Reviewed Preprint
v1 • July 24, 2026
Not revised

For correspondence:
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Competing interests: No competing interests declared

Funding: See page 19

Reviewing editor: Jalees Rehman, University of Illinois Chicago, United States

ITK Deficiency Attenuates Alveolar Hemorrhage by Enhancing Regulatory T Cell-Mediated Tissue Resilience

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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 a **valuable** addition to the fields of autoimmunity, inflammation, and T-cell biology in the lung. However, the experimental evidence supporting the underlying cellular and molecular mechanisms and the integration of foundational background literature to provide the necessary context are **incomplete**.

https://doi.org/10.7554/eLife.111915.1.sa3

Abstract

Pulmonary hemorrhage (PH) is a life-threatening manifestation of systemic autoimmunity characterized by immune-mediated disruption of the alveolar-capillary barrier. Despite mortality rates exceeding 50%, the molecular checkpoints that govern the transition from destructive inflammation to protective immune regulation remain poorly defined. Building on our discovery that interleukin-2-inducible T cell kinase (ITK) uncouples pathogenic inflammation from protective immunity, we investigated ITK as a central regulator of autoimmune lung injury. Using the pristane-induced PH model, we show that ITK deficiency confers near-complete protection against PH and associated multi-organ injury. This protection is accompanied by marked remodeling of the T cell compartment, including expansion of $CD44^+CD122^+Eomes^+T\text{-}bet^+$ memory-like subsets and significant enrichment of $Foxp3^+$ regulatory T cells (Tregs). Notably, adoptive transfer of ITK-deficient Tregs was sufficient to rescue PH and suppress systemic proinflammatory cytokine production in wild-type recipients, identifying these cells as key mediators of tissue protection. Transcriptomic profiling further revealed that loss of ITK signaling reprograms Tregs toward a metabolically and functionally enhanced state, with enrichment of oxidative phosphorylation (OXPHOS), mTORC1, STAT5 signaling, and tissue-repair-associated programs. Together, these findings identify ITK as a critical regulator of the balance between pulmonary injury and reparative immunity and provide a mechanistic rationale for 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 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%. PH occurs in autoimmune disorders such as Goodpasture syndrome, systemic lupus erythematosus (SLE), and antiphospholipid syndrome, in which immune-mediated injury to the alveolar-capillary interface triggers rapid and often fatal pulmonary bleeding[1–3]. Current standard of care relies on high-dose corticosteroids together with systemic immunosuppression, including cyclophosphamide and rituximab, and in some cases antifibrinolytic agents to stabilize clot formation[3,4]. Despite these aggressive interventions, outcomes remain poor, highlighting a major unmet need to define the immune mechanisms that drive organ-specific injury versus those that promote tissue protection and repair[5–8].

Interleukin-2-inducible T cell kinase (ITK), a member of the Tec family of tyrosine kinases, has emerged as a key checkpoint in shaping T cell responses. We previously demonstrated that targeting 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 functions[9]. We have also shown that ITK deficiency gives rise to highly suppressive regulatory T cells (Tregs) with enhanced functional capacity[10]. However, whether ITK-dependent Treg reprogramming can be harnessed to preserve the alveolar-capillary barrier during acute autoimmune lung injury remains unknown.

Defining the mechanisms by which ITK deficiency protects against pulmonary hemorrhage is a central objective of this study. ITK functions as a critical pro-inflammatory 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 constraining the development or function of regulatory T (Treg) cells[11,12]. Through these effects, ITK shifts the immune balance away from tolerance and toward sustained inflammation and tissue injury. Increased ITK activation in $CD4^+$ T cells have been implicated in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA) and multiple sclerosis (MS), positioning ITK as an important molecular checkpoint and a potential therapeutic target for restoring immune homeostasis[13,14]. Despite these established roles, its contribution to pristane-induced pulmonary hemorrhage remains unknown. Here, using ITK-deficient ($ITK^{-/-}$) mice[9,15], we show that loss of ITK drives marked remodeling of the T cell compartment. Specifically, both $CD8^+$ and $CD4^+$ T cells from $ITK^{-/-}$ mice exhibited significant expansion of central memory (CM) and effector memory (EM) subsets compared with wild-type (WT) controls. These populations were characterized by increased expression of CD44 and CD122, together with the T-box transcription factors Eomesodermin (Eomes) and T-bet, consistent with a shift toward a memory-like and potentially protective immune state.

To define the functional contribution of ITK to autoimmune lung injury, we challenged WT and $ITK^{-/-}$ mice with pristane. $ITK^{-/-}$ mice were fully protected from pristane-induced pulmonary hemorrhage, showing a striking absence of alveolar bleeding compared with WT controls. This protective effect extended beyond the lung, as $ITK^{-/-}$ mice also exhibited significantly reduced pathology in multiple organs, indicating that loss of ITK broadly attenuates systemic autoimmune injury. This protection was accompanied by a marked reduction in the infiltration of proinflammatory immune cells into the lung and other affected tissues. Consistent with diminished inflammatory cell accumulation, $ITK^{-/-}$ mice also displayed significantly lower levels of proinflammatory cytokines than WT mice. A key cellular feature associated with this phenotype was the expansion of regulatory T cell populations. Specifically, ITK-deficient mice exhibited significantly increased frequencies of both canonical ($CD4^+CD25^+Foxp3^+$) and non-canonical ($CD4^+CD25^-Foxp3^+$) Tregs, consistent with our previous findings[10,16]. Together, these data suggest that loss of ITK signaling establishes a dominant regulatory immune environment that preserves tissue integrity during autoimmune injury.

To determine whether the protection observed in $ITK^{-/-}$ mice was intrinsic to the regulatory T cell compartment, we performed adoptive transfer experiments[17]. Tregs isolated from ITK-deficient donors and transferred into WT recipients at the time of pristane challenge conferred robust

protection against PH. This transfer significantly reduced pulmonary inflammation, suppressed systemic proinflammatory cytokine production, and increased anti-inflammatory mediator expression. These findings demonstrate that ITK-deficient Tregs possess enhanced suppressive capacity and are sufficient to override pathogenic inflammation in a WT host environment. To define the molecular basis of this heightened regulatory state, we performed unbiased RNA sequencing (RNA-seq). Transcriptomic profiling revealed that ITK-deficient Tregs were significantly enriched for programs associated with immune homeostasis, cytokine regulation, metabolism, and tissue repair. Collectively, these findings identify a previously unrecognized role for ITK signaling in regulating alveolar-capillary barrier integrity. More broadly, our work establishes the ITK axis as a promising therapeutic target for pulmonary hemorrhage and other severe inflammatory lung diseases characterized by dysregulated cytokine responses.

