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ITK deficiency protects against autoimmune pulmonary hemorrhage by promoting Amphiregulin-producing regulatory T cells

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eLife Assessment

This study investigates the role of Interleukin-2-inducible T cell kinase (ITK) deficiency in autoimmune lung injury using a pristane-induced pulmonary hemorrhage (PH) model, suggesting that ITK-deficient regulatory T cells (Tregs) restrict severe tissue pathology. The work represents an **important** addition to the fields of autoimmunity, inflammation, and T-cell biology in the lung. The experimental evidence supporting the core ideas is **solid**, but there is additional work needed to define the underlying mechanisms.

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Abstract

Pulmonary hemorrhage (PH) is a life-threatening manifestation of systemic autoimmunity caused by immune-mediated disruption of the alveolar capillary barrier. Despite high mortality, the molecular checkpoints that shift destructive inflammation toward protective immune regulation remain poorly defined. Here, using the pristane-induced PH model, we identify interleukin-2-inducible T cell kinase (ITK) as a critical regulator of autoimmune lung injury. ITK deficiency (*Itk*^{-/-}) conferred near-complete protection from PH and associated multiorgan injury, accompanied by reduced proinflammatory monocytes and neutrophils and increased Foxp3⁺ regulatory T cells (Tregs). Adoptive transfer of *Itk*^{-/-} Tregs protected wild-type recipients from PH and suppressed systemic proinflammatory cytokines, identifying Tregs as key mediators of tissue protection. Mechanistically, lung *Itk*^{-/-} Tregs exhibited increased amphiregulin, a mediator of tissue repair. Transcriptomic profiling further showed that ITK loss reprogrammed Tregs toward a metabolically and functionally enhanced state, with enrichment of oxidative phosphorylation, mTORC1 and STAT5 signaling, and tissue-repair programs. These findings establish ITK as a regulator of the balance between pulmonary injury and reparative immunity and support targeting the ITK axis in severe inflammatory lung disease.

Highlights

- ITK deficiency protects against pristane-induced pulmonary hemorrhage (PH)
- Loss of ITK expands "super-fit" canonical and non-canonical Tregs
- ITK-deficient Tregs from lungs exhibit enriched Amphiregulin production
- ITK-deficient Tregs exhibit enriched mTORC1, OXPHOS, and IL-10 production
- Transfer of ITK-deficient Tregs rescues established PH and reverses proteinuria
- ITK uncouples pathogenic inflammation from reparative tissue immunity

Introduction

Pulmonary hemorrhage (PH) is a life-threatening manifestation of systemic autoimmunity, with reported mortality rates ranging from 40% to 90%. It can occur in autoimmune diseases such as anti-glomerular basement membrane disease (Goodpasture syndrome), systemic lupus erythematosus (SLE), and antiphospholipid syndrome, in which immune-mediated disruption of the alveolar–capillary interface causes rapid and potentially fatal pulmonary bleeding^{1–3}. Current treatment generally relies on high-dose corticosteroids combined with systemic immunosuppressive agents, including cyclophosphamide and rituximab, with antifibrinolytic therapy used as an adjunct in selected cases to promote hemostasis^{3,4}. Despite these aggressive interventions, clinical outcomes remain poor, underscoring a major unmet need to distinguish the immune mechanisms that drive organ-specific tissue injury from those that promote protection, repair, and recovery^{5–8}.

Interleukin-2-inducible T cell kinase (ITK), a member of the Tec family of tyrosine kinases, is an important checkpoint in the regulation of T-cell responses^{9–11}. In CD8⁺ T cells, ITK positively regulates T-cell receptor (TCR) signaling while restraining homeostatic proliferation, metabolic activity, and effector function¹². Accordingly, CD8⁺ T cells from ITK-deficient mice acquire an innate memory-like phenotype characterized by increased expression of CD44 and CD122, together with the transcription factors Eomes and T-bet^{11,13,14}. ITK also has important functions within the CD4⁺ T-cell compartment, where calcium-dependent ITK signaling regulates the balance between T helper 17 (Th17) and regulatory T-cell (Treg) differentiation¹⁵. In addition, ITK restrains IL-2-driven expansion of Foxp3⁺ Tregs¹⁶. Consistent with these functions, *Itk*^{−/−} CD4⁺ T cells intrinsically generate a higher frequency of functional Foxp3⁺ Tregs than their wild-type counterparts¹².

We previously demonstrated that disruption of ITK signaling uncouples graft-versus-host disease (GVHD) from graft-versus-leukemia (GVL) activity, supporting the concept that ITK differentially regulates pathogenic and protective T-cell responses^{17–19}. We further showed that ITK deficiency promotes the development of highly suppressive Tregs with enhanced functional capacity²⁰. However, whether ITK-dependent Treg reprogramming can protect the alveolar–capillary barrier during acute autoimmune lung injury remains unknown.

ITK functions as a proinflammatory amplifier in autoimmunity by strengthening T-cell receptor (TCR) signaling, promoting the differentiation of pathogenic T helper 17 (Th17) and T follicular helper (Tfh) cells, and restraining the development and suppressive function of Tregs^{21,22}. Through these effects, ITK shifts the immune balance away from tolerance and toward persistent inflammation and tissue injury. Consistent with this role, enhanced ITK signaling in CD4⁺ T cells has been implicated in several autoimmune diseases, including rheumatoid arthritis and multiple sclerosis, positioning ITK as an important molecular checkpoint and a potential therapeutic target for restoring immune homeostasis^{10,23}.

Despite these established functions, the contribution of ITK signaling to pristane-induced PH has not been investigated. Therefore, the central objective of this study was to define the cellular and molecular mechanisms through which ITK deficiency protects against severe autoimmune lung injury. Using *Itk*^{−/−} mice^{17,19}, we show that loss of ITK profoundly remodels the T-cell compartment. Consistent with previous studies, both CD8⁺ and CD4⁺ T cells from *Itk*^{−/−} mice exhibited significant expansion of central memory and effector memory populations compared with wild-type controls.

To define the functional contribution of ITK to autoimmune lung injury, we challenged wild-type (WT) and *Itk*^{−/−} mice with pristane. We found that loss of ITK signaling established a dominant regulatory immune environment that protected mice from pulmonary hemorrhage and broadly attenuated systemic autoimmune injury²⁴. This protection was characterized by reduced infiltration of proinflammatory immune cells and decreased circulating inflammatory cytokines, together with significant expansion of canonical CD4⁺CD25⁺Foxp3⁺ and noncanonical CD4⁺CD25⁺Foxp3⁺ Treg populations. Notably, Tregs from *Itk*^{−/−} mice exhibited increased expression of amphiregulin (AREG), an important mediator of tissue protection and repair^{25,26}.

Adoptive transfer of $\text{Itk}^{-/-}$ Tregs protected WT recipients from PH and suppressed pathogenic inflammation, demonstrating their direct contribution to disease control. Transcriptomic profiling further revealed that $\text{Itk}^{-/-}$ Tregs were reprogrammed toward a metabolically and functionally enhanced state. Together, these findings identify ITK as a previously unrecognized regulator of the balance between alveolar–capillary injury and reparative immunity and highlight the ITK pathway as a potential therapeutic target in severe inflammatory lung disease.

Results

ITK Deficiency Alters the Distribution of Naïve and Memory T-Cell Subsets

ITK is a Tec-family tyrosine kinase required for optimal T-cell receptor (TCR) signaling and plays an important role in T-cell development, activation, and effector function²⁷.

To determine how ITK deficiency alters the T-cell compartment before autoimmune challenge, we analyzed splenic T-cell subsets from wild-type (WT) and $\text{Itk}^{-/-}$ mice on a C57BL/6 background^{17,18}. Because the differentiation state of $\alpha\beta$ T cells strongly influences their effector potential and contribution to disease^{28–31}, freshly isolated splenic CD3^{+} T cells were profiled by flow cytometry. CD3^{+} T cells were first separated into CD4^{+} and CD8^{+} populations and subsequently classified as naïve ($\text{CD44}^{\text{lo}} \text{CD62L}^{\text{hi}}$), central memory (CM; $\text{CD44}^{\text{hi}} \text{CD62L}^{\text{hi}}$), or effector memory (EM; $\text{CD44}^{\text{hi}} \text{CD62L}^{\text{lo}}$) subsets^{20,32}.

Within the CD8^{+} T-cell compartment, $\text{Itk}^{-/-}$ mice exhibited a significantly increased frequency of CM cells compared with WT controls, whereas the frequency of EM cells did not differ significantly between the two groups (Fig. 1A–D [↗](#)). Differences were more pronounced within the CD4^{+} T-cell compartment, in which $\text{Itk}^{-/-}$ mice displayed significant increases in both CM and EM populations, accompanied by a corresponding reduction in the naïve population (Fig. 1E–H [↗](#); gating strategy shown in Figure 1 [↗](#)—figure supplement 1 [↗](#)). Together, these findings demonstrate that ITK deficiency alters the steady-state distribution of naïve and memory T-cell subsets, consistent with previous observations from our laboratory and others^{12,14,16,33,34}.

ITK Deficiency Increases the Expression of CD44, CD122, T-bet, and Eomes in T Cells

We and others have previously shown that attenuation of T-cell receptor (TCR) signaling can alter T-cell activation and differentiation without necessarily promoting pathogenic alloimmune responses^{17,18,20,35,36}. Consistent with these findings, flow cytometric analysis demonstrated significantly increased CD44 expression in splenic $\text{CD3}^{+} \text{CD8}^{+}$ T cells from $\text{Itk}^{-/-}$ mice compared with wild-type (WT) controls (Fig. 2A–B [↗](#)).

We next examined transcription factors and surface receptors associated with T-cell differentiation and cytokine responsiveness. The T-box transcription factors T-bet and Eomes regulate multiple aspects of effector and memory T-cell development and are associated with the expression of CD122 (IL-2R β), a shared component of the IL-2 and IL-15 receptor complexes^{37,38}. CD8^{+} T cells from $\text{Itk}^{-/-}$ mice exhibited significantly increased expression of T-bet, Eomes, and CD122 compared with WT controls (Fig. 2C–H [↗](#)). A similar pattern was observed in the CD4^{+} T-cell compartment, in which $\text{Itk}^{-/-}$ T cells displayed increased expression of CD44, Eomes, CD122, and T-bet (Figure 2 [↗](#)—figure supplement 1A–H [↗](#)). Together, these findings demonstrate that ITK deficiency increases the expression of differentiation-associated transcription factors and surface markers in both CD8^{+} and CD4^{+} T-cell populations. These results further indicate that reduced ITK-dependent TCR signaling does not uniformly diminish the expression of molecules associated with T-cell differentiation and responsiveness to IL-2 and IL-15.

Figure 1. ITK deficiency promotes a predominantly memory-like T cell phenotype.

(A–D) Analysis of the CD8⁺ T cell compartment in WT and *Itk*^{-/-} mice. Freshly isolated splenocytes were gated on CD3⁺ lymphocytes and further subdivided into CD8⁺ and CD4⁺ T cell populations. (A) Representative flow cytometry plots showing CD44 and CD62L expression within CD8⁺ T cells. (B–D) Quantification of effector memory (EM; CD44⁺CD62L⁻), central memory (CM; CD44⁺CD62L⁺), and naïve (CD44⁻CD62L⁺) CD8⁺ T cell subsets. (E–H) Analysis of the CD4⁺ T cell compartment. (E) Representative flow cytometry plots showing CD44 and CD62L expression within CD4⁺ T cells. (F–H) Quantification of EM, CM, and naïve CD4⁺ T cell frequencies. Data are presented as mean ± SEM (*n* = 10–15 mice per group). Results are representative of 6 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's *t*-test. *****p* ≤ 0.0001; ns, not significant (*p* > 0.05).

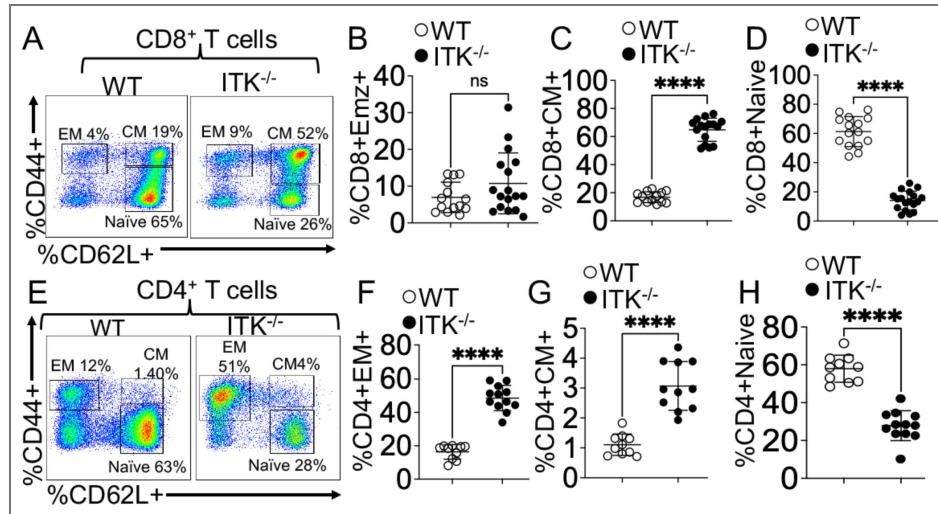
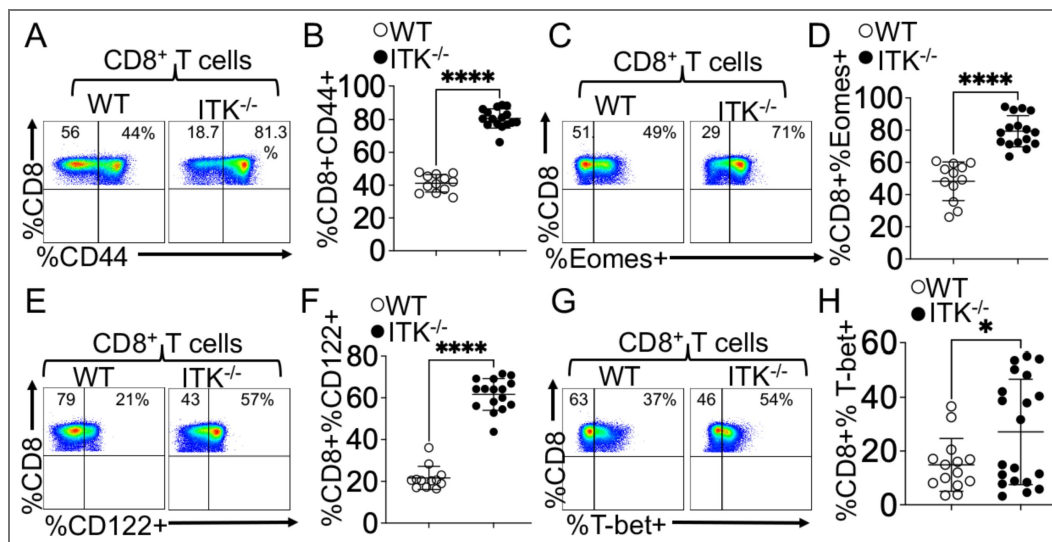


Figure 2. ITK deficiency enhances expression of memory-associated activating markers and transcriptional regulators.

(A and B) Surface expression of CD44 on splenic T cells. Representative flow cytometry plots (A) and quantification of frequencies (B) in WT and *Itk*^{-/-} mice. (C and D) Expression of the T-box transcription factor Eomes. Representative flow cytometry plots (C) and quantification of total Eomes⁺ T cells (D). (E and F) Surface expression of CD122 (IL-2Rβ). Representative flow cytometry plots (E) and quantification of CD122 frequencies on T cells (F) from WT and *Itk*^{-/-} mice. (G and H) Intracellular expression of the transcription factor T-bet. Representative flow cytometry plots (G) and quantification of T-bet frequencies within the indicated population (H). Data are presented as mean ± SEM (*n* = 15–20 mice per group, as indicated in the panels). Results are representative of at least 3 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's *t*-test. **p* ≤ 0.05; *****p* ≤ 0.0001.



ITK Deficiency Protects Against Pristane-Induced Pulmonary Hemorrhage and Systemic Pathology

We and others have previously shown that disruption of ITK signaling attenuates pathogenic immune responses in multiple models of alloimmunity [17,18,20,35,36](#). To investigate the role of ITK in autoimmune acute lung injury, we used the pristane-induced PH model, which reproduces key features of immune-mediated alveolar–capillary injury observed in systemic autoimmune diseases^{1–3}. WT and *Itk*^{−/−} mice received a single intraperitoneal injection of pristane (0.5 mL) and were evaluated 14 days later, as established in previous studies^{24,28,39,40} (Fig. 3A [3A](#)). Neither group exhibited significant weight loss during this acute period. However, gross and histopathologic analyses at day 14 revealed marked differences in pulmonary injury. Lungs from WT mice exhibited extensive diffuse hemorrhage, intra-alveolar erythrocyte accumulation, and disruption of normal lung architecture. In contrast, *Itk*^{−/−} mice were protected from PH and maintained preserved alveolar architecture with minimal evidence of hemorrhage or tissue injury (Fig. 3B–E [3E](#)).

To exclude the possibility that these differences reflected preexisting abnormalities, we examined lungs from untreated WT and *Itk*^{−/−} mice. Both groups exhibited normal pulmonary architecture, indicating that the protection observed in *Itk*^{−/−} mice emerged following pristane challenge rather than from baseline histologic differences (Figure 3 [3](#)—figure supplement 1A–D [3D](#)). We next assessed longer-term disease progression. All pristane-treated WT mice died within 20 days, whereas *Itk*^{−/−} mice exhibited significantly prolonged survival (Figure 3 [3](#)—figure supplement 2 [2](#)). Together, these findings demonstrate that ITK deficiency protects against acute pristane-induced PH and substantially improves survival.

Because the progression of PH is driven in part by the recruitment of pathogenic innate immune cells^{41–43}, we next quantified inflammatory monocyte and neutrophil accumulation in the lungs. WT mice exhibited a marked influx of CD11b⁺Ly6C⁺ inflammatory monocytes following pristane challenge, whereas this population was significantly reduced in *Itk*^{−/−} mice (Fig. 3F–H [3H](#)). Because neutrophils are early responders to inflammatory tissue injury^{43,44}, we also assessed their accumulation in the lungs at day 14 after pristane administration. CD11b⁺Ly6G⁺ neutrophils were significantly reduced in *Itk*^{−/−} mice compared with WT controls (Fig. 3I [3I](#)). Together, these findings demonstrate that protection from pristane-induced PH in *Itk*^{−/−} mice is associated with reduced pulmonary accumulation of proinflammatory innate immune cells.

