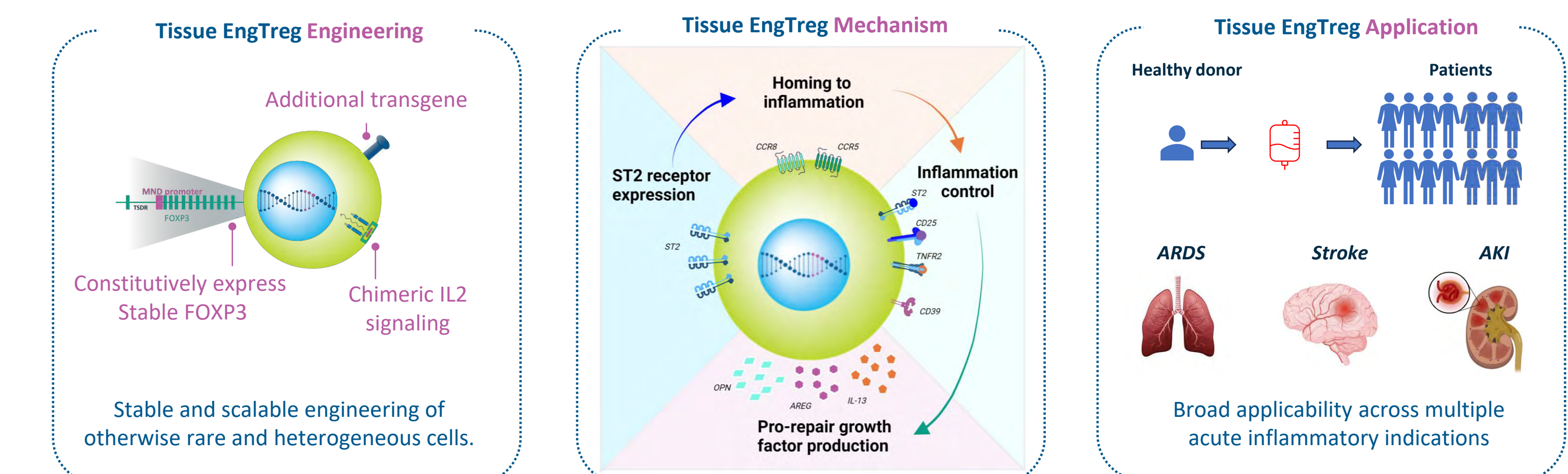


A Tissue Engineered Treg Approach for the Treatment of Acute Ischemic and Inflammatory Diseases

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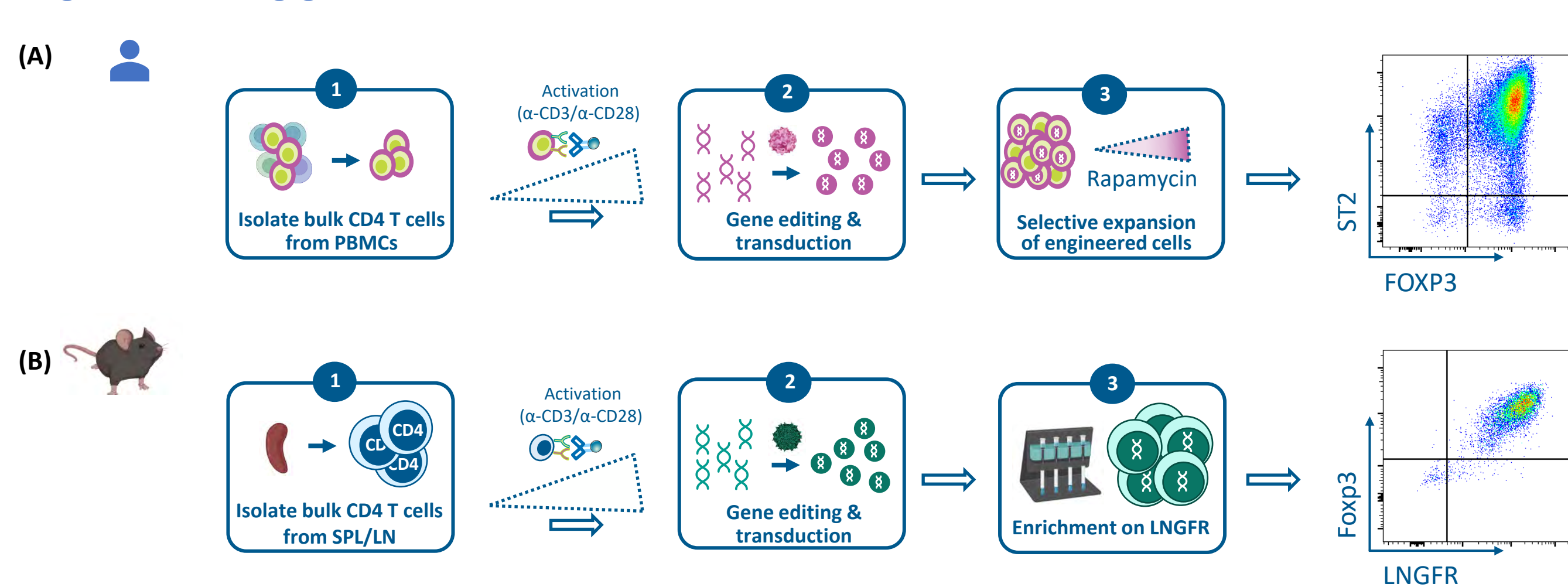
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Premise



