Protective Role of γδ T Cells in Cigarette Smoke and Influenza Infection

M. J. Hong[1], **B. H. Gu**[1,2], **M. Madison**[1], **C. Landers**[1], **H. Y. Tung**[1], **M. Kim**[3], **X. Yuan**[1,2], **R. You**[1,2], **A. A. MacHado**[3], **B. E. Gilbert**[3], **P. Soroosh**[4], **M. Elloso**[5], **L. Song**[1], **M. Chen**[2], **D. B. Corry**[1,2,6,7,*], **G. Diehl**[2,3,7,*], and **F. Kheradmand**[1,2,6,7,*]

[1]Department of Medicine, Section of Pulmonary and Critical Care, Baylor College of Medicine. Houston, TX, 77030

[2]Department of Pathology and Immunology, Baylor College of Medicine. Houston, TX, 77030

[3]Department of Molecular Virology and Microbiology Baylor College of Medicine Houston TX 77030

[4]Janssen R&D Immunology, San Diego, CA 92121

[5]Janssen R&D Immunology Spring House, PA 19002

[6]Center for Translational Research in Inflammatory Diseases, Michael E. DeBakey VA, Houston TX 77030

[7]Biology of Inflammation Center, Baylor College of Medicine, Houston, TX 77030

Abstract

Airborne pathogens commonly trigger severe respiratory failure or death in smokers with lung disease. Cigarette smoking compromises the effectiveness of innate immunity against infections but the underlying mechanisms responsible for defective acquired immune responses in smokers remains less clear. We found that mice exposed to chronic cigarette smoke recovered poorly from primary Influenza A pneumonia with reduced type I and II interferons (IFNs) and viral-specific immunoglobulins, but recruited gamma delta (γδ) T cells to the lungs that predominantly expressed interleukin 17A (IL-17A). *Il-17a*^{-/-} mice exposed to smoke and infected with Influenza A also recruited γδ T cells to the lungs, but in contrast to wild type mice, expressed increased IFNs, made protective influenza specific antibodies, and recovered from infection. Depletion of IL-17A with blocking antibodies significantly increased *T-bet* expression in γδ T cells and improved recovery from acute Influenza A infection in air, but not smoke exposed mice. In contrast, when exposed to smoke, γδ T cell deficient mice failed to mount an effective immune

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*Corresponding authors: Farrah Kheradmand, MD, farrahk@bcm.edu, Gretchen Diehl PhD, Gretchen.Diehl@bcm.edu, David B Corry, MD, dcorry@bcm.edu.

response to Influenza A and showed increased mortality. Our findings demonstrate a protective role for γδ T cells in smokers and suggest that smoke-induced increase in IL-17A inhibits the transcriptional programs required for their optimal anti-viral responses.

Introduction

Seasonal and pandemic influenza A infections are among the most important cause of chronic obstructive pulmonary disease (COPD) exacerbation, hospitalization, and respiratory failure [1]. Epidemiological studies support increased influenza-associated all-cause mortality in current and former smokers compared to life-time nonsmokers[2]. The extent of lung disease is an important contributing factor for hospitalization and the need for mechanical ventilation in COPD patients, while failure to mount appropriate anti-viral immune responses has also been shown to play a role in poor clinical outcomes [3,4]. Active smokers without COPD inoculated with live attenuated influenza show reduced IL-6, and increased viral RNA in the nasal mucosa when compared to nonsmokers, indicating that cigarette smoke may directly inhibit lung epithelial responses to influenza [4]. Despite the influx of innate and acquired immune cells to the lungs, active smokers are highly susceptible to viral and bacterial infections [5]. The adverse response to cigarette smoke may be in part, due to the excessive accumulation of particulate matter in lung macrophages and airway epithelial cells [6] that could render them ineffective against infection and the lungs more susceptible to epithelial injury [7,8]. Therefore, while cigarette smoke could alter local innate immune responses to infection in the lungs, the effect of smoke on acquired immunity in the lung mucosa remains unknown.

Animal models of cigarette smoke-induced lung disease have shown reduced expression of IFNs and increased IL-6, and TNF-α in the lungs in response to influenza infection [9,10]. Further, enhanced lung inflammation and cytokine expression in smoke-exposed mice has shown to promote susceptibility to influenza infection [11,12]. Several experimental models [9,13,14], together with clinical studies of viral-induced COPD exacerbation [15,16] support strong evidence for ineffective anti-viral immunity in response to cigarette smoke which can be augmented by genetic susceptibility factors [17-19].

Systemic and lung-specific cellular responses to cigarette smoke result in activation of several key innate immune cells and complement proteins that accelerate pathogenic myeloid-derived dendritic cell (mDC) recruitment into the lungs [20-23]. Activated mDCs promote the differentiation of Th1, Th17 cells in human emphysema [24], and chronic cigarette smoke enhances IL-17 expression in γδ T cells in the lungs [22]. Cytokines (e.g. IL-17A, IFN-γ, IL-1β, etc.) and chemokines such as CXCL1 (KC), CXCL8 (IL-8), CXCL9 (Mig), and CXCL10 (IP-10) have been shown to induce proteinases in the lungs that are associated with emphysema development [25-27]. We have previously shown that *Il-17a* deficiency attenuates, while constitutive overexpression of this cytokine exacerbates, smoke-induced emphysema [21]. Further, a significant subset of γδ T cells express IL-17A and have a tropism for lung mucosa in mice exposed to smoke, while deficiency in γδ T cells results in exaggerated lung inflammation and emphysema in mice [21]. Several cytokines and chemokines expressed by innate immune cells in chronic smoke exposure and respiratory

viral infection have been shown to induce lung injury [28], however the role of cigarette smoke-induced recruitment of γδ T cells and IL-17A expression in response to respiratory viral infection remains less clear.

Although influenza infection often causes a mild to moderate degree of respiratory illness in immunocompetent hosts, increased morbidity and mortality in response to acute infection in human smokers has been well documented [29]. In this study, we set out to examine the impact of chronic smoke on lung immune cells and physiological outcome following influenza infection using a well-established mouse model of smoke induced lung disease [22]. We have previously found that Th17, and γδ T cells play critical roles in smoke induced lung disease [21], therefore in this study, we examined their role in response to cigarette smoke and influenza infection. We found that mice exposed to smoke recover poorly from influenza pneumonia and fail to produce high titers of hemagglutinin (HA)-specific protective antibodies. These findings were associated with the reciprocal expression of IFN-γ and IL-17A in CD4 and γδ T cells in the lungs. Mice that lack γδ T cells (*Tcrδ^{-/-}*) exposed to smoke and influenza pneumonia showed increased mortality indicating a protective role for γδ T cells in smoke and influenza infection. Finally, we determined the mechanism for the failure of mucosal γδ T cells in protective immunity in mice exposed to chronic smoke.

Results

Increased morbidity in smoke-exposed mice infected with influenza A

Smokers with structural lung disease are at an increased risk for hospitalization and death when infected with respiratory viruses [16,30-32]. To determine how cigarette smoke-induced lung disease can affect acquired immune responses to influenza, we designed a preclinical model that closely emulates acute respiratory infection in smokers. Mice were exposed to three months of Smoke (Smk) or ambient air (Air), followed by primary infection with influenza A virus (H3N2; sublethal dose of 25 $TCID_{50}$)[33]. Two days following inoculation, mice received two additional weeks of cigarette smoke prior to analysis (Fig. S1A). Mice exposed to 12-weeks of cigarette smoke had lower weight, and initially lost less body mass when compared to air exposed mice (Fig. S1B). As expected, smoke-exposed mice infected with Influenza A (Smk/Flu), lost significantly more weight on day 10, and showed increased time to recovery from the acute infection, as measured by weight gain, when compared to mice exposed to Air and infected with the same dose of Influenza A (Air/Flu) (Fig. 1A). Consistent with an exaggerated but ineffective immune response, bronchoalveolar lavage (BAL) fluid collected in Smk/Flu treated mice showed increased neutrophilic inflammation (Fig. 1B), and matrix metalloproteinase (MMP)2 and MMP9 secretion, and MMP12 expression compared to Air/Flu and uninfected air or smoke exposed groups (Fig. S2A, B). Moreover, semi-quantitative histological scoring of the lungs demonstrated more severe peri-bronchial inflammation, in the lungs of Smk/Flu treated mice compared to the Air/Flu group, and increased mucin production as detected by Periodic Acid-Schiff (PAS) staining (Fig. 1C, D, and Fig. S3). As mice were infected with a sublethal dose of influenza, we expected no or limited mortality after influenza infection. Instead, we observed increased mortality in Smk/Flu treated mice with 14% (6 of 43) of the Smk/Flu treated group died compared to 1.75% (1 of 58) of the Air/Flu group.

