Supplementary Material

Methods

Gross appearance, histopathology, and rejection pathology scoring

Grafts were considered to have "survived" if they appeared grossly normal (i.e. via naked eye or low-powered microscope, nearly identical to the untransplanted host lung in architecture, vascularity, color, and inflation/deflation with mechanical ventilation) at the time of harvest. We used this evaluation as a screening technique when determining the regimen components required to achieve graft acceptance. Grafts were considered "accepted" only if they appeared similar to the native lung by both gross and histologic evaluation. For histologic evaluation, grafts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin & eosin (H&E). One investigator (A. P-M.) blinded to lung identity graded the H&E-stained sections of transplanted and native lungs for severity of rejection pathology using the following scoring system: 0 = no inflammation, 0.5 = minimal perivascular infiltrate, 1 = minor perivascular infiltrate with minimal parenchymal infiltrate, 1.5 = minor perivascular infiltrate with minor parenchymal consolidation, 2.0 = moderate perivascular infiltrate, minor peribronchiolar infiltrate, moderate parenchymal consolidation, 2.5 = moderate perivascular infiltrate, moderate peribronchiolar infiltrate, moderate parenchymal consolidation, 3 = extensive perivascular infiltrate, moderate peribronchiolar infiltrate, moderate parenchymal consolidation, minor areas of parenchymal and peribronchiolar fibrosis, 3.5 = extensive perivascular infiltrate, extensive peribronchiolar infiltrate, moderate parenchymal consolidation, areas of

parenchymal and peribronchiolar fibrosis affecting 25% of the lung, 4 = extensive perivascular infiltrate, extensive peribronchiolar infiltrate, extensive parenchymal consolidation, extensive hemorrhaging, areas of parenchymal and peribronchiolar fibrosis affecting 50% of the lung with moderate obliterative bronchiolitis, 4.5 = extensive perivascular infiltrate, extensive peribronchiolar infiltrate, extensive parenchymal consolidation, extensive hemorrhaging, areas of parenchymal and peribronchiolar fibrosis affecting 75% of the lung with extensive obliterative bronchiolitis, 5 = complete lung destruction, complete consolidation of all areas with hemorrhaging, infiltrating cells and fibrosis and airway occlusion.

Tissue isolation, blood preparation, and flow cytometry

Surface antibody staining and intracellular cytokine staining were carried out as previously described [15,19]. Briefly, harvested organs were minced, incubated at 37°C for 1 h in RPMI medium containing 2.4 mg/ml collagenase I and 20µg/ml DNAse (Life Technologies, Grand Island, NY) and passed through a 70-µm cell strainer (BD Falcon). Isolated cells from allograft, native lung, spleen, and heparinized peripheral blood were surface-stained with fluorochrome-labeled anti-$H2K^d$, anti-$H2K^b$, anti-CD3, anti-B220, and anti-CD11b mAbs to determine multi-lineage chimerism. For Vβ T-cell receptor analysis, blood samples were surface-stained with fluorochrome-labeled mAbs against $H2K^d$, $H2K^b$, CD3, CD4, Vβ5.1/5.2, Vβ8.1/8.2 and Vβ11. $CD3^+$ $CD4^+$ $H2K^{b+}$ gated events were acquired on a BD LSR II flow cytometer (Becton Dickinson, San Jose, CA), and analyzed with BD FACS Diva software (BD Biosciences, San Jose, CA).[15] Antibodies were

purchased from BD PharMingen (San Diego, CA), eBioscience (San Diego, CA), or BioLegend (San Diego, CA).

Mixed lymphocyte reaction

$CD4^+$ T-cells were purified with Dynabeads FlowComp mouse CD4 beads (Life Technologies, Carlsbad, CA) from spleens of regimen-treated allograft recipients, naive BALB/c mice, and naive C57BL/6 mice. After labeling with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) for 15 min at 37°C, cells were washed and incubated at 37°C for 30 min to ensure total probe modification. CFSE-labeled responder T-cells were cultured for 96 h with irradiated CD3-depleted splenocytes (stimulators) from naïve BALB/c mice (allogeneic), C57BL/6 mice (syngeneic), or FVB/N mice (third party). Cells were cultured in RPMI 1640 (Mediatech Inc., Manassas, VA) supplemented with 10% heat-inactivated fetal calf serum (HIFCS; Life Technologies), 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50μM β-mercaptoethanol. CFSE dilution on gated H-$2K^{b+}$, $CD4^+$ T-lymphocytes was measured on a BD FACS Calibur (Becton Dickinson).