Beyond the lung, ITK deficiency also attenuated systemic manifestations of pristane-induced injury^{45,46}. Following pristane challenge, WT mice developed pronounced splenomegaly accompanied by extensive accumulation of megakaryocytes, whereas spleens from *Itk*^{−/−} mice retained near-normal size and histologic architecture (Fig. 3J–L [3L](#); Figure 3 [3](#)—figure supplement 3E–F [3F](#)). Histologic examination of spleens from naïve WT and *Itk*^{−/−} mice revealed no baseline abnormalities, indicating that these differences emerged following pristane exposure (Figure 3 [3](#)—figure supplement 3A–C [3C](#)).

We next examined the small intestine. Naïve WT and *Itk*^{−/−} mice exhibited normal intestinal architecture without evidence of preexisting tissue injury (Figure 3 [3](#)—figure supplement 4A–B [3B](#)). Following pristane administration, WT mice developed substantial small-intestinal pathology characterized by epithelial hyperplasia and focal acute inflammation. These changes were markedly attenuated in *Itk*^{−/−} mice (Fig. 3M [3M](#); Figure 3 [3](#)—figure supplement 4C–D [3D](#)).

Together, these findings demonstrate that ITK deficiency provides multiorgan protection against pristane-induced systemic inflammatory injury. Having established this protective phenotype, we next examined whether loss of ITK also reduced systemic proinflammatory cytokine production.

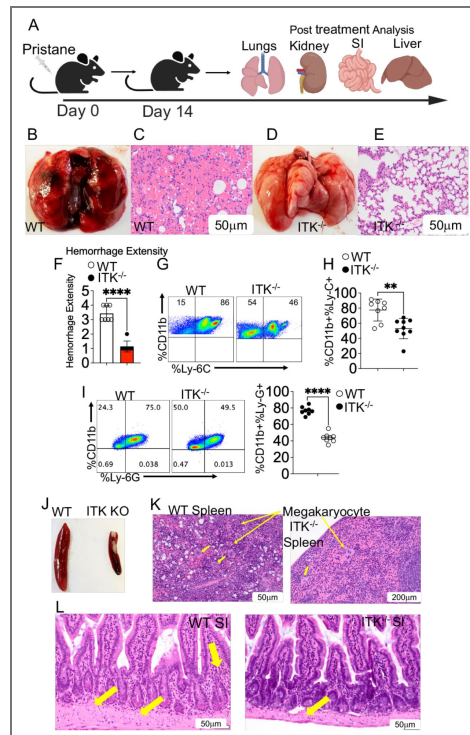


Figure 3. TK deficiency confers protection against pristane-induced PH and systemic pathology.

(A) Experimental schematic: WT and *Itk*^{-/-} mice received a single i.p. injection of 0.5 mL pristane to induce pulmonary hemorrhage (PH) and were euthanized on day 14 for tissue analysis. (B–E) Assessment of lung pathology. Representative gross morphology (B, D) and H&E-stained lung sections (C, E) from WT and *Itk*^{-/-} mice after pristane injection. WT mice exhibit severe pulmonary hemorrhage and loss of architectural integrity, whereas lungs from *Itk*^{-/-} mice remain largely free of blood and retain preserved alveolar structures. (F) Quantification of blinded histopathologic scoring. Lung injury was graded by a board-certified pathologist blinded to genotype and treatment. (G and H) Analysis of lung-infiltrating proinflammatory monocytes. Representative flow cytometry plots (G) and quantification (H) of inflammatory monocytes, gated as CD11b⁺Ly6C⁺ within live singlets. (I) Representative flow cytometry plots and quantification of neutrophils (CD11b⁺Ly6G⁺) within live singlets from WT and *Itk*^{-/-} mice after pristane injection. (J and K) Evaluation of splenic pathology. Representative gross images (J) and H&E-stained sections (K) of spleens from WT and *Itk*^{-/-} mice after pristane injection. (L) Histopathological analysis of systemic inflammation. Representative H&E-stained sections of the small intestine from WT and *Itk*^{-/-} mice. Data are presented as mean ± SEM (*n* = 10 mice per group). Results are representative of 3 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's *t*-test, except for panel F, which was analyzed using the Mann-Whitney *U* test. *p* ≤ 0.01; ** *P* ≤ 0.01, *****p* ≤ 0.0001.

ITK Deficiency Attenuates Proteinuria and Shifts the Systemic Cytokine Balance Toward Resolution

Proteinuria is a clinical hallmark of pulmonary-renal syndrome and frequently accompanies PH in systemic autoimmune disease, serving as an important indicator of glomerular involvement⁴⁷. To determine whether the protection observed in *ITK*^{-/-} mice extended to the kidney, we quantified urinary protein levels at baseline (day 0) and at the study endpoint (day 14). Whereas WT mice developed a significant increase in proteinuria following pristane challenge, *ITK*^{-/-} mice showed a markedly attenuated response, indicating that loss of ITK signaling protects against PH-associated renal dysfunction (Fig. 4A-C [↗](#)). Histologic analysis at this acute 14-day time point did not reveal overt structural kidney injury, although WT mice displayed focal inflammatory cell infiltration that was absent in *ITK*^{-/-} mice (Supplementary Fig. 5 [↗](#)). These findings suggest that although glomerular injury remains at an early stage in this acute model, the ITK axis already regulates the inflammatory processes that precede overt renal damage.

Because PH is associated with a robust systemic inflammatory response, we next quantified circulating cytokines to determine how the ITK axis influences the balance between pathogenic and reparative immunity. We measured key mediators implicated in PH pathogenesis, including IFN- γ and TNF- α , which promote procoagulant activity and tissue factor expression⁴⁸, as well as IL-6 and IL-17, which drive neutrophil recruitment and vascular permeability^{49–53}. Serum analysis by ELISA by (LEGENDplex/multiplex cytokine analysis), showed that *ITK*^{-/-} mice had significantly lower levels of all four proinflammatory cytokines than WT controls (Fig. 4D-G [↗](#)). Conversely, because anti-inflammatory cytokines can restrain pathogenic immune responses^{54,55}, we asked whether ITK deficiency also promotes a pro-resolving cytokine environment. Indeed, *ITK*^{-/-} mice produced significantly higher levels of IL-10 and IL-13 during PH (Fig. 4H-I [↗](#)).

Together, these findings indicate that ITK deficiency does not simply impose broad immunosuppression, but instead redirects the systemic immune response toward regulation and resolution, further supporting ITK as a promising therapeutic target in PH and related autoimmune diseases.

Itk^{-/-} Tregs Exhibit Enhanced AREG Expression and More Effectively Ameliorate PH

Regulatory T cells (Tregs) are key mediators of immune suppression and tissue repair, acting in part through anti-inflammatory cytokines such as IL-10 to restrain neutrophils and inflammatory monocytes^{56,57}. We previously demonstrated that ITK deficiency increases the frequency of both canonical (CD4⁺CD25⁺Foxp3⁺) and non-canonical (CD4⁺CD25⁻Foxp3⁺) Tregs^{19,58}. To determine whether these regulatory populations are similarly altered during autoimmune lung injury, we analyzed splenic Tregs from WT and *Itk*^{-/-} mice following pristane challenge. *Itk*^{-/-} mice exhibited significantly higher frequencies of both canonical and non-canonical Treg subsets compared with WT controls (Fig. 5A [↗](#); Supplementary Fig. 7A-B [↗](#)). These findings are consistent with the established role of ITK in constraining the regulatory T-cell compartment and demonstrate that this altered Treg distribution is maintained during pristane-induced systemic inflammation. Notably, the increased representation of the non-canonical CD25⁻Foxp3⁺ population is consistent with our previous studies demonstrating enrichment and suppressive activity of this population in the setting of ITK deficiency^{12,59,60}. We next examined whether this altered regulatory phenotype was also present in the lung.

Analysis of lung tissue under homeostatic conditions revealed an increased frequency of Tregs in *Itk*^{-/-} mice compared with WT controls, with the most pronounced increase observed in the non-canonical CD25⁻Foxp3⁺ subset (Fig. 5E [↗](#)). From approximately 1×10^7 total viable lung cells recovered per mouse, approximately 8×10^5 CD3⁺CD4⁺ T cells were identified in both WT and *Itk*^{-/-} mice, indicating comparable overall CD4⁺ T-cell abundance between genotypes. Absolute Treg numbers were calculated from the total CD3⁺CD4⁺ T-cell recovery and the corresponding Treg frequencies determined by flow cytometry. In WT lungs, canonical CD25⁺Foxp3⁺ Tregs comprised

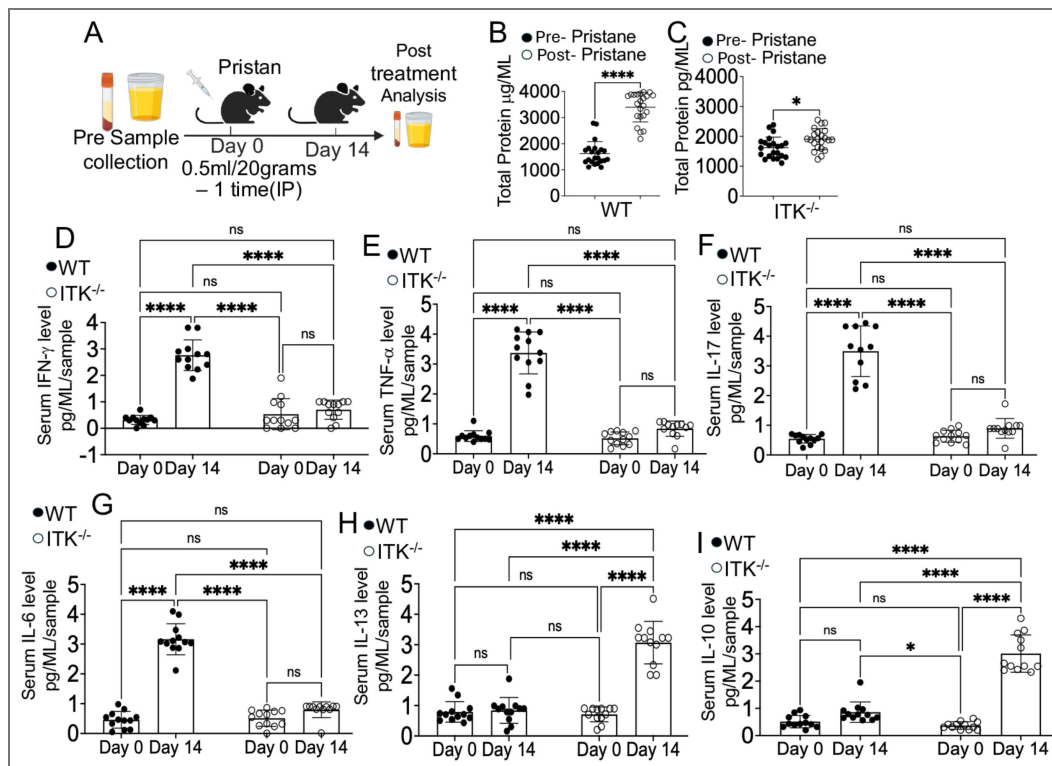


Figure 4. ITK deficiency attenuates proteinuria and shifts the systemic cytokine balance toward resolution.

(A) Experimental schematic: WT and *Itk*^{-/-} mice were challenged with pristane. Urine and blood were collected at baseline (day 0) and at the day 14 endpoint to assess systemic pathology. (B and C) Assessment of renal function by proteinuria. Total urinary protein was quantified by BCA assay in WT (B) and *Itk*^{-/-} mice (C). *Itk*^{-/-} mice show significant protection against the glomerular leakage observed in WT controls following pristane injection. (D–I) Multiplex serum cytokine profiling. Serum levels of proinflammatory mediators (IFN- γ , TNF- α , IL-17, and IL-6) and pro-resolving cytokines (IL-13 and IL-10) were quantified by bead-based immunoassay. *Itk*^{-/-} mice exhibited marked suppression of proinflammatory cytokines together with preservation of regulatory cytokines. Data are presented as mean $\pm$ SEM ($n = 15$ –25 mice per group). Results are representative of 3 independent experiments. Statistical significance was determined by a two-tailed Student's *t*-test for single comparisons or a two-way ANOVA with Tukey's *post hoc* correction for time-course data. $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, ** $P \leq 0.01$

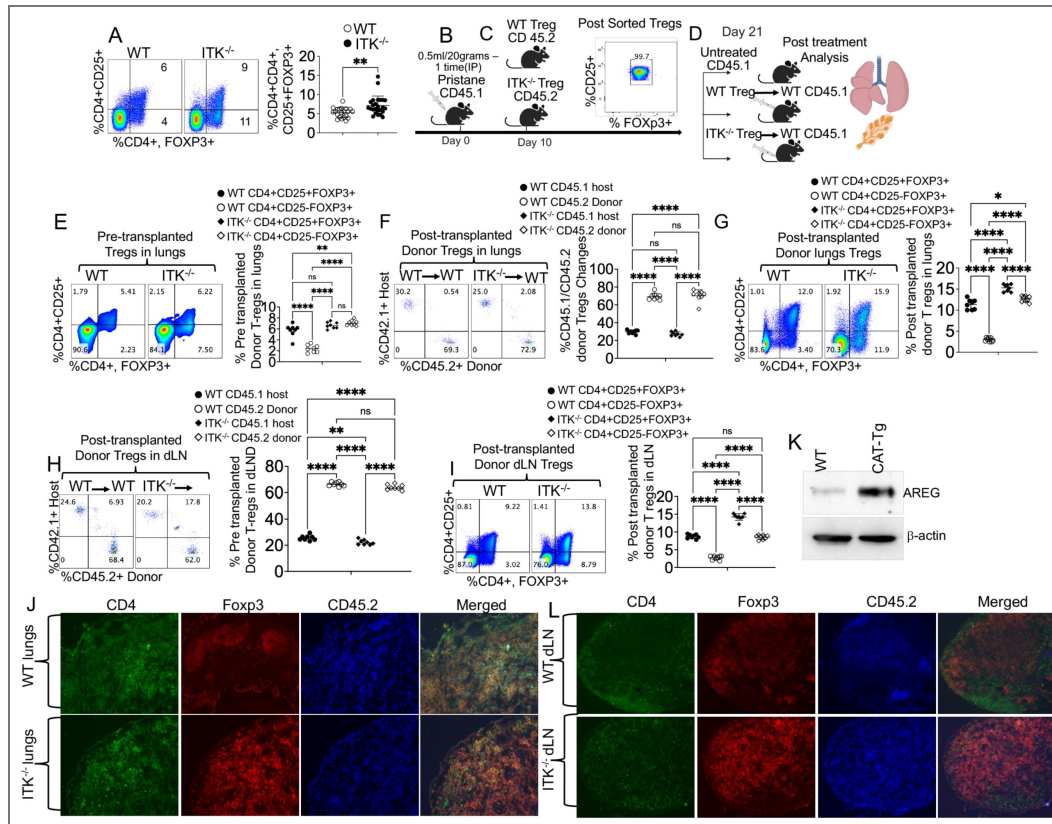


Figure 5. Adoptively transferred *Itk*^{-/-} Tregs exhibit enhanced AREG expression and more effectively ameliorate PH.

(A) Representative flow cytometry plots and quantitative analysis of splenic CD4⁺Foxp3⁺ Tregs in WT and *Itk*^{-/-} mice. (B–D) Experimental schematic for Treg adoptive transfer. (B) WT mice (CD45.1⁺) received a single i.p. injection of 0.5 mL pristane. (C) On day 10, CD4⁺Foxp3⁺ Tregs were FACS-sorted from WT or *Itk*^{-/-} donor mice (CD45.2⁺), with post-sort purity confirmed as CD25⁺Foxp3⁺. These cells were injected intravenously (i.v.) into the pristane-treated CD45.1⁺ recipients as a therapeutic intervention. (D) On day 21, CD45.1⁺ host mice were euthanized for examination of the lungs and draining lymph nodes (dLNs). (E) Representative flow cytometry plots and quantitative analysis of lung CD4⁺Foxp3⁺ Tregs in WT and *Itk*^{-/-} mice at baseline (without pristane injection). (F and G) Representative flow cytometry plots and quantitative analysis of total donor CD45.2⁺ cells (F) and donor-derived Tregs (G) engrafted in the lungs of CD45.1⁺ recipient mice. (H and I) Representative flow cytometry plots and quantitative analysis of total donor CD45.2⁺ cells (H) and donor-derived Tregs (I) in the dLNs of CD45.1⁺ recipient mice. (J) Immunofluorescence microscopy of lung sections confirming the presence of donor Tregs in inflamed lung tissue. Sections were stained for CD4, the donor congenic marker (CD45.2), and Foxp3, with merged images demonstrating colocalization; nuclei were counterstained with DAPI. (K) CD25⁺Foxp3⁺ Tregs were FACS-sorted from lung tissue and analyzed by immunoblot for amphiregulin (AREG), with β -actin as a loading control. Data are presented as mean $\pm$ SEM; sample sizes ($n = 15$ – 25 mice per group) are indicated in the panels. Each experiment was repeated 3 times. Statistical significance was determined using a two-tailed Student's *t*-test, one-way ANOVA, or two-way ANOVA as appropriate; $p < 0.01$, **** $p < 0.0001$, ** $P \leq 0.01$, **** $p < 0.0001$, ** $P \leq 0.01$.

approximately 6% of CD4⁺ T cells, corresponding to approximately 4.8×10^4 cells, whereas non-canonical CD25⁺Foxp3⁺ Tregs comprised approximately 2.3%, corresponding to approximately 1.8×10^4 cells. In *Itk*^{-/-} lungs, canonical Tregs comprised approximately 6–7%, corresponding to approximately 4.8 – 5.6×10^4 cells, whereas non-canonical Tregs increased to approximately 8%, corresponding to approximately 6.4×10^4 cells. Thus, the increased Treg frequencies in *Itk*^{-/-} mice were not attributable to reduced overall CD4⁺ T-cell abundance and were accompanied by a marked increase in the absolute number of non-canonical CD25⁺Foxp3⁺ Tregs.

To determine whether *Itk*^{-/-} Tregs possess enhanced protective activity *in vivo*, we performed adoptive-transfer experiments in WT recipients following initiation of pristane-induced disease. WT CD45.1 congenic mice were challenged with pristane and monitored for 10 days. On day 10, mice were assigned to three groups: a pristane-challenged control group receiving no Tregs, a group receiving FACS-purified WT CD45.2⁺ Tregs (CD3⁺CD4⁺CD25⁺Foxp3⁺), and a group receiving an equivalent number of *Itk*^{-/-} CD45.2⁺ Tregs (1×10^6 cells per mouse; Fig. 5B–D). Tregs were isolated from the spleens of **unchallenged** WT or *Itk*^{-/-} Foxp3-RFP reporter mice, allowing purification of viable CD25⁺Foxp3⁺ Tregs as previously described^{19,24}. On day 21, recipient mice were euthanized, and the lungs and draining lymph nodes (dLN) were analyzed (Fig. 5D).

