



Interleukin-2-Inducible T-Cell Kinase Deficiency Impairs Early Pulmonary Protection Against *Mycobacterium tuberculosis* Infection

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Interleukin-2 (IL-2) inducible T-cell kinase (ITK) is a non-receptor tyrosine kinase highly expressed in T-cell lineages and regulates multiple aspects of T-cell development and function, mainly through its function downstream of the T-cell receptor. *Itk* deficiency can lead to CD4 lymphopenia and Epstein-Bar virus (EBV)-associated lymphoproliferation and recurrent pulmonary infections in humans. However, the role of the ITK signaling pathway in pulmonary responses in active tuberculosis due to *Mtb* infection is not known. We show here that human lungs with active tuberculosis exhibit altered T-cell receptor/ITK signaling and that *Itk* deficiency impaired early protection against *Mtb* in mice, accompanied by defective development of IL-17A-producing $\gamma\delta$ T cells in the lungs. These findings have important implications of human genetics associated with susceptibility to *Mtb* due to altered immune responses and molecular signals modulating host immunity that controls *Mtb* activity. Enhancing ITK signaling pathways may be an alternative strategy to target *Mtb* infection, especially in cases with highly virulent strains in which IL-17A plays an essential protective role.

Keywords: active tuberculosis, transcriptomic analysis, non-receptor tyrosine kinase, IL-17A, IFN- γ , $\gamma\delta$ T cells

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is the causative agent of what remains one of the most insidious and invasive human infections, responsible for millions of cases of active lung diseases and deaths per year in the world [WHO Global Tuberculosis Report, (1) and O'Garra et al. (2)]. The immune responses by macrophages, neutrophils, and T-cell populations contribute to protective immunity controlling *Mtb* expansion in the host and transmission to others (2, 3). Genetic and environmental factors of the host associated with primary and acquired immunodeficiency can lead to an increased risk of developing active tuberculosis that presents severe pulmonary illness in the clinic (2, 4). Our knowledge of the molecular pathways of innate and adaptive immune effector functions remains incomplete, and a better understanding of potential host factors underlying the pulmonary complications could lead to the development of more innovative therapeutic strategies.

Interleukin-2 (IL-2)-inducible T-cell kinase (ITK) is a non-receptor tyrosine kinase highly expressed in T cells. ITK functions downstream of the T-cell receptor (TCR) and regulates multiple aspects of T-cell development and function (5). *ITK* deficiency in humans is associated with primary immunodeficiency, progressive natural killer T (NKT) and CD4⁺ T-cell lymphopenia, elevated susceptibility to Epstein-Bar virus (EBV), and EBV-driven lymphoproliferative diseases, in which frequent pulmonary involvement has emerged as a clinical hallmark (6–13). Recurrent progressive pulmonary infection, airway obstruction, and respiratory failure in *ITK*-deficient patients pose significant threats that can eventually result in deaths of the patients at their early ages (12). Human T cells from patients with *ITK* deficiency exhibit impaired responses to TCR activation, with reduced generation of Th17 cells and production of the associated cytokines IL-17A, IL-22, and granulocyte-macrophage colony-stimulating factor (14). A murine model of *Itk* deficiency reveals a similar NKT and T-cell lymphopenia as observed in human patients with *ITK* deficiency. Mice lacking *Itk* have a marked reduction in NKT cells (15–18). Despite relatively normal number (trending the lower range) of CD8⁺ αβ T cells, *Itk*^{-/-} mice exhibited CD4⁺ αβ T-cell lymphopenia, with reduced proportion of naive and increased memory αβ T cells (19–31). In the absence of *Itk*, mouse CD4⁺ T cells are impaired in Th2 (producing IL-4/5/13) (32–38), Th9 (producing IL-9) (39), Th17 (producing IL-17) (35, 40–42), and Tr1 (producing IL-10) cell responses (43), while they are enhanced in Th1 (producing IFN-γ) cell response (32, 34, 38, 44, 45). Analysis of *Itk*^{-/-} mice also reveals altered γδ T-cell development (46–49); however, the presence and function of γδ T cells has not been evaluated in *ITK*-deficient humans.

Epidemiological analysis of single-nucleotide polymorphism has also revealed a connection between greater *ITK* promoter activity and higher risk of asthmatic incidence in humans, which might be associated with the function of ITK in promoting T-cell activation (50). In murine models of allergic asthma, the expression of ITK is critical for the activation and development of Th2 and Th17 cells and the associated airway and tracheal inflammation (40, 51). Interestingly, a genome-wide association study of susceptibility to *Mycobacterium avium* subspecies *paratuberculosis* in Holstein cattle identified chromosomal regions that included the *ITK* gene (52). However, the role of ITK signaling pathway in pulmonary responses in active tuberculosis due to *Mtb* infection is unknown.

Here, we show that the TCR/ITK signaling pathway is enriched in human lungs with active tuberculosis and that *Itk* deficiency impaired early protection against *Mtb* in mice, accompanied by defective development of IL-17A-producing γδ T cells in the lungs. Furthermore, ITK appears to regulate the dynamics of lung myeloid cells, which may further contribute to immune control of *Mtb* at the early stage of infection.

Abbreviations: CFU, colony-forming unit; GSEA, gene set enrichment analysis; ITK, IL-2-inducible T-cell kinase; *Mtb*, *Mycobacterium tuberculosis*; NK, natural killer; TCR, T-cell receptor; Treg cells, Foxp3-expressing regulatory T cells.

MATERIALS AND METHODS

Mice

All mice were on the C57BL/6 background. Both female and male mice at the age of 6–12 weeks were used. All experiments were approved by the Office of Research Protections Institutional Animal Care and Use Committee at Cornell University.

Microarray and Data Analyses

Microarray data from lung normal tissue and caseous granulomas from active tuberculosis (TB) patient was generated as previously described (53, 54). Microarray data is available in the Gene Expression Omnibus under accession number GSE20050. In brief, tissues were fixed, and areas of interest were dissected using laser capture microdissection on the Leica AS LMD system (Leica, Buffalo Grove, IL). Total RNA was isolated and used on the GeneChip Human X3P Array (Affymetrix, Santa Clare, CA) following the manufacturer's instruction. Data analysis was performed in R (version 3.5.3) and Bioconductor (version 3.8). Probe intensities were log₂ transformed and median centered. Differentially expressed genes were identified with limma (version 3.38.3) (55). In the case of multiple probes mapped to a gene, the probe with the maximum fold change was selected to represent the gene. Gene set enrichment analyses (GSEA) to determine over- and underrepresented gene sets were performed using the Kyoto Encyclopedia of Genes and Genomes pathway database as reference in gage (version 2.32.1) (56). All pathways that exhibited an up- or downregulated trend in caseum samples compared to normal tissues are summarized in the Supplementary Material (**Supplementary Table 1**). Enrichment score and core genes that drive the score are determined using the GSEA platform developed by the Broad Institute (57). Visualization of TCR signaling pathway with differential gene expression was performed in pathview (version 1.22.3) (**Supplementary Figure 1**) (58).

