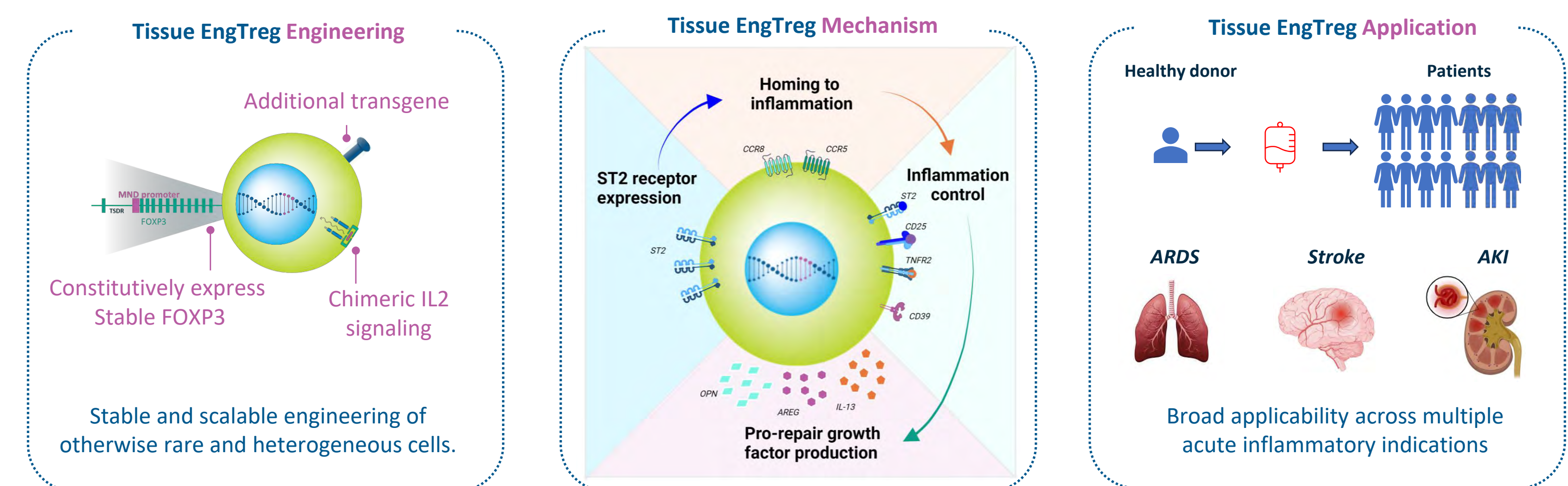


Development of Allogeneic Tissue Engineered Tregs for the Treatment of Ischemic Stroke and Acute Lung Injury

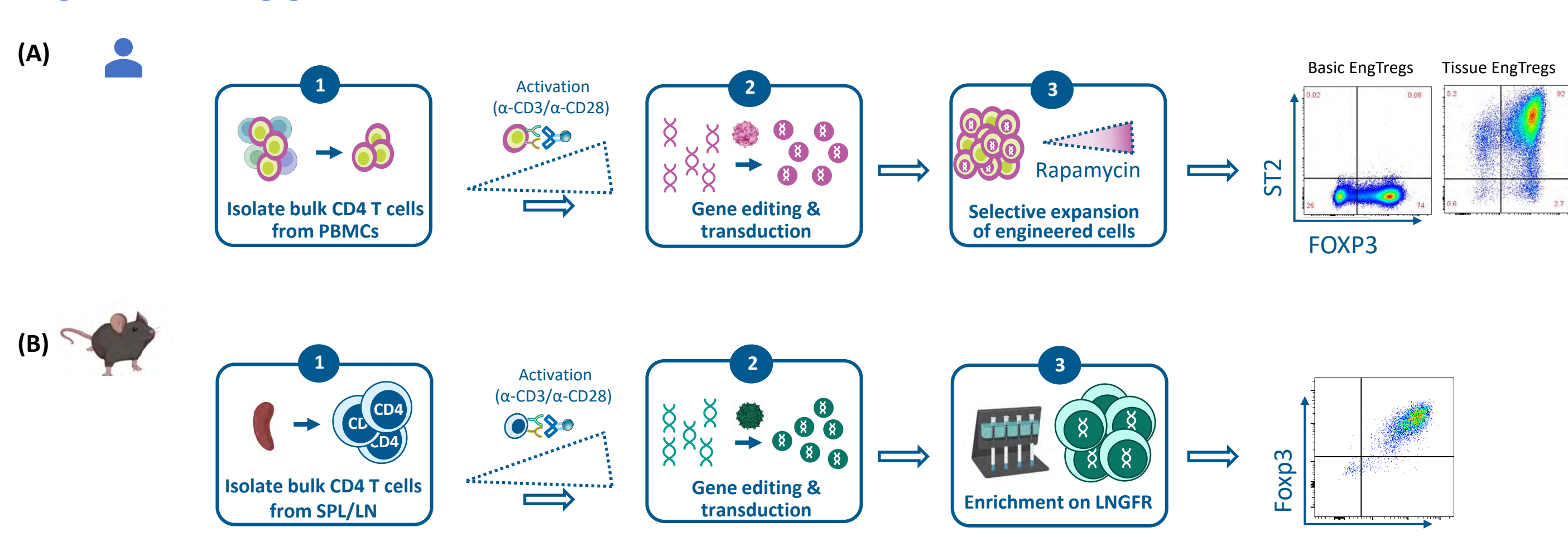
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Premise



