REGULATION OF IMMUNE RESPONSES DURING AIRWAY INFLAMMATION

By

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To my father

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INTRODUCTION

Overview

The goal of this introductory chapter is to provide background information on concepts relevant to the original scientific data presented in this manuscript. As such, the following text will first describe the scope, current treatments, and immunologic mechanisms of airway inflammation studied in this manuscript, including allergic airway inflammation, bacterial infection and asthma, and respiratory syncytial virus (RSV) infection. Subsequent sections will provide an overview of the signaling pathways, biology, and role in disease of immune modulators that are the therapeutic targets for inflammatory airway disease in this work. The final section will highlight what this work contributes to our knowledge of the regulation of immune responses during airway inflammation.

Scope, current treatments, and immunologic mechanisms of airway inflammation

Allergic airway inflammation

In the last several decades, the prevalence of allergic rhinitis, asthma, and atopic eczema increased markedly in developed countries.[1,2] Allergic disease has become one of the most common causes of chronic illness and affects 300 million people worldwide, including 40-50 million Americans.[1] Epidemiologic investigations reveal that roughly a quarter of the U.S. population suffers from allergic diseases.[3] Similar trends have begun

to emerge in developing countries that have witnessed progressive westernization.[4,5] In particular, the Center for Disease Control and Prevention reported that among persons of all ages, the prevalence of asthma increased by 12.3% from 2001 to 2009, with 25 million Americans being affected with this disease.[6] Direct costs from asthma are approximately $50.1 billion and the indirect costs resulting from lost earnings due to illness or death are roughly $5.9 billion.[7] In children ages 5 to 17, asthma is one of the leading causes of school absences and accounts for an annual loss of 10.5 million school days.[7]

The most common form of asthma is allergic asthma which is characterized by increased eosinophilic infiltration, IgE production, and the production of $CD4^+$ Th2 cytokines, such as IL-4, IL-5, and IL-13. Initiation of the allergic immune response starts with immune recognition of common environmental antigens by specialized antigen-presenting cells (APCs) such as DCs, macrophages, and B lymphocytes among several other cells types (**Figure 1.1**).[8,9] Antigens processed by APCs through the endocytic pathway are presented as 8-10 amino acid epitopes in MHC class II molecules to $CD4^+$ T lymphocytes. $CD4^+$ T lymphocytes are generally divided into subsets including Th1, Th2, Th17, and induced-T regulatory cells (iTregs). $CD4^+$ T cell subsets are characterized by the cytokine array produced by the cell (**Figure 1.2**). Both Th1 and Th2 lymphocytes cooperate in generating the allergic phenotype.[10] Th1 cells are important in the immune response to intracellular pathogens such as viruses and mycobacteria and produce IFN-γ, lymphotoxin, and IL-2.[11] Th2 cells are differentiated in the presence of IL-4 produced by cells including basophils and mast cells, and Th2 lymphocytes cooperate in generating the allergic phenotype. Once differentiated, Th2 cells produce

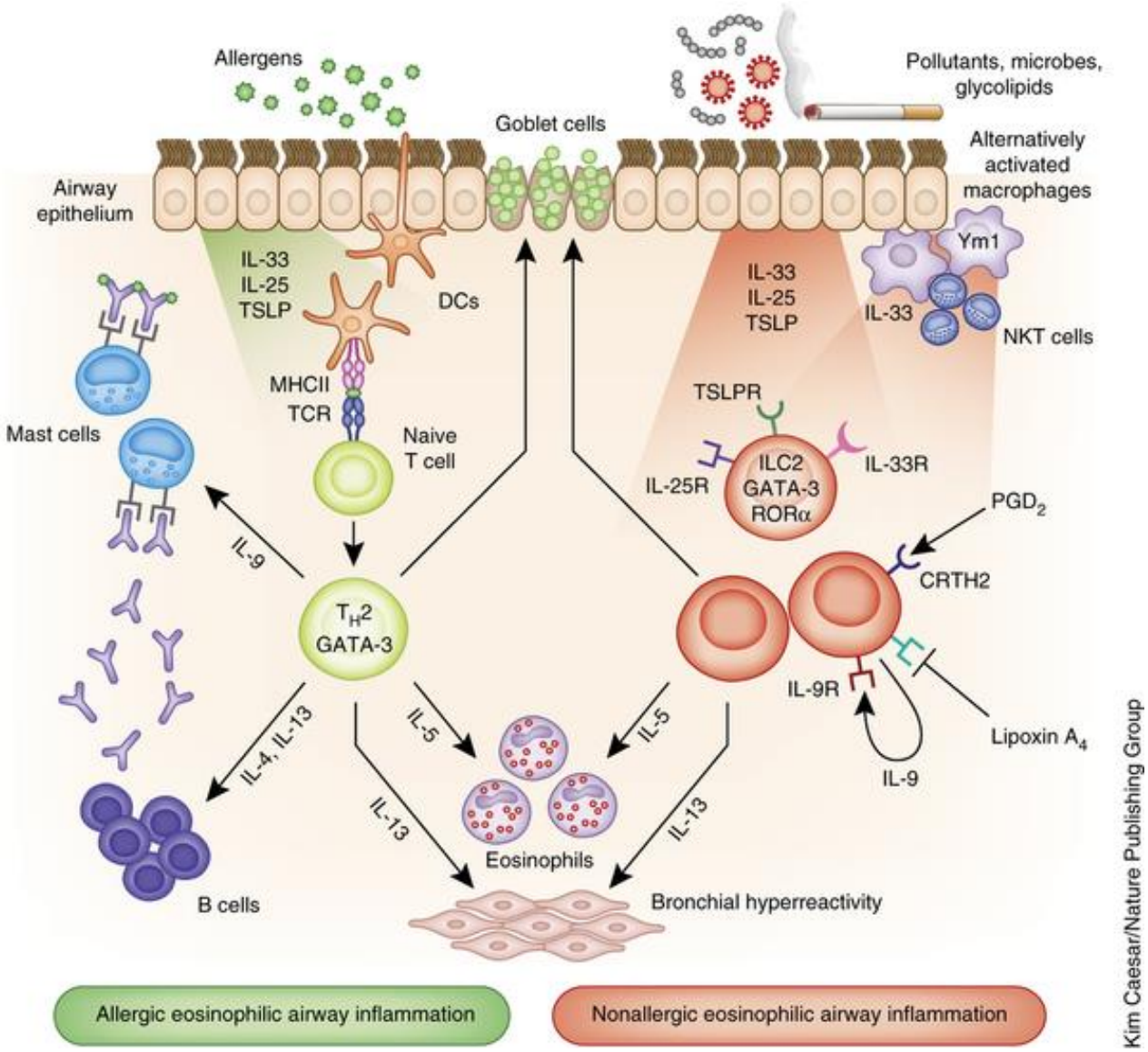

Figure 1.1. The immunology of asthma. Adaptive Th2 cells are stimulated by DCs to produce IL-5, IL-13, and IL-4, resulting in eosinophilic airway inflammation and AHR. ILC2 produce IL-5 and IL-13 which leads to eosinophilia and AHR. Adapted from Lambrecht BN and Hammad H, *Nat Immunol*, 2015.[12]

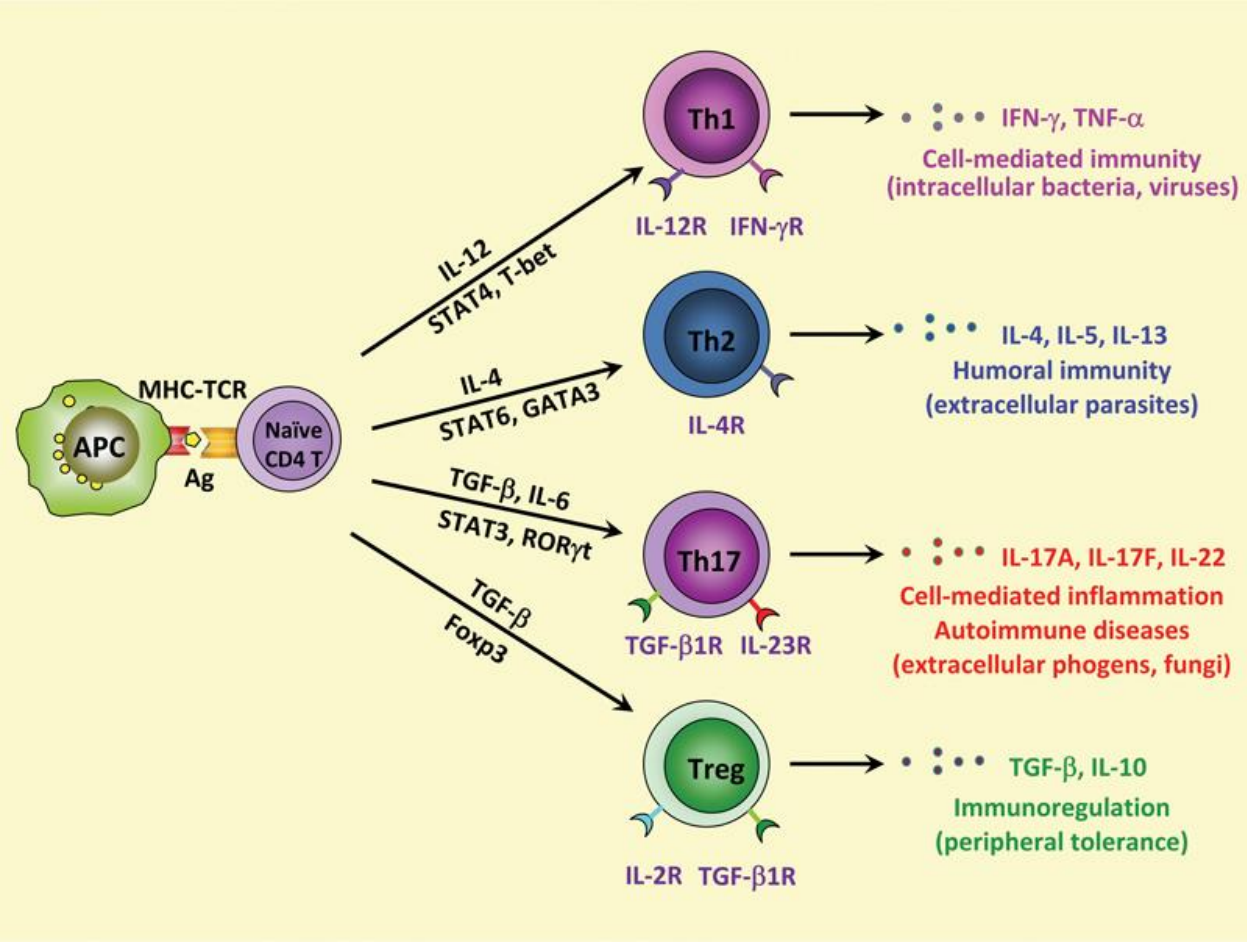

Figure 1.2. Th differentiation from naïve CD4+ T cells. Naïve CD4+ T cells differentiate into Th1 cells in the presence of IL-12, Th2 cells in the presence of IL-4, Th17 cells in the presence of TGF-β and IL-6, and Tregs in the presence of TGF-β. Adapted from Leung S et al. *Cell Mol Immunol*, 2010.[13]

IL-4, an important factor for B lymphocytes to switch antibody production to the IgE isotype.[11] Th2 cells additionally make a variety of other pro-allergic inflammatory cytokines such as IL-5 and IL-13. IL-5 is the most important cytokine in eosinophil growth, differentiation, and survival, and eosinophilic inflammation is one of the hallmarks of the allergic inflammatory response in the airway.[14] IL-13 is a central mediator in airway hyperreactivity (AHR) and mucus expression.[11,14] The allergic response to an aerosolized antigen is characterized by antigen-specific IgE production by B lymphocytes, whereupon IgE can bind to IgE receptors on tissue mast cells and peripheral blood basophils. When these antigen-specific IgE molecules bound to mast cells and basophils are cross-linked by the specific antigen on antigenic re-exposure, the mast cells and basophils undergo a degranulation process. With degranulation, preformed mediators within the mast cell and basophil such as histamine and tryptase are released.[14]

Group 2 innate lymphoid cells (ILC2) are early, important mediators of airway responsiveness, eosinophilia, and mucus production.[15–19] ILC2 are temporally and functionally distinct from Th2 cells. Lung ILC2 are lineage negative (Lin^-) in that they do not express CD3ε, CD19, CD11b, Gr-1, NK1.1, or Ter119.[15] There is some controversy about the surface expression of a variety of proteins including SCA-1, c-kit, ICOS, CD45, and ST2, largely because different groups have used different combinations of these molecules to phenotype ILC2.[15,16,19,20] However, there seems to be complete consensus that ILC2 have surface expression of CD25 (IL-2Rα) and CD127 (IL-7Rα).[15,16,19,20] Lung resident ILC2 express high levels of IL-5 and IL-13, but minimal IL-4.[20] This is in contrast to mature effector Th2 cells that express all three cytokines.

Comparatively, early reports suggest that ILC2 are significantly more potent than Th2 cells, elaborating greater than 10 times the amount of Th2 cytokines on a per cell basis.[20] ILC2 are critical for generating type 2 inflammation because they recruit dendritic cells into the draining lymph node to promote Th2 cell differentiation locally in the airway wall.[21] ILC2 also help establish the adaptive type 2 response by expressing MHC and costimulatory signals that trigger type 2 cytokine production.[22] $CD4^+$ Th2 cell and basophils concomitantly enhance ILC2 responses by promoting ILC2 proliferation and cytokine production via IL-2 and IL-4 production respectively.[22,23] Unlike T and B cells, ILC2 do not have specific antigen receptors. Rather, ILC2 act primarily as early innate effector cells that respond directly to cytokine stimulation.[24–26] Specifically, the epithelial-associated cytokines IL-25, IL-33, and TSLP induce ILC2 proliferation and activation.[15,24–26]

IL-33 is constitutively expressed in the nucleus of epithelial and endothelial cells, and is released upon cell injury as an alarmin.[27] As a result of being expressed by cells that interface with the environment, IL-33 functions as an early initiator of inflammation. In addition, IL-33 expression is stimulated in inflamed tissues and can further amplify inflammatory responses. It is a member of the IL-1 family and extracellular IL-33 binds to its receptors, ST2 and IL-1 Receptor Accessory Protein (IL1RAP), inducing NF-κB and MAPK activation. IL-33 activates cells that produce type 2 cytokines including ILC2, Th2 cells, and basophils to secrete IL-13. IL-33 additionally potentiates type 2 immune responses by polarizes naïve $CD4^+$ T cells to differentiate into type 2 cytokine-producing effector Th2 cells.[28] As a central mediator of both innate and adaptive immunity-

regulated lung inflammation, IL-33 has been identified as an important therapeutic target in inhibiting airway disease.[29]

In many patients, allergic asthma can be controlled by a combination of an inhaled corticosteroid (which acts to suppress the inflammation) and a short- or long-acting β_2-adrenergic agonist (which acts to relax the constricting bronchial smooth muscle). However, in 5–10% of patients, allergic asthma is refractory to corticosteroid treatment and often leads to hospital admissions caused by respiratory viral and/ or bacterial infections. Treatment with an antibody to the α-chain of the receptor for IL-4 (dupilumab; Regeneron Pharmaceuticals), which blocks downstream signaling via the receptors for IL-4 and IL-13, improves lung function and reduces the frequency of exacerbation in people with moderate to severe asthma with high levels of eosinophils in the blood.[30] In a phase 2 trial, blocking IL-13 with lebrikizumab (Genentech) also improved lung-function parameters, particularly in patients with the $Th2^{hi}$ endotype of asthma.[31] Eliminating eosinophils through the use of an antibody to IL-5 (mepolizumab; GlaxoSmithKline) led to a reduction in exacerbation frequency in a subset of patients with high levels of circulating blood eosinophils and frequent exacerbations, even in those receiving inhaled steroids.[32] As exacerbations often require systemic steroids that have serious side effects, blocking IL-5 can therefore reduce the use of oral steroids. In people with asthma in whom IL-5 was blocked, there was also a significant reduction in the deposition of extracellular matrix components tenascin, lumican and procollagen III, possibly leading to less airway remodeling.[33] A new drug that targets eosinophils and is currently under phase 3 clinical development is an antibody to the receptor for IL-5 (benralizumab; AstraZeneca/MedImmune), which effects depletion of eosinophils for

months after a single injection.[34] The drug omalizumab (Xolair; Genentech/Novartis) is a humanized monoclonal antibody that binds to the constant domain of IgE, inhibiting its interaction with the high-affinity receptor FcεRI, and has proven efficacy in a subset of patients. However, omalizumab must be administered for a few weeks before showing clinical effects in patients, suggesting that omalizumab does not work by inhibiting the early degranulation of mast cells or basophils. Prolonged treatment with omalizumab also results in a reduction in Th2 cytokine production in lung tissues, suggesting that it affects the generation of effector Th2 responses.[35]

IL-17A levels and IL-17A-producing cells are increased in the sputum of asthmatic patients with bronchial reactivity.[36,37] IL-17A expression was also significantly greater in bronchial biopsy specimens in the airways of subjects with moderate-to-severe asthma compared to subjects with mild asthma or controls.[38] There are six family members of IL-17, designated IL-17A-F. IL-17F is produced by the same cells that produce IL-17A and also signals through the IL-17RA/ IL-17RC heterodimeric complex; therefore, IL-17F has very similar functions as IL-17A.[39] The first described IL-17 receptor was designated IL-17R and primarily binds IL-17A.[40] Surprisingly, a clinical trial with a antibody that neutralizes the receptor for IL-17 (brodalumab; Amgen/AstraZeneca), blocking the activity of IL-17A, IL-17F and IL-25, has shown only minimal effects on outcome measures of asthma in mild to moderate disease.[41] It is possible that subgroups of patients, particularly those with large numbers of sputum neutrophils or with a high degree of lung function reversibility, would respond more favorably.[42]

While allergic diseases including asthma have risen considerably in prevalence in the last 50 years, a concomitant rise in autoimmune disease suggests a defect in immunoregulation, rather than a reduction in Th1 immunity, in the growing prevalence of both diseases. Moreover, people without allergic diseases have antigen-specific immune tolerance to common aeroallergens. Some studies support the possibility that Tregs suppress allergic diseases and contribute to allergen-specific immune tolerance.[43,44] Tregs are a subtype of $CD4^+$ T cells that are critical for the maintenance of self-tolerance and tolerance to foreign antigens by inhibiting effector T cell responses.[45] Tregs constitute 5-10% of all $CD4^+$ T cells. Natural Tregs (nTregs) differentiate in the thymus before their exit into the peripheral circulation. Inducible Tregs (iTregs) are generated following exposure to cognate antigen in the context of transforming growth factor (TGF)-β and interleukin (IL)-2. The establishment and maintenance of tolerance induced by repeated airway exposures to low-dose ovalbumin (OVA) were dependent on Treg cells that express both cell surface and soluble TGF-β.[44] Therefore, Treg instability and/ or defective Th2 regulation may be mechanisms by which immune tolerance prevents the immune system from responding to innocuous environmental antigens.[46–52]

Bacterial infection and asthma

Patients with asthma are at increased risk for bacterial infections and bacterial pneumonia.[53–57] For instance, in a population of Tennessee Medicaid enrollees, asthma patients had an almost four-fold increased risk of invasive pneumococcal disease compared to nonasthmatics, even when subjects were controlled for medication usage

such as inhaled or systemic corticosteroids.[55] Furthermore, mouse studies have shown that allergic airway inflammation impairs host defense against *Streptococcus pneumoniae* as well as *Pseudomonas aeruginosa*.[58,59]