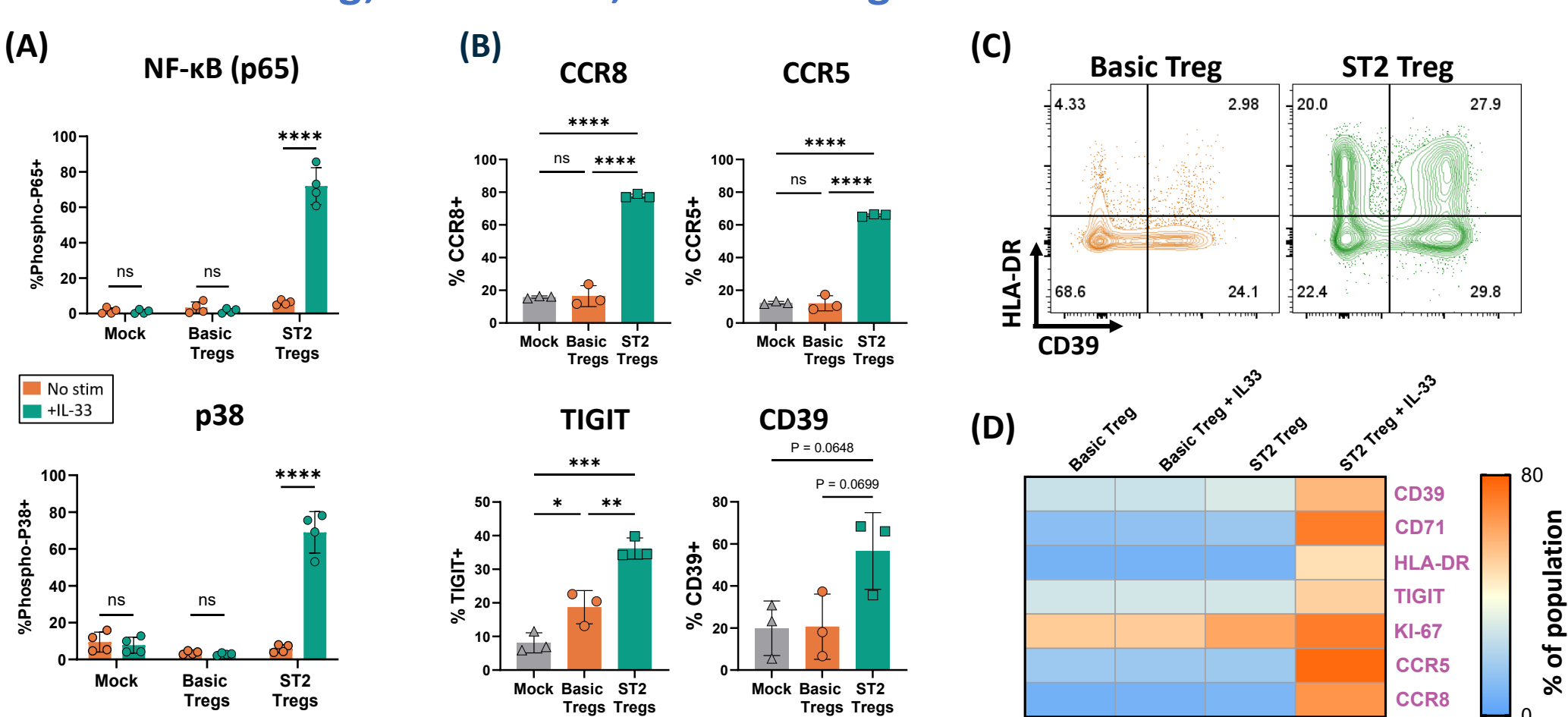
Overview of human Engineered Tissue Tregs as a therapeutic approach for acute inflammatory and ischemic diseases. Gene editing of PBMC isolated CD4⁺ T cells leads to the expression of stable FOXP3 and a rapamycin-activated signaling complex (CISC) that provides tunable IL-2 signal, effectively divorcing 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). Expression of further transgenes, such as the IL-33 receptor ST2, enable effective tissue homing and enhanced expression of programs associated with activation, function, and repair in response to inflammatory signals. 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 often associated with poor prognosis.