Mtb Infection and Colony-Forming Unit Counts

Mice were inoculated intranasally with ~1,000 CFUs of Erdman *Mtb* constitutively expressing mCherry (*mCherry-Mtb*) (59) in 25 μl of phosphate-buffered saline (PBS) containing 0.05% Tween-80. Mice were euthanized after 2 and 4 weeks of infection. The left lung lobe and the accessory lobe of the right lung were removed and homogenized in PBS containing 0.05% Tween-80. Bacterial loads were determined by plating serial dilutions of the homogenates on 7H10 agar plates.

Histology and Pathogenic Scoring

Lung samples were fixed in 4% paraformaldehyde overnight, followed by hematoxylin and eosin (H&E) staining. Histological images were analyzed using DP2-BSW software (Olympus, Waltham, MA) to quantify the percentage of affected tissue area and score the severity of pathology (60).

Isolation of Lung Cells

Mice were euthanized at the indicated time points, and lungs were aseptically removed. To obtain a single-cell suspension, lungs were minced and digested in 5% fetal bovine serum/PBS solution containing 250 U/ml collagenase IV (Worthington, Lakewood, NJ) and 20 U/ml DNase (Roche, Indianapolis, IN) for 30 min at 37°C. Lung digestions were then passed through a 70- μ m cell strainer, and red blood cells were lysed with ammonium-chloride-potassium buffer.

Fluorescent Mouse Antibodies

Fluorescent antibodies are listed in the format of “Fluorophore-target (clone)”: eFluor 450-CD4 (GK1.5), Phycoerythrin (PE)-Foxp3 (FJK-16s), PE-eFluor 610-NK1.1 (PK136), allophycocyanin (APC)-IL-17A (eBio17B7), APC-CD11c (N418), PerCP-eFluor 710-CD49b (DX5), PE-Cy7-NK1.1 (PK136), PE-Cy7-IFN- γ (XMG1.2), and APC-eFluor 780-MHCII (M5/114.15.2) were from eBioscience (San Diego, CA). CD16/32 (93; i.e., Fc block), Brilliant Violet 421-CD64 (X54-5/7.1), Alexa Fluor 488-TCR $\gamma\delta$ (GL3), and APC-Cy7-TCR β (H57-597) were from BioLegend (San Diego, CA). FITC-Ly6G (1A8), PE-Siglec-F (E50-2440), PE-TNF- α (MP6-XT22),

PE-CF594 CD8 α (53-6.7), Alexa Fluor 700-Ki67 (B56), PerCP-Cy5.5-CD8 α (53-6.7), and PerCP-Cy5.5-CD11b (M1/70) were from BD Biosciences (San Jose, CA).

T-Cell Stimulation

To activate bulk T cells, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Sigma) and Ionomycin (0.5 μ M, Sigma); to activate *Mtb*-specific CD4⁺ T cells, cells were stimulated with ESAT-6_{4–17} peptide (MHCII-restricted presentation; synthesized by GenScript, purity > 95%; 5 μ g/ml); to activate *Mtb*-specific CD8⁺ T cells, cells were stimulated with TB10.4_{4–11} peptide (MHCI-restricted presentation; synthesized by GenScript, purity > 95%; 5 μ g/ml). All stimulations were done in full RPMI-1640 media in the presence of Brefeldin A (5 μ g/ml, Sigma) and Monensin (2 μ M, Sigma), at 37°C for 5 h.

Flow Cytometry

Surface protein staining was done with antibodies for surface markers in PBS, in the presence of Fc Block (BioLegend) and fixable viability dye (Tonbo Biosciences). To determine cytokine production, cells were stimulated as indicated, followed by surface staining, then were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and permeabilized and stained with antibodies in PBS containing 0.3% saponin