Klebsiella pneumonia (KP) is a Gram-negative pathogen that causes pneumonia and invasive disease in healthy and immunocompromised adults and children.[60–63] The continued emergence and spread of multidrug-resistant strains of *KP* highlight the importance of developing innovative therapeutic approaches to these infections. Both IL-17A and IL-17F were produced as result of *KP* lung infection. Moreover, mice deficient for IL-17R had greater bacteremia and increased mortality to *KP* infection compared to wild type (WT) mice.[64] Additional studies have similarly found that IL-17A is critical for the clearance of *KP* from the lung and recovery of the host from infection in mouse models.[65–67] These findings were likely related to decreased chemotaxis of neutrophils into the bronchoalveolar compartments as a consequence of a blunted production of granulocytes in the spleen, secondary to inhibited G-CSF expression.[66] The importance of IL-17A for general pulmonary host defense was demonstrated in a study showing that anti-IL-17A antibody treatment decreased endotoxin-induced neutrophil migration into the lung.[68]

γδ17 cells constitute a unique γδ T cell subset that produces IL-17A, IL-17F, and IL-22, and are important in innate antibacterial immunity.[69] γδ17 cells reside at the interface of epithelial-environmental interfaces such as the respiratory tract. Strong early γδ17 cell responses are generated against diverse pathogens including *KP*, *Mycobacterium tuberculosis, Mycobacterium bovis, Staphylococcus aureus, Listeria monocytogenes,* and *Escherichia coli.* γδ T cells responses are critical for immunity

against these pathogens.[70–75] Following acute infection with *KP*, IL-17A is rapidly expressed within 2 days by γδ T cells and mice deficient in γδ T cells succumb to *KP* infection.[70,76,77] IL-17A stimulates neutrophil recruitment, production of antibacterial peptides, and maintenance of epithelial barriers.[78] In contrast to naïve $CD4^+$ T cells that become Th17 cells in the periphery in the context of an immune response, γδ17 cells are preprogrammed in the thymus to produce IL-17A.[79] In the thymus, lack of T cell receptor (TCR) engagement or weak TCR signaling programs developing γδ T cells to secrete IL-17A once they enter the periphery. Alternatively, strong TCR engagement biases the cell towards an IFN-γ-secreting disposition.[79] *In vitro,* treating γδ T cells with IL-1β and IL-23 optimally induces IL-17A production without the need for TCR engagement, since γδ17 cells constitutively express the receptors for IL-1 (IL-1R1) and IL-23 (IL-23R, **Figure 1.3**).[80] The constitutive expression of IL-1R1 and IL-23R provides an efficient mechanism to induce γδ T cell production of IL-17A.[81] The development of γδ17 cells is controlled by transcription factors RORγT and AhR.[69,82] γδ T cells that exit the thymus without going through TCR engagement do not repress RORγT expression and preferentially develop into γδ17 cells. This differs from Th17 cells, which repress RORγT function and require IL-6 and TGF-β to induce the re-expression of RORγT for their differentiation.[82–84]

RSV infection

RSV is the most common cause of severe bronchiolitis in infants in the United States and throughout the world.[85] Several long-term epidemiologic studies reveal that severe RSV bronchiolitis in infancy is associated with recurrent wheeze and asthma

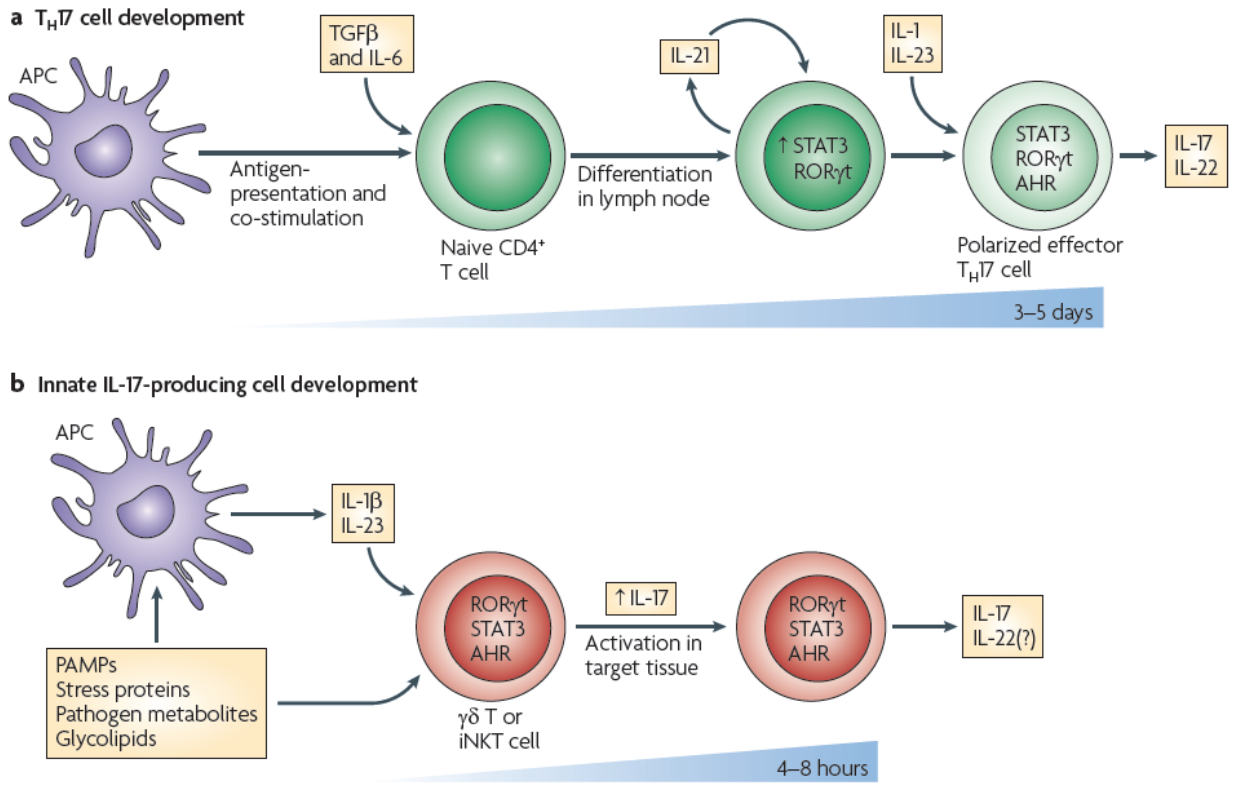

Figure 1.3. γδ17 cells are innate, potent producers of IL-17. Adaptive Th17 cell development and polarization takes 3-5 days. γδ17 reside in peripheral tissues and can produce high levels of IL-17 within hours of stimulation. Adapted from Cua D and Tato CM, *Nat Rev Immunol* 2010.[81]

during childhood. In the prospective Tuscon Children's Respiratory Study of over 1,200 infants enrolled from 1980-1984, RSV lower respiratory tract illness in early childhood was an independent risk factor for the subsequent development of wheezing up to 11 years of age.[86] In a case-control study of Swedish infants born in the winter of 1989-1990, RSV bronchiolitis which was so severe as to lead to hospitalization resulted in a greater than four-fold (39% vs 9%) increased prevalence of asthma at age 18 compared to control subjects.[87] In a population-based retrospective birth cohort study of term healthy infants born between 1995 and 2000 and enrolled in the Tennessee Medicaid program, a significant dose-response relationship was demonstrated between the severity of infant bronchiolitis and the increased odds of both early childhood asthma and asthma-specific morbidity between the ages of 4 and 5½ years of age.[88] Among the 90,341 children examined, 18% had an infant health care visit or hospital admission for bronchiolitis, and these infants contributed to 31% of early childhood asthma diagnoses. These studies demonstrate the very strong association between severe RSV bronchiolitis and the inception of childhood asthma in geographically, economically, and temporally diverse populations.

Conversely, there appears to be synergism between viruses and aeroallergens in the risk of hospital admission for asthma exacerbation.[89] Allergic disease is the strongest identifiable predisposing factor for the development of asthma, and exposure to allergens can cause acute asthma symptoms.[90] Comparatively, viruses were detected in approximately 80% of children with asthma exacerbations,[91] and in over half of adults with worsening asthma symptoms.[92] Although RSV has long been recognized as the most common infectious agent that caused wheezing in infants from

bronchiolitis,[93] more recently this virus has been identified as an important cause of asthma exacerbations in both older children and adults as well.[94,95] For instance, RSV was detected in over 20% of children aged 1-3 years that were hospitalized with wheezing, and in approximately 10% of children 3-16 years old that were admitted with asthma exacerbations.[95] In persons over 65 years of age, RSV was detected in 7% of patients admitted to the hospital diagnosed with an asthma exacerbation.[94]

Well-timed, appropriately located, and stringently regulated host immune responses are essential for protection from RSV infection and limiting symptoms, while poorly regulated inflammation contributes to tissue damage and disease (**Figure 1.4**). The responsiveness of the airway epithelium to the initial stages of RSV infection is critical to the subsequent development of adaptive immunity. RSV infects the respiratory epithelium, which can recognize pathogen-associated molecular patterns (PAMPs) from the virus via pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and intracellular sensors including RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors.[96] Ligation of these receptors leads to type I and III interferon upregulation. Type I and III interferons subsequently trigger the inflammatory response, contribute to the differentiation of adaptive immune cells, and promote an antiviral state locally. Inflammatory mediators recruit innate cells, including neutrophils and natural killer (NK) cells. NK cells can kill RSV-infected cells via perforin-granzyme-dependent mechanisms and the type II transmembrane protein cytokine FasL via its receptor Fas (CD95). NK cells also produce interferon-γ (IFN-γ) as do Th1 cells, which potentiates the polarization of recruited T cells to an antiviral, cytolytic type 1 response which is required for viral clearance.[97,98] Following engagement of the T cell

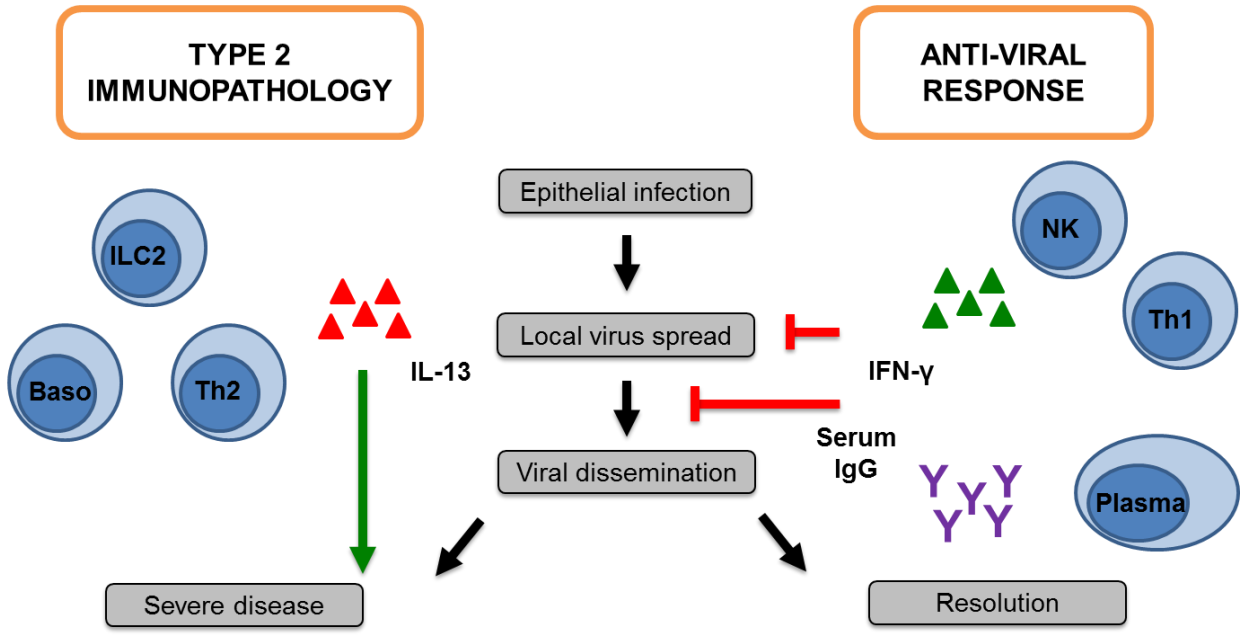

Figure 1.4. The host immune response during RSV infection. Anti-viral IFN and IgG production lead to disease resolution while poorly regulated immune responses can lead to damaging immunopathology. Adapted from Chiu C and Openshaw P, *Nat Immunol* 2015.[99]

antigen receptor and appropriate costimulatory signals, $CD8^+$ T cells acquire cytolytic activity and upregulate chemokine receptors that allow them to migrate to inflamed sites, where they detect RSV-infected cells by binding to viral peptides presented in the context of major histocompatibility complex class I (MHC I). $CD4^+$ T cells recognize viral peptides presented by MHC II-bearing cells and differentiate, depending on the environmental context and strength of interaction, into helper T cell subsets. Following antigen recognition, B cells differentiate with the help of cognate $CD4^+$ Tfh cells to form antibody-secreting plasma cells. Class switching to IgG is followed by the secretion of large amounts of high-affinity antibody that can directly neutralize RSV, block entry of virus into the cell, fix complement, promote phagocytosis, and allow antibody-dependent cellular cytotoxicity. However, pathogenic viral mediators can dysregulate host immune responses during infection. For example, RSV NS1 and NS2 proteins suppress the induction and signaling of type I interferons, DC maturation, and T lymphocyte activation; the NS proteins also inhibit apoptosis, prolonging the life of infected cells and increasing viral yields.[100] In addition, RSV G protein promotes inappropriate inflammatory responses via CX3C chemokine mimicry.[101] Reduced viral control by defective host immunity and over activation of the type 2 host response lead to severe RSV infection. The type 2 response mediates airway mucus production, which directly contributes to the airway obstruction and respiratory failure.[102]

Many studies have examined genetic and environmental factors that modulate severity of illness resulting from RSV infection in human infants. Proposed risk factors for severe RSV bronchiolitis include age less than one year of age, pre-existing bronchopulmonary dysplasia, congenital heart disease, prematurity, age < 3 months at

the start of the RSV season, co-habitation with older siblings, maternal smoking, and abbreviated breast feeding.[103–105] Host genetic factors have also been identified to predispose for severe RSV bronchiolitis and include polymorphisms in the genes for IL-10, CCR5, TGFB1, IL-13, IL-4, and IL-4 receptor.[106] Polymorphisms in the toll like receptor (TLR) 4 gene also have been associated with differential severity of illness.[107] RSV strain differences are a determinant of disease phenotypes and severity. Very different antigenic subgroup A viruses co-circulate each year, and the response to infection with different viral strains is fundamentally different in genetically identical mice.[108] One of the major criticisms about the mouse model of RSV infection is that it does not represent the pathologic features seem in humans with high fidelity, namely that airway obstruction resulting from mucus production present in humans is not present in the mouse. Therefore, it is critical to use clinical strains that recapitulate key features of RSV disease in humans when studying RSV pathology in mice.

Unfortunately, there are no interventions that significantly improve clinical outcomes for RSV bronchiolitis once infection has occurred. Randomized, prospective clinical trials of the anti-viral ribavirin in infant RSV bronchiolitis have yielded disappointing results,[109,110] while studies examining the effect of corticosteroid and bronchodilator therapy have routinely resulted in no improvement compared to placebo.[111–113] Prophylactic administration of the anti-RSV monoclonal antibody palivizumab to high-risk populations, such as premature infants and those with underlying cardiopulmonary disease, significantly reduced hospitalizations.[114,115] Two studies suggest that prevention of severe RSV bronchiolitis with prophylactic administration of RSV immune globulin may impact the subsequent development of

asthma. A prospective multicenter, matched, double cohort study of premature infants less than 36 weeks gestation conducted in 27 centers in Europe and Canada revealed that nonatopic subjects who received palivizumab in the first year of life had an 80% decrease in the relative risk of recurrent wheezing through ages 2 to 5 compared to those of gestational age-matched controls.[116] These results not only showed a protective effect of an anti-RSV monoclonal antibody in the later development of wheezing, but also suggested that RSV predisposes to recurrent wheezing in an atopy-independent mechanism. Another study also suggests that preventing RSV bronchiolitis significantly diminishes the likelihood of asthma exacerbations and improves pulmonary function later in childhood. In a retrospective case-control study of children at high risk of respiratory disease, prophylaxis with RSV immune globulin during infancy significantly reduced the risk of an asthma attack in the year prior to the children being studied at age 7 to 10 years of age.[117] In addition, there was a significant improvement in the ratio of forced expiratory volume in 1 second to forced viral capacity (FEV_1/FVC), a measure of airway obstruction.[117] These studies suggest that preventing severe RSV bronchiolitis may reduce the incidence of asthma in childhood. Two recent trials of antiviral RSV therapies have shown early results of more rapid viral clearance and partial attenuation of disease severity.[118,119] These promising results highlight the importance of developing anti-inflammatory therapies that can be used in combination with anti-virals to achieve complete disease abrogation.