Engineered Treg generation



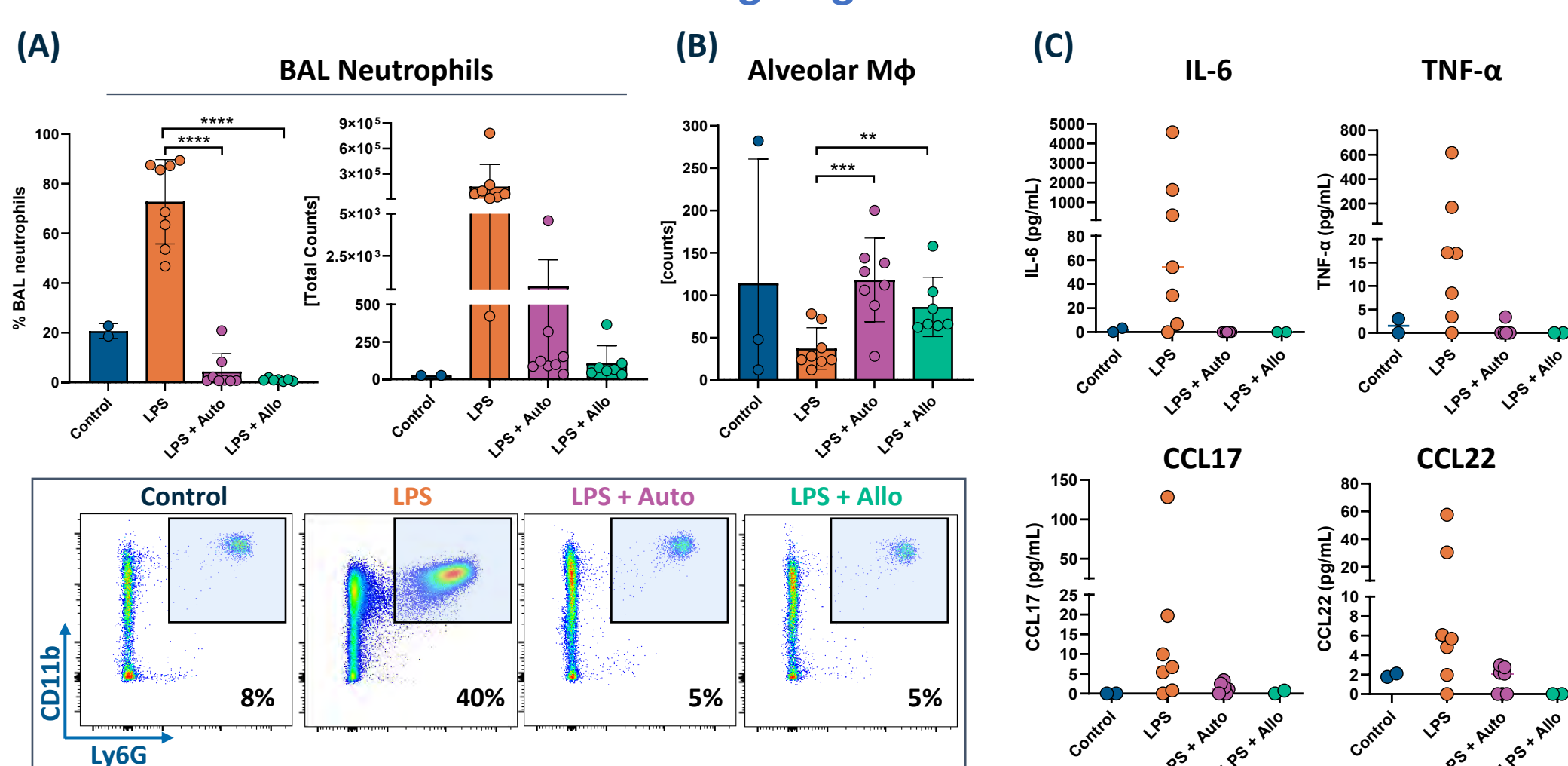
Engineered Treg production processes. (A) For human Engineered Tregs (EngTregs), CD4⁺ T cells were isolated via magnetic enrichment from PBMCs and activated via α -CD3/ α -CD28 microbead stimulation. Cells were then genetically modified using lentiviral transduction and/or CRISPR-Cas9 mediated knock-in of transgenes delivered by AAV vectors. CISC receptor expression enables selective expansion of the intended, edited cell population. (B) For murine surrogate Engineered Tregs (mEngTregs), CD4⁺ T cells were isolated from the spleens and inguinal lymph nodes of 8-12-week-old male mice before activation by α -CD3/ α -CD28 microbeads. Activated cells were genetically modified by CRISPR-Cas9 mediated knock-in of transgenes delivered by AAV vectors into the *Foxp3* locus. Following an additional round of activation/expansion, mEngTregs were enriched via the extracellular LNGFR tag.

1. Results: Tissue EngTregs signal through ST2 and express higher levels of homing, activation, and tolerogenic markers



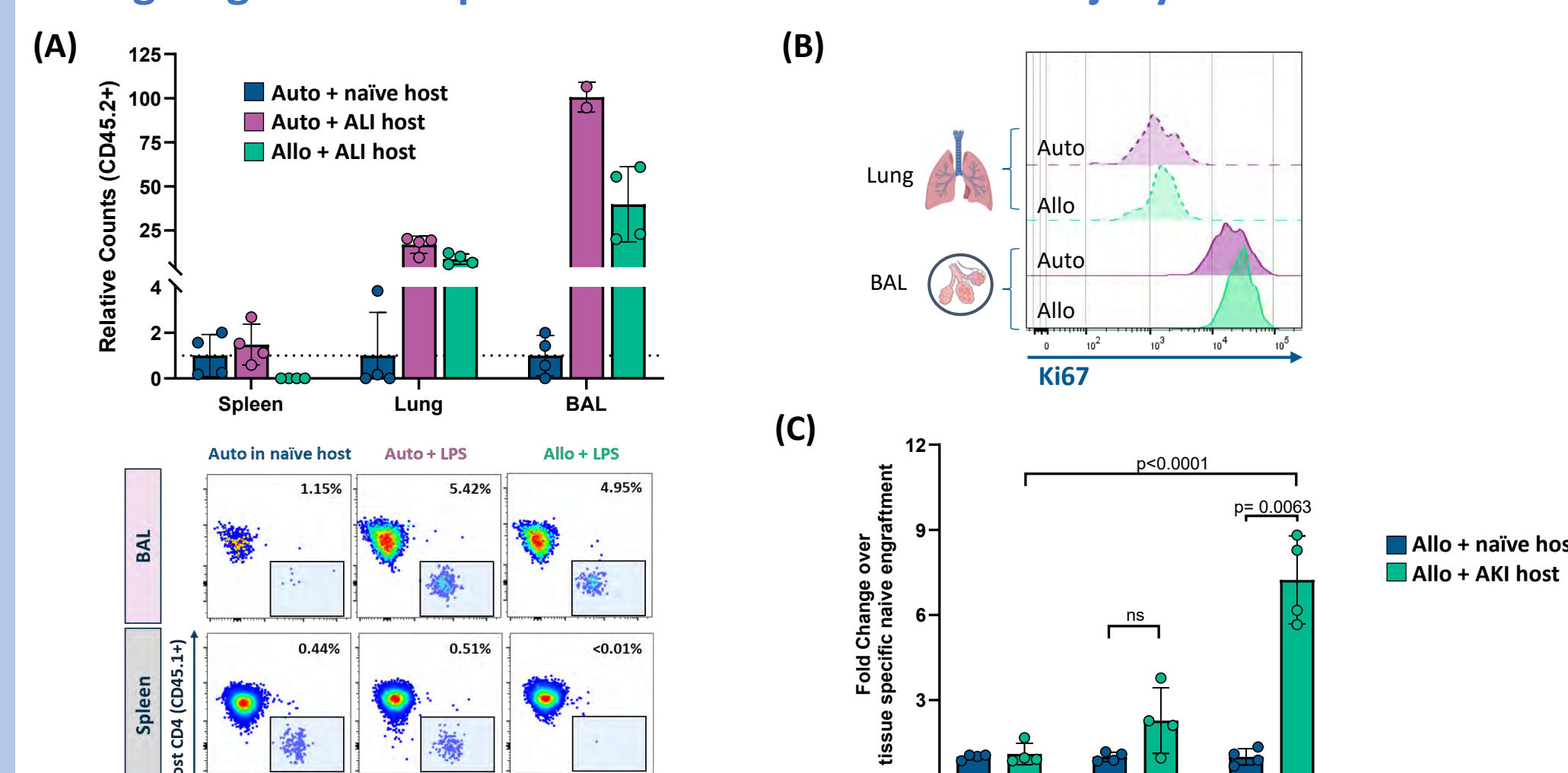
Tissue EngTregs signal downstream of ST2 in response to IL-33 provision, driving a pro-repair phenotype. (A) Phospho-flow assay to measure p38 and p65 signaling downstream of the ST2 receptor with (green bars) or without (orange bars) 50ng/ml r-IL-33 in culture. Statistics by two-way ANOVA. (B)(C) Flow 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). Statistics by One-way ANOVA. (D) Heat map of average frequency (n=3) of indicated tissue Treg markers determined by flow cytometric analysis.

