Role of Itk in Th17 mediated inflammation model Hypersensitivity Pneumonitis

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Hypersensitivity Pneumonitis (HP) is a lung disease caused by repeated inhalation of environmental antigens leading to inflammation, tissue scarring, and some loss of lung function. This pathology is believed to be due to the increased IL-17A, a cytokine secreted predominantly by a subset of T cells, Th17 cells, that induces recruitment of inflammatory cells such as neutrophils and leads to pathology in this disease. The thermophile *Sacharopolyspora rectivigula* (SR) causes HP in humans, and we have used a murine model of HP, exposure to SR, to study the molecular mechanism of disease development. Using novel IL-17A-GFP reporter mice, our preliminary data suggests that the high levels of IL-17A induced in response to SR are produced in part by $CD4^+$ T cells and not by neutrophils. The Tec family tyrosine kinase Itk is a pharmaceutical target and regulates T cell activation and cytokine production, including Th2 cytokines and IL-17A in conventional Th17 cells. Mice lacking Itk are therefore resistant to developing Th2 cytokine driven allergic lung inflammation. However our experiments indicate that mice lacking Itk develop HP. Histology from these mice indicates an increase in inflammatory cells in lung airways as well as deterioration of lung tissue architecture. Mice lacking Itk also have significant levels of IL-17A mRNA expression in the lung, and an increase in the number of $CD4^+$ IL-17A producing cells. Furthermore, the lack of Itk signaling led to the absence of IL-17A producing γδ T cells in the early stage of HP, which recover over the later stage of disease. This phenomenon directly coincides with the emergence

of IL-17A producing $CD4^{+}\gamma\delta$ T cells in the lungs of $Itk^{-/-}$ mice exposed to SR. We conclude that Itk regulated signals are not critical for the production of IL-17A in response to SR, and Itk may therefore differentially regulate the production of IL-17A in different types of T cells. We also show that the inhibition of Itk kinase signaling can be inhibited by targeting allele sensitive Itk*as*. The block in kinase activity leads to decrease response of response in the BAL and lungs. We investigate the relationship between Th17 cell and T regulatory T cells in an IL-17A driven disease as it relates to Itk signaling. We find that Itk signaling is not altering the proportion or numbers of T regulatory cells. Additionally, we show that iNKT cells are a possible source of IL-17A and this primarily independent of Itk signal as well. Lastly, we investigate the role of IL-4Ra signaling and indirectly innate memory phenotype (IMP) cells in HP. Our data shows IL-4 signaling as well as IMP cells play a protective role in the BAL during HP. Furthermore, IL-4Ra and IMP cells are partially responsible for the number of $CD4^{+}$ T cells seen in HP, which is independent of $Itk^{-/-}$ mice ability to make IL-17A cytokine in SR induced HP. Together, you data show Itk mediate differential immune responses in SR induced HP.

BIOGRAPHICAL SKETCH

Chavez was born and raised in Summit MS and attended Alcorn State University where he majored in Biology. He was very active in student governance and student body President his senior year heading the Student Government Association in 2005-06. While at Alcorn, Chavez discovered his passion for research. He received a full scholarship in the Minority Access to Research Careers (MARC) Program to research full time in an immunology lab.

After earning a bachelor's degree from Alcorn, Chavez earned Master's Degree in Biology and was the first student selected to participate in the Alcorn State:Penn State Bridges to Doctoral Degree Program. After the successful completion of this program he began pursuing his PhD in Immunology at Pennsylvania State University. Chavez entered into Immunology and Infectious Disease graduate Field at Cornell University in 2010. He has presented his work at many national conferences and other form of media. While participating in the Yale University Edward Bouchet National Conference for Minority PhD's, Chavez won first place in the Natural Science oral presentation division. He also was a recipient of the 2014 MARC FASEB Travel Award to attend the American Association of Immunologists National Conference. He is a Alfred P. Sloan Fellow and the recipient of the Cornell Provost Diversity Fellowship.

Chavez has served as the President of the Black Graduate and Profession Association at Cornell University as well as Chair of the Diversity and International Student Committee in the Graduate and Professional Assembly. He received the Cornell University Distinguished Leader Award and Student of Color Exemplary Service Award.

Dedicated to father the late Honorable Mayor Charles E Carter, who passed away from Renal Cell Carcinoma inspired my passion to investigate immunology. He is and will always be my inspiration and my hero.

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Hypersensitivity pneumonitis and the immune response

Hypersensitivity pneumonitis

Hypersensitivity Pneumonitis (HP) is induced by an overly aggressive immune response to inhaled particulate antigens (Joshi et al., 2009; Takemura et al., 2008). Farmer's lung, which is a type of HP, is common in populations that are subject to chronic inhalation of foreign emissions, dust, and mold. Farmer's Lung is caused by repeated inhalation of the thermophilic bacteria *Saccharopolyspora Rectivirgula* (SR), which resides in moldy hay (Joshi et al., 2009; Pepys, 1969). Thermophilic actinomycetes are respiratory allergens capable of producing thousands of tiny spores that reach deep into human lungs (Lacey and Crook, 1988). As a thermophilic actinomycete, SR requires 55^{o}C temperature conditions to grow (Kim et al., 2010; Lacey, 1980). Another common form of hypersensitivity pneumonitis occurs after repeated inhalation of bird droppings in a condition known as bird fanciers lung (Kokkarinen et al., 1994). In farmer's lung, the occupational routine of harvesting moldy hay or straw by farmers in the early 20th century would induce HP in this population, hence the name. The dust or mold residue is inhaled and eventually reaches the small air sacs in the lung (**Fig. 1.1**). This response leads to inflammation and infiltration of interstitial mononuclear cell infiltration, bronchiolitis, fibrosis, and sometimes formation of granulomas within the lung **(Fig. 1.1)** (Takemura et al., 2008). Lung inflammation leads to symptoms of shortness of breath, dry cough, and fatigue after initial exposure. Long-term exposure later develops into more severe symptoms of chronic coughing, difficulty breathing, and weight loss. The most effective treatment for HP is to simply avoid exposure to the specific agitator, although this only applies for the early stages of disease, due to the fact that scarring and fibrosis from long-term exposure are both irreversible.

HP is clinically presented in acute, subacute, and chronic phases with inflammatory infiltrates that later proceed to loose formed granulomas and finally fibrosis (Woda, 2008). The granulomas are composed of foamy alveolar macrophages, lymphocytes and cholesterol clefts (Fisher et al., 1992). This is the body's defense mechanism to isolate foreign particles such as mycobacteria and fungi that immune cells have failed to clear. The early onset of HP is associated with a high population of $CD8^+$ T cells, whereas the later stages are more $CD4^+$ T cell rich (Girard et al., 2009). The T cells recognize the inhaled foreign particles presented by APC's and activate inflammatory cells, which lead to the reduction in lung function including asthma-like symptoms and accumulation of fibrosis in the lung (Girard et al., 2009). T cells contribute to the inflammatory process and are the main cause of alveolar lymphocytosis (Lo Re et al., 2010), which is the abnormal increase in lymphocytes in the lung. Nude mice, which lack αβ T cells, do not show complete elimination of pathology but development of HP is less severe, which suggests that αβ T cells may play a critical role in HP (Takizawa et al., 1992). These inflammatory cells enter into the lung and reside in large numbers around the alveolar sac and lung airways. The accumulation of inflammatory cells is pathogenic and this process not only physically obstructs the airways and air sac, but also leads to the alveolar destruction also known as honeycombing.

T cells are essential regulators of cell-mediated immunity. Naïve $CD4^+$ T Helper (Th) cells recognized the presence of foreign antigen presented by innate immune cells. This recognition leads to cytokine production and differentiation into distinct populations of T helper cells with individual effector function including Th1, Th2, Th9, and Th17 (Luckheeram et al., 2012). IL-2 and IFNγ are essential in initiating the downstream

cascades that lead to the development of Th1 T cell subsets, while IL-6, IL-21, IL-23, and TGFβ are critical in committing cells to become Th17 (Trinchieri et al., 2003; Veldhoen et al., 2006; Volpe et al., 2008). The Th1 cytokine IFNγ, produced by T cells was thought to be critical for the development of HP, however more recent investigations using murine models indicate a prominent role for Th17 cytokine IL-17A in this process. Mice lacking IL-17A exhibit reduced number of infiltration of inflammatory cells, as well as reduced inflammatory mediators IL-12p70, CCL3, and CXCL9 in murine models of HP (Joshi et al., 2009; Mroz et al., 2008). Previous studies using the SR induced model of HP in mice have shown that while there is significantly elevated levels of IL-17A in the lung, there is a surprisingly small percentage of $CD4^+$ Th17 cells in the lung, suggesting that IL-17A may be produced by other sources as well (Joshi et al., 2009). However, $CD4^+$ T cells have been identified as a source of IL-17A and in the mice lacking IL-17 receptors, studies have shown decreased cell infiltration and fibrosis in HP (Simonian et al., 2009b). γδ T cells are also a well-known source of IL-17A in response to antigen and bacterial infection (Girard et al., 2009; Mroz et al., 2008). Studies have shown that in a *B. subtilis* induced model of HP in mice, the concentration of IL-17A was significantly increased in the lung, and Vγ6Vδ1 γδ expressing T cells were the predominant population of IL-17A producing cells (O'Brien et al., 2009). Furthermore, neutrophils have recently been reported to be the major producers of IL-17A in HP (Hasan et al., 2013). Further investigation of the role of IL-17A expressing T cells' in the pathological process of HP is needed to better understand their contribution to SR induced HP. Thus while variety of cell types are able to produce IL-17A in response to cytokines or antigen presentation **(Fig. 1.2)**, the cellular source of IL-17A in HP is unclear.

iNKT cell Development and Function

Invariant natural killer T cells (iNKT cells) are an innate subset of T cells identified by their restricted invariant T cell receptor (TCR, Vα24-Jα15 in humans and Vα14-Jα18 in mice) (Godfrey et al., 2010; Lantz and Bendelac, 1994). These cells have characteristics of both NK cells and T cells, and express surface markers NK1.1 and the restricted semi-invariant TCR, as well as other NK and T cell surface molecules such as Ly-49 and CD4 respectively. iNKT cells recognize glycolipid antigen α-galactosylceramide (α-GalCer) presented by the non-classical MHC class I molecule CD1d (Bendelac, 1995; Godfrey et al., 2010). These cells are further characterized by their ability to rapidly produce Th1-associated cytokine IFNγ, Th2-associated cytokine IL-4 and Th17-associated cytokine IL-17A in response to stimulation with glycolipid antigen, as well as inflammatory cytokines. iNKT cells that have the ability to produce IL-17A cytokine have been identified as $CD4^{-}NK1.1^{-}$ (Michel et al., 2007). Furthermore, indirect activation of iNKT cells can occur during the recognition of LPS from bacteria and extracellular cytokines (Tupin et al., 2007). iNKT cells develop in the thymus where they are believed to acquire innate effector function, following which they migrate to the periphery (Wei et al., 2005). These cells have also been reported to have regulatory function in HP, since the production of IFNγ by iNKT cells, triggered by TLR4, can negatively regulate the production of IL-4, which suppresses pulmonary fibrosis but increases HP inflammation (Kim et al., 2012). The opposite occurs where IL-4 secreted from iNKT cells significantly reduces the HP by restricting neutrophil recruitment to the lungs (Hwang et al., 2006). These data suggest that iNKT cells sit at a delicate balance of immunopathogenic vs. immunosuppressive activity in HP.

Tec Family Kinase in T cells

The Tec protein kinase family is the second largest Protein Tyrosine Kinase subfamily and includes Btk, Itk, Tec, Rlk and Bmx (Schwartzberg et al., 2005; Yang et al., 1999). These kinases are significantly involved in specific signals mediated by a range of cytokines and antigen receptors, and are critical in the development of cells in the immune system. Studies have shown that Tec kinases are important in lymphocyte function and are important regulators of the immune response (Schaeffer et al., 2001; Schwartzberg et al., 2005).

Itk is a member of the Tec kinase family and plays an important role in T cell development as well as function (Cua and Tato, 2010; Fowell et al., 1999; Schaeffer et al., 1999; Schaeffer et al., 2001). Itk is activated by the TCR, which then leads to expression of multiple cytokines, which are critical in orchestrating the immune response (**Fig. 1-3)**. In the absence of Itk, mice and humans show reduced number of T cells, with $CD4^+$ T cells being most affected, and activation of naïve T cells are impaired (Fowell et al., 1999; Liao and Littman, 1995; Serwas et al., 2014). These $Itk^{-/-}$ $CD4^+$ T cells also show impaired in Th2 cytokine production, where they produce significantly less IL-4, IL-5, and IL-13 (Fowell et al., 1999; Schaeffer et al., 2001). A potential explanation of this is found in the fact that the lack of Itk correlates with defective activation of Ca^{2+} dependent transcription factor NFAT, which then leads to a decrease in IL-4 transcription (Fowell et al., 1999). Increases in intracellular Ca^{2+} is also required to mount a robust IL-17A response, and T cells lacking Itk show a decrease in IL-17A production by naïve $CD4^+$ T cells that have been conditioned to the Th17 subset (Gomez-Rodriguez et al., 2009). Itk has also been reported to play an important role in the development and function of γδ T

cells, with Itk deficient mice showing an increase in $CD4^+$ γδ T cells possessing Vγ1.1/Vγ6.2/3 γδ TCR (Felices et al., 2009; Qi et al., 2009). These cells develop independently of MHC class I and class II, which would suggest distinctly different development as compared to conventional T cells (Qi et al., 2009).

In mice lacking Itk, studies also show a block in the development of iNKT cells, as well as reduced function of iNKT cells (Felices and Berg, 2008; Gadue and Stein, 2002; Qi et al., 2012; Qi et al., 2011a; Yin et al., 2013). It has also been suggested that Itk plays a distinct role in iNKT cell survival since $Itk^{-/-}$ iNKT cells display increased apoptosis (Felices and Berg, 2008; Qi et al., 2012). Although Itk may not be required for complete maturation of iNKT cells, it is vitally important for their functional capabilities, since Itk is required for cytokine secretion in iNKT cells (Au-Yeung and Fowell, 2007; Gadue and Stein, 2002; Qi et al., 2012; Qi et al., 2011b).

Asthma is an allergic lung disease characterized by T cell regulated inflammation in the airways of the lungs. The two diseases differ in that asthma characterized by large populations of Th2 cells along with high IgE levels (Bogaert et al., 2009; Robinson, 2000). Similar to HP, the T cell mediated inflammatory response in asthma is heavily driven by cytokine produced by Th2 cells in asthma and Th1/Th17 cells in HP (Steinke and Borish, 2001; Villar et al., 2014) (Sahu et al., 2008b). The tyrosine kinase Itk expressed in T cells has been found to play an important role in the development of asthma (August and Ragin, 2012; Mueller and August, 2003; Sahu and August, 2009; Sahu et al., 2008a).

T regulatory cells

T regulatory cells (T_{reg}) are a subset of αβ $CD4^+$ T cells that have been classified as either naturally occurring or inducible populations. Natural T_{reg} cells express surface markers expressing $CD4^+$ and $CD25^+$ as well as transcriptional factor Forkhead box P3 (Foxp3). They develop and mature in the thymus and later migrate to peripheral tissue (Bluestone and Abbas, 2003). Inducible cells develop from either naïve $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells in the periphery that are have been active by antigen presenting dendritic cells in addition to exposure to IL-10 and TGFβ (Mills, 2004). $CD4^+CD25^+Foxp3^+$ constitute about 5-10% of T cells in the periphery and are pivotal in regulating immune cell homeostasis and maintaining a balance between immune response protection and tissue damage (Mills, 2004). Th17 and $T_{reg's}$ share a requirement of TGFβ-1 for their development (Rudensky, 2011; Zhou et al., 2007), and studies have shown that while IL-2 promotes the activation of Foxp3 in T_{reg} cells, IL-2 inhibits the differentiation Th17 cells subset (Laurence et al., 2007). This is important in investigating the role of Itk as previous studies have identified Itk as a negative regulator of IL-2 induced expansion of T_{reg} cells (Huang et al., 2014b).

In the absence of Itk, $CD4^+$ T cells exhibit an increase in the induction of Foxp3 expression under Th17 conditions (Gomez-Rodriguez et al., 2014; Huang et al., 2014b). Furthermore, T_{reg} cells from mice lacking Itk exhibited a significant reduction in TCR-induced activation of mTOR, which is required to generate inducible Th17 cells (Conti et al., 2009). Studies have shown an increase in pathological severity as denoted by increased inflammation and histological score in the absence of $CD4^+CD25^+$ T_{reg} cells in murine models of HP (Park et al., 2009). These investigators further show that restoring

T_{reg} cells enhances protection against HP (Park et al., 2009). The delicate balance of protection vs. pathology is dependent on an antithetical relationship between Th17 and T_{reg} cells in a health immune response.

Aims:

Pathology due to Hypersensitivity pneumonitis is believed to be driven by $CD4^+$ αβ T cells and the production of inflammatory cytokine IL-17A, whereas in the absence of IL-17A, lung infiltration and fibrosis is significantly decreased. Conventional αβ T cells from Itk deficient mice are defective in the production of IL-17A, and the hypothesis being tested is that mice lacking Itk will be resistant to developing HP. Using cytokine reporter mice that have a GFP tag that reports the expression of IL-17A, we have investigated the role of Itk in IL-17A production during HP.

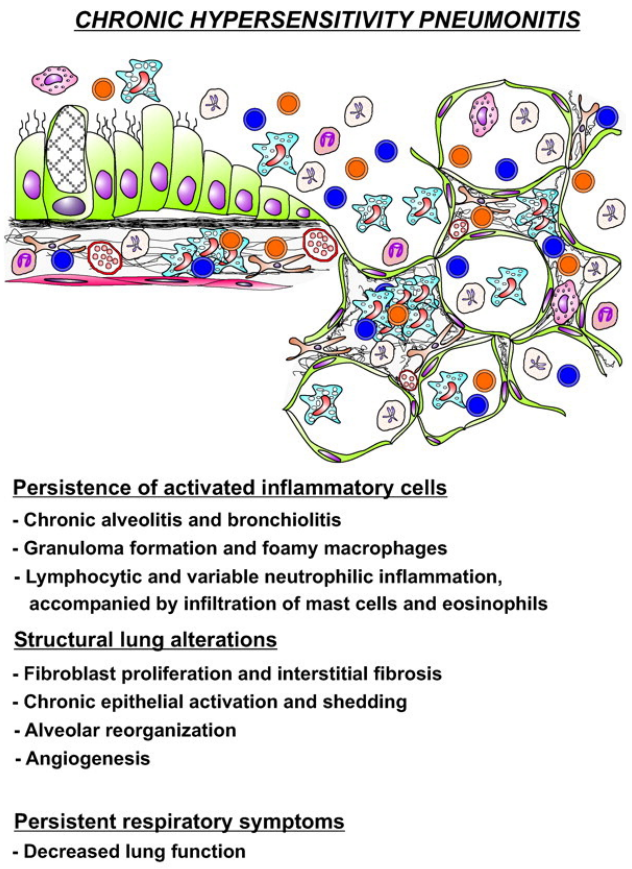

Fig. 1-1. Schematic of Hypersensitivity Pneumonitis. SR is inhaled into the airways and leads to inflammation, alveolaritis and inflammation of the bronchiole. This also leads to the formation of granulomas and influx of immune cells that lead to fibrosis, which consequently cause, epithelial shedding, alveolar epithelial reorganization, and decrease lung function.

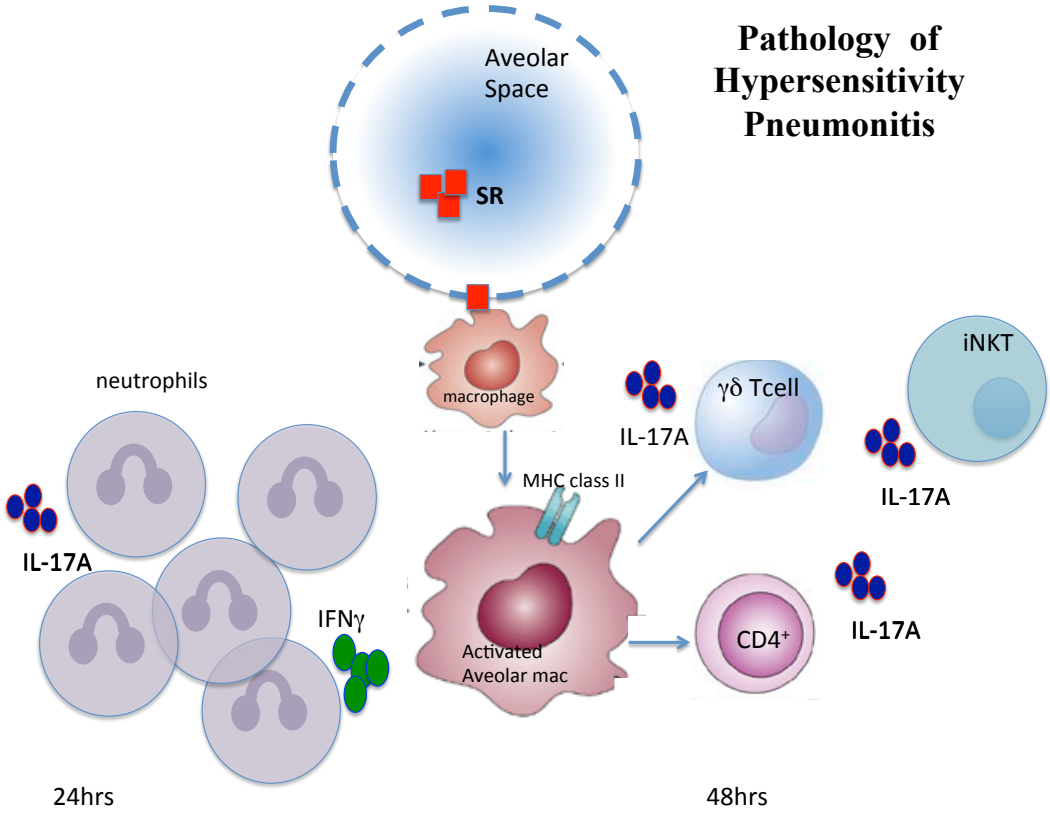

Fig. 1-2. Pathology of Hypersensitivity Pneumonitis. SR is inhaled into the airways of the lungs and processed by alveolar macrophages. Neutrophils are recruited to the lung and secrete IFNγ cytokine. Within 48 hours of exposure, initiation of the cell-mediated pathway occurs. Antigen is presented to $CD4^+$ T cell after activation of alveolar macrophages, and differentiates to Th17 cells and makes IL-17A cytokine. Also, γδ T cells are proposed to be a possible source of IL-17A. iNKT cells have the potential to secrete IL-17A and contribute to the pathology of HP.

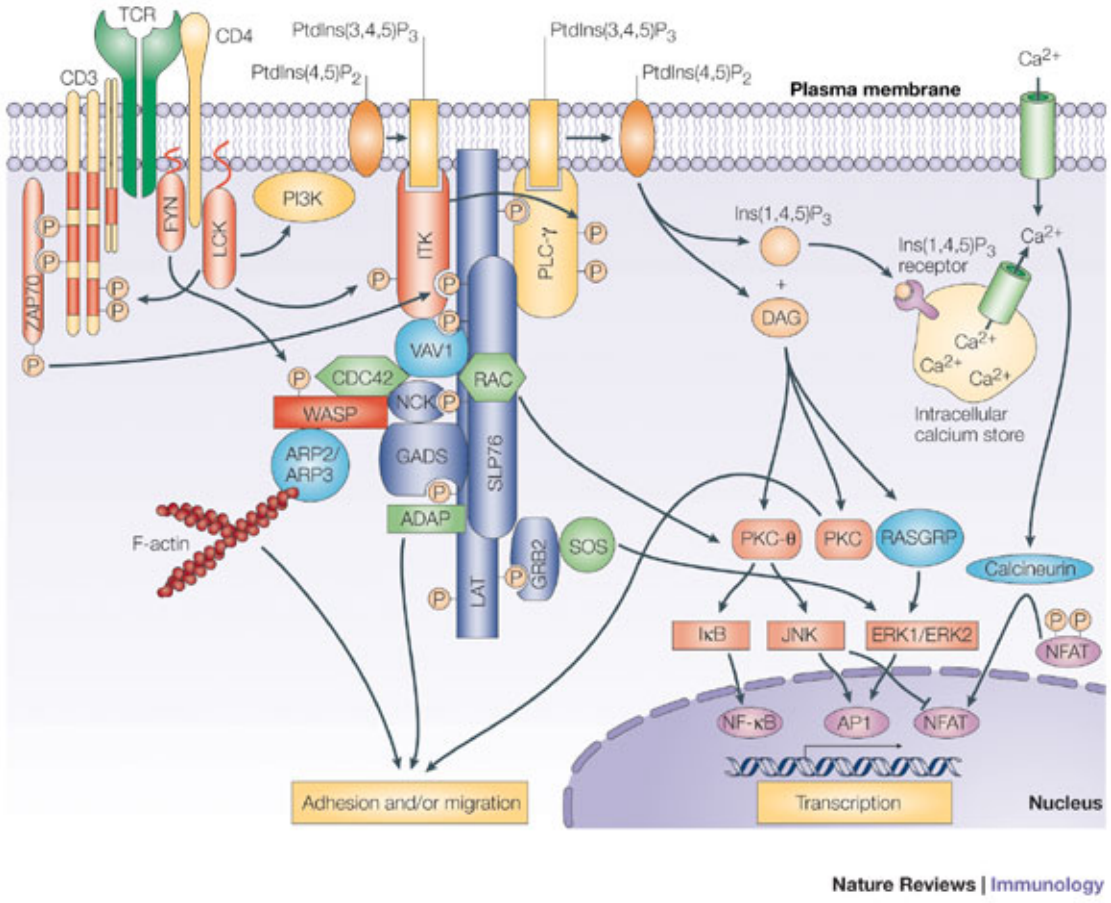

Fig. 1-3. Central role of Itk in T cell activation. Tec kinase signaling pathway is essential to T cell function downstream of the T cell receptor (TCR). Itk plays a pivotal role is this pathway after being recruited from the cytosol to form a complex signaling platform anchored by LAT (linker for activation of T cells) and SLP76 (SRC-homology-2-domain-containing leukocyte protein of 76 kDa). After activation of the TCR, Itk is phosphorylated by LCK, which then phospholipases C-γ (PLC-γ). This activation leads to the formation of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$ (required for Ca^{2+} mobilization) and diacylglycerol (DAG) (initiates activation of PKC θ and Ras guanyl-releasing protein, RASGRP). Afterwards, protein kinases JNK (JUN amino-terminal kinase), ERK1 (extracellular-signal-regulated kinase 1) and ERK2 are activated. This activation is important in orchestrating the transcription of NFκ-B (nuclear factor-κB), AP1 (activator protein 1), and NFAT (nuclear factor of activated T cells). Reference: (Schwartzberg et al., 2005)

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Chapter 2

Materials and Methods

Mice. All mice were on a C57Bl/6 background. IL-17A-GFP mice were purchased from Biocytogen, $Itk^{-/-}$ were from our previously established colony, $TCR\delta^{-/-}$ (γδ T cell deficient) mice were from Jax Labs, $IL4R\alpha^{-/-}$ mice were a kind gift from Dr. Frank Brombacher (University of Cape Town, South Africa, via Dr. Fred Finkelman, University of Cincinnati, (Herbert et al., 2008)). IL-17A-GFP/$Itk^{-/-}$, TCRδ/$Itk^{-/-}$ and $IL4R\alpha^{-/-}$/$Itk^{-/-}$ mice were generated by crossing with $Itk^{-/-}$ mice. *Tg(CD2-hItkas)* mice were generated by cloning the cDNA of human Itk*as* into a transgenic expression cassette driven by the CD2 promoter and enhancer as described (Kannan et al., 2014) All mice were housed in specific pathogen free environment, and were between 6-8 weeks of age when used. The Office of Research Protection's Institutional Animal Care and Use Committee at The Pennsylvania State University and Cornell University approved all experiments.