Statistical analysis

Data were analyzed with GraphPad PRISM software (version 6.0, La Jolla, CA). In all experiments, unless otherwise specified, the two-tailed Student's t-test was used. A p value <0.05 was considered statistically significant. Values are presented as means ± standard errors of the mean.

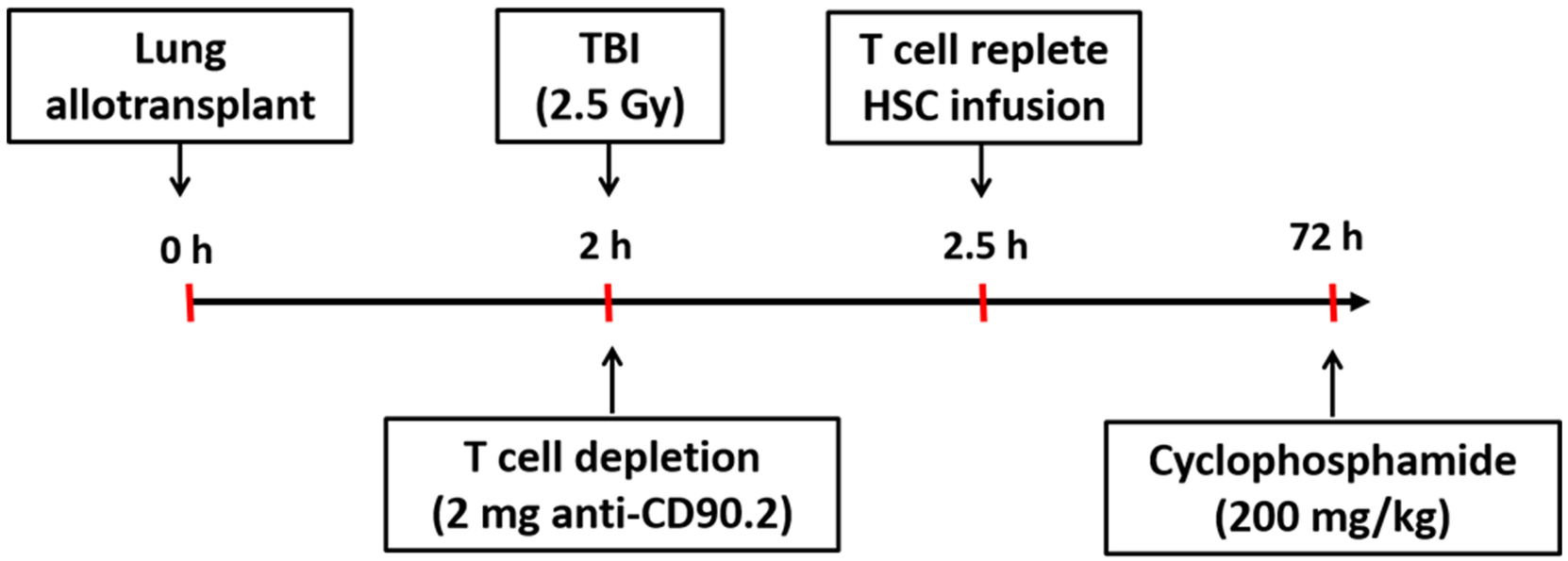
Figure S1. Same-day post-transplant conditioning results in allograft rejection.

(A) Schematic depiction of same-day conditioning regimen, where total body irradiation and T cell depletion in sequence was administered 2 hours after surgery. **(B)** Gross photograph of rejected allograft (left) from a recipient that received the regimen as outlined above. **(C)** Representative lung histology with H&E-staining (20x) at day 60 following same-day post-transplant conditioning. Top panel = allograft; Bottom panel = native lung. **(D)** Mean pathohistologic scores (n = 4-7 per group) of lung allografts and native lungs from pre-conditioned and post-transplant same-day conditioned recipients.

Figure S2. Recipient lymphocyte infusion (RLI) causes allograft rejection in PTTT-PTB/PTCy-treated mice via increased infiltration of effector host CD4$^+$ T cells.