4. Results: Reduced inflammatory infiltrates and cytokines in the BAL of LPS ALI mice treated with mEngTregs



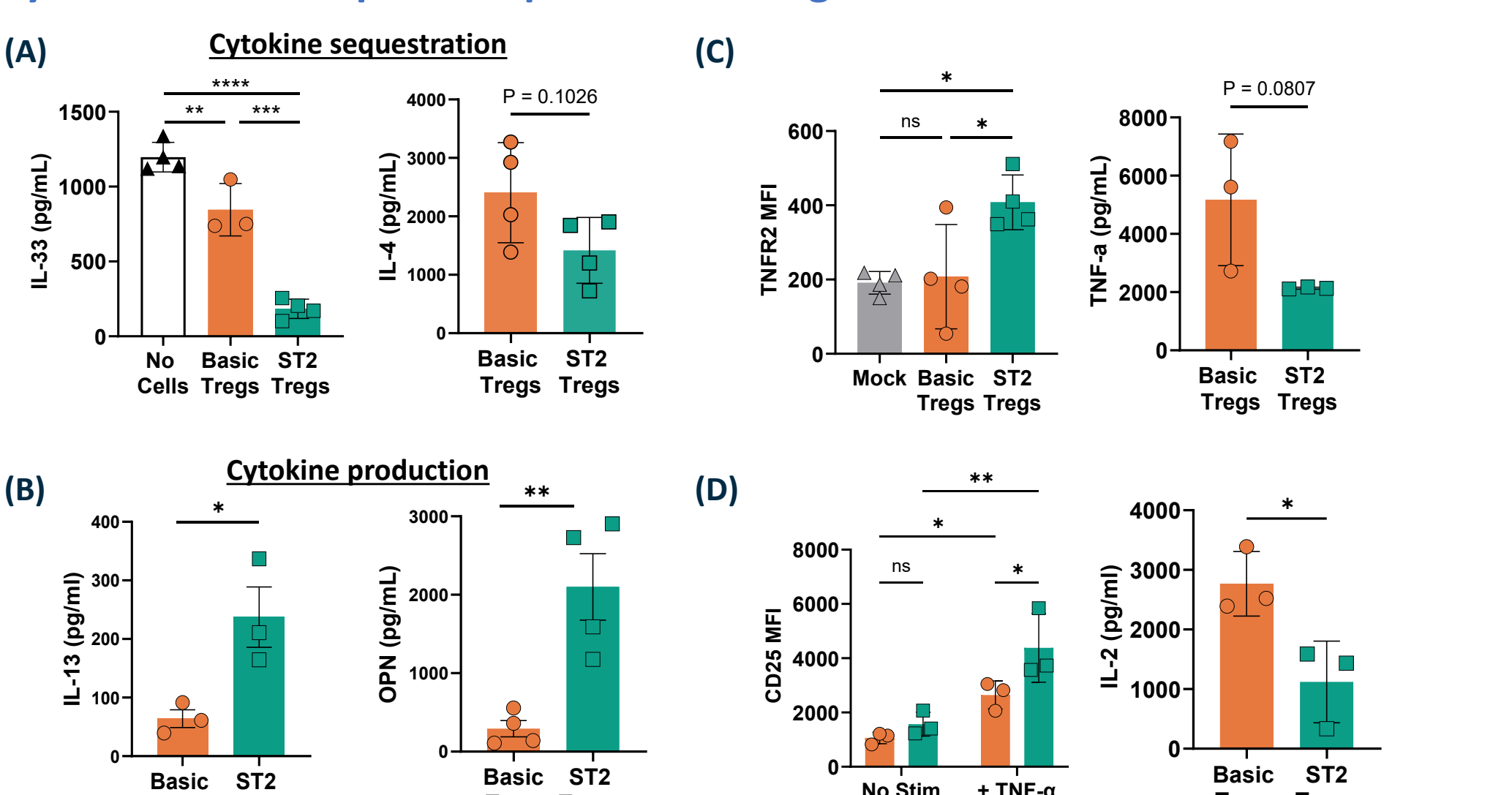
Decreased immune infiltrates, restoration of alveolar macrophage compartment, and reduction of key pro-inflammatory cytokines and chemokines in the bronchoalveolar lavage (BAL) of mEngTreg treated ALI mice. Decreased neutrophil infiltration (A) and normalization of pro-repair alveolar macrophages (B) at Day 11 in the BAL of autologous and allogeneic mEngTreg treated animals. Statistics by unpaired T test. (C) Lower levels of inflammatory cytokines (top) and CCR4 ligands (bottom) detected in the BAL of mEngTreg treated ALI animals on Day 6.