We next examined donor-derived Tregs by distinguishing CD45.1⁺ recipient cells from CD45.2⁺ donor cells. At the experimental endpoint, a significantly higher frequency of donor-derived *Itk*^{-/-} Tregs was detected in the lungs compared with WT donor Tregs (Fig. 5F). To determine the phenotypic composition of the CD45.2⁺ donor population after transfer, we assessed Foxp3 and CD25 expression. Both canonical CD25⁺Foxp3⁺ and non-canonical CD25⁻Foxp3⁺ donor-derived populations were detectable, with higher frequencies observed among cells derived from *Itk*^{-/-} donors compared with WT donors (Fig. 5G).

Consistent with the lung findings, we examined donor-derived Tregs in the dLN of WT CD45.1 recipients^{18,19}. A significantly higher frequency of CD45.2⁺ donor-derived *Itk*^{-/-} Tregs was detected in the dLN compared with WT donor Tregs (Fig. 5H). Within the donor-derived CD45.2⁺CD3⁺CD4⁺Foxp3⁺ population, both canonical CD25⁺ and non-canonical CD25⁻ subsets were identified, with greater representation among *Itk*^{-/-} donor cells (Fig. 5I). These flow-cytometric findings were further supported by immunofluorescence microscopy demonstrating donor-derived CD4⁺Foxp3⁺CD45.2⁺ cells in both the lungs and dLN of recipient mice (Fig. 5J).

To further characterize the tissue-repair-associated phenotype of *Itk*^{-/-} Tregs, we evaluated Amphiregulin (AREG), an epidermal growth factor receptor ligand implicated in tissue repair independently of classical Treg suppressive functions^{25,61–63}. CD25⁺Foxp3⁺ Tregs were FACS-sorted from the lungs of WT and *Itk*^{-/-} Foxp3-RFP reporter mice, and AREG protein expression was assessed by immunoblotting. Tregs isolated from *Itk*^{-/-} lungs expressed substantially higher levels of AREG than their WT counterparts (Fig. 5K), consistent with an enhanced tissue-repair-associated phenotype.

Collectively, these findings demonstrate that *Itk*^{-/-} Tregs are more prominently represented in disease-relevant tissues following transfer and exhibit increased AREG expression, providing additional phenotypic features associated with their enhanced protective activity during PH.

Adoptively Transferred *Itk*^{-/-} Tregs Protect Mice from PH, Reduce Proinflammatory Cytokine Levels, and Increase Anti-inflammatory Cytokine Levels

Tregs suppress autoimmune disease primarily by secreting inhibitory cytokines. These specialized signaling proteins actively dampen harmful immune responses, maintain self-tolerance, and protect healthy tissues from autoimmune destruction^{61,62}. In addition to their suppressive function, Tregs contribute directly to the restoration of the alveolar-capillary barrier following inflammatory injury^{56,63}.

To determine whether the adoptive transfer of Tregs from WT or *Itk*^{-/-} mice reduced proinflammatory cytokines or increased anti-inflammatory cytokines, we repeated the *in vivo* transfer experiments described above (Fig. 5C). WT (CD45.1) congenic mice were injected intraperitoneally with pristane (0.5 mL) to induce disease. On day 10, following the establishment of PH, CD25⁺Foxp3⁺ Tregs were FACS-sorted from the spleen of WT (CD45.2) and *Itk*^{-/-} (CD45.2) donor mice utilizing Foxp3-RFP reporter expression, and were subsequently transferred into the affected recipients (Fig. 6A-D).

By day 21 post-pristane injection, untreated WT mice developed severe gross pulmonary pathology alongside a significant increase in proteinuria relative to their baseline (day 0) levels (Fig. 6E). Recipients of WT Tregs exhibited only partial protection; while they showed a moderate reduction in proteinuria compared with untreated controls, their protein levels remained significantly elevated above baseline (Fig. 6F). In striking contrast, WT mice treated with *Itk*^{-/-} Tregs were fully protected from PH, with their proteinuria maintained at pre-challenge baseline levels (Fig. 6G). Together, these findings demonstrate that *Itk*^{-/-} Tregs possess enhanced suppressive and tissue-protective capacity *in vivo*, further supporting the modulation of the ITK axis as a promising therapeutic strategy for PH and severe autoimmune inflammatory injury.

Consistent with the reduced cytokine levels observed in *Itk*^{-/-} mice (Fig. 4C), we next asked whether the adoptive transfer of *Itk*^{-/-} Tregs could similarly reshape the systemic cytokine milieu in WT recipients. Serum ELISA analysis confirmed that pristane-challenged WT mice developed marked increases in IFN- γ , TNF- α , IL-6, and IL-17 (Fig. 6H-K). Although the transfer of WT Tregs produced a modest reduction in these proinflammatory cytokines, recipients of *Itk*^{-/-} Tregs exhibited significantly greater suppression of this inflammatory response. Notably, WT mice treated with *Itk*^{-/-} Tregs also showed a significant increase in serum IL-10, a key mediator of immune regulation and resolution, exceeding the levels observed in both untreated and WT Treg-treated groups (Fig. 6L).

These findings indicate that *Itk*^{-/-} Tregs not only suppress pathogenic Th1- and Th17-associated inflammatory responses more effectively than WT Tregs, but also actively promote an anti-inflammatory systemic environment. To determine whether *Itk*^{-/-} Tregs provide better long-term disease resolution, the recipient mice were monitored for survival. Our data confirmed that WT mice transplanted with *Itk*^{-/-} Tregs exhibited significantly prolonged survival compared with recipients of WT Tregs. Collectively, these results demonstrate that *Itk*^{-/-} Tregs confer enhanced protection against PH by shifting the cytokine balance from a proinflammatory toward a pro-resolving state.

ITK Deficiency Reprograms the Treg Transcriptome Toward a Metabolically and Functionally Enhanced State

Having established that *Itk*^{-/-} Tregs confer enhanced protection against PH, we next sought to define the global transcriptomic programs underlying this phenotype. We performed RNA sequencing (RNA-seq) on FACS-purified canonical (CD3⁺CD4⁺CD25⁺Foxp3⁺) Tregs from WT and *Itk*^{-/-} mice, as well as non-canonical (CD3⁺CD4⁺CD25⁻Foxp3⁺) Tregs from *Itk*^{-/-} mice. Notably, the non-canonical subset was selectively expanded in *Itk*^{-/-} mice, precluding direct comparison with an equivalent WT population. Hierarchical clustering of normalized gene expression profiles revealed distinct transcriptomic segregation, with samples partitioning into two principal modules: Module 1, consisting of genes downregulated in *Itk*^{-/-} subsets, and Module 2, comprising genes upregulated in *Itk*^{-/-} Tregs (Fig. 7A-B).

To identify the biological programs associated with the enhanced function of *Itk*^{-/-} Tregs, we performed Gene Set Enrichment Analysis (GSEA) and hallmark pathway profiling. Dot plot and ridge plot analyses of normalized enrichment scores (NES) revealed significant enrichment of multiple pathways in both canonical and non-canonical *Itk*^{-/-} Tregs, including MYC targets, mTORC1 signaling, oxidative phosphorylation (OXPHOS), and glycolysis (Fig. 7C-H). Additional enrichment was observed in pathways related to DNA repair, E2F targets, and immune homeostasis, alongside the differential regulation of IFN- γ and NF- κ B signaling. GSEA enrichment

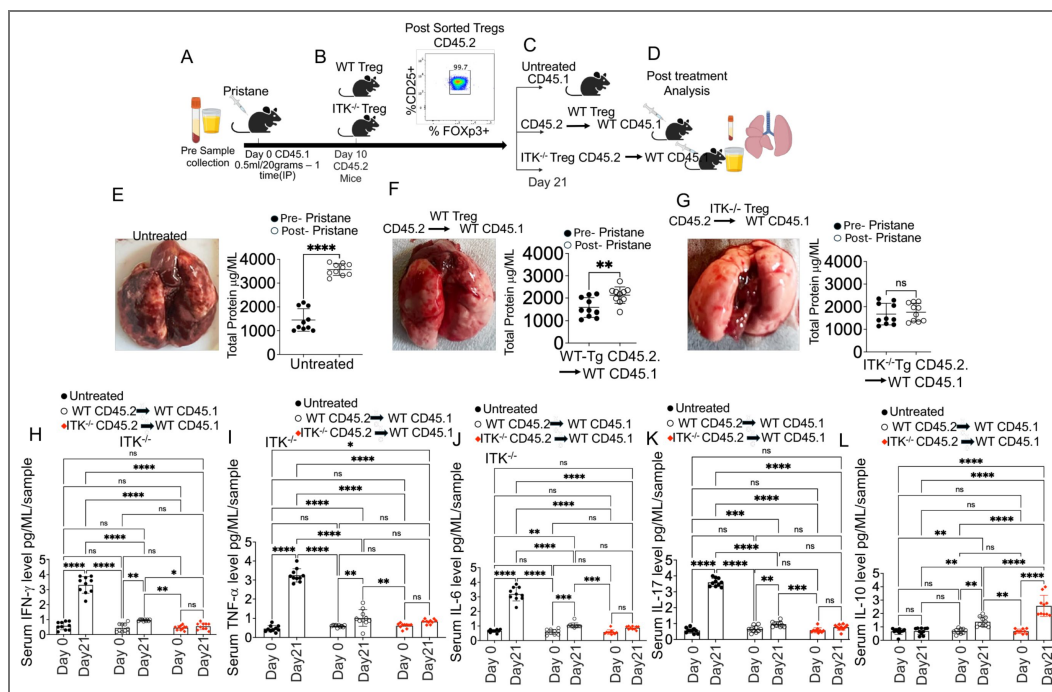


Figure 6. Adoptively transferred *Itk*^{-/-} Tregs protect mice from PH, reduce proinflammatory cytokine levels, and increase anti-inflammatory cytokine levels.

(A–D) Experimental schematic for the therapeutic adoptive transfer model. (A) Baseline serum and urine were collected from WT host mice (CD45.1⁺), followed by a single i.p. injection of 0.5 mL pristane. (B and C) On day 10, CD4⁺Foxp3⁺ Tregs were FACS-sorted from WT or *Itk*^{-/-} donor mice (CD45.2⁺), confirmed positive for CD25 and Foxp3, and injected i.v. into the pristane-treated CD45.1⁺ recipients. A control group of pristane-challenged CD45.1⁺ mice was left untreated. (D) On day 21, host mice were euthanized and analyzed for urinary protein and gross anatomical damage to the lungs. (E–G) Evaluation of therapeutic efficacy in lung and kidney injury. Representative gross lung images and longitudinal quantification of urinary protein (day 0 versus day 21) are shown for (E) untreated pristane-challenged mice, (F) mice treated with WT Tregs, and (G) mice treated with *Itk*^{-/-} Tregs. (H–L) Suppression of systemic inflammatory responses. Serum levels of proinflammatory cytokines (IFN- γ , TNF- α , IL-6, and IL-17) and the pro-resolving mediator IL-10 were quantified at baseline and day 21. *Itk*^{-/-} Tregs promoted a high-IL-10 regulatory environment while effectively suppressing the proinflammatory cytokine response. Data are presented as mean $\pm$ SEM ($n = 15$ –25 mice per group). Results are representative of 3 independent experiments. Statistical significance was determined by a two-tailed Student's *t*-test or a two-way ANOVA with Tukey's *post hoc* correction. ns, $p > 0.05$; * $p \leq 0.05$; $p \leq 0.01$; **** $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$.

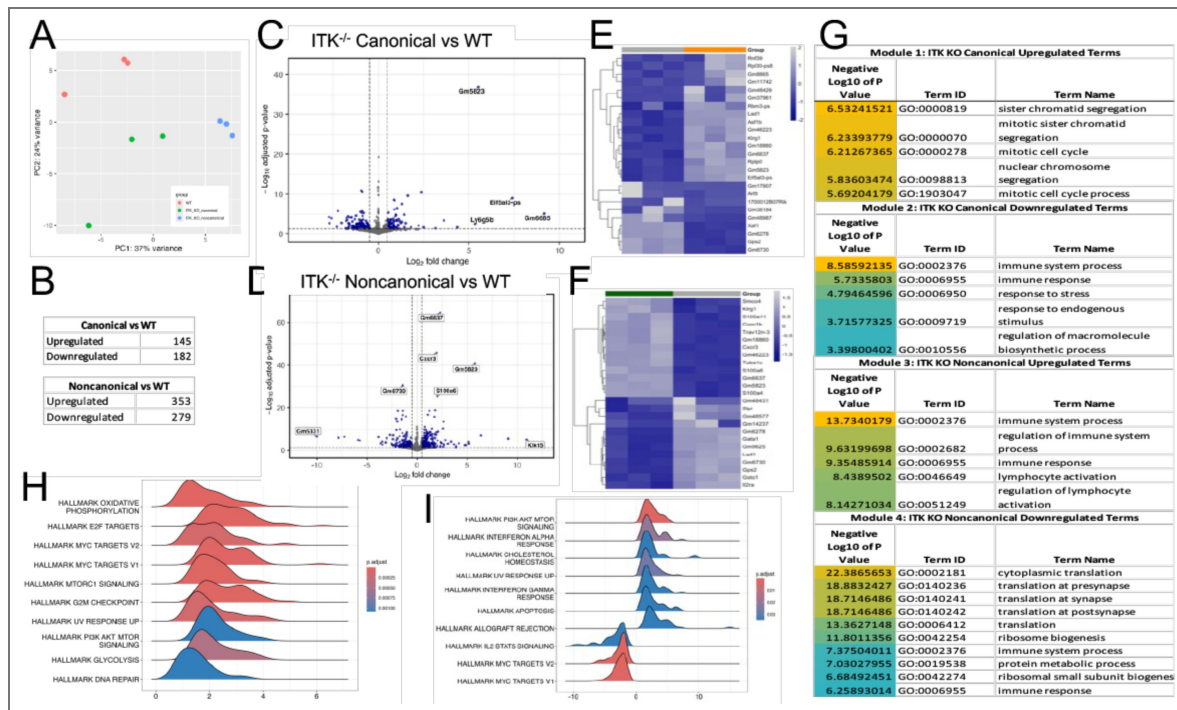


Figure 7. ITK deficiency reprograms the Treg transcriptome toward a metabolically and functionally altered state.

(A) Global transcriptomic relationships. Principal component analysis (PCA) of transcriptomes from WT and *Itk*^{-/-} canonical (CD25⁺) and non-canonical (CD25⁻) Tregs. All replicates are shown (*n* = 3). (B) Table showing the number of up- or downregulated differentially expressed genes (DEGs) between groups. (C and D) Transcriptomic landscape of ITK-deficient Tregs. Volcano plots identify the top DEGs between *Itk*^{-/-} and WT Tregs. Genes meeting the thresholds of FDR ≤ 0.05 and |log₂FC| ≥ 0.5 are highlighted, with a positive log₂FC indicating enrichment in *Itk*^{-/-} canonical (C) and non-canonical (D) Tregs. (E and F) Hierarchical clustering heatmaps showing row-scaled normalized expression of all significant DEGs in canonical CD4⁺CD25⁺Foxp3⁺ Tregs from WT and *Itk*^{-/-} mice (*n* = 3 per group). A grey bar indicates WT, orange indicates the canonical group, and green indicates the non-canonical group. (G) Tables showing GO enrichment analysis of the DEGs from *Itk*^{-/-} canonical (CD25⁺) and non-canonical (CD25⁻) Tregs. Using the online tool gProfiler and the ordered g:GOST query, we assessed which biological processes (BP) were linked to the genes in the different modules from *Itk*^{-/-} and WT mice. In the table, *p*-values are color-coded from light blue (not significant) to orange (highest significance) (*n* = 3 mice per group). (H and I) Gene Set Enrichment Analysis (GSEA) of regulatory pathways in canonical (H) and non-canonical (I) samples. Ridge plots show the distribution of ranked *DESeq2* Wald statistic values for selected Hallmark pathways, including oxidative phosphorylation (OXPHOS), mTORC1 signaling, and MYC targets, which together reflect enhanced metabolic fitness in ITK-deficient regulatory subsets.

plots further supported the conclusion that the loss of ITK signaling promotes a metabolically active and functionally enhanced Treg state, characterized by increased cell cycle- and metabolism-associated programs (Supplementary Fig. 9A-K [↗](#)). Together, these data indicate that ITK deficiency does not simply expand Treg numbers, but instead reprograms the Treg transcriptome toward a state consistent with greater metabolic fitness and regulatory function. These findings provide a mechanistic framework for the potent tissue-protective activity of *Itk*^{-/-} Tregs in PH.

Gene-Level Analyses Reveal Distinct Transcriptional Programs in Canonical and Non-Canonical *Itk*^{-/-} Tregs

To address whether these pathway level differences reflect coordinated changes in identifiable genes, we examined the leading-edge genes of each enriched pathway (Figure 8 [↗](#)). In canonical *Itk*^{-/-} Tregs the oxidative phosphorylation signature was carried by ninety-two leading edge genes, including subunits of complex I (Ndufs8, Ndufb8), complex III (Uqcrc1, Cyc1), complex IV (Cox5b) and the mitochondrial ATP synthase (Atp5f1b, Atp5f1d, Atp5mc1, Atp5mc3). None of these genes reached statistical significance on its own, so this signature consists of small increases distributed across the respiratory chain rather than a change in any single enzyme, and it would not have been recovered by differential expression testing alone. Glycolysis and DNA repair were also enriched in canonical *Itk*^{-/-} Tregs. The E2F and MYC target signatures, by contrast, were driven by genes with larger individual changes, including the replicative helicase components Mcm3, Mcm5 and Mcm7 together with Ccnb2, Birc5, Espl1, Asf1b and Mybl2. The four pathways drew on largely distinct gene sets, with 207 leading edge genes assigned to a single pathway and only three shared across three pathways, indicating that they are not restatements of a single signature.