***Saccharopolyspora rectivirgula* (SR) induced Hypersensitivity Pneumonitis.** SR (obtained from American Type Tissue Collection catalogue #153347) was prepared by growing at 55^{o}C in tryptic soy broth with constant agitation. The SR was then washed in sterile water three times, centrifuged and suspended in sterile PBS. Protein concentration is measured using protein quantification kit (QB Perbio). Mice were exposed intranasally to 150 μg extract of SR 3 times a week for 3 weeks (**Fig. 2-1)**. Twenty-four hours after the last exposure, mice were sacrificed and analyzed. In order to assess the pathology of HP, it is necessary to utilize techniques that will effectively determine the types of cells in the lung to determine lung infection. Bronchoalveolar lavage (BAL) is a prominent method of investigation that is used to assess fluid recovered from patients with HP (Reynolds et al., 1977). A saline solution is delivered to the lung, which allows

for sampling of the epithelial lining fluid, as well as composition of the pulmonary airways where much of the inflammation takes place. In some cases mice were treated intranasally with vehicle (Dimethyl sulfoxide (DMSO)+ethanol+Sterile water) or kinase inhibitor 4-Amino-1-tert-butyl-3-(3-methylbenzyl)pyrazolo[3,4-d]pyrimidine (3-MBPP1) 3 hrs. prior to SR exposure to inhibit the kinase activity of Itk*as* in the relevant mice. In other cases, IL-17A GFP mice were treated with 25 mg/kg of Rapamycin (i.p.) delivered every other day while receiving SR (i.n.) for 3 consecutive days per week for 2 weeks.

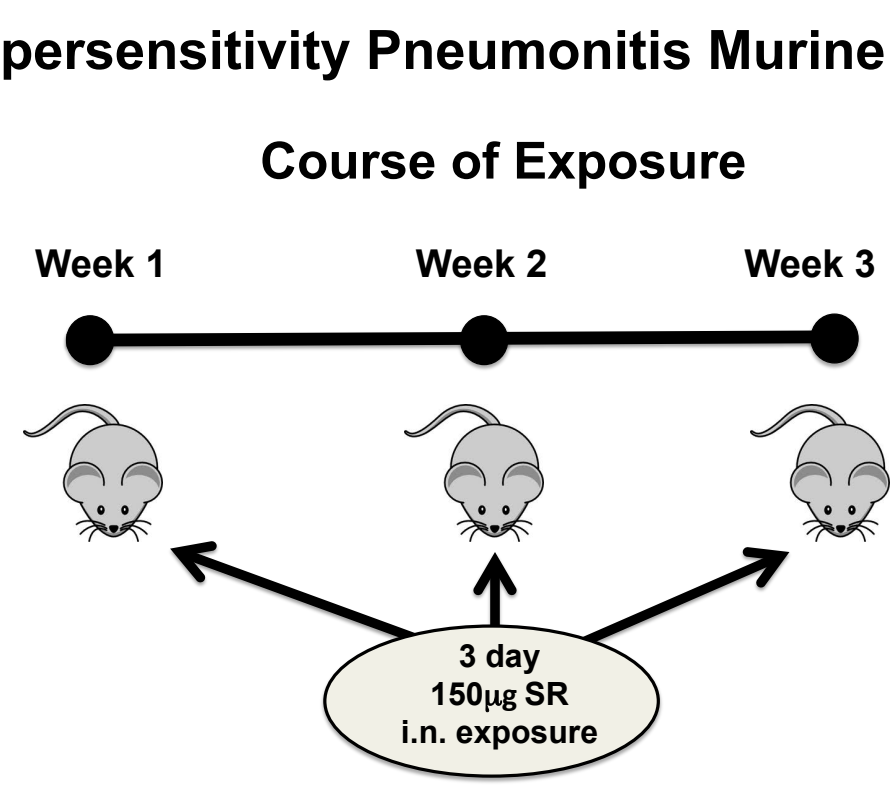

Fig. 2.1. Model of exposure for Hypersensitivity Pneumonitis. Mice were exposed to 150 μg of SR intranasally 3 consecutive days for 3 weeks as indicated. Mice were scarified 24 hrs. after final injection.

Cell stimulation and flow cytometry. Live cells were collected from BAL, lung tissue homogenates, spleen, and draining lymph nodes. Cells were stimulated with P/I (100 ng/ml/0.5 μM) and Brefeldin A (BFA, 10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 6 h, followed by fixation/permeabilization and staining with indicating antibodies using a FoxP3 staining buffer kit (eBioscience). Cells were stained as previously described with the following antibodies: *Ebioscience* (anti-Ly6G PE-C7, anti-IL-17A PerCP-CY5.5) *Biolegend* (anti-TCRβ *APC-CY7)*, *BD Pharm* (anti-CD4 PE, anti-CD8α PE-CF594, anti-IFNγ FITC) , anti-NK1.1 anti-CD11b Alexa 647). Cells were analyzed using a BD FACS Aria II flow cytometer and analyzed with Flojo software.

Reverse transcription/Quantitative PCR. RNA was extracted either using TRIZOL reagent (Invitrogen) or a RNAeasy kit (Invitrogen) and cDNA was generated using a You Prime First-Strand beads kit (GE Healthcare). q-PCR was then performed using a 7500 Fast Real-Time PCR instrument (Applied Biosystems). Data was analyzed using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method, normalized to respective and the values were expressed as fold change compared to WT mice.

Histopathological analysis of lung sections. Lung samples were harvested from mice after pulmonary perfusion. Mice were sacrificed 24 hrs after final SR exposure. Lung tissues were collected, stored in 10% formalin embedded in paraffin, and sections were stained with H&E by the Cornell University histology lab.

Data analysis. Student's *t* test or two-way ANOVA analysis were performed using GraphPad Prism version 5.00 for Windows (GraphPad, San Diego, CA). Differences with probability $p \leq 0.05$ were considered statistically significant.

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Chapter 3

Characterizing the role of Itk in the immune response during development of Hypersensitivity Pneumonitis

Introduction

Hypersensitivity Pneumonitis (HP), also known as extrinsic allergic alveolitis, is a complex disease, involving both innate and adaptive immunity. There are several categories of HP which include but are not exclusive to bagassosis, mushroom worker's disease, suberosis, malt worker's lung, and farmer's lung (Yi, 2002). The specific environmental agent causing the inflammation normally categorizes the HP disease (Lacasse et al., 2003). The inflammation in the airways of the lung in HP is due to the repeated exposure of allergic environmental antagonist such as moldy hay, moldy sugar, moldy compost, and contaminated barley (Yi, 2002).

To replicate the inhalation process of repeated antigen exposure observed in human HP in a laboratory setting, mice are exposed intranasally, or in some cases, intratracheally to the *Saccharopolyspora rectivirgula* (SR). This process induces a similar response to that seen in human farmer's lung (Roberts and Moore, 1977; Semenzato et al., 1985). Studies of BAL fluid from patients with HP have shown the composition of immune cells within the lung of patients at multiple stages of disease (Lacasse et al., 2003; Navarro et al., 2003). What we know is that neutrophils and T cells are major components of the cells recovered from BAL in patients suffering from HP (Woda, 2008). T cells are vital contributors to host immune responses against pathogens, bacteria, viruses and other invasive organisms. Neutrophils, macrophages, natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells are critical for the development of HP, and contribute to the pathology (Girard et al., 2009; Lacasse et al., 2009; Woda, 2008). A recent study even declared that neutrophils are the major source of IL-17A during HP in mice (Hasan et al., 2013). A better understanding of how this disease develops will lead to the

development of better therapy, since mechanisms involved in Th17 driving inflammatory lung disease are far less understood in comparison to the classic Th2 mediated asthma.

The recognition of pathogens by T cells initiates a downstream signaling cascade to appropriately respond to the organism (Smith-Garvin et al., 2009). Tec kinases are pivotal in the signaling pathways leading to the activation of immune cells and in specific cases, are needed to complete a full immune response by not only T cells, but also B cells and Mast cells (Berg et al., 2005; Schwartzberg et al., 2005; Yang et al., 1999). Itk signaling has been shown to be necessary for the development of Th2 and Th17 cells (Berg et al., 2005; Fowell et al., 1999; Gomez-Rodriguez et al., 2009; Schaeffer et al., 2001). Indeed, mice lacking Itk do not develop Th2 driven allergic asthma (Mueller and August, 2003; Sahu and August, 2009).

HP was originally described as a Th1 mediated disease, driven by IFNγ (Gudmundsson and Hunninghake, 1997), since depletion of IFNγ was able to reduce the pathology of HP. Neutrophils were also identified as a source of IFNγ (Nance et al., 2005). However, in murine models of HP driven by SR, IFNγ is no longer detectible in lungs of mice after 2 weeks of exposure (Simonian et al., 2009b). Furthermore, more recent studies have identified a strong Th17 response from $CD4^+$ T cells in HP (Joshi et al., 2009). Itk has been shown to be important for the production of IL-17A by Th17 cells (Gomez-Rodriguez et al., 2009), and we therefore hypothesize that Itk regulates the production of IL-17A in conventional Th17 cells, and regulates the development of HP.

We have utilized IL-17AGFP reporter mice in a model of SR-induced HP (Simonian et al., 2009b), to investigate the role of the Tec kinase Itk in regulating

development of IL-17A dependent airway immune response. We show that γδ and later $CD4^+$ αβ T cells are the predominant sources of IL-17A during SR-induced development of HP. Furthermore, contrary to previous reports, we find that neutrophils are not major producers of this cytokine in vivo. Additionally, while Itk plays a critical role in Th2 mediated allergic airway inflammation, and is required for the development and cytokine production of IL-17A by Th17 cells, we found that Itk is not required for the development of Th17-driven lung inflammation in HP. These data indicate that Itk differentially regulates Th17 cytokine production dependent on the type of lung inflammation. This work has implications for understanding T helper differentiation programs and Itk as a therapeutic target for T cell-driven inflammatory diseases.

Results

The absence of Itk does not affect development of lung inflammation induced by SR

To determine the role of Itk in a Th17-driven model of HP, we compared $Itk^{-/-}$ mice to WT mice following exposure to SR intranasally for 3 consecutive days over a 3 week period as previously reported (Simonian et al., 2009c). A hallmark of HP is severe inflammation in the lungs after repeated exposure to inhaled antigen. We found that there was severe inflammation in WT mice exposed to SR, which was surprisingly also seen in mice lacking Itk (**Fig. 3-1**). Given the fact that SR induced HP has been shown to be dependent on IL-17A (Joshi et al., 2009), these data suggest that contrary to expectations, Itk may not be required to generate a Th17 mediated inflammatory response in the lung in response to SR exposure.

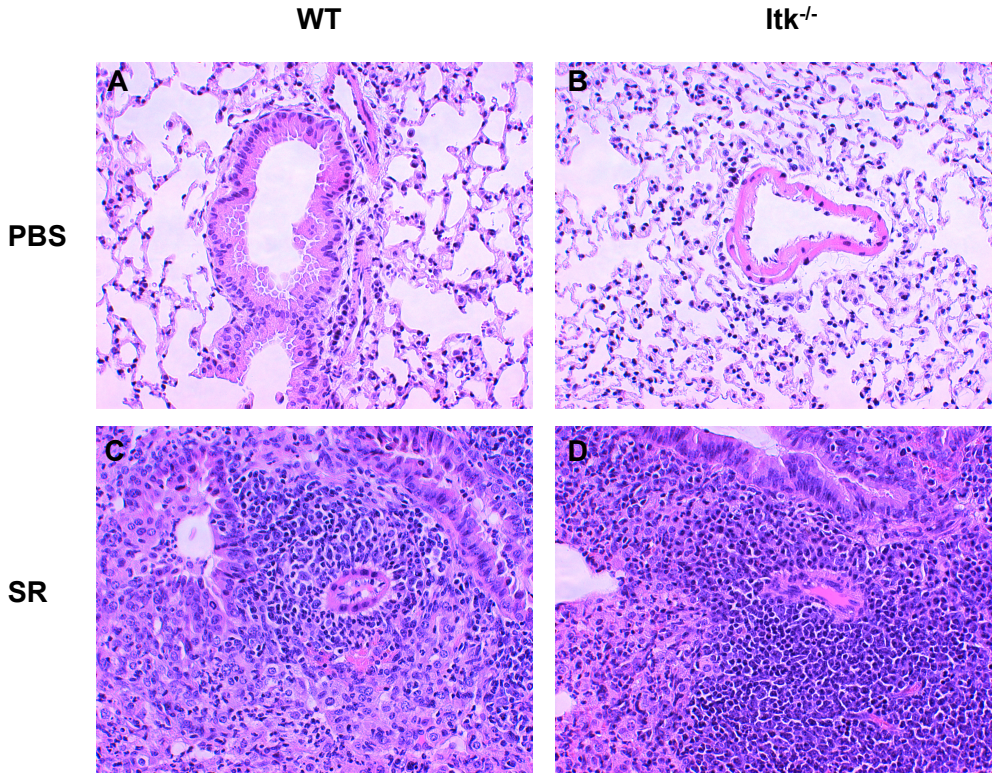

Fig. 3-1. The absence of Itk does not affect development of lung inflammation in HP model. 5-7 week old WT and Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week over a period of 3 weeks. **(A)** WT mice were exposed to PBS i.n. for 3 weeks **(B)** Itk$^{-/-}$ mice were exposed to PBS i.n. for 3 weeks. **(C)** WT mice were exposed to SR i.n. for 3 weeks. **(D)** Itk$^{-/-}$ mice were exposed to SR i.n. for 3 weeks. Tissue from lung sections from the indicated mice treated as indicated were stained with Hematoxylin and Eosin (H&E) staining and analyzed for severity.

Itk is not required for induction of IL-17A mRNA in lungs

To determine if the absence of Itk affected the expression of IL-17A in the lung, we investigated cytokine mRNA in lung tissue by quantitative RTPCR. We found that there was no induction of IFNγ mRNA compared to the PBS control in either WT or $Itk^{-/-}$ mice (**Fig. 3-2A**). By contrast, WT mice as well as $Itk^{-/-}$ mice exposed to SR show a significant increase in IL-17A mRNA levels as compared to PBS exposed mice (**Fig. 3-2B**). Further analysis of other cytokines revealed that IL-17A mRNA was induced to the highest level compared to Th1 and Th2 cytokines IFNγ and IL-4, confirming the IL-17A driven nature of this disease (**Fig. 3-3A**). Surprisingly, $Itk^{-/-}$ mice express higher levels of IL-6 during HP, commonly associated with increased cellular recruitment to the lungs (**Fig. 3-3B**). Furthermore, we observed induction of IL-17F in SR induced HP was not statistically different in the absence of Itk **(Fig. 3-3C)**. These findings suggest that the absence of Itk does not affect the development of lung inflammation or induction of IL-17A due to exposure to SR. Conversely, the increase in IL-6 may be associated with an increase in the recruitment of inflammatory cells during HP.

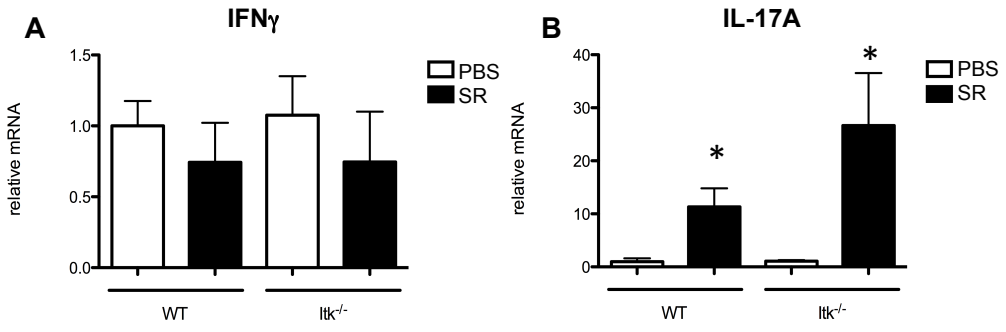

Fig. 3-2. The loss of Itk does not impair expression of IL-17A in the lung. 5-7 week old WT and Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week for 3 weeks. Lung tissue was collected and analyzed for mRNA levels of **(A)** IFNγ or (**B**) IL-17A. *$p<0.05$ compared to PBS controls. Data are +/- SEM, n= 3 mice per group.

Itk does not affect the recruitment of αβ T cells to the lung during development of HP

To determine whether Itk affects the recruitment of T cells to the lung during SR-induced inflammation, we harvested cells from BAL fluid and lung tissue along with cells from secondary lymphoid organs spleen and draining lymph nodes. The cells are then stained for αβ TCR surface markers and quantified using flow cytometry. The results show that a large number of αβ T cells were recruited to the airways (via analysis of BAL) over the course of 3 weeks of exposure to SR (**Fig. 3-4A**), and the loss of Itk did not affect this recruitment. We also saw an increase in the number of αβ T cells from lung samples in mice lacking Itk over the WT mice, but there was no difference in cell number at the end of the third week of inflammation (**Fig. 3-4B**). This increase in T cell numbers was mainly compartmentalized in the lung, since similar increases were not found in the lymph nodes or spleens of exposed mice (**Fig. 3-4C,D**). These data collectively suggest that αβ T cells are migrating and/or proliferation in the lung during exposure to SR and that the absence of Itk does not regulate this process. This also suggests that the immune response to SR is mainly restricted to the lung.

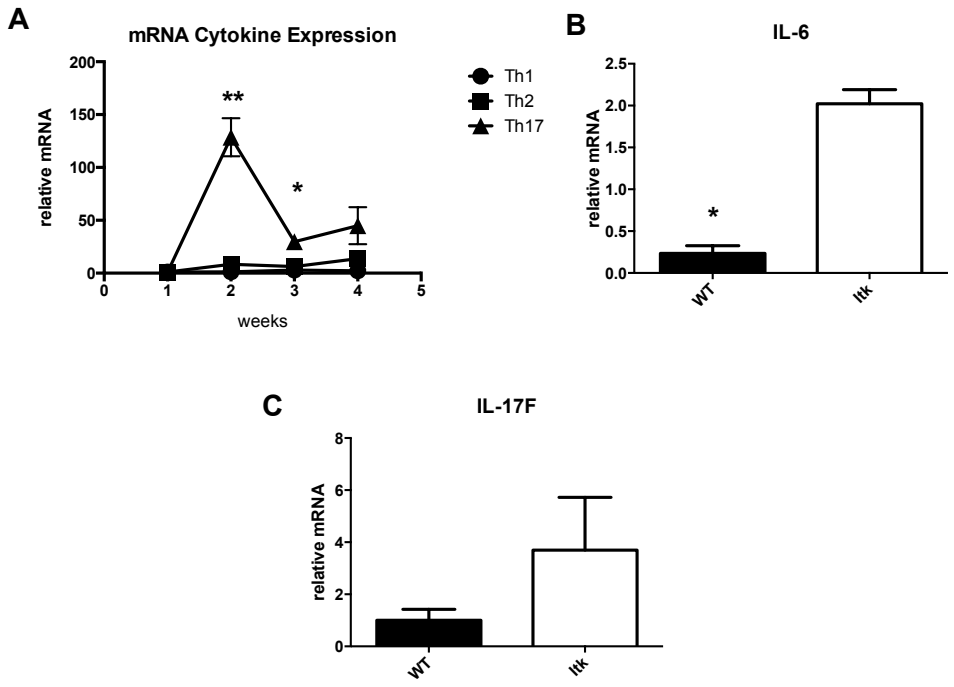

Fig. 3-3. SR induces an Itk independent increase in Th17 but not Th1 or Th2 cytokines in the lung. 5-7 week old WT and Itk$^{-/-}$ mice received SR intranasally 3 times a week for 4 weeks. Lung tissue was collected and analyzed for mRNA levels of **(A)** Th1 cytokine IFNγ**,** Th2 cytokine IL-4**,** and Th17 cytokine IL-17A. **(B)** Lungs were analyzed at week 3 of exposure for mRNA expression of IL-6, and **(C)** IL-17F. **p< 0.05 Th17 vs. Th1&2 at week 2, p< 0.05 Th17 vs. Th1&2 at week 3, *p< 0.05 WT vs. Itk$^{-/-}$ IL-6 mRNA expression. Data +/- SEM, n= 3 mice per group.

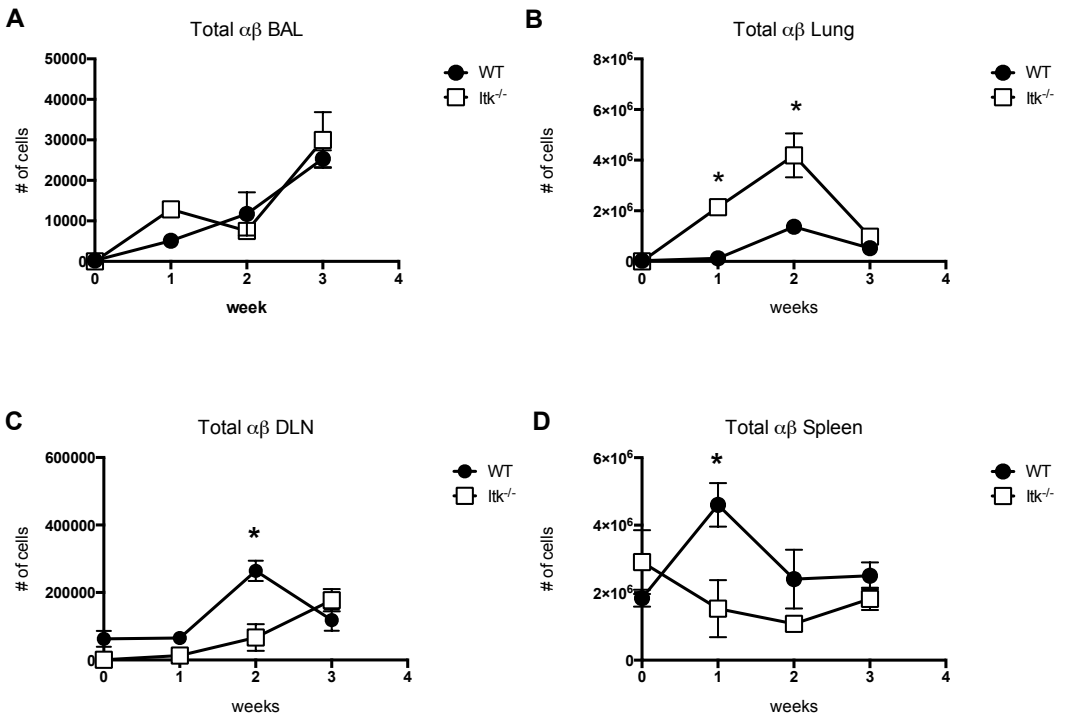

Fig. 3-4. Itk is not required for recruitment of αβ T cells to the lung during SR induced airway inflammation. 5-7 week old WT and Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week for 3 weeks. Cells from the indicated tissues were stained for TCRβ and analyzed using flow cytometry for number of αβ T cells. BAL (**A**), lungs (**B**), *p< 0.05 WT vs. Itk$^{-/-}$ at weeks 1 and 2), draining lymph nodes (**C**), *p< 0.05 WT vs. Itk$^{-/-}$ at week 2, and spleen (**D**), *p< 0.05 WT vs. Itk$^{-/-}$ at week 1). Data +/- SEM, n= 3 mice per group.