7. Results: Inflammation tuned homing and persistence of allogeneic mEngTregs in multiple models of acute tissue injury



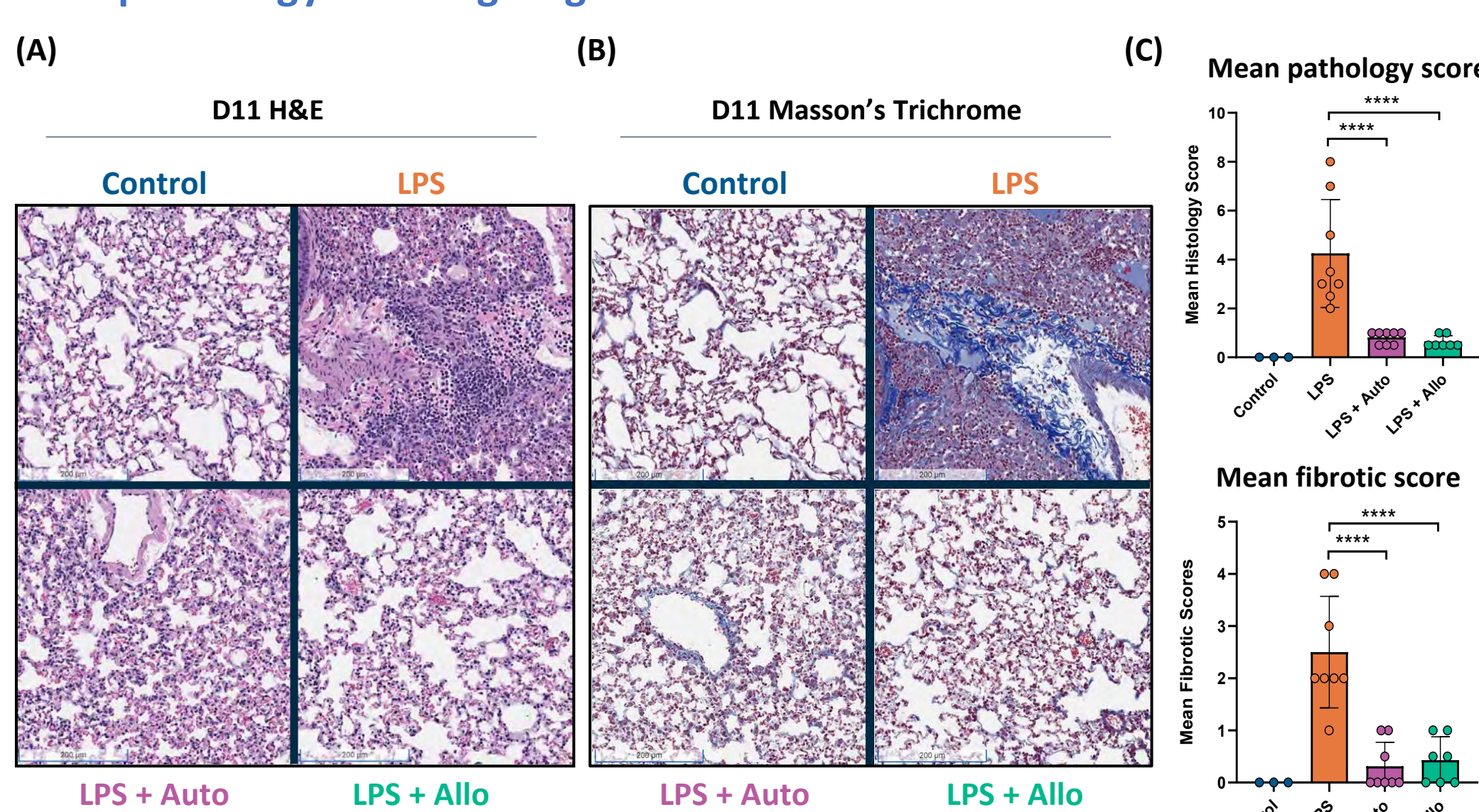
Specific homing and persistence of allogeneic mEngTregs to site of tissue damage. (A) Increased counts (top) and representative flow cytometry plots (bottom) of mEngTregs in the BAL and lungs, but not spleen, of ALI mice at Day 5 compared to naive hosts receiving autologous mEngTregs. (B) mEngTregs are proliferating at the site of tissue injury. (C) Preferential kidney engraftment of mEngTregs in the context of cisplatin-induced acute kidney injury.