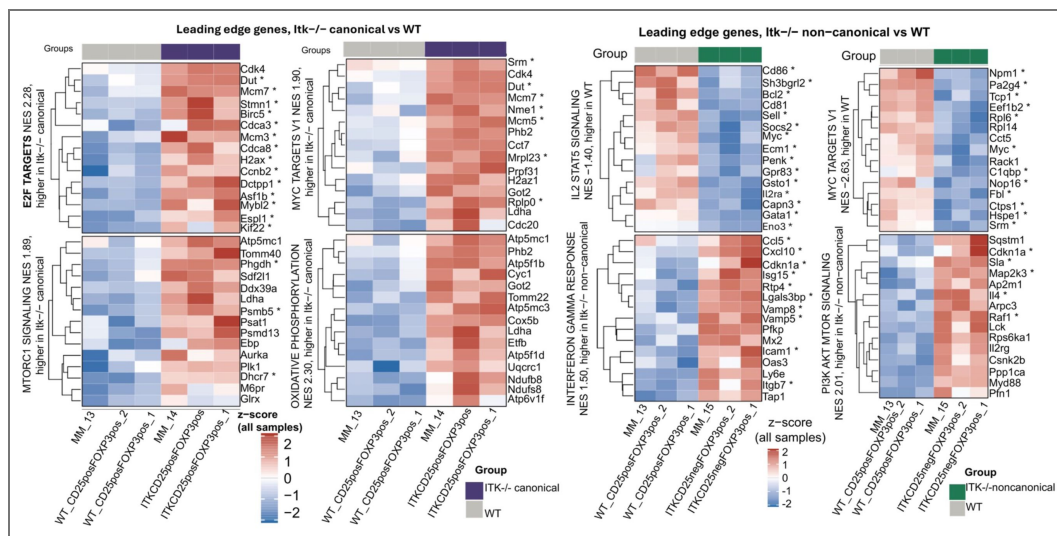
Gene Ontology analysis of the genes upregulated in canonical *Itk*^{-/-} Tregs returned terms centered on sister chromatid segregation, mitotic nuclear division and spindle attachment, driven by Espl1, Xrcc3, Spag5, Birc5, Ncaph, Mybl2, Cdca8 and Cdk1. No biological process term reached significance among the downregulated genes in this comparison.

Non-canonical *Itk*^{-/-} Tregs showed a partly opposing profile. MYC targets and IL2/STAT5 signalling were enriched in wild type rather than in *Itk*^{-/-} cells, the latter consistent with the CD25 negative phenotype used to define this subset, while PI3K/AKT/mTOR signalling and the interferon gamma response were enriched in *Itk*^{-/-} cells. The upregulated genes were annotated to lymphocyte and alpha beta T cell activation, leukocyte cell-cell adhesion and chemokine-mediated signalling, with *Ascl2* showing by far the largest change, followed by *Il4*, *Cxcr3*, *Prdm1*, *Ccr2*, *Tigit*, *Icos*, *Cxcr5*, *Tbx21* and *Runx3*. The downregulated genes were dominated by ribosome biogenesis and cytoplasmic translation, including *Npm1*, *Dkc1*, *Nop58* and numerous ribosomal protein genes. Gene significant hallmark, leading Edge gene among *Itk*^{-/-} Non-canonical Tregs and Canonical, leading, plotted Canonical, GSVE per samples, GO *Itk*^{-/-} Non-canonical Tregs and Canonical are listed in supplementary tables

Discussion

Pulmonary hemorrhage (PH) remains a life-threatening clinical emergency in systemic autoimmune disease. With mortality rates frequently exceeding 50%, there is a pressing need to move beyond broad immunosuppression toward therapeutic strategies that promote immune resolution and tissue protection^{64–68}. In this study, we identify ITK as a key molecular checkpoint that regulates the balance between destructive pulmonary inflammation and reparative immunity. Our findings demonstrate that ITK deficiency confers marked protection against PH and is associated with a broad remodeling of the T cell compartment toward a dominant regulatory state with features of enhanced metabolic fitness.

ITK has long been viewed as a signaling rheostat, whereby its absence or inhibition attenuates T cell receptor (TCR) signaling in ways that favor the development of memory CD8⁺ T cells and regulatory T cell lineages over pathogenic Th17 and Tfh populations^{9,12,19,23,69,70}. Although inherited ITK deficiency in humans is associated with impaired IFN-γ immunity and defective



viral control^{71,72}, our findings suggest that in the setting of acute autoimmune injury, reduced ITK signaling does not result in immune failure, but instead supports a more protective regulatory response. In particular, we show that *Itk*^{-/-} Tregs are not only expanded in number, but are also sufficient to rescue established PH in wild-type recipients. This regulatory reprogramming may help explain why ITK-deficient T cells, which we previously showed retain anti-tumor activity without inducing graft-versus-host disease (GVHD)¹⁷, are also capable of protecting the alveolar-capillary barrier during autoimmune injury.

Our study further shows that ITK deficiency reshapes the T cell compartment, characterized by the expansion of effector memory (EM) and central memory (CM) populations in both the CD4⁺ and CD8⁺ lineages. This memory-like shift is accompanied by increased expression of activation-associated markers and the T-box transcription factors Eomes and T-bet, which are known to support effector competence and durable memory differentiation^{37,73}. Interestingly, although T-bet is typically induced downstream of TCR, IL-12, and mTOR signaling, and can promote Eomes expression through IL-2/STAT5-dependent pathways^{34,37,74–76}, our findings suggest that ITK deficiency creates a distinct signaling context that favors the acquisition of an Eomes-rich, memory-like phenotype. While the role of ITK in T cell development is well established^{11,13,77}, our results extend its significance to systemic autoimmunity and inflammatory lung injury. We propose that this preconfigured memory-like state may contribute to protection during acute injury by enabling more controlled and effective immune responses, thereby limiting the unchecked innate inflammation that characterizes pristane-induced PH.

Utilizing the pristane-induced murine model, we show that ITK deficiency confers marked protection against PH, as evidenced by the preservation of lung architecture and a striking reduction in pulmonary hemorrhage. Acute PH is associated with robust recruitment of proinflammatory innate immune cells that disrupt the alveolar-capillary barrier^{33,78}. In this context, the loss of ITK signaling significantly reduced the accumulation of CD11b⁺Ly6C⁺ inflammatory monocytes and CD11b⁺Ly6G⁺ neutrophils in the lung, and was also associated with reduced inflammatory pathology in the spleen and gastrointestinal tract. These findings suggest that ITK contributes broadly to the inflammatory cascade that drives both pulmonary and systemic tissue injury in PH. This tissue protection was paralleled by a marked suppression of systemic inflammatory cytokines. ITK deficiency significantly reduced circulating levels of IFN- γ , IL-6, TNF- α , and IL-17—mediators known to promote vascular permeability, inflammatory amplification, and tissue injury during severe autoimmune inflammation^{19,79}. By limiting this proinflammatory milieu, modulation of the ITK axis may provide a dual therapeutic benefit: preserving alveolar-capillary barrier integrity while reducing the systemic inflammatory injury that contributes to poor outcomes in PH.

Beyond suppressing proinflammatory mediators, ITK deficiency also promoted a pro-resolving cytokine environment, including increased IL-10, IL-13, and, in some settings, IL-4^{80,81}. This shift is particularly relevant in PH, where early containment of inflammatory injury is likely critical for preserving alveolar-capillary barrier integrity. These mediators are known to act not only on immune cells but also directly on the lung epithelium and endothelium. This observation suggests a dual-action mechanism in which *Itk*^{-/-} Tregs suppress inflammatory infiltration while simultaneously signaling to the alveolar-capillary unit to enhance barrier integrity and promote tissue resilience. A central finding of our study is that the adoptive transfer of *Itk*^{-/-} Tregs was sufficient to rescue established PH in WT recipients^{20,82}. Because the therapeutic efficacy of Tregs is often limited by impaired stability or function under inflammatory stress^{83,84}, these findings suggest that the loss of ITK signaling supports a more durable regulatory program in vivo.

To define the mechanisms underlying this protective response, we examined the expression of Amphiregulin (AREG), a key regulator of tissue repair and immune stability^{26,85–90}. We demonstrated that Tregs isolated from the lungs of *Itk*^{-/-} mice express significantly higher levels of AREG, which actively protects mice from PH^{91,92}. Functional relevance was further supported by our adoptive transfer experiments, in which *Itk*^{-/-} Tregs conferred robust protection, suppressing IFN- γ , TNF- α , and IL-17 while simultaneously enhancing IL-10 production (Fig. 6 [↗](#)).

Our transcriptomic analysis provides a mechanistic framework for this phenotype. RNA sequencing revealed that ITK deficiency reprograms the Treg transcriptional landscape, with enrichment of pathways linked to oxidative phosphorylation (OXPHOS)⁹³, mTORC1^{94,95}, STAT5 signaling^{96,97}, and tissue repair. Together with the observed changes in glycolytic and cell cycle-associated programs, these data suggest that *Itk*^{-/-} Tregs acquire a state of enhanced metabolic fitness that may support their persistence and suppressive activity in inflamed tissue. Thus, the loss of ITK signaling appears to generate not simply more Tregs, but Tregs with molecular features consistent with heightened regulatory capacity.

Collectively, these findings establish ITK as a promising therapeutic target in PH. Modulating the ITK axis may offer a strategy to suppress systemic inflammation while promoting tissue-protective immune regulation and the restoration of alveolar-capillary barrier integrity. More broadly, our study provides a mechanistic rationale for developing ITK-directed approaches in severe inflammatory lung disease. While our findings in the pristane-induced model are striking, further studies are required to determine whether pharmacological ITK inhibition can fully phenocopy the genetic deficiency observed here, and to evaluate the long-term stability of these reprogrammed Tregs in chronic models of systemic autoimmunity.

Materials and Methods

Mice

Both male and female mice were included as a biological variable in all studies, as previously described^{35,98}. C57BL/6 mice were obtained from Charles River Laboratories or The Jackson Laboratory. All experiments were performed using mice 8–12 weeks of age, with age- and sex-matched controls included throughout. *ITK*^{-/-} mice and WT mice were bred onto the C57BL/6 background and crossed with the *Foxp3*^{tm1Flv/J} strain, an X-linked knock-in reporter in which *Foxp3*-expressing cells are co-marked with monomeric red fluorescent protein (mRFP). In this model, mRFP faithfully reports endogenous *Foxp3* expression. *ITK*^{-/-} and WT *Foxp3*-RFP mice were generously provided by Dr. Avery August (Cornell University)^{14,17,19}.

Reagents, cell lines, flow cytometry

Spleen or lung tissues were processed into single-cell suspensions. For surface staining, cells were incubated with an Fc receptor- blocking antibody (anti-CD16/32) followed by fluorochrome-conjugated monoclonal antibodies at a 1:100 dilution (unless otherwise specified) for 30 minutes at 4°C. Antibodies were purchased from BioLegend, eBioscience, or BD Pharmingen. Surface-staining antibodies included anti-mouse CD3 (BV605, 100237), CD4 (PE, 100408; BV510, 100559), CD8 (PE/Cy7, 100722; APC/Cy7, 140421), CD11b (FITC, 101205; PerCP-Cy5.5, 450112-82), CD25 (FITC, 101907; FITC, 101908; Pacific Blue, 102021), CD44 (Pacific Blue, 156006), CD62L (APC, 161218; APC/Cy7, 304814), CD122 (APC, 105912; APC, 123213), and Ly-6C (APC, 128015). PE anti-mouse Ly-6G Antibody Cat#27608 For transcription factor and intracellular cytokine staining, cells were fixed and permeabilized using the

Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Intracellular antibodies included Eomes (PE, 12-4875-80; PE/Cy7, 25-4877-42), *Foxp3* (Pacific Blue, 126410), T-bet (FITC, 64481; BV421, 644816), TCF-1 (PE, 564217), IFN-γ (APC, 505810), and TNF-α (FITC, 502906). Dead cells were excluded using a LIVE/DEAD Fixable Viability Dye (BioLegend). Data were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star), with singlets identified by forward- and side-scatter properties as previously described^{19,58}.

Consumables

All general laboratory consumables and plasticware were sourced from EIMMUNA Medical Supply.

Proteinuria Assays

To assess renal involvement, urine was collected from mice at baseline (Day 0) and at specified intervals (Day 14 or Day 21) following pristane administration. Total urinary protein concentrations were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Cat# 23225) according to the manufacturer's instructions. Briefly, urine samples were diluted 1:10 in PBS, and 25 μ L of the diluted sample was combined with 200 μ L of the BCA working reagent (50:1 ratio of Reagents A:B) in a 96-well microplate. The plate was briefly centrifuged (30 s at 1,250 rpm) to ensure uniform mixing and incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm using a BioTek FLx800 plate reader. Protein concentrations (mg/mL) were determined using Gen5 software by interpolation from a bovine serum albumin (BSA) standard curve. Data were normalized to baseline values to account for physiological variation as previously shown by our lab and others²⁴.

Serum Cytokine Profiling

Systemic cytokine concentrations were assessed at baseline (day 0) and at the study endpoints (day 14 or day 21). Whole blood was collected via cardiac puncture, and serum was isolated by centrifugation. A comprehensive panel of cytokines—including IFN- γ , TNF- α , IL-5, IL-12, IL-6, IL-10, IL-9, IL-17A, IL-17F, IL-22, and IL-13—was quantified using the LEGENDplex™ Mouse Th Cytokine Panel (13-plex) (BioLegend, Cat# 741011) according to the manufacturer's instructions^{17,18}. Briefly, serum samples were incubated with capture beads, followed by detection antibodies and streptavidin-phycoerythrin (SA-PE). Data were acquired on a BD LSRFortessa (BD Biosciences). Absolute cytokine concentrations (pg/mL) were calculated using the LEGENDplex Data Analysis Software (BioLegend) by interpolation from a 5-parameter logistic (5PL) standard curve. All samples were analyzed in duplicate to ensure technical reproducibility, as previously described^{17,18,24,32,58,99}.

Histopathological Evaluation

Following euthanasia at day 14 or day 21 post-pristane challenge, systemic organs—including the lungs, liver, spleen, kidneys, and small intestine—were harvested and fixed in 10% neutral-buffered formalin for 24–48 hours. Tissues were processed for paraffin embedding, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) by the Histology Core Facility at SUNY Upstate Medical University. Histopathological evaluation, specifically focusing on the severity of PH, alveolar-capillary disruption, and systemic inflammatory infiltration, was performed by a board-certified pathologist (L.C.) who was blinded to the experimental groups. Tissue injury was graded according to previously established semi-quantitative criteria^{24,100,101}. Representative images were captured using a light microscope, and histopathology scores were analyzed using the Mann-Whitney *U* test for non-parametric comparisons.

Fluorescence microscopy and histologic analysis

WT mice carrying the CD45.1 congenic marker were induced to develop PH. On day 10 post-induction, these CD45.1 recipients were adoptively transferred with Tregs from either WT or *Itk*^{-/-} mice carrying the CD45.2 congenic marker. On day 21 recipient mice were euthanized. Circulating blood was cleared by gentle cardiac perfusion with 2 mL of warm PBS using a 25-gauge needle, followed by tracheal inflation with a 1:1 mixture of PBS and O.C.T. compound to preserve alveolar architecture. Lungs were excised, embedded in O.C.T. compound, flash-frozen, and cryosectioned at 10 μ m. Sections were fixed in acetone (5 minutes, room temperature), blocked for endogenous biotin, and stained overnight at 4°C.

For immunohistochemistry (IHC), primary antibodies included anti-CD4 (clone GK1.5, Thermo Fisher Scientific; Cat# 14-0041-82), anti-CD45.2 (Thermo Fisher Scientific; Cat# 14-0454-82), and anti-Foxp3 (Thermo Fisher Scientific; Cat# 14-5773-82). Additional reagents included an Avidin/Biotin blocking kit (Thermo Fisher Scientific; Cat# 001303), a rat IgG2a isotype control (Thermo Fisher Scientific; Cat# PA533213), Streptavidin Alexa Fluor 594 (Thermo Fisher Scientific;

Cat# S11227), DAPI (4',6-diamidino-2- phenylindole dihydrochloride; Thermo Fisher Scientific; Cat# 62247), and a goat anti-mouse IgG secondary antibody, Alexa Fluor 647 (Invitrogen; Cat# A32728TR). Images were acquired using a Zeiss Axioplan microscope equipped with AxioVision 4.8 software. Histologic scoring was performed by a board-certified pathologist blinded to the experimental groups, as previously described^{24,102}.

Isolation and Purification of Regulatory T Cells

To facilitate the live isolation of regulatory T cells, we utilized *Itk*^{-/-} and WT Foxp3-RFP reporter mice on the C57BL/6 background. In this X-linked knock-in model, monomeric red fluorescent protein (mRFP) faithfully reports endogenous Foxp3 expression. Foxp3-RFP expression was confirmed in both WT and *Itk*^{-/-} cohorts prior to experimentation. Single-cell suspensions were prepared from spleens, or lungs and CD4⁺ lymphocytes were pre-enriched using anti-CD4 magnetic microbeads and MACS column-based separation (Miltenyi Biotec, Cat# 130-114-043). For high-purity isolation, pre-enriched cells were stained with antibodies against CD3 and CD25. Canonical Tregs were identified and purified as CD3⁺CD4⁺CD25⁺Foxp3(RFP)⁺ cells, while non-canonical Tregs were identified as CD3⁺CD4⁺CD25⁻Foxp3(RFP)⁺ cells. Fluorescence-activated cell sorting (FACS) was performed on a BD FACSaria IIIu (BD Biosciences), with sorted Treg purity routinely exceeding 98%. Purified cells were used immediately for RNA sequencing or adoptive transfer. Unless otherwise noted, all cell culture reagents were obtained from Sigma- Aldrich or Invitrogen, consistent with previously established protocols^{20,24,58}.

Western blotting

For the immunoblotting data shown in Figure 5K²⁴, Tregs from either WT or *Itk*^{-/-} mice were isolated from lung tissue. Briefly, lungs were gently homogenized, and total lung cellularity was quantified. Lung CD4⁺ T cells were then enriched by MACS using CD4 microbeads (Miltenyi Biotec; Cat# 130-114-043). These cells were further purified by FACS for CD4⁺, CD25⁺, and Foxp3⁺ (via the RFP reporter). The sorted Tregs were lysed in freshly prepared RIPA buffer (Fisher Scientific; Cat# PI9900) supplemented with a complete protease inhibitor cocktail (Sigma-Aldrich; Cat# 11697498001) and clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C. Lysates equivalent to 1 × 10⁶ cells per sample were resolved on 12%–18% SDS–polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting. Unless otherwise specified, reagents were obtained from Invitrogen (Grand Island, NY) or Sigma-Aldrich (St. Louis, MO). For analyses, membranes were probed with antibodies against Amphiregulin (AREG; clone AREG559, Thermo Fisher Scientific; Cat# 14-9999-82) and β-actin (Cell Signaling Technology; Cat# 4970), as previously described²⁴.