Pathways of immune modulators of inflammation

STAT6 signaling

The Th2 cytokines IL-4 and IL-13 utilize the STAT6 signaling pathway. IL-4 is the most important cytokine in Th2 differentiation and inhibits Th1 and Th17 differentiation. IL-4 suppression of Th1 development is mediated by GATA-3; this transcription factor is induced by IL-4 and also acts as the master regulator of Th2 differentiation.[120] IL-4 inhibits Th1 differentiation by decreasing IL-12R expression.[121] IL-4, as well as IL-13, induces goblet cell metaplasia and mucus expression, alternative macrophage activation, and airway remodeling.[122] IL-4 can signal through either the type I IL-4R which consists of IL-4Rα and the common γ chain, or the type II IL-4R which consists of IL-4Rα and IL-13Rα (**Figure 1.5**).[123,124] Low concentrations of IL-4 are presumed to signal more effectively through the type II IL-4Rα than do low concentrations of IL-13. IL-4 binds to the IL-4Rα portion of the IL-4 heterodimer receptor with high affinity and causes activation of JAK1 and JAK3 leading to the phosphorylation and dimerization of the transcription factor STAT6 at the Tyr-641 residue.[125,126] STAT6 is then able to translocate into the nucleus and turn on the transcription of STAT6-dependent genes. IL-4 suppression of IL-17A protein expression from Th17 cells is dependent on STAT6.[127–129] IL-4 attenuates IL-17A protein expression by T cells.[127–129]

In addition to dupilumub and lebrikizumab, the IL-4Rα antibody, AMG 317, was recently reported to reduce IgE levels and the number of exacerbations in patients with moderate-to-severe asthma.[130] The rhIL-4 variant, pitrakinra, which competitively inhibits IL-4 or IL-13 binding to IL-4Rα, resulted in a smaller decrease in FEV_1 in patients with mild asthma.[131]

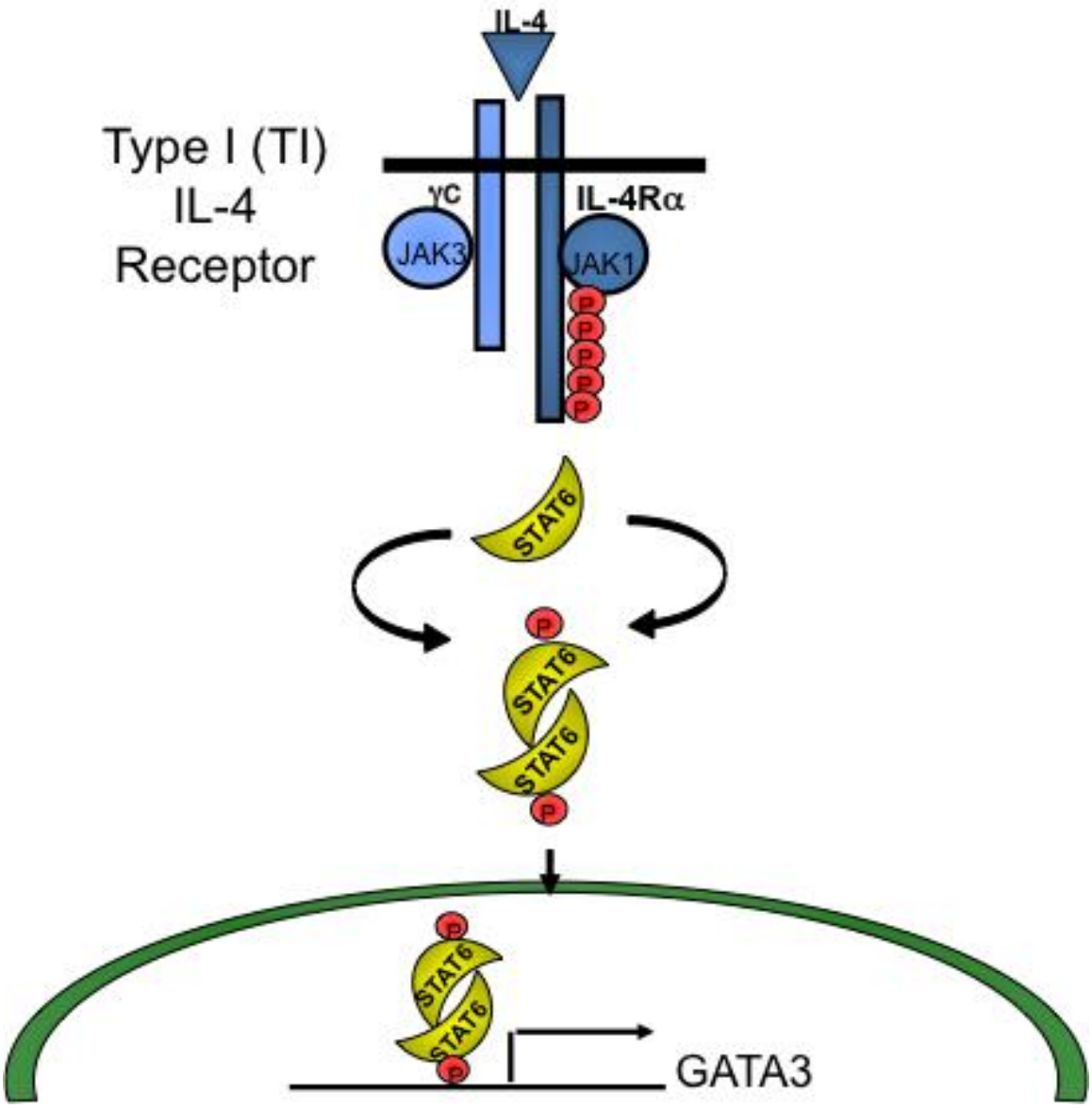

Figure 1.5. STAT6 signaling. IL-4R activation activates JAK1 and JAK3 leading to the phosphorylation and dimerization of STAT6, which then translocates into the nucleus to turn on the transcription of STAT6-dependent genes.

PGI_2 signaling

One of the primary byproducts of acute allergic (IgE-mediated type I hypersensitivity) reactions is the generation of arachidonic acid from inflammatory cell membranes.[132] The cyclooxygenase family of enzymes convert arachidonic acid substrate into prostaglandins and thromboxane. Arachidonic acid can also be metabolized by the enzyme 5-lipoxygenase to produce the leukotrienes.[132] In particular, prostaglandins influence many important inflammatory processes.[132] These mediators are produced by the phospholipase A_2 cleavage of arachidonic acid from membrane phospholipids, followed by the COX conversion of arachidonic acid to prostaglandin H_2 (PGH_2). PGH_2 may then be converted by specific synthases or isomerases to either prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2), prostaglandin E_2 (PGE_2), or thromboxane (**Figure 1.6**).[132] The prostanoids have diverse effects on the inflammatory cascade and are presumed to have modulatory roles in asthma.[133,134] PGD_2 is increased in the bronchoalveolar lavage (BAL) fluid of asthmatic subjects, while both PGD_2 and $PGF_{2\alpha}$ contract human airways smooth muscle *in vitro* and are potent bronchoconstrictors *in vivo*.[135,136] Thromboxane also causes human airway smooth muscle contraction *in vitro*.[137] PGE_2 inhalation inhibits the pulmonary immediate and late phase responses to inhaled allergen.[138,139] Compared to vehicle inhalation, inhaled PGE_2 also decreases the change in methacholine airway reactivity and reduces the number of eosinophils after inhaled allergen challenge.[138] Interestingly, although PGE_2 has significant effects on pulmonary function in challenge models, it has no effect on baseline FEV_1 or methacholine reactivity.[139] The results from these studies suggest that PGE_2 has a greater immunomodulatory effect than direct effect on airway

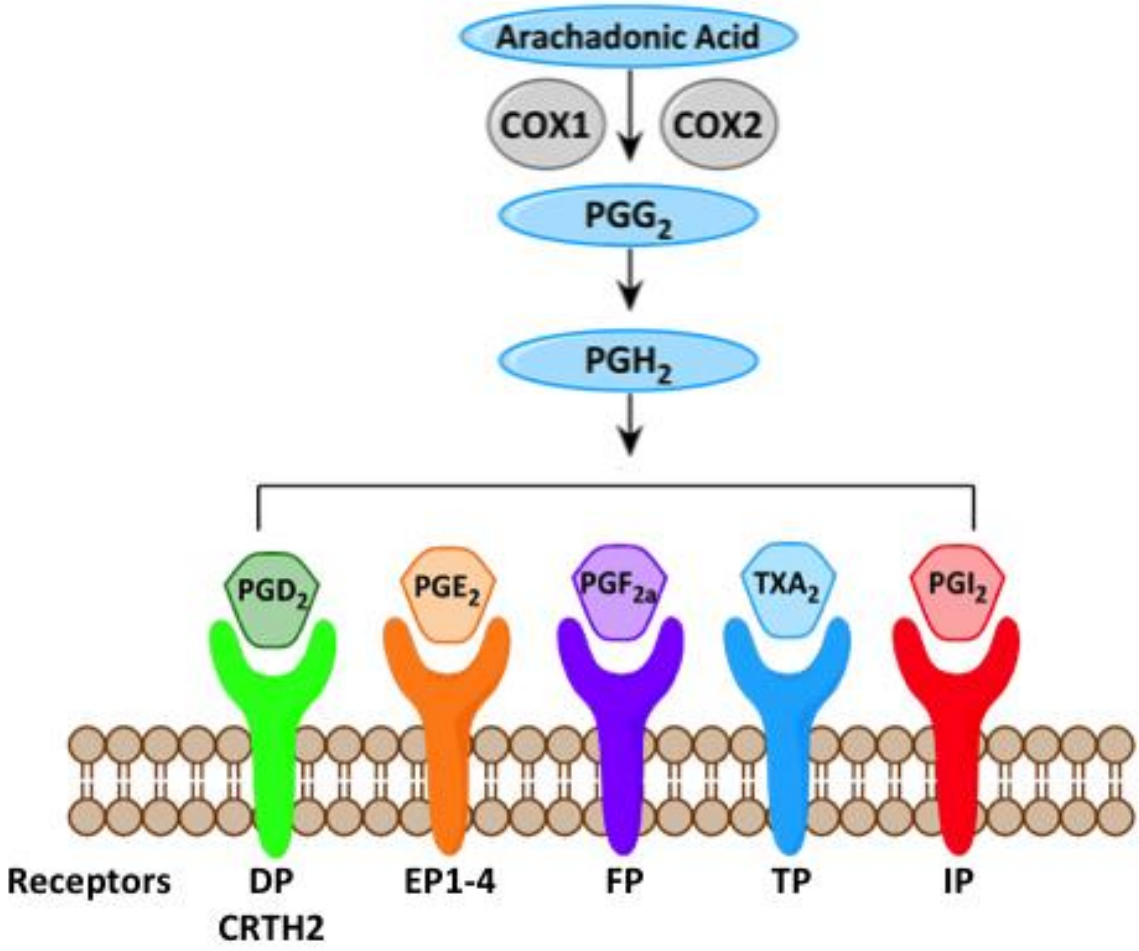

Figure 1.6. PGI_2 production and signaling. PGI_2 is one of 5 primary prostaglandins generated by COX-mediated conversion of arachidonic acid. PGI_2 signals through the seven transmembrane G protein-coupled receptor IP. Adapted from Stitham et al., *Front Pharmacol* 2011.[140]

caliber. This notion is supported by the fact that PGE_2 inhalation prior to segmental allergen challenge significantly reduced the BAL levels of PGD_2, an important product of mast cell degranulation, and the cysteinyl leukotrienes.[141] Thus, these prostanoids have important immunomodulatory effects in allergic lung inflammation and asthma.

PGI_2 is a prominent COX product produced in the lung as a result of antigen challenge. PGI_2 and PGD_2 were the predominant COX products produced in antigen-induced anaphylactic reactions of human lung parenchyma, on the order of 3- to 7-times greater concentrations than of the other prostanoids.[142] The PGI_2 metabolite 6-keto-PGF_1 was produced in concentrations two-to-three-fold higher than all the other prostanoids in both airway and subpleural lung fragments in an *in vitro* anaphylaxis assay of passively sensitized human lung.[143] Plasma 6-keto-PGF_1 was also increased following antigen challenge in which asthmatic subjects were pretreated with indomethacin.[144] Thus, PGI_2 is produced in abundance in allergic inflammatory responses in the lung. For many years, PGI_2 was thought to primarily have effects on vascular tissues as these were the tissues that produced PGI_2 in greatest abundance and were the ones that most highly expressed IP. PGI_2 production is highest in the heart, lung, smooth muscle, kidney, and ovary and is expressed at moderate levels in the brain, pancreas, and prostate.[145] Low level production of PGI_2 has been reported in the placenta, spleen, and leukocytes.[145] One study also reported that PGI_2 was produced by the human alveolar basal epithelial cell line A549.[146] In these experiments, *in vitro* basal level expression of the PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ was approximately 25 ng/10^6 A549 cells and this increased to 75 ng/10^6 A549 cells after culture with IL-1β for 24 hours.[146] Therefore, human alveolar basal epithelial cells produce PGI_2 which

may regulate immune responses in the lung. IP is a member of a family of eight prostanoid receptors that have conserved homology in mammals, including mice and humans, through which PGI_2 and PGI_2 analogs signal.[147] All of the prostanoid receptors are encoded on different genes. IP is a G protein-coupled rhodopsin-type receptor that has seven transmembrane domains. Binding of PGI_2 to its receptor activates adenylate cyclase via G_s in a dose-dependent manner, increasing the production of cyclic adenosine monophosphate (cAMP).[148] This increase in intracellular cAMP mediates PGI_2's effect of inhibiting platelet aggregation, and dispersing existing platelet aggregates both *in vitro* and in human circulation.[148] Northern blot analysis reveals that IP mRNA is expressed to the greatest degree in the thymus, while high level of IP mRNA expression is also found in spleen, heart, lung, and neurones in the dorsal root ganglia. IP is also expressed in T cells of mice, along with the PGE_2 receptor (EP) subtypes and the thromboxane receptor (TP).[149] IP has also been found in kidney smooth muscle and epithelial cells.[150]

The Food and Drug Administration (FDA) has approved epoprostenol (the sodium salt of PGI_2), treprostinil, and iloprost for treatment of primary pulmonary hypertension on the basis of their vasodilatory effects. Epoprostenol has also been used safely and effectively in patients with other causes of pulmonary hypertension, including scleroderma, systemic lupus erythematosus, congenital heart disease, HIV, and Gaucher's disease.[151] Analogs, such as treprostinil, cicaprost, and iloprost, are used because the half-life of PGI_2 is extremely short in circulation (3-5 minutes). Epoprostenol is administered intravenously and is converted quickly to stable metabolites through rapid hydrolysis in the circulation and extensive metabolism in the liver.[152] Treprostinil

can be administered either intravenously or directly to the lungs by inhaler, and iloprost is inhaled. Treprostinil has similar physiologic properties to epoprostenol, but has the advantage in that the half-life is significantly longer at three hours. Inhaled treprostinil, administered four times a day, has recently been shown to be effective in the treatment of severe pulmonary hypertension.[153] Iloprost has a half-life of approximately 20 minutes and because of its short duration of action it must be inhaled 6-9 times per day. Therefore, newer PGI_2 analogs offer a significant pharmacologic, and potentially therapeutic, advantage over epoprostenol.

PGI_2 modulates the intersection of innate and adaptive immunity. The PGI_2 analogs iloprost, cicaprost, and treprostinil differentially modulated activation-induced responses of murine bone marrow-derived dendritic cells (BMDC) in an IP-dependent manner.[154] These PGI_2 analogs decreased activated-induced BMDC production of the proinflammatory cytokines IL-12, TNF-α, IL-1α, and IL-6, while also inhibiting the expression of chemokines known to be important in recruitment of inflammatory cells to the lung, including MIP-1α and MCP-1.[154] While reducing these pro-inflammatory cytokines and chemokines, PGI_2 analogs increased the production of the anti-inflammatory cytokine IL-10 by BMDCs, an important finding since DC production of IL-10 has been shown to promote the induction of CD4+ CD25+ Foxp3+ T regulatory cells (Tregs).[155] The modulatory effect of PGI_2 analogs on BMDCs was associated with IP-dependent upregulation of intracellular cAMP and down-regulation of NF-κB activity.[154] Iloprost and cicaprost also suppressed activated BMDC expression of CD86, CD40, and MHC class II molecules and inhibited the ability of BMDCs to stimulate antigen-specific CD4 T cell proliferation and production of the Th2 cytokines IL-5 and IL-13. These

findings suggest that PGI_2 signaling through the IP may exert anti-inflammatory effects by acting on DC.[154] PGI_2 analogs potently modulate CD4 T cell function. Purified CD4 T cells were activated with anti-CD3 and anti-CD28 antibodies under Th1 and Th2 polarizing conditions for 4 days and restimulated the T cells with anti-CD3 in the presence of PGI_2 analogs for 2 days. Cicaprost and iloprost inhibited the production of Th1 cytokines (IFN-γ) and Th2 cytokines (IL-4 and IL-13) in a dose-dependent pattern. The inhibitory effect was partially dependent on the IP receptor signaling and was also correlated with elevated intracellular cAMP and down-regulated NF-κB activity. PGI_2 analogs have potent anti-inflammatory effects on activated DCs and previously activated and differentiated CD4 T cells *in vitro*.

PGI_2 and signaling through IP downregulates allergic inflammation in mouse models of ovalbumin-induced allergic inflammation. Acute ovalbumin-induced allergic inflammation, as defined by lung production of IL-4 and IL-5, serum antigen-specific and total IgE levels, and airway leukocyte accumulation were increased in IP-/- mice compared to wild type mice.[156] In a model of chronic ovalbumin airway exposure, IP-/- mice had greater airway eosinophils and lymphocytes, Th2 cytokine levels, and hydroxyproline concentrations compared to wild type mice.[157] In addition, treatment of polarized murine Th2 CD4+ T lymphocytes with a stable PGI_2 analog augmented IL-10 production, which is conjectured to restrain allergic responses.[158] Intranasal administration of the PGI_2 analog iloprost protected against the development of allergic lung inflammation and methacholine-induced airway responsiveness (AR).[159] Thus, published work indicates that signaling through IP downregulates allergic inflammation in the lung and improves lung physiology. Finally, absence of PGI_2 signaling during

sensitization broke immune tolerance elicited by prior mucosal allergen exposure.[160] Another study revealed that endogenous PGI_2 signaling through IP regulates lung disease expression in a model different from allergic inflammation. Mice that lacked the ability to signal through IP had augmented inflammatory and physiologic changes compared to WT mice in the model of bleomycin-induced fibrosis.[161]

GLP-1R signaling

Incretins are gut hormones that potentiate insulin secretion after meal ingestion in a glucose-dependent manner. While the insulinotropic properties of incretins were identified more than 25 years ago, new actions of incretin hormones continue to be identified. The two best-studied incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), exert their actions through distinct G-protein-coupled receptors. Two strategies encompassing potentiation of incretin receptor signaling have been pursued for the treatment of type 2 diabetes. Inhibition of dipeptidyl peptidase-4 (DPP-4), the enzyme responsible for N-terminal cleavage and inactivation of GIP and GLP-1, has been achieved through the use of orally available medications with high selectivity for the catalytic subunit of DPP-4. A second class of incretin-based therapies is comprised of injectable GLP-1R agonists that exhibit structural homology to human GLP-1 or to nonmammalian GLP-1R agonists.