(A) Gross photograph of rejected allograft (left) from a transplanted chimeric mouse that received PTTT-PTB/PTCy followed by RLI 4 weeks later. Organs were harvested 2 weeks after RLI administration. **(B)** Representative lung histology with H&E-staining (20x) at day 60 of the same animal. Left panel = allograft; Right panel = native lung. **(C)** Host (H-2K^{b+}) CD4$^+$ T cells were evaluated for their PMA+Ionomycin-induced production of Interferon-γ and IL-17 together with Foxp3 expression. Representative flow-cytometry dot plots of host-type (H-2K^b) CD4$^+$ cells from allografts of transplanted mice that received PTTT-PTB/PTCy and were accepted or PTTT-PTB/PTCy followed by RLI resulting in rejection. Data are representative of 4-6 animals per group. **(D)** Ratio of H-2K^{b+} CD4$^+$ IFN-γ$^+$ effectors: FoxP3$^+$ Tregs or (IFN-γ$^+$ + IL-17$^+$): FoxP3$^+$ Tregs are shown for host-type CD4$^+$ T cells derived from lung allograft, native lung and spleen. N= 4-6 per group.

A



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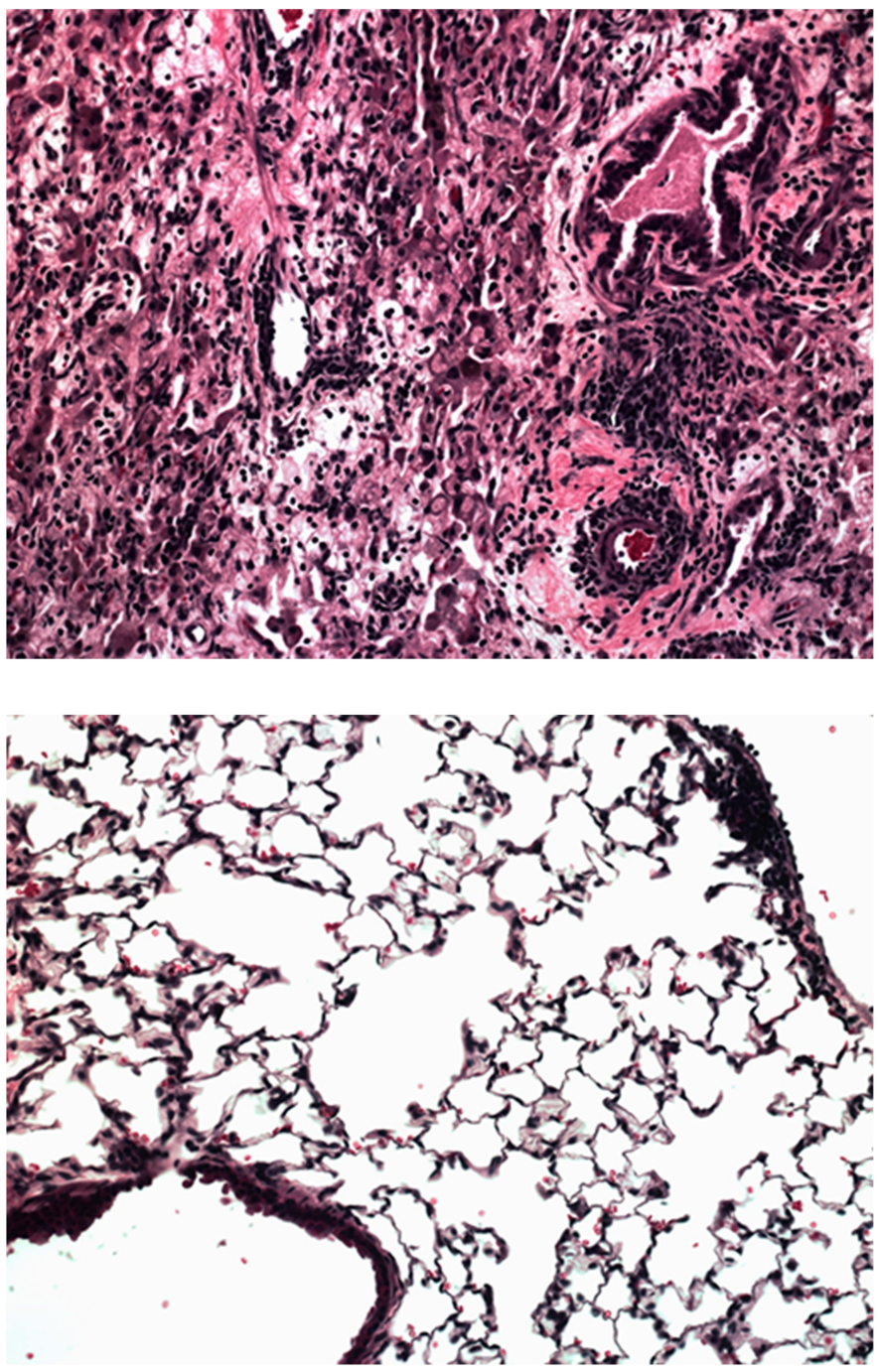