Itk is not required for the production of IL17A by T cells in the lungs in response to SR

We next investigated whether Itk affects the ability of the recruited T cells to make IL-17A. We harvested cells and stimulated them with PMA+Ionomycin/BFA ex vivo in order to induce intracellular cytokine secretion. We found that the number and percentage of $CD4^+$ T cells that express IL-17A cells was significantly higher in BAL from $Itk^{-/-}$ mice compared to WT mice exposed to SR, correlating with our analysis of the mRNA results (**Fig. 3-5A,C,E**). This was different from the lungs where there was no difference in the number or percentage of cells making IL-17A in the absence of Itk (**Fig. 3-5A,C,E**). This suggests that Itk does not regulate IL-17A production in conventional $CD4^+$ Th17 cells in the lung during HP. By contrast, there was little production of IFNγ by $CD4^+$ cells in the BAL and lung, however as has been previously reported by our lab, we saw an increase of IFNγ producing $CD4^+$ cells in spleens of mice lacking of Itk compared to WT mice following stimulation with PMA/Ionomycin (**Fig. 3-5A,B,D**) (Hu and August, 2008).

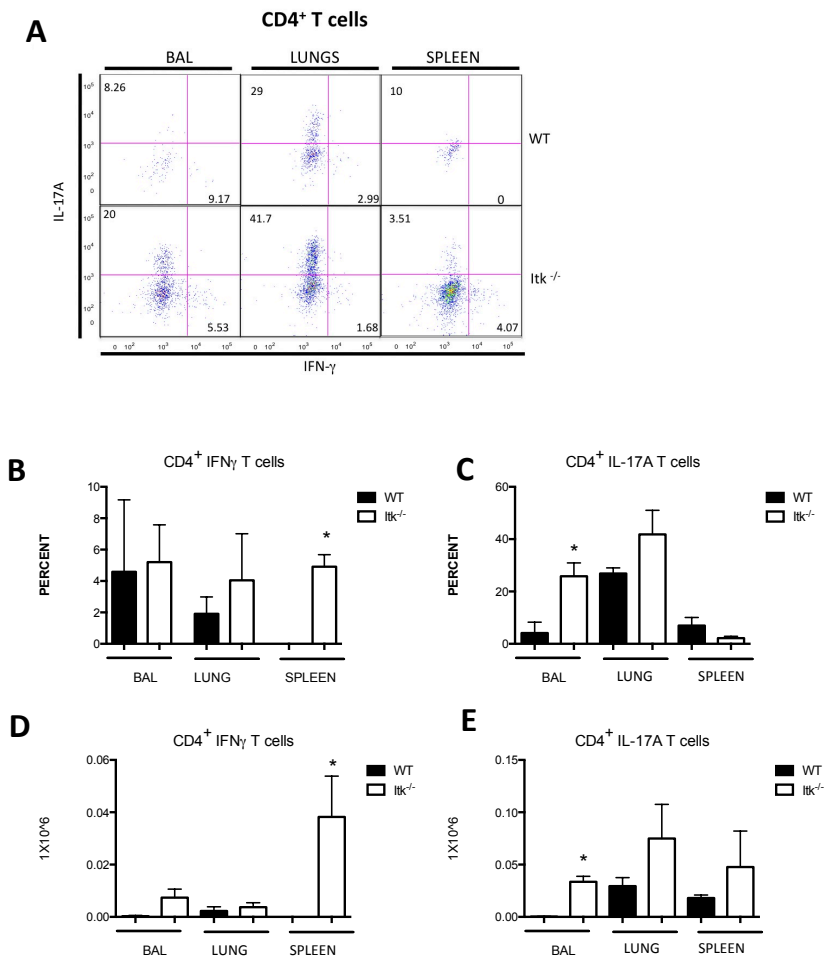

Fig. 3-5: Itk is not required for production of IL17A by CD4$^+$ T cells in the lungs in response to SR exposure. 5-7 week old WT and Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week for 3 weeks. **(A)** Cells from BAL, lungs, and spleen were harvested and stimulated with PMA/Ionomycin + BFA for 5 hrs., then stained for CD4 and intracellular IFNγ and IL-17A, and analyzed using flow cytometry gated on CD4$^+$ cells. Representative plots of IFNγ and IL-17A secreting CD4$^+$ cells are shown. (**B**)(**C**) Percentage of CD4$^+$ IFNγ$^+$ and CD4$^+$ IL-17A$^+$ cells. (**D**)(**E**) reflects number of cells shown in **(B)** and **(C)**. *p < 0.05 WT vs. Itk$^{-/-}$ percent spleen, *p < 0.05 WT vs. Itk$^{-/-}$ number BAL spleen, *p < 0.05 WT vs. Itk$^{-/-}$ percent BAL IFNγ , *p value < 0.05 WT vs. Itk$^{-/-}$ number BAL IL-17A. Data +/- SEM, n= 3 mice per group.

Itk is not required for the production of IL-17A in vivo during the development of IL-17A mediated HP

To examine this IL-17A response in more detail, we used flow cytometry and IL-17A-GFP reporter mice to identify the specific cell types producing IL-17A during the development of HP. The use of IL-17A-GFP reporter mice allows the identification of IL-17A producing cells *in vivo* during development of disease without resorting to ex vivo stimulation to identify cells, which may misidentify IL-17A producers in vivo. We generated IL-17A-GFP reporter mice lacking Itk, and exposed them, and their WT counterparts to SR as described above. We found that both WT and mice lacking Itk exhibit IL-17A producing cells in the lung following exposure to SR (**Fig. 3-6, 3-7**). Further analysis of these cells by flow cytometry showed that there were similar numbers of IL-17A producing αβ T $CD4^+$ cells in BAL and lungs in WT and $Itk^{-/-}$ mice, and $CD4^+$ T cells and not $CD8^+$ T cells were the major producers of IL-17A (**Fig. 3-8C, D)**. $Itk^{-/-}$ mice exhibited similar proportion of IL-17A GFP producing $CD4^+$ cells compared to WT mice in the lung over the course of disease **(Fig. 3-8A,B)**. These data indicate that $CD4^+$ αβ T cells are the dominant IL-17A producing αβ T cell population in the lung and BAL during HP, and that Itk is not required for the production of IL-17A in these cells.

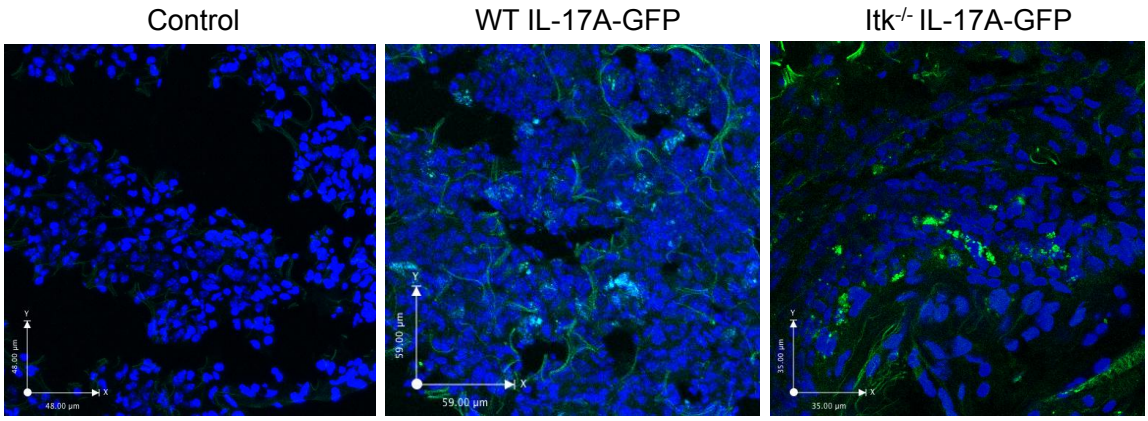

Fig. 3-6. IL-17A-GFP expression in lungs of SR exposed mice. 5-7 week old WT IL-17A-GFP and Itk$^{-/-}$ IL-17A-GFP mice received PBS or SR intranasally 3 times a week for 3 weeks. Lung tissue was collected, suspended and frozen using OTC and sectioned for staining. Lung sections was stained with DAPI to show cell nuclei and imaged for IL-17A GFP expression using a fluorescence microscope. Blue represents DAPI and green represents IL-17A GFP. Green staining identifies GFP expression in lung sections. Controls were exposed to WT GFP mice exposed to PBS for 3 weeks.

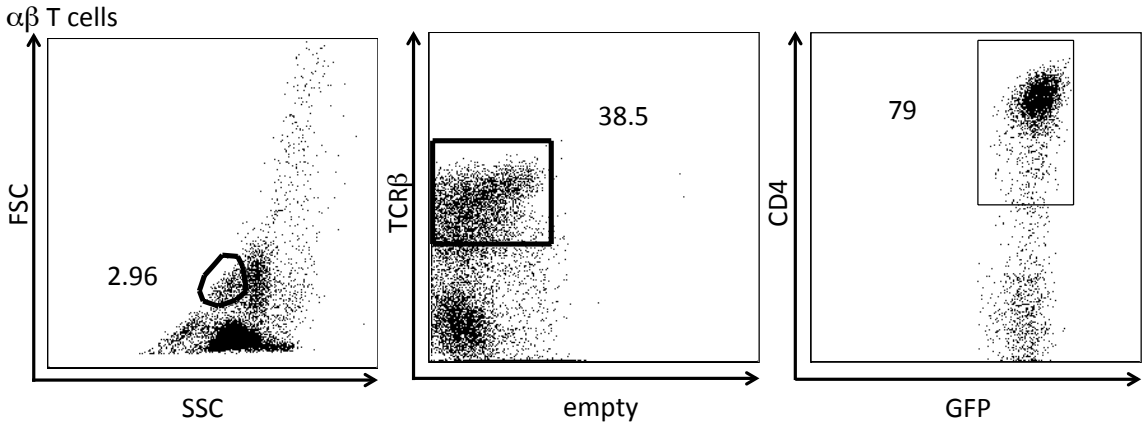

Fig. 3-7. CD4$^+$ T cells are making IL-17A during the development of HP. 5-7 week old WT IL-17-GFP and Itk$^{-/-}$ IL-17-GFP mice received SR intranasally 3 times a week for 3 weeks. Representative plots, gating strategy and staining for TCRβ, CD4, and IL-17A-GFP expression. Lymphocytes population was gated on by FSC vs. SSC. TCRβ$^+$ lymphocytes were gated on TCRβ vs. empty channel, then TCRβ vs. CD4$^+$ *not shown.* IL-17A producing cells were identified by gating on CD4 vs. GFP. Numbers represent percentage of gated events.

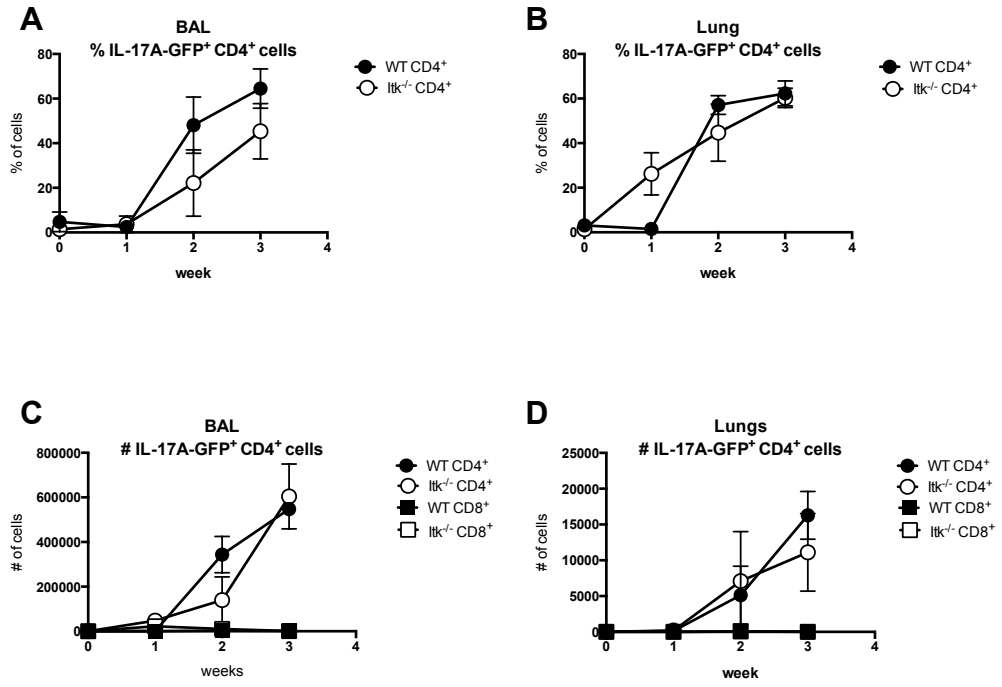

Fig. 3-8. Itk$^{-/-}$ CD4^{+} T cells are still able to produce IL-17A in response to SR in vivo. 5-7 week old WT IL-17-GFP and Itk$^{-/-}$ IL-17A GFP mice received SR intranasally 3 times a week for 4 weeks. Cells from BAL and lungs were harvested and the percent (**A), (B**) and numbers (**C, D**) of IL17A GFP^{+} CD4^{+} T cells from **(A, C)** BAL, (**B, D**) Lungs, were harvested and characterized using flow cytometry. Data +/- SEM, n= 3 mice per group.

Neutrophils are not a major source of IL-17A *in vivo* in SR-induced HP

A recent study reported that the major source of IL-17A in HP are not T cells but neutrophils, partly due to the fact that neutrophils from HP induced mice have the ability to make large amounts of IL-17A cytokine after in vitro stimulation (Hasan et al., 2013). However, when we analyzed the neutrophil population to determine whether they produce IL-17A in vivo (i.e. whether they were GFP^+), we found that neutrophils produced little if any IL-17A over the course of disease, indicating that they are not significant producers of IL-17A in vivo in HP (**Fig. 3-9 A-C**). Other cells such as γδ T cells have also been shown to make IL-17A, and we explore these cells in Chapter 4.

Thus, our data suggest that $CD4^+$ αβ T cells are the dominant source of endogenous IL-17A in SR induced HP. Furthermore, our data suggest that Itk does not affect the ability of αβ T cells to produce IL-17A during exposure to SR.

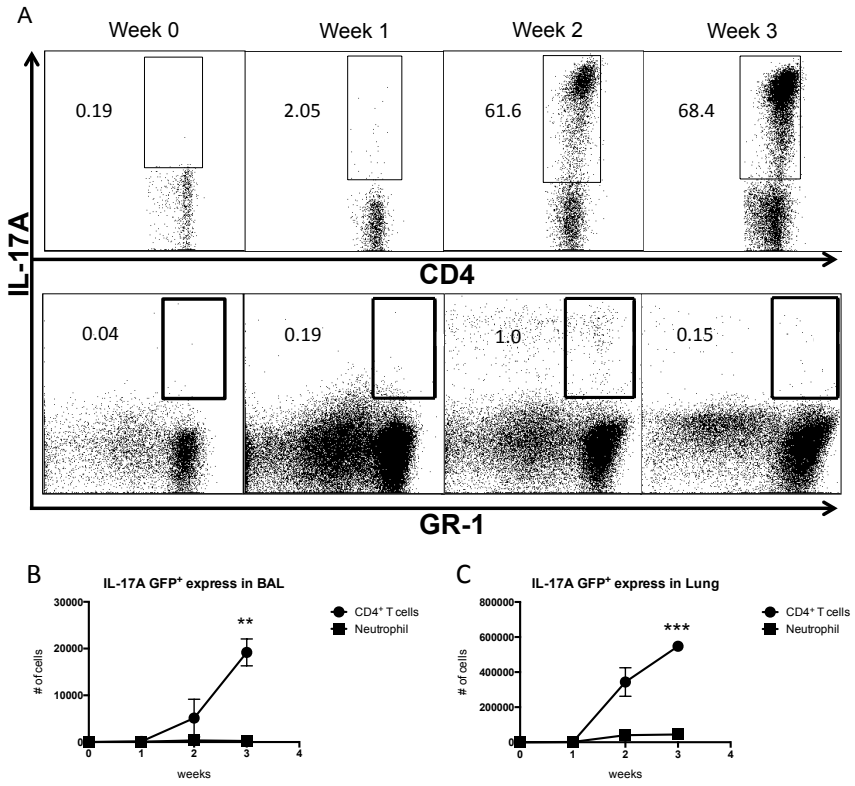

Fig. 3-9. CD4$^+$ T cells and not neutrophils are producing IL-17A in vivo during HP. 5-7 week old WT and Itk$^{-/-}$ mice expressing IL-17A GFP received SR intranasally 3 times a week for 3 weeks. **(A)** Lung cells were collected and stained for αβ CD4$^+$ T cells or Gr-1 expressing neutrophils and analyzed for IL-17A GFP expression. (**B**) Number of WT CD4$^+$ GFP$^+$ T cells vs. GPF$^+$ neutrophils in BAL. (**C**) Number of WT CD4$^+$ GFP$^+$ T cells vs. GPF$^+$ neutrophils in lung. **p value < 0.05 CD4$^+$ T cells vs. Neutrophil number BAL IL-17A GFP$^+$, ***p value < 0.05 CD4$^+$ T cells vs. Neutrophil number lung IL-17A GFP$^+$. Data +/- SEM, n= 3 mice per group.

Inhibition of Itk leads to a decrease in immune response during HP

Our results so far indicate that the absence of Itk does not affect the development of robust Th17 responses during HP. This is despite the finding that $Itk^{-/-}$ $CD4^+$ T cells are defective in developing into Th17 cells and produce IL-17A (Gomez-Rodriguez et al., 2009). These data would suggest that although Itk is a unique target for pharmaceutically inhibiting T cell mediated inflammatory lung diseases such as Th2 mediated allergic asthma, this may not be useful in targeting Th17 driven lung inflammation (Mueller and August, 2003; Sahu and August, 2009). However, the absence of the kinase in T cells may be different from the inhibition of the kinase, as would be the case with a pharmaceutical approach. We therefore wanted to determine whether inhibiting Itk's kinase activity would effect the development of HP in the same manner as the absence of the kinase. We therefore utilized a unique mouse expressing an allele sensitive Itk (Itk*as*) in the place of WT Itk (Kannan et al., 2014). Unlike an $Itk^{-/-}$ mouse, this Itk*as* expressing mouse allows T cells to develop normally, and allows us to temporarily inhibit the kinase activity utilizing small molecule inhibitors such as 3-MBPP1 (Gregan et al., 2007; Shokat and Velleca, 2002). This allows us to inhibit the kinase activity of Itk during development of disease, to determine whether inhibiting the kinase activity of Itk affects the production of IL-17A in T cells and the development of HP. Itk*as*/IL-17A-GFP mice were exposed to SR for 3 weeks and treated with kinase inhibitor 3-MBPP1. We found that inhibiting Itk kinase activity resulted in reduced lung inflammation in SR exposed mice compared to control treated mice (**Fig. 3-10**). Furthermore, we found that inhibition of Itk kinase activity blocked the increase in $CD4^+$ αβ T cells in BAL and the lungs (**Fig. 3-11B, C, D**). Blocking Itk kinase activity also inhibited αβ $CD4^+$ T cells from making

IL-17A although this inhibition was not statistically significant; there was a trend towards significance (**Fig. 3-12B,D**).

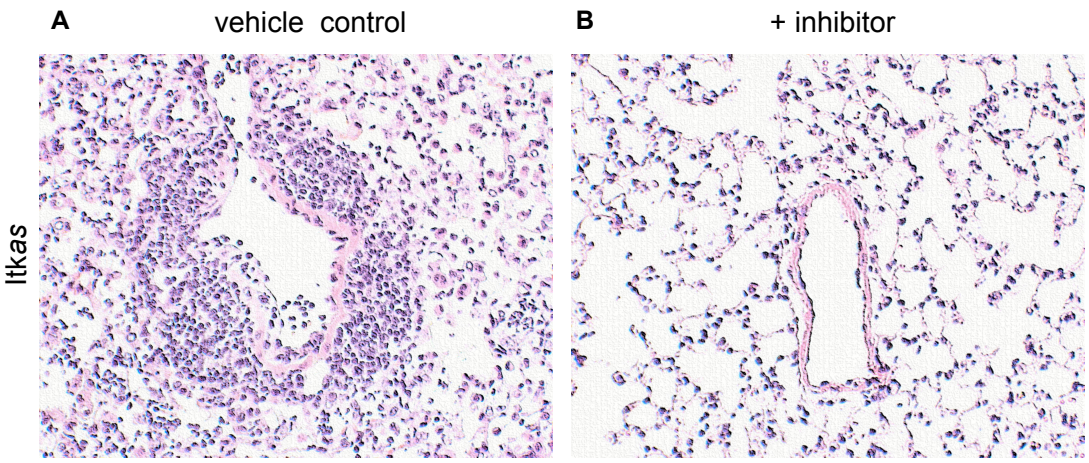

Fig. 3-10. Inhibition of Itk decreases inflammation in the lungs of HP mice. H&E stain of lung sections of 5-7 week old Itk*as* IL-17A-GFP mice exposed to SR 3 times a week intranasally for 2 weeks after receiving an i.n. injections of vehicle before i.n. SR exposure (**A**) or treatment with 3-MBPP1 (**B**).

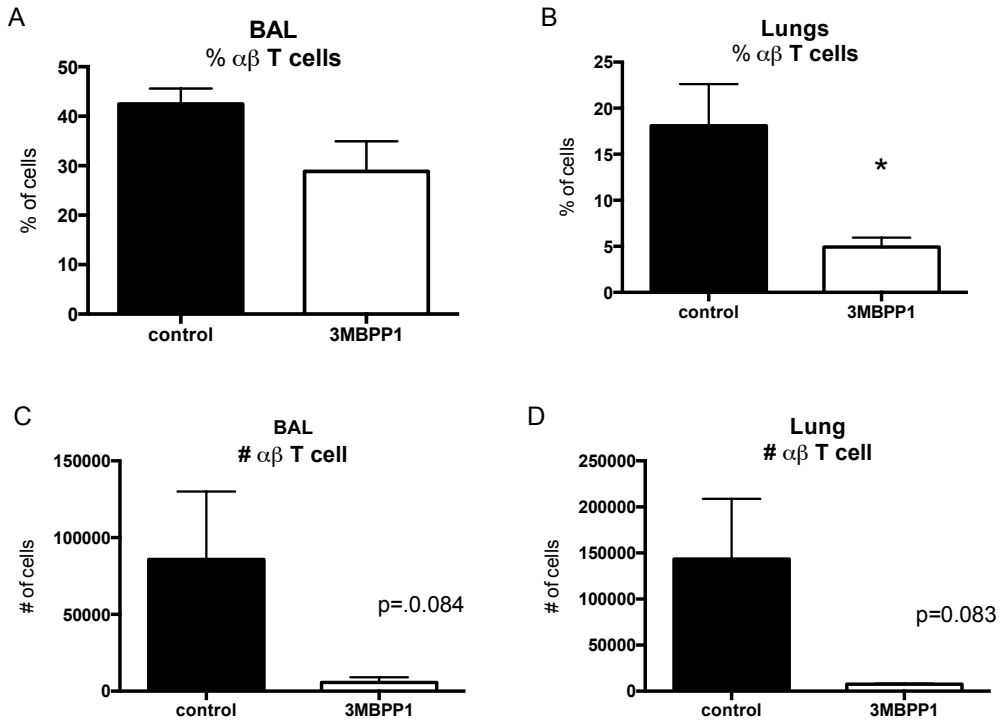

Fig. 3-11. The inhibition of Itk leads to a decrease in total αβ T cells in the lung during HP following SR exposure. 5-7 week old Itk*as* IL-17A-GFP mice exposed to SR along with vehicle or with 3-MBPP1 to inhibit Itk kinase activity. Percent (**A, B**) and number (**C, D**) of $CD4^+$ αβ T cells from BAL (**A, C**) and lung (**B, D**) were determined using flow cytometry. *p= 0.084 Control vs. 3-MBPP1 number BAL, *p= 0.083 Control vs. 3-MBPP1 number lung. Data +/- SEM, n= 3 mice per group.

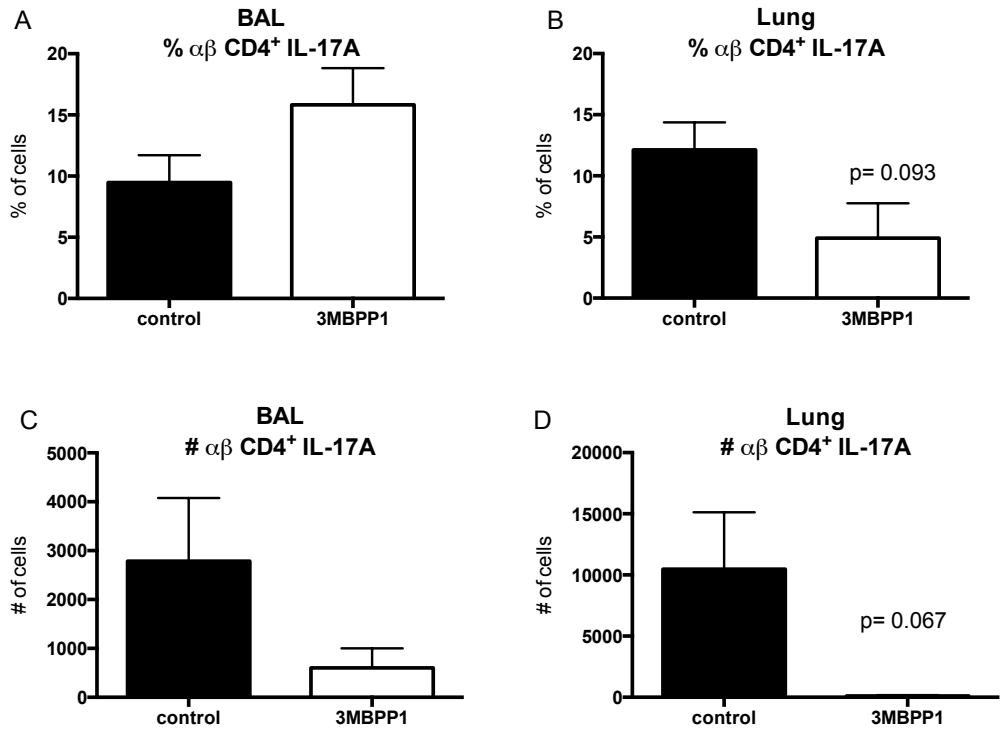

Fig. 3-12. The inhibition of Itk leads to a decrease in production of IL-17A by CD4$^+$ T cells during HP. 5-7 week old Itk*as* IL-17A-GFP mice exposed to SR along with vehicle or with 3-MBPP1 to inhibit Itk kinase activity. Percent (**A, B**) and number (**C, D**) of IL-17A GFP$^+$ CD4$^+$ αβ T cells from BAL (**A, C**) and lung (**B, D**) were determined using flow cytometry. *p = 0.093 Control vs. 3-MBPP1 percent lung, * p = 0.067 Control vs. 3-MBPP1 number cells lung. Data +/- SEM, n = 3 mice per group.