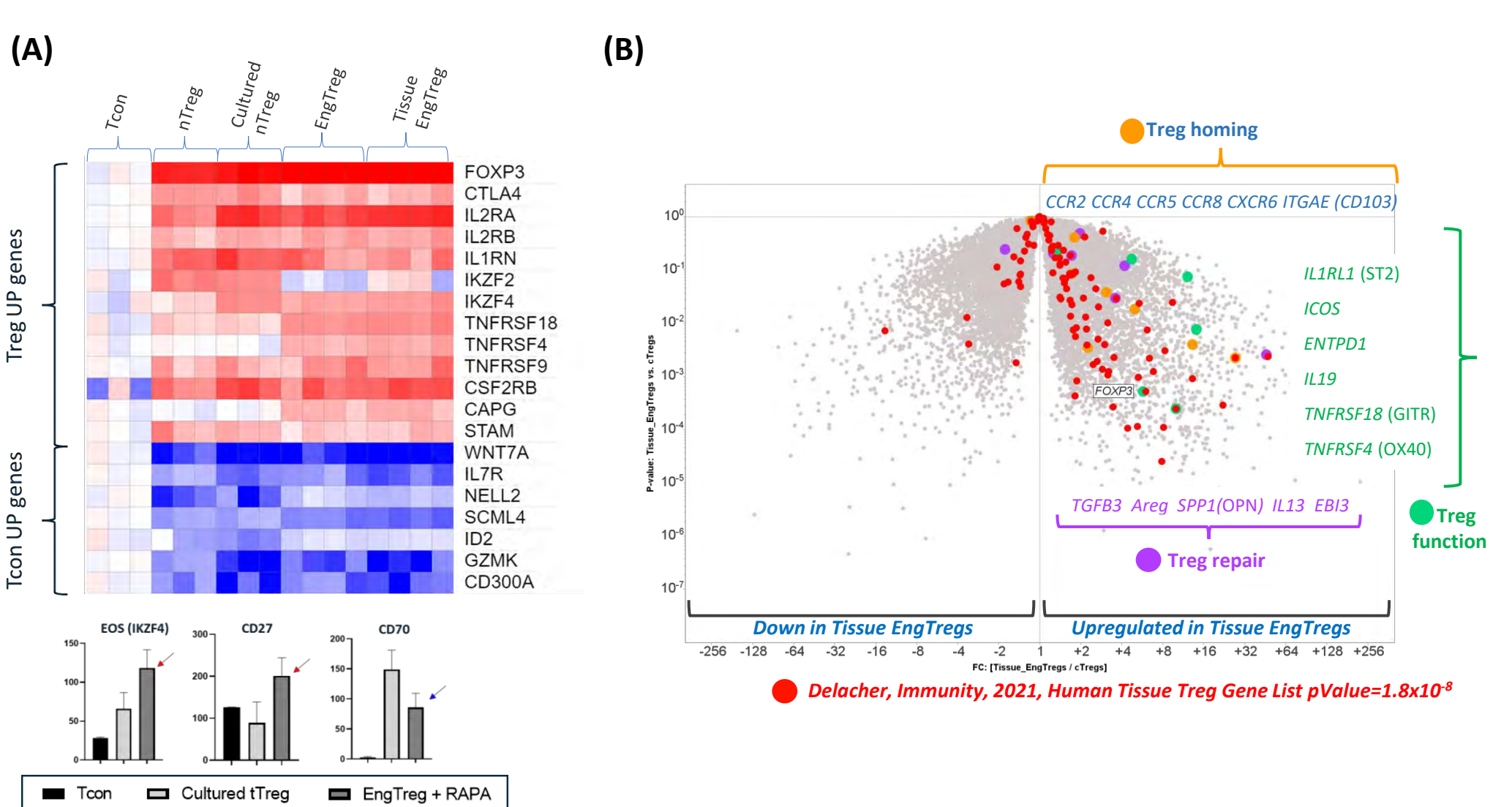
Engineered Treg generation



Human Engineered Tissue Tregs as a therapeutic approach for acute inflammatory and ischemic diseases. Panel 1: Gene edited CD4⁺ T cells express 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). Panel 2: Expression of further transgenes, such as the IL-33 receptor, ST2, enable effective tissue homing and a transcriptional program enriched in activation, function, and repair in response to inflammatory signals. Panel 3: Scalable manufacturing of an allogeneic cell product from healthy donors enables an off-the-shelf treatment for a broad range of acute inflammatory and ischemic diseases often associated with poor prognosis.