GLP-1 is synthesized by and secreted from enteroendocrine L cells present throughout the small and large intestines.[162] GLP-1 is a product of post-translational processing of proglucagon by prohormone convertase 1/3.[162] GLP-1 is also produced by the CNS, predominantly in brainstem nuclei with projections throughout the brain to

regulate metabolic, cardiovascular, and neuroprotective activities.[163] For instance, neuronal GLP-1R signaling reduced hypothalamic reactive oxygen species production.[164] Indeed, GLP-1 is a pleiotropic hormone with salutary action in multiple tissues and organs. The section of GLP-1 from enteroendocrine cells is stimulated by the gastrointestinal ingestion of nutrients such as carbohydrates, fats, and proteins.[165] Inflammatory signals also regulate GLP-1 synthesis, secretion, and action. For instance, the inflammatory cytokines IL-1, IL-6, and LPS increased plasma levels of GLP-1.[166,167] IL-6 may also be important for the synthesis, secretion, and anorectic actions of GLP-1 in the brain, as almost 40% of the GLP-1+ neurons within the nucleus of the solitary tract express the IL-6 receptor α, and these PGDP+ neurons respond to exogenous IL-6 with an increase in cytosolic Ca^{2+}.[168] The GLP-1R is a G protein coupled cytoplasmic membrane receptor. The GLP-1R is expressed on the beta cells of the pancreas, in the brain, on the myocardial cells of the heart, in the liver, and on airway epithelial cells in the lung. GLP-1 binding to its receptor results in activation of G_s which activates adenylate cyclase to increase cyclic AMP and activate the protein kinase A pathway (**Figure 1.7**). GLP-1R activation also results in β-arrestin-1 association with ERK1/2, leading to sustained activation and sequestration of phosphorylated ERK1/2 in the cytoplasm. In the pancreatic islets, GLP-1 potentiates insulin and suppresses glucagon secretion in a glucose-dependent fashion.[169] It is this activity of GLP-1 that has been capitalized upon for the development of blockbuster diabetes drugs that target the GLP-1 axis.[170] There are several forms of these drugs that are commercially available including liraglutide, exenatide, and albiglutide. While GLP-1R signaling decreases serum glucose concentrations in people with diabetes, it does not stimulate insulin

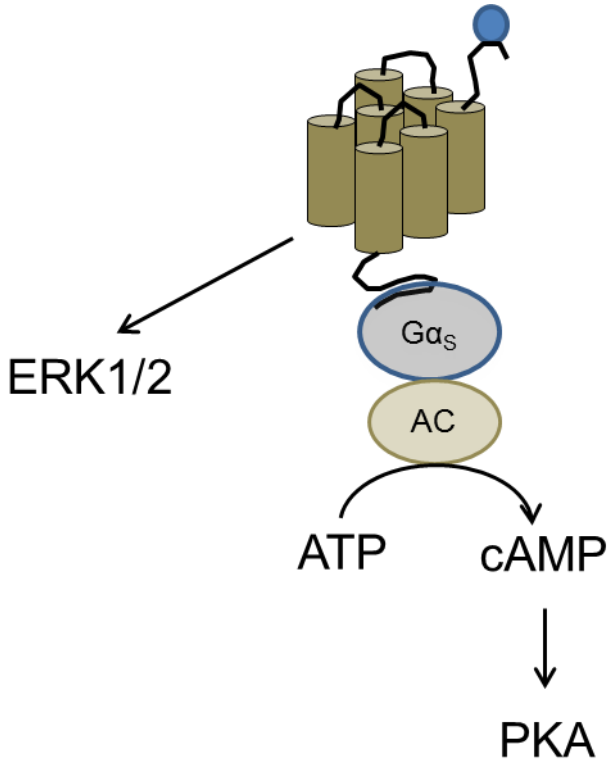

Figure 1.7. GLP-1 signaling. GLP-1 is one of two well-studied incretin hormones. GLP-1 signals through the seven transmembrane receptor GLP-1R which results in G_s activation, adenylate cyclase activation, increased cyclic AMP, and activation of the protein kinase A pathway. GLP-1R activation also signals through the ERK pathway.

secretion or decrease glucose levels in people without diabetes under euglycemic conditions, an important safety component of these therapies.[170]

In addition to its metabolic effects, GLP-1R signaling also has immunoregulatory functions that decrease inflammatory responses. GLP-1R is expressed on a variety of hematopoetic cells, including thymocytes, splenocytes, and T regulatory cells (Tregs).[171] A GLP-1R agonist decreases human $CD4^+$ cell migration to both RANTES and CXCL12 (SDF-1). CXCL12 is strongly chemotactic for lymphocytes.[172] GLP-1R signaling increases levels of cAMP and significantly inhibits IL-4 production by human invariant natural killer (iNKT) cells.[173] GLP-1R signaling decreases TNF-α-induced reactive oxygen species (ROS) production, protein kinase C activation, and NF-κB activation in primary human endothelial cells.[174] GLP-1R-deficient mice have decreased numbers of T regulatory cells, suggesting that GLP-1 receptor signaling promotes Treg development.[171] The intestinal mucosa of GLP-1R-deficient mice also exhibited dysregulated anti-inflammatory gene profiles, which was reversed by the reconstitution of GLP-1R+ intestinal intraepithelial lymphocytes through bone marrow transplantation.[175]

Anti-inflammatory actions of GLP-1R agonists have also been described in diabetes, psoriasis, cardiovascular disease, and chronic lung disease such as allergic airway inflammation and bleomycin-induced fibrosis. Activation of the GLP-1R in T2D patients significantly reduced serum levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6.[176] GLP-1 infusion decreased circulating levels of IL-6 during normal or increased insulinemia in the presence of euglycemia, demonstrating that GLP-1 exerts anti-inflammatory actions independent of changes in insulin levels.[177] A recent

randomized control trial demonstrated the effectiveness of liraglutide in reducing rates of first occurrence of death from cardiovascular disease, nonfatal myocardial infarction, or nonfatal stroke in patients with Type II diabetes presenting with a high risk for cardiovascular disease.[178] The number of first microvascular events, largely reflecting a reduction in kidney disease, was also significantly lower in liraglutide-treated subjects. In addition, the low-frequency missense *GLP1R* variant rs10305492 was associated with significantly lower risk of coronary artery disease independent of any changes in blood pressure or body mass index.[179] A recent prospective cohort study evaluated the effectiveness of liraglutide in reducing disease severity in patients with psoriasis and Type II diabetes.[180] Patients on liraglutide therapy had decreased Psoriasis Area and Severity Index (PASI) scores. GLP-1R signaling also decreased inflammatory cell infiltration and goblet cell hyperplasia in models of chronic lung inflammation such as allergic airway inflammation and bleomycin-induced fibrosis.[181,182]

Significance of these studies

Chapter II demonstrates the ability of STAT6 to regulate innate γδ17 cell immune responses. STAT6 suppression of γδ17 cell function may provide one explanation for why asthmatic patients have significantly greater risk for invasive bacterial disease, including pneumonia, than nonasthmatic subjects. Chapter III demonstrates that a GLP-1R agonist, an FDA-approved agent currently used for Type II Diabetes, attenuates the type 2 immune response to RSV and attenuates RSV illness. The current availability of GLP-1R agonists for human treatment highlights the clinical significance of these studies as this therapy could be immediately transferrable to RSV disease. Chapter IV

demonstrates that PGI_2, an FDA-approved agent currently used for pulmonary hypertension, protects against autoimmunity; enhances Treg stability and function; renders T effector cells more susceptible to Treg- mediated suppression; and promotes iTreg differentiation. PGI_2 may therefore represent a novel treatment strategy for diseases that result from Treg dysregulation.

CHAPTER II

STAT6 SIGNALING ATTENUATES IL-17-PRODUCING γδT CELLS DURING ACUTE *KLEBSIELLA PNEUMONIAE* INFECTION

Introduction

γδ T cells compose approximately 50% of the intraepithelial lymphocyte population and constitute a critical first line of defense against bacterial and fungal pathogens.[183] In contrast with adaptive αβ T cells, γδ T cells are capable of immediate cytokine release, providing an initial innate layer of protection at mucosal surfaces while influencing the development of subsequent adaptive responses.[80,184] γδ17 cells are a subset of γδ T cells that produce large quantities of IL-17A, a cytokine crucial to anti-bacterial and anti-fungal defense.[69] γδ17 cells also produce high levels of IL-17A in various models of inflammation and autoimmunity, including experimental autoimmune encephalitis, ischemic brain injury, and psoriasis.[80,184–187] While these data highlight the importance of understanding how γδ17 cell function is regulated, this process remains poorly understood.

γδ17 cell function is controlled by multiple immune cell populations and soluble molecules, particularly cytokines. Within 4-8 hours in the presence of the inflammatory cytokines IL-23 and IL-1β, γδ17 cells secrete IL-17A without the need for TCR engagement.[80] γδ17 cells constitutively express IL-23R and IL-1R1, providing for an efficient mechanism to induce rapid effector cytokine production. A recent study showed

that the serine/threonine kinase Sgk1 is a novel, critical regulator of IL-23R expression.[188]

Studies from our group and others have established that STAT6 negatively regulates IL-17A expression in Th17 cells.[127–129,189,190] By extension, we hypothesized that STAT6 also inhibits innate γδ17 cell cytokine secretion. STAT6 is a transcription factor important for Th2 differentiation, inhibiting Th1 differentiation, and activating the B cell response.[191] IL-4 signals through both the type I IL-4R, which consists of IL-4Rα and the common γ chain, and the type II IL-4R, which consists of IL-4Rα and IL-13Rα, while IL-13 signals through only the type II IL-4R.[123,124] IL-4 binds to the IL-4Rα portion and IL-13 binds to the IL-13Rα subunit of the IL-4 heterodimer receptor with high affinity, leading to the phosphorylation of STAT6.[125,126] STAT6 is expressed at high levels in the settings of parasitic infections and asthma,[192] during which STAT6 induces Th2 differentiation, IgE antibody class switching, globlet cell metaplasia, alternative macrophage activation, mucus expression, and airway remodeling.[193] Thus, STAT6 attenuation of γδ17 cell function may impair host defenses against bacterial and fungal infections in people with asthma or parasitic infections.

We found that γδ17 cells expressed the type I IL-4R, and that IL-4 increased STAT6 phosphorylation in γδ17 cells. Furthermore, IL-4 signaling attenuated γδ17 cell production of IL-17A and IL-17F. IL-4 also decreased γδ17 cell expression of the IL-23R as well as Sgk1. To determine whether STAT6 regulates γδ17 cell cytokine expression *in vivo*, we used a mouse model of *Klebsiella pneumoniae* lung infection in mice deficient in STAT6. We chose *K. pneumoniae* for our *in vivo* model since *K. pneumoniae* increases IL-17A expression and the number of γδ17 cells.[65–

[67,70,76,77,194,195] We found a significant increase in γδ17 cell numbers in STAT6- deficient mice following acute lung infection with *K. pneumoniae in vivo* compared to WT mice. Together, these studies reveal that STAT6 negatively regulates γδ17 cells, a cell population that plays a front line role in mucosal immunity.

Materials and Methods

Mice

8- to 12-week old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). STAT6 KO, IL-4 KO, and IL-13 KO mice on a BALB/c background were purchased from The Jackson Laboratory, and breeding colonies were established (Bar Harbor, ME). In caring for the animals, investigators adhered to the revised 1996 Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

T cell isolation and culture

γδ T cells from the spleens of BALB/c WT mice were enriched using anti-murine γδ TCR magnetic microbeads (Miltenyi Biotec, Auburn, CA). γδ T cells were purified by flow cytometry by blocking with anti-FcR antibody (2.4G2, BD Biosciences, San Jose, CA), and using propidium iodide viability stain and surface markers against CD3 (145-2C11) and γδ TCR (GL3, BD Biosciences). The purified CD3+γδ TCR+ cells were resuspended at 3 x 10^5 cells/mL and were induced to produce IL-17A using IL-1β (10

ng/mL) and IL-23 (10 ng/mL) in 96-well plates for 3 days.[80] CD3+ T cells were activated with plate-bound anti-CD3 (5 ug/mL) and anti-CD28 (1 ug/mL). IL-4 (0.01-1 ng/mL) or IL-13 (10 ng/mL) was also added in select cultures. Mouse rIL-23 was purchased from R&D Systems. Mouse rIL-1β, mouse rIL-4, and mouse rIL-13 were purchased from PeproTech (Rocky Hill, NJ).

Cytokine measurements

The secretion of cytokines (IL-17A, IL-17F, and IL-22) in cell culture supernatants were analyzed by commercially available enzyme-linked immunoabsorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Any value below the lower limit of detection was assigned half the value of the lowest detectable standard for statistical comparisons.

Quantitative PCR

Total RNA was isolated using a Qiagen RNAesy micro kit (Valencia, CA) and cDNA was generated. A two-step real-time PCR assay using SYBR green mix (Bio-Rad, Hercules, CA) was used to detect IL-23R, Sgk1, Foxo1, MyD88, TRAF6 (proprietary information available by Qiagen), RORγT, and AhR as previously described [196], and normalized to GAPDH. Primer sequences were as follows: IL-23R: forward, 5'-GGTCCAAGCTGTCAATTCCCTAGG-3', reverse, 5'-AGCCCTGGAAATGATGGACGCA-3'; RORγT: forward, 5'-GCGGCTTTCAGGCTTCATGGAG-3', reverse, 5'-GGGCGCTGAGGAAGTGGGAAAA-3'; AhR: forward, 5'-CGGGGTACCAGTTCATCCACGCT-3', reverse, 5'-

GCAAACATGAAGGGCAGCGACGT-3'; GAPDH: forward, 5'-GGCCCCTCTGGAAAGCTGTGG-3', reverse, 5'-CCCGGCATCGAAGGTGGAAGA-3'.

Klebsiella infection

WT and STAT6 KO mice were anesthetized with ketamine/xylazine and then infected with 0.3-1x10^3 CFU of serotype 2 *K. pneumoniae* (ATCC 43816) in 50 µL PBS or 50 µL PBS alone by retropharyngeal instillation. Bacterial stocks of *K. pneumoniae* were inoculated into 100 ml of tryptic soy broth (TSB) and incubated for 18 h at 37°C and 225 rpm. One milliliter of this culture was transferred to 100 ml of TSB, and bacteria were grown to mid-log phase for 2 h at 37°C. Bacteria were pelleted, washed in sterile PBS, and diluted to the appropriate concentration as previously described.[195,197] The inoculum concentration was verified by serial dilution in PBS, plating on tryptic soy agar (TSA), and visual determination of colony counts.

Harvest and bacterial quantitation

Lungs were harvested 36 hours after *K. pneumoniae* infection. Mice were euthanized by intraperitoneal injection of pentobarbital sodium (Vortech Pharmaceuticals, Dearborn, MI). The right lung was harvested into 1 mL of RPMI medium and analyzed by flow cytometry. The left lung was harvested into 1mL of sterile PBS and organs were homogenized. Serial dilutions in PBS were plated onto TSA, grown overnight at 37°C, and colonies were counted.

Flow cytometry

CD3+ T and γδ17 cells were harvested and cells were blocked with anti-

antibody (BD Biosciences) and in select experiments stained with surface markers against CD3, γδ TCR, IL-4Rα (mIL4R-M1) or IgG2a, κ isotype control (BR2a), and common γ-chain (TUGm) or IgG2b, κ isotype control (27-35, BD Biosciences). For experiments examining STAT6 phosphorylation, select wells were stimulated with IL-4 (10ng/ml) for one hour. Cells were blocked with normal mouse serum (eBioscience, San Diego, CA) and stained with surface markers against CD3 and γδ TCR. Cells were then permeabolized and fixed with 4% paraformaldehyde and methanol, respectively, washed thoroughly, and stained for phospho-STAT6 (J71-773.58.11) or IgG1κ isotype control (MOPC-31C, BD Biosciences).

Lungs were harvested, minced, and digested in RPMI media containing 5% FBS, 1 mg/mL collagenase type IV, and 0.02 mg/mL DNase I for 40 min at 37°C. The digestion was stopped with 100 µl of 0.5 M EDTA, and a single cell suspension was generated by straining these digestions through a 70-µm strainer. Cells were restimulated in RPMI media containing, 10% FBS, 50 ng/mL PMA (Sigma-Aldrich, St. Louis, MO), 1 µM ionomycin (Sigma-Aldrich), and 0.07% GolgiStop (BD Biosciences) for 4 hours at 37°C and 5% CO_2. Following restimulation, six million cells were stained with Live/Dead Blue (Life Technologies, Carlsbad, CA), blocked with anti-FcR antibody and surface markers against CD3, CD4 (H129.19), and γδ TCR. Cells were fixed/ permeabolized for 12 hours in Cytofix/ Cytoperm (BD Biosciences) and stained for IL-17A (TC11-18H10, BD Bioscience). Anti-FcR antibody (BD Biosciences) was used to prevent nonspecific staining. A total of one million cells were analyzed using a LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analyses

Data are presented as mean ± SEM. For Figs. 1-4, data shown represent a combined analysis of cell culture wells from three independent experiments. For Fig. 5, data shown represent a combined analysis of individual mice from three independent experiments. Groups were compared using one-way analysis of variance (ANOVA) with Bonferroni's post-test or Student's T-Test using Prism (version 5; GraphPad Software, San Diego, CA) with values being considered significant when $p < 0.05$.

Results

The type I IL-4R is expressed on γδ17 cells

STAT6 negatively regulates IL-17A expression in CD4+ Th17 cells [127–129]. By analogy, we hypothesized that γδ17 cells express the IL-4R, that IL-4 phosphorylates STAT6 in γδ T cells, and that IL-4 inhibits γδ17 cell cytokine secretion. To test this hypothesis, we first enriched γδ T cells from mouse spleens using a commercially available magnetic sorting kit (Miltenyi Biotec, Auburn, CA), and cell sorted for viable CD3+ γδ TCR+ cells (**Figure 2.1**). IL-4Rα was clearly detected on γδ T cells, and also on CD3+ T cells, which are known to express the IL-4R. Common γ-chain expression was also clearly detected on both γδ Tcells and CD3+ T cells (**Figure 2.2*A***). As optimal IL-17A production from γδ17 cells is induced following culture with both IL-1β and IL-23,[80] γδ T cells were cultured with IL-1β (10 ng/mL) and IL-23 (10 ng/mL) for three days. γδ17 cells or CD3+ T cells were then collected and IL-4R expression was examined by flow cytometry. Both IL-4R and common γ-chain were detected on induced γδ17 cells and activated CD3+ T cells (**Figure 2.2*B***). Combined, these data demonstrate that γδ17

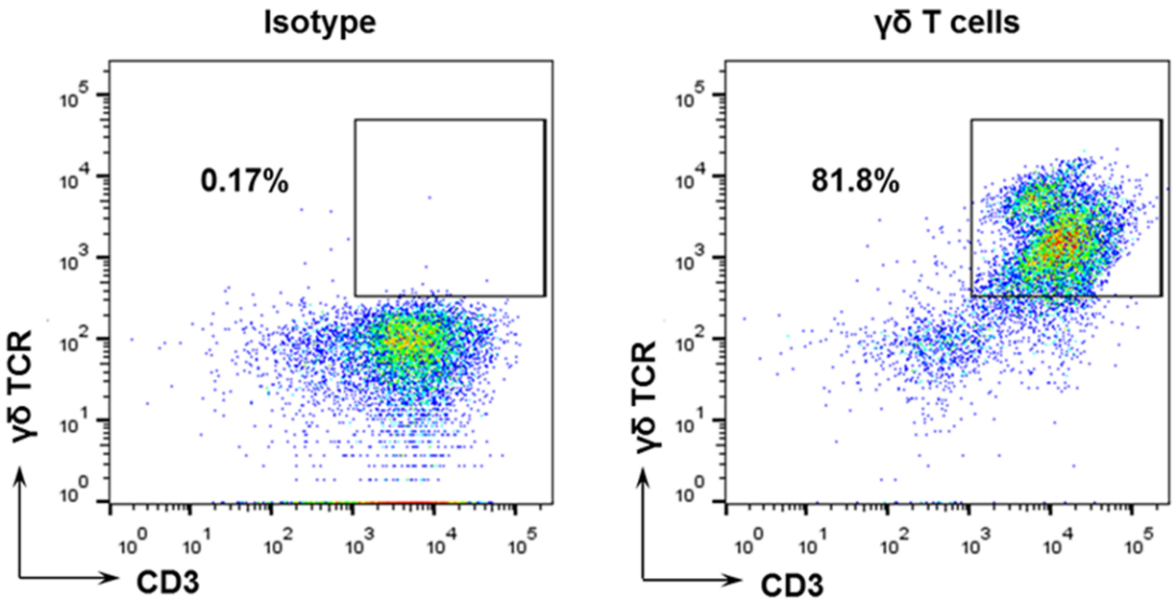

Figure 2.1. **Isolated cells are CD3+ γδ TCR+.** Representative plots after magnetic enrichment and during cell sorting are shown. Cells were gated on live cells.