Discussion

In this chapter we investigated the role of Itk in the production of IL-17A by αβ T cells during the inflammatory lung disease hypersensitivity pneumonitis. Our data confirms that IL-17A is the most highly expressed cytokine in the lung during HP as compared to Th1 cytokine IFNγ and Th2 cytokine IL-4. Our data also indicates that unlike what has been previously reported, neutrophils are not major producers of IL-17A during HP, but that $CD4^+$ αβ T cells are the major producers of IL-17A during development of disease. Furthermore, despite our predictions, the expression of Itk was found to be unimportant for the development of a strong $CD4^+$ Th17 response to SR in the development of HP.

In an effort to investigate the endogenous expression of IL-17A, we utilized mice able to express the GFP marker when they express IL-17A (IL-17A GFP mice). This method allows us the opportunity to observe cytokine expression in vivo without ex vivo stimulation, which only displays the potential of a cell to express cytokine and can be misleading. One group showed that ex vivo stimulated neutrophils had the potential to express IL-17A during HP and proposed that neutrophils and not T cells are responsible for the increased IL-17A (Hasan et al., 2013). Our studies using the IL-17A GFP reporter confirmed that IL-17A is being expressed during HP and that a large number of αβ $CD4^+$ T cells are responsible for this expression. However, IL-17A GFP expression was not seen in the neutrophil population. These findings support the use of such cytokine reporter mice to more accurately determine the dynamics of cytokine expression in vivo during disease.

αβ T cells are recognized as important mediators of pathology in HP, and studies have shown that nude mice lacking T cells display reduced pulmonary fibrosis in HP (Takizawa et al., 1992). IL-17A has been established as the predominant cytokine in SR induced HP, and these T cells are important sources of IL-17A in response to the repeated exposure to SR (Simonian et al., 2009c; Takizawa et al., 1992). Our results confirm that $CD4^+$ αβ T cells are major producers of IL-17A, unlike what has previously been reported suggesting that neutrophils are the major source of this cytokine in HP (Hasan et al., 2013). Thus we suggest that HP develops due to a predominant Th17 response driven by IL-17A from $CD4^+$ αβ T cells. This IL-17A may then recruit neutrophils to the lung to contribute to the disease, but these neutrophils contribute little if any IL-17A to the process.

In our original hypothesis, we proposed that the absence of Itk would lead to a reduction in response in HP due to the fact that naïve $CD4^+$ T cells in $Itk^{-/-}$ mice have impairment in the development of Th17 cells as well as exhibit a decrease in corresponding IL-17A cytokine (Berg et al., 2005; Gomez-Rodriguez et al., 2009). Thus, in an IL-17A driven lung disease, $Itk^{-/-}$ mice would lack IL-17A to mediate disease pathology. However, this was not the case, since the absence of Itk did not lead to any alteration in inflammation in the lung tissue. $Itk^{-/-}$ mice also displayed significant induction of IL-17A mRNA in lung tissue, as well as a significant number of inflammatory αβ T cells in the lung. Furthermore, a large proportion of these cells in the $Itk^{-/-}$ mice making IL-17A cytokine were found to be $CD4^+$ αβ T cells. A possible explanation for $Itk^{-/-}$ mice possessing this Th17 population is that the impairment seen in the $Itk^{-/-}$ phenotype is not a complete ablation, and due to the nature of the repeated

exposure of these cells to SR antigen, the small population that does exist is then able to expand and initiate the proinflammatory environment necessary to drive disease. Indeed, evidence to support this view is found in the fact that there is a slight reduction in the $CD4^+$ αβ T cells making IL-17A in the $Itk^{-/-}$ mice early during the response, although this was not significant and the $Itk^{-/-}$ mice were able to catch up. In addition, there are other sources of IL-17A in the absence of Itk that we will discuss in the following chapters.

We next asked whether the development of HP would be altered if we blocked the kinase activity of Itk. In order to do this, we generated an allele sensitive Itk (Itk*as*) in place of WT Itk that can be targeted by small molecule inhibitor 3-MBPP1. The benefit of using this experimental method is that we can specifically target Itk kinase activity without the caveats of the $Itk^{-/-}$ phenotypes of IMP cells and increased population of immune cells not seen in normal WT mice (Hu and August, 2008). Our data shows that targeting Itk kinase activity significantly reduces the immune response in HP. We observed a trend toward decreases in cells in the lungs as well as decreased inflammation in the lung tissue, although this did not reach statistical significance. We suggest that in the absence of Itk, $CD4^+$ T cells may develop alternative ways of becoming Th17 cells, however in the presence of Itk, they may be dependent on Itk and thus this process can be inhibited by inhibiting Itk kinase activity.

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Chapter 4

The role of Itk signaling in γδ T cells during Hypersensitivity Pneumonitis

Introduction

Hypersensitivity Pneumonitis has been shown to been regulated by the master cytokine IL-17A (Simonian et al., 2009b; Simonian et al., 2009c). Th17 cells are able to produce IL-17A but there are many other cells that may be responsible for the production of this cytokine. A potential candidate for this activity are γδ T cells, which express innate and adaptive features in response to antigen and environmental cues such pathogen-associated molecular patterns (Zheng and Yang, 2014). γδ T cells migrate early during their developmental stage and persist as residential cells in areas lining respiratory, digestive, and reproductive track, as well as within layers of the skin (Bonneville et al., 2010). These cells also have the ability to rapidly produce cytokine, including IL-17A, in response to lung epithelial injury, which leads to inflammation and recruitment of neutrophils that are suggested to promote bacterial clearance in HP (Simonian et al., 2009c; Stark et al., 2005).

While αβ T cells have been investigated for their role in SR induced HP pathology, significantly less is known about the contribution of γδ T cells to this process. Studies have shown that in the absence of γδ T cells in murine models of SR, αβ $CD4^+$ cells have a compensatory increase of IL-17A that reflect the levels seen in WT mice (Simonian et al., 2009c). What is known is that Vγ6Vδ1 expressing γδ T cells produce IL-17A and accumulate in large numbers in a *B. subtilis* model of HP (O'Brien and Born, 2010; Simonian et al., 2006). In this model of *B. subtilis* induced HP, Vγ6Vδ1 T cells are also a source of IL-22, which has been shown to suppress fibrosis in the lung, although these cells are found in lower frequency than the IL-17A expressing population

(Simonian et al., 2009b). Further investigation is therefore is needed to determine the role of γδ T cells in IL-17A production that leads to HP.

Studies have shown that $Itk^{-/-}$ mice exhibit enhanced development of $CD4^{+}$ γδ T cells (Qi et al., 2009). In our previous data shown in chapter 3, we showed that $CD4^{+}$ T cells were significant producers of IL-17A in HP. We also noted a population of $CD4^{-}$ T cells in both WT and $Itk^{-/-}$ mice able to produce IL-17A as well. In this chapter, we investigate the nature of these cells and show that $CD4^{-}$ as well as $CD4^{+}$ γδ T cells produce IL-17A during HP in the lung. However, we also find that γδ T cells are not required for inflammation during HP, since γδ deficient mice still develop lung inflammation upon exposure to SR, suggesting that although γδ T cells can produce IL-17A during HP, they are not required for development of disease.

Results

A distinct population of γδ T cells produces IL-17A in the absence of Itk in response to intranasal SR

To determine a more complete profile of the cells that produce IL-17A during the development of HP, we isolated cells from the lung, spleen, and lymph nodes of mice exposed to SR and stimulated them with PMA/Ionomycin in the presence of BFA, and analyzed their expression of IL-17A and IFNγ by intracellular cytokine staining (**Fig. 4-1A)**. We found that γδ T cells are significant producers of IL-17A, but not of IFNγ **(Fig. 4-1B,C)**. Mice lacking Itk exhibited similar behavior **(Fig. 4-1B,C)**. In fact, the absence of Itk resulted in an enhanced population of γδ T cells expressing IL-17A, particularly in the BAL **(Fig. 4-1C,E)**. Furthermore, an increased frequency of $CD4^+$ γδ T cells produced IL-17A in the absence of Itk **(Fig. 4-1B,D)**. It should be noted that the absence of Itk results in the expansion of this population of $CD4^+$ γδ T cells (Qi et al., 2009), suggesting that these cells are able to produce IL-17A in the absence of Itk.

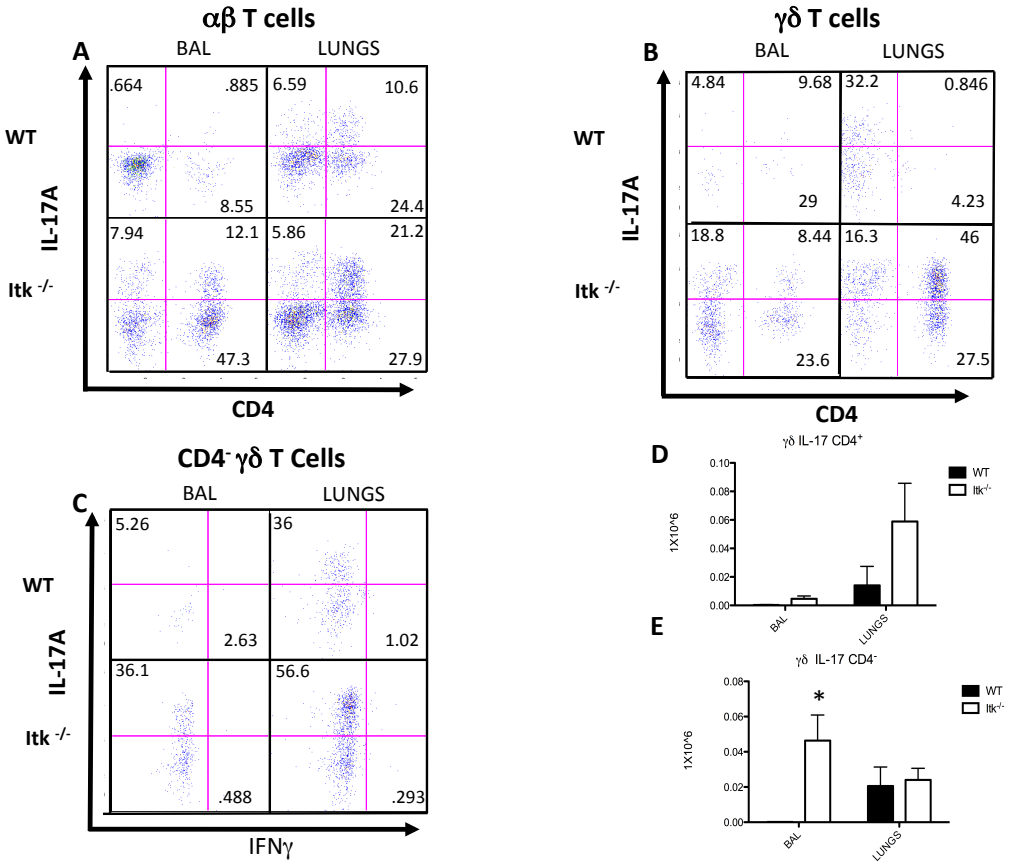

Fig. 4-1. The absence of Itk leads to increased IL-17A in CD4$^+$ and CD4$^-$ γδ T cells in response to SR exposure. 5-7 week old WT and Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week for 4 weeks. (**A**) Cells from BAL and lungs were stained for CD4 and intracellular IL-17A. (**B**) γδ cells were gated on from total lymphocytes and analyzed for CD4 and intracellular IL-17A. (**C**) CD4$^-$ γδ cells were gated on from total lymphocyte and analyzed for intracellular IL-17A and IFNγ. (**D**)(**E**) Number of cells displayed in **(B)**. *p <0.05 WT vs. Itk$^{-/-}$ γδ CD4$^-$ IL-17A. Data +/- SEM, n = 3 mice per group.

To confirm the behavior of IL-17A expressing γδ T cells, we once again utilized IL-17A GFP reporter mice to determine if the expression of IL-17A observed in ex vivo stimulation reflected the endogenous IL-17A frequency. Our data showed that the proportion of γδ T cells that produce IL-17A significantly increased over the course of HP, and in mice lacking Itk, we found a similar trend of increased proportion of γδ T cells producing IL-17A by during HP, although this was significantly less that WT mice **(Fig. 4-2A,B)**. The number of IL-17A producing γδ T cells was increased in both WT and $Itk^{-/-}$ mice in the BAL of HP mice **(Fig. 4-2C,D)**. We also observed an increase in the number of IL-17A producing cells in lung tissue of WT mice but mice lacking Itk displayed significantly lower proportion of IL-17A producing γδ T cells **(Fig. 4-3A,B)**. Together, this data shows that two populations of γδ T cells, $CD4^-$ and $CD4^+$ are producing IL-17A during the immune response to HP. Furthermore, in WT mice, the $CD4^-$ population of γδ T cells are the predominant producers of IL-17A during HP. In addition, the results show that the absence of Itk lead to reduced production of IL-17A by γδ T cells during HP.

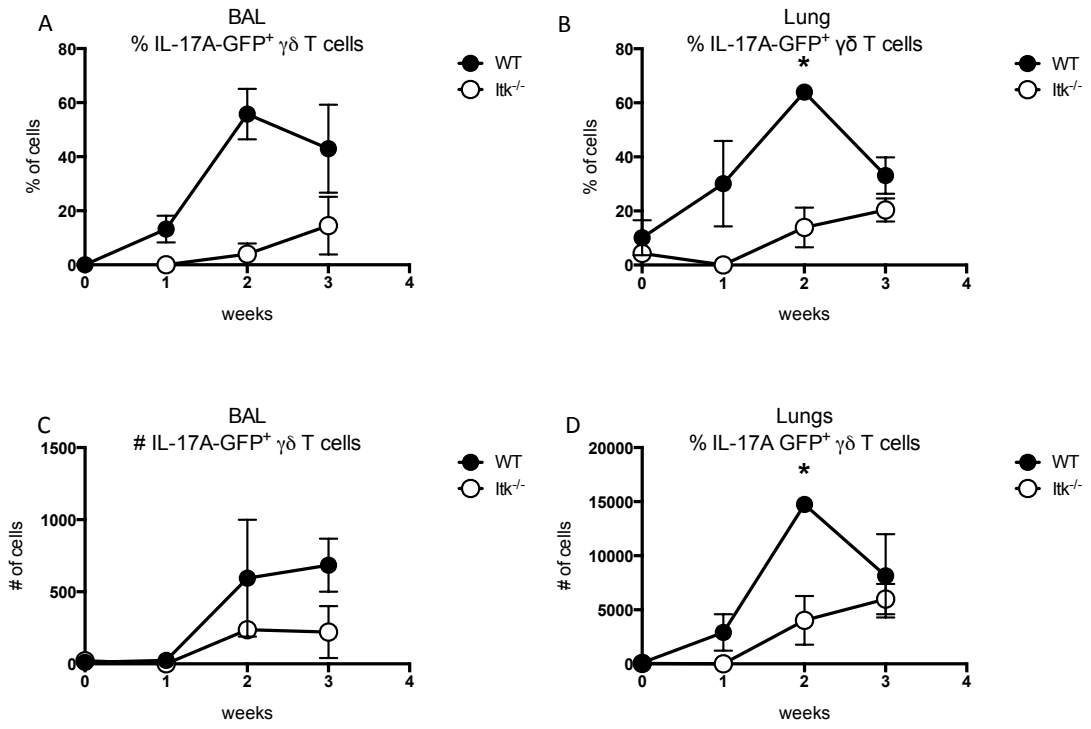

Fig. 4-2. Loss of Itk leads to reduced IL-17A production by γδ T cells during HP. 5-7 week old WT and Itk$^{-/-}$ IL-17A/GFP reporter mice received SR intranasally 3 times a week for 3 weeks. Cells from BAL and lungs were collected and analyzed for the percent (**A, B**) and number (**C, D**) of IL17A GFP^{+} γδ T cells from BAL **(A, C)**, lungs **(B, D)** by flow cytometry. *p <0.05. Data +/- SEM, n = 3 mice per group.

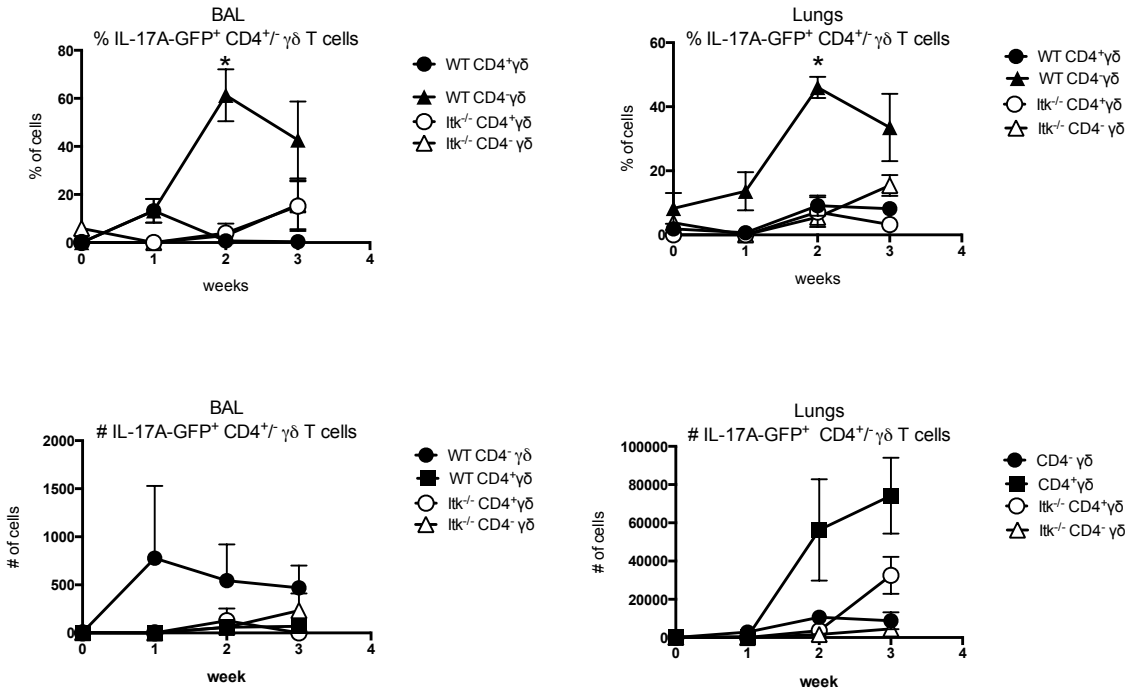

Fig. 4-3. The absence of Itk affects the proportion of γδ T cells that produce IL-17A during HP. 5-7 week old WT and Itk$^{-/-}$ IL-17A/GFP reporter mice received SR intranasally 3 times a week for 3 weeks. Percent of (**A, B**) and numbers (**C, D**) IL17A GFP^{+}γδ T cells from BAL **(A, C)**, lungs (**B, D**) were characterized using flow cytometry. *p value <0.05 BAL WT CD4^{-} γδ vs. Itk$^{-/-}$ CD4^{+} γδ WT CD4^{-} γδ vs Itk$^{-/-}$ CD4^{-} γδ, WT CD4^{-} γδ vs. WT CD4^{+} γδ, *p value <0.05 lung WT CD4^{-} γδ vs. Itk$^{-/-}$ CD4^{+} γδ WT CD4^{-} γδ vs Itk$^{-/-}$ CD4^{-} γδ, WT CD4^{-} γδ vs. WT CD4^{+} γδ. Data +/- SEM, n = 3 mice per group.

γδ T cells account for small number of IL-17A producing cells in HP

To investigate the overall contribution of γδ T cells to total IL-17A production in HP, we looked at endogenous IL-17A GFP expression by γδ $CD4^+$, γδ $CD4^-$, and αβ $CD4^+$ T cells, along with neutrophils in response to SR in HP **(Fig. 4-4)**. The data shows αβ $CD4^+$ T cells have the highest proportion of IL-17A GFP expression in HP, while $CD4^-$ γδ T cells display a moderate proportion of IL-17A GFP^+ cells as well in the lungs of HP mice (**Fig. 4-4**). Analysis of the total number of IL-17A-GFP^+ cells in HP revealed that the number of γδ T cells is lower than αβ $CD4^+$ T cells, which was also the case in mice lacking Itk (**Fig. 4-4A-D**). Together, these results suggest that although a large percent of γδ T cells make IL-17A in SR induced HP, the numbers of cells is low in comparison to αβ $CD4^+$ T cells, indicating that they are only a minor contributor to IL-17A production in HP.

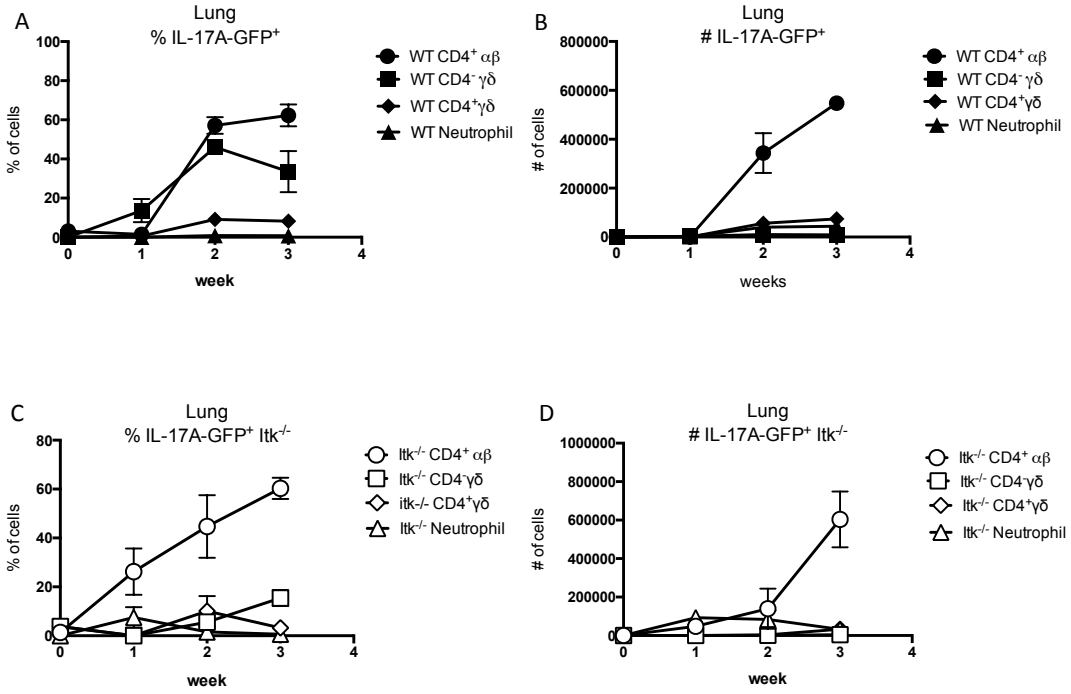

Fig. 4-4. αβ CD4$^+$ T cells are the predominant producers of IL-17A during HP. 5-7 week old WT and Itk$^{-/-}$ IL-17A/GFP reporter mice received SR intranasally 3 times a week for 3 weeks. Percent (**A, C**) and number (**B, D**) of IL17A GFP$^+$ γδ T cells from lungs were determined using flow cytometry. αβ and γδ WT and Itk$^{-/-}$ CD4$^+$/CD4$^-$ IL-17A-GFP$^+$ T cells represent data from Fig. 3. Chapter 4. Data +/- SEM, n = 3 mice per group.

γδ T cells are required for neutrophil infiltration not cytokine expression during development of HP in the presence or absence of Itk

Our data indicates that a large proportion of γδ T cells produce IL-17A in SR induced HP. In addition, the $Itk^{-/-}$ mice have an increased proportion of $CD4^+$ γδ T cells that also produce IL-17A. These populations of cells may affect the IL-17A response by compensating for defects in the $Itk^{-/-}$ mice during HP. We therefore generated $TCR\delta^{-/-}$ (lacking functional γδ T cells) and $TCR\delta^{-/-}/Itk^{-/-}$ (lacking γδ T cells and Itk) mice to determine their contribution to the SR induced inflammation. We exposed these mice for 3 weeks to SR. After harvesting cells, we stimulated them before characterized them by staining cells surface markers and intracellular cytokine expression. We found that in the absence of γδ T cells, IL-17A is still being produced in the BAL and lung by $CD4^+$ αβ T cells **(Fig. 4-5)**. We also did not see any changes in the frequency of IL-17A producing $CD4^+$ αβ cells in the absence of Itk **(Fig. 4-5)**. We did, however, see a noticeable difference in the percentage of neutrophils in the BAL and lungs during HP **(Fig. 4-6)**. This would support the theory that γδ T cells are important in assisting neutrophilic migration to the lungs (O'Brien et al., 2009). This was confirmed by analyzing lungs histological lung sections from $TCR\delta^{-/-}$ and $TCR\delta^{-/-}/Itk^{-/-}$ exposed to SR **(Fig. 4-7)**. Furthermore, we found that the absence of Itk and γδ T cells leads to a significant reduction in the percentage of neutrophils in the BAL and lung. This data suggest that γδ T cells affect HP not by cellular secretion of cytokine IL-17A but by facilitating neutrophilia in response to SR in HP.