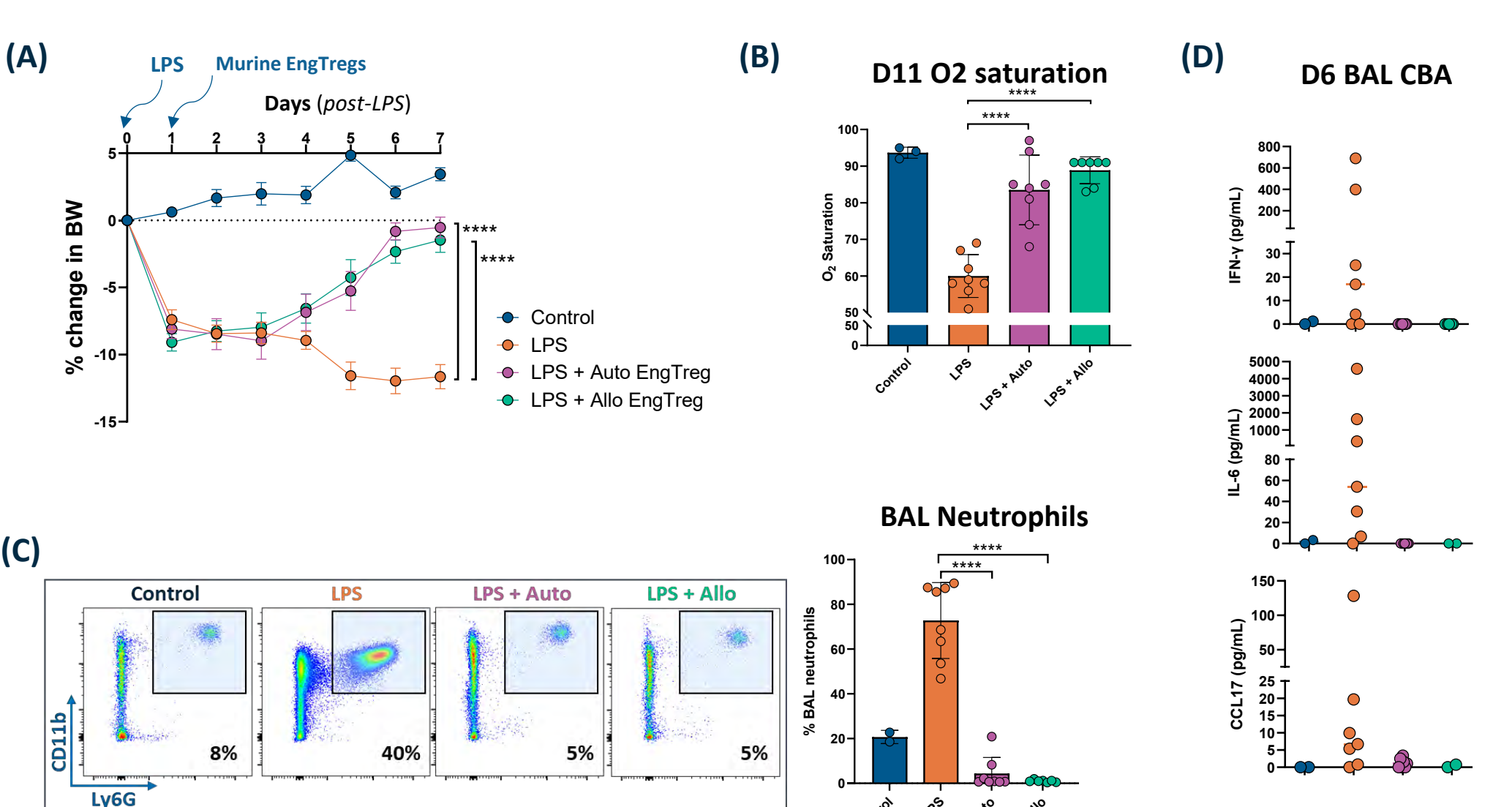
(A) For human Engineered Tregs (EngTregs), CD4⁺ T cells were magnetically isolated from PBMCs (peripheral blood mononuclear cells) and activated via α -CD3/ α -CD28 microbead stimulation. Cells were then engineered using lentiviral transduction and/or CRISPR-Cas9 mediated knock-in of transgenes delivered by AAV vectors. CISC receptor expression enables selective expansion of the 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 LNNGFR tag.

1. Enrichment of Tissue Treg gene signature in Tissue EngTregs



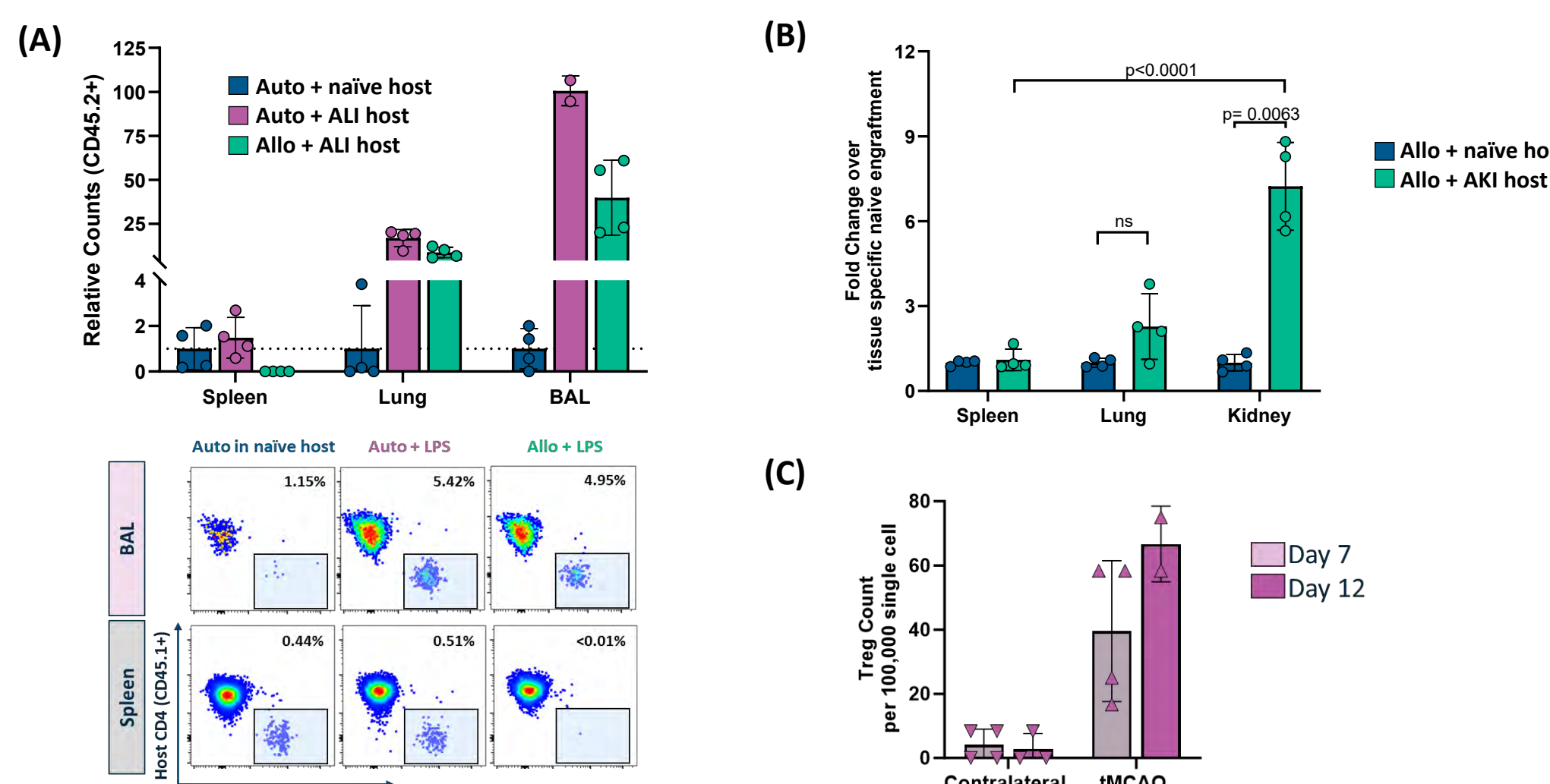
Tissue EngTregs express ST2 and fortifies a Tissue Treg signature (A) Transcriptome profiling of EngTregs show relative expression of core Treg genes compared to Tcon (conventional T cells) fresh PBMC sorted nTregs and cultured nTreg. Gene signatures adapted from Pesenacker *et al*, *Diabetes*, 2016. (B) Volcano plot visualizes bulk RNA transcripts from Tissue EngTregs (ST2+) in comparison to cultured sorted nTregs.