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



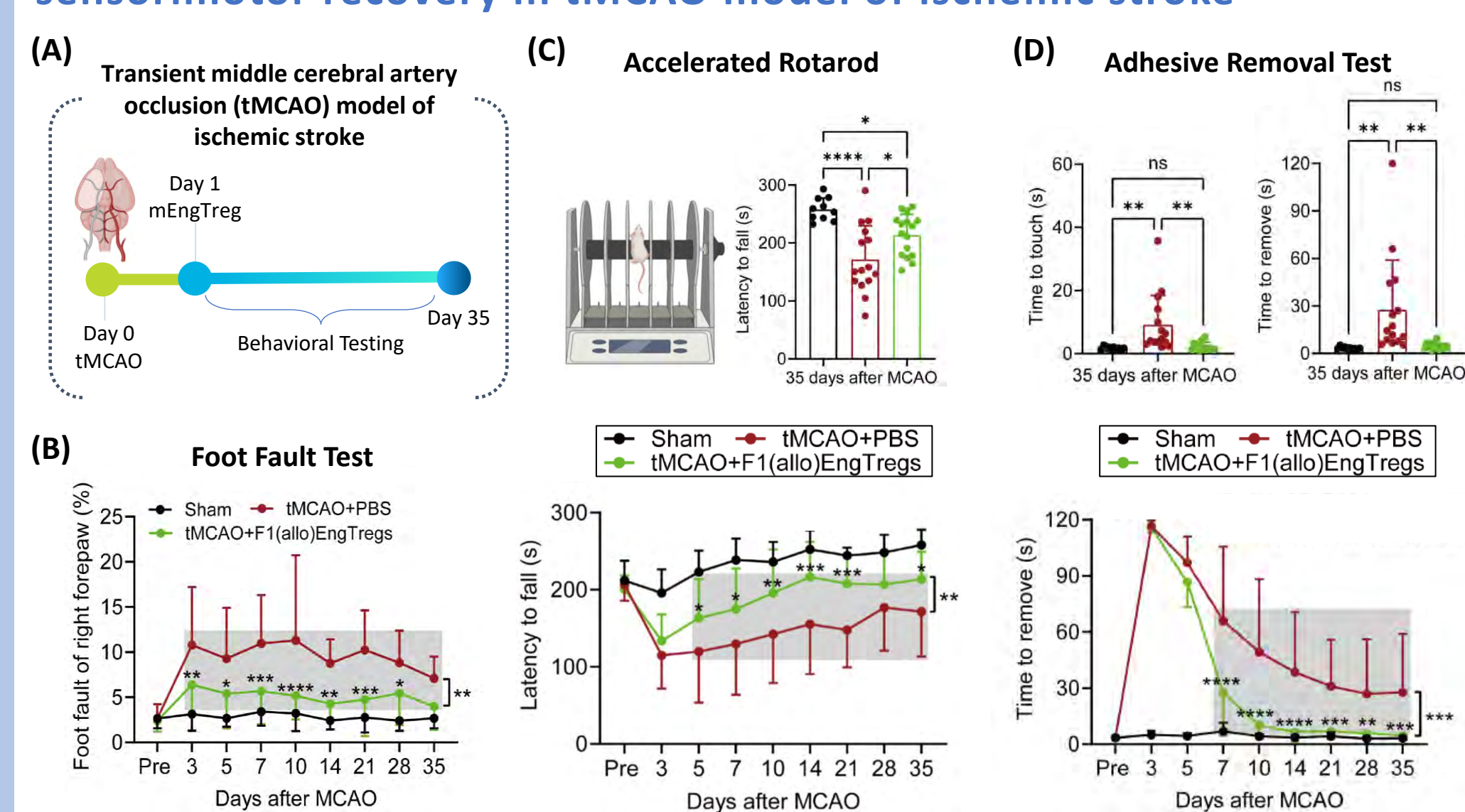
Tissue EngTregs show enhanced response to acute inflammatory cytokines. (A) Higher clearance of IL-33 and IL-4 by Tissue EngTregs in co-culture experiment as detected by ELISA. (B) Higher expression of IL-13 and Osteopontin (OPN) by Tissue EngTregs vs Basic EngTregs co-cultured with IL-33 (ELISA). (C) 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.

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



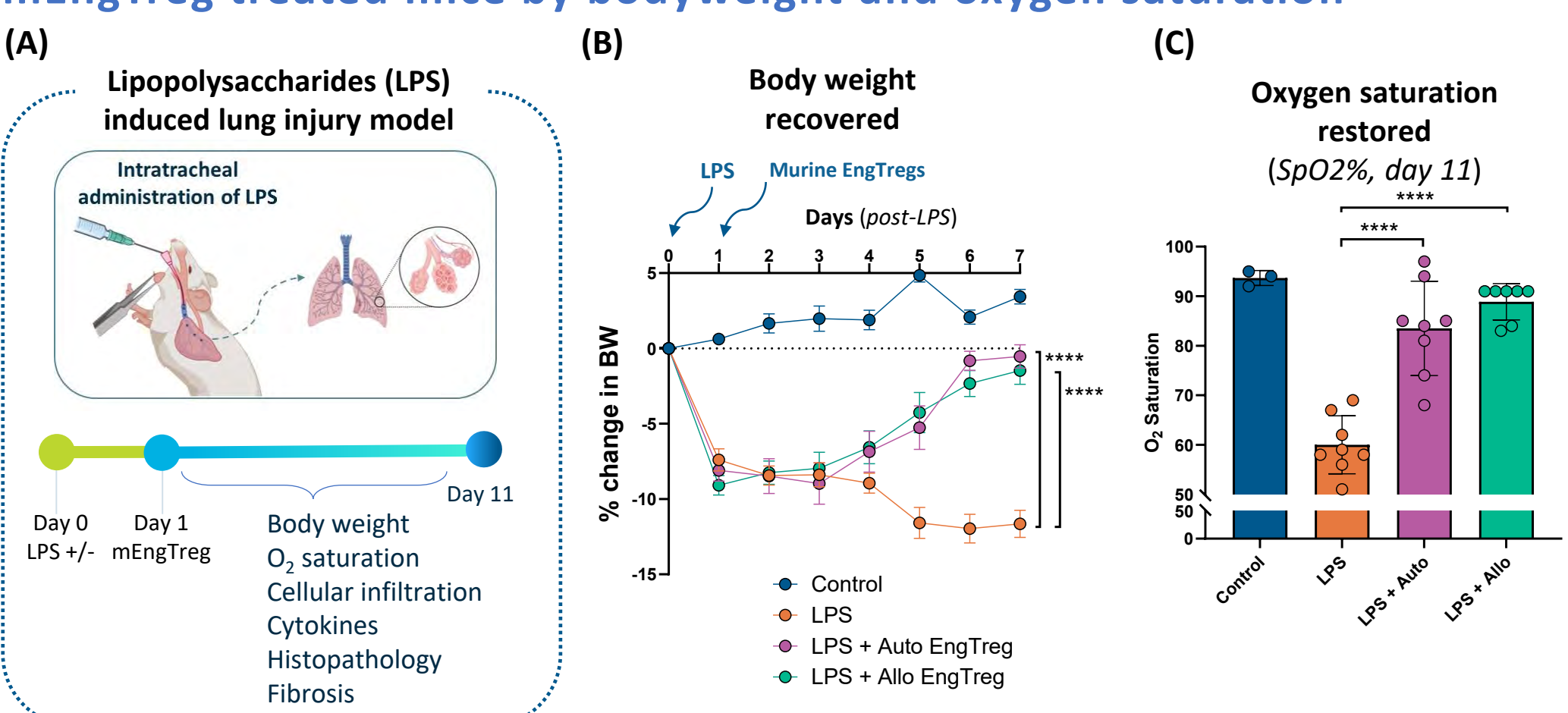
Amelioration of LPS induced ALI in autologous and allogeneic mEngTreg treated mice. (A) Fewer infiltrating leukocytes observed in H&E stained lung sections at Day 11 from LPS treated mice receiving autologous or allogeneic mEngTregs. (B) Reduced collagen deposition in mEngTreg treated ALI animals as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