Statistics

Statistical analyses were performed using GraphPad Prism 10. Unless otherwise specified in the figure legends, data are presented as mean ± SD. For comparisons between two groups, an unpaired two-tailed Student's *t*-test was used. For comparisons involving multiple groups, a one-way or two-way ANOVA was performed, followed by Tukey's *post hoc* test for multiple comparisons. Non-parametric data, such as histopathology scores, were analyzed using the Mann-Whitney *U* test. A *p* value of ≤ 0.05 was considered statistically significant (**p* ≤ 0.05, *p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001). For *in vivo* studies, cohorts of *n* = 10–22 age- and sex-matched mice were used per group. All experiments were independently repeated at least twice to ensure reproducibility. Sample sizes were determined based on power calculations to achieve a minimum power of 0.8, and the experimental design was consistent with previously established protocols^{17-20,24,32,35,36,58,99,102-113}.

RNA Sequencing and Transcriptomic Analysis

For transcriptomic profiling, canonical and non-canonical Tregs were FACS-purified from the spleens of WT (*n* = 3) and *Itk*^{-/-} (*n* = 3) mice, as described above. Total RNA was extracted, and library preparation and high-throughput sequencing were performed by the Molecular Analysis

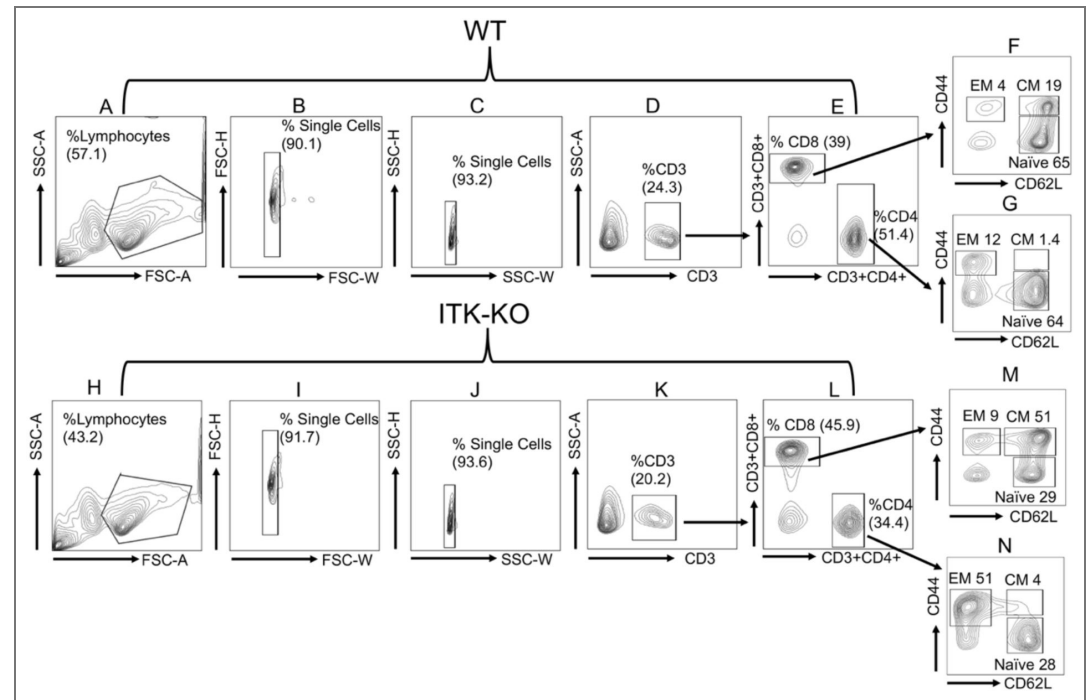
Core Facility at SUNY Upstate Medical University. Raw sequencing data were processed in R (v4.5.1) using RStudio (v2025.05.1) and Bioconductor packages. Transcript abundance was quantified via pseudoalignment with Kallisto (v0.46.2) ¹¹⁴. Transcript abundance estimates were imported with tximport ¹¹⁵, summarized to the gene level, and converted to scaled TPM values.

Genes with low expression were filtered prior to model fitting. Differentially expressed genes (DEGs) were identified using DESeq2 ¹¹⁶, with significance defined using a Benjamini-Hochberg ¹¹⁷, adjusted false discovery rate (FDR) ≤ 0.05 and a $|\log_2 \text{fold change}| \geq 0.5$. Variance-stabilized expression data were visualized using principal component analysis (PCA) and hierarchical clustering heatmaps generated with pheatmap ¹¹⁸. Functional annotation of identified up- and downregulated genes was performed by Gene Ontology (GO) enrichment analysis using gprofiler2 (gost) ¹¹⁹. Gene Set Enrichment Analysis (GSEA) was conducted using clusterProfiler against the MSigDB Hallmark gene set, with the DESeq2 Wald statistic as the ranked input list. Normalized enrichment scores (NES) and ridge plots were used to identify metabolic and signaling pathways unique to ITK-deficient Treg subsets. All RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE185327.

Ethics Statement and Animal Welfare

All animal housing and experimental procedures were performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of SUNY Upstate Medical University (Protocol #443). All mice were maintained in a pathogen-free facility under a 12-hour light/dark cycle with *ad libitum* access to food and water. Every effort was made to minimize animal suffering during the pristane challenge and subsequent procedures.

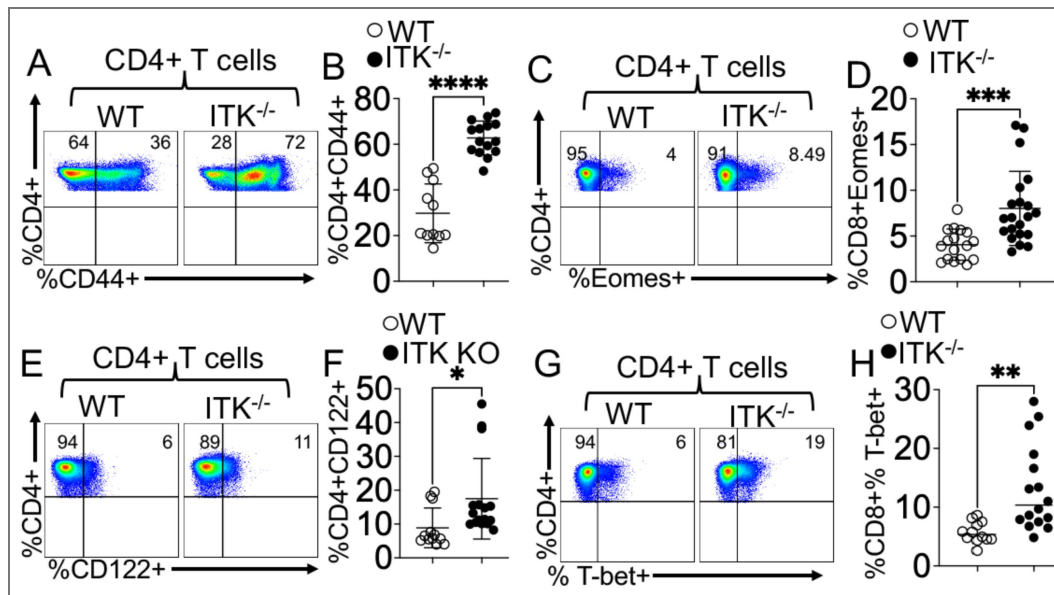
Supplemental Figures



Supplemental Figure 1. Gating strategies for effector and central memory phenotypes. Freshly isolated splenocytes from WT (A–G) and *Itk*^{-/-} (H–N) mice were analyzed by flow cytometry. (A, H) Lymphocyte populations were initially gated based on side scatter area (SSC-A) versus forward scatter area (FSC-A). (B, I) Lymphocytes were then gated for single cells using forward scatter height (FSC-H) versus forward scatter width (FSC-W) to discriminate doublets. (C, J) Additional singlet gating was performed using side scatter height (SSC-H) versus side scatter width (SSC-W) to exclude any remaining doublets. (D, K) CD3⁺ T lymphocytes were identified from the single-cell population based on SSC-A versus CD3 expression. (E, L) The CD3⁺ compartment was further subdivided into CD8⁺ and CD4⁺ T cell populations. (F, M) CD8⁺ and (G, N) CD4⁺ T cells were then analyzed to identify and quantify effector memory (EM; CD44⁺CD62L⁻), central memory (CM; CD44⁺CD62L⁺), and naive (CD44⁻CD62L⁺) subsets, as depicted in the contour plots.

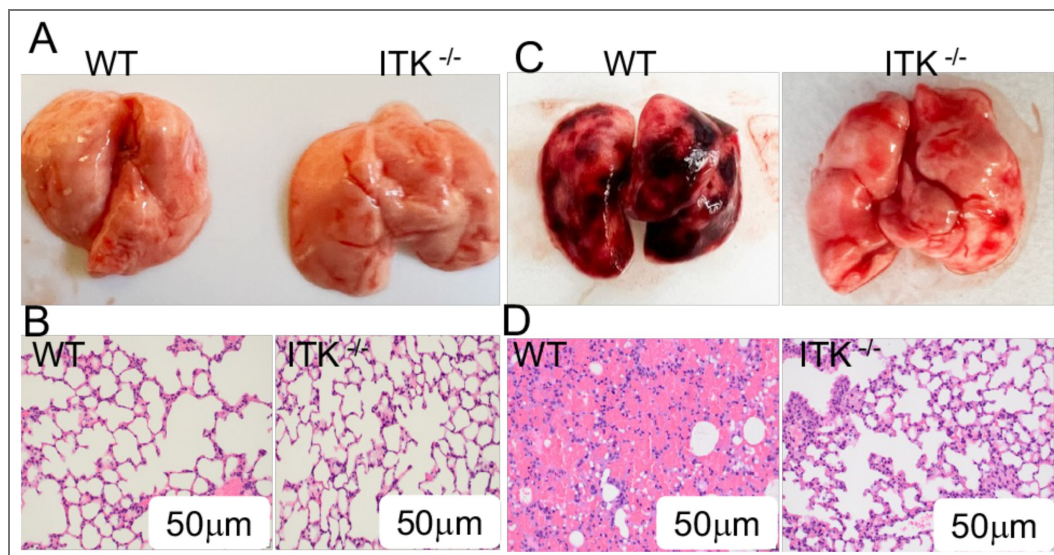
Supplemental Figure 2. ITK deficiency promotes an activated, memory-like program in CD4⁺ T cells.

(A and B) Surface expression of CD44 on splenic CD4⁺ T cells. Representative flow cytometry plots (A) and quantification of CD44⁺ frequencies (B) in WT and *Itk*^{-/-} mice. (C and D) Expression of the T-box transcription factor Eomes. Representative flow cytometry plots (C) and quantification of total Eomes⁺ CD4⁺ T cells (D) within the CD3⁺CD4⁺ gate. (E and F) Surface expression of CD122 (IL-2R β). Representative flow cytometry plots (E) and quantification of CD122 frequencies on CD4⁺ T cells (F) from WT and *Itk*^{-/-} mice. (G and H) Intracellular expression of the transcription factor T-bet. Representative flow cytometry plots (G) and quantification of T-bet frequencies within the CD3⁺CD4⁺ population (H). Data are presented as mean $\pm$ SEM ($n = 15$ –20 mice per group). Results are representative of at least 3 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's *t*-test *** $p < 0.001$, * $p \leq 0.05$ **** $p < 0.0001$, ** $p \leq 0.01$.



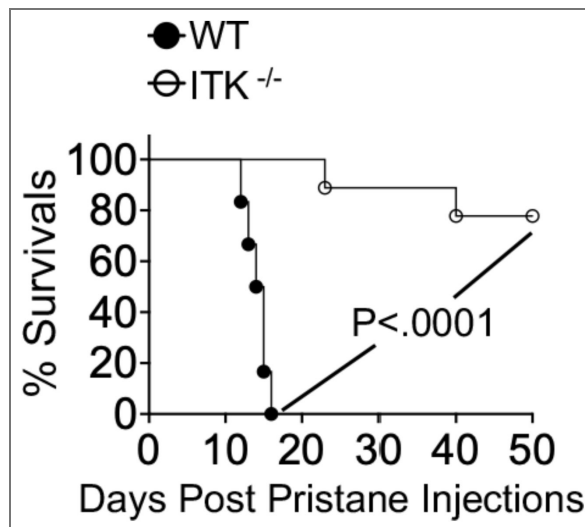
Supplemental Figure 3. ITK deficiency protects mice from pristane-induced PH and systemic pathology.

(A and B) Representative gross morphology of lungs and H&E-stained lung sections from WT and *Itk*^{-/-} mice without pristane injection. (C and D) Representative gross morphology and H&E-stained lung sections from WT and *Itk*^{-/-} mice 14 days after pristane injection.



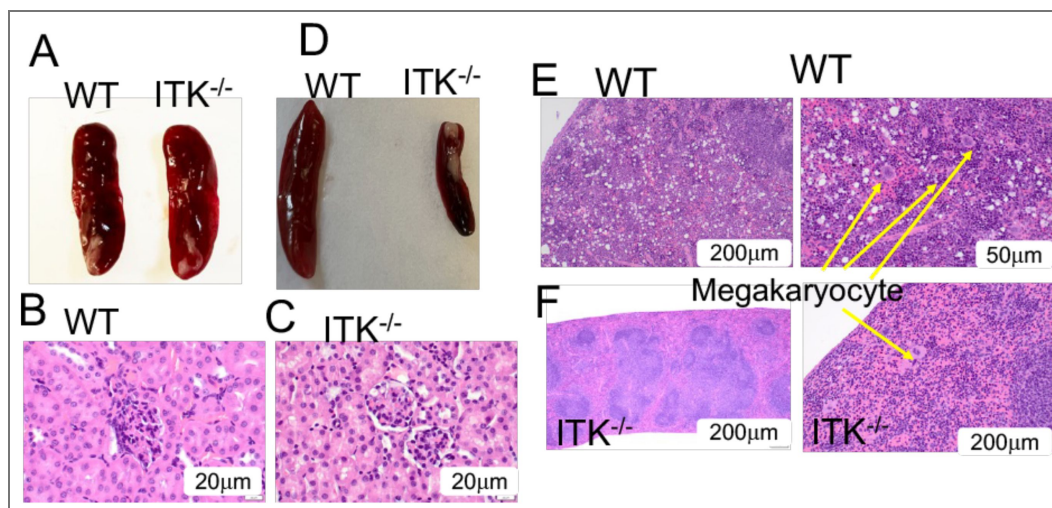
Supplemental Figure 4. ITK deficiency prolongs survival following pristane-induced PH.

WT and *Itk*^{-/-} mice received a single i.p. injection of 0.5 mL pristane to induce pulmonary hemorrhage (PH). Survival was monitored for 50 days post-injection.



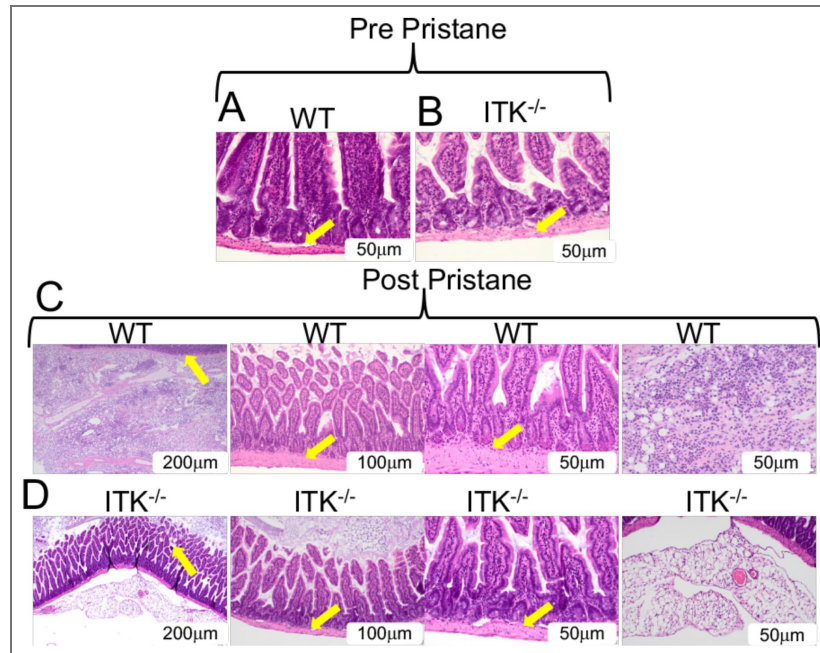
Supplemental Figure 5. ITK deficiency protects mice from pristane-induced PH and systemic pathology.

(A–C) Representative gross morphology of lungs and H&E-stained lung sections from either WT or *Itk*^{-/-} mice without pristane injection. (D–F) Representative gross images and H&E-stained sections of spleens from WT and *Itk*^{-/-} mice after pristane injection



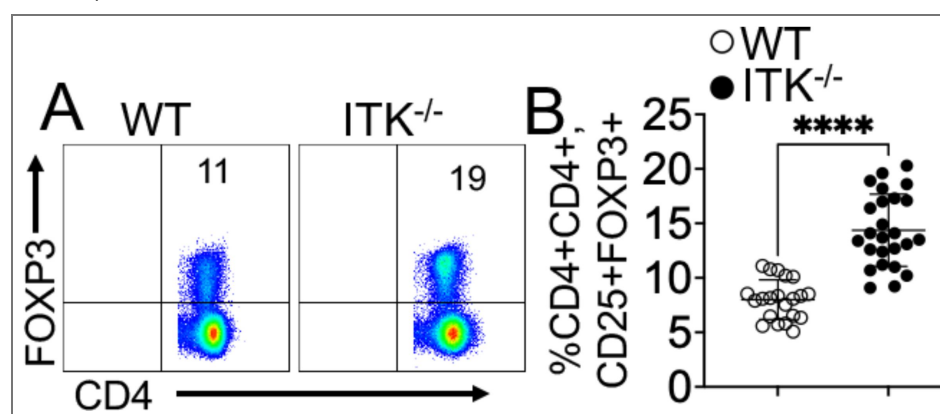
Supplemental Figure 6. ITK deficiency protects mice from pristane-induced small intestine pathology.

(A and B) Representative gross morphology and H&E-stained sections of the small intestine from either WT or *Itk*^{-/-} mice without pristane injection. (C and D) Histopathological evaluation of the small intestine. Representative H&E-stained sections of the small intestine from WT (C) and *Itk*^{-/-} (D) mice at day 14 post-pristane administration. In WT mice, systemic inflammatory injury is associated with evident mucosal damage, characterized by villus architecture disruption and increased inflammatory cell infiltration (black arrows). In contrast, *Itk*^{-/-} mice exhibited significant protection against this systemic injury, with preservation of mucosal integrity. Results are representative of 3 independent experiments.