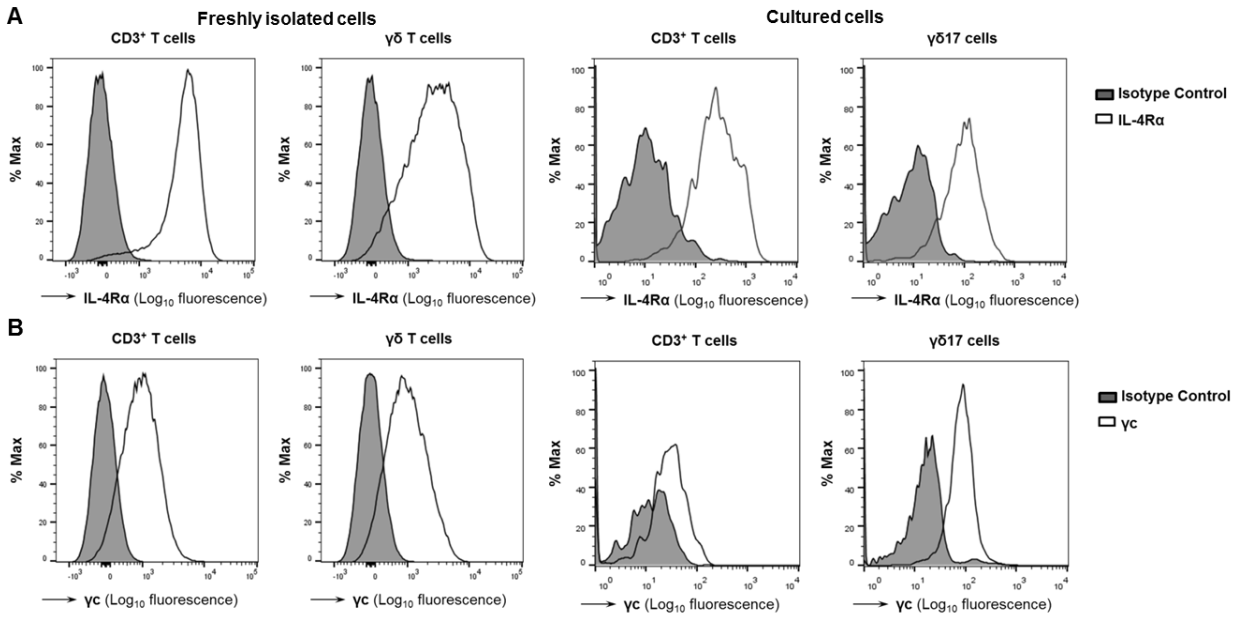

Figure 2.2. IL-4Rα and common γ-chain are expressed in freshly isolated (**A**) **and induced γδ17** (**B**). CD3+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28, and γδ T cells were induced to produce IL-17A with IL-1β and IL-23 for 3 days. Representative histograms of IL-4Rα and common γ-chain expression in CD3+ or γδ TCR+ cells compared with the isotype control. Histograms are representative of 3 independent experiments; n = 5.

cells express the subunits of the type I IL-4R, suggesting a potential role for IL-4 in regulating these cells.

IL-4 increases STAT6 phosphorylation in γδ17 cells

Since the type I IL-4R was expressed on γδ17 cells, we hypothesized that the downstream transcription factor STAT6 is phosphorylated in the presence of IL-4. To test this hypothesis, we stimulated cultured γδ T cells or CD3+ T cells for one hour with IL-4 (0-10 ng/mL). CD3+ T cells stimulated with IL-4 had increased STAT6 phosphorylation compared with that seen in CD3+ T cells with no IL-4 added. Similarly, γδ17 cells stimulated with IL-4 had increased STAT6 phosphorylation compared with that seen in γδ17 cells with no IL-4 added (**Figure 2.3**). These data demonstrate that a functional IL-4R is expressed by γδ17 cells.

IL-4 but not IL-13, directly attenuates IL-17A production from γδ17 cells

Based on the expression of a functional type I IL-4R on γδ17 cells and the known inhibitory effect of IL-4 on CD4+Th17 cells, we hypothesized that IL-4 directly decreases γδ17 cell cytokine secretion. Treatment of γδ17 cells with IL-4 *in vitro* (0.01-1 ng/mL) induced a significant dose-dependent decrease in γδ17 cell IL-17A protein expression (**Figure 2.4*A***). IL-4 also attenuated γδ17 cell production of IL-17F (**Figure 2.4*B***) in a dose-dependent manner. In three independent experiments, IL-4 also decreased γδ17 cell IL-22 production in a dose-dependent manner, though the decrease in IL-22 production was not statistically different when the three experiments were combined (data not shown).

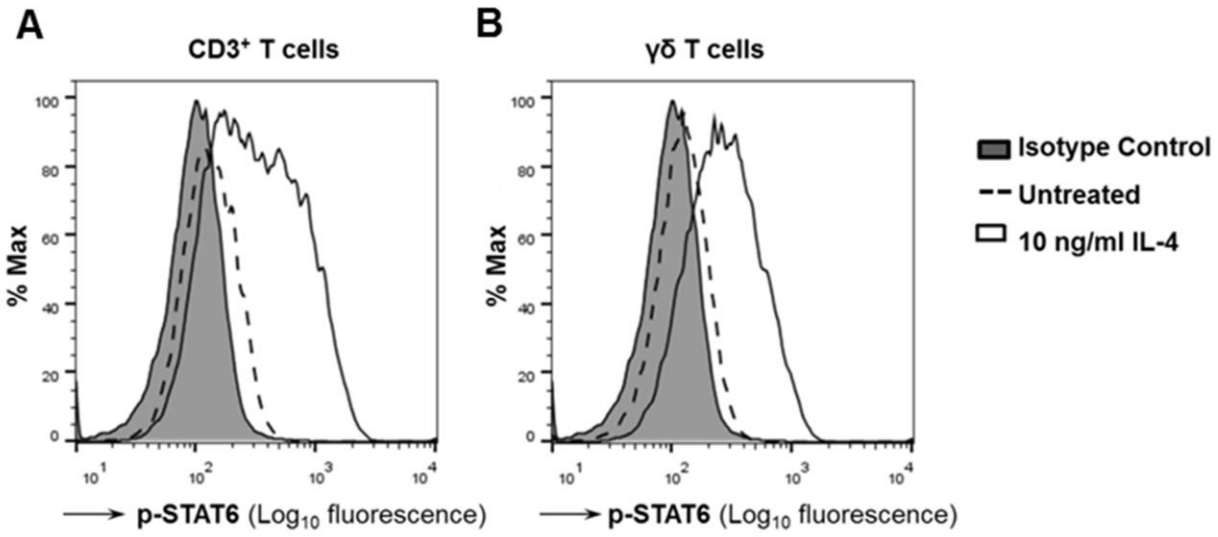

Figure 2.3. IL-4 increases STAT6 phosphorylation in γδ T cells. CD3+ T cells or γδ T cells were stimulated with IL-4 (10 ng/ml) for 1 hour and examined for phospho-STAT6 expression by flow cytometry. Representative histograms of phospho-STAT6 expression in CD3+ (**A**) or γδ TCR+ (**B**) cells compared with untreated cells or isotype control. Histograms are representative of 3 independent experiments; n = 5.

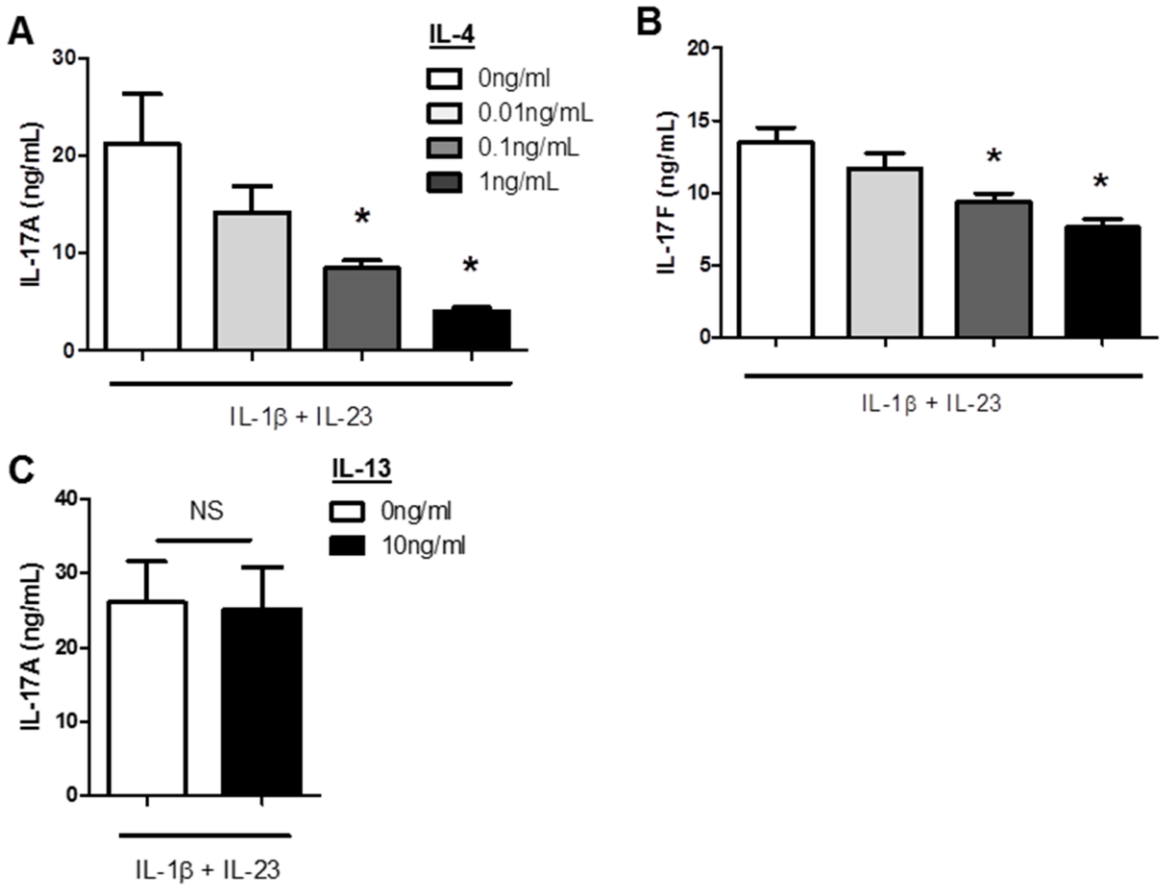

Figure 2.4. IL-4 but not IL-13 attenuates IL-17A and IL-17F production from γδ17. γδ T cells were induced to produce IL-17, and IL-4 (0.01 ng/ml- 1ng/ml) and IL-13 (10ng/ml) were added at the time of γδ T cell induction. IL-17A (**A**) IL-17F (**B**), or IL-13 (**C**) protein expression in cultured supernatants were quantified by ELISA 3 days after induction. Data are combined from 3 independent experiments; n = 5; *$p < 0.05$, versus untreated γδ T cells (no IL-4), ANOVA (**A-B**), Student's T-Test (**C**).

Since the Th2 cytokine IL-13 also negatively regulates IL-17A expression in CD4+ Th17 cells,[128,198] we hypothesized that IL-13 also directly inhibits γδ17 cell cytokine secretion. However, we found that IL-17A expression was not statistically different between γδ17 cells treated with IL-13 and vehicle (**Figure 2.4*C***). These data demonstrate that IL-4, but not IL-13, negatively regulated IL-17A and IL-17F protein expression from γδ17 cells at the time of induction.

IL-4 decreases IL-23R and Sgk1 expression in γδ17 cells

IL-4 reduced γδ17 cell IL-17A protein expression. Since IL-23 signals through the IL-23R to induce IL-17A production in γδ T cells, we hypothesized that IL-4 administered during γδ17 cell induction decreases the expression of IL-23R and Sgk1, a critical regulator of IL-23R expression.[188] We found that IL-4 decreased IL-23R mRNA expression in γδ17 cells three days following γδ17 cell induction (**Figure 2.5*A***). We also found that γδ17 cells exposed to IL-4 had decreased Sgk1 mRNA expression one day following γδ17 cell induction (**Figure 2.5*B*)**. The decreases in IL-23R and Sgk1 mRNA expression paralleled the IL-4-mediated decrease in γδ17 cell IL-17A production. Sgk1 positively regulates IL-23R expression by deactivating Foxo1, a direct repressor of IL-23R,[188] however we did not find statistically significant differences in Foxo1 mRNA expression between IL-4 and vehicle- treated γδ17 cells *in vitro* (data not shown). We also determined mRNA expression for downstream components of the IL-1R signaling pathway, MyD88 and TRAF6, as well as for the canonical Th17-related transcription factors RORγT and AhR, which control γδ17 cell development.[69,82] We did not find statistically significant differences in MyD88, TRAF6, RORγT, nor AhR mRNA

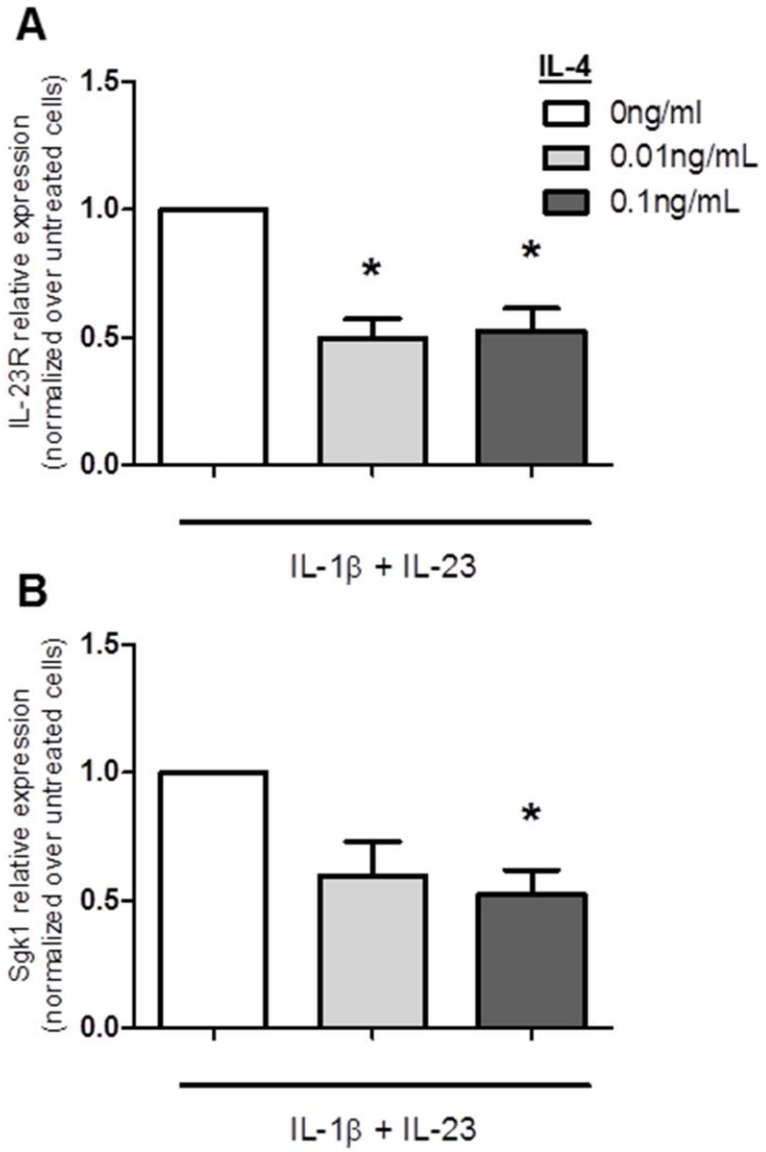

Figure 2.5. IL-4 signaling through IL-4R decreases IL-23R and Sgk1 mRNA expression in γδ17. Total RNA was isolated from γδ17 cells 3 (**A**) or 1 (**B**) days after induction. IL-23R (**A**) and Sgk1 (**B**) mRNA expression in γδ T cells induced in the presence of IL-4. All mRNA levels were assayed by quantitative real-time RT-PCR, with each sample normalized to GAPDH and relative expression compared with γδ T cells induced without IL-4 present. Data are combined from 3 independent experiments; n = 5; *$p < 0.05$, versus untreated γδ T cells (no IL-4), ANOVA.

expression between IL-4 and vehicle- treated γδ17 cells *in vitro* (data not shown). Combined, these data suggest that IL-4 may attenuate γδ17 cell IL-17A production by decreasing IL-23R expression.

STAT6 deficiency increases the number of mouse lung γδ17 cells in response to K. pneumoniae *infection*

Based on our *in vitro* data that IL-4 inhibits IL-17A protein expression by γδ17 cells, we hypothesized that STAT6 reduces the number of lung γδ17 cells *in vivo.* We chose mice deficient in STAT6, which is essential for signaling downstream of the IL-4R. We infected mice with *K. pneumoniae* administered by retropharyngeal instillation and sacrificed the mice 36 hours later. We chose *K. pneumoniae* for our *in vivo* model because *K. pneumoniae* increases IL-17A expression and γδ17 cell numbers.[65–67,194,195] We chose this time course with the consideration that there was unlikely to be a robust adaptive immune response after only 36 hours of *K. pneumoniae* infection, and that instead this protocol would allow us to assess the innate response during which γδ T cells are important producers of IL-17A. We determined the number of lung γδ17 cells following 36 hours of *K. pneumoniae* infection. Following digestion of the lung, the number of γδ17 cells was determined following cell surface and intracellular cytokine staining in *K. pneumoniae*- infected WT and STAT6 KO mice (**Figure 2.6*A***). There was a statistically significant two-fold increase in the percentage of lung γδ T cells that expressed IL-17A in the *K. pneumoniae*- infected STAT6 KO mice compared to the WT mice (**Figure 2.6*C***). In addition, there was a statistically significant three-fold increase in the total numbers of lung γδ T cells that expressed IL-17A in the *K. pneumoniae*-

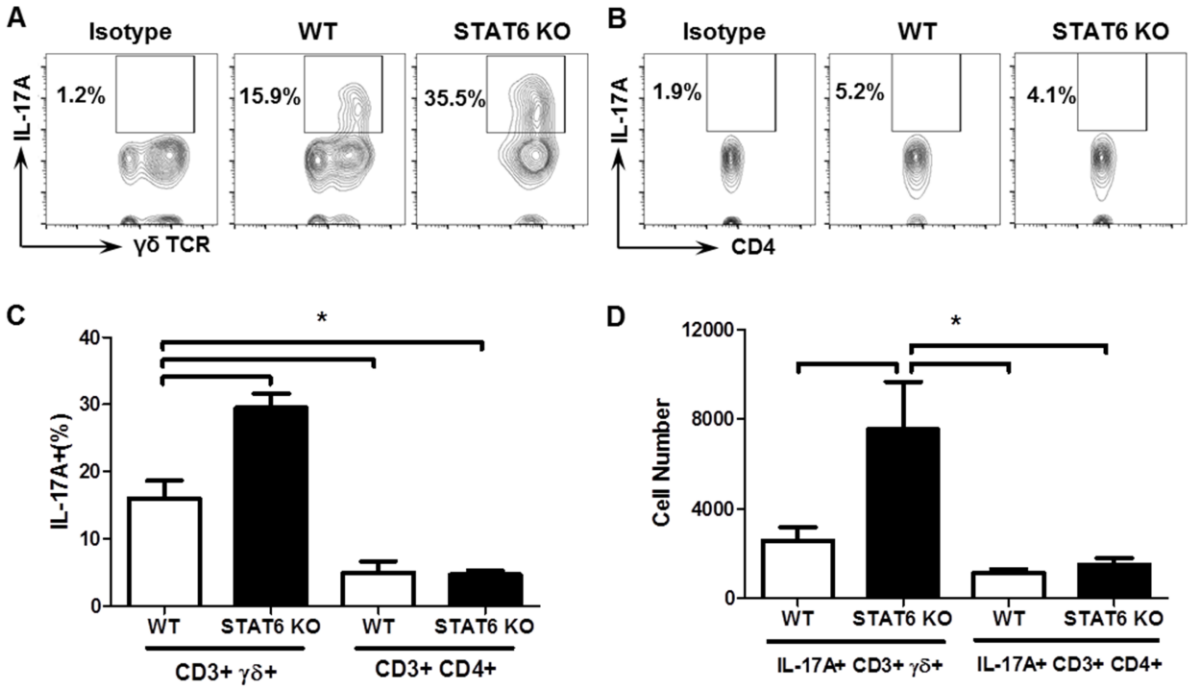

Figure 2.6. Increased airway γδ17 cell numbers in STAT6 KO compared to WT mice following acute lung infection with *K. pneumoniae*. Lung cells were restimulated with PMA and ionomycin in the presence of Golgi-stop. Representative contour plots that are CD3-gated (**A and B**). Percentages of IL-17A+CD3+γδ TCR+ or IL-17A+CD3+CD4+ cells (**C**). Total number of cells that are IL-17A+CD3+γδ TCR+ or IL-17A+CD3+CD4+ (**D**). Total cell numbers were calculated by multiplying the percentage of IL-17A+CD3+γδ TCR+ or IL-17A+CD3+CD4+ cells by the total cell number. Data are combined from 3 independent experiments; n = 15 mice for each group. *$p < 0.05$, ANOVA.

infected STAT6 KO mice compared to the WT mice (**Figure 2.6*D***). In our experiments with *K. pneumoniae*, we were unable to detect IL-4 or IL-13 protein. However, our data would suggest that there was sufficient endogenous IL-4 and/ or IL-13 expression to decrease γδ17 cell numbers. To determine whether IL-4 or IL-13 alone had effects on γδ17 cells during acute *K. pneumoniae* infection, we also detected γδ17 cells in *K. pneumoniae*- infected IL-4 KO and IL-13 KO mice. The deletion of either IL-4 or IL-13 alone did not have an effect on γδ17 cell numbers during acute *K. pneumoniae* infection (**Figure 2.7**). Therefore, STAT6 suppression of γδ17 cells during acute *K. pneumoniae* infection is likely due to the combined effect of both IL-4 and IL-13 signaling through STAT6. There were no significant differences in the percentage or number of γδ17 cells in STAT6 KO, IL-4 KO, or IL-13 KO mice compared to WT mice at a baseline level in mice not infected with *K. pneumoniae* (**Figure 2.8**).