Results

ITK Deficiency Promotes a Predominantly Memory-like T Cell Phenotype

Interleukin-2-inducible T cell kinase (ITK) is a Tec-family tyrosine kinase required for optimal T cell receptor (TCR) signaling and is therefore critical for T cell development, activation, and effector function[18]. To determine how ITK deficiency reshapes the T cell compartment prior to autoimmune challenge, we analyzed splenic T cell subsets in WT and ITK$^{-/-}$ mice on a C57BL/6 background[9,19]. Because the differentiation state of αβ T cells strongly influences effector potential and disease outcome[20–23], we used flow cytometry to profile freshly isolated splenic CD3$^+$ T cells. T cells were first divided into CD4$^+$ and CD8$^+$ populations and then classified as naïve (CD44^{lo} CD62L^{hi}), central memory (CM; CD44^{hi} CD62L^{hi}), or effector memory (EM; CD44^{hi}CD62L^{lo}) subsets[10,24]. ITK$^{-/-}$ mice displayed a significantly increased proportion of CM CD8$^+$ T cells compared with WT controls (Fig. 1A-D). Within the CD4$^+$ compartment, this shift was even more pronounced, with ITK$^{-/-}$ mice showing significant expansion of both EM and CM subsets, accompanied by a corresponding reduction in the naïve T cell population (Fig. 1E-H). Together, 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.

ITK Deficiency Enhances Expression of Memory-Associated Activating Markers and Transcriptional Regulators

We have previously shown that T cells with attenuated T cell receptor (TCR) signaling can acquire an activated phenotype without driving pathogenic alloimmunity[9,10,19,25,26]. Consistent with this, flow cytometric analysis revealed significantly increased CD44 expression on CD3$^+$CD8$^+$ T cells from ITK$^{-/-}$ mice compared with WT controls (Fig. 2A-B). We next examined transcriptional regulators associated with memory differentiation. The T-box transcription factors T-bet and Eomes cooperatively promote memory formation in part through induction of CD122 (IL-2Rβ), a key component of the IL-2 and IL-15 receptor complexes[27,28]. Consistent with an enhanced memory-like state, CD8$^+$ T cells from ITK$^{-/-}$ mice expressed significantly higher levels of T-bet, Eomes, and CD122 than WT controls (Fig. 2C-H). Notably, a similar pattern was observed in the CD4$^+$ T cell compartment, where T cells from ITK$^{-/-}$ mice also exhibited increased expression of CD44, Eomes, CD122, and T-bet (Supplementary Fig. 1A-H). Together, these data indicate that ITK deficiency reprograms both CD8$^+$ and CD4$^+$ T cell lineages toward a memory-like phenotype. Importantly, these findings suggest that attenuated TCR signaling does not produce a loss-of-function state, but instead preserves—and in some respects enhances—features associated with T cell fitness and cytokine responsiveness.

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, p > 0.05.

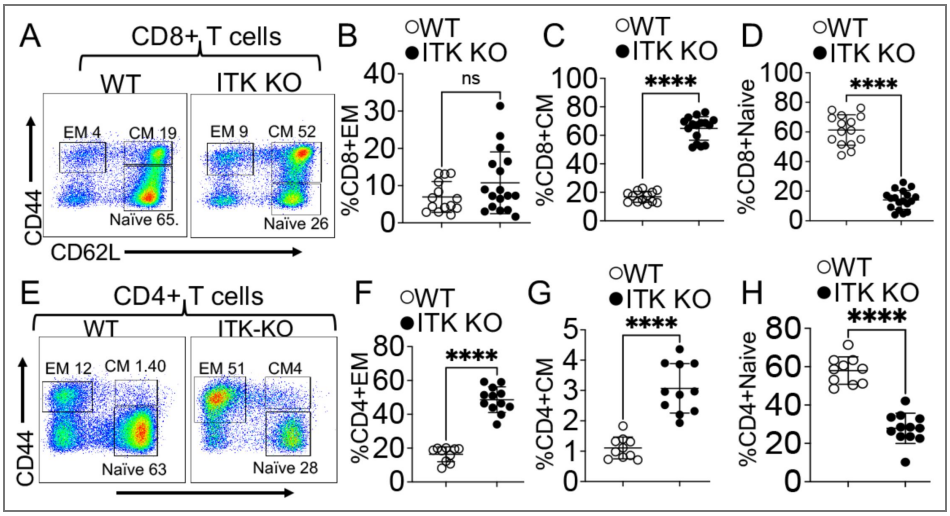
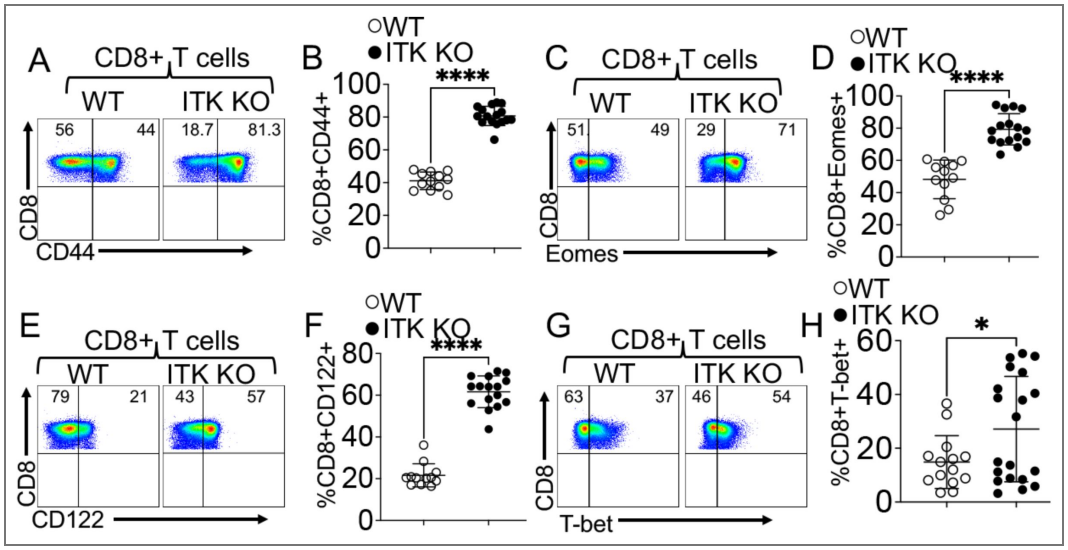


Figure 2. ITK Deficiency Enhances Expression of Memory-Associated Activating Markers and Transcriptional Regulators.