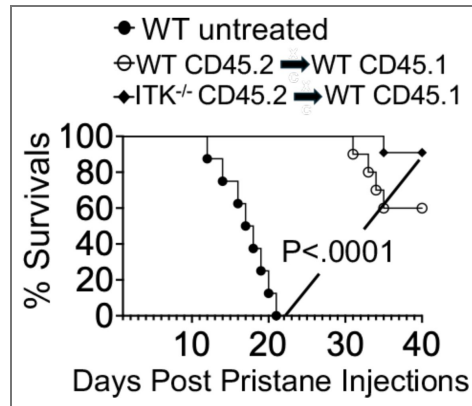
Supplemental Figure 7. ITK deficiency promotes the expansion of non-canonical Tregs.

(A and B) Quantitative analysis of Treg subsets at baseline. Representative flow cytometry plots (A) and statistical quantification (B) of freshly isolated splenic regulatory T cells from WT and *Itk*^{-/-} mice. Tregs were identified as CD3⁺CD4⁺Foxp3⁺ cells. ITK deficiency led to a significant increase in the frequency and absolute number of non-canonical (CD25⁻) Tregs compared with WT controls, while maintaining the canonical (CD25⁺) population. This expansion contributes to the broader regulatory reservoir observed in *Itk*^{-/-} mice. Data are presented as mean ± SEM (*n* = 15–20 mice per group). Results are representative of 3 independent experiments. Statistical significance was determined by an unpaired two-tailed Student's *t*-test. *****p* ≤ 0.0001.



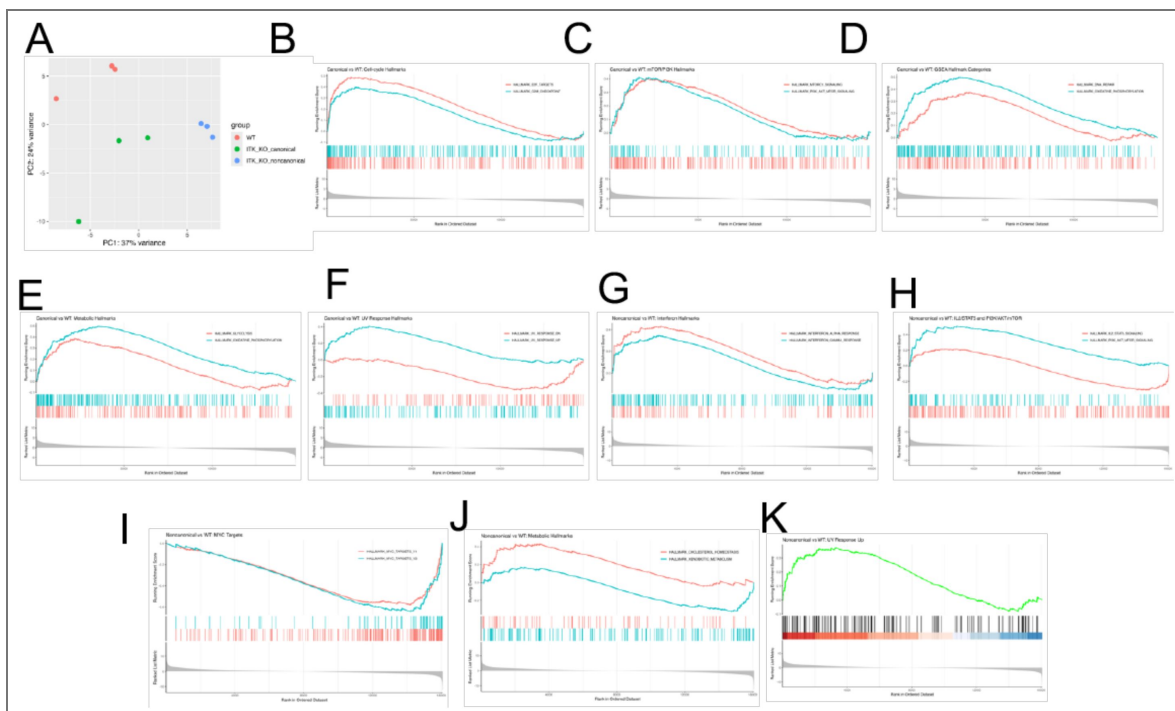
Supplemental Figure 8. Adoptive transfer of *Itk*^{-/-} Tregs prolongs survival following pristane-induced PH.

WT host mice (CD45.1⁺) received a single i.p. injection of 0.5 mL pristane. Mice were divided into three groups: left untreated, or adoptively transferred with Tregs isolated from either WT or *Itk*^{-/-} donor mice (CD45.2⁺). Survival was monitored for over 40 days post-injection.



Supplemental Figure 9. ITK Deficiency Reprograms Regulatory T Cells through Enhanced Metabolic and Signaling Circuitry.

(A–J) Enrichment of metabolic and survival pathways. Summary of MSigDB Hallmark pathway enrichment in canonical and non-canonical *Itk*^{-/-} Tregs relative to WT controls. GSEA enrichment score plots show running enrichment scores for selected Hallmark gene sets. Representative GSEA plots are shown for high-impact pathways, including cell cycle progression, mTORC1 signaling, PI3K/Akt/mTOR signaling, IL-2/STAT5 signaling, MYC targets, and oxidative phosphorylation. Notably, *Itk*^{-/-} Tregs exhibit coordinated upregulation of metabolic, survival, and stress-adaptation pathways, including interferon response and UV response/DNA repair signatures, consistent with enhanced resilience in inflammatory environments. Data are derived from transcriptomic analysis of n = 3 independent biological replicates per group.



Data availability

NCBI Gene Expression Omnibus (GEO) under accession number GSE185327.

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Additional information

Author contributions

M.S.H., H.X., A.M., and R.T.: Investigation, methodology, and data acquisition. **L.C. and L.S.:** Formal analysis and histological evaluation. **M.K.:** Conceptualization, supervision, project administration, funding acquisition, resources, data curation, and writing— original draft preparation, review, and editing.

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Peer reviews

Reviewer #1 (Public review):

In this study, Hossain et al. investigated the role of Interleukin-2-inducible T cell kinase (ITK) in autoimmune lung injury, demonstrating that ITK-deficient (*Itk*^{-/-}) mice are protected against pristane-induced pulmonary hemorrhage (PH). Authors suggest that this protection correlates with a significant remodeling of the T cell compartment in *Itk*^{-/-} mice, including increased frequency of memory-like CD4⁺ and CD8⁺ T cells (CD44⁺CD62L⁺) as well as higher frequency of Treg populations. Furthermore, adoptive transfer of ITK-deficient Treg isolated from injured ITK-deficient mice confers protection against pulmonary hemorrhage in WT recipients.

Strengths:

The adoptive transfer of congenically marked wildtype and *Itk*^{-/-} Treg populations clearly demonstrates that ITK-deficient Treg can actively rescue pre-existing lung injury and reverse systemic secondary metrics like proteinuria in wild-type recipients, providing proof-of-concept validation for the therapeutic utility of the ITK-Treg axis.

Weaknesses:

The molecular analysis of Tregs isolated from unchallenged wild-type ITK-deficient mice has limited value to understand how the ITK-deficient Tregs protect against autoimmune pulmonary hemorrhages in lungs. Detailed molecular analysis of Tregs isolated from the lung will be helpful in understanding the mechanistic regulation and identifying further therapeutic targets.

<https://doi.org/10.7554/eLife.111915.2.sa3>

Reviewer #2 (Public review):

Summary:

In this manuscript, Hossain and colleagues investigate the role of the ITK kinase in modulating inflammation in a pristane-induced lung hemorrhage model. Using a germline ITK KO mouse, they report that loss of ITK skews the T cell compartment toward a memory-like state, expanding Tregs, and conferring protection against alveolar hemorrhage, inflammatory monocyte recruitment, proteinuria, and systemic cytokine elevation. They further show that transfer of ITK-deficient Tregs into wild-type hosts with established disease attenuates injury and shifts the cytokine balance toward resolution, and that ITK-deficient

Tregs carry a transcriptional signature enriched for OXPHOS, mTORC1, MYC, and cell-cycle programs. While these observations are interesting for the development of potential immunotherapies, there are several issues with the methodological approach that support the authors' claims, tempering my enthusiasm for this manuscript.

Strengths:

(1) The clinical motivation and potential targeted therapies are relevant.

(2) The murine phenotype seems robust.

Comments on revised version.

The authors have addressed my comments appropriately.

<https://doi.org/10.7554/eLife.111915.2.sa2>

Reviewer #3 (Public review):

Summary:

Hossain et al. investigate the role of ITK as a central regulator of autoimmune lung injury. They used ITK-deficient mice and the pristane-induced pulmonary hemorrhage (PH) model to show that ITK deficiency confers protection against PH. The adoptive cell transfer experiment suggests a possible role for altered Treg cells in ITK-deficient mice in regulating the inflammatory response in the lungs of pristane-injected mice. This study shows that targeting the ITK axis may be beneficial by reducing systemic inflammatory injury that contributes to poor outcomes in PH.

Strengths:

This study highlights the importance of ITK in regulating pulmonary hemorrhage. The enrichment of Treg cells is known to confer protection in autoimmunity-mediated alveolar damage. However, the involvement of ITK in regulating the Treg cells' function is interesting and could be explored as a novel therapeutic approach for chronic inflammation.

Weakness:

The novelty of this study lies in the association between ITK-deficient Tregs and pulmonary hemorrhage in autoimmunity. One limitation of this study is that the authors performed all experiments in ITK global knockout mice, in which not only T cells but all other cell types are deficient in ITK. Therefore, further studies are required for a mechanistic rationale for targeting ITK in severe inflammatory lung disease.

Comments on revised version:

The authors have answered my comments by incorporating new results and clarification to their observation.

<https://doi.org/10.7554/eLife.111915.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

A primary limitation of this manuscript is its omission of foundational literature from the Schwartzberg and Littman laboratories, which originally established the indispensable role of IL-2-inducible T cell kinase (ITK) in proximal T-cell receptor (TCR) signaling dynamics and thymic lineage commitment. Because classic studies demonstrate that ITK is a critical regulator of thymic T cell development and cellular proliferation (PMID: 8777721, 10213685), the authors' claim that "these findings indicate that ITK deficiency skews the T cell compartment toward a memory-like state, establishing a distinct immune baseline that may favor protective and regulatory responses over pathogenic inflammation" is not substantiated by evidence and requires more robust validation.

We thank the reviewer for highlighting the foundational studies defining the role of ITK in proximal TCR signaling, thymic T-cell development, and proliferation. We agree that these studies provide essential context for interpreting the altered T-cell phenotype observed in *Itk*^{-/-} mice. Accordingly, we have revised the Introduction and Discussion to incorporate this established literature, including PMID: 8777721 and 10213685, and to more clearly acknowledge the established role of ITK in T-cell development and differentiation.

We also agree that our original statement suggesting that the memory-associated phenotype of *Itk*^{-/-} mice may establish an immune baseline favoring protection from pulmonary inflammation was not directly supported by our experiments. Our data demonstrate altered relative distributions of naïve and memory-phenotype CD4⁺ and CD8⁺ T cells, together with increased expression of CD44, CD122, Eomes, and T-bet, but do not establish that these baseline differences mediate protection from pulmonary hemorrhage.

We have therefore removed this causal interpretation and now present these findings as baseline characterization consistent with established ITK biology.

The exclusive reliance on splenic immunophenotyping is a major limitation, as it fails to capture the local cellular dynamics within the primary organs of injury (the lung and kidney). Evaluating canonical and non-canonical Treg expansion solely in the spleen overlooks the distinct functional programming of tissue-resident subsets. The authors should extend their characterization of regulatory T cell compartments directly to the lungs and draining lymphoid structures.

We thank the reviewer for this important suggestion. We agree that splenic analysis alone does not adequately capture the local regulatory T-cell response during pulmonary injury. We therefore expanded the revised manuscript to directly examine Treg populations in the lungs and draining lymph nodes.

We now quantify canonical (CD4⁺CD25⁺Foxp3⁺) and non-canonical (CD4⁺CD25⁻Foxp3⁺) Tregs in the lungs of WT and *Itk*^{-/-} mice, demonstrating that the altered regulatory phenotype associated with ITK deficiency is also evident locally in the lung (Figure 5E). In addition, using CD45.1 recipients and CD45.2 WT or *Itk*^{-/-} donor Tregs, we directly tracked transferred cells and observed significantly greater accumulation of *Itk*^{-/-} donor Tregs in both the lungs and draining lymph nodes (Figure 5F–I). Donor-derived CD4⁺Foxp3⁺CD45.2⁺ cells were also confirmed in these tissues by immunofluorescence microscopy (Figure 5J).

These new experiments extend our analysis beyond the spleen and provide direct evidence that the enhanced regulatory phenotype associated with ITK deficiency is present within tissues relevant to pulmonary inflammation.

More importantly, the authors overlook key historical publications that explicitly established ITK as a negative "rheostat" or gatekeeper for regulatory T cell (Treg) differentiation. Specifically, Huang et al. (PMID: 25063868) previously demonstrated that Treg abundance is inversely correlated with ITK expression, and that ITK activity serves as a vital negative tuner of IL-2-driven Foxp3⁺ Treg expansion. Since it is already well-

established that suppressing or deleting ITK promotes Treg accumulation and function, and that these cells are intrinsically vital to suppressing systemic autoimmunity, it is unclear how these findings expand upon our existing mechanistic understanding of ITK regulatory biology.

We thank the reviewer for highlighting the important study by Huang et al. (PMID: 25063868), which established ITK as a negative regulator of IL-2-driven Foxp3⁺ Treg expansion. We agree that increased Treg abundance following loss of ITK is established biology and should not be presented as a novel finding. We have revised the Introduction and Discussion accordingly and incorporated this foundational literature. The present study extends this established ITK–Treg relationship into autoimmune pulmonary hemorrhage by demonstrating functional and tissue-associated differences between WT and *Itk*^{−/−} Tregs. Equal-dose adoptive-transfer experiments showed that purified *Itk*^{−/−} Tregs provide substantially greater protection than WT Tregs in an otherwise ITK-sufficient host, including reduced pulmonary pathology and proteinuria and improved survival (Figures 5 and 6). Congenic CD45.1/CD45.2 tracking further demonstrated greater accumulation of *Itk*^{−/−} donor Tregs in the lungs and draining lymph nodes (Figure 5F–J).

In addition, lung-derived *Itk*^{−/−} Tregs expressed higher levels of Amphiregulin (AREG), identifying a tissue-repair-associated phenotype (Figure 5K), while RNA-seq demonstrated a distinct transcriptional state enriched for metabolic, proliferative, and regulatory programs (Figure 7). Thus, the advance of this study is not increased Treg abundance itself, but the demonstration that ITK-deficient Tregs exhibit enhanced protective activity and distinct tissue-associated properties during severe autoimmune pulmonary injury.

Reviewer #2 (Public review):

(1) All loss-of-function experiments are from a global ITK knockout. This is a major limitation and weakness of this study. The protection observed in the intact knockout, therefore, cannot be attributed to Tregs specifically. The Treg-intrinsic claim rests almost entirely on a single adoptive-transfer experiment. In order to show that this effect is Treg-specific, the authors would need to generate a Treg-specific ITK-deficient mouse.

We thank Reviewer #2 for highlighting this important limitation. We agree that experiments performed in global *Itk*^{−/−} mice cannot establish that the protective phenotype is specifically attributable to Treg-intrinsic loss of ITK, because other ITK-deficient immune populations may also contribute. We have revised the manuscript to make this limitation explicit and agree that a Treg-specific conditional model, such as Foxp3-Cre × *Itk*^{fl/fl} mice, would be required to establish Treg-specific necessity in the intact disease model. To directly assess the contribution of Tregs independently of the global knockout environment, we transferred equal numbers (1 × 10⁶) of FACS-purified WT or *Itk*^{−/−} CD4⁺CD25⁺Foxp3⁺ Tregs into pristane-challenged WT CD45.1 recipients. Thus, all endogenous immune and stromal compartments in the recipients remained ITK-sufficient. Under these conditions, *Itk*^{−/−} Tregs provided substantially greater protection than WT Tregs, including reduced pulmonary pathology and proteinuria and improved survival (Figures 5 and 6). Congenic tracking further demonstrated greater accumulation of *Itk*^{−/−} donor Tregs in the lungs and draining lymph nodes. We therefore conclude that purified *Itk*^{−/−} Tregs are sufficient to confer enhanced protective activity in an ITK-sufficient host, while recognizing that these experiments do not establish that Tregs are solely responsible for the protection observed in global *Itk*^{−/−} mice.

(2) In their sufficiency experiment (adoptive Treg cell transfer), donor and/or host cells are not congenially marked, so persistence, lung trafficking, and in vivo expansion of transferred Tregs are not demonstrated.

We thank Reviewer #2 for this important comment. To directly address this limitation, we performed new adoptive-transfer experiments using CD45.1 WT recipients and CD45.2 WT or

Itk^{-/-} donor Tregs, allowing transferred cells to be clearly distinguished from endogenous host cells. Following transfer, flow cytometric analysis demonstrated significantly greater accumulation of donor-derived *Itk*^{-/-} Tregs in the lungs and draining lymph nodes compared with WT donor Tregs (Figure 5F–I). Within the CD45.2⁺ donor population, cells retained Foxp3 expression, and both CD25⁺Foxp3⁺ and CD25⁻Foxp3⁺ regulatory populations were detectable at the experimental endpoint. The presence of donor-derived CD4⁺Foxp3⁺CD45.2⁺ cells within the lungs and draining lymph nodes was independently confirmed by immunofluorescence microscopy (Figure 5J).

These new experiments directly demonstrate localization and enhanced accumulation/recovery of *Itk*^{-/-} Tregs in disease-relevant tissues. However, because these are endpoint measurements, we have avoided attributing the increased donor-cell recovery specifically to proliferation or trafficking, as differences in survival, retention, or recruitment may also contribute.

(3) The authors claim that ITK-deficient Tregs possess enhanced metabolic fitness. This conclusion is based on transcriptional profiling of isolated splenic Tregs from unchallenged mice, yet it concerns lung protection during active disease narrative. Additional functional validation would be needed (Seahorse assay, mitochondrial mass/potential, etc). Some of these GSEA programs enriched in ITK-deficient Tregs could reflect a more general proliferative signature.

We thank Reviewer #2 for this thoughtful critique. We agree that enrichment of metabolic pathways by RNA-seq does not, by itself, demonstrate enhanced metabolic fitness and that direct functional assays, such as Seahorse analysis or measurements of mitochondrial mass and membrane potential, would be required to establish altered metabolic function.