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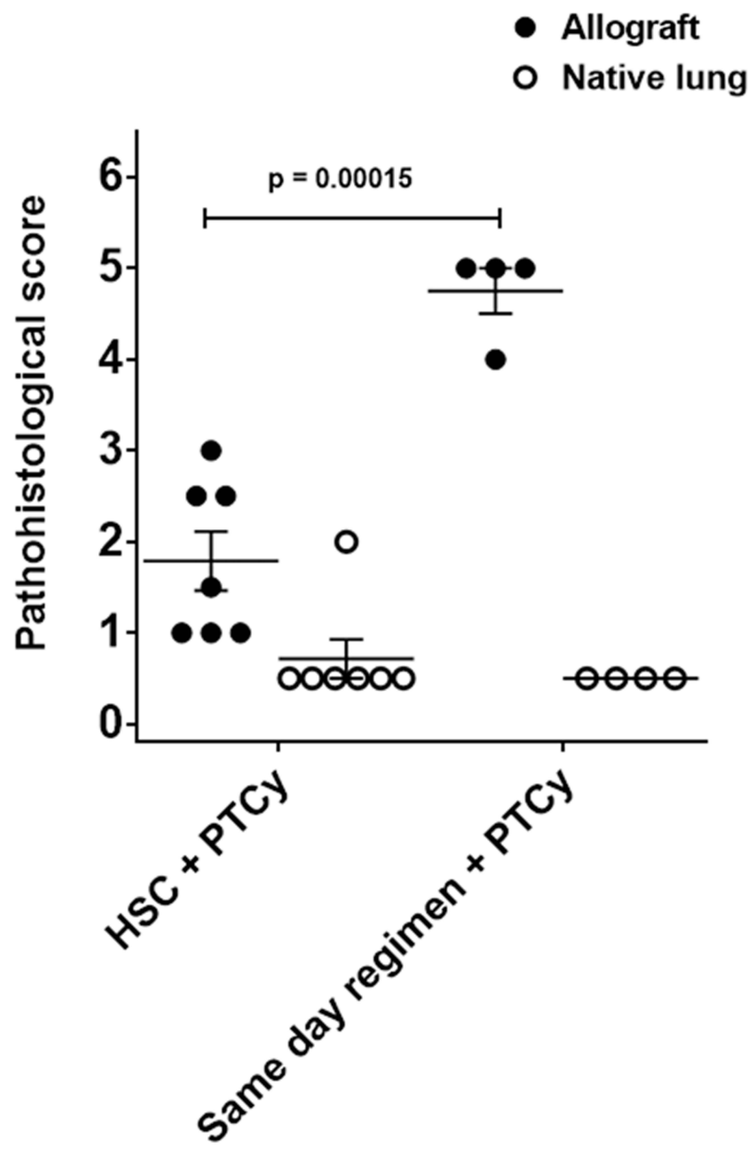