We also used flow cytometry to determine the number of CD4+ T cells producing IL-17A in our model (**Figure 2.6*B***). We found that there was a two-fold increase in γδ17 cells compared to CD4+ IL-17A-expressing cells in WT mice, and a six-fold increase in γδ17 cells compared to CD4+ IL-17A-expressing cells in STAT6 mice, showing that γδ17 cells were more abundant than CD4+IL-17A+ cells at this time point after infection in both WT and STAT6 KO mice (**Figure 2.6*D***). The percentage of CD4+IL-17A+ cells was not statistically different between the *K. pneumoniae*- infected STAT6 KO and WT mice. Similarly, the number of CD4+IL-17A+ cells was not statistically different between the *K. pneumoniae*- infected STAT6 KO and WT mice (**Figure 2.6*D***). Lung *K. pneumoniae* burden did not differ significantly between the *K. pneumoniae*- infected STAT6 KO and WT mice (data not shown). These results reveal that endogenous

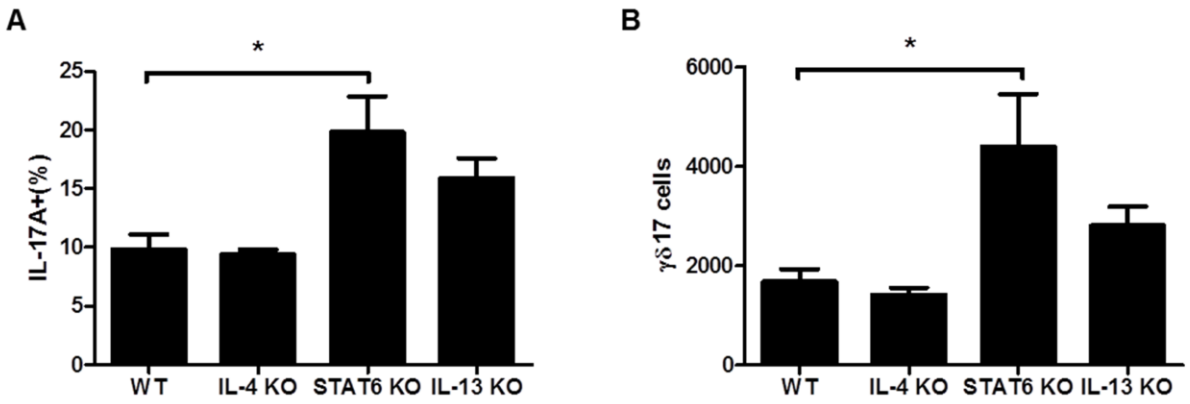

Figure 2.7. Increased airway γδ17 cell numbers in STAT6 KO compared to WT mice following acute lung infection with *K. pneumoniae*. Lung cells were restimulated with PMA and ionomycin in the presence of Golgi-stop. Percentages of IL-17A+CD3+γδ TCR+ (**A**). Total number of cells that are IL-17A+CD3+γδ TCR+ (**B**). Total cell numbers were calculated by multiplying the percentage of IL-17A+CD3+γδ TCR+ cells by the total cell number. Data are representative of 2 independent experiments; n = 8-10 mice for each group. *$p < 0.05$, ANOVA.

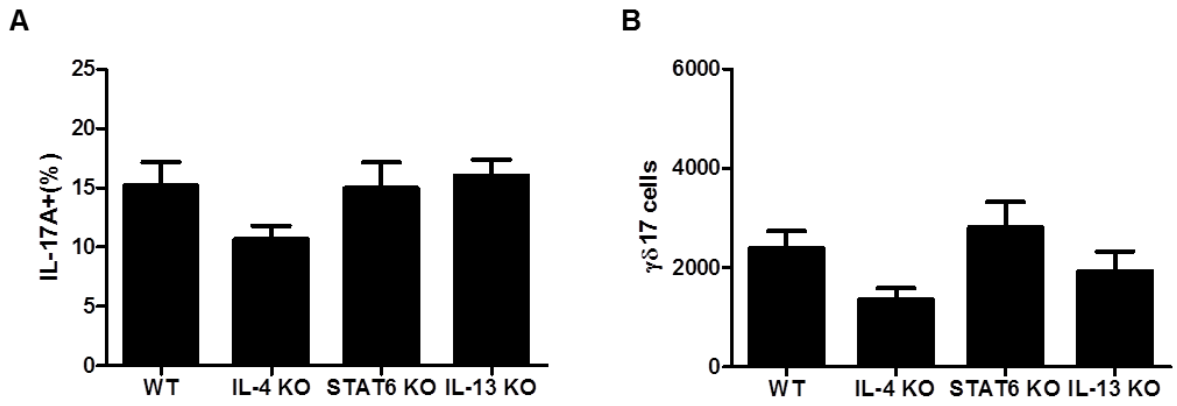

Figure 2.8. Baseline γδ17 cell numbers in STAT6 KO compared to WT mice. Lung cells were restimulated with PMA and ionomycin in the presence of Golgi-stop. Percentages of IL-17A+CD3+γδ TCR+ (**A**). Total number of cells that are IL-17A+CD3+γδ TCR+ (**B**). Total cell numbers were calculated by multiplying the percentage of IL-17A+CD3+γδ TCR+ cells by the total cell number. Data are representative of 2 independent experiments. n = 10 mice for each group. ANOVA.

STAT6 signaling significantly inhibited the number of γδ17 cells in response to *K. pneumoniae* infection. The results also support that γδ17 cells, and not CD4+ cells, were the primary producers of IL-17A in response to this acute *K. pneumoniae* infection protocol.

Discussion

Our data are the first to demonstrate that γδ17 cells expressed both IL-4Rα and common γ chain, the heterodimeric components of the type I IL-4R, and that γδ17 cell expression of the type I IL-4R was functional, as IL-4 induced STAT6 phosphorylation in γδ T cells. IL-4 negatively regulated γδ17 cell production of IL-17A and IL-17F *in vitro*. IL-4-mediated attenuation of IL-17A production also paralleled decreases in IL-23R and Sgk1 expression. Since IL-23 is required for γδ17 cell production of IL-17, IL-4 may inhibit γδ17 cell function by decreasing IL-23R expression. This mechanism of γδ17 cell inhibition via IL-23R downregulation could mirror how IL-4 inhibits Th1 differentiation by decreasing IL-12R expression.[121] The serine/ threonine kinase, Sgk1, was recently found to stabilize the Th17 cell phenotype by stabilizing IL-23R expression.[188] We found that Sgk1 expression was decreased one day following γδ17 cell induction in culture, while IL-23R expression was decreased three days following γδ17 cell induction. Therefore, the observed IL-4-mediated decreases in IL-17A production could potentially be caused by decreased Sgk1 expression leading to destabilized IL-23R expression on γδ17 cells.

An important finding in this report is that IL-4 and IL-13 differentially regulate CD4+ Th17 and γδ17 cell cytokine production. While a concentration as low as 0.1

ng/mL of IL-4 significantly decreased γδ17 cell IL-17A production, 100 times more IL-4 is required to inhibit IL-17A production from Th17 cells.[128] This indicates that the IL-4R on γδ17 cells is either more sensitive to the presence of IL-4 or more robustly induces downstream IL-4 signaling compared to the IL-4R on Th17 cells. While IL-4 directly inhibited IL-17A production from both Th17 and γδ17 cells, IL-13directly inhibited IL-17A production from Th17 cells but not from γδ17 cells.[128,198] These results suggest that IL-4 and IL-13 may use different mechanisms to inhibit γδ17 cells.

We show that STAT6 signaling reduced lung γδ17 cell numbers *in vivo*. IL-4 binds to the IL-4Rα portion and IL-13 binds to the IL-13 α subunit of the IL-4 receptor, leading to the phosphorylation of STAT6. STAT6 deficiency increased both the percentage of lung γδ T cells that expressed IL-17A as well as the total number of γδ T cells in the lung that expressed IL-17A protein in response to acute *K. pneumoniae* infection. We confirmed that γδ17 cells, and not CD4+ T cells, were the major contributors of IL-17A in our model of acute *K. pneumoniae* infection. To our knowledge, there are no studies in which CD4+ T cells or IL-13 has been depleted during acute *K. pneumoniae* infection to determine whether this cell type or cytokine has a role during the immune response. IL-13 did not have a direct effect on γδ17 cell cytokine production *in vitro*. However, the genetic deletion of either IL-4 or IL-13 alone did not have an effect on γδ17 cell numbers during acute *K. pneumoniae* infection. Therefore, STAT6 suppression of γδ17 cells during acute K. pneumoniae infection is likely due to the combined effect of both IL-4 and IL-13 signaling through STAT6. IL-13 may regulate γδ17 cells indirectly, as IL-13 suppresses dendritic cell expression of γδ17 cell-promoting factors such as IL-1β and IL-23.[199]

Future lines of research should address STAT6 downregulation of γδ17 cell function in other tissues and in additional models of immune-mediated inflammation and infection. Tissue localization dictates γδ TCR diversity.[200] Similarly, γδ17 cells may differentially express the IL-4R and STAT6 may distinctly regulate γδ17 cell cytokine production in other sites where γδ T cells are prevalent, such as in the gut or skin. Increasing STAT6 activity could represent an immunotherapeutic strategy to treat γδ17-mediated diseases, such as psoriasis and multiple sclerosis, by decreasing inflammatory IL-17A production.[201,202] In contrast, STAT6 is expressed at high levels in patients with asthma,[193] and phase II randomized clinical trials have investigated the effects of blocking IL-4 and IL-13 signaling. The IL-4Rα antibody, AMG 317, reduced IgE levels and the number of exacerbations in patients with moderate-to-severe asthma.[130] The rhIL-4 variant, pitrakinra, which competitively inhibits IL-4 or IL-13 binding to IL-4Rα, resulted in a smaller decrease in FEV_1 in patients with mild asthma.[131] However, the impact of these anti-IL-4 and anti-IL-13 therapies on γδ17 cells is unknown. Based on the findings of our study, anti-IL-4 and anti-IL-13 therapies used to treat asthmatic patients could potentially have the adverse effect of exacerbating co-existing immune-mediated inflammatory diseases, such as multiple sclerosis, in these patients. Blocking IL-4 and IL-13 activity could also decrease infection severity from pathogens for which strong, early γδ17 cell responses are critical for immunity. During the immune response to pathogens such as *Mycobacterium tuberculosis, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli,* and *Candida albicans,*[71,73–75] γδ17 cells stimulated neutrophil recruitment, antibacterial peptide production, and maintenance of epithelial barriers.[78] Notably, people with asthma, who

express high levels of STAT6,[203] are at increased risk for invasive bacterial infections and bacterial pneumonia.[53–56] However, the mechanisms by which asthma impairs host defense against bacterial infection are unknown. Our results may provide one explanation for why asthmatic subjects have a significantly greater risk of invasive bacterial disease than nonasthmatic subjects.

In summary, we are the first to show that γδ17 cells express a functional IL-4R, and demonstrate a novel role for the transcription factor STAT6 in negatively regulating γδ17 IL-17A production. These findings have significant and broad implications for how γδ17 cells, an important cell population that plays a front-line role in mucosal immunity, are regulated in the spleen and lung.

CHAPTER III

GLP-1R SIGNALING ATTENUATES RSV-INDUCED TYPE 2 RESPONSES AND IMMUNOPATHOLOGY

Introduction

Respiratory syncytial virus (RSV) is the leading cause of infant hospitalization.[204] RSV induces bronchiolitis and viral pneumonia, and in severe cases, can lead to death. RSV is the second most frequent cause of "excess death" during the winter months in elderly and high-risk adults, with a disease burden similar to that of influenza.[94] Though expensive, the only FDA-approved therapy, palivizumab, is effective when administered prophylactically.[205] Given the frequency and potential severity of RSV infection, as well as the current lack of a vaccine or cost-effective therapies, the development of novel preventative and therapeutic measures is imperative.

RSV infection activates multiple arms of the immune response, resulting in severe and prolonged lung inflammation. By infecting the respiratory epithelium, RSV upregulates type I interferons. Both Th1 and natural killer (NK) cells produce interferon-γ (IFN-γ), which potentiates the polarization of incoming T cells to an antiviral, cytolytic type 1 response which is required for viral clearance.[97,98] However, pathogenic viral mediators can dysregulate host immune responses during infection. For example, RSV NS1 and NS2 proteins suppress type I interferons,[100] and RSV G protein promotes inappropriate inflammatory responses via CX3C chemokine mimicry.[101] Reduced viral control by defective host immunity and over activation of the type 2 host response lead

to severe RSV infection. The type 2 response mediates airway mucus production, which directly contributes to airway obstruction and respiratory failure.[102] Group 2 innate lymphoid cells (ILC2), $CD4^+$ Th2 cells, and basophils produce type 2 cytokines, which mediate airway responsiveness and mucus production during severe infection.[108,206–209] The epithelial-associated cytokine IL-33 activates cells that produce type 2 cytokines including ILC2, Th2 cells, and basophils to secrete IL-13, and polarizes naïve CD4+ T cells to differentiate into type 2 cytokine-producing effector Th2 cells.[28] As a central mediator of both innate and adaptive immunity-regulated lung inflammation, IL-33 has been identified as an important therapeutic target in inhibiting airway disease.[29]

Glucagon-like peptide-1 (GLP-1) is an emerging anti-inflammatory therapeutic target. GLP-1 is a peptide hormone that is synthesized and released by enteroendocrine L cells in the ileum and large intestine following oral food intake.[162,165] As GLP-1 induces insulin and inhibits glucagon secretion in a glucose-dependent fashion,[169] GLP-1R agonists are approved by the Food and Drug Administration (FDA) for the treatment of Type II diabetes (T2D).[170] Importantly, GLP-1R signaling does not stimulate insulin secretion or decrease glucose levels in people without diabetes under euglycemic conditions, carries little risk for hypoglycemic events, and is administered at doses that do not produce weight loss.[210] Recent reports demonstrate that GLP-1R signaling attenuates inflammation during cardiovascular disease, psoriasis, and chronic lung disease such as allergic airway inflammation and bleomycin-induced fibrosis.[181,182] However, no studies have investigated the effect of GLP-1R signaling during viral-associated immunopathology.

We found that GLP-1R agonist treatment decreased airway inflammation, airway responsiveness, and airway mucus production in mice infected with a strain of RSV that was isolated from a hospitalized infant with severe lower respiratory tract infection and bronchiolitis. GLP-1R agonist treatment decreased lung IL-13 and IL-33 levels, with concurrent decreases in IL-13^{+} group 2 innate lymphoid cells (ILC2), CD4^{+} Th2 cells, and basophils, as well as IL-33^{+} epithelial cells. Moreover, the GLP-1R agonist prevented airway inflammation and did not impact viral load, anti-viral interferon and antibody production during secondary RSV infection. Relative to the respective mock-infected groups, RSV-infected GLP-1R agonist-treated mice did not have increased weight loss compared to vehicle treated mice. Lastly, a phenome-wide association study (PheWAS) identified a link between GLP-1R signaling and acute bronchitis and bronchiolitis in humans. This report is the first to identify an FDA-approved pharmacologic agent that inhibits lung IL-33 release and demonstrate that GLP-1R signaling may be a novel treatment strategy for RSV bronchiolitis.

Materials and Methods

Virus and Mice

RSV strain 12/12-6 was isolated in 2012 from a hospitalized infant with severe lower respiratory tract infection and bronchiolitis as part of the INSPIRE study.[211] RSV was propagated and titrated in HEp-2 cells as previously described.[212] Mock inoculum was prepared by collecting cell culture supernatant from lysed, uninfected HEp-2 cells. 8 week old female BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Il33*$^{Citrine/+}$ reporter mice were generated by crossbreeding WT BALB/c

mice and *Il33*$^{Citrine/Citrine}$ mice that were the kind gift of Dr. Andrew N.J. McKenzie.[213] Mice were maintained under specific pathogen free conditions and used in compliance with the revised 2011 Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.[214] For infection, mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution and inoculated via intranasal delivery with 9 x 10^5 PFU of RSV 12/12-6 or an equal volume of mock inoculum as previously described.[215] Weight loss was measured daily. The GLP-1R agonist liraglutide (Novo Nordisk, Plainsboro, NJ) was initiated at the same dose that is used in patients with Type II Diabetes. This was increased in two-fold increments daily until the final dose of 0.2 mg/kg weight was reached. The vehicle for liraglutide, 0.1% BSA in PBS, was used as a control. Treatment was given subcutaneously beginning 2 days prior to infection or beginning on the same day as infection and given twice daily until the mice were euthanized.

BAL and PAS Staining

0.8 mL saline was instilled through a tracheostomy tube and withdrawn via syringe to obtain BAL fluid. Total cells were counted on a hemocytometer by using trypan blue exclusion. For cell differentials, 0.1 mL of BAL fluid was prepared via cytospin (Thermo Fisher Scientific, Waltham, MA) and subsequently fixed and stained using DiffQuik (American Scientific Products, Columbus, OH). Differential counts were based on counts of 200 cells, using standard morphological criteria to visualize neutrophils, eosinophils, lymphocytes, or macrophages. For PAS staining, lungs were

perfused with PBS, inflated with 10% neutral buffered formalin, and fixed in 10% neutral buffered formalin for 24 hours at room temperature. Lungs were then paraffin embedded, sectioned (5 μm), and stained with periodic acid-Schiff (PAS) to visualize mucus. Small and medium sized airways were scored for mucus by a trained pathologist blinded to the experimental information using the following scoring scheme: (0) no PAS positive cells observed in cross sections of medium to small airways; (1) less than 10 PAS positive cells observed in cross sections of medium to small airways; (2) greater than 10 PAS positive cells observed in cross sections of medium to small airways; or (3) greater than 10 PAS positive cells observed in cross sections of medium to small airways with mucous strands observed in air spaces.

