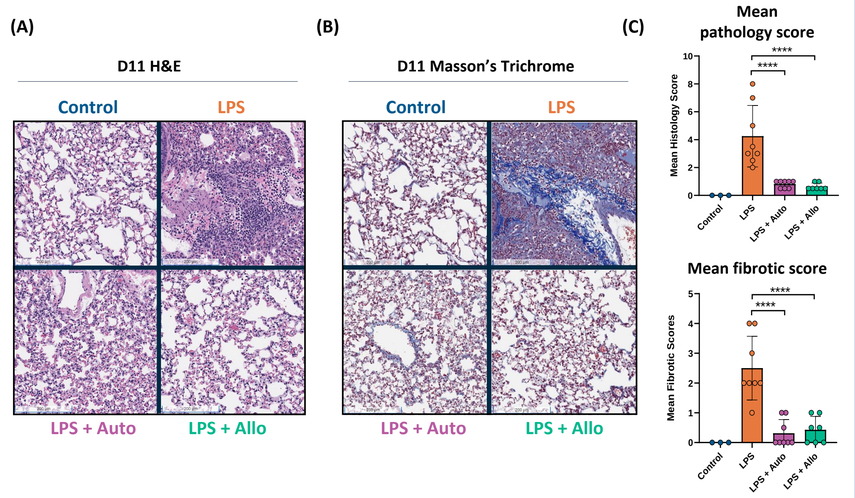
mEngTreg surrogate production process. (A) Protocol outline detailing stimulation and gene editing timelines prior to magnetic enrichment using LNGFR surface protein tag edited into the *Foxp3* gene locus. (B) Day 7 mEngTregs co-express Foxp3 and LNGFR. (C) Autologous (C57BL/6 derived) and allogeneic (CB6F1 derived) mEngTregs also express high levels of key Treg effector markers (e.g. CD25), homing receptors (e.g. CCR4) and tissue Treg markers (e.g. ST2) compared to unstimulated conventional T cells.

5. Results: Reduced ALI disease severity in autologous and allogeneic mEngTreg treated mice by bodyweight and oxygen saturation



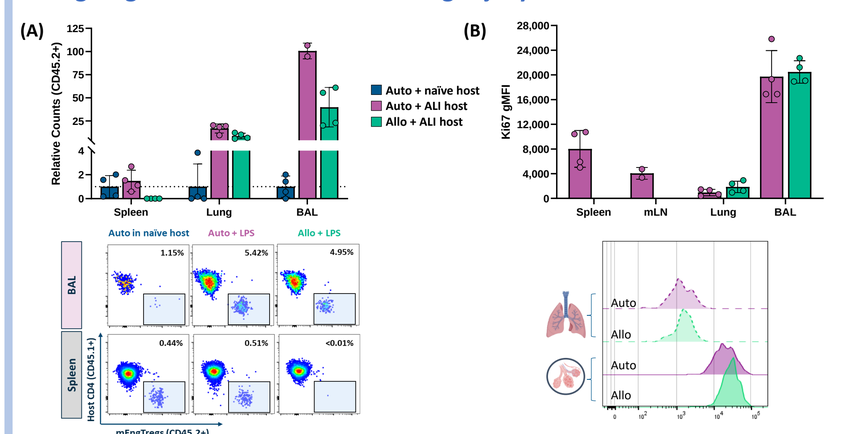
Reduced disease severity in mEngTreg treated mice. (A) LPS-induced acute lung injury (ALI) model for investigating mEngTreg efficacy *in vivo*. Significant improvements in body weight recovery (A) and O_2 saturation (B) of autologous and allogeneic mEngTreg treated animals compared to LPS controls. n=9-10 mice in ALI groups. Statistics by two-way repeated measures ANOVA (Day 11 bodyweight statistics reported) and unpaired T test (O_2 Day 11).

6. Results: Reduced disease severity and fibrosis based on histopathology in mEngTreg treated ALI mice



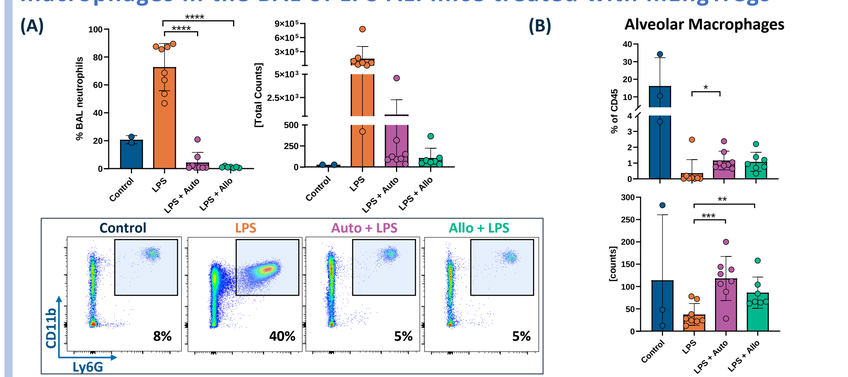
Amelioration of LPS induced ALI in autologous and allogeneic mEngTreg treated mice. (A) Fewer infiltrating leukocytes observed in H&E stained lung sections at Day 11 from LPS treated mice receiving allogeneic or autologous mEngTregs. (B) Reduced collagen deposition in mEngTreg treated ALI animals as measured by Masson's Trichrome staining at Day 11. (C) Graphs of combined pathology and fibrotic histopathology scores. Statistics by One-way ANOVA.