(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 Confers Complete Protection Against Pristane-Induced PH and Systemic Pathology

We and others have previously shown that targeting ITK signaling attenuates pathogenic immune responses across multiple models of alloimmunity[9,10,19,25,26]. To investigate the role of ITK in autoimmune lung injury, we used the pristane-induced pulmonary hemorrhage (PH) model, which recapitulates key features of immune-mediated capillary damage observed in clinical conditions such as systemic lupus erythematosus (SLE) and Goodpasture syndrome[1–3]. WT and ITK$^{-/-}$ mice were challenged with a single intraperitoneal injection of pristane (0.5 mL) and monitored for 14 days, as previously established in our studies and others[17,20,29,30] (Fig. 3A). Although neither group exhibited significant weight loss during this acute phase, gross and histologic analyses at day 14 revealed striking differences in disease severity. Lungs from WT mice showed severe diffuse pulmonary hemorrhage, with extensive erythrocyte accumulation and marked tissue injury on histologic examination. In contrast, ITK$^{-/-}$ mice were fully protected from PH, preserving normal lung architecture and clear alveolar spaces (Fig. 3B-E). Because progression of PH is driven in part by recruitment of pathogenic innate immune cells[31–33], we next quantified inflammatory monocyte infiltration. WT mice displayed a marked influx of CD11b^{+}Ly6C^{+} inflammatory monocytes into the lung, whereas this population was significantly reduced in ITK$^{-/-}$ mice (Fig. 3F-G). Beyond the lung, ITK deficiency also mitigated systemic manifestations of pristane-induced injury[34,35]. WT mice developed pronounced splenomegaly associated with megakaryocyte infiltration, whereas spleens from ITK$^{-/-}$ mice remained normal in size and histologic appearance (Fig. 3H-I; Supplementary Fig. 2). Similarly, WT mice exhibited substantial small intestinal injury, including epithelial hyperplasia and focal acute inflammation, both of which were markedly attenuated in ITK$^{-/-}$ mice (Fig. 3J; Supplementary Figs. 3-4). Together, these findings establish that ITK deficiency confers multi-organ protection against the systemic inflammatory injury triggered by pristane. Having established this protective phenotype, we next asked whether loss of ITK also restrains systemic proinflammatory cytokine production.

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 involvement[36]. 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. 4). 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 expression[37], as well as IL-6 and IL-17, which drive neutrophil recruitment and vascular permeability[38–42]. Serum analysis by ELISA 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[43,44], 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$^{-/-}$ does not simply

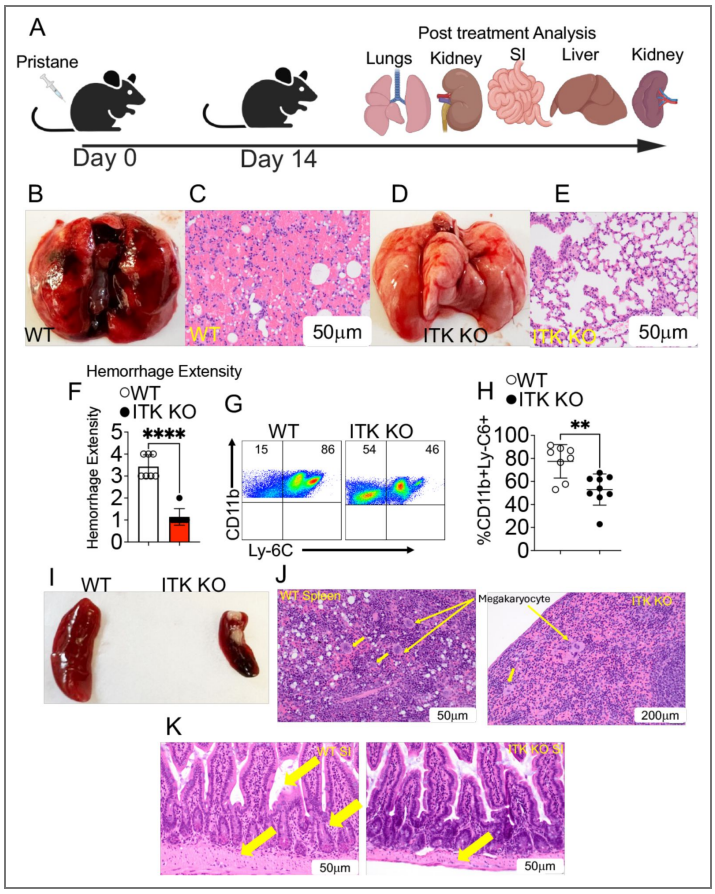


Figure 3. ITK Deficiency Confers Complete Protection Against Pristane-Induced PH and Systemic Pathology.

(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 innate immune cells. Representative flow cytometry plots (G) and quantification (H) of inflammatory monocytes, gated as CD11b^{+}Ly6C^{+} within live singlets. (I and J) Evaluation of splenic pathology. Representative gross images (I) and H&E-stained sections (J) of spleens from WT and ITK$^{-/-}$ mice after pristane injection. (K) 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 ≤ 0.0001; **P ≤ 0.01.