Whole lung mRNA expression showed significantly decreased type I IFNs, and cytotoxic enzymes including Granzyme b (*Gzmb*) in Smk/Flu compared to Air/Flu treated group (Fig. 1E). In contrast, the concentration of three pro-inflammatory cytokines, TNF-α, IL-6, and KC were increased in the lungs, while we found relative suppression of monocyte-related factors in particular *Ccl3*, and *Ccl4* (Macrophage Inflammatory Protein-1 alpha (MIP-1α) and MIP-1β), and *Ccl5* (RANTES), in Smk/Flu treated group when compared to Air/Flu treated mice (Fig. 1F).

Reciprocal expression of type I -II IFNs, and IL-17A

We next examined lung specific protective immune responses to influenza in our smoke/flu model. Comprehensive characterization of single cells isolated from lungs showed increased IL-17A, but decreased IFN-γ expression in Smk/Flu mice when compared to Air/Flu (Fig. 2A, Fig. S4). Whole lung mRNA expression showed significantly decreased type II IFNs and their associated genes including IFN-induced protein with tetra-tricopeptide repeat 3 (*Ifit3*), transcription factor *Irf7* (Fig. 2B) that is linked to susceptibility to influenza [34]. To further characterize the cellular expression profile of IFN-γ and IL-17A in the lungs, we next performed intracellular cytokine (ICC) of CD3-, and CD3+ immune cells in whole lung, spleen and draining lymph node. We found a reciprocal expression of IL-17A and IFN-γ in the lung CD3+ T cells but not spleen or lung draining lymph nodes (Fig. S6A-F). Further analysis of cytokine expression revealed significant changes in IL-17A and IFN-γ expression in CD3+ but not CD3- cells in the lungs (Fig. S7). To determine the contribution of different IL-17A expressing T cell subsets, we next used flow cytometry to assess IL-17A and IFN-γ expression in γδ, CD4, and CD8 T cells in each of the four experimental groups. Overall there were no significant differences in the relative abundance of CD4, CD8, or γδ T cells between Air/Flu and Smk/Flu treated mice (Fig. S8A-B), and γδT cells were the predominant IL-17A expressing T cells. (Fig. S8C). However, ICC analyses of lung CD3+ T cells showed increased abundance of γδ and CD4 T cells that express IL-17A in Smk and Smk/Flu compared to Air or Air/Flu mice (Fig. 2C, D). Influenza infection induced significant IFN-γ production in γδ, CD4, and CD8 T cells; however, this response was severely blunted in CD4 and γδ T cells from smoke-exposed mice (Fig. 2C, D). Interestingly, smoke did not blunt IFN-γ production by CD8 T cells (Fig. 2C, D), indicating that the phenotypic differences we observed were primarily due to the impact of smoke on CD4 and γδ T cells.

Because neutralizing antibodies play an important role in immune defense against influenza [35], we next examined whether chronic cigarette smoke could adversely affect antigen-specific antibody production in our model. Total IgG and IgA concentrations in the lung homogenates of uninfected smoke-exposed mice were significantly greater than the Air control group (Fig. 2E). However, protective influenza antibody (e.g. HA-specific IgG and IgA) titers in the Smk/Flu treatment group were significantly reduced when compared to the Air/Flu group (Fig. 2F). These data indicate that despite chronic inflammatory conditions, specific anti-viral immunity (e.g. HA-specific IgG and IgA antibodies) is compromised in mice exposed to chronic smoke.

IL-17A deficiency protects smoke-exposed mice from flu

We have previously shown that IL-17A deficient (*Il-17a^-/-*) mice develop attenuated lung inflammation in response to cigarette smoke and are protected against emphysema, while airway overexpression of IL-17A results in exaggerated responses to cigarette smoke [21]. As we detected a reciprocal relation between IL-17A and IFN-γ expression in γδ and CD4 T cells in the lungs of Smk/Flu treated mice, we next sought to determine whether lack of IL-17A could protect smoke exposed mice from severe and/or ineffective immune response to acute flu infection. In contrast to wildtype (WT) animals, Smk/Flu treated *Il-17a^-/-* mice were phenotypically similar to air treated mice and did not show increased weight loss, did not develop respiratory failure, and showed rapid recovery from influenza infection (Fig. 3A, B). Consistent with improved recovery in the absence of IL-17A, Smk/Flu and Air/Flu treated *Il-17a^-/-* mice showed comparable infiltration of inflammatory cells in BAL (Fig. 3C). In sharp contrast to WT mice exposed to Smk/Flu (Fig. 1E), lung histology also showed lack of exaggerated inflammatory cells in the lung parenchyma and attenuated peri-bronchiolar inflammation and airway wall thickening (Fig. 3D, E). Whole-lung gene expression analyses of *Il-17a^-/-* Smk/Flu and Air/Flu treated mice also showed similar expression of type I and II IFNs, and their associated transcription factors (e.g. *Irf7, Ifit3*) as well as *Gzmb* indicting that appropriate viral-specific immune responses are not inhibited by smoke in the absence of IL-17A expression (Fig. 3F).

To determine if lack of IL-17A concomitant with smoking led to recovered viral specific T cell responses, we next examined IFN-γ production in different T cell subsets as described above. ICC analyses of lung lymphocytes from influenza infected smoke or air exposed *Il-17a^-/-* mice showed comparable induction of IFN-γ in CD4, and CD8 T cells (Fig. 3G, Fig. S9A). In particular, γδ T cells in smoke-exposed *Il-17a^-/-* mice infected with influenza showed significantly increased IFN-γ expression when compared to air exposed animals infected with influenza (Fig. 3H). Moreover, *Il-17a^-/-* Smk/Flu treated mice produced protective HA-specific IgG and IgA antibodies (Fig. 3I, Fig. S9B) which is consistent with intact IFN-γ producing cells in the lungs. Together these findings indicate that in the absence of systemic IL-17A, smoke exposed mice infected with influenza can appropriately increase type I and II IFNs leading to protection from influenza.

IL-17A mediates early influenza clearance but causes excessive lung injury

To determine whether IL-17A plays an important role in viral clearance, we next measured lung Influenza A viral titers during peak, mid, and late viral replication phases (6, 8, and 10 days post infection, respectively) [36]. At peak replication (day 6), compared to WT, *Il-17a^-/-* Air/Flu or WT Smk/Flu treated mice, *Il-17a^-/-* Smk/Flu showed a 10-fold increase in viral titers indicating IL-17A is required for early clearance of influenza in smoke exposed mice (Fig. 4A). However, during the late viral replication phase (day 10), WT Smk/Flu treated mice showed approximately 100-fold higher viral titers indicating significant failure to clear viral infection compared to WT Air/Flu treated mice (Fig. 4A).

All groups at peak influenza amplification time points showed a similar increase in IFN-γ expression, notably however when compared to WT Air/Flu condition, we found significant increase in IL-17a concentration only in the lungs of WT mice in Smk/Flu group indicating

an inappropriate cytokine response to influenza infection (Fig. S5). WT mice treated with Smk/Flu showed lower viral titers on day 6 when compared to Il-$17a^{-/-}$ group (Fig. 4A), however by day 10, and despite continued exposure to cigarette smoke, influenza was not detectable in nearly half of Il-$17a^{-/-}$ mice (4/10), compared to only 1 out of 10 in WT mice in Smk/Flu group (Fig. 4A). We also found that by day 8, viral titers were similar between the groups while lung weight, and hemorrhage score were less severe in Il-$17a^{-/-}$ mice (Fig. 4C, D).

We next measured lung weight and assigned hemorrhagic scores [37,38] to quantify acute lung inflammation and injury using the same viral replication time points (e.g. day 6-, 8-, and 10-day) in WT and Il-$17A^{-/-}$ mice exposed to Air/Flu and Smk/Flu. Consistent with the notion that IL-17A induces lung damage after influenza infection, IL-$17a^{-/-}$ Smk/Flu treated mice showed significantly lower lung hemorrhage score at day-10 compared to WT mice treated the same way (Fig. 4B, C, D). Further, IL-$17a^{-/-}$ Smk/Flu treated mice showed significantly lower lung weight than WT mice (Fig. 4D).