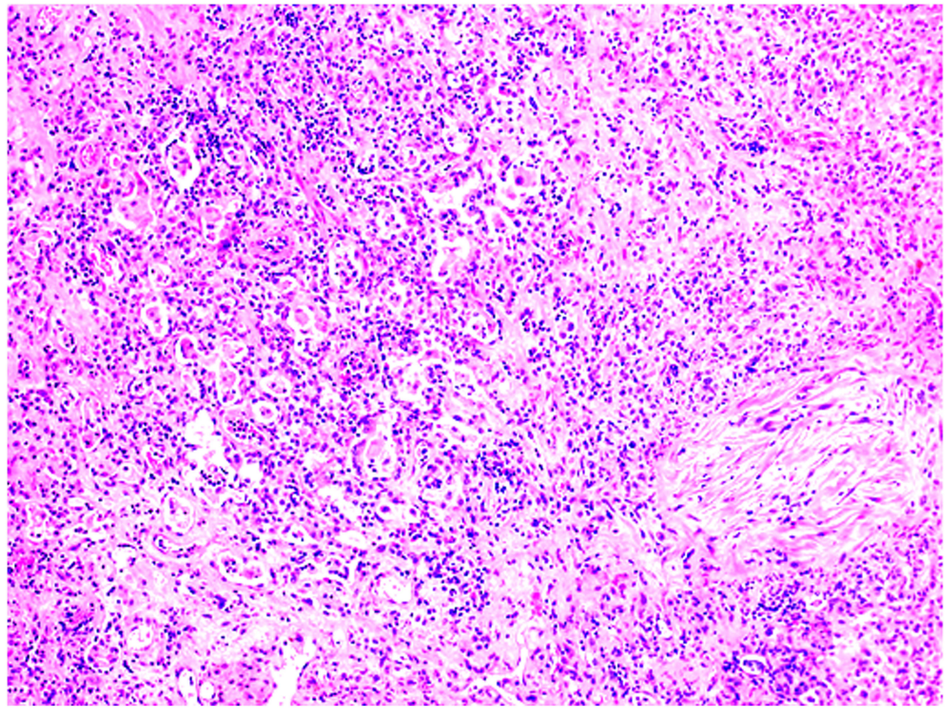
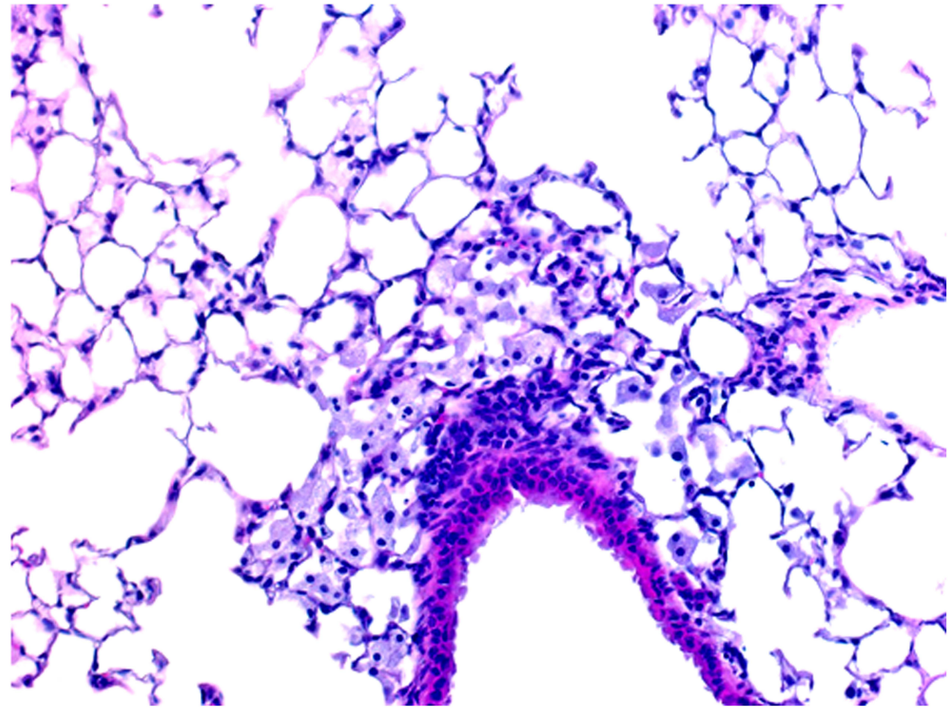