4. Improved disease recovery in LPS ALI mice treated with Allo mEngTregs



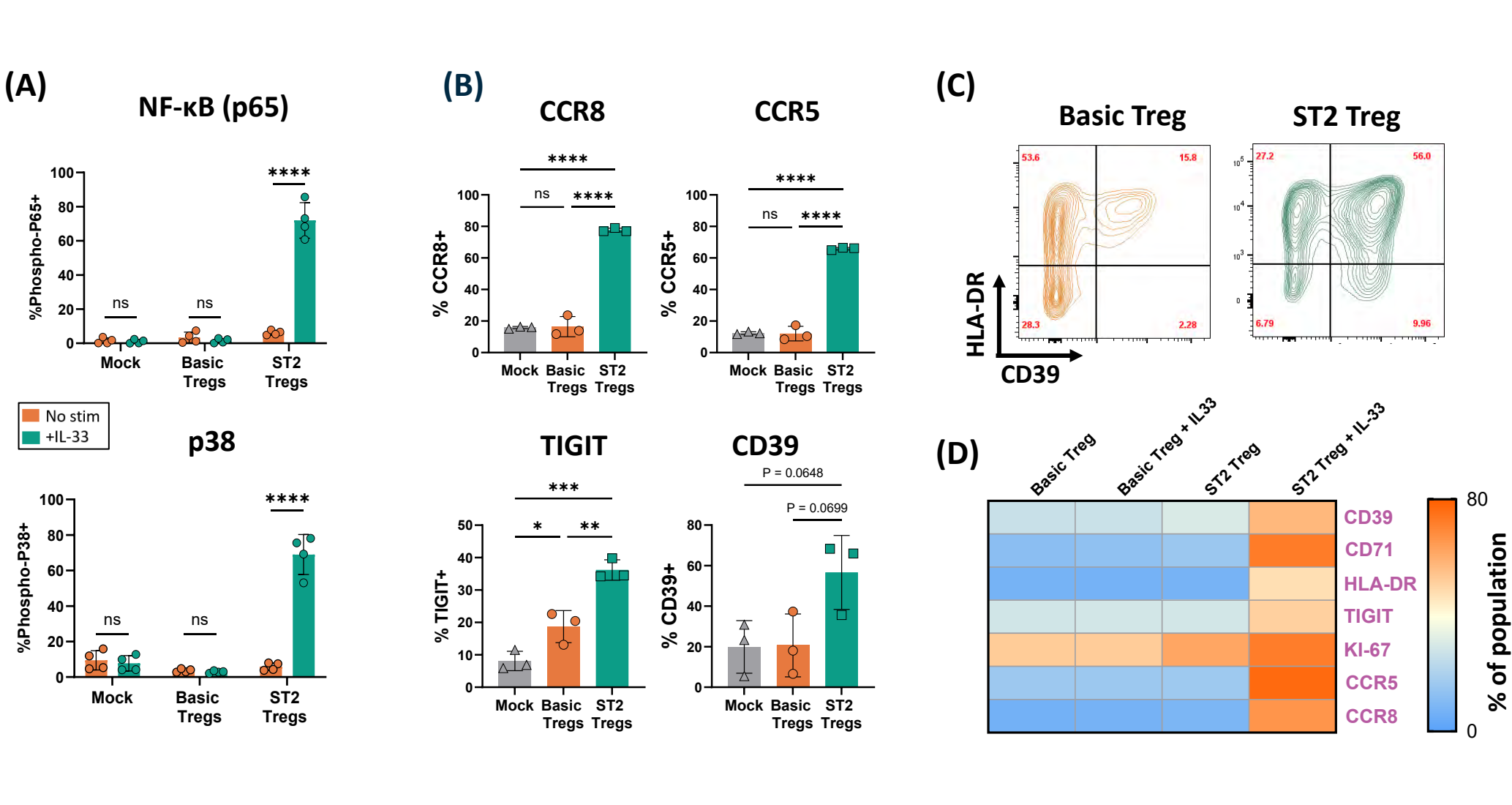
(A) and (B) Significant improvements in body weight recovery and O₂ saturation of autologous and allogeneic mEngTreg treated animals compared to i.t. LPS treated B6 animals. (C) Decreased D11 neutrophil frequency in the BAL. (D) D6 BAL CBA analysis of IL-6, IFN γ and CCL17. Stats: two-way repeated measures ANOVA and unpaired T test (O₂ Day 11).