Anti-IL-17A treatment attenuates lung inflammation

The above results suggested a potential role for IL-17A in limiting effective immune defense against primary influenza pneumonia. Therefore, we next examined the therapeutic effects of IL-17A inhibition in our preclinical model of smoke and influenza infection. WT mice were exposed to cigarette smoke or air for 3 months and were treated with anti-IL-17 antibody or control IgG every three days for two weeks, with the first dose given 3 days prior to influenza A infection (Fig. S10A). Neutralizing anti-IL-17 antibody treatment efficiently reduced available circulating IL-17A as determined by ELISA assay of serum and BAL fluid in air- and smoke-exposed mice when compared to isotype control IgG2 treatment (Fig. S10B). Air/Flu mice treated with anti-IL-17A antibody showed significantly improved recovery (e.g., less weight loss) when compared with isotype control antibody-treated mice (Fig. 5A, left panel). Inhibition of IL-17A in Smk/Flu treated mice did not result in improved recovery when compared to the isotype control group, indicating that downstream effects of IL-17A induction in the smoke group were not affected by late inhibition of this cytokine after infection (Fig. 5A, right panel). Consistent with these observations there was a significant decrease in lung inflammatory cells in the BAL fluid (Fig. 5B), lung histology (Fig. 5C, 5D), and hemorrhagic score in Air/Flu but not Smk/Flu treated mice with anti-IL-17A antibody (Fig. 5E). Analysis of BAL fluid also showed reduced pro-inflammatory chemokines in Air/Flu mice that received anti-IL-17A antibody, while smoke-exposed mice showed relatively similar cytokine and chemokine levels (e.g. KC, TNF-α, MCP-1) (Fig. S11A).

We next examined the relative abundance of T cells in each group and found that in vivo IL-17A blockade in the Air/Flu group resulted in significant increase in the relative abundance of γδ T cells when compared to isotype control, while the Smk/Flu group showed no significant changes in this cell population (Fig. 5F, G). As blocking IL-17 in smoke treated animals did not offer protection from influenza, we hypothesized that smoke altered the ability of γδ T cells to produce IFN-γ after viral infection. To test this, we isolated lung CD45$^+$ from influenza infected mice exposed to smoke or air for three months.

Cells were treated ex vivo with anti-IL-17A antibodies or isotype control antibodies for 24hrs. Consistent with the in vivo data, inhibition of IL-17A in air but not smoke exposed mice resulted in an increase in transcriptional expression of *T-bet* in γδ T cells, while no significant difference were noted in CD4^{+} T cells and CD45^{+}/γδ T-/CD4 T cells (Fig. 5H, I; Fig. S11 B, C; Fig. S12A-G). Together these findings indicate that smoke-mediated induction of IL-17A is critical for effective γδ T cell anti-viral IFN-γ responses in the lungs.

γδ T cells are required for recovery from Influenza A in smoke-exposed mice

Our findings indicate a reciprocal relation between IFN-γ and IL-17A expression in recovery from acute influenza infection, whereby smoke-induced increased IL-17A expression in γδ and CD4 T cells leads to reduced IFN-γ and decreased survival after influenza infection. Because *Il-17a*^{-/-} mice exposed to smoke and influenza showed attenuated lung injury and increased IFN-γ expression especially in γδ T cells, we next examined the role of γδ T cells in the smoke-flu model. We had previously shown that *Tcrδ*^{-/-} mice develop an exaggerated inflammatory phenotype when exposed to four months of smoke [21]. Consistently, we found *Tcrδ*^{-/-} Smk/Flutreated mice exhibited significantly increased morbidity (as assessed by greater weight loss) (Fig. 6A) and experienced higher mortality (30% versus 14%) when compared to WT mice treated the same way. Similar to Smk/Flu *Il-17*^{-/-} mice, Smk/Flu *Tcrδ*^{-/-} mice showed transient increases in viral titers at peak infection (day 6), indicating that IL-17A derived from γδ T cells plays a critical role in early viral containment (Fig. 6B). Further, we found increased inflammatory cells in the BAL of Smk/Flu *Tcrδ*^{-/-} compared to WT mice (Fig. 6C). Histological analyses demonstrated extensive tissue destruction and macrophage infiltration in the lungs of Smk/Flu *Tcrδ*^{-/-} mice (Fig. 6D, 6E). To determine if lack of γδ T cells in mice exposed to smoke fail to induce viral specific T cell responses, we next examined IL-17A and IFN-γ production in different T cell subsets. ICC analyses of lung T cells showed no significant changes in CD4 and CD8 T cells expressing IL-17A in WT or *Tcrδ*^{-/-} mice in Air/Flu or Smk/Flu group (Fig. 6F, G). Further, Smk/Flu group significant decrease in IFN-γ in CD4 T cells in both WT and *Tcrδ*^{-/-} mice, while this response was also reduced in CD8 T cells in Smk/Flu group *Tcrδ*^{-/-} mice (Fig. 6G). In the absence of γδ T cells, both CD4^{+} T cells and CD8^{+} T cells show reduced IFN-γ in the Smk/Flu treated groups.

Despite the increase in inflammatory cells, we found decreased expression of IFN-γ and its regulated genes (e.g. *Irf7, Gzmb*, etc.) and significant reduction of protective HA specific IgG and IgA in Smk/Flu treated *Tcrδ*^{-/-} mice (Fig. 7A, B). Similarly, lung homogenates of *Tcrδ*^{-/-} mice contained increased inflammatory cytokines (e.g. MCP-1 and IL-6), while anti-viral immune responses were significantly reduced (Fig. 7C). Thus, in the context of smoke-exposed mice, lack of γδ T cells limits production of IFN-γ and related genes, lack of which correlates with poor survival after influenza infection.

Discussion

Seasonal and pandemic influenza pneumonia account for the largest number of COPD exacerbations and hospitalizations, and are in turn, associated with increased mortality [32]. Survival after acute lung infection heavily depends on the recruitment of specialized

immune cells that resolve the infection while simultaneously protecting barrier functions in the lungs [39,40]. Specifically, eradication of acute viral infections requires the rapid induction of type I IFNs to inhibit viral replication and reduce epithelial cell injury [8]. Human respiratory epithelial cells and mice exposed to smoke fail to express protective type I IFNs in response to influenza infection [9,11,40], indicating the presence of suppressive factors in the lungs that could mediate poor immune responses. We found that WT mice exposed to chronic smoke developed exaggerated lung inflammation marked by enhanced IL-17A expression and respiratory failure when infected with influenza. Increased mortality and morbidity in these mice was associated with decreased type I and II IFNs expression, and reduced HA-specific antibody production that resulted in inadequate viral clearance during the late stage of influenza infection.

The primary intention of our protocol was to simulate behavior in addicted smokers who continue to smoke despite development of acute lower respiratory tract infection. Our findings (e.g. reduced anti-HA specific IgG, or IgA antibody, IFN-γ, and its related chemokines), suggest that chronic smoke significantly inhibits anti-viral responses. In a model of side-stream smoke and high-dose influenza infection, lung viral clearance was shown to remain intact, although smoke-exposed mice showed increased mortality, which was associated with increased TNF-α, IL-6, and type 1 IFN expression [9]. Although influenza-specific memory antibody formation in response to influenza re-challenge was not altered in that model [9], our studies reported here did not specifically examine the role of IL-17a in B cell memory response to influenza re-infection. Whether global loss of IL-17a could provide better secondary immune response upon influenza re-challenge should be an interesting area of new investigation in the future.

Prior studies have shown that under homeostatic conditions, IL-17A has a limited role in acute influenza viral clearance, because IL-17RA deficient mice showed no defect when infected with influenza[41]. We show here that under steady state conditions, IL-17A is minimally expressed by CD4 and γδ T cells in the airway mucosa, but after influenza infection, they express IFN-γ. However in response to cigarette smoke, lung both γδ and αβ population of T cells increase IL-17 production[24], which we show here blunts IFN-γ responses after influenza infection. Notably, on day 6 post infection, we observed a transient decrease in influenza viral clearance in *Il-17a⁻/⁻* mice however this difference was no longer evident by day 8, and influenza-related lung injury as measured by lung weight and hemorrhagic scores, were significantly improved in smoke exposed infected *Il-17a⁻/⁻* mice. Together, these findings suggest that in this model of Smk/Flu treatment, absence of IL-17a promotes improved recovery and survival by day 14 post infection.