Figure S1

A

B





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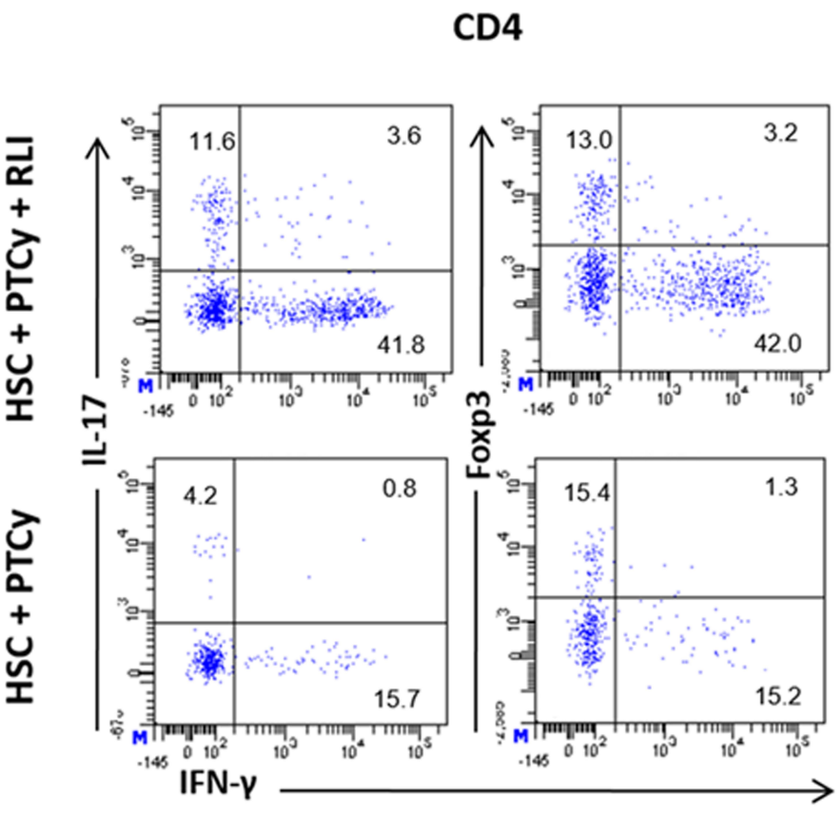

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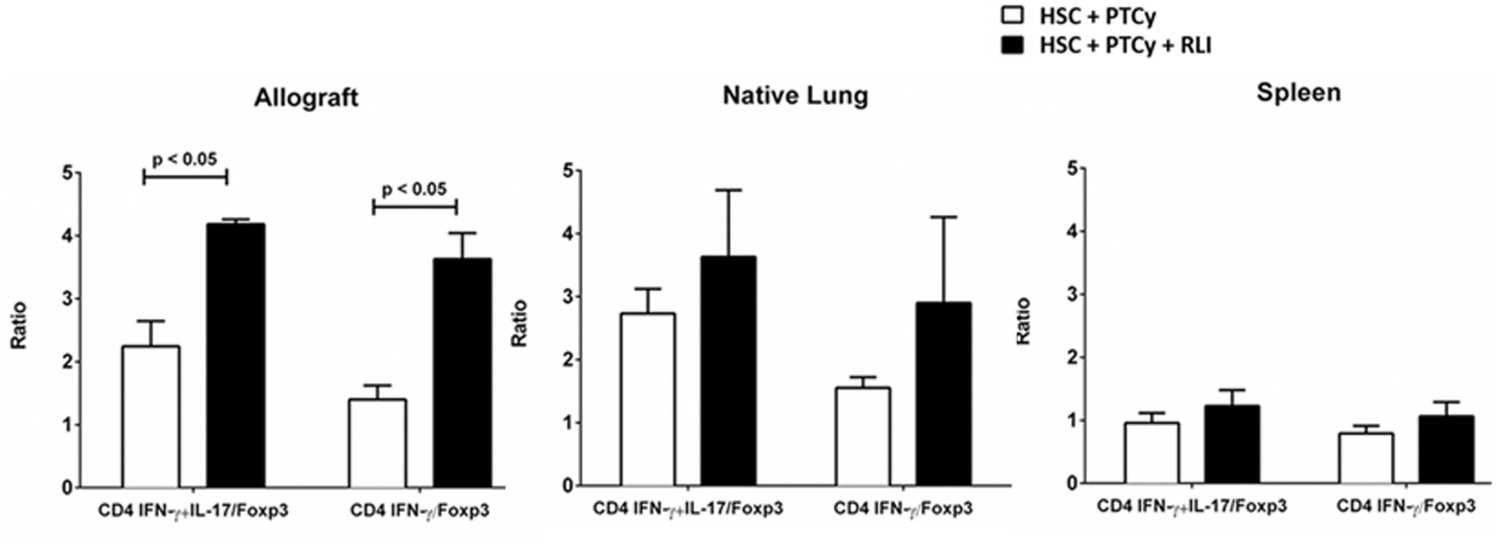

Figure S2