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



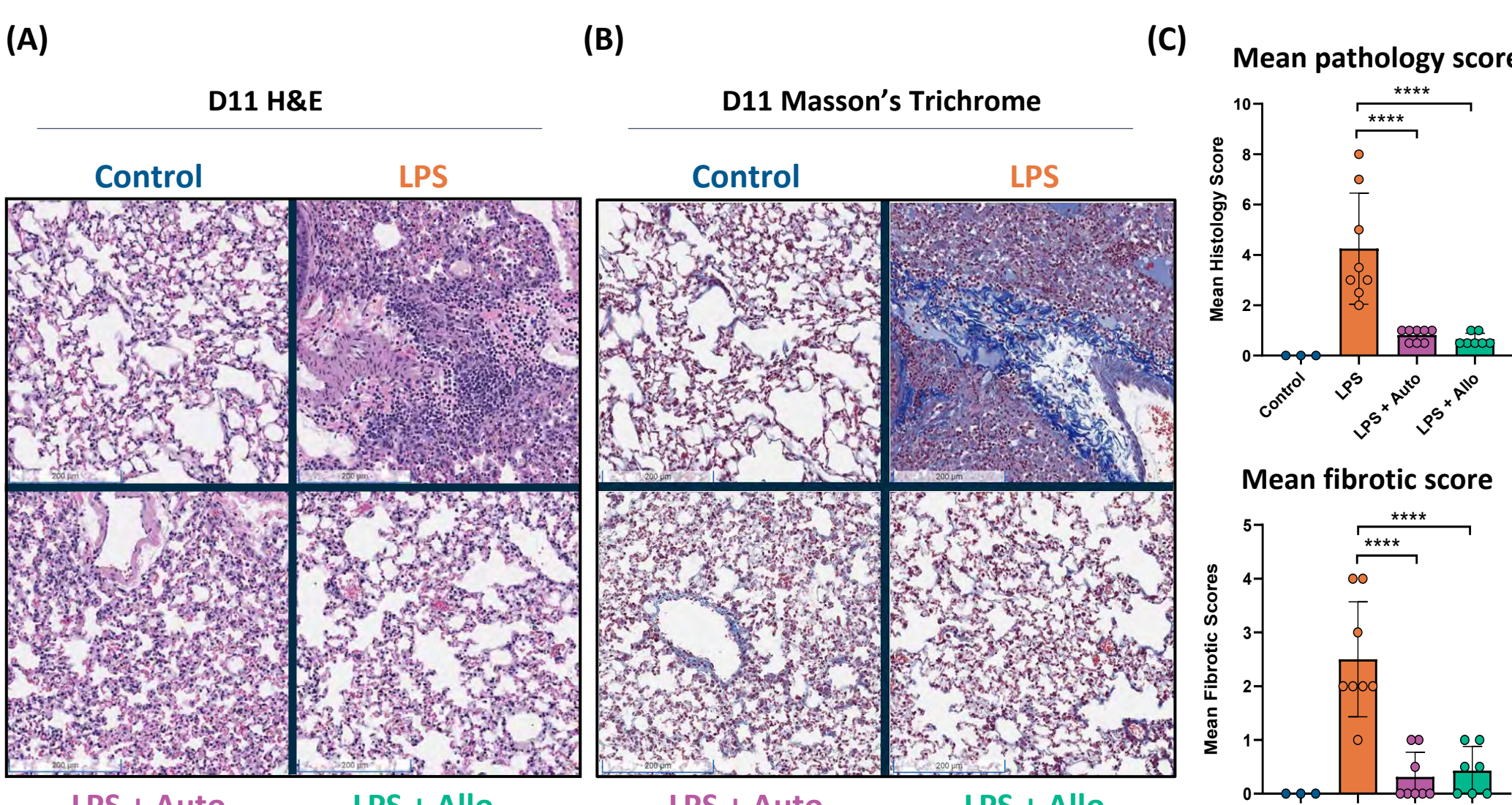
(A) Increased counts (top) and representative flow cytometry plots (bottom) of mEngTregs in the BAL and lungs, but not spleen, of ALI mice at Day 5 compared to naive hosts receiving autologous mEngTregs. (B) Preferential kidney engrftment of mEngTregs in the context of cisplatin-induced acute kidney injury (C) Autologous mEngTregs are detected in the brain of tMCAO animals on both Day 7 and Day 12 following mEngTreg dosing.