8. Results: Allogeneic mEngTreg treatment significantly improves sensorimotor recovery in tMCAO model of ischemic stroke



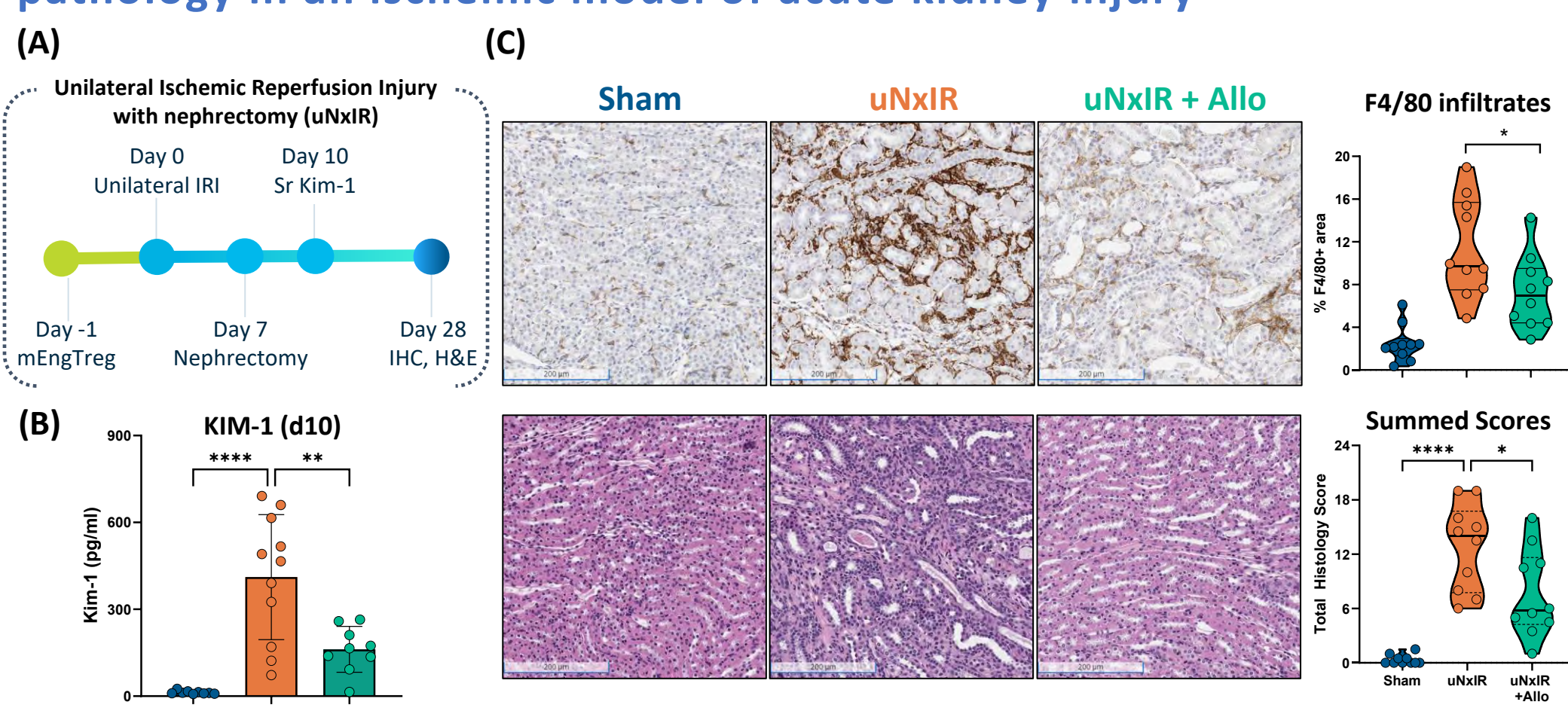
Improved sensorimotor readouts in allogeneic mEngTreg treated tMCAO animals. (A) tMCAO model of ischemic stroke, with mEngTreg dosing at + Sh. (B - D) Sensorimotor readouts from D-1 to D35: (B) Reduced foot fault frequency in tMCAO mice treated with allo mEngTregs. (C) Increased latency to fall in the rotarod test for tMCAO mice treated with allo mEngTregs. (D) Increased sensitivity to somatosensory neglect and motor impairment in tMCAO mice treated with mEngTregs based on removal time of tape applied to forepaws contralateral to the injury.