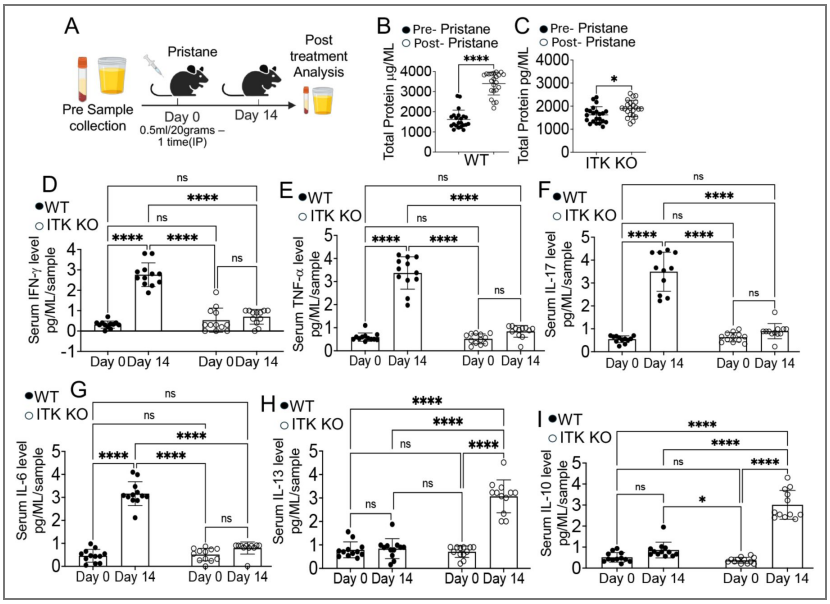


Figure 4. ITK Deficiency Attenuates Proteinuria and Shifts the Systemic Cytokine Balance Toward Resolution.

(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–L) 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 ± 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 two-way ANOVA with Tukey's post hoc correction for time-course data. **P < 0.01; ***P < 0.001; ****P < 0.0001.

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 disease.

ITK Deficiency Expands Canonical and Non-canonical Treg Populations During 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[45,46]. In addition to their suppressive function, Tregs contribute directly to restoration of the alveolar-capillary barrier following inflammatory injury[45,47]. We have previously shown that ITK deficiency increases the frequency of both canonical ($CD4^+CD25^+Foxp3^+$) and non-canonical ($CD4^+CD25^-Foxp3^+$) Tregs[15,48]. To determine whether these subsets expand during autoimmune lung injury, we analyzed splenic Tregs from WT and $ITK^{-/-}$ mice following pristane challenge. $ITK^{-/-}$ mice exhibited significantly higher absolute numbers and frequencies of both canonical and non-canonical Treg subsets than WT controls (Fig. 5A-B; Supplementary Fig. 6A-B). These findings suggest that ITK signaling normally constrains Treg expansion during systemic inflammation and that its absence establishes a broader regulatory reservoir capable of counteracting PH pathogenesis. Furthermore, the expansion of non-canonical $CD25^-Foxp3^+$ Tregs—a population often considered less stable or transient in other inflammatory contexts—suggests that ITK deficiency creates a uniquely permissive environment for robust, multi-lineage regulatory responses.

To determine whether the expanded Treg populations in $ITK^{-/-}$ mice possess enhanced therapeutic activity in vivo, we performed adoptive transfer experiments in WT recipients with established disease. WT mice were challenged with pristane and monitored for 10 days to allow initiation of systemic inflammation and lung injury. On day 10, mice were assigned to three groups: an untreated control group, a group receiving FACS-purified WT Tregs ($CD3^+CD4^+CD25^+Foxp3^+$), and a group receiving $ITK^{-/-}$ Tregs (1×10^6) cells per mouse; Fig. 5C-D). By day 21 after pristane injection, untreated WT mice developed severe gross pulmonary pathology and a significant increase in proteinuria relative to baseline (day 0) (Fig. 5E). Recipients of WT Tregs showed partial protection, with a moderate reduction in proteinuria compared with untreated controls, although levels remained significantly elevated above baseline (Fig. 5F). In contrast, WT mice treated with $ITK^{-/-}$ Tregs were fully protected from PH, with proteinuria returning to baseline levels comparable to those observed before challenge (Fig. 5G). Together, these findings demonstrate that ITK-deficient Tregs possess enhanced suppressive and tissue-protective capacity in vivo and support 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. 4), we next asked whether adoptive transfer of ITK-deficient 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. 5H-K). Although 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 levels observed in both untreated and WT Treg-treated groups (Fig. 5L). 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. Collectively, these results show that $ITK^{-/-}$ Tregs confer enhanced protection against PH by shifting the cytokine balance from a proinflammatory toward a pro-resolving state.

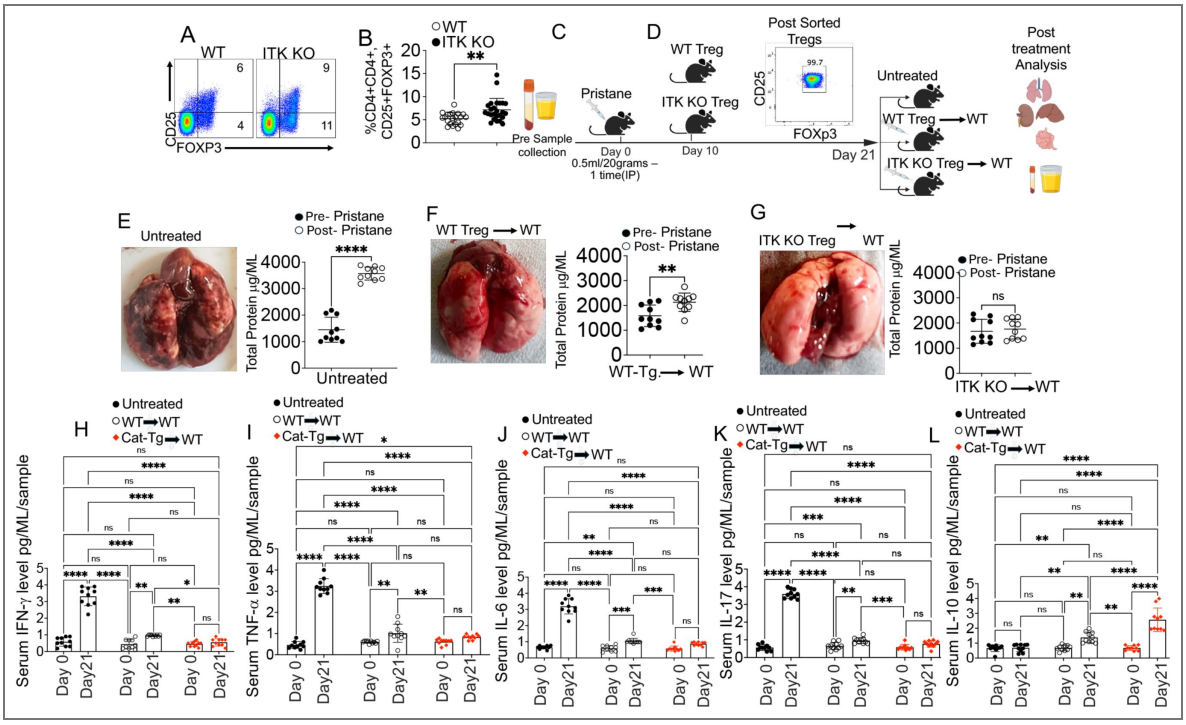


Figure 5. ITK Deficiency Expands Canonical and Non-canonical Treg Populations During PH.