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



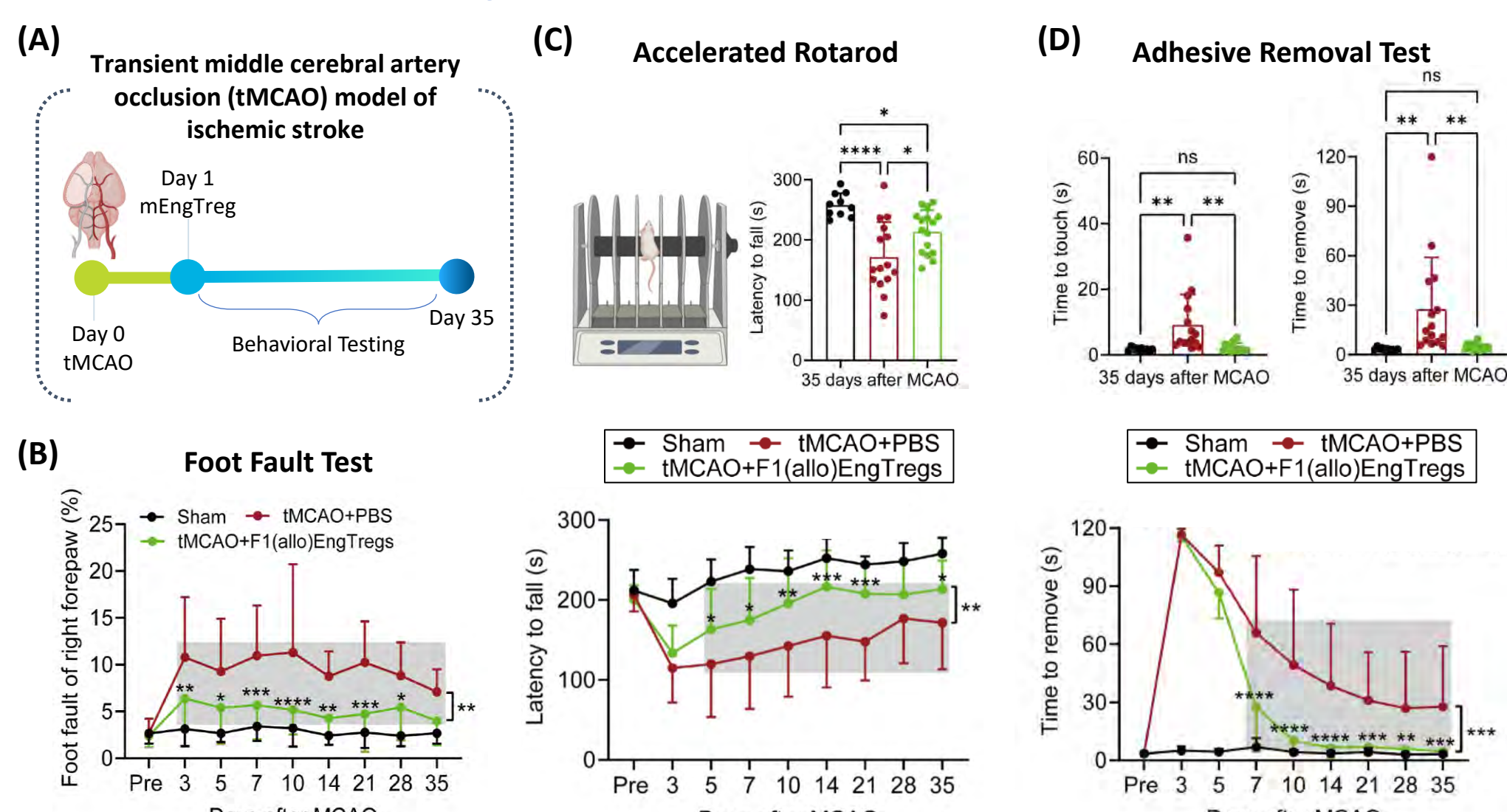
(A) Phos-flow assay to measure P38 and P65 signaling downstream of the ST2 receptor with (green bars) or without (orange bars) 50ng/mL r-IL-33 in culture. Statistics by two-way ANOVA. (B)(C) Flow cytometric analysis of tissue homing, activation and tolerogenic markers comparing Tissue EngTregs (ST2 Tregs), 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.