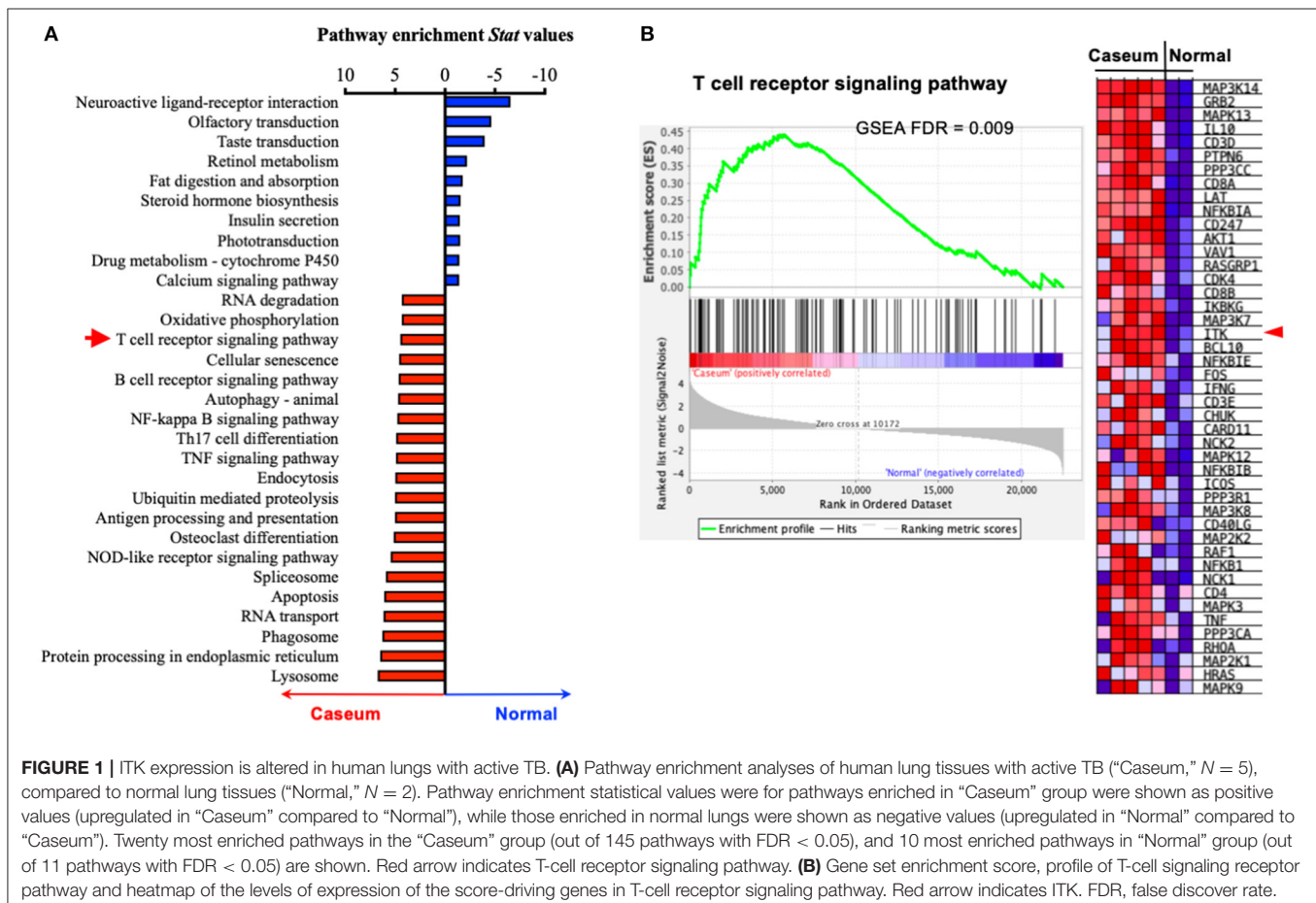


FIGURE 1 | ITK expression is altered in human lungs with active TB. **(A)** Pathway enrichment analyses of human lung tissues with active TB (“Caseum,” $N = 5$), compared to normal lung tissues (“Normal,” $N = 2$). Pathway enrichment statistical values were for pathways enriched in “Caseum” group were shown as positive values (upregulated in “Caseum” compared to “Normal”), while those enriched in normal lungs were shown as negative values (upregulated in “Normal” compared to “Caseum”). Twenty most enriched pathways in the “Caseum” group (out of 145 pathways with FDR < 0.05), and 10 most enriched pathways in “Normal” group (out of 11 pathways with FDR < 0.05) are shown. Red arrow indicates T-cell receptor signaling pathway. **(B)** Gene set enrichment score, profile of T-cell signaling receptor pathway and heatmap of the levels of expression of the score-driving genes in T-cell receptor signaling pathway. Red arrow indicates ITK. FDR, false discover rate.

(Sigma). To stain for nuclear transcription factors Foxp3 and Ki67, following surface staining, cells were fixed, permeabilized, and stained using Foxp3 staining buffer set (eBioscience). All flow cytometry data were acquired on LSRII (BD Biosciences) and analyzed in FlowJo (Tree Star, Ashland, OR).

Statistical Analysis

Two-tailed Student's *t*-test and two-way analysis of variance (ANOVA) between groups were performed using Prism (GraphPad, San Diego, CA), with $p < 0.05$ considered statistically significant. "NS" indicates differences that are not significant.

RESULTS

TCR/ITK Signaling Components Are Upregulated in Active Tuberculosis in Humans

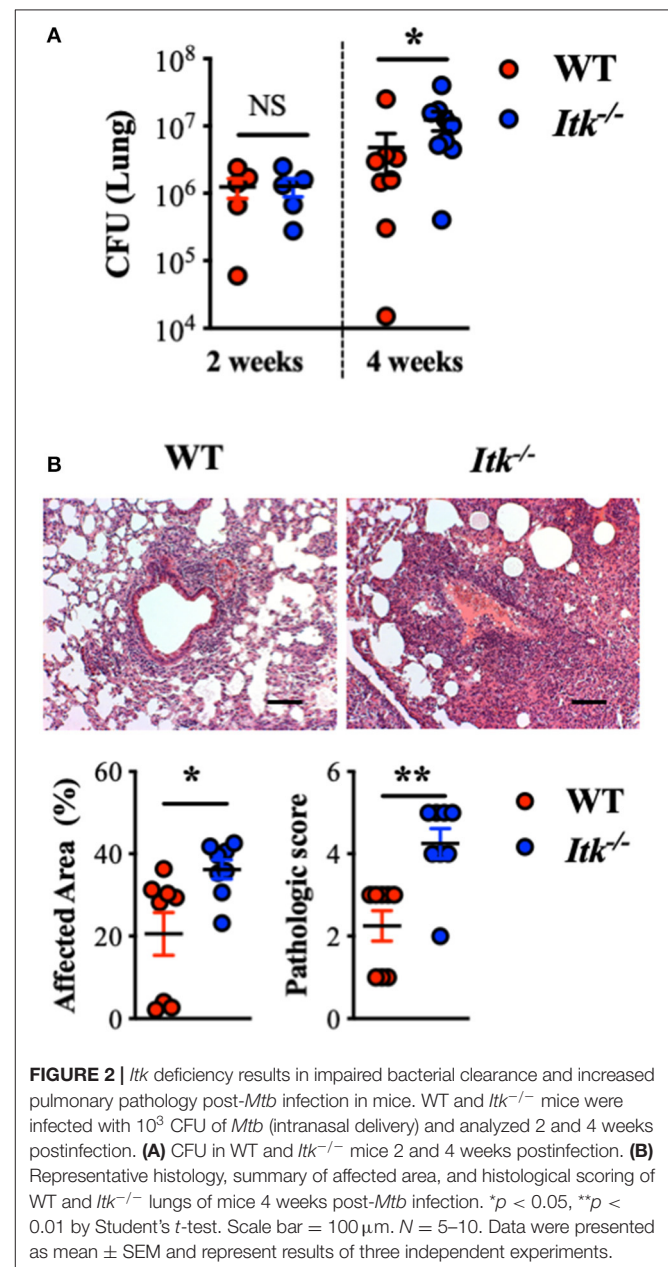
The progression of human active TB disease and transmission involves the development of the caseous granuloma, in which both *Mtb* and the immune response are active (53). We have previously isolated human granulomata from patients with active TB (Caseum) and analyzed the transcriptomic profile in comparison to uninvolved lung tissue (normal) (53). Using pathway enrichment analyses, we found that genes of the TCR signaling were significantly enriched in the caseum tissue that was subjected to active TB, compared to uninvolved lung tissue (Figure 1). Among the enrichment score-driving critical genes of the TCR signaling, the levels of transcripts for ITK and its signaling components (5) such as LCK, GRB2, SLP76, NCK1, FYN, and PLCG are significantly upregulated in caseated granulomas compared to uninvolved lung tissue (Figure 1B). Among the genes that are significantly enriched in active TB, ITK locates in the hub of the TCR signaling pathway (Supplementary Figure 1). These data imply a role for ITK signaling in host immune activity during active TB.