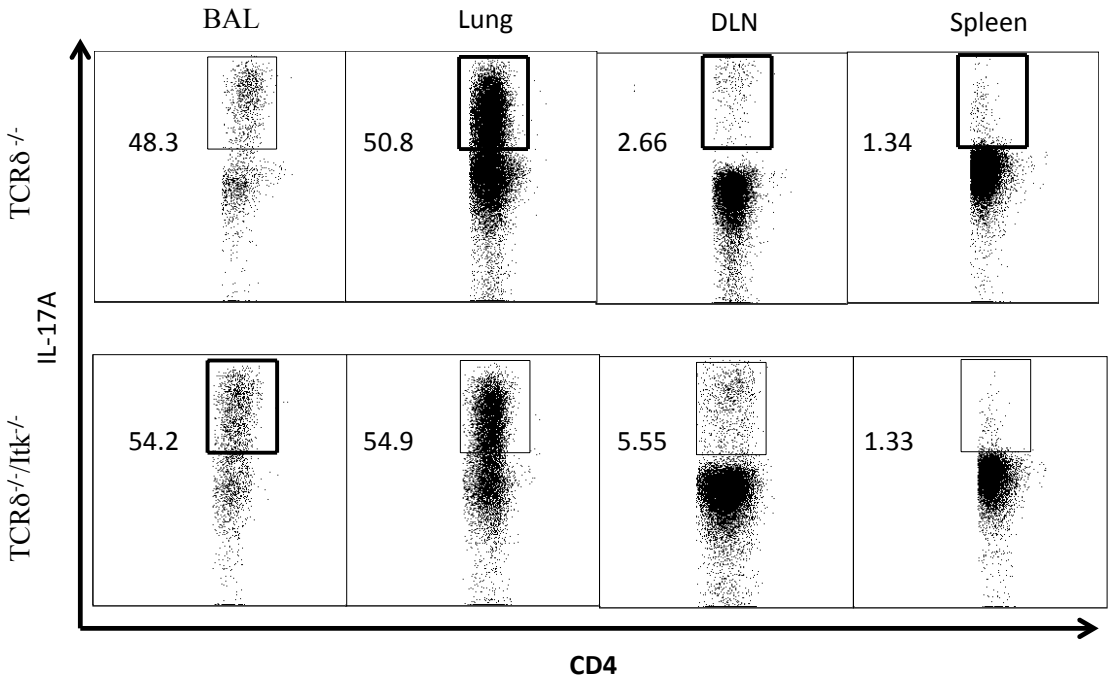

Fig. 4-5. The loss of γδ T cell and/or Itk does affect production of IL-17A in αβ CD4$^+$ T cells in HP. 5-7 week old TCRδ$^{-/-}$ and TCRδ$^{-/-}$/Itk$^{-/-}$ mice received SR intranasally 3 times a week for 3 weeks. Cells from BAL, lungs, and spleen were harvested and stimulated with PMA/Ionomycin + BFA for 5 hrs., then stained for CD4 and intracellular IL-17A, and analyzed using flow cytometry gated on CD4$^+$ cells. Representative plots of IL-17A secreting CD4$^+$ cells are shown. Representative plots from 3 mice.

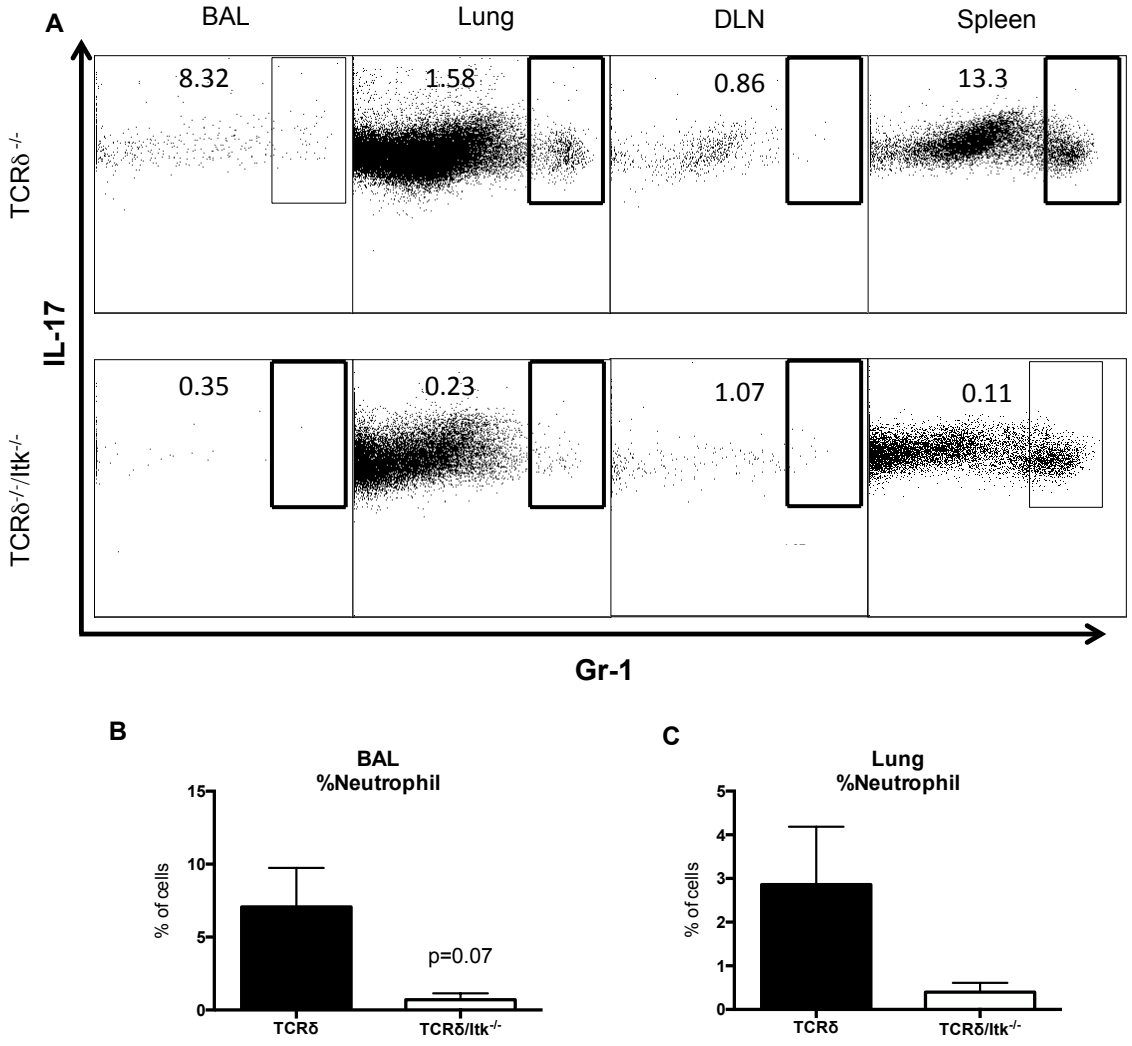

Fig. 4-6. Absence of Itk in γδ null mice leads to reduced percentage of neutrophils in the lung. 5-7 week old TCRδ$^{-/-}$ and TCRδ$^{-/-}$/Itk$^{-/-}$ mice received SR intranasally 3 times a week for 3 weeks. (**A**) Cells from BAL, lungs, lymph nodes and spleen were harvested and stimulated with PMA/Ionomycin + BFA for 5 hrs, then stained for surface markers TCRβ, TCRδ, CD4, CD8, NK1.1, and Gr-1. Neutrophils were identified by gating on FSC vs. SSC then Gr-1^{+} cells and intracellular IL-17A, followed by analysis by flow cytometry gated on Gr1^{+} cells. Representative plots of IL-17A neutrophils are shown. Representative plots from 3 mice. Percentage of neutrophils in **(B)** BAL and **(C)** Lung were calculated. *p=0.07 Data +/- SEM, n = 3 mice per group.

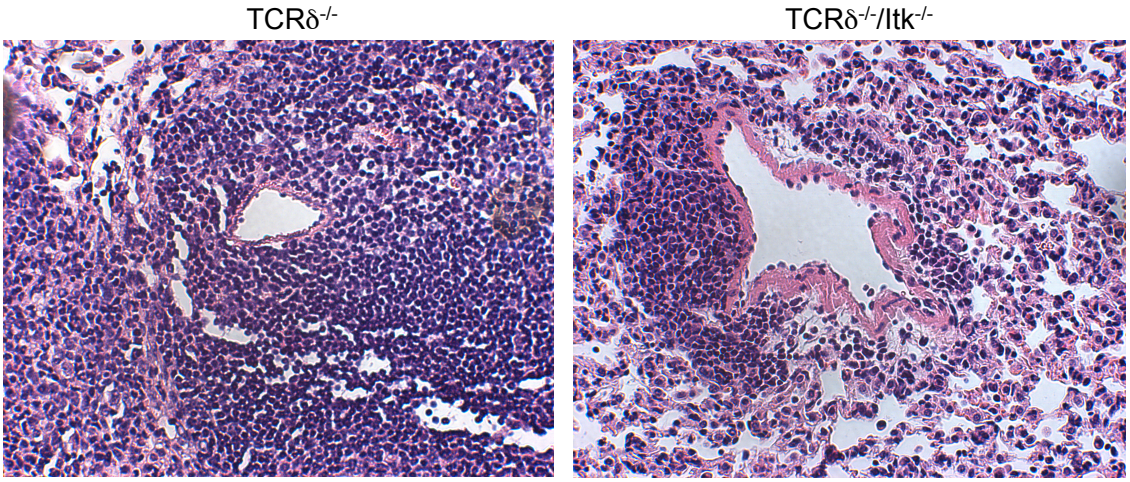

Fig. 4-7. Decreased infiltrating neutrophils in the absence of Itk and γδ T cells.
5-7 week old TCRδ$^{-/-}$ and TCRδ$^{-/-}$ /Itk$^{-/-}$ mice received SR intranasally 3 times a week for 3 weeks. Tissue from lungs sections from the indicated mice were stained with Hematoxylin and Eosin (H&E) staining and analyzed for severity.

Discussion

In this chapter we investigated the role of Itk in γδ T cells during an inflammatory response leading to HP. In our studies, we recognized that there was a sizable population of IL-17A expressing cells that were negative for CD4, which would exclude the conventional $CD4^+$ Th17 cells. We begin by asking if γδ T cells could serve as another possible source of IL-17A in this response. Other groups have shown that γδ T cells are able to produce IL-17A during autoimmune disease (Cui et al., 2009). They have also been shown to appear in large numbers in the lung and express IL-17A in response to *Bacillus subtilis* induced HP (Simonian et al., 2009a). Our data shows not only that both $CD4^-$ as well as $CD4^+$ γδ T cells have the capacity to make IL-17A during HP in vivo, and the absence of Itk also affected the number and proportion of these cells making IL-17A. We also found that the loss of γδ T cells has little affect on the ability of αβ T cells to make IL-17A but there is an affect on the proportion of neutrophils in the lung in the absence of Itk.

Our data shows that both $CD4^+$ and $CD4^-$ γδ T cell populations were expressing IL-17A after in vivo stimulation, as characterized by intracellular cytokine staining and flow cytometry. Additionally, there was significant increase in the proportion of cells making IL-17A in the BAL of $Itk^{-/-}$ mice after the fourth week of exposure. There seemed to be a differential level of IL-17A cytokine expression in $CD4^-$ γδ T cells between the BAL and lung, where we observed significant increases in IL-17A producing cells in BAL but not in the lung after ex vivo stimulation. Furthermore, we did not see upregulation of Th1 cytokine IFNγ by γδ T cells during HP reinforcing the view that IL-17A cytokine is the master regulatory cytokine in HP. Our data correlated with previous

studies that show an increased number of γδ T cells in the lungs after HP (Simonian et al., 2009a). We observed γδ T cells increasing over the course of HP inflammation and the loss of Itk signaling led to a significant decrease in the proportion and number of cells in the lung earlier in the disease, which later recover to levels comparable to WT. This would suggest that the absence of Itk results in a delayed response of γδ T cells in making IL-17A response during development of HP. We also compared the profile of the $CD4^+$ and $CD4^-$ γδ T cells in WT and $Itk^{-/-}$ mice and confirmed that this delay in response is seen mostly in the $CD4^-$ γδ T cells. $CD4^-$ γδ T cells have the highest expression of IL-17A GFP in the lungs and BAL over the course of HP. We also observed a proportion of those cells expressing IL-17A GFP shows a significant reduction in expression that later recovers by week 3 of SR exposure. The absence of Itk further exacerbates this loss of expression, and may allude to Itk being important in this delayed immune response. Lastly we noted that in the absence of functional γδ T cells, IL-17A is being highly expressed by αβ $CD4^+$ T cells in both BAL and lung and the loss of Itk does not alter this response. Our data shows that in mice lacking γδ T cells, there are still functional αβ T cells able to make IL-17A and may compensate for any cytokine lost by the absence of γδ T cells. Mice lacking γδ T cells expressed IL-17A similar to WT mice in the BAL, and lungs of mice during HP. However, surprisingly, the loss of Itk on this background led to a decrease in the proportion of neutrophils that are recruited, suggesting that Itk may be required for neutrophil recruitment during HP in the absence of γδ T cells.

In conclusion, our data confirms that γδ T cells can make IL-17A during HP. In our earlier experiments, we noted a population of $CD4^-$ T cells making IL-17A along with the conventional $CD4^+$ T cells after ex vivo stimulation. We provided conclusive

evidence identifying these cells as $CD4^-$ γδ T cells. We also concluded that the ex vivo stimulated $CD4^+$ γδ population expressing IL-17A in the absence of Itk was absent when we looked at IL-17A expression using our reporter mice. This further shows that ex vivo stimulation reflects the capacity of immune cells to make cytokine but does not necessarily accurately depicts the endogenous cytokine expression. We found that the absence of γδ T cells does not affect the ability of $CD4^+$ T cells to make IL-17A, but may affect the development of neutrophilia during HP in the absence of Itk. Together we conclude that γδ T cells play a minor role in HP and the absence of Itk in γδ T cells does not affect this overall outcome.

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Chapter 5

Characterizing the role of Itk in Regulatory T cells and iNKT cells during HP

Introduction

There are various cells involved in the recognition of SR antigen during HP and that drive the immune response in HP. Regulation of this immune response is a vital aspect of this response. T regulatory (T_{reg}) cells are a subset of immune cells that help in maintaining the balance between the damaging and protective effect of this response (Fontenot and Rudensky, 2005). These cells are identified by expression of CD4 and CD25 surface markers and expression of the transcription factor Foxp3 (Fontenot and Rudensky, 2005). These cells have been are suggested to have a protective role in HP as mice depleted of T_{reg} cells display an exacerbated pathology in HP, with an increase in inflammatory cell number in BAL as well as more severe lung inflammation (Park et al., 2009). Th17 and T_{reg} cells share a similar differentiation pathway (e.g. Th17 cells require IL-6 and TGFβ, while T_{reg} cells only require TGFβ)(Rudensky, 2011). Furthermore, T_{reg} cells may be able to covert to pathogenic pro-inflammatory Th17 cells in the presence of proinflammatory cytokines (Noack and Miossec, 2014).

Mice lacking Itk exhibit enhanced development of $CD4^+$ T regulatory cell, suggesting an important function for Itk in these cells (Gomez-Rodriguez et al., 2014; Huang et al., 2014b). Itk has also been suggested to play a role in the differentiation between Th17 and T_{reg} cells (Gomez-Rodriguez et al., 2014; Huang et al., 2014b). These findings suggest that the absence of Itk signaling leads to an increase levels of $CD4^+$ $Foxp3^+$ T_{reg} cells, and that in vivo $CD4^+$ cells from $Itk^{-/-}$ mice preferentially develop into T_{reg} cells. Furthermore, when differentiated in vitro under Th17 conditions, naïve $Itk^{-/-}$ $CD4^+$ T cells preferentially develop into T_{reg} cells, with reduced Th17 differentiation.

These data suggests that Itk controls the choice that T cell make to become Th17 or T_{reg} cells.

As discussed in the previous chapters, HP is an inflammatory disease that is mediated by the cytokine IL-17A. While we have shown that the source of this IL-17A cytokine is largely from αβ and to a minor extent γδ T cells, iNKT cells can also produce IL-17A (Michel et al., 2007). These cells have innate function of rapid secretion of pro and anti-inflammatory cytokines within minutes or hours of antigenic stimulation, and they can have outsized effects on disease, such as the case in allergic asthma, where small numbers of iNKT cells can influence the development of disease (Godfrey et al., 2010; Iwamura and Nakayama, 2010). Studies suggest the IL-4 producing NKT cells also play a protective role in SR induced HP (Kim et al., 2005). However, it is not known whether iNKT cells produce IL-17A in hypersensitivity pneumonitis.

Previous studies have identified an important role for Itk in the survival and maturation of iNKT cells, and Itk null iNKT cells are also defective in secreting cytokine when stimulated by α-GalCer, an iNKT cell ligand (Au-Yeung and Fowell, 2007; Felices and Berg, 2008; Huang et al., 2014a; Qi et al., 2012; Qi et al., 2011b). In this chapter, we address the questions of the role of Itk in the presence of T_{reg} cells in the lung during HP, as well as production of IL-17A by iNKT cells in HP.

Results

No difference in T regulatory cell numbers in the lungs in the absence of Itk in HP

T_{reg} cells are distinguished by their ability to down regulate inflammatory immune response in the body, and are suggested to provide protection in HP since mice lacking T regulatory cells having more severe pathology during disease (Park et al., 2009). Given the relationship between Th17 cells and T regulatory cells, as well as the role of Itk in regulating differentiation of both cell types, we examined the behavior of T regulatory cells in WT and Itk deficient mice exposed to SR. WT or $Itk^{-/-}$ IL-17A-GFP mice were exposed to SR for 3 weeks and then lung and airway cells were characterized for the presence of $Foxp3^{+}$ T regulatory cells **(Fig. 5-1, 5-2)**. We found no difference in the proportion or number of T regulatory cells in BAL from mice lacking Itk as compared to WT **(Fig. 5-2A)**. This was similar to the results from lung samples **(Fig. 5-2B-D)**. This suggests that Itk is not important in the behavior of T regulatory cells in HP.

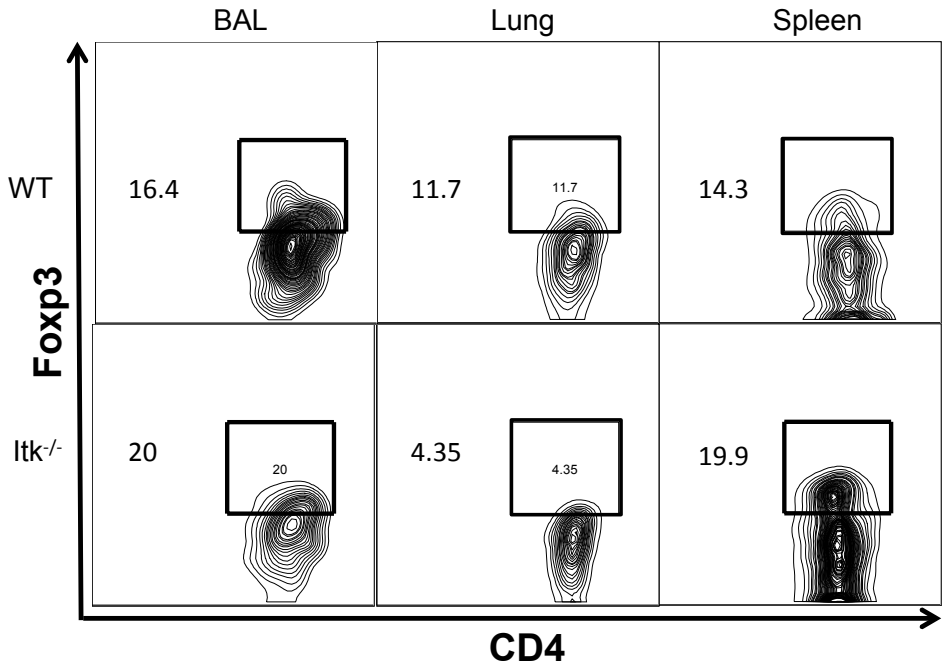

Fig. 5-1. Regulatory T cells were characterized in SR induced HP. 5-7 week old WT and $Itk^{-/-}$ IL-17A GFP reporter mice received SR intranasally 3 times a week for 3 weeks. Cells from BAL, lungs, and spleen were harvested then stained for $CD4^+$ and Foxp3, and analyzed using flow cytometry gated on $CD4^+$ cells. Representative plots of $CD4^+$ $Foxp3^+$ T regulatory cells are shown at the end of 3 weeks.

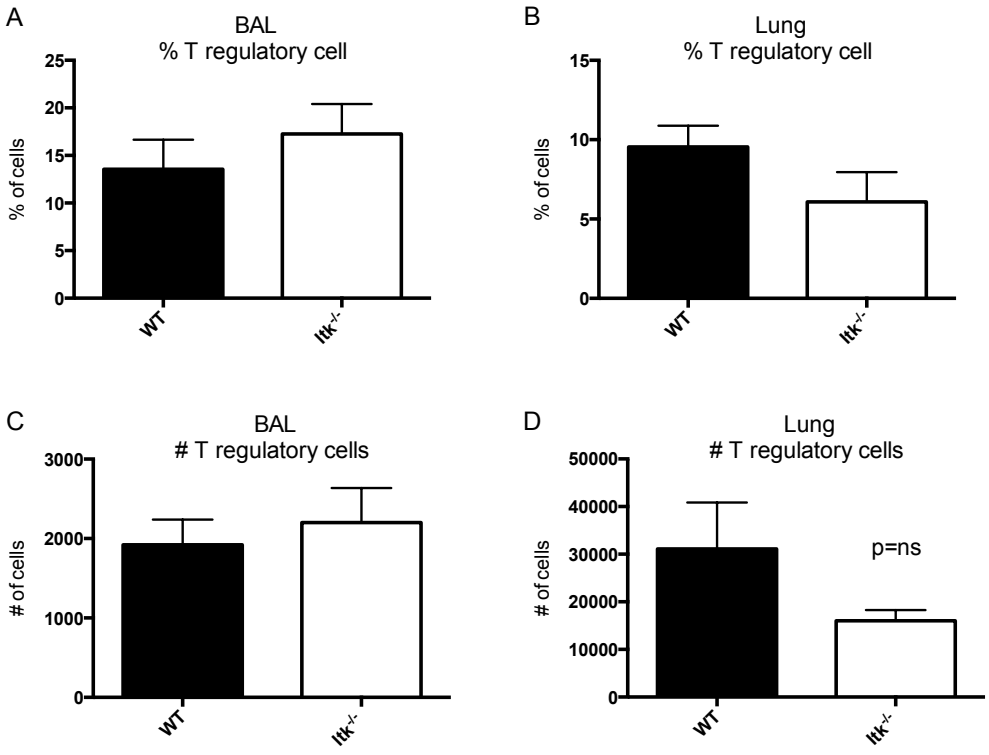

Fig. 5-2: Itk is not required for T regulatory cells to get into the lungs. 5-7 week old WT and $Itk^{-/-}$ IL-17A GFP reporter mice received SR intranasally 3 times a week for 3 weeks. Percent of $Foxp3^{+}$ Regulatory T cells from BAL **(A)**, lungs (**B**), and number of cells from BAL (**C**), and lung (**D**) were harvested, stained and characterized using flow cytometry. Plots of percentage and number of $CD4^{+}$ $Foxp3^{+}$ T regulatory cells are shown at 3 weeks. Data +/- SEM, n = 3 mice per group.

Itk is not required for iNKT cell production of IL-17A in the lung during development of HP

Given the role of Itk in regulating iNKT cell development and function, we next examined the behavior of iNKT cells in the SR-induced HP model. We found that there was no difference in the proportion of iNKT cells in the BAL and lung of WT and $Itk^{-/-}$ mice **(Fig. 5-3, 5-4A,B).** This correlated with the numbers of iNKT cells in the BAL and lung where we did not observe any significant changes **(Fig. 5-4C,D)**. Furthermore, we found that there was no difference in the proportion or number of iNKT cells producing IL-17A between WT and $Itk^{-/-}$ mice **(Fig. 5-5B,D)**. The proportion of iNKT cells making IL-17A in the BAL was significantly higher in the absence of Itk, although IL-17A expressing iNKT cells in the lung displayed proportions similar to WT during HP **(Fig. 5-5A, 5B)**. Although the number of IL-17A expressing iNKT cells in BAL of $Itk^{-/-}$ mice were marginally reduced, there were similar numbers of iNKT cells expressing IL-17A in the lung **(Fig. 5-5C, 5D)**. Together, the data suggest that iNKT cells are a source of IL-17A in HP, but that Itk is not required for these cells to produce IL-17A in SR induced HP.