We and other have shown that γδ T cells represent less than 5% percent of total lung leukocytes, but their relative abundance is increased in response to chronic smoke exposure [21,42]. In influenza infected animals, *Irf7*, a transcription factor that is critical for the induction of type I IFN production and recovery from primary influenza infection in humans is increased [40]. The importance of this transcription factor in protection from acute viral infection has been underscored by the discovery of compound homozygous mutations in IRF7 in a patient who developed acute respiratory failure after primary influenza infection [34]. In the context of COPD and active smoking with its associated chronic lung

inflammation and loss of lung structural integrity, we found that smoke exposed mice showed significantly reduced *Irf7* expression after infection with influenza, whereas air exposed infected mice treated the same way showed a robust enhancement of this transcription factor. In response to chronic smoke exposure γδ T cells produce increased IL-17A and lose the capacity to secrete IFN-γ during Influenza A infection. IL-17 production has been shown to inhibit IFNγ production by CD4^{+} T cells in an autocrine manner [43]. We hypothesized that the enhanced IL-17 production after chronic smoke exposure reprogrammed γδ T cells inhibiting their ability to produce IFN-γ. Supporting this, IL-17A deficient mice showed intact immune response, expanded γδ T cell population which retain the ability to produce IFN-γ after influenza infection despite chronic exposure to smoke. We also showed that exposure to smoke consistently inhibited *Irf7* expression except in mice lacking IL-17A where its expression was independent of smoke exposure, indicating the inhibitory role of IL-17A in the regulation of *Irf7* and both type I and Type II interferons.

We have shown here that inhibition of IL-17A using blocking antibodies results in increased relative abundance of γδ T cells, and enhanced type I IFNs production during Influenza A infection. Further, we have found that blocking IL-17A in lung CD45^{+} cells, can reprogram γδ T cells in infected mice to increase *T-bet* expression. Conversely, exposure to cigarette smoke blocks this expression of *T-bet*. Together, these findings support a mechanism whereby chronic smoke exposure promotes persistent production of IL-17 by lung γδ T cells while simultaneously inhibiting *T-bet* and IFN-γ expression. However, because we used lung CD45^{+} cells in our in vitro assays, we speculate that inhibition of IL-17 under our culture conditions could be indirect. The exact mechanism(s) for this immune response in the lungs would be an interesting area of future investigation.

Although our studies confirm that both conventional CD4 (αβ) and γδ T cells predominantly express IL-17A in the lungs of mice exposed to smoke, and that these cells play non-redundant roles conferring protection against lethal Influenza A, we show here that γδ T cells specifically protect against influenza pneumonia under aberrant (e.g. smoke-induced) lung inflammatory conditions. In our model, the absence of γδ T cells resulted in worse outcomes in mice exposed to smoke and Influenza A as assessed by increased weight loss, mortality, lung inflammation and viral burden. We found γδ T cell deficient animals exposed to smoke had significantly compromised anti-viral immunity with reduced type I and II IFN production and failure to secrete HA specific antibody. In addition to their previously described role in controlling cytomegalovirus (CMV) infection [44], our findings indicate that γδ T cells potentially play a critical role in recovery from influenza infection in smokers with COPD. The protective role of γδ T cells were further confirmed in our preclinical model because deletion of this subset of T cells resulted in exaggerated lung inflammation and death in mice exposed to smoke and influenza infection. Increased lung inflammation (e.g. macrophages, neutrophils) may play a significant role in increased mortality in *Tcrδ*^{-/-} mice treated in Smk/Flu model, but specific contribution of ineffective innate cells was not evaluated in this study. Together, our findings suggest a model whereby cigarette smoke induces recruitment of activated γδ T cells that are biased towards IL-17A expression, and fail to produce protective immunity in the context of influenza infection. In this scenario, smoke exposure promotes constitutive expression of IL-17A in γδ T cells, fail

to produce IFN-γ expression which is required for induction of protective HA-specific antibodies. Consistent with our findings, a critical role for γδ T cells has been shown where immunodeficient mice reconstituted with human peripheral blood mononuclear cells (PMBCs) specifically expanded γδ T cells in response to aminobisphosphonate pamidronate (PAM) treatment and showed enhanced immunity against influenza [45]. The protective effect of PAM against influenza was lost in mice reconstituted with γδ T cells depleted human PBMC [45].

Smoke induced IL-17 responses limit Th1 cell differentiation that is required for effective antiviral protection. Mice exposed to smoke show baseline weight difference which might in part, account for the observation that following infection weight loss is more pronounced in the air group. The slope of average weight loss per day in flu infected mice was similar in air or smoke exposed group, and do not account for lack of appropriate immunological response to influenza infection. Notably however, IL-17A deficient mice exposed to smoke recovered faster from primary influenza infection, regained weight and showed robust type I and II IFN responses in γδ T cells following influenza infection. Highlighting the detrimental impact of IL-17 in influenza responses, neutralizing antibody to IL-17A protected air-exposed mice from primary influenza pneumonia, while blocking IL-17 after smoke exposure did not offer similar protection. The mechanism for the protective effects of IL-17A neutralization was in part mediated through enhanced induction of *T-bet* and IFN-γ in γδ T cells. Recovery of *T-bet* expression was not evident in cells from mice exposed to smoke demonstrating epigenetic changes induced by smoke that limit the immune system's ability to respond to subsequent challenges. Together, our findings highlight a novel immune mechanism underlying respiratory failure in smokers with COPD, and suggest that exploring natural and induced properties of γδ T cells in the lungs could provide new therapeutic option to treat viral infection in smokers.

Materials and Methods

Mice

Female wild-type (WT), and *Tcrδ⁻/⁻* mice (C57BL/6 background) were originally purchased from the Jackson Laboratory and all mice used in our experiments were bred at our facilities. *Il17a⁻/⁻* mice were acquired from Dr. Chen Dong's laboratory at University of Texas M. D. Anderson Cancer Center, were backcrossed six to eight generations to C57BL/6 as we have described previously [21] . All mice were bred in the transgenic animal facility at Baylor College of Medicine. All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and followed the National Research Council Guide for the Care and Use of Laboratory Animals.

Cigarette smoke exposure

Mice were exposed to cigarette smoke as we have previously reported [21,23]. Six- to 8-week-old female mice were exposed to active smoke from commercial cigarettes (Marlboro 100's). Exposure to 4 cigarettes (approximately 4 to 5 minutes/cigarette) per day, 5 days a week was carried out by intermittently forcing air (4 liters/minute) through the burning cigarette. Intermittent cycles were designed to mimic puffing cycles of actual human smokers and to

prevent CO2-induced asphyxiation. Puffing cycles consisted of 5 seconds of active cigarette smoke followed by 25 seconds of forced air by a timer-controlled 2-way valve (Humphrey). Mice were given 10 minutes of rest between each cycle of cigarette smoke exposure. In total, mice were given 4 cigarettes each day (1 hour), 5 days a week for 3 months. Specific details regarding our validated whole body smoke exposure are previously published [22]. Two days following influenza infection, mice were exposed to 2 cigarettes each day for two additional 12 days; mice were euthanized 14 days following influenza infection.

Flow cytometry and antibodies

Flow cytometry was performed with a BD LSRII (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc.). The following mouse-specific antibodies were purchased from BD Pharmingen: phycoerythrin (PE)-conjugated anti-IL-17A (559502); allophycocyanin (APC)-conjugated anti-interferon-γ (554413); Pacific Blue-conjugated anti-CD3e (558214); APC-Cy7-conjugated anti-CD8a (557654), and PE-C$\gamma\delta$-conjugated anti-CD4 (553050). In addition, FITC-conjugated anti-$\gamma\delta$ T cell receptor (11-5711-82), PE-conjugated anti-ROR$\gamma\tau$ (12-6981-80), PE-conjugated anti-CD11b (12-0112-82), and APC-conjugated anti-CD11c (17-0114-82) were purchased from eBioscience. PE/Cy7-conjugated anti-Tbet (644824) from Biolegend. Goat Anti-Mouse Ig (H+L) (1010-01), horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse IgG (1130-05) and IgA (1140-05) for ELISA were purchased from Southern Biotechnology. HA Recombinant Influenza A Virus protein, Subtype H3N2(A/Aichi/2/1968), His Tag (11707V08H50) for HA-specific ELISA were purchased from Sino Biological. Anti-IL-17A (CNTO8096) and isotype control (CNTO6601) rat/murine IgG2a/kappa were provided by Janssen R&D.

Gelatin gel zymography

MMP2 and MMP9 proteins in BAL fluid were detected using gelatin zymography [46]. Briefly, 10 μl of BAL fluid was separated using 10% SDS-polyacrylamide/2% gelatin and were washed in 2.5% Triton X-100 prior to incubation at 37°C in developing buffer (50 mM Tris HCL at pH 8, 5 mM $CaCl_2$, and 0.02% NaN_3). Gels were then fixed and stained with 50% methanol and 10% acetic acid that contained 0.3% w/v Coomassie blue. MMP2 and MMP9 appear as clear bands at (x0025A1)68 and 102 kDa, respectively [46].