7. Results: Inflammation tuned homing and persistence of allogeneic mEngTregs in LPS induced acute lung injury



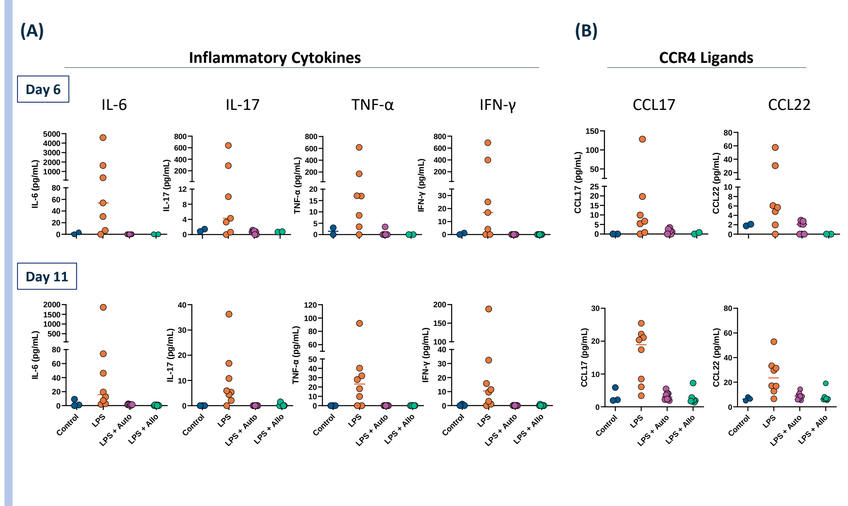
Specific homing and persistence of allogeneic mEngTregs to site of tissue damage. (A) Increased counts (top) and representative flow cytometry plots (bottom) of mEngTregs in the BAL and lungs but not spleen of LPS ALI mice (D5) compared to naïve mice receiving autologous mEngTregs. (B) Allogeneic and autologous mEngTregs show equivalent proliferative capacity at the site of tissue injury (BAL and lung) based on flow cytometric analysis of Ki67 expression.

8. Results: Reduced neutrophil infiltrates and restored alveolar macrophages in the BAL of LPS ALI mice treated with mEngTregs



Decreased immune infiltrates and restoration of alveolar macrophage compartment in the BAL of mEngTreg treated LPS ALI mice. (A) Decreased neutrophil frequency and counts at Day 11 in the BAL of both allogeneic and autologous mEngTreg treated animals. (B) Normalized frequency and counts of pro repair alveolar macrophages in the late phase (D11) of acute lung injury in mEngTreg treated ALI animals. Statistics by unpaired T test.

9. Results: Lower concentration of pro-inflammatory cytokines and chemokines detected in the BAL of mEngTreg treated mice



Decreased pro-inflammatory cytokines and chemokines as measured by CBA in the BAL of mEngTreg treated animals. (A) Lower levels of key inflammatory cytokines IL-6, TNF-α, IL-17, and IFN-γ detected in the BAL of mEngTreg treated animals on days 6 and 11 following i.t. LPS. (B) Decreased levels of CCR4 ligands, CCL17 and CCL22 in the BAL of autologous and allogeneic mEngTreg treated ALI mice compared to LPS only controls.

CONCLUSIONS

- GentiBio's Engineered Treg platform overcomes scaling and stability limitations of Treg therapeutics by starting with more abundant T cell sources and enriching FOXP3+ edited cells with an engineered IL-2 signaling receptor.
- Human Tissue EngTregs express higher levels of activation, tolerogenic and tissue homing receptors and are better able to sequester inflammatory cytokines while expressing repair cytokines *in vitro*.
- In preclinical studies, equivalent efficacy and improved disease outcome observed in LPS induced acute lung injury mice treated with allogeneic or autologous mEngTregs based on body weight and pulse oximetry measurements.
- Improved histopathology in the lungs of mEngTreg treated ALI animals as measured by H&E and Masson's Trichrome staining reflecting reduced tissue damage and potential protection from fibrotic disease, respectively.
- High frequency of allogeneic mEngTregs detected during the inflammatory phase of disease and at the site of inflammation while lower persistence is observed in distal sites with lower inflammation during ALI.
- Fewer inflammatory infiltrates in the lung and BAL of mEngTreg treated mice, with normalized alveolar macrophage counts, and reduced BAL inflammatory cytokines suggesting a return towards pulmonary immune homeostasis.
- These data lend support to the use of allogeneic CD4 derived Engineered Tregs as a powerful off-the-shelf therapeutic approach for acute onset inflammatory and ischemic diseases including ARDS.

ACKNOWLEDGEMENT: The laboratory of Dr. David Rawlings at Seattle Children's Hospital pioneered the gene editing approach to produce engineered Tregs. The research groups of Dr. Jason Mock and Dr. Heth Turnquist contributed scientific discussions regarding models of acute lung injury.

# Denotes equal contribution.

*, **, ***, **** = p-value < 0.01, 0.005, 0.001 and 0.0001 respectively.

We make Tregs. *Better.*