Itk Deficiency Results in Impaired *Mtb* Clearance and Increased Lung Pathology

Given the observation that the TCR signaling pathway was upregulated in the face of active TB in human lungs and that ITK is a critical score-driving gene for the pathway enrichment (Figure 1), we sought to determine the role of ITK in host responses to *Mtb* infection. In murine models of *Itk* deficiency, despite no difference in animal mortality, we found that *Mtb* bacterial burden was significantly higher in the lungs in the absence of ITK 4 weeks post-*Mtb* infection (Figure 2A). Moreover, pulmonary pathology was elevated in the absence of ITK, with significantly larger areas in the airway affected at higher pathological scores (Figure 2B). Notably, compared to wild-type (WT) mice, the relative kinetics of bacterial growth in the lungs of *Itk*^{-/-} and *Rag*^{-/-} mice are similar (61, 62). These data suggest that the TCR/ITK signaling pathway regulates immune responses that contribute to limiting *Mtb* growth and controlling pulmonary inflammation 4 weeks postinfection.

Itk-Deficient Mice Exhibit Altered Early Immune Responses to *Mtb* Infection

The progression of human active TB disease and transmission involves the development of both innate and adaptive immunity. The relative abundance of lung phagocyte populations is extremely dynamic at the early stage of *Mtb* infection (63). Importantly, the various phagocytes in the lung provide different environments for *Mtb* and reveal distinct permissiveness for the growth of *Mtb* (64, 65). We thus analyzed populations of innate immune cells in both WT and *Itk*-deficient mice infected with *Mtb* that constitutively express fluorescent protein mCherry, which allows mapping of the cellular location of intracellular bacteria. During early-stage infection, we



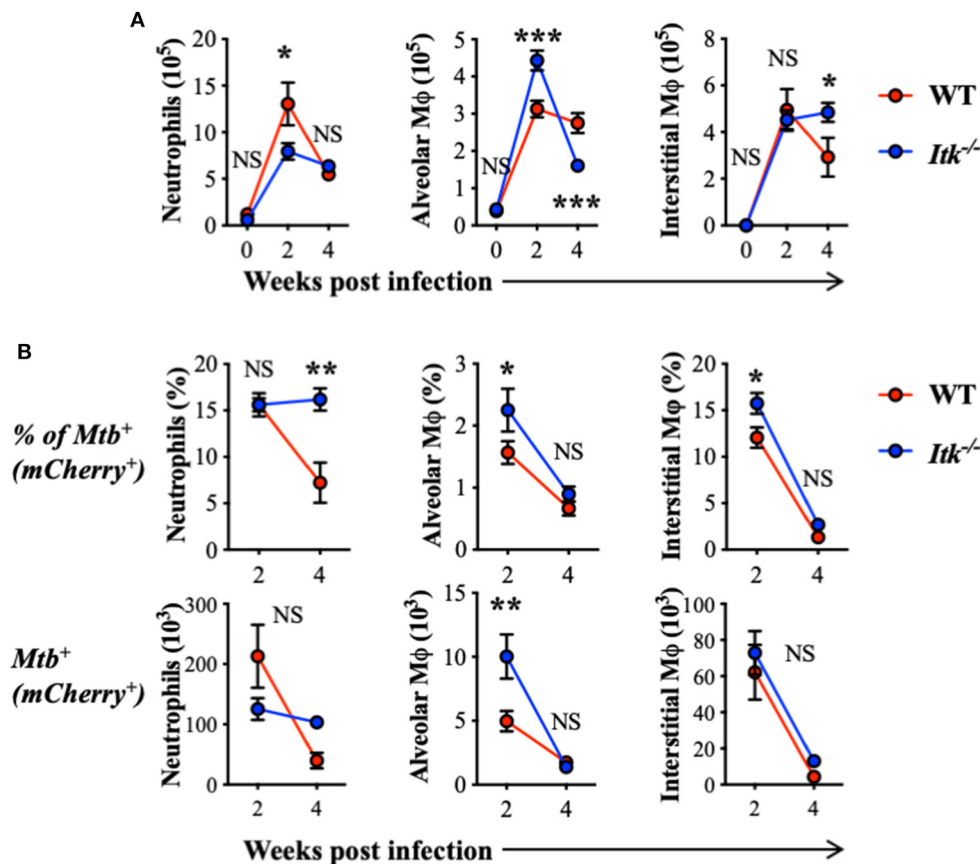
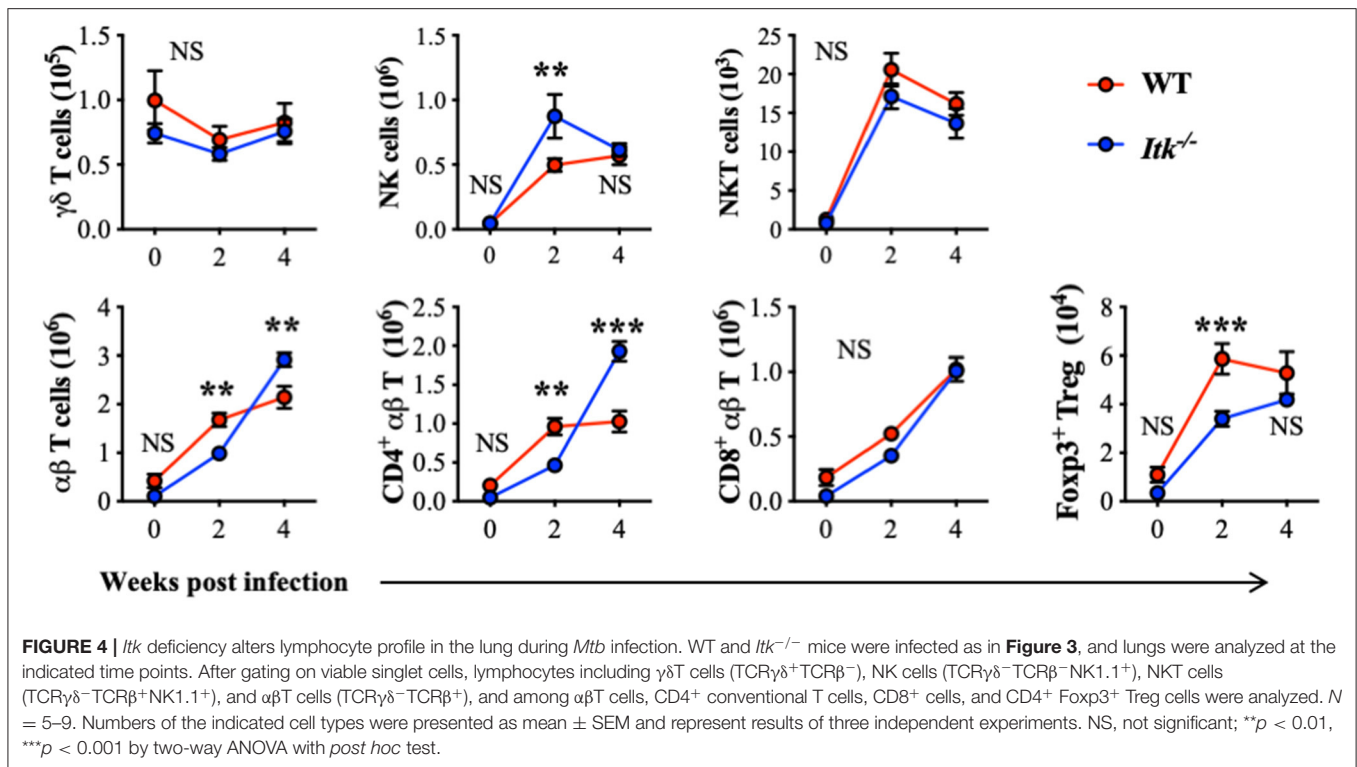