Intracellular cytokine staining and ELISpot

Mouse lung RBC-free single-cell suspensions were stimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) (Sigma) and ionomycin (1 mg/ml) (Sigma) supplemented with brefeldin A (10 mg/ml) (Sigma) for 6 hours. Cells were fixed with BD FACS lysing solution, and then stained for surface markers with anti-CD3, anti-CD4, anti-CD8, and anti–$\gamma\delta$ TCR antibodies and then fixed with 1% paraformaldehyde, permeabilized with 0.5% saponin (Sigma), and stained with anti-IFN-γ and anti-IL-17A antibodies for analysis of intracellular cytokine production by flow cytometry. Enzyme linked immune-spot (ELISpot) assay used to detect IFN-γ, IL-4, IL-17 as we have previously described [47].

mRNA isolation and qPCR

Cell pellets were treated with TRIzol (Invitrogen), and mRNA was extracted with chloroform (Sigma-Aldrich), precipitated in isopropanol (Sigma-Aldrich), and washed in 70% alcohol (Sigma-Aldrich). The mRNA concentration was measured using Nano-Drop 2000 (Thermo Scientific). RNA was used to prepare cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Real-time RT-PCR was performed using Taqman Universal PCR Master Mix with specific primers to determine relative gene expression using the ABI PerkinElmer Prism 7500 Sequence Detection System (Applied Biosystems). The following primers of the analyzed genes, were all purchased from Applied Biosystems: *Il17a* (Mm00439619_m1), *Ifnγ* (Mm01168134_m1), *Mmp9* (Mm00600164_g1), *Mmp12* (Mm00500554_m1), *Irf7* (Mm00516788_m1), *Ifit3* (Mm01704846_s1), *Ifnb1* (Mm00439552_s1) and *Gzmb* (Mm00442834_m1) were purchased from Applied Biosystems. All data were normalized to 18S ribosomal RNA (Hs99999901_s1) expression.

Sub lethal influenza infection and anti-Il-17A blocking antibody treatment

Sex and age-matched 6 to 8-week old mice with exposed to air or smoke were given influenza A/Hong Kong/8/68 (H3N2) (A/HK/68) at a sub lethal dose of 25 $TCID_{50}$/mouse using aerosol administration[48]. A clinical isolate of influenza A/Hong Kong/8/68 (H3N2) (A/HK; Mouse Lung Pool) virus was stored as frozen stock (2.8×10^7 $TCID_{50}$/ml). Stock was diluted 1:1,000 in 0.05% gelatin in Eagle's minimal essential medium (Sigma-Aldrich) and animals were placed in an enclosed chamber and flu virus was aerosolized for 20 min. Viral concentration in the nebulizer before and after aerosolization and in lung homogenates was determined by hemagglutination assay of infected MDCK cells. Mouse weight changes, an indication of infection severity, recovery, and survival were monitored prior to and daily for 14 days post influenza infection. Mice were euthanized on day 14 unless indicated.

In selected experiments, mice were treated with 100 µg/mouse anti-IL-17A (CNTO8096), or equal quantity of isotype control (CNTO6601) i.p. [49] three days prior to influenza A inoculation, and continued every three days for two weeks as described in Fig S10A.

Determination of lung influenza viral titer

Some mice were euthanized on days 6, 8 and 10 after intranasal inoculation with influenza A as we have previously reported [33]; lungs were collected and rinsed in sterile water to lyse excess red blood cells. Lungs were placed in Dulbecco's modified Eagle's medium (DMEM) and homogenized using a glass bead beater (Biospec Products, Bartlesville, OK). Samples were diluted in DMEM containing 0.05 % trypsin (Worthington Biochemical, Lakewood, NJ), were centrifuged for 10 min at 9,000 rpm, and supernatants were serially diluted in 96-well round-bottom plates (Fisher Scientific, Atlanta, GA). Samples were then transferred to 96-well round-bottom plates containing Madin Darby canine kidney (MDCK) cell monolayers. Lung dilutions and MDCK cells were incubate for 4 days, and then visualized for characteristic adherence of turkey red blood cells (Fitzgerald Industries, Concord, MA).

Analysis of experimental model of COPD

Acellular bronchoalveolar lavage (BAL) fluid collection and quantitation of airway cells were carried out as previously described [21]. BAL fluid was collected using 0.8 ml of sterile phosphate-buffered saline (PBS) twice. Total and differential cell count in the BAL fluid were determined with the standard hemocytometer and HEMA3 staining (Biochemical Sciences Inc.) of 200 μl of BAL fluid prepared using the cytospin [21]. Cytokine and chemokine concentrations in the BAL fluid and lung homogenate supernatant were measured with Milliplex kit (Millipore). In some experiments, mouse lungs were dissected and single-cell suspensions were cultured overnight to measure cytokines or were used directly to measure intracytoplasmic cytokines (ICC) using the flow cytometer. Alternatively, some lungs were fixed with instillation of 4% paraformaldehyde solution via a tracheal cannula at 25 cm H_2O pressure followed by paraffin embedding and were sectioned for histopathological studies. Hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining were performed as described [21].

Semi-quantitation of lung inflammation scores

Hematoxylin and eosin-stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial and endothelial inflammation, bronchitis, edema, thrombi, pleuritic, and percentage of the lung surface demonstrating confluent (diffuse) inflammatory infiltrate [50]. The total lung inflammation score was expressed as the sum of the scores for each parameter.

Hemorrhagic score

Briefly quantification of lung hemorrhage was performed immediately after mice were euthanized at the indicated time points. Lung surface area were visually inspected by two blinded observers, and the extent of hemorrhage defined by percent of discolored lung surface area (e.g. from normal pink to maroon color), were scored using the following ranges: 0 (pink; no evidence of discoloration), 25, 50, 75, 100% (complete maroon discoloration) [37,38]

Mouse immune cell isolation from lung, draining lymph nodes, and spleen

Mouse lung, draining lymph node or spleen single-cell suspensions were prepared by mincing through a 40-μm Falcon cell strainer, followed by RBC lysis (ACK lysis buffer) for 3 minutes. To isolate lung APCs, RBC-free whole lung cells were labeled with paramagnetic bead– conjugated anti-CD11c (Miltenyi Biotec) and isolated using autoMACS.

Mouse lung CD45⁺cell isolation and IL-17 blocking studies

Single-cell suspensions of whole moue lung were prepared by mincing through a 40-μm Falcon cell strainer, followed by RBC lysis (ACK lysis buffer) for 3 minutes. Lung CD45$^+$ cells were labeled using microbeads-conjugated to monoclonal anti-mouse CD45 (130-052-301, Miltenyi Biotec) and isolated using autoMACS separator. Sorted cells (0.3×10^6 cells per well) were cultured in the presence of anti-IL-17a (30 μg/ml) or isotype control (30 μg/ml) at 37°C for 24 hr. *T-bet* and *Rorγt* expression in γδ and CD4 T cells were quantified using fluorescently labeling (PE/Cy7-*T-bet* or PE-*Rorγt).

ELISA

Serial dilutions of lung homogenate supernatants were added to 96-well plates coated with 5 μg ml^{-1} goat anti-mouse Ig (H+L) (Southern Biotechnology), and were incubated at room temperature for 2 h, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse IgG (1:8,000 dilution) and IgA (1:2,000 dilution) (Southern Biotechnology). TMB Peroxidase Substrate Kit (Bio-Rad Laboratories, Hercules, CA) was used for detection of bound antibodies, and optical densities at 450 nm were measured. Influenza A H3N2-specific antibodies in the lung homogenate supernatants were measured similarly as above, except that 96-well plates were coated with 2 μg ml^{-1} H3N2 HA (Sino Biological) and detected. A mixture of lung homogenates from wild-type smoke exposure mice infected with influenza virus A was used to establish standard curves in each plate, and antibody levels were shown as relative titers.