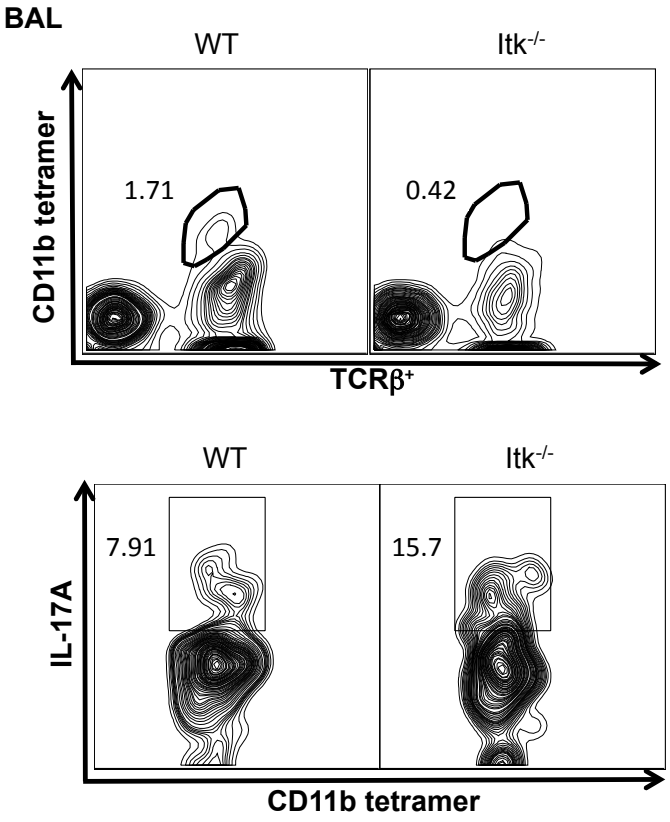

Fig. 5-3. iNKT cells were characterized in SR induced HP. 5-7 week old WT and Itk$^{-/-}$ IL-17A GFP mice received SR intranasally 3 times a week for 3 weeks. Cells from BAL, lungs, and spleen were harvested then stained for TCRβ and α-GalCer/CD1b tetramer, and analyzed using flow cytometry gated on IL-17A tetramer^{+} cells. Data from the 3 week time point. Representative plots of iNKT cells are shown.

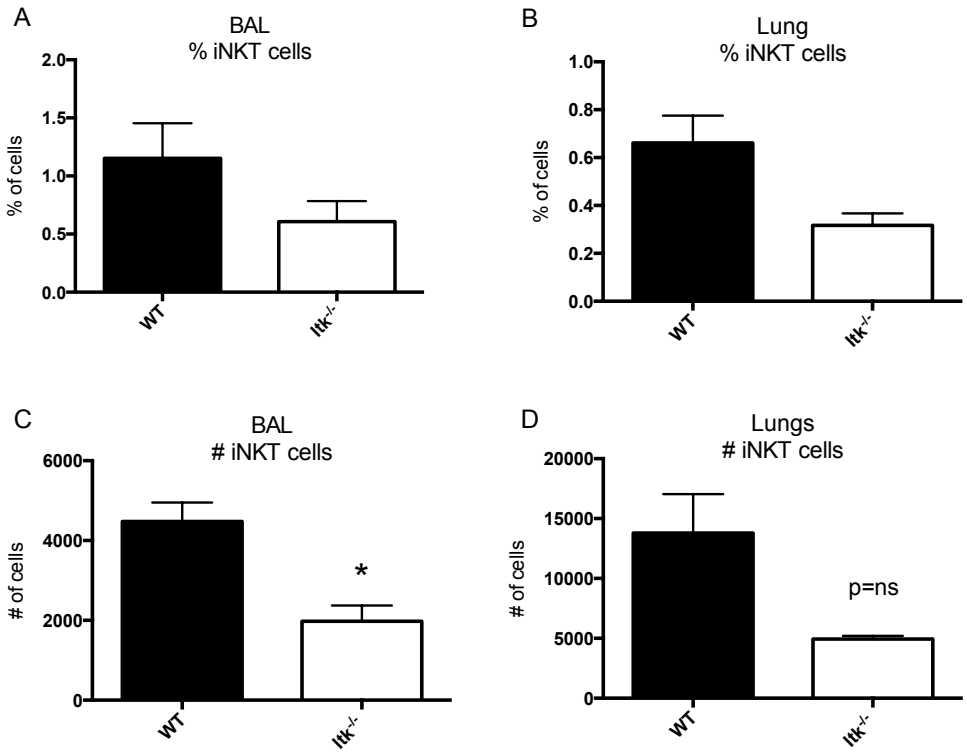

Fig. 5-4. Itk is important for iNKT cells to enter the BAL during SR. 5-7 week old WT and Itk$^{-/-}$ mice expressing IL-17A GFP received SR intranasally 3 times a week for 3 weeks. Percent (**A, B**) and numbers (**C, D**) of iNKT cells from BAL **(A, C)**, lungs (**B, D**), characterized using flow cytometry. Representative plots of iNKT cells are shown. *$p<0.05$, WT vs. Itk$^{-/-}$ BAL number. Data +/- SEM, n = 3 mice per group.

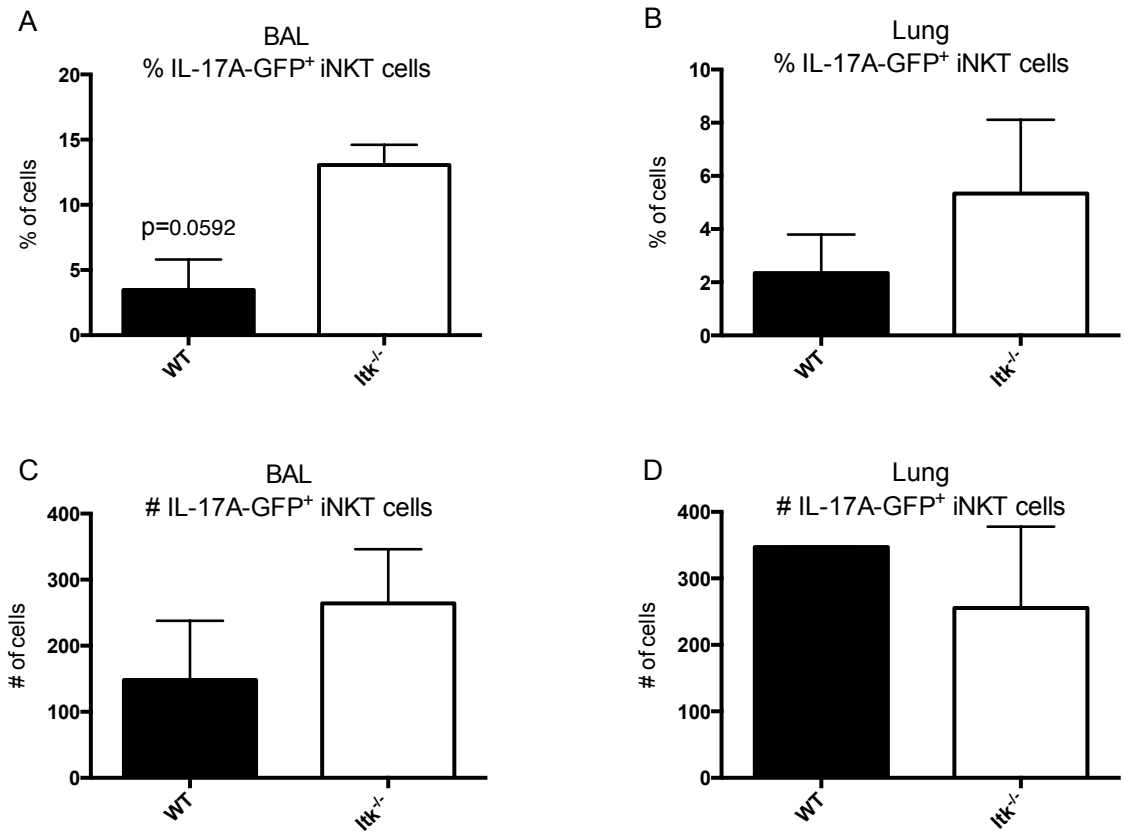

Fig. 5-5. iNKT cells are able to produce IL-17A GFP in the absence of Itk. 5-7 week old WT and Itk$^{-/-}$ IL-17A GFP mice received SR intranasally 3 times a week for 3 weeks. Percent of IL-17A GFP^{+} iNKT cells from BAL **(A)**, lungs (**B**), and number of cells from BAL (**C**), and lung (**D**) as characterized using flow cytometry. Data from week 3. Data +/- SEM, n = 3 mice per group.

Discussion

HP is hallmarked by a strong Th17 induced inflammatory response in the lung (Simonian et al., 2009b). In this chapter we have examined the role of Itk in the response of T regulatory cells in HP, as well as iNKT cells that are able to produce IL-17A. We found that there is no difference in the proportion or numbers of regulatory T cells during HP in the absence of Itk, We also found that $Itk^{-/-}$ iNKT cells are able to produce IL-17A in this inflammatory model. These would suggest that Itk is not important regulator for T regulatory cells to get into the lung, nor control of IL-17A production by iNKT cells.

The fact that we find no effect of the absence of Itk on T_{reg} cells is not surprising since previous studies have shown that proinflammatory environments can down regulate T regulatory differentiation, which may also occur in the absence of Itk (Hatton and Weaver, 2009; Lee et al., 2009). Indeed, other studies have shown that T regulatory cells from HP patients are nonfunctional and fail to inhibit cell proliferation (Girard et al., 2011). HP results from the development of a very strong IL-17A response, caused by repeated agitation of the immune system, and it is possible that this proinflammatory environment prevents T regulatory cells from playing a more prominent role that would be revealed by the absence of Itk. We do note that these T_{reg} cells were not producing IL-17A (data not shown), suggesting that they were not converted to pathogenic IL-17A producing T_{reg} cells as has been seen in other diseases (Noack and Miossec, 2014). Naïve $Itk^{-/-}$ T cells display impaired Th17 differentiation along with increased T_{reg} differentiation, and we would therefore expect to see an increase in T_{reg} cells and a decrease in Th17 responses, as suggested by Gomez-Rodriguez et al (Gomez-Rodriguez et al., 2014). However, this was not observed. Furthermore, our lab has also recently

shown that Itk$^{-/-}$ T_{reg} are less functional in suppressing Th17 mediated Inflammatory Bowel Disease, and that Itk$^{-/-}$ T_{reg} produce IL-17A under conditions of inflammation in that model (Huang et al., 2014b). Given the lack of IL-17A production by the T_{reg} in either WT or Itk$^{-/-}$ mice, these data suggest that perhaps T_{reg} may indeed be less functional in HP as has been suggested (Girard et al., 2011). More detailed analysis of the function of these cells will be required to determine whether this is the case.

Itk has been suggested to play a role in the development and function of both iNKT cells and T_{reg} cells (Au-Yeung and Fowell, 2007; Felices and Berg, 2008; Gomez-Rodriguez et al., 2014; Huang et al., 2014b; Qi et al., 2012; Qi et al., 2011b). We therefore also investigated the behavior of iNKT cells in HP and whether the loss of Itk affects the response of these cells. The data shows that there is no difference in the proportion of iNKT cells that are in the BAL and lung of mice during HP. The number of iNKT cells in the lungs was also comparable between WT and Itk$^{-/-}$ mice, although Itk$^{-/-}$ had significantly less iNKT cells in the BAL. However, these differences did not translate into a decrease in expression of IL-17A in the absence of Itk. Our lab and others have shown that Itk is important for the complete maturation of iNKT cells as well as survival and cytokine production in response to NKT cell ligand α-GalCer (Au-Yeung and Fowell, 2007; Qi et al., 2012; Qi et al., 2011b). In the context of this study, we note that even with reduced maturation and survival, Itk$^{-/-}$ iNKT cells are still able to produce IL-17A response in HP. This may be due to Itk not being required for the production of this cytokine in these cells when stimulated by the TCR via NKT cell ligands as we saw for αβ CD4^{+} T cells. Alternatively the elevated inflammatory environment or bacterial derived ligands may induce IL-17A production by iNKT cells independently of the TCR,

and thus Itk, since iNKT cells can respond to inflammatory cytokines and PAMPS to produce cytokines (Watarai et al., 2012). Altogether, our findings suggest that iNKT cells are making IL-17A cytokine during HP and while Itk may be important for these cells to get into the BAL, it is not required for functional production of IL-17A. One caveat is that we were only able to look at this at week 3, as we may see differences at different time points in the disease during a time course.

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Chapter 6

Characterizing the role of Innate Memory Phenotype T cells in HP

Introduction

As we investigate the role that Itk signaling plays in hypersensitivity pneumonitis, we must take into account the phenotypical anomalies that exist in this strain of mice. Specifically, our lab has observed the emergence of $CD4^+$ and $CD8^+$ innate memory phenotype (IMP) cells in mice lacking Itk. Unlike naïve $CD4^+$ T cells, these cells have innate like functions such as rapid secretion of cytokine (Berg et al., 2005; Huang et al., 2014a; Huang et al., 2014c; Prince et al., 2014). These $CD4^+$ IMP cells have also been shown to display protective properties against autoimmunity (Huang et al., 2014c). The development of these IMP cells has been shown to be largely dependent on IL-4 from the thymic environment, and is suggested to be due to the conversion conventional T cells to cells that acquire the memory phenotype (Huang et al., 2014a; Huang et al., 2014c; Prince et al., 2014; Weinreich et al., 2010).

Since the presence of these cells may affect the response in the $Itk^{-/-}$ mice, we have investigated whether removal of these cells affects IL-17A driven inflammatory model of HP. Since these cells depend on IL-4 for their development, to remove IMP cells, we generated mice lacking the IL-4Rα receptor (Itk/IL4Rα double knock out, DKO) and compared their response to SR exposure to control mice lacking the IL-4Rα. Our results uncovered a role for IL-4 in enhancing T cell infiltration into the lung during the inflammatory response to SR, but showed that the absence of IL-4Rα does not affect the ability of $Itk^{-/-}$ mice to develop Th17 responses to SR. These data suggest that the ability of $Itk^{-/-}$ mice to develop strong Th17 responses to SR is not due to IL-4 or the presence of IMP cells.

Results

The IL-4Rα and IMP cells are not required for development of lung inflammation to SR in Itk$^{-/-}$ mice

IMP cells develop in the absence of Itk and may affect the ability of these mice to respond to SR. Since these cells depend on IL-4 for their development, to remove IMP cells, we generated mice lacking the IL-4α receptor (Itk/IL4Rα double knock out, DKO) and compared their response to control mice lacking the IL-4Rα. We then exposed these mice to SR to induce HP and examined cells for their ability to produce IL-17A. As discussed in the previous chapters, we found that there was significant inflammation in the lungs of WT and Itk$^{-/-}$ mice **(Fig. 6-1A,C)**. Analysis of mice lacking the IL-4Rα, as well as the Itk/IL-4Rα DKO mice, which lack IMP cells, revealed that these mice also had significant inflammation in the lungs **(Fig, 6-1B,D)**. Thus the loss of IL-4α signaling and IMP cells in the Itk deficient mice did not alter the ability of SR to induce lung inflammation and HP.

The IL-4Rα inhibits CD4^{+} T cell influx in the lung during SR induced HP, and regulates T cell recruitment to the lung in the absence of Itk

We next analyzed the proportions of CD4^{+} T cells in the lung and found that there is a significant reduction in the proportion of CD4^{+} T cells in the BAL in the absence of IL-4α signaling in both WT and Itk$^{-/-}$ mice **(Fig. 6-2A)**. The decrease in proportion of CD4^{+} T cells in the BAL did not affect the total number of CD4^{+} cell present in the BAL during HP **(Fig. 6-2C)**. When we compared the mice lacking the IL-4Rα on Itk$^{-/-}$ backgrounds to IL-4α$^{-/-}$ on WT backgrounds, we found that the Itk/IL4Rα DKO mice had

a lower proportion and number of $CD4^+$ T cells in the lung, suggesting that the IL-4Rα or IMP cells affects the recruitment of $CD4^+$ T cells to the lung during SR induced HP **(Fig. 6-2B,D)**.

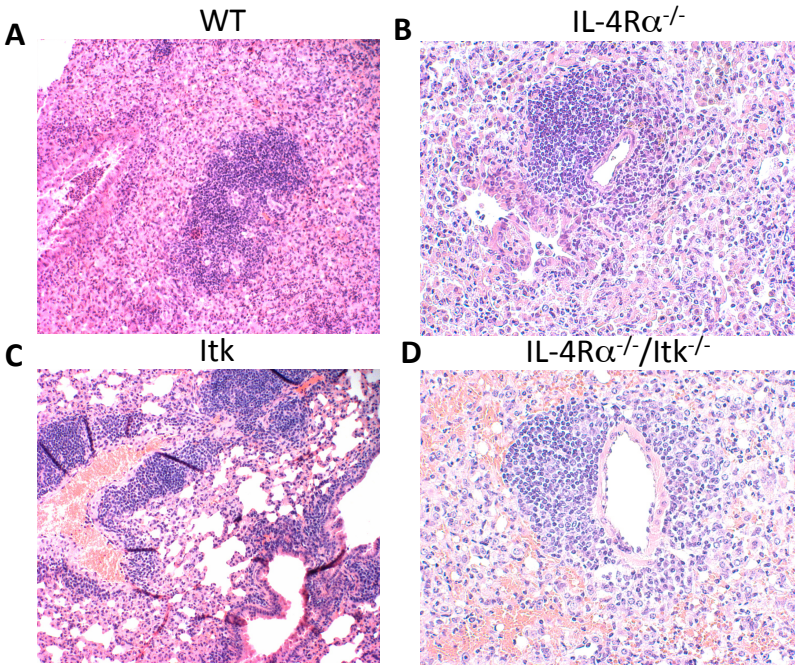

Fig. 6-1. IL-4Rα and IMP cells are not required for the development of lung inflammation during SR induced HP. 5-7 week old **(A)** WT, **(B)** IL-4Rα$^{-/-}$, **(C)** Itk$^{-/-}$, and **(D)** IL-4Rα$^{-/-}$/Itk$^{-/-}$ mice received SR intranasally 3 times a week over a period of 3 weeks. Tissue from lungs sections were stained with Hematoxylin and Eosin (H&E) staining and analyzed for severity of inflammation.

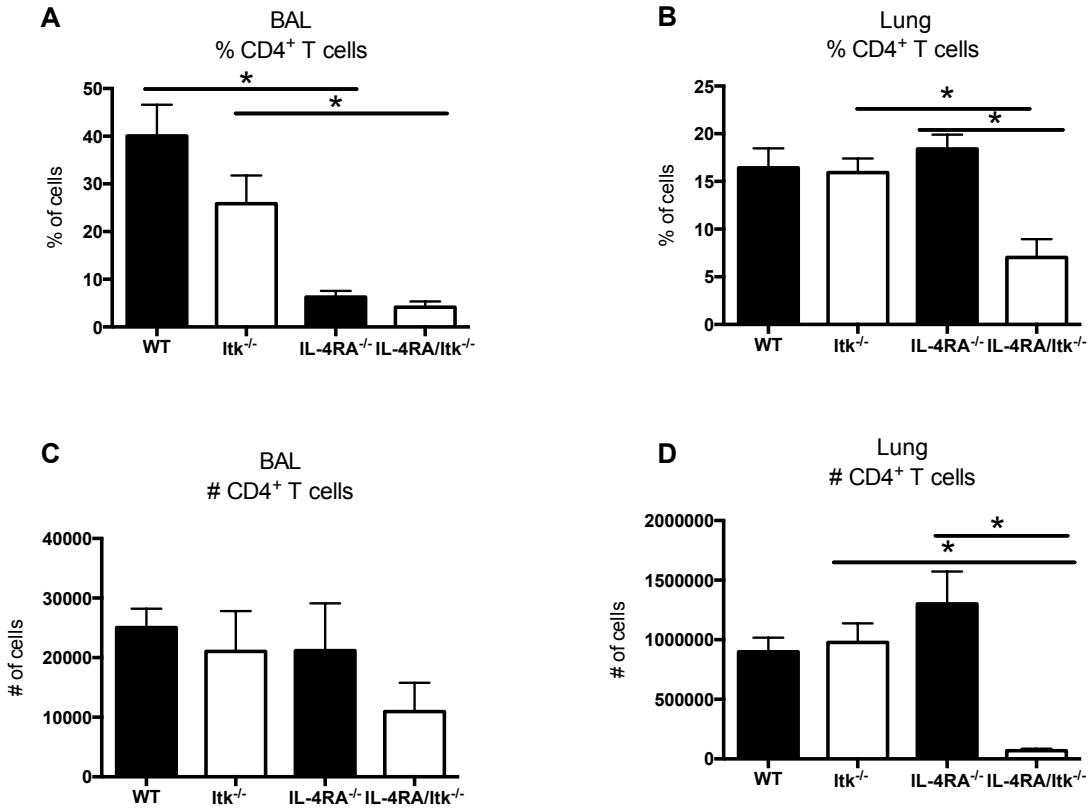

Fig. 6-2. The loss of IL-4Rα signaling leads to a decrease in the proportion of CD4$^+$ T cells in HP in the lung the absence of Itk. 5-7 week old WT IL-17A-GFP, Itk$^{-/-}$/IL-17A-GFP, IL-4Rα$^{-/-}$, IL-4Rα$^{-/-}$/Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week for 3 weeks. Cells from IL-4Rα$^{-/-}$, IL-4Rα$^{-/-}$/Itk$^{-/-}$ mice BAL, and lung were harvested and stimulated with PMA/Ionomycin + BFA for 5 hrs., then stained for TCRβ and CD4 and analyzed using flow cytometry gated on CD4$^+$ cells. Plots reflect the percent of CD4$^+$ T cells in BAL **(A)**, and lung **(B)**, and number of CD4$^+$ T cells in BAL **(C)** and lung (**D)**. *p< 0.05 Itk$^{-/-}$ vs. Itk$^{-/-}$/IL-4Rα$^{-/-}$ percent BAL, *p< 0.05 WT vs. IL-4Rα$^{-/-}$ percent BAL, *p<0.05 Itk$^{-/-}$ vs. IL-4Rα$^{-/-}$/Itk$^{-/-}$ percent lung, *p=0.062 Itk$^{-/-}$ vs. IL-4Rα$^{-/-}$/Itk$^{-/-}$ number BAL, and *p< 0.05 IL-4Rα$^{-/-}$ vs. IL-4Rα$^{-/-}$/Itk$^{-/-}$ number lung. Data +/- SEM, n = 3 mice per group.

Differential regulation of IL-17A production in CD4$^+$ T cells in the airway and lung by the IL-4Rα during SR induced HP

We next investigated the ability of these CD4$^+$ T cells recruited to the lung to express IL-17A in the absence of IL-4Rα signals alone (or IMP cells). We first compared the WT and IL-4Rα$^{-/-}$ mice and found that there is a significant difference in IL-17A cytokine expression from CD4$^+$ T cells in the BAL **(Fig. 6-3A)**. To further investigate this, we quantified the number of CD4$^+$ T cells expressing intracellular IL-17A and found that the loss of IL-4Rα signaling led to a significant decrease in the number of IL-17A expression in the BAL. This would suggest that in the BAL, IL-4 enhances the production of IL-17A by CD4$^+$ T cells in WT mice. Similar to the BAL, the absence of IL-4 signaling significantly decreases the number of IL-17A expressing CD4$^+$ T cells in the lung, suggesting that IL-4 regulates IL-17A production by CD4$^+$ T cells in a location dependent manner (**Fig. 6-3C, D**).

Next, we compared the Itk$^{-/-}$ mice to mice lacking both Itk and the IL-4Rα (as well as IMP cells) and saw no differences in in the proportion of CD4$^+$ T cells that produced IL-17A in the BAL though IL-17A expression in the lung was significantly decreased **(Fig. 6-3A)**. In the BAL there is a decrease, which is trending towards significant in the numbers of these cells in the absence of the IL-4Rα (and IMP cells) on Itk$^{-/-}$ background (**Fig. 6-3C**). The absence of IL-4α signaling in Itk$^{-/-}$ mice also led to a decrease in the number and proportion of IL-17A expressing CD4$^+$ T cells, suggesting that IL-4 regulates IL-17A production by CD4$^+$ T cells (**Fig. 6-3B, D**).

Comparing the two mouse strains that lack the IL-4Rα (IL-4Rα$^{-/-}$ vs. Itk/IL4Rα DKO), however, revealed that in the absence of IL-4Rα signaling, mice lacking Itk

exhibited reduced proportion of $CD4^+$ T cells in the lung, and the BAL (**Fig. 6-2**). However, the proportion of IL-17A expressing $CD4^+$ T cells in the BAL did not reflect this reduction as seen in the lung tissue **(Fig. 6-3B)**. We did determine that there is a significant reduction in the number of IL-17A expressing cells in the lung in comparing IL-4R$\alpha^{-/-}$ vs. Itk/IL-4Rα DKO). Since the $CD4^+$ T cells were unable to express a robust IL-17A response to SR, these results suggest that the loss of IL-4Rα (and IMP cells) affect the ability of $CD4^+$ T cells to express IL-17A during the SR induced development of HP.