Airway Responsiveness

Airway responsiveness was measured as previously described.[216,217] Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (85 mg/kg). A tracheostomy tube was inserted for ventilation. The internal jugular vein was cannulated for intravenous delivery of acetyl-β-methacholine. The mice were then placed in a whole body plethysmography chamber and mechanically ventilated. Precision glass microsyringes were used to deliver increasing doses of acetyl-β-methacholine. Baseline airway resistance measurements were collected followed by measurements with 137, 411, 1233, and 3700 μg/kg body weight of acetyl-β-methacholine (Sigma-Aldrich, Saint Louis, MO). Peak airway resistance measurements for each dose were recorded.

ELISA

Lungs were snap-frozen in liquid nitrogen at the time of harvest. Lungs were mechanically disrupted using 1 mL of MEM media and homogenized via BeadBeater (BioSpec Products, Bartlesville, OK). Protein measurements were performed using either Duoset (IL-33), Quantikine (IL-13, IFN-γ, and IL-27), or Verikine (IFN-α and IFN-β) enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer instructions (R&D Systems, Minneapolis, MN). Serum RSV F-protein-specific antibody was measured as previously described.[212]

Flow Cytometry

Lungs were harvested, minced, and digested in RPMI media with 5% FBS, 1 mg/mL collagenase, and 0.02 mg/mL DNase I for 40 minutes at 37°C. The digestion was stopped with 100 µl of 0.5 M EDTA, and a single cell suspension was generated by straining these digestions through a 70 µm filter. RBC lysis (BioLegend, San Diego, CA) was performed according to manufacturer instructions. Cells were stimulated in IMDM media with 10% FBS, 0.01 mM non-essential amino acids, penicillin/streptomycin, 1 mM sodium pyruvate, 10 ng/mL PMA, 1 µM ionomycin, and 0.07% monensin for 4 hours at 37°C. Cells were stained with Live/Dead Blue (Life Technologies, Carlsbad, CA) and combinations of the following surface markers: CD45 (30-F11), CD25 (PC61.5), FcεR1 (MAR-1), DX5 (DX5), FcεR1 (MAR-1), and NKG2D (CX5) from eBioscience (San Diego, CA); CD127 (SB/199), CD3 (17A2), CD146 (ME-9F1), and EpCAM (G8.8) from BioLegend (San Diego, CA); CD4 (H129.19) from BD Biosciences (San Jose, CA); and/or a surface marker cocktail containing CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-

4, and Ter-119 from Miltenyi (Bergisch Gladbach, Germany). Cells were fixed/ permeabolized for 12 hours in Cytofix/ Cytoperm (BD Biosciences) and stained with combinations of the following intracellular markers: IL-13 (eBio13A) and IFN-γ (XMG1.2) from eBioscience. Anti-FcR antibody (BD Biosciences) was used to prevent nonspecific staining. All samples were run on a BD LSR II Flow Cytometer and analyzed using FlowJo (Version 10; Treestar, Ashland, OR). ILC were defined as Lineage$^-$ CD45$^+$ CD25$^+$ CD127$^+$ cells where Lineage (Lin) includes (CD3, CD5, CD45R [B220], CD11b, Gr-1 [Ly-6G/C], 7-4, and Ter-119). ILC2 were defined as ILC that expressed IL-13. Th cells were defined as CD3$^+$ CD4$^+$ cells, basophils were defined as DX5$^+$ FcεR1$^+$ cells, NK cells were defined as CD3$^-$ DX5$^+$ cells, and epithelial cells were defined as CD45$^-$ CD146$^+$ EpCAM$^+$ cells. MFI was determined as the geometric mean.

Viral Load

Lungs were snap-frozen in liquid nitrogen at the time of harvest. Thawed lungs were resuspended in 1 mL of sterile MEM media or TRIzol reagent (Invitrogen, Carlsbad, CA) and homogenized via BeadBeater (BioSpec Products). Total RNA was isolated using TRIzol reagent and cDNA was generated for qPCR analysis of RSV-M and GAPDH. Commercially available primers and probes for GAPDH were used (Applied Biosystems, Foster City, CA). Custom primers were designed to measure RSV-M [218]. Gene expression was normalized to GAPDH before the fold change was calculated. The fold change in gene expression was calculated via the comparison of gene expression to that of lungs from RSV-infected, vehicle-treated mice.

Antibody ELISA

Ectodomain F protein from RSV A2 was fused to a GCN4 trimerization domain and a His tag and expressed in mammalian cells, as previously described. [219] Immulon 2B (Thermo Scientific, Rochester, NY) plates were coated with 150 ng of RSV F protein in PBS overnight at 4°C. Plates were blocked with 1% BSA in PBS for 1 hour at room temperature. Supernatants were serially diluted 1:2 starting at 1:80 over 6 total dilutions, and plates were incubated for 1 hour at room temperature. RSV-specific antibody was detected using horseradish peroxidase-conjugated goat anti-mouse antibody specific for mouse IgG (1:5,000), IgG1 (1:500), or IgG2a (1:500) for 1 hour at room temperature (Southern Biotech, Birmingham, AL). Plates were developed in Ultra-TMB (Pierce, Rockford, IL) and the reaction was stopped with 1M HCl. Absorbance values (450 nm) were measured and assessed using Gen 5 software (BioTk, Vinooski, VT). The serum endpoint dilution at 0.2 absorbance units above background (PBS blank) was calculated for each antibody type.

Statistical Analysis

Groups were compared using unpaired t-test, one-way analysis of variance (ANOVA) with Bonferroni post test, or two-way ANOVA with Dunn's multiple comparison test, as appropriate with GraphPad Prism (Version 5; GraphPad Software, San Diego, CA). Measurements below the limit of detection were assigned half of the value of the limit of detection for statistical comparisons.

PheWAS Study

The rs7578597 variant of *THADA*, a loss-of-function single polymorphism nucleotide (SNP) encoding thyroid adenoma-associated protein, associated with lower beta-cell response to GLP-1.[220] To investigate associations with *THADA*, we used a population of 29,713 individuals (age >18 years) of European ancestry with genotyping on Illumina HumanExome BeadChip version 1.1 and available electronic health record (EHR) data from the Vanderbilt BioVU DNA biobank.[221] This platform contained the *THADA* SNP rs7578597, which corresponds to a threonine to alanine in multiple splice variants. The minor T allele of rs7578597 was present in 11% of individuals. We then evaluated all phenotypes defined using a PheWAS of this SNP using previously described methods.[222] Briefly, the method defines cases for 1,000 phenotypes by the presence of specific *International Classification of Diseases, Ninth Revision* (ICD9), codes on at least two different days. Controls for each phenotype are defined as individuals who lack case ICD9 codes and other codes that are related. For example, cases of the “acute bronchitis and bronchiolitis” phenotype are defined with the ICD9 code 483, while its controls are defined as absence of the 483 ICD9 codes. We used version 1.2 of the PheWAS code terminology system and the R PheWAS package to calculate the PheWAS and graph results,[223] both of which can be downloaded from http://phewascatalog.org. We used logistic regression for each phenotype with 40 cases or more, adjusted for age and sex, assuming an additive genetic model.

Results

GLP-1R signaling attenuates RSV-induced airway inflammation, airway responsiveness, and airway mucus

We first evaluated whether the lungs of mice infected with RSV 12/12-6, a clinical isolate strain that was isolated from a hospitalized infant with severe lower respiratory tract infection and bronchiolitis, exhibited features of type 2-mediated immunopathology. Eight week old mice were infected with 9×10^5 PFU of RSV and lungs were harvested on days 6 or 8 for ELISA or PAS staining of histologic sections. RSV 12/12-6 induced significant lung IL-13 and airway mucus production, mimicking what is seen in patients with severe infection (**Figure 3.1**).

We hypothesized that GLP-1R signaling attenuates RSV-induced immunopathology. To test this hypothesis, GLP-1R agonist or vehicle was administered subcutaneously beginning 2 days prior to RSV infection and given twice daily until all endpoints (**Figure 3.2**). To assess the physiological effect of GLP-1R agonist treatment on cellular inflammation, we assessed BAL cell counts and differentials on day 6. RSV infection significantly increased the number of total BAL cells compared to mock-infected mice. GLP-1R agonist treatment significantly decreased the number of RSV-induced total BAL cells and lymphocytes compared to vehicle treatment (**Figure 3.3*A***). We next sought to determine the effect of GLP-1R signaling on airway obstruction and responsiveness which are physiological mediators of respiratory failure during severe infection. To allow time for IL-13 to induce physiologic changes in the airways, we performed methacholine challenge at day 8 post-infection. There was a significant decrease in airway responsiveness in the RSV-infected GLP-1R agonist-treated group

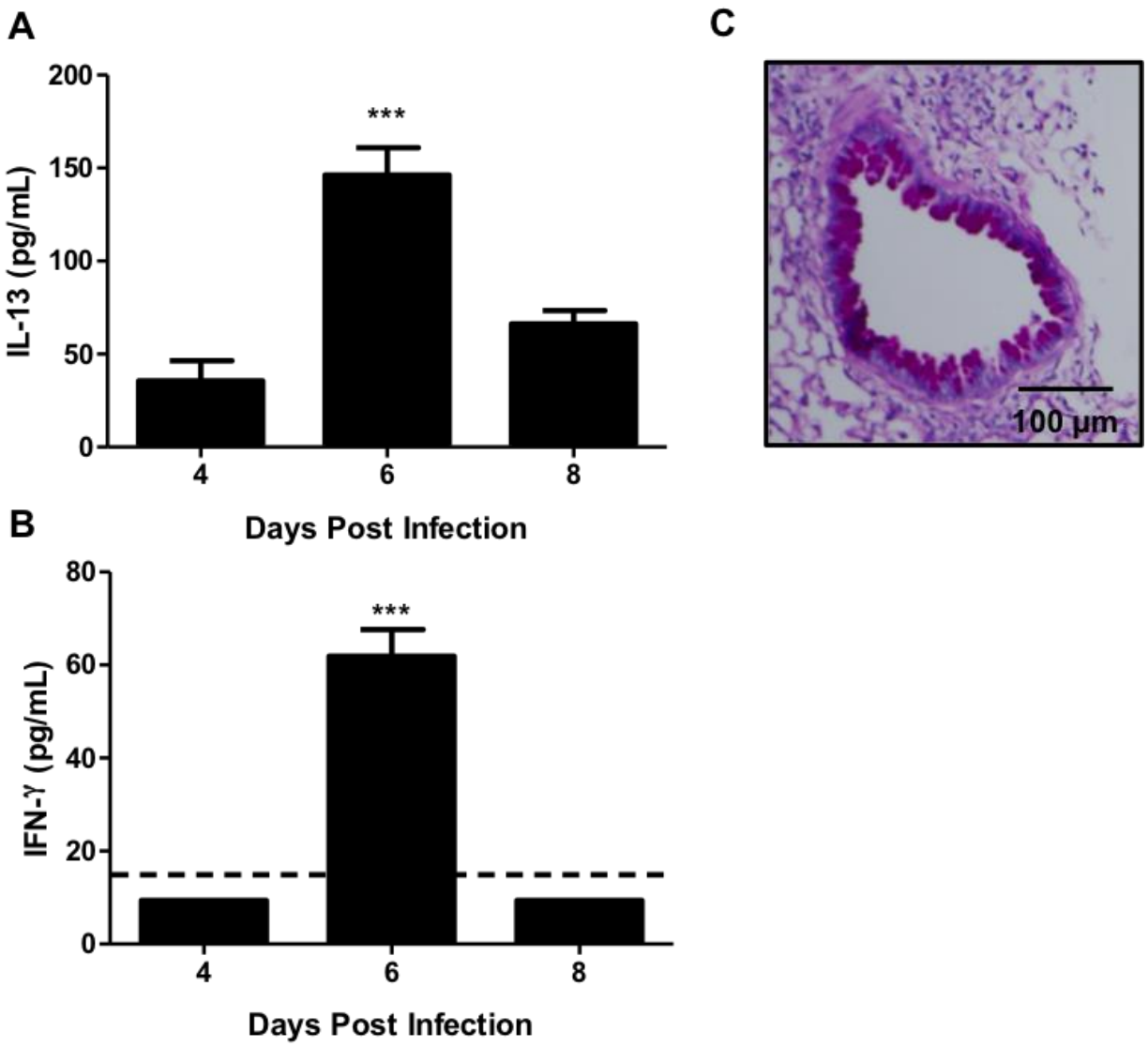

Figure 3.1. RSV 12/12-6 induces lung IL-13 and mucus production. BALB/cJ mice were infected with 9 x 10^5 PFU of RSV strain 12/12-6 and lungs were harvested for ELISA or histopathology. ELISA for (**A**) IL-13 and (**B**) IFN-γ in the whole lung homogenate (both lungs) 6 days after infection. (**C**) Representative PAS-stained section of mucus-containing airway in the lungs 8 days after infection. Data plotted as mean + SEM. n = 5 mice per group. ***p < 0.001 by one-way ANOVA. Dashed line is the limit of detection of the assay.

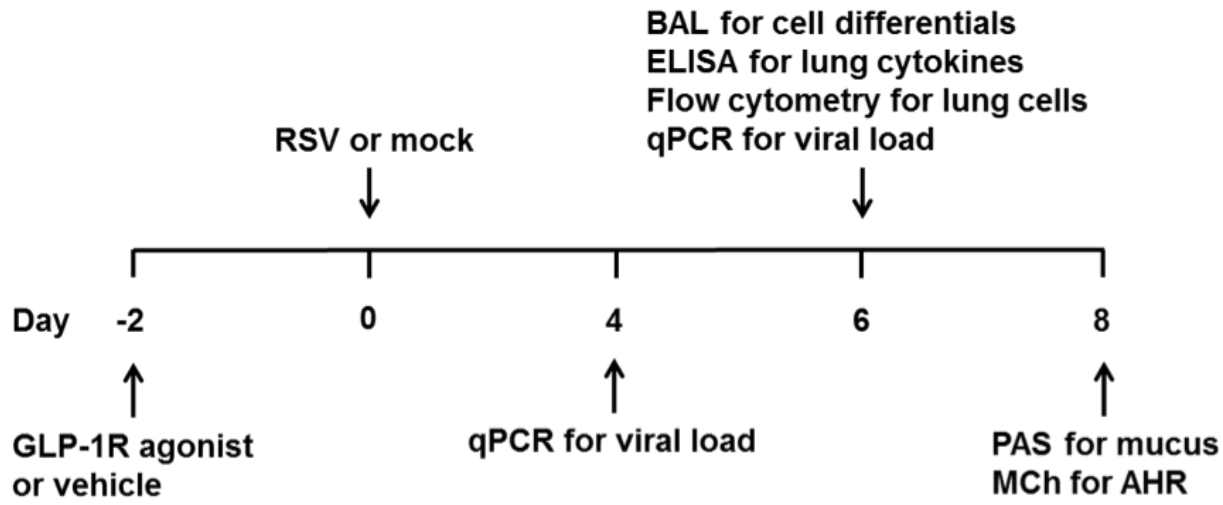

Figure 3.2. Protocol for *in vivo* administration of GLP-1R agonist or vehicle and subsequent infection with RSV or mock preparation. BALB/cJ mice were infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum on day -2. On day 0, **t**he GLP-1R agonist liraglutide or vehicle (0.1% BSA in PBS) was initiated. Treatment was given twice daily until the mice were euthanized.

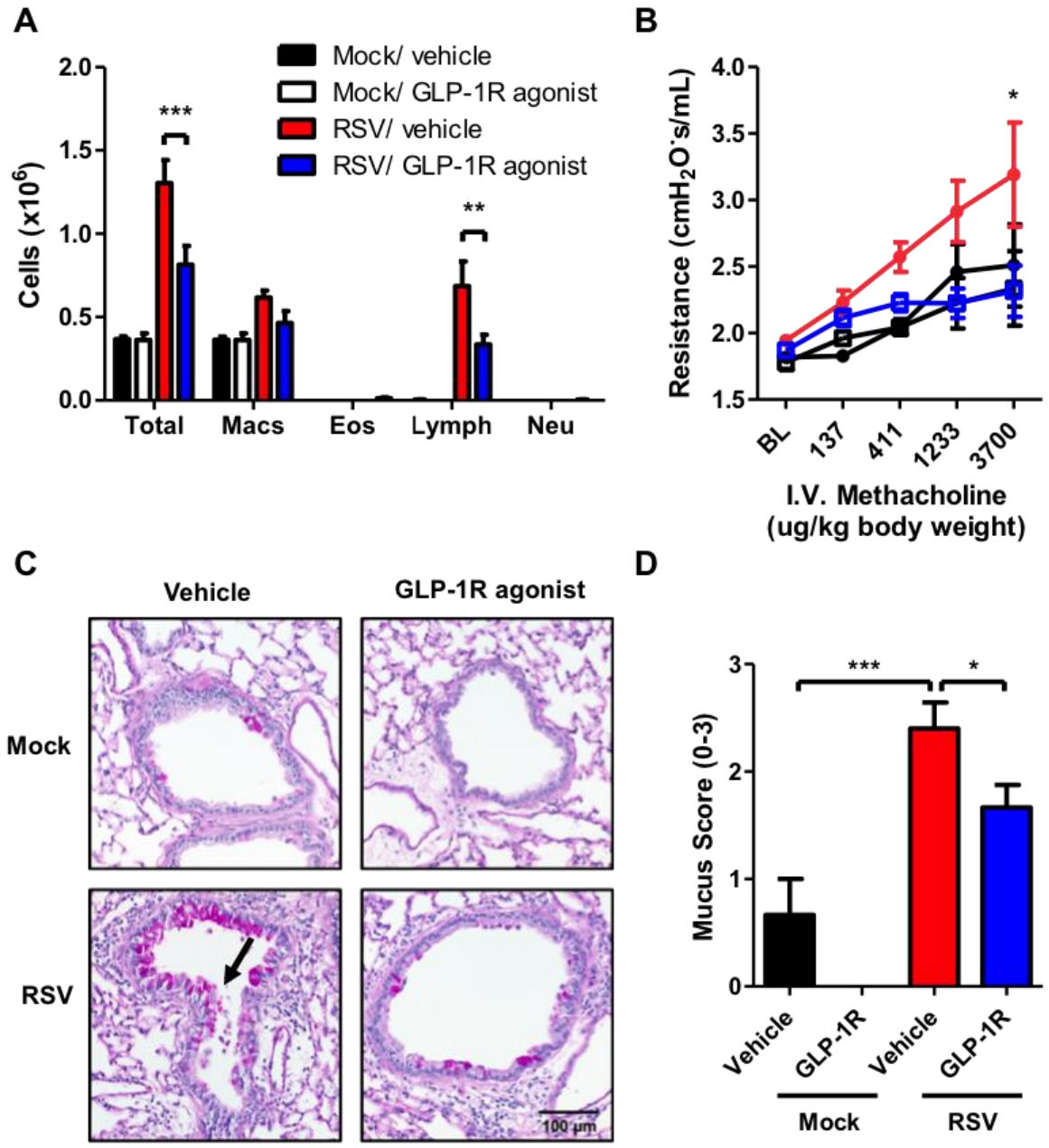

Figure 3.3. GLP-1R agonist decreases RSV-induced airway inflammation, airway responsiveness, and airway mucus accumulation. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. (**A**) BAL cell counts 6 days post-infection. (**B**) Airway responsiveness measured at baseline and increasing doses of methacholine 8 days post-infection. (**C**) Representative PAS-stained section of mucus-containing airway in the lungs 8 days post-infection (40x magnification); arrowhead denotes intraluminal mucus strand. (**D**) Quantification of airway mucus from the experiment in **C**. Data plotted as mean + SEM. n = 3-6 mice per group representative of 3 (**A**) or 2 (**C&D**) independent experiments. n = 6-12 mice per group combined from 2 independent experiments (**B**). *p < 0.05, **p < 0.01, ***p < 0.001 by two-way (**A-B**) or one-way (**D**) ANOVA. BL = baseline.

compared to RSV-infected vehicle-treated mice with increasing methacholine concentration (**Figure 3.3*B***). No significant differences in airway responsiveness were observed between mock-infected GLP-1R agonist and vehicle-treated mice. To determine the effect of GLP-1R signaling on mucous cell metaplasia and airway mucus accumulation, we evaluated histologic sections of lungs stained with PAS to detect mucus at day 8 post-infection. In mock-infected mice, there was minimal or absent mucous cell metaplasia (**Figure 3.3*C***). In RSV-infected vehicle-treated mice, there was appreciable mucous cell metaplasia and airway mucus accumulation with intraluminal mucus strands. RSV-infected GLP-1R agonist-treated mice also demonstrated some mucous cell metaplasia but they had no substantial intraluminal mucus accumulation. Collective scoring of airways from multiple mice showed a significant decrease in mucus severity score in RSV-infected GLP-1R agonist-treated mice compared to RSV-infected vehicle-treated mice (**Figure 3.3*D***). Collectively, these data show that GLP-1R signaling attenuates RSV-induced immunopathology.