(A and B) Characterization of the regulatory T cell reservoir. Representative flow cytometry plots (A) and quantification (B) of splenic $CD4^+Foxp3^+$ Tregs in WT and $ITK^{-/-}$ mice, illustrating expansion of both canonical ($CD25^+$) and non-canonical ($CD25^-$) populations in the absence of ITK signaling. (C and D) Experimental schematic for therapeutic rescue. WT recipient mice were challenged with pristane. At day 10 post-injection, a time point at which pulmonary injury was already established, mice were either left untreated or received an adoptive transfer of 1×10^6 FACS-purified $CD3^+CD4^+CD25^+Foxp3^+$ Tregs from either WT or $ITK^{-/-}$ donor mice. All cohorts were euthanized at day 21 for systemic analysis. (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) for untreated mice (E), WT Treg-treated mice (F), and $ITK^{-/-}$ Treg-treated mice (G). Transfer of ITK-deficient Tregs resulted in near-complete resolution of gross hemorrhage and significant attenuation of proteinuria. (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 ± 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 two-way ANOVA with Tukey's post hoc correction. NS, $p > 0.05$; *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$

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

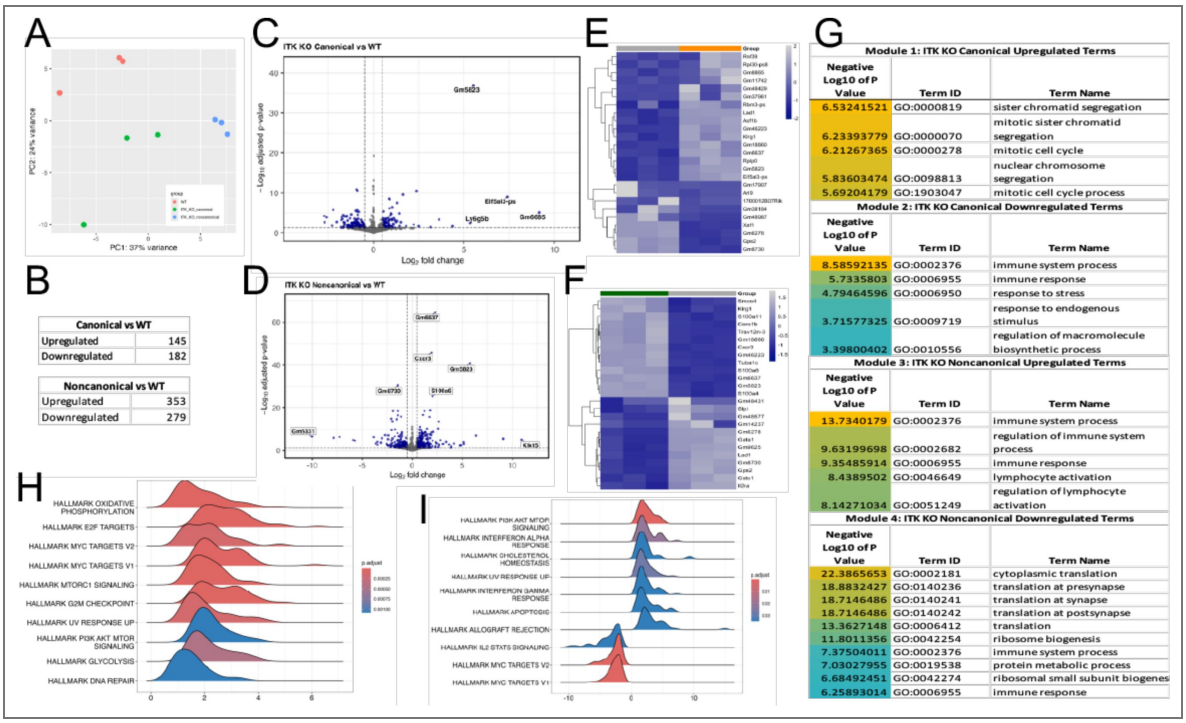
Having established that ITK-deficient Tregs confer enhanced protection against PH, we next sought to define the global transcriptomic programs underlying this phenotype. We performed RNA sequencing (RNA-seq) on FACS-purified canonical ($CD3^+CD4^+CD25^+Foxp3^+$) Tregs from WT and $ITK^{-/-}$ mice, as 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. 6A-B). To identify the biological programs associated with the enhanced function of ITK-deficient 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. 6C-H). Additional enrichment was observed in pathways related to DNA repair, E2F targets, and immune homeostasis, together with differential regulation of IFN-γ and NF-κB signaling. GSEA enrichment plots further supported the conclusion that loss of ITK signaling promotes a metabolically active and functionally enhanced Treg state, characterized by increased cell cycle- and metabolism-associated programs (Supplementary Fig. 7A-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-deficient Tregs in PH.

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[49–53]. 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 broad remodeling of the T cell compartment toward a dominant regulatory state with features of enhanced metabolic fitness.