We also acknowledge that our RNA-seq was performed on FACS-purified splenic Tregs from unchallenged mice and therefore does not directly define the transcriptional state of Tregs within the inflamed lung during active pulmonary hemorrhage. Our intent was to determine whether ITK deficiency is associated with baseline transcriptional differences prior to disease challenge. The analysis identified enrichment of oxidative phosphorylation, mTORC1, glycolysis, MYC, E2F, and cell-cycle-associated programs in *Itk*^{-/-} Tregs (Figure 7). We agree that some of these signatures may reflect proliferative or activation-associated transcription rather than enhanced metabolic function. We have revised the RNA-seq analysis, please updated Figure 8 and supplementary Tables. Accordingly, we have revised the Results and Discussion to describe these data as a distinct transcriptional state enriched for metabolic and proliferative programs, rather than direct evidence of enhanced metabolic fitness. We also note that disease-state lung Treg transcriptomics and direct metabolic assays will be required to determine the functional significance of these pathways during pulmonary injury.

Reviewer #3 (Public review):

The novelty of this study lies in the association between ITK-deficient Tregs and pulmonary hemorrhage in autoimmunity. The weakness of the manuscript is the lack of sufficient experiments to support the claim that ITK-deficient mice show protection specifically mediated by Treg cells, and to demonstrate that ITK-deficient Treg cells are more efficient than WT Treg cells in regulating other immune cells that drive pulmonary damage. The authors performed all the experiments in ITK global knockout mice, in which not only T cells but all other cell types are deficient in ITK. Furthermore, they have not performed any functional analysis to demonstrate the functional differences between WT Treg and ITK-deficient Treg cells, undermining the novelty of this study.

We sincerely thank Reviewer #3 for this constructive critique. We agree that experiments performed in global *Itk*^{-/-} mice alone cannot establish that protection is specifically mediated

by Tregs, because other ITK-deficient immune populations may also contribute. We have revised the manuscript to clearly acknowledge this limitation.

To directly compare the functional protective capacity of WT and *Itk*^{-/-} Tregs independently of the global knockout environment, we transferred equal numbers of FACS-purified CD4⁺CD25⁺Foxp3⁺ Tregs into pristane-challenged WT recipients. Under these conditions, *Itk*^{-/-} Tregs provided substantially greater protection than WT Tregs, including reduced pulmonary pathology and proteinuria, a more regulatory systemic cytokine profile, and improved survival (Figures 5 and 6). These experiments provide direct *in vivo* evidence that purified *Itk*^{-/-} Tregs have enhanced protective activity in an otherwise ITK-sufficient host.

We further performed CD45.1/CD45.2 congenic tracking and observed significantly greater accumulation of donor-derived *Itk*^{-/-} Tregs in the lungs and draining lymph nodes compared with WT donor Tregs (Figure 5F–J). Donor-derived cells retained Foxp3 expression following transfer. In addition, canonical Tregs isolated directly from *Itk*^{-/-} lungs expressed substantially higher levels of Amphiregulin (AREG), identifying a tissue-repair-associated phenotype that accompanies their enhanced protective activity (Figure 5K). We have also incorporated our previous functional studies demonstrating enhanced suppressive activity of *Itk*^{-/-} regulatory T-cell populations compared with WT Tregs (Mammadli et al., Clinical and Translational Medicine, 2021).

Thus, the advance of the present study is not simply increased Treg abundance after loss of ITK, but the demonstration that purified *Itk*^{-/-} Tregs provide greater protection than WT Tregs in an ITK-sufficient host, accumulate more prominently in disease-relevant tissues, and exhibit increased AREG expression. We nevertheless acknowledge that a Treg-specific conditional *Itk* knockout will ultimately be required to establish Treg-specific necessity in the intact disease model.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

Major concerns

(1) The authors demonstrate that the frequency of memory-like T cells is elevated in the spleens of Itk^{-/-} mice, but the absolute cellular numbers are notably absent. This proportional observation lacks conceptual novelty, as highly similar findings regarding the baseline expansion of memory-like/innate-like T cells under ITK-deficient conditions have been extensively established, most notably by the Schwartzberg laboratory (PMID: 16860760, 16860760). These early reports should be cited in the introduction and not presented as novel findings. More critically, the direct relevance of this baseline phenotype to the active autoimmune disease model remains unaddressed. The frequencies of these effector and memory-like compartments are evaluated exclusively in unperturbed, naïve animals and are never tracked or revisited during the subsequent kinetics of the pristane-induced lung injury model.

We thank Reviewer #1 for this important critique and for highlighting the foundational studies describing memory-like/innate-like T-cell development in ITK-deficient mice. We agree that this phenotype is well established and should not be presented as a novel finding. We have therefore incorporated the relevant literature, including PMID: 16860760, and now present Figures 1 and 2 as baseline characterization of the established *Itk*^{-/-} T-cell phenotype. We also agree that the frequency measurements in these figures do not demonstrate expansion in absolute cellular terms. Accordingly, we have removed the term “expansion” for this baseline naïve, central-memory, and effector-memory populations and now describe the findings as differences in their relative frequencies or distribution. Importantly, these analyses were performed in unchallenged mice, and we did not longitudinally track these memory-associated CD4⁺ and CD8⁺

populations during pristane-induced disease. We therefore no longer suggest that this baseline phenotype directly mediates protection from pulmonary hemorrhage. The revised manuscript now clearly separates this established baseline ITK phenotype from the disease-focused findings, with the mechanistic emphasis placed on the regulatory T-cell compartment, for which we provide direct functional and tissue-level analyses during pulmonary injury.

(2) This memory-like cell population expansion is observed strictly within naïve, SPF mice that have had no prior exposure to foreign antigens; it raises an important question of whether these are bona fide, antigen-experienced memory cells, or do they represent a "virtual memory" population (CD44⁺CD122⁺CD49d⁻) that typically arises from aberrant T cell development and homeostatic dysregulation? Prior work has already demonstrated that compromised TCR signal strength via ITK deficiency alters critical aspects of homeostatic proliferation, metabolic programming, and non-conventional effector differentiation in unexposed environments (Huang et al., 2018; bioRxiv, <https://doi.org/10.1101/359604>). Without providing absolute counts during active disease and ruling out a virtual memory phenotype, the authors' conclusion that this baseline skewing directly orchestrates active protection against pathogenic inflammation is currently unsupported.

We thank Reviewer #1 for this important comment. We agree that the CD44⁺CD122⁺ phenotype observed in naïve SPF *Itk*^{-/-} mice cannot be assumed to represent conventional antigen-experienced memory. Prior studies have shown that reduced ITK-dependent TCR signaling can promote innate-like or virtual-memory-associated T-cell populations under antigen-naïve conditions, and we have incorporated the relevant literature, including Huang et al., into the revised Introduction and Discussion. Because our experiments did not include CD49d or other markers required to distinguish conventional memory from virtual-memory cells, we have avoided assigning a definitive origin to these populations and now refer to them as memory-phenotype or memory-associated T cells. We also agree that these baseline measurements were obtained from unchallenged mice and that we did not determine absolute numbers or longitudinally track these populations during pristane-induced disease. We have therefore removed the interpretation that baseline memory-associated skewing directly promotes protection from pulmonary inflammation. Accordingly, Figures 1 and 2 are now presented as characterization of an established consequence of ITK deficiency, while our disease-focused mechanistic conclusions are based on the Treg experiments performed during pulmonary injury.

(3) The manuscript relies predominantly on qualitative histological representations to assess autoimmune tissue pathology. To substantiate the study's claims, the authors should provide standardized quantification of the histological changes. Additionally, direct measurement of autoimmune T cell responses, rather than descriptive tissue overviews, is required to definitively prove that ITK deficiency suppresses pathogenic autoimmunity.

We thank Reviewer #1 for these important comments. Regarding tissue pathology, the lung histology was evaluated using standardized semi-quantitative scoring performed by a board-certified pathologist blinded to genotype and treatment group. These quantitative scores are presented in Figure 3 and described in the Methods. We have revised the manuscript to make this analysis more explicit. The histopathologic findings are further supported by quantitative measurements of pulmonary CD11b⁺Ly6C⁺ inflammatory monocytes and CD11b⁺Ly6G⁺ neutrophils, as well as proteinuria as a measure of renal involvement (Figures 3 and 4). We also agree that direct analysis of antigen-specific pathogenic T-cell responses would provide additional mechanistic information. Such populations were not directly measured in the present study, and we have therefore revised the manuscript to avoid implying that ITK deficiency specifically suppresses autoreactive conventional T cells.

Instead, our data support the conclusion that ITK deficiency is associated with reduced inflammatory tissue injury and that purified *Itk*^{-/-} Tregs are sufficient to confer enhanced protection in WT recipients. This is supported by reduced pulmonary pathology, decreased inflammatory-cell accumulation, improved proteinuria, and the adoptive-transfer experiments. We have revised the Discussion accordingly to distinguish these directly demonstrated effects from the broader question of how ITK regulates antigen-specific autoimmune T-cell responses.

(4) While the adoptive transfer experiments suggest that Tregs from injured ITK-deficient animals possess enhanced protective capacity, the interpretation of these data is heavily confounded by a lack of absolute cellular quantification. Prior literature establishes that while ITK deficiency increases Treg frequency, the absolute number of Tregs remains unchanged due to concurrent reductions in global T cell cellularity (PMID: 25063868). Consequently, the authors must provide the absolute numbers of Tregs infiltrating the lungs both at baseline and post-injury. Since the authors adoptively transfer matched numbers of WT and KO Tregs, they are testing an artificial cellular ratio that may completely bypass the actual physiological abundance, contraction, or recruitment dynamics operating within the lung tissue in vivo. Furthermore, the current experimental design is insufficient to establish whether this protection depends specifically on injury-primed effector Treg programming or whether naïve ITK-deficient Tregs are sufficient.

We thank Reviewer #1 for this important critique. We agree that Treg frequencies alone do not establish physiological abundance, particularly because previous studies have shown that increased Treg frequency in *Itk*^{-/-} mice may occur without a proportional increase in absolute Treg number.

To address this concern, we have added absolute quantification of canonical (CD4⁺CD25⁺Foxp3⁺) and non-canonical (CD4⁺CD25⁻Foxp3⁺) Tregs in the lung at baseline and following adoptive transfer. Absolute numbers were determined from the total viable cellular recovery from each individual tissue together with the complete sequential flow-cytometric gating hierarchy. These analyses provide both frequency and absolute cellular abundance and demonstrate greater recovery of donor-derived *Itk*^{-/-} Tregs in the inflamed lung and draining lymph nodes following transfer (Figure 5E–I).

We also agree that equal-dose adoptive transfer does not reproduce the endogenous WT: *Itk*^{-/-} Treg ratio. Rather, this experiment was intentionally designed as a controlled functional comparison in which identical numbers of purified WT or *Itk*^{-/-} Tregs were introduced into the same ITK-sufficient recipient environment. The greater protection and tissue accumulation observed with *Itk*^{-/-} Tregs therefore reflect differences between the transferred populations under equivalent starting conditions, rather than differences in the number of cells administered. Importantly, the donor Tregs used for these experiments were isolated from unchallenged WT and *Itk*^{-/-} Foxp3-reporter mice. Thus, prior pristane-induced injury was not required for the enhanced protective activity of *Itk*^{-/-} Tregs. We have clarified this point in the revised manuscript.

Together, these analyses distinguish Treg frequency from physiological abundance, provide quantitative assessment of donor-cell recovery in disease-relevant tissues, and demonstrate that the enhanced protective capacity of *Itk*^{-/-} Tregs does not require prior injury-dependent priming.

(5) The transcriptomic analyses suggest metabolic and functional reprogramming of ITK-deficient Tregs. However, the interpretation of these findings currently remains speculative, as it relies on pathway analysis and no key genes involved in any of these programs were identified/highlighted as key differentially expressed genes. Lastly, there is no functional validation to suggest that these transcriptional changes are functionally relevant.

We thank Reviewer #1 for this important critique. We agree that pathway-level enrichment alone does not establish functional metabolic reprogramming and that the genes underlying these signatures should be provided more clearly. To address this concern, we have expanded the transcriptomic analysis to identify the individual differentially expressed genes contributing to the major pathways observed in *Itk*^{-/-} Tregs, including mTORC1, oxidative phosphorylation, MYC/E2F, glycolytic, and regulatory programs. We have revised Figure 8 and added supplementary tables providing the gene-level differential-expression data for the canonical and non-canonical Treg comparisons, allowing the underlying molecular changes to be evaluated directly rather than relying solely on pathway enrichment scores. We also agree that these transcriptional signatures do not demonstrate enhanced metabolic fitness. Direct functional assays, such as extracellular flux analysis, mitochondrial mass or membrane-potential measurements, would be required to establish altered metabolic function. We have therefore revised the Results and Discussion to describe *Itk*^{-/-} Tregs as exhibiting a distinct transcriptional state enriched for metabolic, proliferative, and regulatory programs, while acknowledging that some pathways may reflect proliferation or activation.

Separately, lung-derived *Itk*^{-/-} Tregs showed increased AREG protein expression (Figure 5K), which we interpret as a tissue-repair-associated phenotype rather than functional validation of the metabolic pathways.

Thus, we have added gene-level resolution to the RNA-seq analysis while narrowing our mechanistic interpretation to what is directly supported by the data. Accordingly, we have revised the Results and Discussion to describe these data as a distinct transcriptional state enriched for metabolic and proliferative programs, rather than direct evidence of enhanced metabolic fitness.

Minor concerns

(1) The introduction is structurally imbalanced, with three extensive paragraphs dedicated to describing and discussing the study's results. On the contrary, critical historical studies are missing from this section.

We thank Reviewer #1 for this constructive comment. We agree that the original Introduction devoted excessive space to summarizing our findings while providing insufficient historical context for ITK biology. We have therefore substantially shortened and reorganized the Introduction. The revised version now incorporates the foundational studies defining ITK function in TCR signaling and T-cell development, as well as the work establishing ITK as a regulator of Foxp3⁺ Treg differentiation and expansion. We also removed the extended discussion of our results and now conclude the Introduction with a concise statement of the knowledge gap and objective of the present study.

(2) In Figure 1A and 1E, a substantial CD44⁺CD62L⁻ population is present in both WT and ITK KO mice, potentially reflecting gating-related issues or loss of CD62L staining.

We thank Reviewer #1 for this careful observation. We agree that the CD44⁺CD62L⁻ population should be interpreted cautiously, as reduced CD62L staining may reflect biological variability or loss/shedding during tissue processing. To improve transparency, we have added the complete flow-cytometric gating strategy to the revised manuscript (Supplementary Figure 1; page 50, lines 845–855), including sequential exclusion of debris, doublets, and non-viable

cells before identification of CD3⁺, CD4⁺, and CD8⁺ T-cell populations. WT and *Itk*^{-/-} samples were processed and analyzed under identical conditions. Importantly, we do not assign biological significance to the CD44⁺CD62L⁺ population. Our conclusions are restricted to the conventionally defined naïve (CD44^{lo}CD62L^{hi}), central-memory (CD44^{hi}CD62L^{hi}), and effector memory (CD44^{hi}CD62L^{lo}) populations.

(3) The characterization of the myeloid compartment is limited given the highly complex nature of pulmonary inflammation in small vessel vasculitis models. To rigorously evaluate the local immune microenvironment, the authors must provide a detailed profiling of key populations within the affected tissues, including alveolar macrophages, interstitial macrophages, neutrophils, and eosinophils.

We thank Reviewer #1 for this important comment. We agree that pulmonary inflammation involves a heterogeneous myeloid compartment and that more comprehensive profiling of alveolar macrophages, interstitial macrophages, eosinophils, and other subsets would provide additional insight. In the present study, our myeloid analysis focused on two populations strongly associated with acute inflammatory lung injury: CD11b⁺Ly6C⁺ inflammatory monocytes and CD11b⁺Ly6G⁺ neutrophils. Quantitative flow cytometry demonstrated significantly reduced accumulation of both populations in the lungs of pristane-treated *Itk*^{-/-} mice compared with WT controls (Figure 3H–I). We acknowledge that this does not represent comprehensive profiling of the pulmonary myeloid compartment, and we have revised the manuscript to make this limitation explicit and to avoid broader claims of global myeloid remodeling. We also note in the Discussion that detailed analysis of macrophage and granulocyte subsets will be important for determining how ITK-dependent regulatory responses influence the broader pulmonary inflammatory environment.

(4) Although the study focuses on suppression of autoimmunity, chronic ITK deficiency may plausibly impair protective immunity against infection or malignancy. Some discussion of these potential tradeoffs would improve the translational interpretation of the findings.

We thank Reviewer #1 for raising this important translational consideration. We agree that chronic or systemic inhibition of ITK could impair protective immunity, particularly against infection and potentially malignancy. We have expanded the Discussion to address these potential trade-offs. In particular, we now note that inherited ITK deficiency in humans has been associated with impaired protective immune responses and increased susceptibility to infection, emphasizing that prolonged systemic ITK suppression cannot be assumed to be without consequence. At the same time, our previous studies in alloimmunity demonstrated that attenuation of ITK signaling can markedly reduce graft-versus-host disease while preserving substantial graft-versus-leukaemia activity (PMIDs: 33324410, 33851101, and 34919342). These findings suggest that pathogenic and protective immune responses may differ in their dependence on ITK signaling. We therefore emphasize that the translational effects of ITK modulation will likely depend on the magnitude, duration, and cellular context of inhibition. Future studies will be needed to determine whether partial, transient, or cell-selective ITK targeting can retain anti-inflammatory and Treg-associated benefits while preserving antimicrobial and antitumor immunity.

Reviewer #3 (Recommendations for the authors):

The manuscript by Hossain et al investigates the role of ITK as a central regulator of autoimmune lung injury, using the pristane-induced pulmonary hemorrhage (PH) model.

The authors claim that the deficiency of ITK confers complete protection primarily by marked remodeling of the T cell compartment, including expansion of CD44⁺, CD122⁺, Eomes⁺, T-bet⁺ memory-like subsets, and significant enrichment of Foxp3⁺ regulatory T cells (Tregs). However, they have used the ITK global KO mouse model, which undermines

their claim. Adoptive transfer of Treg cells to suppress autoimmunity has been shown before in various studies. The authors have performed an adoptive transfer of ITK-deficient Tregs, which they claim are more efficient at suppressing autoimmunity, based solely on two cytokine levels in systemic circulation. Although the authors identified the ITK-deficient Treg as key mediators of tissue protection, they have not provided any functional characterization of ITK-deficient Treg cells as compared to WT Treg cells. There are a few comments that will help to strengthen the findings and establish the importance of ITK regarding autoimmune PH.

We sincerely thank Reviewer #3 for this rigorous and constructive critique. We agree that experiments performed in global *Itk*^{-/-} mice alone cannot establish that protection is specifically mediated by Tregs, because other ITK-deficient immune populations may also contribute. We have revised the manuscript to make this limitation explicit.