Statistical analyses

For the comparison of cytokine production and gene expression from air- or smoke-exposed and with- or without- influenza virus infection mice, we used the Student's t test or 1-way ANOVA and Bonferroni's correction for multiple comparison. All statistical analyses were performed with GraphPad Prism (Graph-Pad Software). The Student's t test was performed using 2-tailed parameters, and a P value of less than 0.05 was considered significant. All data shown are the mean ± s.e.m.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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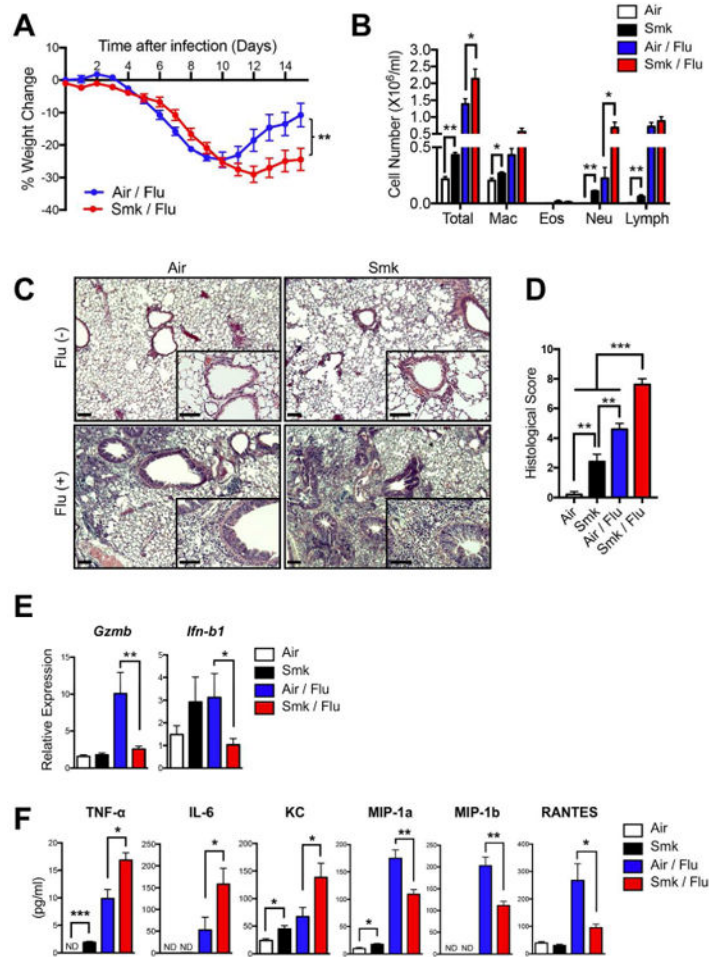
Fig. 1. Cigarettes smoke-exposed mice infected with influenza A show increased lung inflammation

Mice were exposed to air (Air) or smoke (Smk) for three months and were infected with H3N2 (A/HK/8/68) at 25 $TCID_{50}$ (<$LD_{2.5}$; sublethal). (A) Representative body weight reduction (percent change) on day 14 following influenza infection (Air/Flu, $n = 8$; Smk/Flu, $n = 7$). Data are representative of three independent studies, (B) Total bronchoalveolar lavage (BAL) cell number, and differential: macrophages (Mac), eosinophil (Eos), neutrophils (Neu) and lymphocytes (Lym) on day-14 following influenza infection. ($n = 4$ or 5 mice per group). (C) Histopathological analysis of the lung tissue collected on day 11 after influenza infection and pathology scores quantified (D) ($n = 5$ mice per group) **P<0.01, ***P<0.001. Representative (200X) hematoxylin and eosin (H&E) micrographs. Scale bar: 100 μm. (E) Relative expression of anti-viral defense granzyme b (*Gzmb*), and Interferon b1 (*Ifn-b1*) were measured using mRNA isolated from whole lung on day 14 following influenza infection. (F) Cytokines and chemokines were measured in BAL fluid (TNF-α) or whole lung homogenates supernatant on day 14 following influenza infection ($n = 8$ in each group). Results represent mean ± s.e.m, Significance was determined using the Student t test with Bonferroni correction for multiple comparisons *P<0.05, **P<0.01.
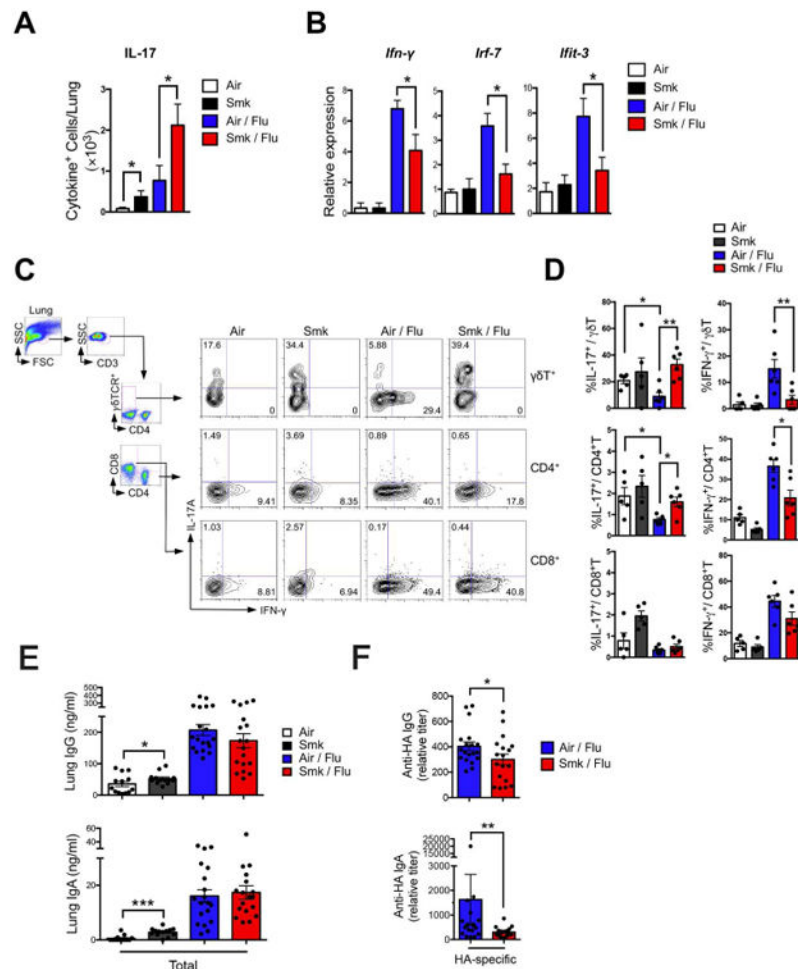
Fig. 2. Decreased protective immunity in influenza infected smoke-exposed mice

(**A**) ELISpot quantification of IL-17A using single cells isolated from whole lung in the same group of mice on day-14 following influenza infection ($n = 4$ per group) *P<0.05. (**B**) Whole lung mRNA expression of *Ifn-γ, Irf7, Ifit-3*, in the same groups of mice describe in Fig 1. (Air, Smk, Air/Flu, Smk/Flu, $n = 5$)(**C**) Representative intracellular cytokine staining analyses of lung γδT^{+}, CD4^{+}T, CD8^{+}T cells gated on total lung CD3^{+} lymphocytes. (**D**) Cumulative data IL-17A and IFN-γ % ICC in γδ, CD4, CD8 T cell subsets isolated from the lungs in the same group of mice ($n = 5$ or 6 per group). (**E**) Quantification of total IgG, IgA or (**F**) anti-HA specific IgG, or IgA antibody in the lung homogenates samples 14 days after influenza infection. Pooled data obtained from at least three different experiments (Air, $n = 13$: Smk, $n = 13$; Air/Flu, $n = 19$, Smk/Flu, $n =18$). Significance was measured using the Student t test with Bonferroni correction for multiple comparisons *P<0.05, **P<0.01, ***P<0.001.
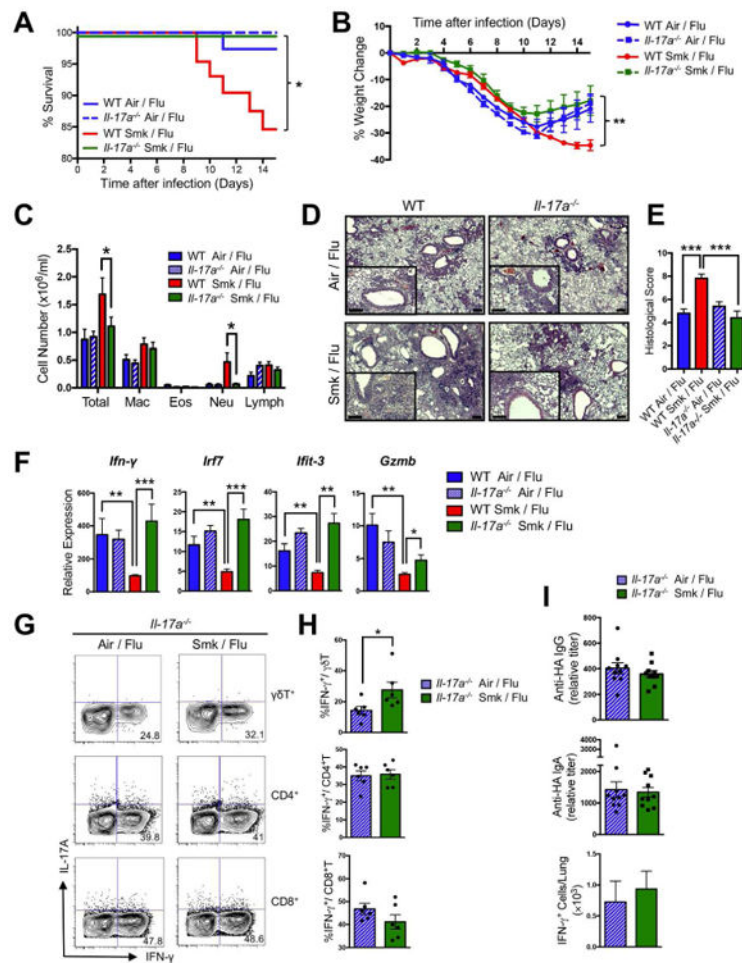
Fig. 3. *Il-17a ⁻* mice exposed to smoke generate protective immune response against influenza
Il-17a⁻/⁻ mice were exposed to air (Air) or smoke (Smk) for three months and were infected
with sub lethal H3N2 per protocol (Fig. S1). (**A**) Kaplan-Myer Survival curve for influenza
infected WT and *IL-17a⁻/⁻* (Air/Flu and Smk/Flu) mice. (WT Air/Flu, *n* = 58; WT Smk/Flu,
n = 43; *Il-17a⁻/⁻* - Air/Flu, *n* = 20, *Il-17a⁻/⁻* Smk/Flu, *n* = 30) determined by log-rank test.
*P<0.05 WT Smk/Flu vs. *Il-17a⁻/⁻* Smk/Flu (B**) Percent body weight changes were
monitored in the same group of mice for 14 days following influenza infection. (**C**)
Representative experiments showing total BAL cell count and differential (Mac, Eos, Lym,
Neu) in the same group of mice described in **A** (*n* = 7 or 8 per group) on day-14 following
influenza infection. (**D**) Representative Hematoxylin and Eosin (H&E) stain of lung tissue
section 14 days after influenza A infection and histology scoring (**E**) (*n* = 5 mice per group).
Scale bars: 100 **tu**m. ***P<0.001 (**F**) mRNA expression in total lung cells from WT and *Il-1
7a⁻/⁻ in* the same group of mice (*n* = 6 or 8 per group). (**G**) Representative flow cytometry
ICC plots and (**H**) cumulative frequencies for IL-17A and IFN-γ production in each of the T
cells subsets isolated from lungs in 2 different groups of *Il-1 7a⁻/⁻* mice as indicated before (*n*
= 6 in each group). (**I**) Quantification of anti-HA specific IgG, or IgA antibody using lung
homogenates in the same groups. Pooled data from two separate experiments (*n* = 5 per
group). IFN-γ measured in whole lung homogenates using ELISpot in the same group of