ITK Signaling 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[14,15,54–57]. Although inherited ITK deficiency in humans is associated with impaired IFN-γ immunity and defective viral control[58,59], 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-deficient 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)[9], are also capable of protecting the alveolar-capillary barrier during autoimmune injury

Our study further shows that ITK deficiency reshapes the T cell compartment, with 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[27,60]. 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[27,61–64]. our findings suggest that attenuation of ITK



Module 1: ITK KO Canonical Upregulated Terms

Negative Log10 of P Value	Term ID	Term Name
6.53241521	GO:0000819	sister chromatid segregation
6.23393779	GO:0000070	mitotic sister chromatid segregation
6.21267365	GO:0000278	mitotic cell cycle
5.83603474	GO:0098813	nuclear chromosome segregation
5.69204179	GO:1903047	mitotic cell cycle process

Module 2: ITK KO Canonical Downregulated Terms

Negative Log10 of P Value	Term ID	Term Name
8.58592135	GO:0002376	immune system process
5.7335803	GO:0006955	immune response
4.79464596	GO:0006950	response to stress
3.71577325	GO:0009719	response to endogenous stimulus
3.39800402	GO:0010556	regulation of macromolecule biosynthetic process

Module 3: ITK KO Noncanonical Upregulated Terms

Negative Log10 of P Value	Term ID	Term Name
13.7340179	GO:0002376	immune system process
9.63199698	GO:0002682	regulation of immune system process
9.35485914	GO:0006955	immune response
8.4389502	GO:0046649	lymphocyte activation
8.14271034	GO:0051249	regulation of lymphocyte activation

Module 4: ITK KO Noncanonical Downregulated Terms

Negative Log10 of P Value	Term ID	Term Name
22.3865653	GO:0002181	cytoplasmic translation
18.8832427	GO:0140236	translation at presynapse
18.7146486	GO:0140241	translation at synapse
18.7146486	GO:0140242	translation at postsynapse
13.3627148	GO:0006412	translation
11.8011356	GO:0042254	ribosome biogenesis
7.37504011	GO:0002376	immune system process
7.03027955	GO:0019538	protein metabolic process
6.68492451	GO:0042274	ribosomal small subunit biogenesis
6.25893014	GO:0006955	immune response

Figure 6. 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 of differentially expressed genes. Plots identify top DEGs between $ITK^{-/-}$ and WT Tregs. Genes meeting the thresholds of FDR ≤ 0.05 and $|\log_2 FC| \geq 0.5$ are highlighted, with positive $\log_2 FC$ 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, green indicates the non-canonical group. (G) Tables showing GO enrichment analysis of the DEG of 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 Tregs mice. On the table, p-values were color-coded as light blue for insignificant findings to orange with highest significance. (n = 3 mice per group). (H and I) Gene Set Enrichment Analysis (GSEA) of regulatory pathways in canonical (H) and noncanonical (I) samples. Ridge plots showing 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.

signaling creates a distinct signaling context that favors acquisition of an Eomes-rich, memory-like phenotype. While the role of ITK in T cell development is well established[65–67], 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 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[68,69]. In this context, loss of ITK signaling significantly reduced the accumulation of $CD11b^{+}Ly6C^{+}$ inflammatory monocytes 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 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[15,70]. By limiting this proinflammatory milieu, modulation of the ITK axis may provide 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 and IL-13, and in some settings IL-4[71,72]. 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-deficient 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 adoptive transfer of ITK-deficient Tregs was sufficient to rescue established PH in WT recipients[10,73]. Because the therapeutic efficacy of Tregs is often limited by impaired stability or function under inflammatory stress[74,75], these findings suggest that loss of ITK signaling supports a more durable regulatory program in vivo.

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), mTORC1, STAT5 signaling, and tissue repair. Together with the observed changes in glycolytic and cell cycle-associated programs, these data suggest that ITK-deficient Tregs acquire a state of enhanced metabolic fitness that may support their persistence and suppressive activity in inflamed tissue. Thus, 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 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[25,76]. ITK$^{-/-}$ mice were generously provided by Dr. Avery August (Cornell University)[9,15,77]. 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.

Reagents, cell lines, flow cytometry

Spleen or lung tissues were processed into single-cell suspensions. For surface staining, cells were incubated with 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 and included: anti-mouse CD3 (BV605, 100237), CD4 (PE, 100408), CD8 (PE/Cy7, 100722), CD44 (Pacific Blue, 156006), CD122 (APC, 105912), CD62L (APC/Cy7, 304814), CD25 (FITC, 101908), CD11b (FITC, 101205), and Ly-6C (APC, 128015). 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/Cy7, 25-4877-42), T-bet (BV421, 644816), TCF-1 (PE, 564217), Foxp3 (Pacific Blue, 126410), TNF-α (FITC, 502906), and IFN-ψ (APC, 505810). We also used Ly-6C (APC, 128015), CD62L (APC, 161218), CD4 (BV510, 100559), CD25 (FITC, 101907), CD25 (PB, 102021), CD122 (APC, 123213), CD11b (Percp 5.5 450112-82), CD8 (APC Cy7 140421), Eomes (PE, 12-4875-80), and T-bet (FITC, 64481), 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[15,48].

Consumable

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 µL of the diluted sample was combined with 200 µL 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 (µg/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[17].

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-ψ, 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[9,19]. 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.

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 5μm, 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[17,78,79]. Representative images were captured using a light microscope, and histopathology scores were analyzed using the Mann-Whitney U test for non-parametric comparisons

Isolation and Purification of Regulatory T Cells

To facilitate live isolation of regulatory T cells, $ITK^{-/-}$ and WT mice were bred onto the C57BL/6 background and crossed with the Foxp3^tm1Flv/J reporter strain (kindly provided by Dr. Avery August, Cornell University). 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, 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[10,17,48].

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, 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 (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). 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[9,10,15–17,19,24–26,48,80–91].

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)[92]. Transcript abundance estimates were imported with tximport[93], summarized to the gene level, and converted to scaled TPM values.