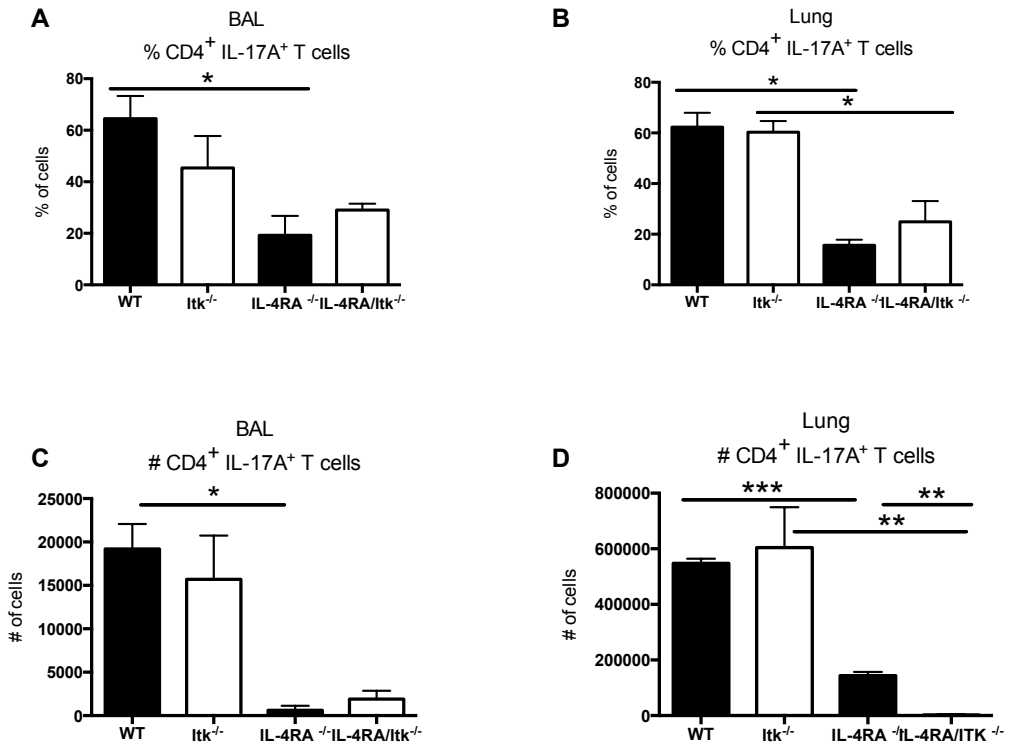

Fig. 6-3: The IL-4Rα enhances CD4$^+$ T cell IL-17A responses in the lung, but does not regulate the IL-17A response in Itk$^{-/-}$ mice. 5-7 week old WT IL-17A-GFP, Itk$^{-/-}$ /IL-17A-GFP, IL-4Rα$^{-/-}$, IL-4Rα$^{-/-}$/Itk$^{-/-}$ mice received PBS or SR intranasally 3 times a week for 3 weeks. Cells from IL-4Rα$^{-/-}$, IL-4Rα$^{-/-}$/Itk$^{-/-}$ mice BAL, and lungs were harvested and stimulated with PMA/Ionomycin + BFA for 5 hrs, then stained for TCRβ and CD4 and intracellular IL-17A, and analyzed using flow cytometry gated on IL-17A expressing CD4$^+$ cells. Plots reflect the percent of IL-17A$^+$ CD4$^+$ T cells in BAL **(A)**, and lung **(B)**, and number of IL-17A$^+$ CD4$^+$ T cells in BAL **(C)** and lung (**D)**. *p < 0.05 WT vs. IL-4Rα$^{-/-}$ percent lung, *p< 0.05 WT vs. Itk$^{-/-}$/IL-4Rα$^{-/-}$ percent lung, *p< 0.05 WT vs. IL-4Rα$^{-/-}$ percent BAL, *p< 0.05 WT vs. IL-4Rα$^{-/-}$ number BAL, ***p< 0.05 WT vs. IL-4Rα$^{-/-}$ number lung, **p<0.05 Itk$^{-/-}$ vs. IL-4Rα$^{-/-}$/Itk$^{-/-}$ number lung and **p< 0.05 IL-Rα$^{-/-}$ vs. IL-4Rα$^{-/-}$/Itk$^{-/-}$. Data +/- SEM, n = 3 mice per group.

Discussion

We and others have shown that a population of $CD4^+$ and $CD8^+$ innate memory phenotype (IMP) cells emerge in mice lacking Itk. These IMP T cells have innate like functions such as rapid secretion of cytokine (Berg, 2007; Broussard et al., 2006; Huang et al., 2014b; Huang et al., 2014c; Prince et al., 2014). The development of these IMP cells has been shown to be largely dependent on IL-4 in the thymic environment (Huang et al., 2014a; Prince et al., 2014; Weinreich et al., 2010). In this chapter, we investigated the role of signaling by the IL-4Rα, and indirectly the role of Innate Memory Phenotype T cells in HP. We found that the loss of IL-4 signaling (IL-4Rα$^{-/-}$) in WT or Itk$^{-/-}$ mice led to comparable lung inflammation to what is seen in normal WT mice after 3 weeks of exposure, suggesting that IL-4Rα signals or IMP cells may not be required for inflammation observed in the lungs of mice during HP. However, we also found that the loss of IL-4Rα signaling on both WT and Itk$^{-/-}$ backgrounds led to a significant decrease in the proportion and number of $CD4^+$ cells in BAL. Furthermore, the absence of IL-4Rα (or IMP cells) Itk$^{-/-}$ mice led to the decrease in the proportion of $CD4^+$ T cells in the lung suggesting that IL-4Rα signaling (or IMP cells) may be important for this response. We also found that the loss of IL-4Rα signaling on the WT as well as Itk$^{-/-}$ background significantly affects the ability of $CD4^+$ T cells to express IL-17A during SR induced lung inflammation. These data suggest that IL-4Rα signaling may contribute to the ability of $CD4^+$ T cells to express IL-17A in the lung following SR exposure.

Our results show that $CD4^+$ T cells from IL-4Rα$^{-/-}$/Itk$^{-/-}$ IMP null mice displayed similar proportion of cells expressing IL-17A cytokine compared to those from equivalent WT IL-4Rα$^{-/-}$ mice. Conversely, the proportion of $CD4^+$ cells in the BAL in

IL-4Rα$^{-/-}$ are significantly reduced as compared to WT, and this loss in IL-4 signaling also led to decreased IL-17A expression in the BAL. This suggests that IL-4 signaling plays an important role in IL-17A production during HP, regardless of the presence or absence of Itk. While clearly additional studies are needed, and the interactions are clearly complex, the summation of these studies would suggest that IL-4Rα and/or IMP cells are partially responsible for the number of CD4^{+} T cells that respond in HP, but that this pathway does not contribute to the ability Itk$^{-/-}$ to produce IL-17A in response to SR.

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Chapter 7

Characterizing the role of natural Th17 cells in HP

Introduction

The cytokine IL-17A produced during hypersensitivity pneumonitis has been shown to come from several different cellular sources (Hasan et al., 2013; Simonian et al., 2009a; Simonian et al., 2009b). The most well studied source of IL-17A is from inducible Th17 cells (iTh17) that differentiate from naïve $CD4^+$ in response to environmental influences such as antigen stimulation and extracellular cytokine secretion. However, more recent studies have identified a new subset of $CD4^+$ T cells that produce IL-17A, referred to as natural Th17 cells (nTh17) cells (Tanaka et al., 2009). These nTh17 cells have innate like function in producing IL-17A, and differ from iTh17 cells in that they have the capacity to produce IL-17A cytokine immediately upon stimulation without having to migrate to the peripheral tissue to undergo further differentiation (Conti et al., 2009; Tanaka et al., 2009). iTh17 and nTh17 are indistinguishable by surface markers, however, they are distinguished by their different pathways of development (Tanaka et al., 2009). Both nTh17 and iTh17 cells are regulated by the serine/threonine kinase AKT, however iTh17 development requires signaling through mTORC1 while nTh17 require mTORC2 for development (Conti et al., 2014). Thus Rapamycin, which inhibits mTORC1, can block the development of iTh17 cells **(Fig. 7-1).** However, blocking mTORC1 does not affect the development of nTh17 cells (Conti et al., 2009).

Given the previous findings that Itk plays a positive role in the development of iTh17 cells, and our findings that $Itk^{-/-}$ mice still develop robust Th17 responses in response to SR, there is a possibility that the Th17 responses observed in these mice are due to nTh17 cells. We therefore investigated whether the Th17 responses observed in the absence of Itk is likely due to nTh17 cells.

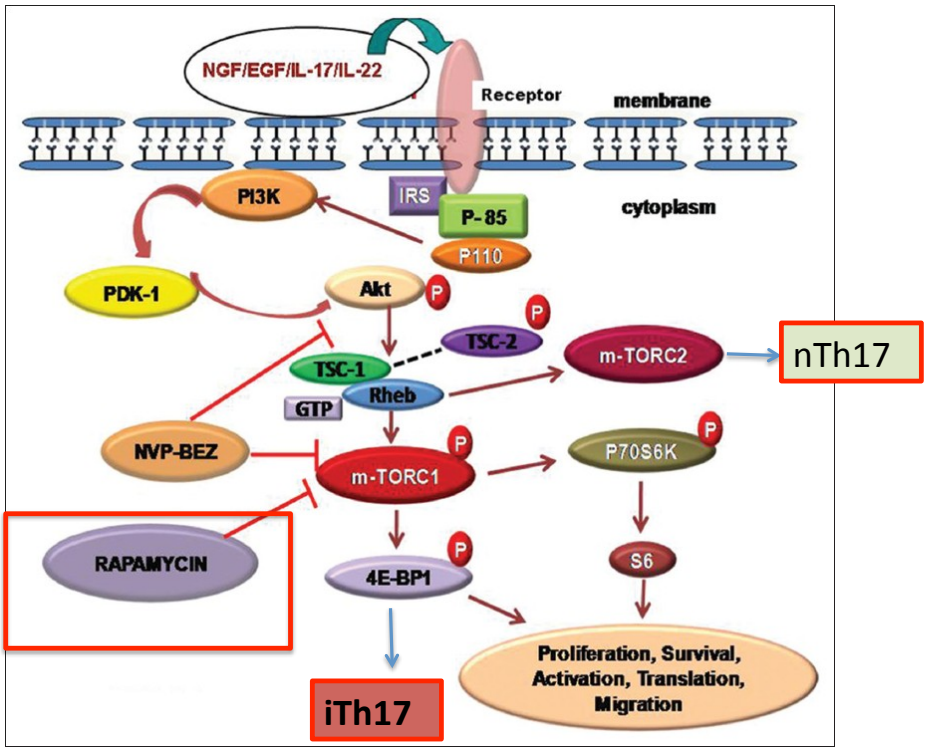

Fig. 7-1. Schematic of mTOR pathway and role in iTh17 and nTh17 development. Receptor tyrosine kinases are activated by ligand resulting in phosphorylation of tyrosine sites, which serve as a platform for the complex of phosphatidylinositol 3-kinase (PI3K) and AKT. This leads to the phosphorylation of mTORC1 (required for inducible Th17) and mTORC2 (required for natural Th17). Rapamycin blocks the mTORC1 pathway therefore inhibiting the development of iTh17 cells, while leaving the nTh17 pathway intact. Modified from (Conti et al., 2009).

Results

$CD4^+$ T cell recruitment to the lung during SR is Rapamycin sensitive

To determine the role of nTh17 cells in the Th17 response to SR, we inhibited mTORC1 using rapamycin during exposure to SR. This should inhibit the development of an iTh17 response, while leaving intact the nTh17 cells. WT and $Itk^{-/-}$ IL-17A-GFP mice were exposed to SR for 3 weeks in the presence or absence of exposure to Rapamycin followed by analysis of IL-17A production. We found that in both WT and $Itk^{-/-}$ mice treated with Rapamycin, the proportion of αβ T cells in the BAL and lung was comparable to the non-treated mice **(Fig. 7-2A,B).** However, the number of αβ T cells in BAL and lungs of both WT and $Itk^{-/-}$ mice were significantly decreased by treatment with Rapamycin **(Fig. 7-2C,D)**, and there was no difference in the response of the WT and $Itk^{-/-}$ mice in the BAL, although the $Itk^{-/-}$ mice had lower numbers of αβ T cells in the lungs after Rapamycin treatment **(Fig. 7-2D)**. Similar results were obtained when the αβ $CD4^+$ T cells were analyzed **(Fig. 7-3A-D)**. These results suggest that the predominant response to SR in both WT and $Itk^{-/-}$ mice is due to the mTORC1 rapamycin sensitive iTh17 cell.

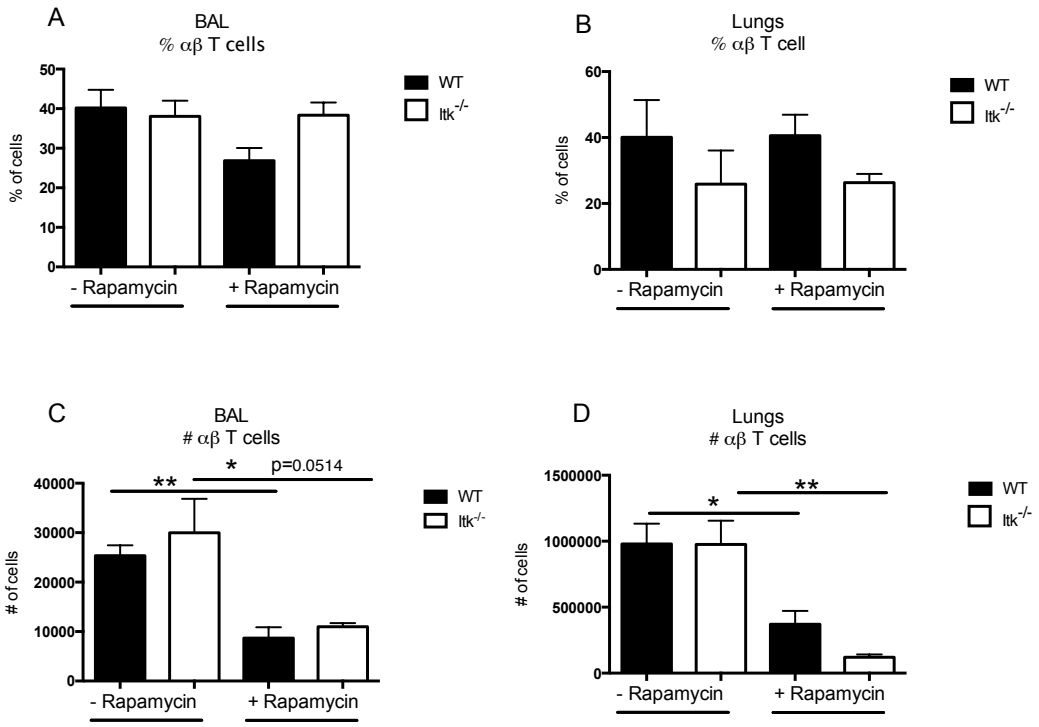

Fig. 7-2. Recruitment of αβ T cells to the lungs during SR induced inflammation is Rapamycin sensitive. 5-7 week old WT and Itk$^{-/-}$ IL-17A GFP mice received Rapamycin every other day i.p. and SR intranasally 3 times a week for 3 weeks. Cells from BAL and lungs were harvested and the percent (**A, B**) and numbers (**C, D**) of αβ T cells from **(A, C)** BAL, (**B, D)** as determined by flow cytometry. **p< 0.05 WT Rap^{-} vs. WT Rap^{+} BAL number, Itk$^{-/-}$ Rap^{-} vs. Rap^{+} lung number, *p< 0.05 WT Rap^{-} vs. WT Rap^{+} lung number, *p=0.0514 Itk Rap^{-} vs. Itk Rap^{+} BAL number. Data +/- SEM, n = 3 mice per group.

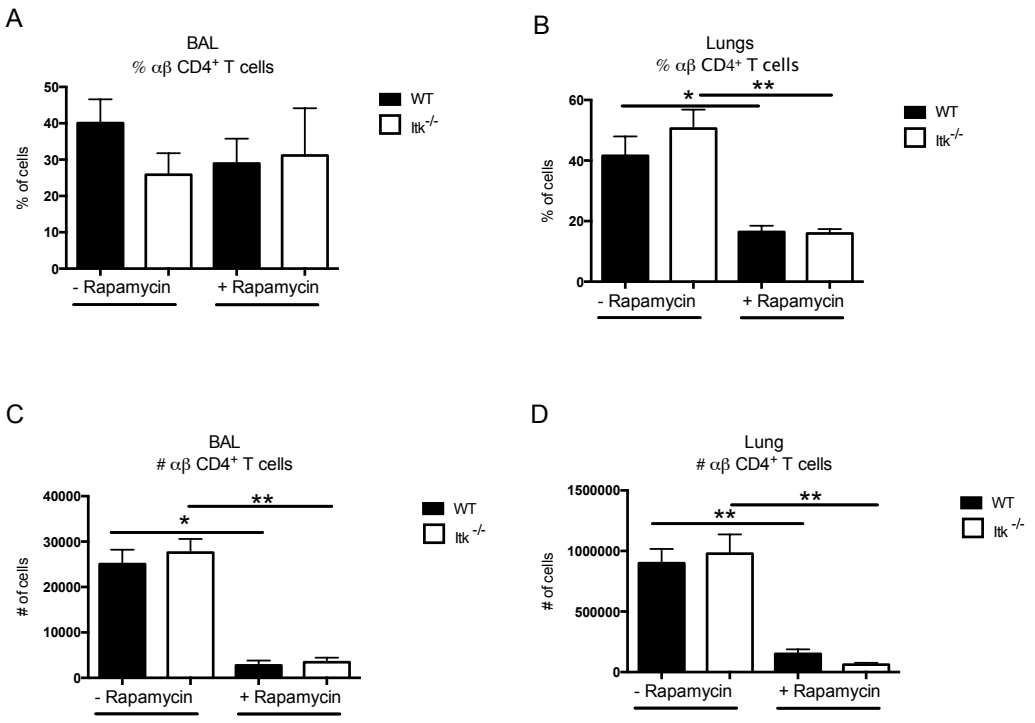

Fig. 7-3. Recruitment of αβ CD4$^+$ T cells to the lungs during SR induced inflammation is Rapamycin sensitive. 5-7 week old WT and Itk$^{-/-}$ IL-17A GFP mice received Rapamycin every other day i.p. and SR intranasally 3 times a week for 3 weeks. Cells from BAL and lungs were harvested and the percent (**A, B**) and numbers (**C, D**) of αβ CD4$^+$ T cells from **(A, C)** BAL, (**B, D**) as determined by flow cytometry. *p< 0.05 Rap$^-$ vs. WT Rap$^+$ lung percent, WT Rap$^-$ vs. WT Rap$^+$ BAL number, **p< 0.05 Itk$^{-/-}$ Rap$^-$ vs. Itk$^{-/-}$ Rap$^+$ lung percent, Itk$^{-/-}$ Rap$^+$ vs. Itk$^{-/-}$ Rap$^-$ BAL number, WT Rap$^-$ vs. WT Rap$^+$ lung number, Itk Rap$^-$ vs. Rap$^+$ lung number. Data +/- SEM, n = 3 mice per group.

Rapamycin sensitive iTh17 cells are the major contributors to the IL-17A producing cells in the lung in response to SR

We next examined whether Rapamycin treatment affected the production of IL-17A by these $CD4^+$ T cells. We found that there was no difference in the proportion of $\alpha\beta$ $CD4^+$ T cells expressing IL-17A GFP in the BAL of WT or $Itk^{-/-}$ mice **(Fig. 7-4A),** although we did observe a significant decrease in the proportion of IL-17A expressing cells in the lungs of $Itk^{-/-}$ mice **(Fig. 7-4B).** Additionally, both WT and $Itk^{-/-}$ mice displayed a significant decrease in the number of $CD4^+$ T cells expressing IL-17A in the BAL and lung in the presence of Rapamycin **(Fig. 7-4C,D).** This suggests the Rapamycin sensitive iTh17 cells may be responsible for the robust $CD4^+$ IL-17A secreting $\alpha\beta$ T cells mediating HP in both WT and $Itk^{-/-}$ mice.

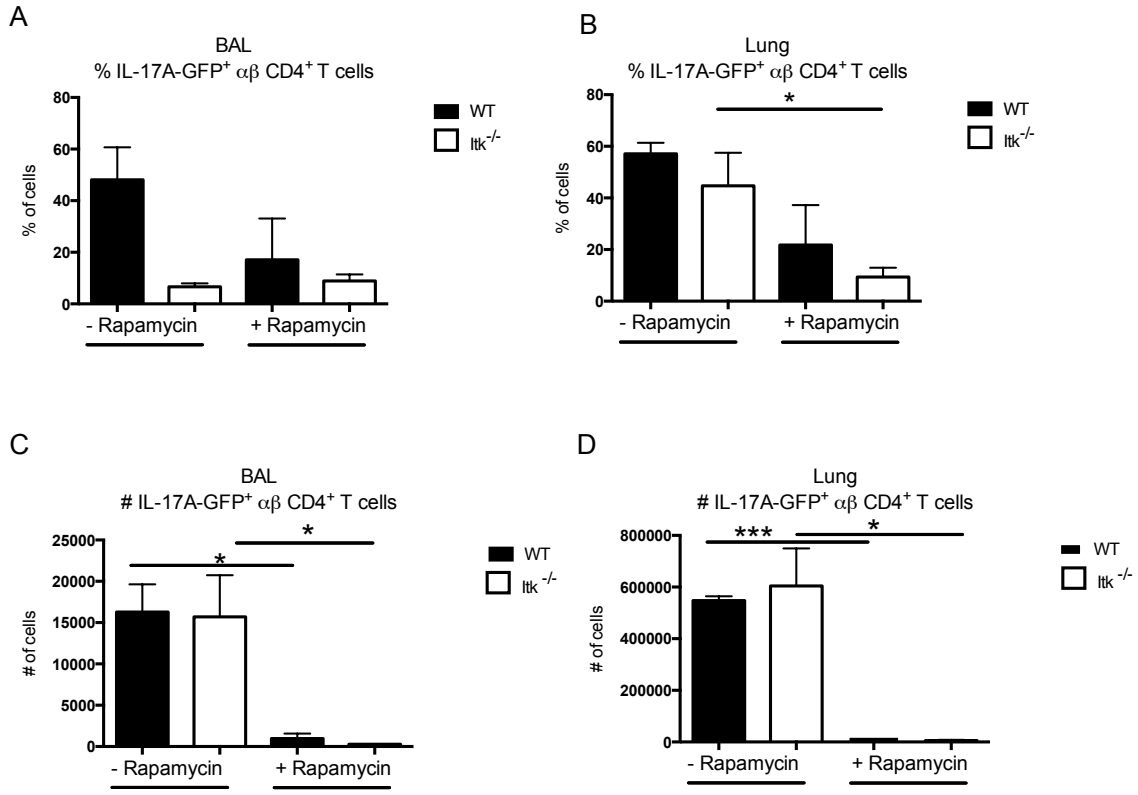

Fig. 7-4. IL-17A production by CD4$^+$ T cells in the lung in response to SR is Rapamycin sensitive. 5-7 week old WT and Itk$^{-/-}$ IL-17A GFP mice received Rapamycin every other day i.p. and SR intranasally 3 times a week for 3 weeks. Cells from BAL and lungs were harvested and the percent (**A, B**) and numbers (**C, D**) of GFP/IL-17A$^+$ αβ CD4$^+$ T cells from **(A, C)** BAL, (**B, D)** as determined by flow cytometry. *p< 0.05 Itk$^{-/-}$ Rap$^+$ vs. Itk$^{-/-}$ Rap$^-$ lung percent, WT Rap$^-$ vs. WT Rap$^+$ BAL number, Itk$^{-/-}$ Rap$^+$ vs. Itk$^{-/-}$ Rap$^-$ BAL number, Itk$^{-/-}$ Rap$^+$ vs. Itk$^{-/-}$ Rap$^-$ lung number, ***p< 0.05 WT Rap$^-$ vs. WT Rap$^+$ lung number. Data +/- SEM, n = 3 mice per group.

Rapamycin does not affect the ability of γδ T cells to produce IL-17A in response to SR

We next examined the behavior of γδ T cells in the Rapamycin treated mice. Our previous data shows that these cells have the capacity to produce IL-17A after exposure SR leading to HP. Cells from airway and lungs were analyzed for γδ T cells and examined for IL-17A GFP expression. We found that the γδ T cells in WT mice exhibited significant production of IL-17A (**Fig. 7-5A**). When comparing WT mice treated with Rapamycin vs. not treated, we observed significantly higher proportion of γδ T cells making IL-17A regardless of background (**Fig. 7-5B**). The number of γδ T cells in both WT and $Itk^{-/-}$ mice were not altered after Rapamycin treatment, suggesting that Rapamycin does not affect IL-17A production by γδ T cells, but is important in regulating the number of γδ T cells making IL-17A **(Fig. 7-5B,C)**. This change in proportion may be caused by the αβ T cells have a lower proportion, thus if the γδ T cell number doesn't change, their proportion will go up. Analyses of $Itk^{-/-}$ mice revealed that there was no difference in IL-17A expression by γδ T cells from the BAL, although there was decrease in IL-17A expression in lung γδ T cells. These results suggesting that Itk may modulate the Rapamycin sensitivity of γδ T cells to maintain their ability to produce IL-17A.