mice (n = 6 per group). Results are mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001 as determined by the Student t test and Bonferroni correction.
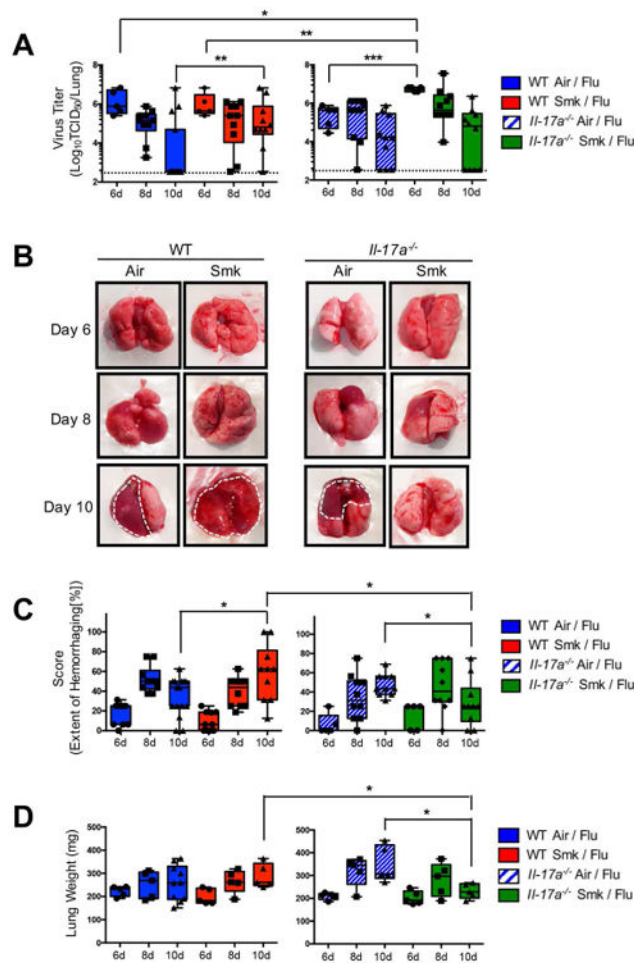
Fig. 4. Increased late stage lung viral load and damage in chronic cigarette smoke

Mice (WT and *Il-17a*^-/- mice) in Air/Flu and Smk/Flu groups were euthanized at peak (day-6), mid (day-8), and late (day-10) post sub-lethal dose (25 $TCID_{50}$) influenza A infection. (**A**) Quantification of viral load determined using standard plaque assay *P<0.05, **P<0.01, ***P<0.001 (day 6, *n* = 5 per group; day 8 and day 10, *n* =10 per group. Determined by two-tailed student's *t*-test. Data are representative of two independent experiments. Dashed line indicates the limit of the viral detection. (**B**) Representative photograph of lung hemorrhage, white dashed lines indicate the boundaries of lung hemorrhagic area (**C**) quantification of the hemorrhagic scores (% of surface area), (**D**) lung weight at 6, 8, and 10-days post influenza A infection in the same group of mice described in **A**. *P<0.05 (*n* =5 to 15 per group) using the Student t test with Bonferroni correction.
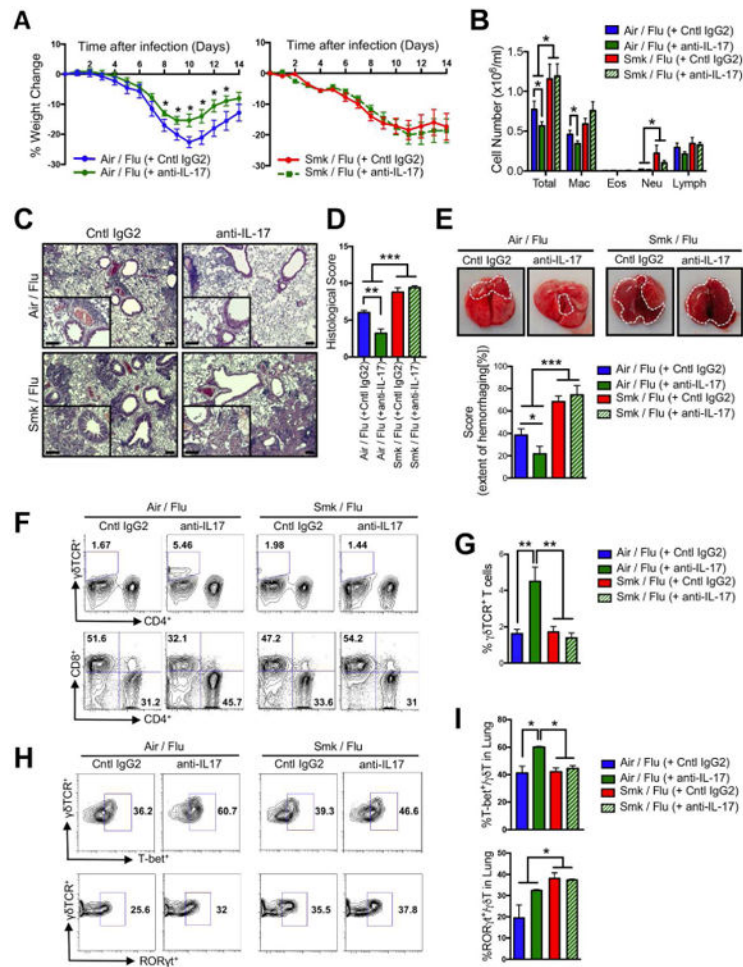
Fig. 5. IL-17 inhibition improves influenza recovery

WT mice were exposed to air (Air) or smoke (Smk) for three months and were infected with sub lethal H3N2 per protocol (Fig. S9A). Mice were treated with anti-IL-17A or isotype control (Cntrl IgG2) 100 ng/mouse for three days prior to flu infection and continued every three days for two weeks. (**A**) Percent body weight changes following influenza ($n = 9$ or 10). (**B**) Total BAL cell count and differential (Mac, Eos, Lym, Neu) in the same group of mice on day-14 following influenza infection described in **A** *P<0.05 ($n = 8$ or 9 mice per group). (**C**) Representative Hematoxylin and Eosin (H&E) stain of lung tissue section 14 days after influenza A infection and pathology score (D) **P<0.01, ***P<0.001. Scale bar: 100 μm. (**E**) Representative lung hemorrhage (top) and cumulative scores (bottom) in the same group of mice. (**F**) Representative flow cytometry, and (**G**) cumulative data showing relative abundance of γδ, CD4, and CD8 T cells in the lungs($n = 6$ in each group. **P<0.01 (**H**) Representative, and cumulative (**I**) lung CD45^{+} cells isolated from Air/Flu and Smk/Flu exposed mice as described in **B**, and were cultured with anti-IL-17 antibody (30 μg/ml) or isotype control (IgG2) for 24hr. *T-bet*, an*d Rorγt* expression in γδ T cells were detected using flow cytometry. Results are mean ± s.e.m ($n = 4$ per group). *P<0.05 using the Student t test with Bonferroni correction.

