Adoptive Transfer of Murine Engineered T Regulatory Cells Ameliorates Disease in a Model of Lipopolysaccharide Induced Acute Lung Injury

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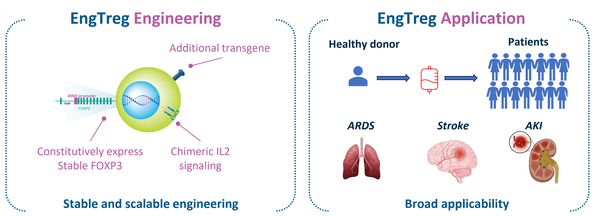
INTRODUCTION & PREMISE OF GENTI PLATFORM

Introduction

- Acute Respiratory Distress Syndrome (ARDS) is a life-threatening condition characterized by diffuse alveolar tissue damage, pulmonary edema, severe hypoxemia, increased inflammatory cytokine response, and immune cell infiltration.
- Despite medical advancements, treatment of ARDS primarily relies on managing symptoms, stabilizing disease progression, and relying on the patient's intrinsic healing processes.
- A major driver of ARDS is the widespread dysregulation of the pulmonary immune response.
- FOXP3+ T regulatory cells play a critical role in lung homeostasis and tolerance, controlling inflammation and promoting tissue repair during resolution of acute inflammatory conditions such as ARDS.
- GentiBio's platform allows for the generation of stable, tissue targeted, selectively activated T regulatory cells from PBMC isolated bulk CD4+ T cells, overcoming the major limitations of current Treg therapeutic approaches.
- The production of a murine surrogate engineered T regulatory cell allows for the evaluation of the GENTI platform in murine preclinical models.
- Intratracheal instillation of lipopolysaccharides (LPS) triggers acute lung injury (ALI) in mice, recapitulating many of the symptoms experienced in human ARDS.
- It is the purpose of the current work to assess the ability of murine engineered T regulatory cells to ameliorate disease in a mouse model of LPS induced acute lung injury.

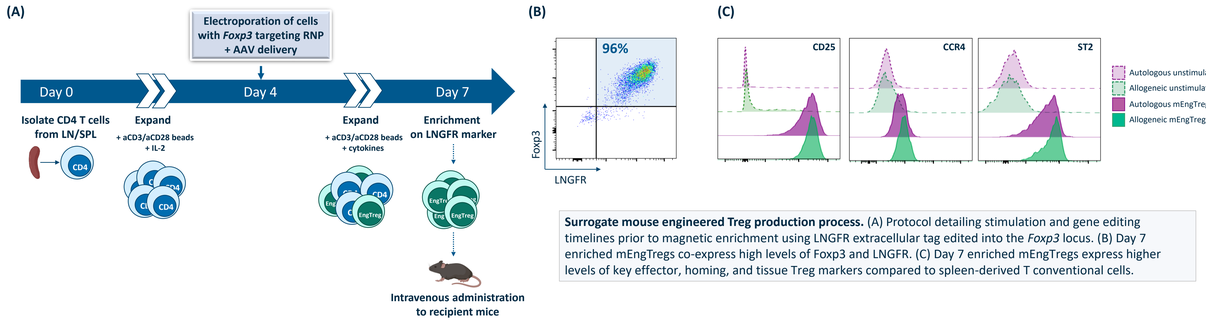
Premise



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, enable effective tissue homing and mediation of tissue Treg capabilities. Scalable manufacturing of an allogeneic cell product from healthy donors would enable an off-the-shelf treatment for a broad range of acute inflammatory and ischemic diseases including Acute Respiratory Distress Syndrome (ARDS).

MURINE SURROGATE SYSTEM

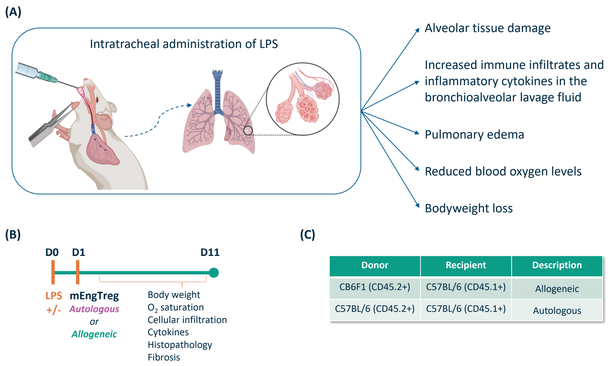
Murine surrogate engineered Treg production



Surrogate mouse engineered Treg production process. (A) Protocol detailing stimulation and gene editing timelines prior to magnetic enrichment using LNGFR extracellular tag edited into the *Foxp3* locus. (B) Day 7 enriched mEngTregs co-express high levels of Foxp3 and LNGFR. (C) Day 7 enriched mEngTregs express higher levels of key effector, homing, and tissue Treg markers compared to spleen-derived T conventional cells.