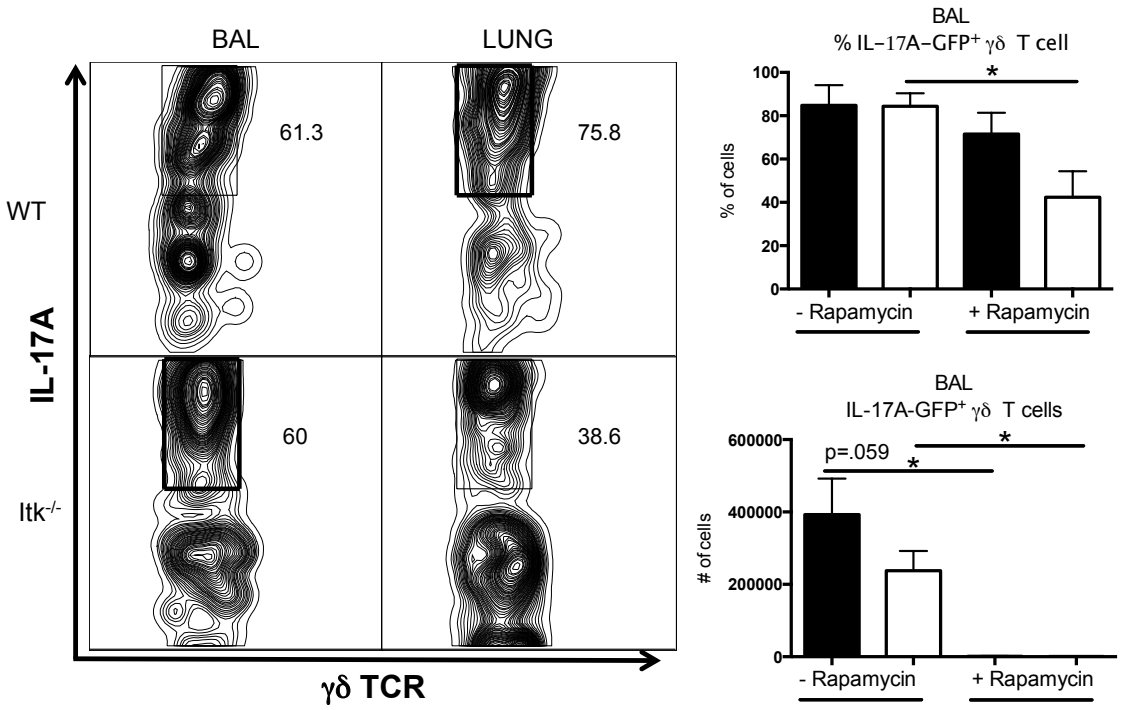

Fig. 7-5. Rapamycin does not affect the ability of γδ T cells to produce IL-17A. 5-7 week old WT (*black bar*) and Itk$^{-/-}$ IL-17A/GFP (*open bar*) mice received Rapamycin every other day i.p. and SR intranasally 3 times a week for 3 weeks. Representative plots show cells from lungs harvested and stained for surface expression of γδ TCR vs. IL-17A GFP (**A**). Analyzed cell number for BAL (**B**), and lung (**C**). *p=0.059 WT Rap^{-} vs Itk$^{-/-}$ Rap^{-} BAL number, *p=0.05 Itk$^{-/-}$ Rap^{-} vs Itk$^{-/-}$ Rap^{+} BAL number, *p=0.05 Itk$^{-/-}$ Rap^{-} vs Itk$^{-/-}$ Rap^{+} BAL Percentage. Data +/- SEM, n = 3 mice per group.

Effect of Rapamycin treatment on T regulatory cells in HP

Rapamycin has been shown to enhance the development of T_{reg} (Battaglia et al., 2006), and so we next investigated whether Rapamycin treatment affected the presence of these cells. Th17 cell and T_{reg} cells share a common developmental lineage. There is a delicate balance that determines the lineage commitment of cells becoming either Th17 or T_{reg} cells. The treatment of mice with Rapamycin inhibits iTh17 cells and the absence of iTh17 cells leads to less IL-17A and consequently decreases in development of HP. To determine if this affected T_{reg} cells, mice treated with Rapamycin were analyzed for the presence of T_{reg} cells **(Fig. 7-6A)**. We found that Rapamycin treatment led to an increase in the proportion of T_{reg} cells in the BAL and lung of WT mice, although the numbers of these cells were decreased **(7-5B,C, 7-6B,C)**.

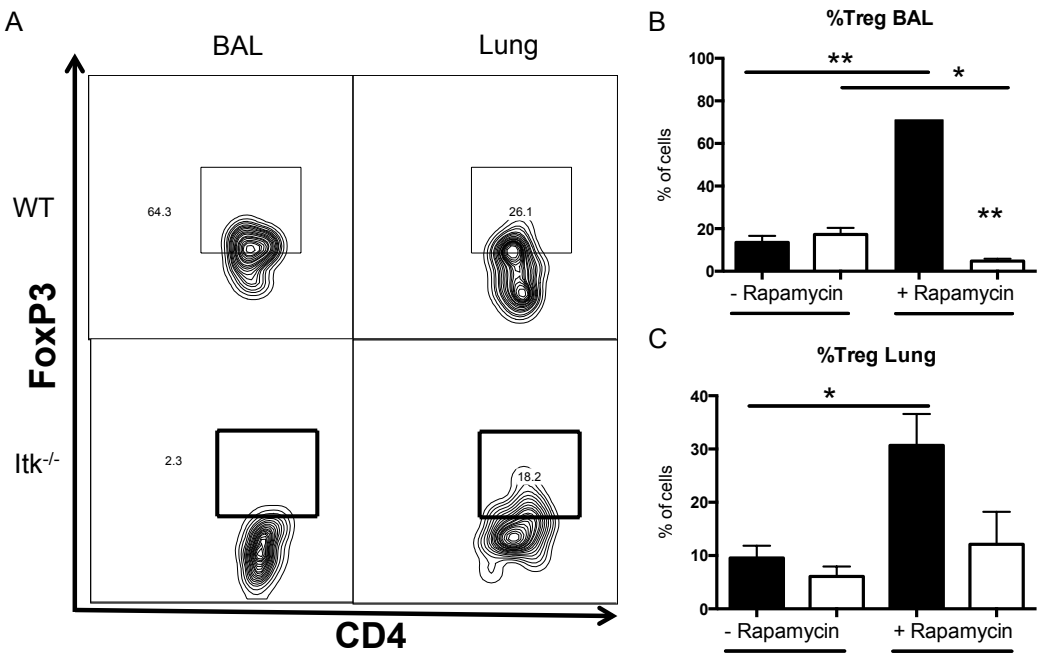

Fig. 7-6. Effect of Rapamycin on the proportion of T regulatory cells in lung during SR induced inflammation. 5-7 week old WT and $Itk^{-/-}$ IL-17A GFP mice received Rapamycin every other day i.p. and SR intranasally 3 times a week for 3 weeks. Representative plots show cells from lungs harvested and analyzed for $Foxp3^{+}$ T regulatory cells. BAL and lung representative plot of $CD4^{+}$ $Foxp3^{+}$ cell (**A**) percentage of BAL T_{reg} cells +/- Rapamycin (**B**) percentage of lung T_{reg} cells +/- Rapamycin (**C**). **p<0.05 WT Rap^{+} vs $Itk^{-/-}$ Rap^{+} BAL percent, *p<0.05 $Itk^{-/-}$ Rap^{-} vs. $Itk^{-/-}$ Rap^{+} BAL percent, **p<0.05 WT Rap^{+} vs. Itk Rap^{+} BAL *p<0.05 WT Rap^{-} vs. WT Rap^{+} lung percent. Data +/- SEM, n = 3 mice per group.

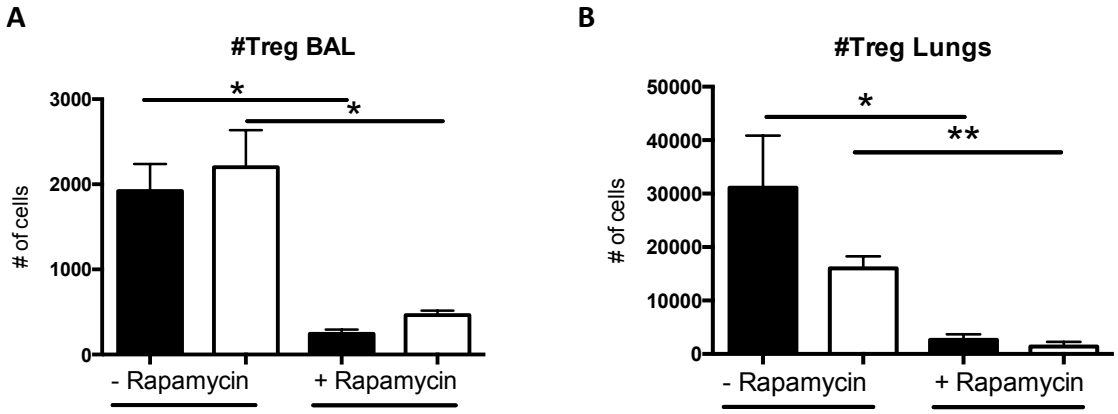

Fig. 7-7. Rapamycin reduces the number of T regulatory cells population in lung during SR induced inflammation. 5-7 week old WT and $Itk^{-/-}$ IL-17A GFP mice received Rapamycin every other day and SR intranasally 3 times a week for 3 weeks. BAL (**A**) and lung (**B**). *$p<0.05$ WT Rap^{-} vs. WT Rap^{+} BAL number, *$p<0.05$ $Itk^{-/-}$ Rap^{-} vs. $Itk^{-/-}$ Rap^{+} BAL number, *$p<0.05$ WT Rap^{-} vs. WT Rap^{+} lung number, **$p<0.05$ $Itk^{-/-}$ Rap^{-} vs. $Itk^{-/-}$ Rap^{+} lung number. Data +/- SEM, n = 3 mice per group.

Discussion

We and others have recently reported that Itk plays a positive role in the development of iTh17 cells, but given our findings that $Itk^{-/-}$ mice still develop robust Th17 responses in response to SR, there is a possibility that the Th17 responses observed are due to a recently described population of nTh17 cells. In this chapter, we explored the role of these nTh17 cells in HP by treating mice with Rapamycin, which should differentially affect the development if iTh17 cells (sensitive) and nTh17 cells (resistant) (Conti et al., 2009). We found that Rapamycin treatment, and by inference, blocking iTh17 cells reduced the proportion of T cells in the lung (but not the BAL) in response to SR induction. Furthermore this block leads to a significant decrease in αβ T cells in both WT and $Itk^{-/-}$ mice. We also found that Rapamycin treatment significantly reduced the proportion and numbers of $CD4^+$ T cells producing IL-17A in the lungs of both WT and $Itk^{-/-}$ mice, suggesting that the SR induced IL-17A response in $CD4^+$ T cells is primarily due to the development of iTh17 cells, and not nTh17 cells. By contrast, Rapamycin treatment enhanced the proportion of T_{reg} cells in the lung, although their numbers were reduced. This is comparable to previous studies that demonstrate a decrease in the absolute number of T cells but not the proportion of $CD4^+$ Foxp3 expressing cells in the thymus after Rapamycin treatment (Coenen et al., 2007).

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Chapter 8

Discussion

In this project we utilized the IL-17A driven airway inflammatory model hypersensitivity pneumonitis to investigate the role of Tec kinase Itk in Th17 differentiation and function. HP results from a complex immune response in the lung and it was important to investigate this disease using a diverse array of approaches. In this study, we investigated the immune response over the course of disease. This was accomplished by characterizing the composition of immune cells involved during HP. We examined the capacity of T cell subsets to make IL-17A cytokine in response to SR exposure, as well as investigated this cytokine production without external stimulation or manipulation. We utilized various murine genetic models to investigate the cell types involved in the production of Il-17A, required for the development of HP. We also examined the role of the kinase activity of Itk, and the caveats of phenotypical differences observed in some of our mouse models. Together, the results provide a new perspective on the requirements for the production of IL-17A in $CD4^+$ T cells, leading to the development of HP.

Hypersensitivity Pneumonitis is characterized by aggressive inflammation of the lung airways in response to repeated inhalation of SR and this inflammation can leads to pulmonary dysfunction in humans and mice (Takemura et al., 2008; Woda, 2008). Early studies of the disease showed that T cells are important mediators of pathology in HP (Takizawa et al., 1992). Our experiments confirm previous studies that show that αβ T cells are important contributors to IL-17A expression and that this cytokine is the most highly expressed in comparison to Th1 cytokine IFNγ and Th2 cytokine IL-4 (Simonian et al., 2009b). Itk has been shown to be important in the differentiation of αβ T cells to Th2 and Th17 cells, and T cells from mice lacking Itk display impairment in the

production of IL-17A (Berg et al., 2005; Gomez-Rodriguez et al., 2009). After establishing our SR model of HP we investigated the role of Itk in this IL-17A response and surprisingly saw that the lack of Itk did not decrease the expression of IL-17A mRNA, nor did it lead to less IL-17A expression in αβ $CD4^+$ T cells. It has been shown that IL-6 is a driver of Th17 differentiation, and our data shows $Itk^{-/-}$ mice display significantly higher levels of mRNA expression of IL-6 in HP after 3 weeks of exposure SR (Kimura and Kishimoto, 2010). That may be partially responsible for the ability of $Itk^{-/-}$ mice to mount a CD4 dependent IL-17A response. Additionally, proinflammatory environments promote the differentiation of IL-17A and if there are alternative sources of this cytokine, this can potentially compensate for the endogenous lack of $CD4^+$ αβ T cells seen in the $Itk^{-/-}$ phenotype. To be noted, although $Itk^{-/-}$ mice have an impairment in Th17 cells, there is not a complete ablation of this population. Thus it is possible that the small number of Th17 cells in $Itk^{-/-}$ mice may have the ability to expand in the lung as well as periphery and induce disease.

The caveat of using intracellular staining to assess cytokine expression is that this experimental model allows you to observe the potential a cell has to make cytokine but is not the true representative of the cytokines that cell is actually making in vivo. In order to bypass these conditions, we utilized WT and $Itk^{-/-}$ mice expressing a GFP tag whenever cells make IL-17A, or have recently made IL-17A. The subsequent experiments confirmed that αβ $CD4^+$ T cells are making expressing IL-17A, and further showed that contrary to expectations, the lack of Itk did not affect this expression. This confirms that without any manipulation, these $CD4^+$ T cells are highly expressing IL-17A in response to SR antigen. This experimental design also allowed us to reanalyze other cells believed

to contributing to IL-17A production, and we found that neutrophils are not making significant amounts of IL-17A contrary to what was previously reported (Hasan et al., 2013). This was important because this previous work had suggested that neutrophils in HP lungs were responsible for the increase in IL-17A cytokine driving inflammation and pathology and not αβ T cells. Although our data show that neutrophils are not responsible for significant IL-17A production during HP, it is very likely that they still have some contribution in overall IL-17A production pathway. Indeed, studies have demonstrated that neutrophils have the ability to prime antigen-specific Th1 and Th17 immune responses by inducing differentiation, even in the absence of exogenous cytokines present in their environment (Abi Abdallah et al., 2011). Together this data gives a broader picture of the mechanisms involved in IL-17A expression in HP.

In order to investigate the role of the kinase activity of Itk in the immune response during HP, we utilized mice expressing an allele sensitive Itk which can be targeted and blocked with 3-MBPP1 inhibitor (Gregan et al., 2007; Kannan et al., 2014; Shokat and Velleca, 2002). The results show that if Itk kinase activity is blocked during the development of HP, inflammation is significantly reduced in the lung. αβ $CD4^+$ T cell function is also greatly reduced, as they made significantly less IL-17A in the lung. This of course was contrary to our previous findings that the absence of Itk does not affect this inflammatory response. We hypothesize that T cells that develop in the presence of Itk (Itk*as*) are dependent on the kinase for developing into Th17 cells, while T cells that develop in the absence of Itk (as in the $Itk^{-/-}$ mice) are not dependent on the kinase activity for the development of a Th17 responses and thus can develop HP independently of Itk.

As previously mentioned, we believed that there may be other sources of IL-17A during the response to SR in HP. It has been shown the γδ T cells expand and expression IL-17A in *B. subtilis* induce HP (Simonian et al., 2009a). Like SR, *B. subtilis* is found in hay but mainly resides as a ubiquitous soil organism whereas, SR is a thermophilic bacteria. As previously reported, our data confirm that γδ T cells express IL-17A in an SR induced model of HP. Furthermore, we also showed that both $CD4^-$ and $CD4^+$ γδ T cell populations make this cytokine after ex vivo stimulation. Additionally, the lack of Itk lead to increased numbers of IL-17A expressing cells compared to WT in IL-17A expressing $CD4^+$ T cells after ex vivo stimulation. As we looked into the endogenous expression of IL-17A, we confirmed that IL-17A expression increased over the course of 3 weeks in WT mice. $Itk^{-/-}$ did not have the same endogenous increase of IL-17A expression in comparison to what was shown during ex vivo stimulation. This is not surprising, as intracellular cytokine staining does not completely reflect the level of cytokine expression, but more reflects the potential of cells to make a certain cytokine under various conditions. With the IL-17A GFP mice we are able to more directly determine the expression of IL-17A in these cells simply by staining for surface markers and determining the levels of GFP. Of the γδ T cell population, $CD4^+$ γδ T cells exhibited the highest expression of IL-17A, and these cells accounted for a higher proportion of cells in the lungs of mice during HP. Previous studies determined that naïve $Itk^{-/-}$ mice have elevated numbers of $CD4^+$ γδ T cells in the thymus as well as spleen (Qi et al., 2009). We confirm this elevated population also occurs in the lung during HP.

We determined that there were different levels of IL-17A expression in the γδ T cells BAL and lungs. We hypothesize that a contributing factor that may explain difference in IL-17A expression in these tissues is that γδ T cells in the BAL may be receiving significantly higher level of exposure to antigen in the airways of the respiratory track of the lungs, where they would be exposed to the inhaled antigen at a higher level than the cells in the lung parenchyma. This increased frequency of exposure in the BAL may compensate for the inadequacy in signaling function exhibited by the absence of Itk. This exposure would not be as frequent in the dense tissue of the lung, thus explaining the decrease in response. In the context of other immune cells producing IL-17A, $CD4^+$ γδ T cells have been determined to have the highest expression of IL-17A although a moderate proportion of $CD4^-$ γδ T cell are also expressing IL-17A.

Given the production of IL-17A by γδ T cells, we next examined whether the absence of γδ T cells would alter the IL-17A driven HP immune response. Our data shows that in the absence of γδ T cells, αβ $CD4^+$ T cells continue to express high levels of IL-17A, and the loss of Itk does not affect the ability of αβ $CD4^+$ T cells to express IL-17A. However, the loss of γδ and Itk did lead to a reduction in the neutrophil population suggesting that γδ T cells may be important in neutrophil recruitment in the absence of Itk. Similar to neutrophils, human γδ T cells have the ability to behave as professional APC's and may be contributing to the overall mechanism of IL-17A production in HP by priming the immune response to make IL-17A cytokine (Moser and Brandes, 2006).

It has been shown that T_{reg} cells impose the balance between proinflammatory and suppressive immune responses (Fontenot and Rudensky, 2005). Foxp3 regulates the differentiation of T regulatory cells and is down regulated by IL-17A-induced

inflammatory conditions (Gao et al., 2012; Li and Zheng, 2012). We therefore also investigated the role both cell types play in IL-17A driven HP. We did not find any difference in the proportion of T regulatory cells in the BAL or lung compared to T_{reg} cells in WT. This suggests that Itk in not required for these cells to be recruited to the lung in response to SR during the development HP. Our data suggest that Itk was not important in these T regulatory cells during HP. Itk has been shown to play a role in iNKT cell development (Au-Yeung and Fowell, 2007; Felices and Berg, 2008; Gadue and Stein, 2002; Huang et al., 2014a; Qi et al., 2012; Qi et al., 2011b). We also investigated the role of Itk in the function of iNKT cells during SR induced HP. However, we found that the lack of Itk did not alter the proportion of iNKT cells in the BAL or lung of mice during HP. Interestingly, the numbers of iNKT cells were significantly less in $Itk^{-/-}$ mice but the proportion that expressed IL-17A remained the same. This would suggest that Itk is important for the number of iNKT cells during HP, but not their production of IL-17A.

Our laboratory has shown a distinct population of innate memory phenotype (IMP) cells in $Itk^{-/-}$ mice that have the capacity to rapidly secrete cytokine upon activation. (Berg et al., 2005; Huang et al., 2014a; Huang et al., 2014c; Prince et al., 2014). The development of these IMP are suggested to be a result of elevated levels of IL-4 cytokine in the thymic environment of $Itk^{-/-}$ mice (Huang et al., 2014a; Huang et al., 2014c). Due to the fact that these IMP cells may be impacting the response in the $Itk^{-/-}$ mice, we examine whether eliminating these IMP cells affects IL-17A driven inflammatory model of HP. Considering these cells are dependent on IL-4 for development, we developed IL-4R$\alpha^{-/-}$ and $Itk^{-/-}$/IL-4R$\alpha^{-/-}$ to remove IMP cells.

The loss of IL-4Rα signaling in WT and Itk$^{-/-}$ mice did not alter the inflammation seen during HP. This data would suggest that IL-4Rα signaling or IMP cells are not required for the immune response seen in SR induced HP. However, the loss of IL-4Rα signaling in WT and Itk$^{-/-}$ mice led to a decrease in the proportion of CD4^{+} T cells in BAL during HP compared to mice with intact IL-4Rα signaling. Moreover, Itk$^{-/-}$ mice lacking IL-4Rα signaling exhibited a decrease in the proportion of CD4^{+} T cells in the lung, which suggest a potentially important role for IL-4Rα signaling (or IMP cells) in the HP response to SR in the lung. These data suggest IL-4Rα signaling/IMP cells are important in IL-17A expression in CD4^{+} T cells during HP. We also determined that the loss of IL-4Rα led to marginal decrease of IL-17A expression in T cells in the BAL, which would suggest IL-4Rα signaling/IMP cells play a minor role in the BAL T cells. We determined that there were decreased proportions of CD4^{+} T cells making IL-17A cytokine in the lung during HP in the absence of IL-4Rα signaling. This suggests that IL-4Rα signaling exhibits differential response and requirements for IL-17A production in BAL and lungs. Collectively the data suggest an important role for IL-4Rα signaling or IMP cells in the CD4^{+} IL-17A expression in WT and Itk$^{-/-}$ mice during HP.

Inducible Th17 cells (iTh17) are the most commonly studied group of IL-17A producing cells types that differentiate from naïve CD4^{+} T cells in response to antigen stimulation and cytokines. Studies have identified CD4^{+} T cell subset with innate-like functions that immediately produce IL-17A known as natural Th17 cells (nTh17) (Conti et al., 2009). Itk has been shown by several groups to be important for the development of iTh17 cells, but our data has suggested that Itk is not important in the Th17 response in HP. We investigated these cells and their possible contribution to in HP by inhibiting

with Rapamycin, which blocks development of iTh17 cells but not nTh17. Rapamycin treatment during HP reduces the proportion of T cells in lungs (but not BAL). αβ T cells in both WT and $Itk^{-/-}$ backgrounds were significantly reduced after Rapamycin treatment, and potential block of iTh17 cells. The ability of $CD4^+$ T cells to produce IL-17A cytokine was also severely inhibited after Rapamycin treatment, suggesting that iTh17 may be responsible for the primary response of $CD4^+$ T cells in HP and not nTh17 cells. Additionally, there were an increase proportion of T_{reg} cells in the lung, while the number of cells decreased during HP. We suggest a possible important role of Itk and TCR signaling in the development of nTh17 cells.

Given the proposed role of Itk in the production of IL-17A by Th17 cells, we probed its role in the Th17 driven lung inflammatory model, HP. Our experiments suggest a model for the Th17 response to SR (**Fig. 8**). SR activates a strong Th17 response resulting in high levels of IL-17A cytokine expression in the lungs (**Fig. 8**). The absence of Itk does not affect IL-17A production in HP by conventional Th17 cells. Additionally, γδ T cells also produce IL-17A in response to SR in vivo during the development of inflammation. $CD4^-$ γδ were identified as the major source of cytokine, whereas Itk regulated this production (**Fig. 8**). Our results suggest that Itk regulation of IL-17A production is complex and is dependent on T cell type.

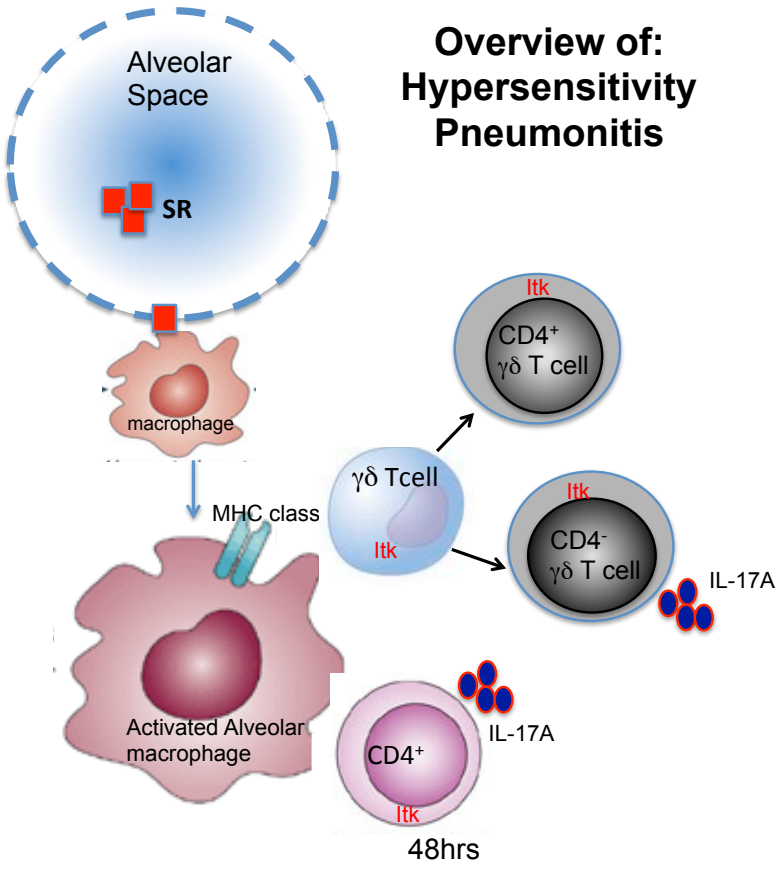

Fig. 8: SR is inhaled into the airways of the lungs and processed by alveolar macrophages. Within the first 24 hours of exposure, neutrophils are recruited. Within 48 hours of exposure, initiation of the cell-mediated pathway occurs. Antigen is presented to $CD4^+$ T cells after activation of alveolar macrophages. $CD4^+$ Th17 cells make IL-17A cytokine in response to activation. In the absence of Itk these cells are still able to make IL-17A. γδ T cells are also a source of IL-17A, and in the absence of Itk $CD4^+$ γδ T cells do not make IL-17A but $CD4^-$ γδ T cells are able to make IL-17A.

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