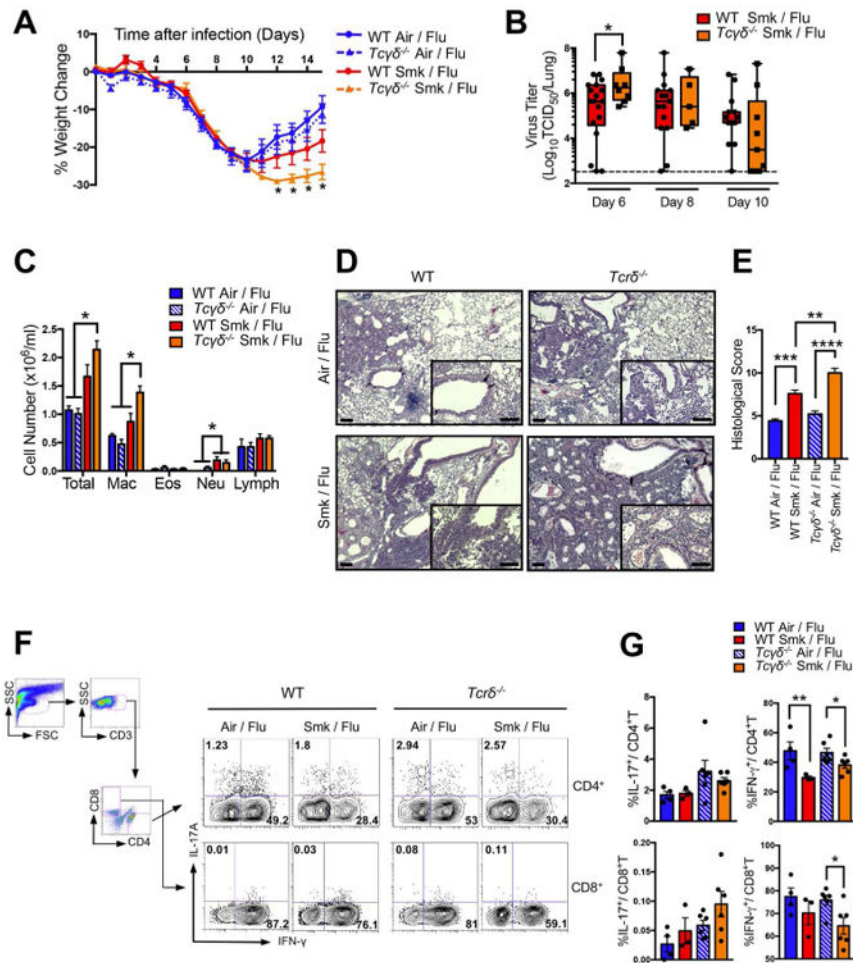**Fig. 6. Increased mortality in *Tcrδ⁻/⁻* mice exposed to smoke and influenza**

WT and *Tcrδ⁻/⁻* mice were exposed to air (Air) or smoke (Smk) for three months and were infected with sub lethal H3N2 per protocol (Fig. S1A). (**A**) Body weight reduction (percent change) following influenza infection (*n* = 7 to 10 mice per group). Data (mean ± s.e.m) are representative of three independent studies using Pearson correlation coefficient. (**B**) Quantification of viral load in the lung from mice (WT and *Tcrδ⁻/⁻* mice) in Smk/Flu groups. Data are presented as mean ± s.e.m. and are representative of two independent experiments. *****P<0.05 (**C**) Representative experiment showing total BAL cell count and differential (Mac, Eos, Lym, Neu) in the same group of mice on day-14 following influenza infection described in **A** (*n* = 4 to 8 mice per group). (**D**) Representative H&E stain of lung tissue section 14 days after influenza A infection and pathology score (E). Scale bars: 100 μm. ******P<0.01, *******P<0.001, ********P<0.0001 (**F**) Representative intracellular cytokine staining analyses of lung CD4⁺ T, CD8⁺ T cells gated on total lung CD3⁺ lymphocytes. (**G**) Cumulative data IL-17A and IFN-*γ*% ICC in CD4, CD8 T cell subsets isolated from the lungs in the same group of mice (*n* = 3 to 6 per group). *****P<0.05, ******P<0.01 using the Student t test with Bonferroni correction for multiple comparisons.
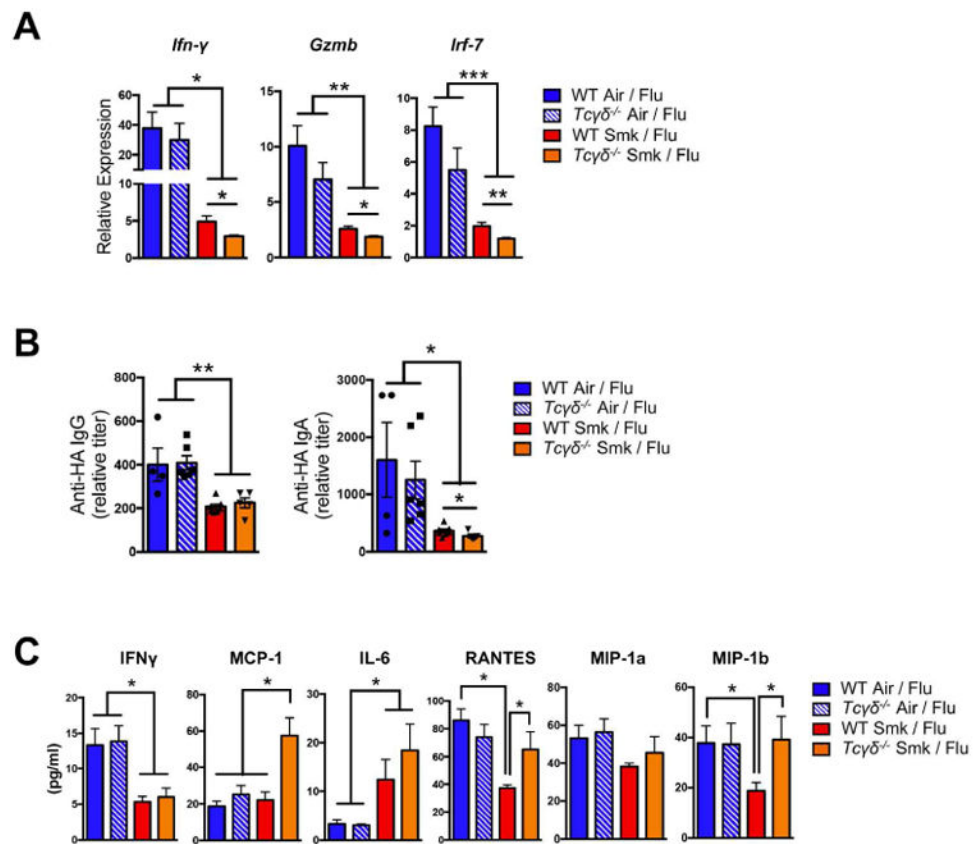
Fig. 7. Reduced anti-viral immune responses in $Tcr\delta^{/-}$ mice exposed to smoke and influenza
(**A**) Total lung mRNA expressions of *Ifn-y, Irf-7,* and *Gzmb* at day 14 in the same group of mice on day 14 following influenza infection ($n = 4$ to 8 mice per group). *P<0.05, **P<0.01, ***P<0.001 (**B**) Quantification of anti-HA specific IgG, or IgA antibody using lung homogenates in the same four groups. Pooled data from two separate experiments are presented as mean ± s.e.m. and significance was assessed using the Student t test. *P<0.05, **P<0.01 ($n = 4$ to 7 mice per group). (**C**) Selective cytokines and chemokines concentration was measured in lung homogenates using Multiplex assay in the same group ($n = 4$ to 8 mice per group). *P<0.05 as determined using the Student t test with Bonferroni correction for multiple comparisons.