METHODS

Overview of LPS induced acute lung injury approach and study design

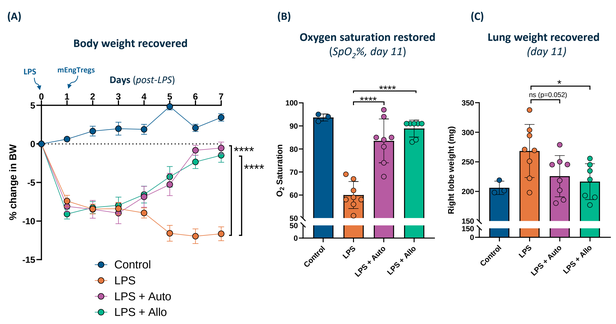


Donor	Recipient	Description
CB6F1 (CD45.2+)	C57BL/6 (CD45.1+)	Allogeneic
C57BL/6 (CD45.2+)	C57BL/6 (CD45.1+)	Autologous

Key Read-outs
✓ Bodyweight change and pulse oximetry
✓ Hematoxylin and eosin (H&E) and Masson's Trichrome stain of lung
✓ Lung weight
✓ Engraftment of mEngTregs in spleen, lung, and bronchoalveolar fluid (BAL)
✓ Proliferation of mEngTregs in spleen, lung, and BAL
✓ Neutrophil infiltration and alveolar macrophage population in lung and BAL
✓ Inflammatory cytokine response in BAL

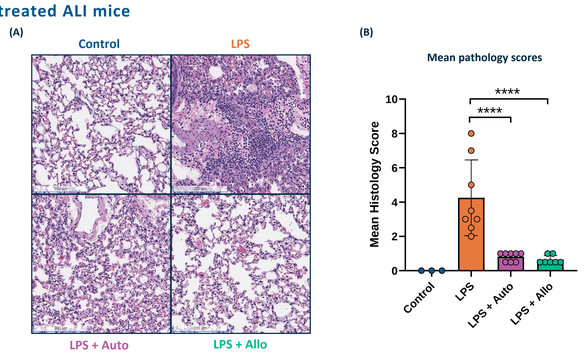
LPS induced ALI introduction and study design. (A) Direct pulmonary insult with LPS by intratracheal (i.t.) instillation triggers acute lung injury (ALI) in mice, recapitulating many pathophysiological conditions visible in the progression of human ARDS. (B) Timeline of study design and disease progression. Adoptive transfer of mEngTregs occurred 24 hours following i.t. administration of LPS from *E. coli* serotype O55:B5. (C) CB6F1 donors were used in the generation of allogeneic mEngTregs to model non-NK cell rejection of transferred cells. CD45.1 expressing congenic hosts were used as recipients to enable *ex vivo* tracking of CD45.2+ mEngTregs. (D) Graphic schematic of experimental groups and key read-outs.

Figure 1. Reduced disease severity in mEngTreg treated ALI mice



Reduced disease severity in mEngTreg treated mice. Significant improvements in body weight recovery (A), O_2 saturation (B), and lung weight (C) of allogeneic and autologous mEngTreg treated animals compared to LPS controls. n=7-10 mice in ALI groups. Statistics by two-way repeated measures ANOVA (Day 11 bodyweight statistics reported) and unpaired T test (O_2 and lung weights at Day 11).

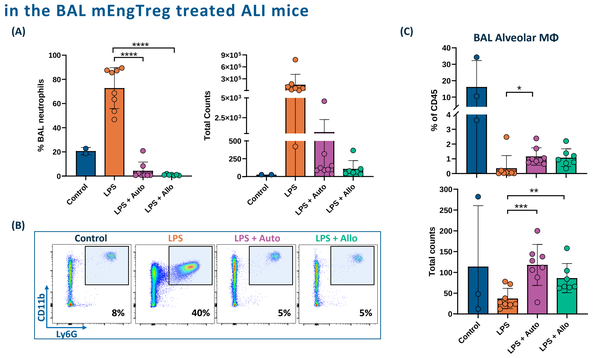
Figure 2. Reduced disease severity based on histopathology in mEngTreg treated ALI mice



Amelioration of LPS induced lung damage in mEngTreg treated mice. (A) Fewer infiltrating leukocytes and improved tissue integrity observed by H&E in the lungs of allogeneic and autologous mEngTreg treated ALI animals at Day 11. (B) Graph of mean histopathology scores across groups. Statistics by one-way ANOVA.