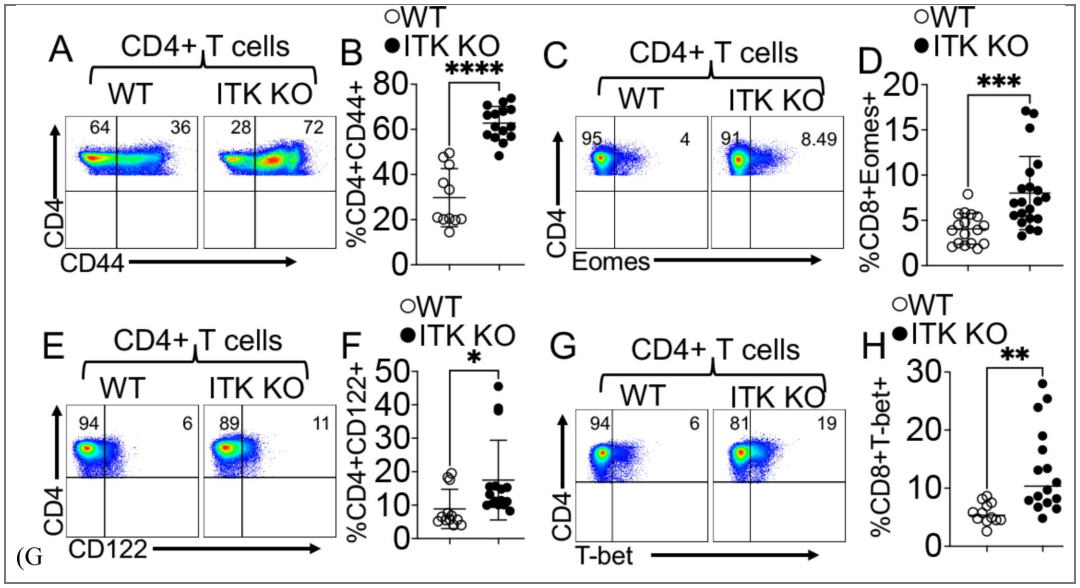
Genes with low expression were filtered prior to model fitting. Differentially expressed genes (DEGs) were identified using DESeq2[94], with significance defined using a Benjamini-Hochberg[95] adjusted FDR ≤ 0.05 and a $|\log_2$ fold change$| \geq 0.5$. Variance-stabilized expression data were visualized using PCA and hierarchical clustering heatmaps generated with pheatmap[96].Functional annotation of identified up- and downregulated genes was performed by Gene Ontology (GO) enrichment analysis using gprofiler2 (gost)[97]. 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.

Data availability

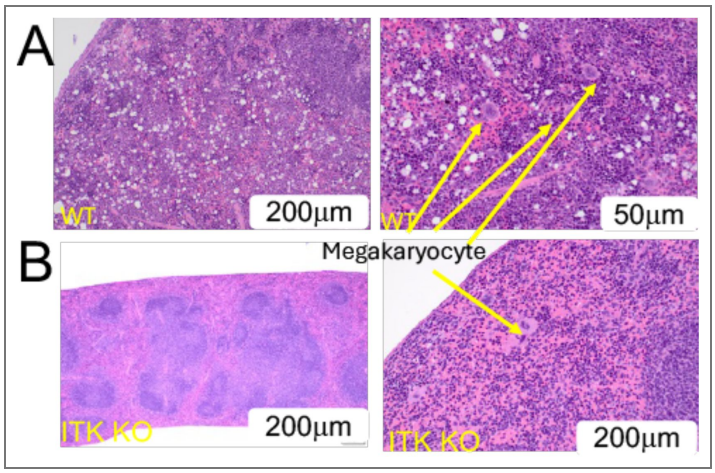
NCBI Gene Expression Omnibus (GEO) under accession number *GSE185327*.



Supplemental Figure 1. 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-2Rβ). 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 ± 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 ≤ 0.0001; *P ≤ 0.05.

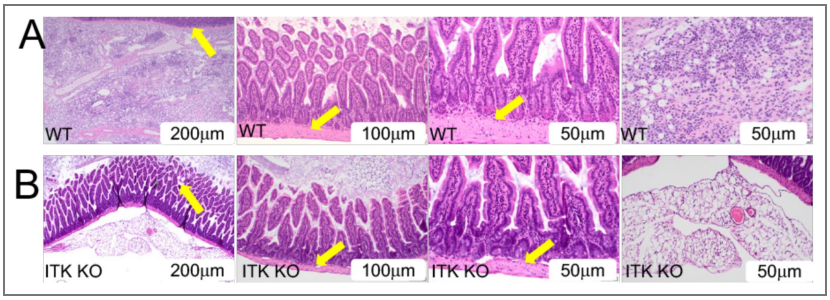
Supplemental Figure 2. **ITK Deficiency Preserves Splenic Architecture and Limits Inflammatory Infiltration.**

(A and B) Histopathological evaluation of the spleen during the systemic inflammatory phase. Representative H&E-stained sections of spleens from WT (A) and ITK$^{-/-}$ (B) mice at Day 14 post-pristane challenge. Note that WT sections exhibit significant architectural disruption and the accumulation of proinflammatory immune cells. Black arrows indicate clusters of infiltrating inflammatory cells and regions of follicular expansion/disruption in the WT, which are markedly attenuated in the ITK$^{-/-}$cohorts.



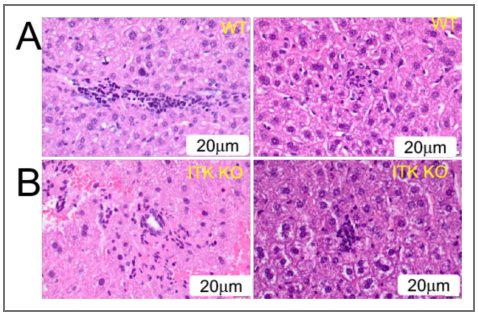
Supplemental Figure 3. **ITK Deficiency Protects Against Systemic Gastrointestinal Injury.**

(A and B) Histopathological evaluation of the small intestine. Representative H&E-stained sections of the small intestine (SI) from WT (A) and ITK$^{-/-}$ (B) 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.



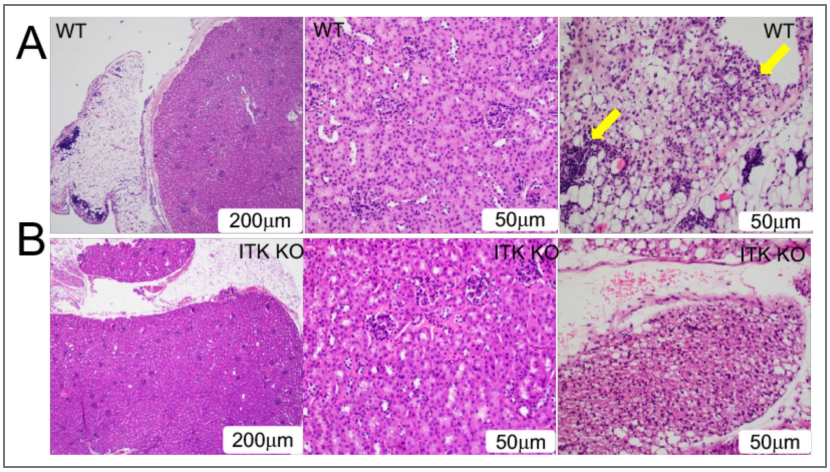
Supplemental Figure 4. **ITK Deficiency Attenuates Hepatic Inflammatory Infiltration.**

(A and B) Histopathological evaluation of the liver. Representative H&E-stained sections of liver tissue from WT (A) and ITK$^{-/-}$ (B) mice at Day 14 post-pristane challenge. Black arrows indicate focal regions of infiltrating proinflammatory immune cells and periportal inflammation in WT mice, which are markedly reduced in ITK$^{-/-}$ cohorts. Notably, while inflammatory infiltration is attenuated in ITK$^{-/-}$ mice, no significant differences in hepatocellular hyperplasia were observed between genotypes at this time point. Results are representative of 3 independent experiments.