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



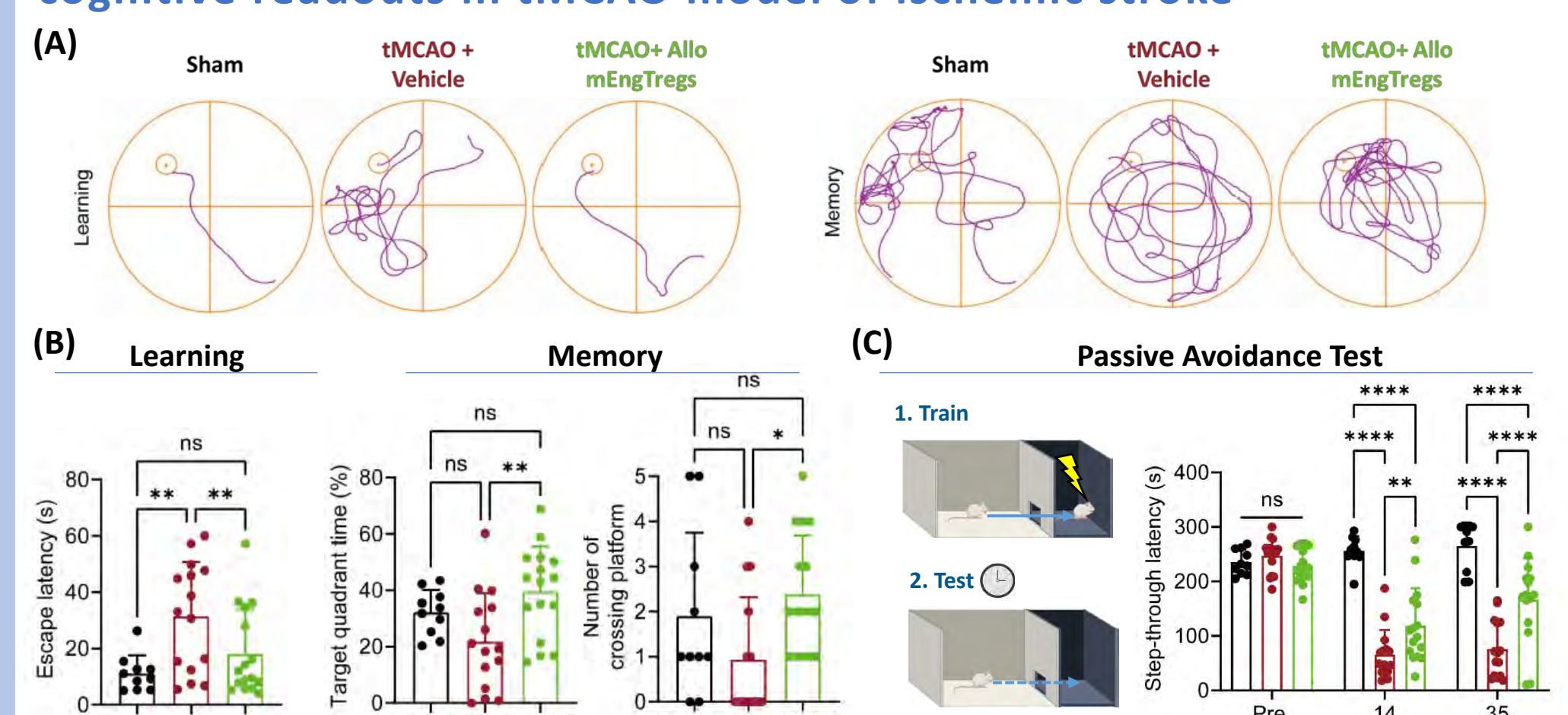
Reduced ALI disease severity in mEngTreg treated mice. (A) LPS-induced acute lung injury (ALI) model for investigating mEngTreg efficacy *in vivo*. Significant improvements in body weight recovery (A) and O₂ saturation (B) of autologous and allogeneic mEngTreg treated animals compared to LPS controls. n=9-10 mice in ALI groups. Statistics by two-way repeated measures ANOVA (Day 11 bodyweight statistics reported) and unpaired T test (O₂ Day 11).

6. Results: Allogeneic mEngTreg treatment significantly reduces kidney pathology in an ischemic model of acute kidney injury



Reduced AKI disease severity in allogeneic mEngTreg treated mice. (A) UIRI model of acute kidney injury (AKI) with Day 7 nephrectomy. (B) Reduction in Kim-1 shedding in the serum of allogeneic mEngTreg treated AKI animals. (C) Improved histopathology in the kidneys of mEngTreg treated AKI animals at Day 28. Significantly reduced F4/80 infiltrates by IHC (top) and improved overall histopathology scores by H&E (bottom). Statistics by One-way ANOVA.

9. Results: Allogeneic mEngTreg treatment significantly improves cognitive readouts in tMCAO model of ischemic stroke



Improved cognitive readouts in allogeneic mEngTreg treated tMCAO animals. (A)(B) Allogeneic mEngTreg treated tMCAO animals display improved spatial cognitive function as measured by the ability to locate and spend time on a hidden platform in a circular water pool (Morris water maze). (C) Improvements in non-spatial learning and memory in allogeneic mEngTreg treated tMCAO animals by measuring avoidance of an aversive stimulus (mild foot shock).

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 of acute lung injury in mice, equivalent efficacy is observed by bodyweight and pulse oximetry measurements following allogeneic or autologous mEngTreg treatment.
- Fewer inflammatory BAL infiltrates and cytokines is associated with improved histopathology in the lungs of mEngTreg treated ALI animals as measured by H&E and Masson's Trichrome staining.
- Reduced severity of disease progression in allogeneic mEngTreg treated mice in an ischemic model of acute kidney injury as measured by serum Kim-1 levels and multiple histopathology readouts.
- In models of both ALI and AKI, there is a high frequency of allogeneic mEngTregs detected during the inflammatory phase of disease at the site of inflammation with lower counts observed in distal sites with lower inflammation.
- Recovery of sensorimotor and cognitive function in allogeneic mEngTreg treated mice undergoing middle cerebral occlusion clamping followed by reperfusion compared to disease controls.
- 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, AKI, and ischemic stroke.

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We make Tregs. Better.