FIGURE 3 | ITK regulates innate myeloid immune response in mice during *Mtb* infection. WT and *Itk*^{-/-} mice were infected with 10³ CFU of mCherry-*Mtb*, and lungs were analyzed at the indicated time points. After gating on viable singlet cells, neutrophils (Ly6G⁺CD11b⁺), alveolar macrophages (Ly6G⁻CD11c⁺Siglec-F⁻), and interstitial macrophages (Ly6G⁻Siglec-F⁻CD11b⁺MHCII⁺CD64⁺) were analyzed. **(A)** Number of total neutrophils, alveolar macrophages, and interstitial macrophages isolated from the lungs of infected mice. **(B)** Percentage and number of mCherry-*Mtb* positive neutrophils, alveolar macrophages, and interstitial macrophages in the lung of the infected mice. NS, not significant; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by two-way ANOVA with *post hoc* test. *N* = 5. Data were presented as mean ± SEM and represent results of three independent experiments.

observed increased total numbers of neutrophils and alveolar macrophages in the lung at 2 weeks postinfection in both WT and *Itk*^{-/-} mice (Figure 3A). Further analysis of the pulmonary immune cell populations revealed that, in the absence of ITK, the proportion of *Mtb*-infected alveolar and interstitial macrophages was significantly higher early after infection (2 weeks); in addition, the proportion of *Mtb*-infected neutrophils was significantly higher 4 weeks after infection (Figure 3). Effective removal of *Mtb*-infected apoptotic neutrophils by macrophages, or efferocytosis, is considered beneficial for host defense (66). The failed clearance of infected neutrophils in *Itk*-deficient mice at 4 weeks suggests that *Itk* may be involved in regulating efferocytosis of lung macrophages. Both alveolar macrophages and neutrophils have been demonstrated as permissive cell types in *Mtb* infection by providing a hospitable environment for optimal bacterial growth (64, 65). The increased proportion of *Mtb*-infected neutrophils in the absence of ITK is associated with the increased bacterial burden at 4 weeks, suggesting

that bacterial burden may be attributed to this difference. Therefore, these data suggest that ITK regulates the dynamic of lung phagocytes and contributes to host protection against *Mtb* infection.

ITK is highly expressed in T-cell lineages including $\gamma\delta$ and $\alpha\beta$ T cells. To further determine whether the absence of ITK affects T-cell and other related lymphocyte responses during *Mtb* infection, we also analyzed the abundance of $\gamma\delta$ T cells, NK cells, NKT cells, and $\alpha\beta$ T cells, including total $\alpha\beta$ T cells, CD4⁺ conventional $\alpha\beta$ T cells, CD8⁺ $\alpha\beta$ T cells, and CD4⁺ Foxp3⁺ regulatory T (Treg) cells. Comparable numbers of $\gamma\delta$ T cells, NKT cells, and CD8⁺ $\alpha\beta$ T cells were observed in WT and *Itk*-deficient mice infected with *Mtb*, while NK cells and CD4⁺ $\alpha\beta$ T cells were significantly increased, and Foxp3⁺ Treg cells were significantly reduced in the absence of ITK (Figure 4). These results seemed surprising, as a reduced number of Treg cells accompanied by increased numbers of CD4⁺ $\alpha\beta$ T cells may suggest a more active immune response. The hosts' ability to limit bacterial growth and control pulmonary



inflammation was, however, impaired in the absence of ITK. A possible explanation could be that the effector activity of the lymphocytes observed in the infected airway against *Mtb* may differ. These effector immune responses could involve innate lymphocyte activities, as well as *Mtb* antigen-specific adaptive lymphocyte functions.

ITK Is Critical for $\gamma\delta$ T-Cell-Derived IL-17A Production During *Mtb* Infection

It has been reported that IL-17A is protective during primary infection of virulent *Mtb* (67), and IL-17A is predominantly produced by $\gamma\delta$ T cells in the lungs early after *Mtb* infection (68). Indeed, while we detected significant IL-17A production by $\gamma\delta$ T cells in the lungs of WT mice infected with *Mtb* (red foreground in Figures 5A–C), very limited IL-17A was produced by the conventional CD4⁺ $\alpha\beta$ Th17 cells (gray background in Figure 5A, as well as in Figure 5D). Interestingly, in the absence of ITK, there was a marked reduction in IL-17A-producing $\gamma\delta$ T cells in the lung (Figures 5A–C). In contrast to the significant levels of IL-17A production in $\gamma\delta$ T cells, IL-17A production by CD4⁺ and CD8⁺ $\alpha\beta$ T cells during *Mtb* infection was minimal (Figure 5B vs. Figure 5D). Furthermore, in the absence of ITK, unlike $\gamma\delta$ T-cell-derived IL-17A that was significantly impaired, IL-17A production by CD4⁺ and CD8⁺ $\alpha\beta$ T cells did not exhibit overt differences, although the proportion of those cells making IL-17A was quite low (Figure 5D).