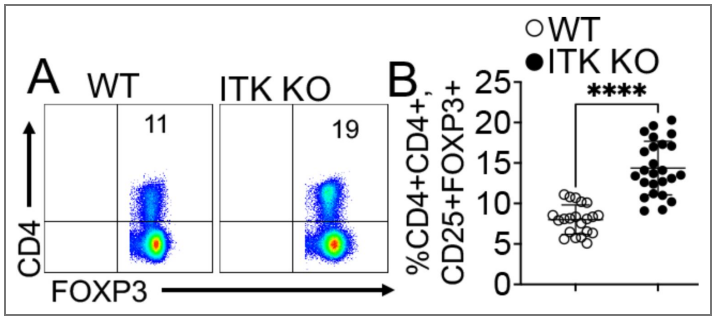
Supplemental Figure 5. **ITK Deficiency Attenuates Renal Inflammatory Infiltration.**

(A and B) Histopathological evaluation of the kidneys during the systemic inflammatory phase. Representative H&E-stained sections of kidney tissue from WT (A) and ITK$^{-/-}$ (B) mice at Day 14 post-pristane challenge. In WT mice, black arrows highlight focal clusters of infiltrating proinflammatory immune cells within the renal interstitium. While ITK$^{-/-}$ mice show a reduction in these inflammatory clusters, the overall glomerular and tubular architecture remains largely preserved in both genotypes at this time point. Notably, while immune cell infiltration is localized, no significant differences in gross renal structural damage or tubular hyperplasia were observed between groups. Results are representative of 3 independent experiments.



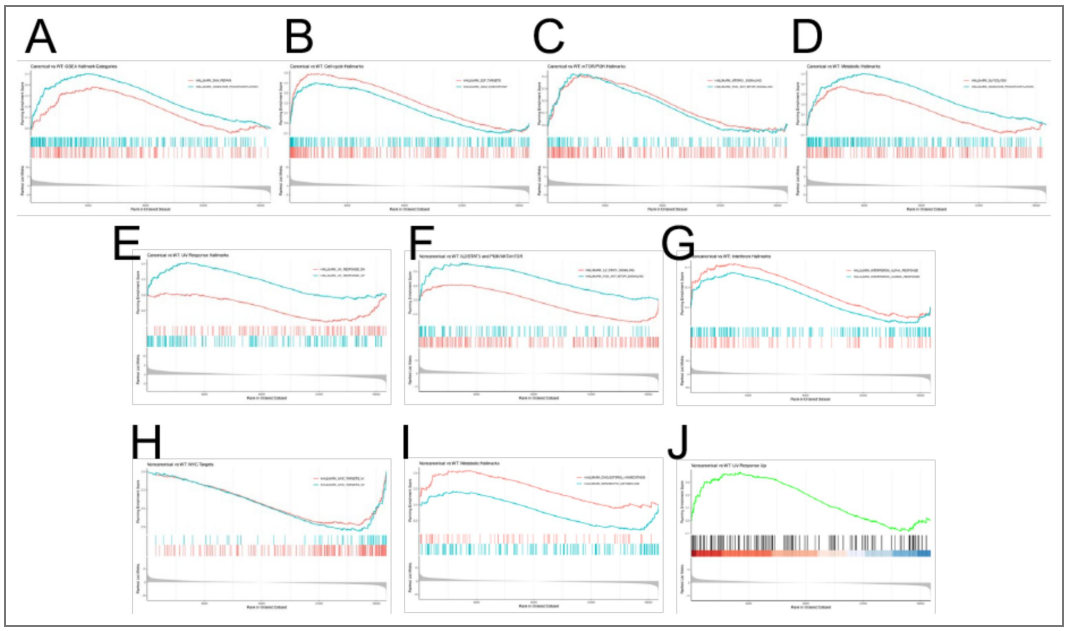
Supplemental Figure 6. ITK Deficiency Promotes the Expansion of Non-canonical Tregs.

(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 7. 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.



Acknowledgements

The authors thank all members of the Karimi Laboratory for their helpful discussions and technical support throughout this study. We also thank the Molecular Analysis Core and the Histology Core Facility at SUNY Upstate Medical University for their expertise and assistance with high-throughput sequencing and pathological processing.

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.

Funding Support

This research was supported by the National Institute on Aging (NIA) of the National Institutes of Health under Award Number 1R21AG098389 (to M.K.). This work was also supported by the Upstate Medical University Cancer Center (PTA 1184369 to M.K.), the Carol M. Baldwin Breast Cancer Research Fund (PTA 1192750 to M.K.), and the Paige's Butterfly Run Fund (Grant #33875 to M.K.). This work is subject to the NIH Public Access Policy. Through the acceptance of federal funding, the NIH has been granted the right to make this work publicly available in PubMed Central.

Funding

Funder	Grant reference number	Author
HHS | NIH | NIH Office of the Director (OD)	1R21AG098389	Mobin Karimi
Upstate Cancer Center (UCC)	PTA 118436	Mobin Karimi
Baldwin Breast Cancer Research	1192750	Mobin Karimi

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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). The 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 wild-type and Itk-/- Treg populations 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:

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.

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.

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.

https://doi.org/10.7554/eLife.111915.1.sa2

Reviewer #2 (Public review):

Summary:

In this manuscript, Hossaim 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.

Weaknesses:

(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

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

(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. A disease-state and ideally lung-relevant transcriptome would more directly support the mechanistic 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.

https://doi.org/10.7554/eLife.111915.1.sa1

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, ITK's involvement in regulating Treg cell function is interesting and could be explored as a novel therapeutic approach for chronic inflammation.

Weaknesses:

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.

https://doi.org/10.7554/eLife.111915.1.sa0