Importantly, the functional comparison between WT and *Itk*^{-/-} Tregs in the revised study extends beyond circulating cytokines. Equal numbers of FACS-purified WT or *Itk*^{-/-} CD4⁺CD25⁺Foxp3⁺ Tregs were transferred into pristane-challenged WT recipients, in which all endogenous immune and stromal compartments remained ITK-sufficient. Under these conditions, *Itk*^{-/-} Tregs provided substantially greater protection than WT Tregs, including reduced pulmonary pathology and proteinuria, a more regulatory systemic cytokine profile, and improved survival (Figures 5 and 6).

These experiments demonstrate enhanced protective activity of purified *Itk*^{-/-} Tregs in an ITK-sufficient host. We further performed CD45.1/CD45.2 congenic tracking and observed greater accumulation of donor-derived *Itk*^{-/-} Tregs in the lungs and draining lymph nodes compared with WT donor Tregs (Figure 5F–J). In addition, lung-derived *Itk*^{-/-} Tregs expressed substantially higher levels of Amphiregulin (AREG), identifying a tissue-repair-associated phenotype (Figure 5K). We have also more clearly incorporated our previous functional study demonstrating enhanced suppressive activity of *Itk*^{-/-} regulatory T-cell populations compared with WT Tregs (PMID: 34919342).

Together, these findings extend previous work by demonstrating that *Itk*^{-/-} Tregs exhibit enhanced protective activity and distinct tissue-associated properties during autoimmune pulmonary haemorrhage. We nevertheless acknowledge that a Treg-specific conditional *Itk* knockout will ultimately be required to establish Treg-specific necessity in the intact disease model.

Major comments:

(1) In Figure 1, the authors have shown the T cell profile in the spleen from WT vs ITK-KO mice under homeostatic conditions. But it will be important to show the immune cell population, both innate and adaptive, in the lung, as it is the organ of focus for their study.

We sincerely thank Reviewer #3 for this important suggestion. We agree that because the lung is the primary site of injury in this model, characterization of the local pulmonary immune compartment is important.

In the revised manuscript, we have expanded our lung-focused analyses. We now directly quantify canonical (CD4⁺CD25⁺Foxp3⁺) and non-canonical (CD4⁺CD25⁻Foxp3⁺) Tregs in the lungs of WT and *Itk*^{-/-} mice (Figure 5E), demonstrating that the altered regulatory phenotype associated with ITK deficiency is also evident locally within the lung.

During pristane-induced disease, we additionally quantified key inflammatory innate populations and found significantly reduced accumulation of CD11b⁺Ly6C⁺ inflammatory monocytes and CD11b⁺Ly6G⁺ neutrophils in *Itk*^{-/-} lungs compared with WT controls (Figure 3H–I). Furthermore, using CD45.1/CD45.2 congenic adoptive transfer, we directly tracked WT

and *Itk*^{-/-} donor Tregs and observed greater accumulation of *Itk*^{-/-} Tregs in the lungs and draining lymph nodes (Figure 5F–J).

We acknowledge that the present study does not provide comprehensive profiling of all pulmonary immune populations, including macrophage subsets, eosinophils, and conventional effector T-cell populations. We have therefore revised the manuscript to make the scope of our lung immune analysis explicit and identify broader pulmonary immune profiling as an important area for future study

(2) The authors need to show the status of Treg cells in the spleen and lung of ITK KO mice under homeostatic conditions.

We thank Reviewer #3 for this important suggestion. We agree that defining the homeostatic Treg compartment in both the spleen and lung is important for interpreting the disease-associated changes.

In the revised manuscript, we now provide baseline characterization of canonical (CD4⁺CD25⁺Foxp3⁺) and non-canonical (CD4⁺CD25⁻Foxp3⁺) Tregs in naïve, unchallenged WT and *Itk*^{-/-} mice. Homeostatic splenic Tregs are shown in Supplementary Figure 7, demonstrating the established increase in the non-canonical Treg compartment in *Itk*^{-/-} mice. We also added direct analysis of lung Tregs under homeostatic conditions (Figure 5E), demonstrating that the altered regulatory phenotype associated with ITK deficiency is also present locally in the lung prior to pristane challenge.

These analyses establish the baseline Treg phenotype in both systemic and pulmonary compartments and provide an appropriate reference for interpreting the subsequent disease and adoptive-transfer experiments.

(3) The authors claim ITK deficiency shifts the memory T cell phenotype based on surface markers and transcription factors TBET and Eomes. It is well established that the memory T cell phenotype depends on a fine balance between TBET and Eomes (Cárdenas et al., Nature, 2024; Kar et al., Science Advances, 2026). Therefore, to support the claim, authors need to co-stain TBET and Eomes to show the relative balance of the TFs.

We thank Reviewer #3 for this important comment and for highlighting the literature emphasizing that memory T-cell differentiation is influenced by the relative balance of T-bet and Eomes. We agree that because T-bet and Eomes were measured independently in our experiments, our data do not establish their relative balance or co-expression at the single-cell level. Definitive analysis would require simultaneous co-staining of these transcription factors.

Accordingly, we have revised the manuscript to avoid interpreting T-bet and Eomes expression as evidence of a specific memory differentiation program. Figures 1 and 2 are now presented as baseline characterization of the established *Itk*^{-/-} T-cell phenotype. We describe the CD44/CD62L data as showing altered relative frequencies of naïve and memory-phenotype populations, while increased T-bet, Eomes, CD44, and CD122 expression is reported separately as a differentiation-associated phenotype.

We no longer infer that a specific T-bet/Eomes balance contributes to the memory phenotype or to protection from pulmonary hemorrhage, and we have incorporated the literature cited by the reviewer into the revised Discussion.

(4) A higher expression of TBET and Eomes could also lead to enhanced production of Th1 cytokines. Have the authors measured the CD4 T cell cytokines or CD8 T cell granzyme/perforin production in ITK KO mice?

We thank Reviewer #3 for this important point. We agree that increased T-bet and Eomes expression may be associated with altered CD4⁺ and CD8⁺ T-cell effector function and that direct functional measurements would be required to establish this.

In the present study, we did not directly measure cytokine production by conventional CD4⁺ or CD8⁺ T cells, nor did we quantify granzyme or perforin expression. Although systemic IFN- γ , TNF- α , IL-6, and IL-17 were measured during pristane-induced disease, these serum measurements do not identify the cellular source of the cytokines.

We have therefore revised the Results and Discussion to avoid inferring enhanced Th1 or cytotoxic function from increased T-bet and Eomes expression. Figures 1 and 2 are now presented as baseline phenotypic characterization of the conventional T-cell compartment, while the mechanistic conclusions of the study remain focused on the regulatory T-cell response during pulmonary hemorrhage.

We have also acknowledged that direct functional analysis of conventional CD4⁺ and CD8⁺ T cells will be required to define the consequences of altered T-bet and Eomes expression in this model.

(5) The authors have shown that ITK KO mice significantly restrict the pulmonary hemorrhage after 14 days post-pristane injection. Have the authors evaluated the findings at a longer time point, such as 6-8 weeks post-pristane, which is considered the optimal time point for disease progression? This will clarify whether ITK deficiency completely inhibits the autoimmunity or delays its progression.

We thank Reviewer #3 for raising this important point regarding disease kinetics. We agree that later time points, such as 6–8 weeks after pristane administration, are commonly used to evaluate chronic manifestations of pristane-induced systemic autoimmunity.

However, the pulmonary hemorrhage phenotype examined in our study follows a much more acute and severe course. We therefore extended our analysis to include survival and added these data to the revised manuscript (Figure 3—figure supplement 2). All pristane-treated WT mice died within approximately 20 days, whereas *Itk*^{-/-} mice exhibited significantly prolonged survival.

Because WT mice do not survive to the 6–8-week time point in this model, a direct WT versus *Itk*^{-/-} comparison at these later stages is not feasible without substantial survivor-selection bias. We have therefore clarified that the present study addresses the acute, severe phase of pristane-induced pulmonary hemorrhage.

Importantly, the survival data demonstrate that ITK deficiency protects against the early lethal phase of disease rather than simply delaying the day-14 pulmonary phenotype. However, these findings do not establish that ITK deficiency completely prevents later chronic manifestations of pristane-induced autoimmunity. We have added this limitation to the Discussion and identify longer-term analysis of surviving *Itk*^{-/-} mice as an important future direction.

(6) In Figure 3, the authors have shown that ITK KO mice show less monocyte infiltration in pristane-injected mice. However, they have not shown effector T cell percentages, such as Th1 and Th17, which are important drivers of disease progression. Given their focus on Treg cells, it will be important to show the effector T-cell population in the lung.

We thank Reviewer #3 for this important comment. We agree that direct quantification of pulmonary Th1 and Th17 populations would provide additional insight into the balance between pathogenic effector T cells and Tregs during pulmonary hemorrhage.

In the present study, we did not directly quantify lung Th1 or Th17 cells by intracellular cytokine or transcription-factor staining. We have therefore revised the manuscript to avoid implying that our experiments demonstrate direct suppression of these effector T-cell populations.

Our lung analysis instead demonstrated significantly reduced accumulation of CD11b⁺Ly6C⁺ inflammatory monocytes and CD11b⁺Ly6G⁺ neutrophils in *Itk*^{-/-} mice (Figure 3), together with increased pulmonary Treg populations (Figure 5). We also observed reduced systemic IFN- γ , TNF- α , IL-6, and IL-17 during disease (Figure 4); however, we recognize that these serum measurements do not establish the cellular source of these cytokines. Accordingly, we now distinguish the directly measured pulmonary cellular changes from broader Th1/Th17-associated inflammatory responses and identify direct profiling of lung effector T-cell populations as an important area for future investigation.

(7) In Figure 4, the authors have shown several plasma cytokine levels in KO vs WT mice to support their hypothesis. However, as their focus is on alveolar hemorrhage, they have also mentioned that these cytokines influence the pulmonary damage by altering neutrophil infiltration and vascular permeability. Showing data on neutrophil and vascular damage will strengthen their claim.

We thank Reviewer #3 for this constructive suggestion. We agree that direct assessment of pulmonary neutrophil accumulation and tissue injury provides important context for interpreting the systemic cytokine changes observed during pulmonary hemorrhage.

To address this concern, we added quantitative flow-cytometric analysis of pulmonary neutrophils. CD11b⁺Ly6G⁺ neutrophils were significantly reduced in the lungs of pristane-treated *Itk*^{-/-} mice compared with WT controls (Figure 3I). Together with the reduced CD11b⁺Ly6C⁺ inflammatory monocytes, these findings demonstrate decreased accumulation of major inflammatory innate-cell populations in the injured lung.

Pulmonary injury was also evaluated using blinded, standardized histopathologic scoring. WT mice exhibited extensive intra-alveolar hemorrhage, erythrocyte accumulation, and disruption of alveolar architecture, whereas *Itk*^{-/-} mice showed markedly reduced hemorrhage and preserved lung architecture (Figure 3B–E). We acknowledge that the present study does not include a direct assay of vascular permeability or endothelial barrier function. We have therefore revised the manuscript to describe these findings as evidence of reduced alveolarcapillary injury and pulmonary hemorrhage rather than as a direct demonstration of improved vascular permeability.

(8) The authors mentioned non-canonical Treg cells without proper characterization. These Treg cells are reported to lack CD25 and, in some cases, FOXP3, and to have reduced suppressive function. Therefore, if ITK KO mice show that both canonical and non-canonical Treg cells are increased, this raises the question of how that balance leads to less pulmonary hemorrhage. The authors need to clarify their findings and discuss the relevance.

We sincerely thank Reviewer #3 for raising this important point. In the present study, we define non-canonical Tregs specifically as CD3⁺CD4⁺CD25⁻Foxp3⁺ cells. These cells were identified using Foxp3-RFP reporter mice, and therefore the population analyzed here is Foxp3⁺CD25⁻ but lacks CD25.

We agree that CD25⁻Foxp3⁺ Tregs may exhibit reduced stability or suppressive function in some settings. However, in our previous study, we performed dedicated phenotypic and functional analyses of this population and demonstrated that *Itk*^{-/-} CD4⁺CD25⁻Foxp3⁺ Tregs possess substantial suppressive activity in both *in vitro* and *in vivo* assays (Mammadli et al.,

Clinical and Translational Medicine, 2021; PMID: 34919342). We have revised the manuscript to more clearly incorporate this prior work.

In the present study, both canonical CD25⁺Foxp3⁺ and non-canonical CD25⁻Foxp3⁺ Treg populations were increased in *Itk*^{-/-} mice, including in the lung. However, we did not independently test the protective function of the non-canonical subset. Importantly, the adoptive-transfer experiments were initiated with purified canonical CD25⁺Foxp3⁺ Tregs.

We have therefore revised the manuscript to avoid implying that non-canonical Tregs independently mediate protection from pulmonary hemorrhage. Instead, we interpret their increase as an additional feature of the altered regulatory T-cell compartment associated with ITK deficiency, while restricting the direct functional conclusion to the enhanced protective activity of purified *Itk*^{-/-} canonical Tregs.

| (9) Do the ITK KO mice have non-canonical Treg in the homeostatic lung?

We thank Reviewer #3 for this specific question. Yes, *Itk*^{-/-} mice contain noncanonical CD4⁺CD25⁻Foxp3⁺ Tregs in the lung under homeostatic conditions.

To directly address this point, we added flow-cytometric analysis of canonical CD4⁺CD25⁺Foxp3⁺ and non-canonical CD4⁺CD25⁻Foxp3⁺ Tregs in the lungs of naïve, unchallenged WT and *Itk*^{-/-} mice. These data demonstrate that non-canonical Tregs are present in the homeostatic lung and are increased in *Itk*^{-/-} mice compared with WT controls (Figure 5E).

Thus, the non-canonical Treg population observed during pulmonary hemorrhage is not exclusively induced by disease but is already present as part of the baseline regulatory T-cell phenotype associated with ITK deficiency

| (10) The authors stated that ITK^{-/-} Tregs confer enhanced protection against PH by shifting the cytokine balance from a proinflammatory toward a pro-resolving state. However, the adoptive transfer of ITK KO Treg results in differences only in IFN γ and IL10 levels compared with WT Treg. The authors need to clearly discuss this.

We thank Reviewer #3 for this careful observation. We agree that our original wording overstated the extent of the cytokine differences between recipients of WT and *Itk*^{-/-} Tregs. Upon direct comparison of the two Treg-treated groups, the clearest statistically significant differences were observed for IFN- γ and IL-10. Although *Itk*^{-/-} Treg treatment was associated with reductions in additional proinflammatory cytokines, these changes should not be interpreted as demonstrating significant superiority over WT Tregs for every cytokine measured.

We have therefore revised the Results and Discussion to emphasize that, compared with WT Tregs, *Itk*^{-/-} Tregs produced a greater reduction in IFN- γ together with increased IL-10, and we have removed the broader statement that they uniformly shift the entire systemic cytokine profile from a proinflammatory to a pro-resolving state.

Importantly, the enhanced protective activity of *Itk*^{-/-} Tregs is supported not only by cytokine measurements, but also by reduced pulmonary pathology and proteinuria, improved survival, and greater accumulation of donor-derived *Itk*^{-/-} Tregs in the lungs and draining lymph nodes.

| (11) The claim that ITK-deficient Treg is better than WT Treg is the novelty of the study. Therefore, the functional characterization, such as cytokine profile and suppressive functions, could be shown to support their claim. Adoptive transfer of WT-Treg cells provides significant protection, as shown by Dall'Era et al. (2019, Arthritis Rheumatol)

and by the authors in Figure 5. Providing supporting data on the advantages of *ITK-KO* Treg will be important for strengthening the study's novelty.

We sincerely thank Reviewer #3 for this important comment. We agree that the novelty of the present study is not simply that Tregs can protect against pulmonary hemorrhage, which has been previously demonstrated, but that *Itk*^{-/-} Tregs exhibit enhanced protective activity compared with WT Tregs in this disease setting.

To directly address this point, we compared equal numbers (1×10^6 cells per recipient) of FACS-purified WT or *Itk*^{-/-} CD4⁺CD25⁺Foxp3⁺ Tregs following transfer into pristane-challenged WT recipients. WT Tregs provided measurable protection, consistent with previous studies. Importantly, however, *Itk*^{-/-} Tregs provided substantially greater protection, including reduced pulmonary pathology and proteinuria and improved survival (Figures 5 and 6). Thus, the functional advantage of *Itk*^{-/-} Tregs is supported by disease outcomes beyond systemic cytokine measurements.

We further performed CD45.1/CD45.2 congenic tracking and observed greater accumulation/recovery of donor-derived *Itk*^{-/-} Tregs in the lungs and draining lymph nodes compared with WT donor Tregs (Figure 5F–J). In addition, *Itk*^{-/-} Treg-treated recipients showed a greater reduction in IFN- γ and increased IL-10 compared with WT Treg-treated recipients. Canonical Tregs isolated directly from *Itk*^{-/-} lungs also expressed substantially higher levels of AREG, identifying a tissue-repair-associated phenotype (Figure 5K).

We have also more clearly incorporated our previous functional study demonstrating enhanced suppressive activity of *Itk*^{-/-} regulatory T-cell populations in *in vitro* and *in vivo* assays (Mammadli et al., Clinical and Translational Medicine, 2021; PMID: 34919342).

Together, these findings demonstrate that the advantage of *Itk*^{-/-} Tregs over WT Tregs is supported by greater protection after equal-dose transfer, enhanced accumulation in disease-relevant tissues, differential regulation of IFN- γ and IL-10, and increased AREG expression. We have clarified that AREG is associated with this protective phenotype but has not been shown here to be required for protection.

Minor comments

(1) In Figure 3, the authors need to provide important experimental control as H & E lung and spleen images under homeostatic conditions, without pristane.

We thank Reviewer #3 for this important suggestion. We agree that homeostatic histological controls are necessary to determine whether the differences observed after pristane challenge reflect pre-existing tissue abnormalities. In the revised manuscript, we have added H&E analyses of lungs and spleens from naïve, unchallenged WT and *Itk*^{-/-} mice. Both genotypes exhibited normal baseline tissue architecture, with no evidence of pulmonary hemorrhage or abnormal splenic pathology (Figure 3—figure supplement 1A–D and Figure 3—figure supplement 3A–C). These controls confirm that the pulmonary and splenic abnormalities observed after pristane administration arise during disease rather than from pre-existing histological differences between WT and *Itk*^{-/-} mice.

(2) In Supplementary Figure 6, the axis legend needs correction.

We sincerely thank Reviewer #3 for the careful review and for identifying this error. We have corrected the axis legend accordingly. The corrected graph is now presented as Supplementary Figure 7 (page 56) in the revised manuscript.

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