Itk Deficiency Has Minimal Impact on Antigen-Specific $\alpha\beta$ T-Cell Responses During *Mtb* Infection

Our data above supports a strong role of ITK in promoting the IL-17A-producing effector $\gamma\delta$ T cells during *Mtb* infection. It is possible that $\alpha\beta$ T-cell effector functions were also altered in the absence of ITK and further contributed to the impaired bacterial clearance and enhanced lung pathology as observed in Figure 2. To determine whether ITK regulates $\alpha\beta$ T-cell effector function, we stimulated cells isolated from the lungs of the infected mice 4 weeks postinfection. We found that bulk T-cell activation by PMA and ionomycin suggested that ITK is not required for CD4⁺ and CD8⁺ $\alpha\beta$ T cells to produce effector cytokines tumor necrosis factor alpha (TNF- α) and interferon-gamma (IFN- γ) during *Mtb* infection (Figure 6A). Moreover, to our surprise, stimulation of *Mtb* antigen-specific CD4⁺ $\alpha\beta$ T cells with ESAT-6_{4–17} (MHCI-restricted epitope), or CD8⁺ $\alpha\beta$ T cells with TB10.4_{4–11} (MHCI-restricted epitope) revealed no difference in production of TNF- α and IFN- γ by these cells in the absence of ITK (Figure 6B). Along with the data above, our results suggest that the major protective role of ITK during *Mtb* infection might be executed through ITK-mediated IL-17A production by $\gamma\delta$ T cells.

Itk Regulates $\gamma\delta$ T Cell but Not $\alpha\beta$ T-Cell Proliferation During *Mtb* Infection

Itk deficiency led to significantly impaired effector $\gamma\delta$ T cells (Figure 5) but not $\alpha\beta$ T cells (Figure 6). These might be

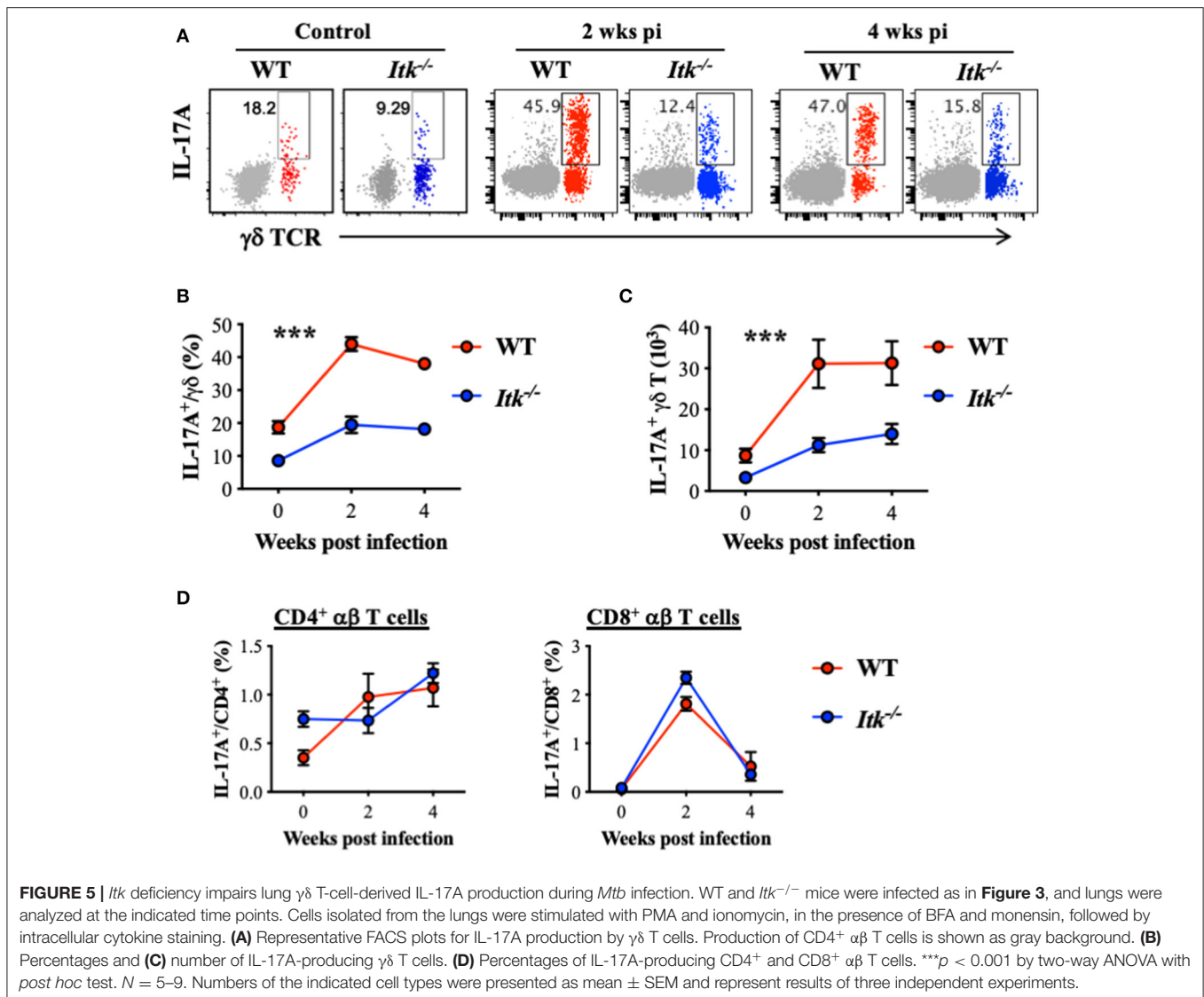


FIGURE 5 | *Itk* deficiency impairs lung $\gamma\delta$ T-cell-derived IL-17A production during *Mtb* infection. WT and *Itk*^{-/-} mice were infected as in **Figure 3**, and lungs were analyzed at the indicated time points. Cells isolated from the lungs were stimulated with PMA and ionomycin, in the presence of BFA and monensin, followed by intracellular cytokine staining. **(A)** Representative FACS plots for IL-17A production by $\gamma\delta$ T cells. Production of CD4⁺ $\alpha\beta$ T cells is shown as gray background. **(B)** Percentages and **(C)** number of IL-17A-producing $\gamma\delta$ T cells. **(D)** Percentages of IL-17A-producing CD4⁺ and CD8⁺ $\alpha\beta$ T cells. ****p* < 0.001 by two-way ANOVA with *post hoc* test. *N* = 5–9. Numbers of the indicated cell types were presented as mean \pm SEM and represent results of three independent experiments.