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



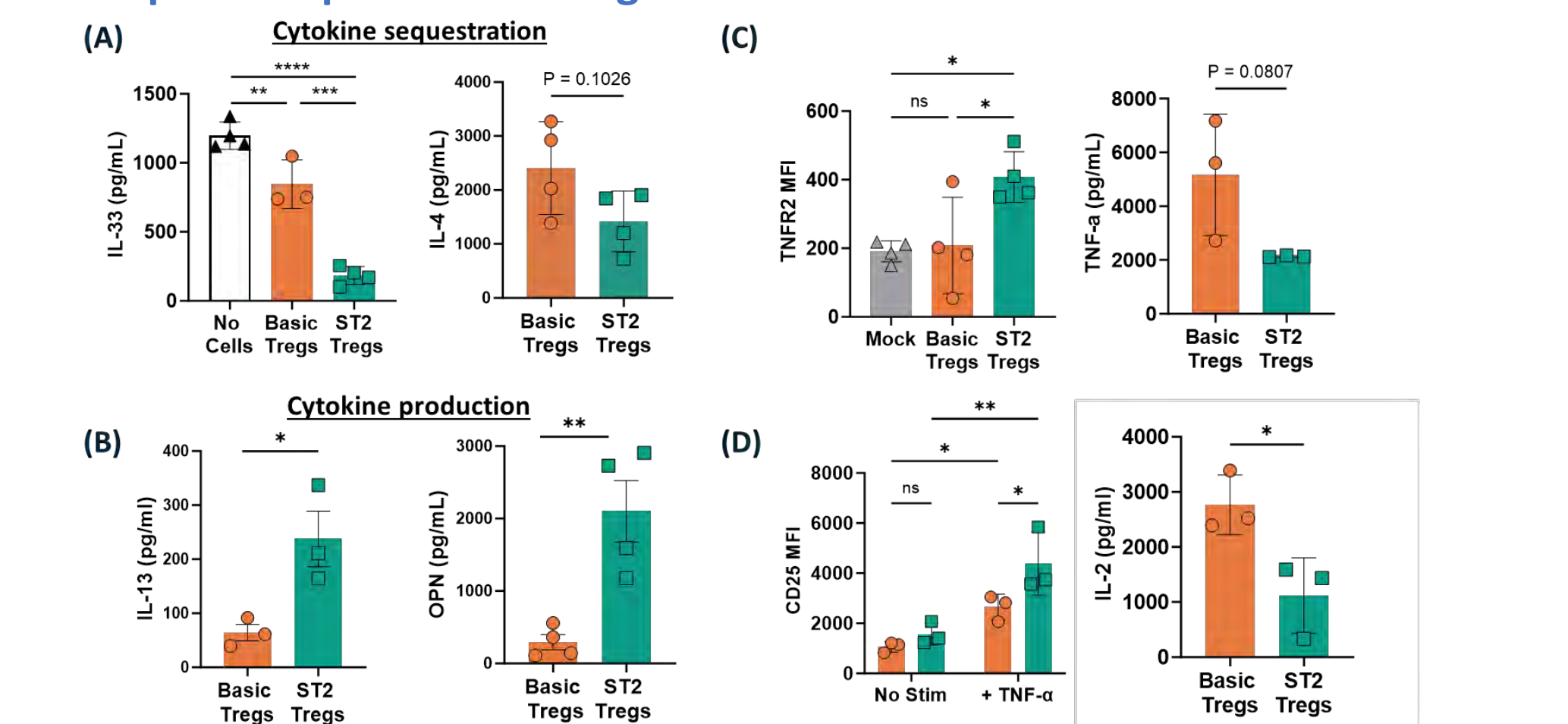
(A) Fewer infiltrating leukocytes observed in H&E stained lung sections at Day 11 from LPS treated mice receiving autologous or allogeneic mEngTregs. (B) Reduced collagen deposition in mEngTreg treated ALI animals as measured by Masson's Trichrome staining at Day 11. (C) Graphs of combined pathology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

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



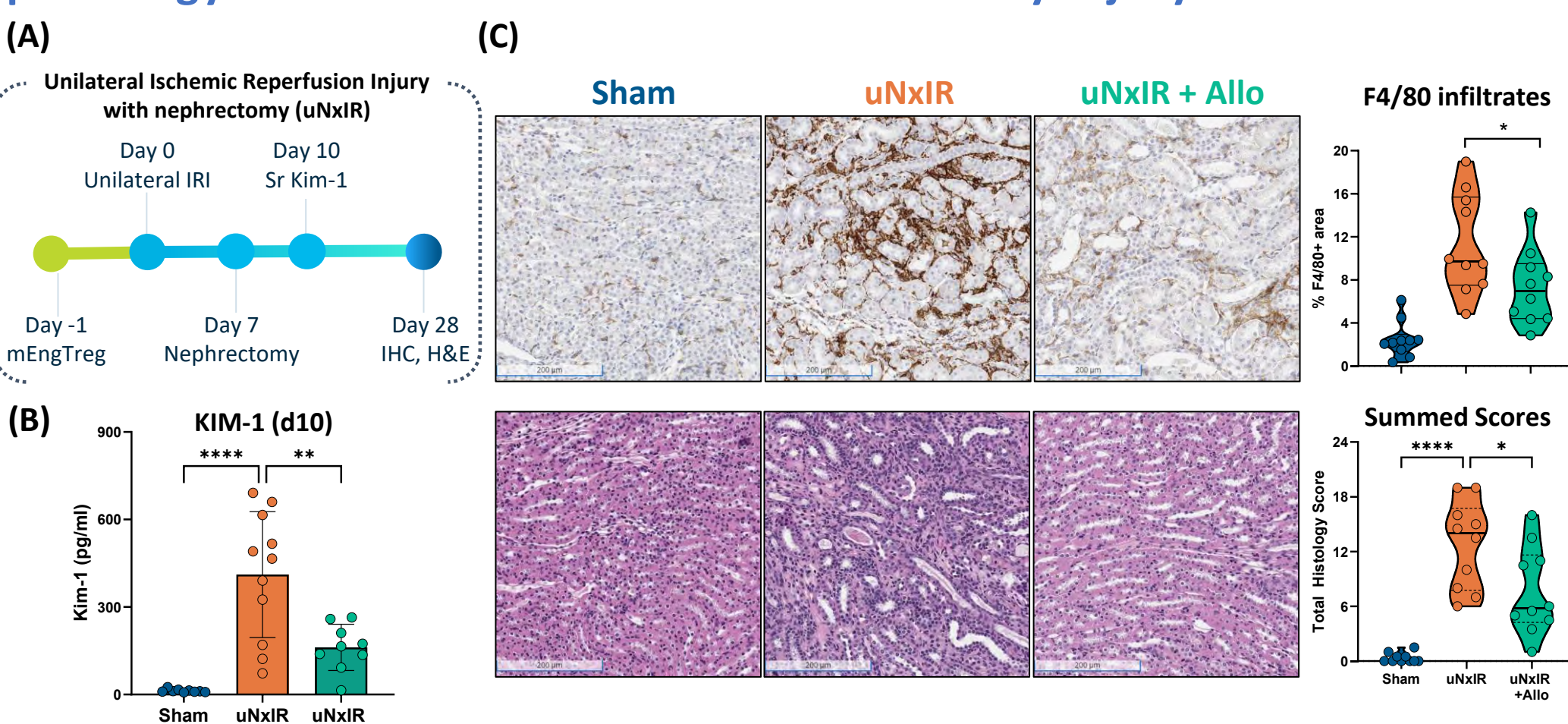
(A) tMCAO model of ischemic stroke, with mEngTreg dosing at +5h. (B - D) Sensorimotor readouts from D-1 to D35: (B) Foot fault: mice placed on a grid floor and walking pattern is recorded for one minute. (C) Rotarod: mice placed on accelerating rotating rod with speed increasing from 4-40rpm over 5 minutes. (D) Sensitivity to somatosensory neglect and motor impairment measured by the removal of adhesive tape applied to forepaws contralateral to the injury.