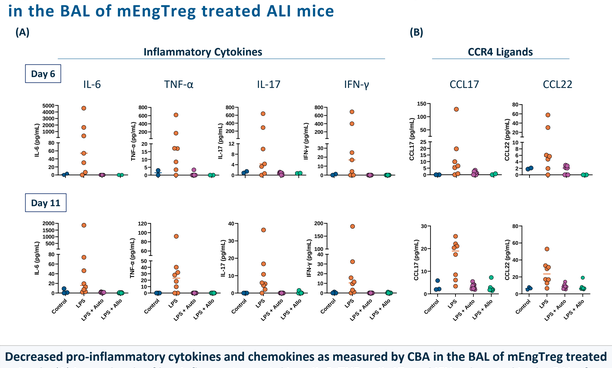
RESULTS

Figure 3. Reduced neutrophil infiltrates and restored alveolar macrophages in the BAL mEngTreg treated ALI mice



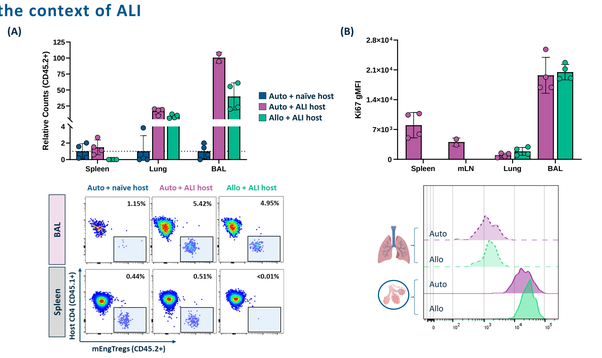
Decreased immune infiltrates and restoration of alveolar macrophage compartment in the BAL of mEngTreg treated ALI animals. Reduced neutrophil frequency and counts at Day 11 in the BAL (A) and lung (B) of allogeneic and autologous mEngTreg treated animals. (C) Normalized frequency and counts of pro repair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

Figure 4. Lower concentration of pro-inflammatory cytokines and chemokines in the BAL of mEngTreg treated ALI mice



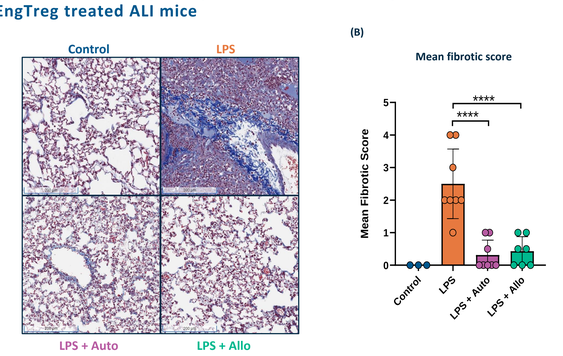
Decreased pro-inflammatory cytokines and chemokines as measured by CBA in the BAL of mEngTreg treated animals. (A) Lower levels of key inflammatory cytokines IL-6, TNF-α, IL-17, and IFN-γ detected in the BAL of allogeneic and autologous mEngTreg treated animals on days 6 and 11 following LPS-induced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

Figure 5. Inflammation tuned homing and persistence of mEngTregs in the context of ALI



Specific homing to, and persistence of, mEngTregs at site of injury. (A) Increased autologous and allogeneic mEngTreg engraftment in the BAL and lungs, but not spleen, of ALI animals (D5) compared to naïve hosts. (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.

Figure 6. Reduced collagen deposition as measured by Masson's Trichrome in mEngTreg treated ALI mice



Reduced fibrosis in mEngTreg treated ALI animals. (A) Reduced collagen deposition in mEngTreg treated ALI animals as measured by Masson's Trichrome staining at Day 11. (B) Graph of mean fibrotic scores across treatment groups. Statistics by one-way ANOVA.

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.**
- **mEngTreg surrogate cells express key markers of pulmonary thymic Tregs including Foxp3, CD25, CCR4 and ST2.**
- **In preclinical studies of acute lung injury in mice, equivalent efficacy is observed by bodyweight, pulse oximetry measurements, and pulmonary edema following allogeneic or autologous mEngTreg treatment.**
- **Improved histopathology in the lungs of mEngTreg treated ALI animals as measured by H&E.**
- **Fewer inflammatory infiltrates in the lung and BAL of mEngTreg treated mice, with normalized alveolar macrophage counts, and reduced BAL inflammatory cytokines suggests a return towards pulmonary immune homeostasis.**
- **High frequency of mEngTregs are detected during the inflammatory phase of disease at the site of inflammation, while lower persistence is observed at distal sites with lower inflammation during ALI.**
- **Reduction of collagen deposition in the lungs of mEngTreg treated ALI animals suggests downstream protection from late-stage fibrotic disease.**
- **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.**

ACKNOWLEDGEMENTS

- 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 to the process of setting up models of acute lung injury.

DISCLOSURES

- All authors employed by GentiBio.

# Denotes equal contribution

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