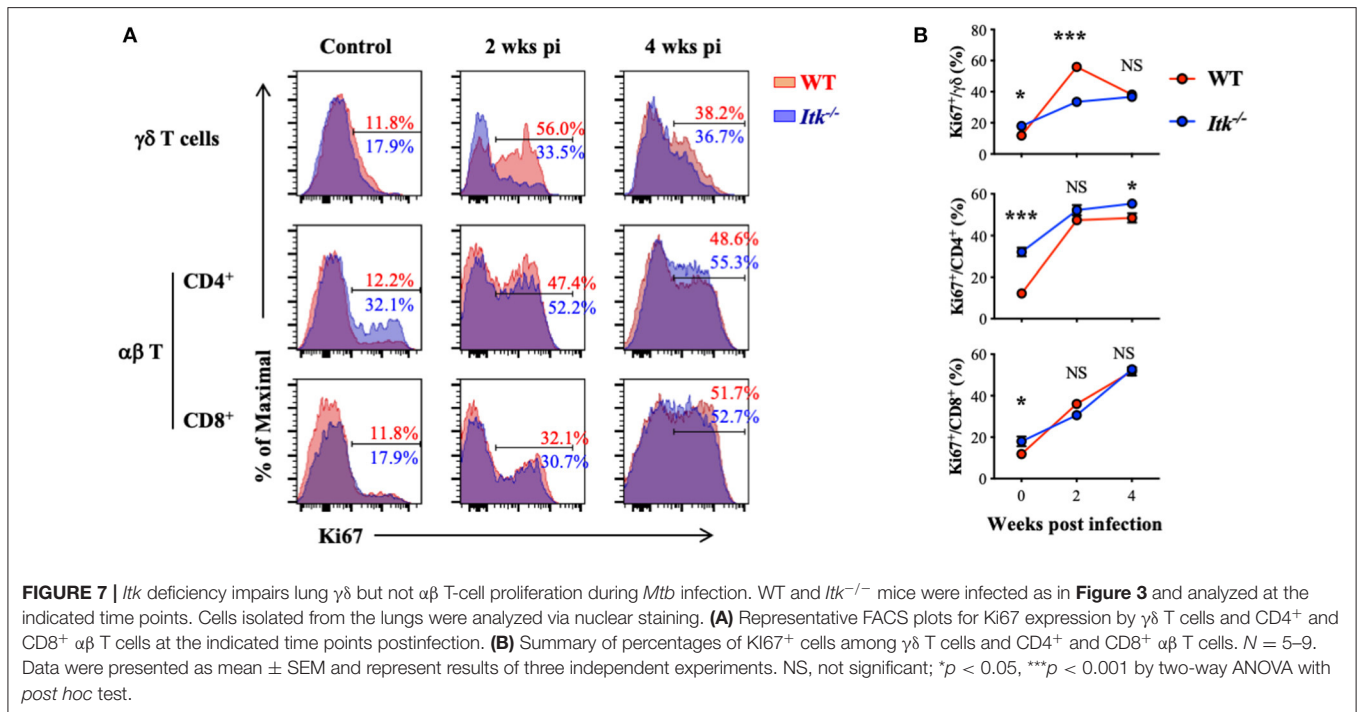
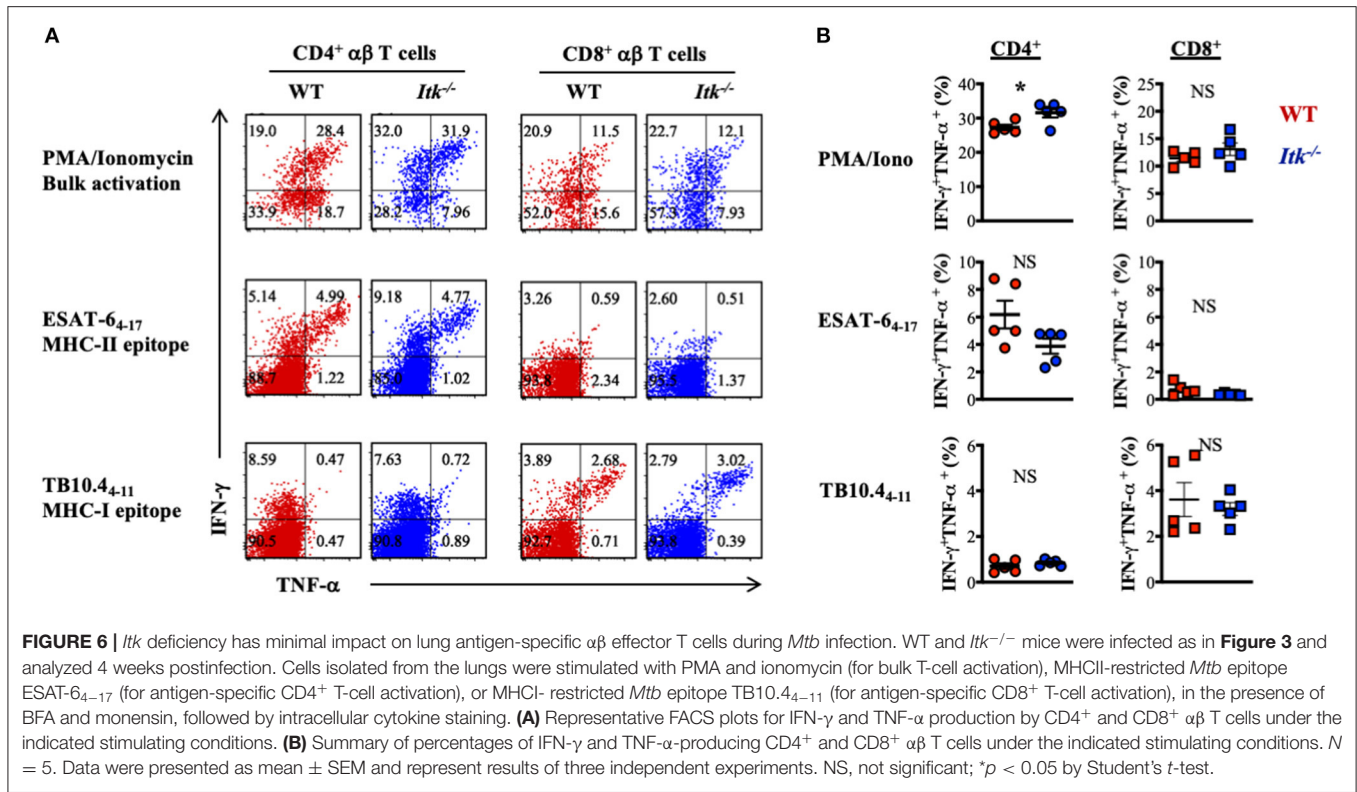
the results of differential requirement of ITK signaling in T-cell expansion. Using proliferative marker Ki67 to detect T-cell proliferation, we found that *Mtb*-driven early proliferation of $\gamma\delta$ T cells was severely impaired in *ITK* deficiency (**Figure 7**, top panel), but not the CD4⁺ and CD8⁺ $\alpha\beta$ T-cell subsets (**Figure 7**, middle and bottom panels). These data, in part, explain the selective defect of effector $\gamma\delta$ T-cell development in *Itk*-deficient mice during *Mtb* infection.