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



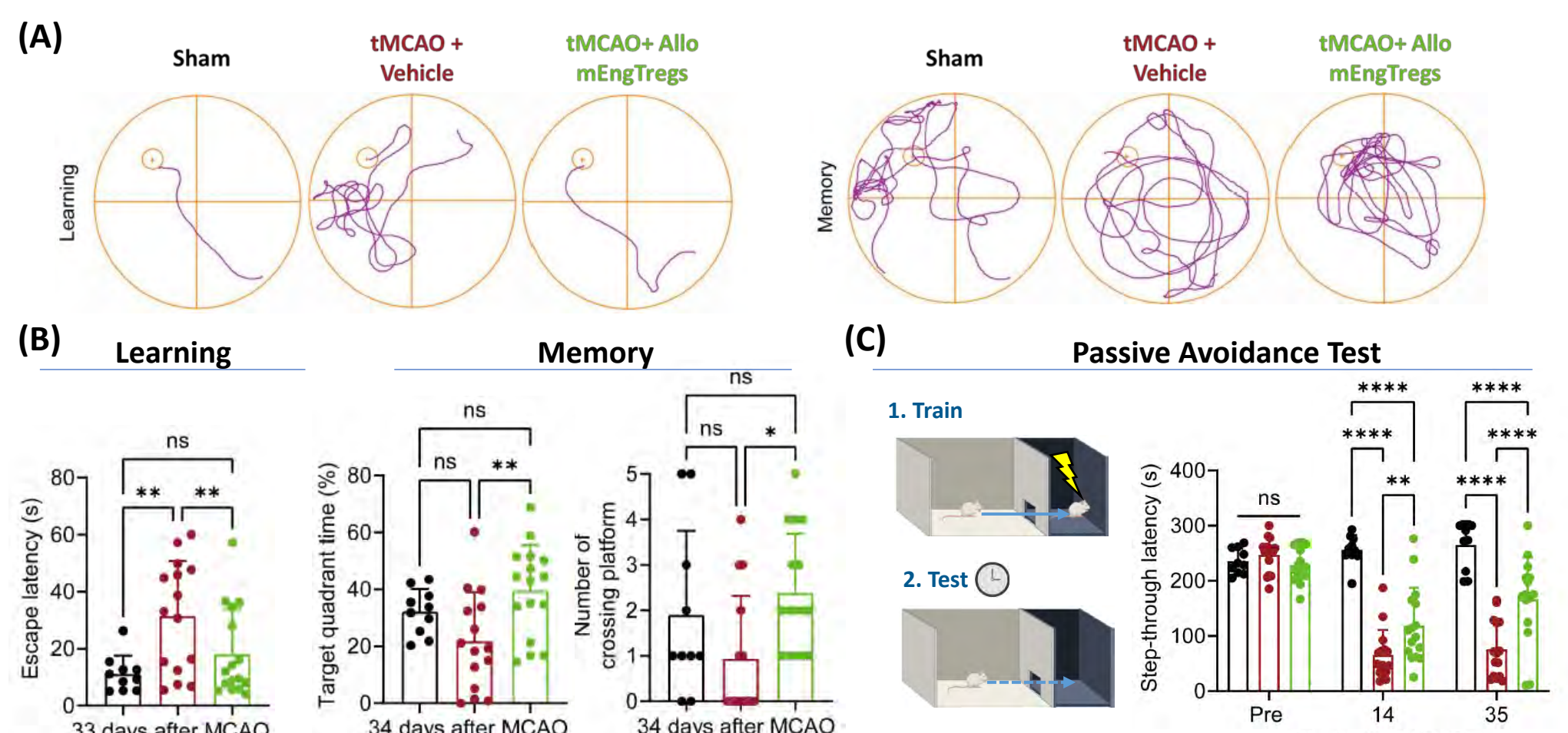
(A) Higher clearance of IL-33 and IL-4 by Tissue EngTregs in co-culture experiment as detected by ELISA. (B) Higher expression of IL-13 and Osteopontin (OPN) by Tissue EngTregs vs Basic EngTregs co-cultured with IL-33 (CBA). (C) TNFR2 MFI (flow cytometry) and TNF- α uptake in co-culture assays (ELISA). (D) TNF- α co-culture of Tissue EngTregs results in greater relative upregulation of CD25 and IL-2 uptake by Tissue EngTregs.

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



(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. Allogeneic mEngTreg treatment significantly improves cognitive readouts in tMCAO model of ischemic stroke



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

We make Tregs. Better.