DISCUSSION

The immune cells that control *Mtb* include macrophages, neutrophils, and T-cell populations (2, 3). The TCR pathway is elevated in active *Mtb* infection in human lung, and ITK is a major component of this pathway; however, its role in the T-cell response to *Mtb* is not known. Here, we demonstrate a protective role of ITK in *Mtb* infections in murine models, with a particular role in the ability of lung $\gamma\delta$ T cells to produce IL-17A, which is associated with *Mtb* residence in lung neutrophils.

These findings have important implications for understanding the T-cell immune response to *Mtb* and the role of ITK in this process.

In humans, *Mtb* infects *via* inhalation of a low dose of aerosolized bacteria; therefore, low-dose aerosol inoculation would better mimic the nature of *Mtb* infection in humans. However, in the absence of the ability to perform aerosolized infections, the standard experimental protocol of *Mtb* infection in mice is 1,000 CFU *via* intranasal inoculation (69–72). Monocytes play an essential role in initiating T-cell responses in the lung against *Mtb* infection (73), and using this standard protocol of *Mtb* infection in mice, we previously observed monocytoysis (64), similar to what has been reported in other studies using aerosolized low-dose infection protocols (74, 75). Using intranasal inoculation with 1,000 CFU of *Mtb* in mice, we observed that the numbers of host myeloid cells infiltrating to the lung during *Mtb* infection are significantly altered in the absence of ITK. Alveolar macrophages are considered as the preferred replicating niche for *Mtb* and promote the early



stage of infection (64, 76). In *Itk*-deficient mice, there are more alveolar macrophages harboring *Mtb*, suggesting a more permissive cellular environment in the lung. Moreover, although fewer neutrophils are present in the lung in the absence of

ITK, there are more *Mtb* resident in neutrophils at 4 weeks postinfection. The redistribution of *Mtb* in different phagocytes in the absence of ITK appears to correlate with the increased bacterial burden in the lung 4 weeks postinfection. Other than

T cells, ITK has been indicated to regulate functions of innate immune cells such as mast cells (77, 78). However, potential intrinsic functions of ITK in lung macrophages and neutrophils during *Mtb* infection would merit further studies.

Activation of $\gamma\delta$ T cells for IL-17A production is severely impaired in *Itk*-deficient mice and very likely responsible for the resultant neutrophil recruitment early after infection(s), as well as the increased neutrophil resident *Mtb* population later in infection. Indeed, we have shown that ITK can regulate $\gamma\delta$ T cell development and function in mouse (48), and while there was no apparent difference in peripheral blood $\gamma\delta$ T-cell numbers in humans carrying *ITK* mutations (12), the normal range for $\gamma\delta$ T cells varies quite widely by anatomical location, as well as by geography and ethnicity (79). Despite relatively normal antigen-specific $\alpha\beta$ T-cell responses, our results identify ITK signaling as an essential player for the IL-17A production by $\gamma\delta$ T cells, the predominant source of IL-17A in *Mtb*-infected lungs (68). ITK exhibited a $\gamma\delta$ T cell-specific function, as compared to $\alpha\beta$ T cells, in driving T-cell proliferation during early immune responses to *Mtb* infections. Our work suggests that *ITK* deficiency in humans may lead to $\gamma\delta$ T-cell deficiency in expansion and production of IL-17A, in the face of *Mtb* infection.

We and others have previously reported a role for ITK signaling in regulating Foxp3⁺ regulatory T (Treg) cell development and function (41, 80). In the absence of ITK, the proportion of Treg cells among CD4⁺ T cells in the lymphoid organs of naive mice is increased, and the differentiation of inducible Treg cells from CD4⁺ naive T cells *in vitro* is also enhanced (41, 80). The role of ITK in Treg cell responses in the lung during *Mtb* infection was, however, unclear. In mouse model of *Mtb* infection, we observed higher *Mtb* burdens, higher lung pathological scores and reduced numbers of Treg cells found in the lungs in *Itk*-deficient mice, compared to those in the infected WT mice (Figure 4). Tissue damage may be explained by excessive growth of the pathogen and/or immunopathology. Indeed, we observed impaired $\gamma\delta$ T cell expansion and $\gamma\delta$ T cell-derived IL-17A production in *Mtb*-infected *Itk*-deficient mice but no overt differences in other immune effectors. The decrease in Treg numbers in the lung during *Mtb* infection in the absence of ITK may explain higher levels of immunopathology potentially due to impaired immunomodulatory function. Future research using murine models with conditional deletion of *Itk* specifically in Foxp3⁺ Treg cells would allow more in-depth investigation of the role of ITK in Treg cells during *Mtb* infection.

Taken together, these findings suggest a potential role for ITK in active TB in humans, in addition to its known connection with primary immunodeficiency, susceptibility to EBV, lymphoproliferative diseases, and asthma (12, 50, 51). These findings also have important implications for human genetics associated with susceptibility to *Mtb* due to altered immune responses and molecular signals modulating host immunity that controls the progression of active tuberculosis.

Our findings support a role of ITK signaling in promoting protective immune responses against *Mtb*, in particular, $\gamma\delta$ T cell expansion and production of IL-17A, which could contribute to the modulation of tuberculosis, especially in infections with highly virulent bacterial strains in which IL-17A has been shown

to play an essential protective role (67). However, our work also sounds a note of caution for the potential use of compounds such as Ibrutinib that inhibit the related kinase BTK as well as ITK (81). Given the potential for inhibition of ITK, patients being treated with Ibrutinib may need to be monitored for infection or potential reactivation of latent *Mtb*.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus under accession number GSE20050.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committees at Cornell University.

AUTHOR CONTRIBUTIONS

LH, MM, NN, JE, CL, and WH performed the experiments. LH, TS, AA, and WH analyzed and interpreted data. LH, AA, and WH wrote the manuscript. KY performed bioinformatic analyses. DR contributed reagents and intellectual input. AA and WH conceived research and designed experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.03103/full#supplementary-material>

Supplementary Figure 1 | Gene enrichment profile of the TCR/ITK signaling in human lungs with active TB. Genes in the TCR signaling pathways were rendered with their positions and connections in the pathway, with colored codes indicating the log₂-fold change comparing levels of gene expression in caseous to those in normal tissues. Red indicates upregulation in caseous samples while blue indicates downregulation. Note that ITK is one of the central components of the TCR signaling pathway.

Supplementary Table 1 | Summary of pathways that exhibited an up- or down-regulated trend in caseum samples compared to normal tissues.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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