GLP-1R signaling decreases the concentration of IL-13 and the numbers of IL-13-producing ILC2, Th2 cells, and basophils in the lungs

Our finding that GLP-1R agonist treatment significantly attenuated RSV-induced immunopathology led us to hypothesize that GLP-1R signaling suppresses RSV-induced type 2 responses. To determine the effect of GLP-1R on type 2 cytokine production, we evaluated lung IL-13 expression 6 days post-infection, at the height of lung IL-13 during RSV infection. RSV infection significantly increased lung IL-13 production compared to mock-infected mice (**Figure 3.4*A***). RSV-infected GLP-1R

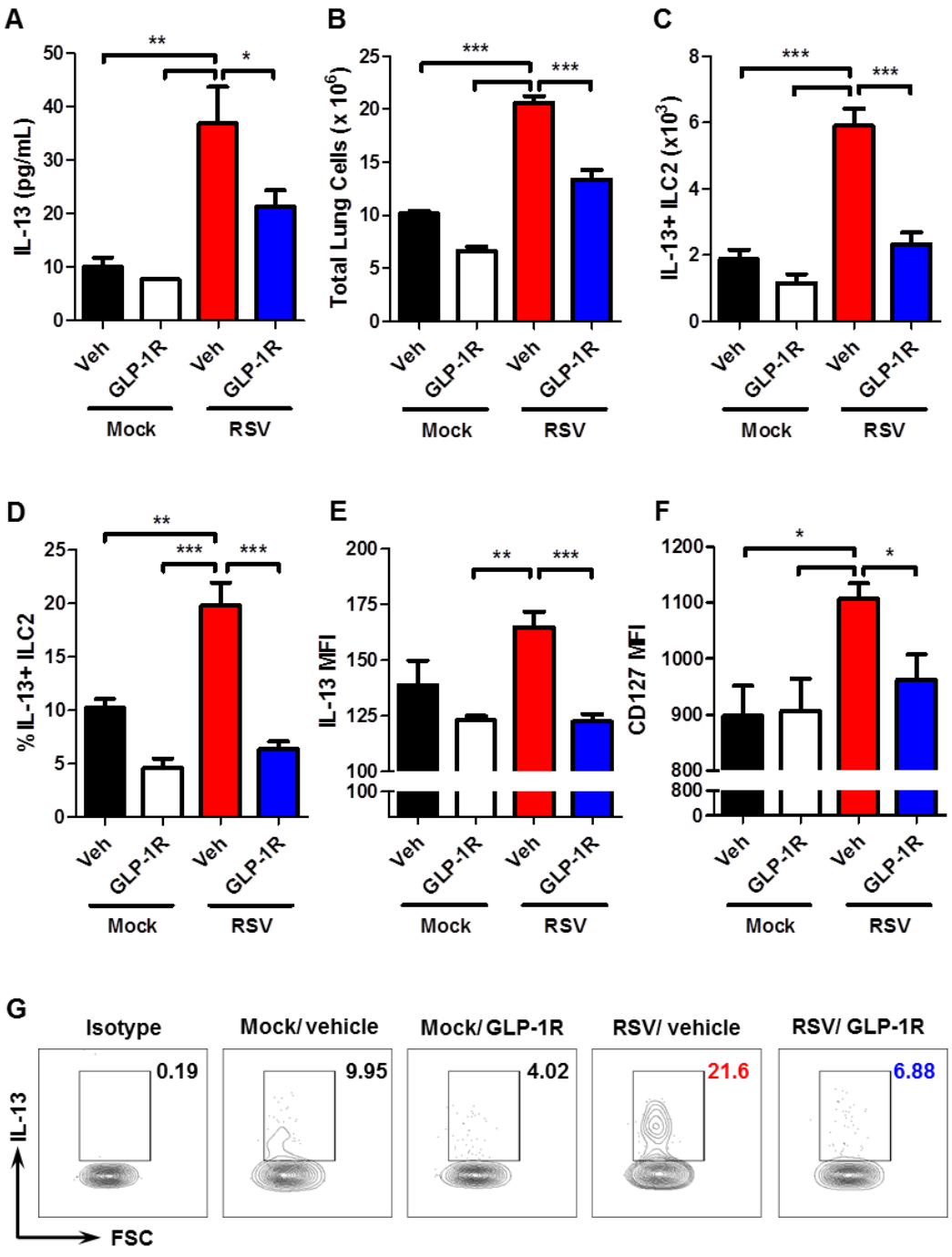

Figure 3.4. GLP-1R agonist decreases whole lung IL-13 accumulation and IL-13-producing ILC2 6 days after RSV infection. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. (**A**) ELISA for IL-13 in whole lung homogenate (right lung only). (**B**) Total number of live lung cells, (**C**) total number of IL-13^+ ILC2, and (**D**) percent of ILC that are IL-13^+. MFI of (**E**) IL-13 and (**F**) CD127 staining in ILC2. (**G**) Representative IL-13 expression measured by flow cytometry in ILC2. Data plotted as mean + SEM. n = 3-6 mice per group representative of 3 (**A**) or 2 (**B-F**) independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA.

agonist-treated mice had significantly decreased levels of lung IL-13 compared to RSV-infected vehicle-treated mice. Administration of GLP-1R agonist beginning on the same day of RSV infection also decreased lung IL-13 and airway mucus production (**Figure 3.5**). We next sought to identify the cellular sources of IL-13 that GLP-1R signaling was inhibiting. ILC2 have recently been identified as important producers of IL-13 during RSV infection.[207,208] No single surface marker(s) is presently known to identify ILC2 exclusively. We identified innate lymphocytes (ILC) by flow cytometry as hematopoietic lineage marker (CD3, CD5, B220, CD11b, Gr-1, 7-4, and Ter-119) negative $CD45^+$ $CD25^+$ $CD127^+$ and ILC2 as ILC that are $IL\text{-}13^+$ (**Figure 3.6*A***).[224] There were significant increases in the total number of cells in the lung, the total number of ILC2, and the percentage of ILC that were $IL\text{-}13^+$ in the RSV-infected group compared to the mock-infected group at day 6 post-infection (**Figure 3.4*B-D***). GLP-1R agonist treatment in RSV-infected mice significantly decreased the total number of cells in the lung, the total number of ILC2, and the percentage of ILC that were $IL\text{-}13^+$ compared to RSV-infected vehicle-treated mice (**Figure 3.4*G***). There was significantly decreased MFI of IL-13 and CD127 in the ILC2 of RSV-infected GLP-1R agonist-treated mice compared to RSV-infected vehicle-treated mice, indicating decreased IL-13 production and CD127 expression on a per ILC2 basis with GLP-1R agonist treatment (**Figure 3.4*E-F*** & **Figure 3.7**). These data demonstrate that GLP-1R signaling inhibits type 2 immune responses, and specifically IL-13-producing ILC2, during RSV infection.

Next we enumerated the number of total $CD4^+$ T cells and basophils to determine the effect of GLP-1R signaling on the other major cellular sources of IL-13 during RSV infection. The gating strategy for $IL\text{-}13^+$ Th2 cells and basophils are shown in **Figure**

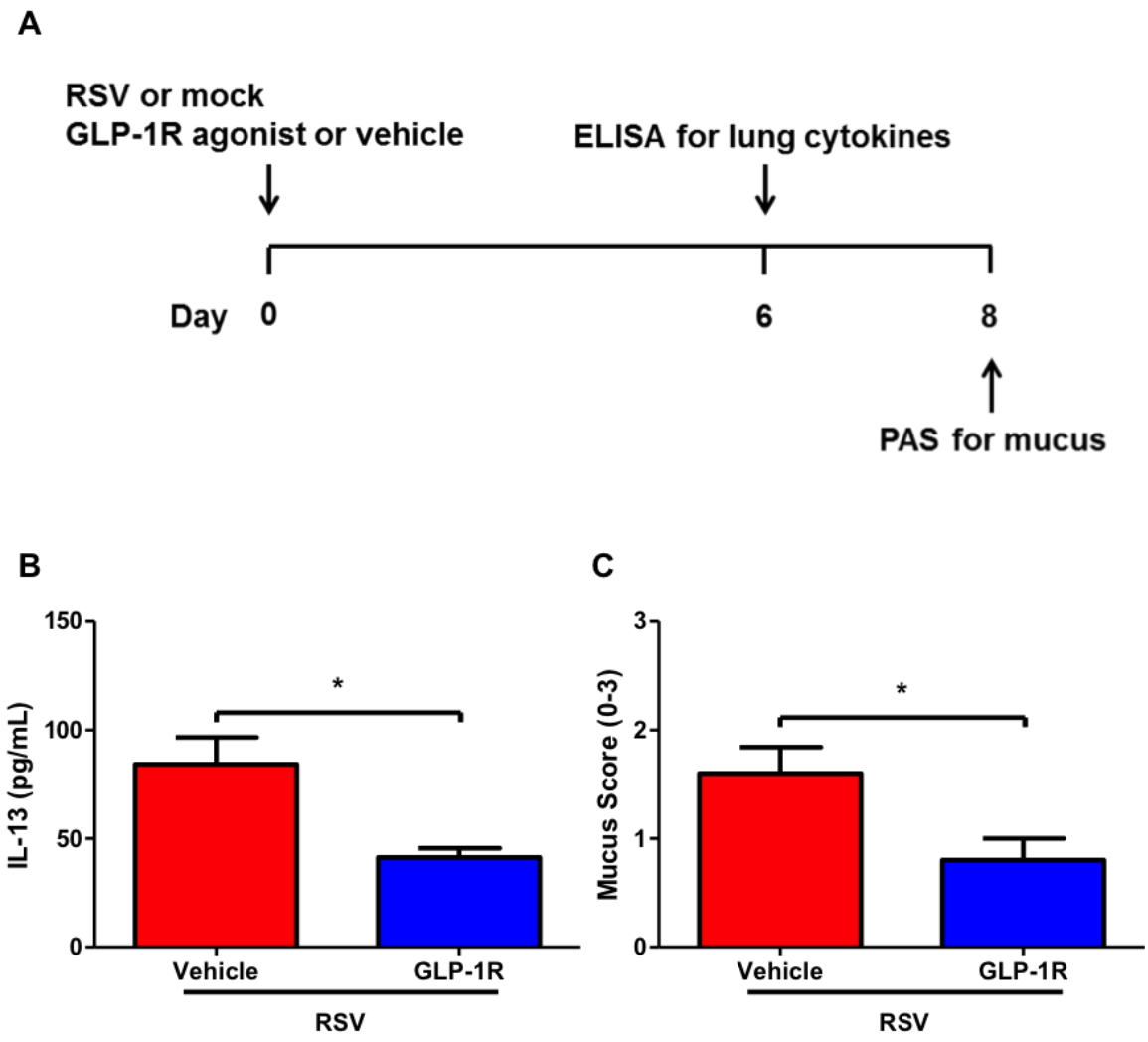

Figure 3.5. GLP-1R agonist decreases RSV-induced whole lung IL-13 accumulation and airway mucus production. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. (**A**) Protocol for *in vivo* administration of GLP-1R agonist or vehicle and simultaneous infection with RSV or mock preparation. (**B**) ELISA for IL-13 in whole lung homogenate 6 days after infection. (**C**) Quantification of airway mucus in the lungs 8 days post-infection. Data plotted as mean + SEM. n = 5 mice per group. *$p < 0.05$ by unpaired t-test.

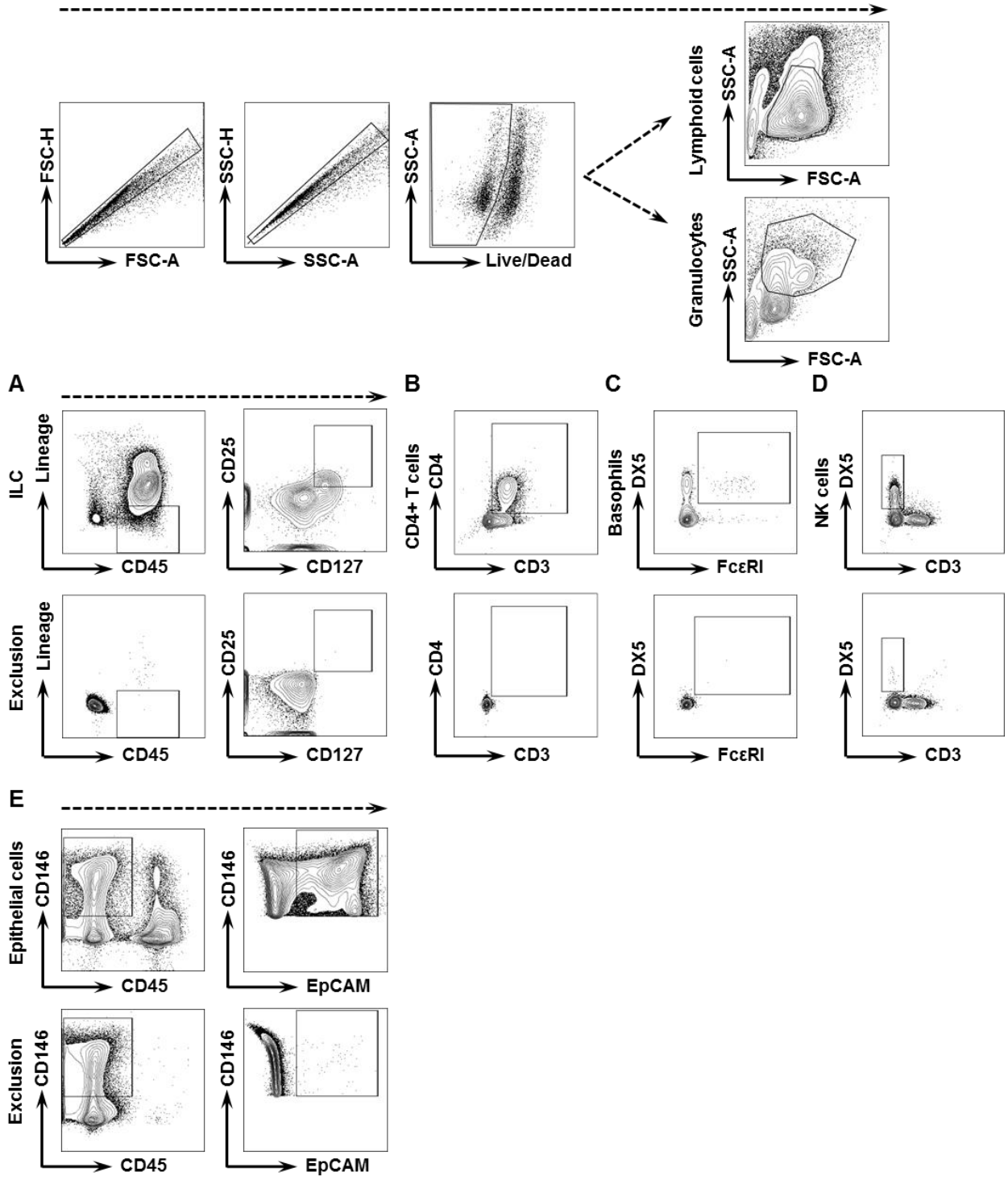

Figure 3.6. Flow gating for ILC, CD4+ T cells, NK cells, basophils, and epithelial cells. (**A**) ILC were defined as viable Lin^- $CD45^+$ $CD25^+$ $CD127^+$ cells. (**B**) CD4+ T cells were defined as viable $CD3^+$ $CD4^+$ cells. (**C**) Basophils were defined as viable $Fc\varepsilon RI^+$ $DX5^+$ cells. (**D**) NK cells were defined as viable $DX5^+$ $CD3^-$ cells. (**E**) Epithelial cells were defined as viable $CD45^-$ $CD146^+$ $EpCAM^+$ cells.

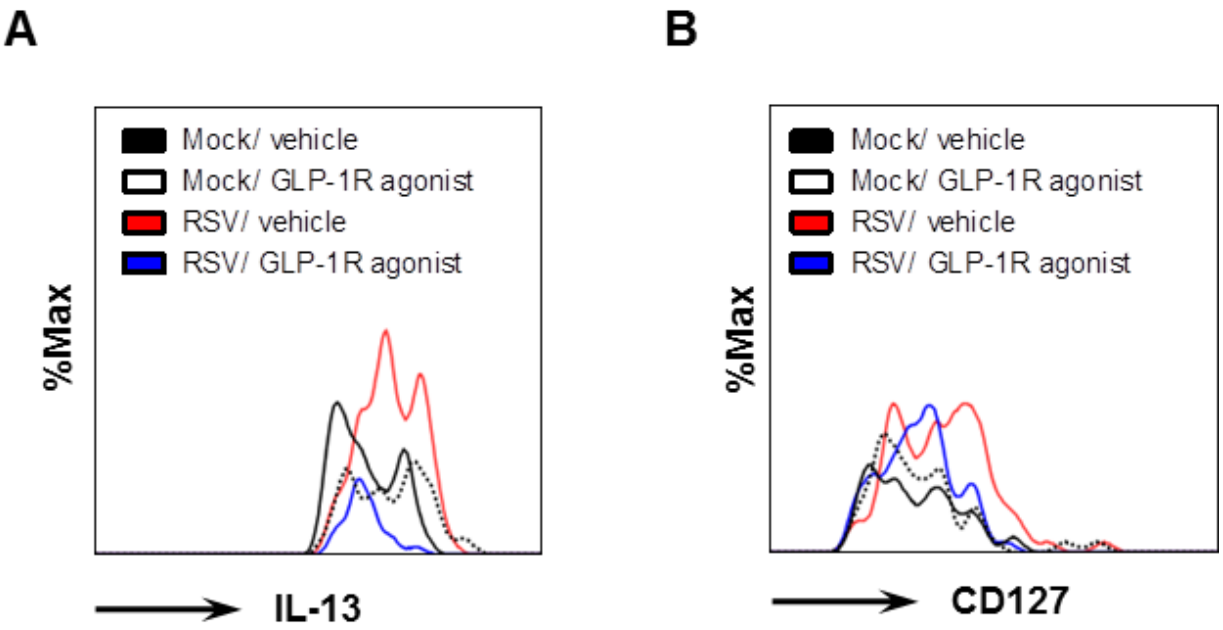

Figure 3.7. GLP-1R agonist decreases MFI of IL-13 and CD127 staining in ILC2 6 days after RSV infection. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. Representative MFI of (**A**) IL-13 and (**B**) CD127 staining in ILC2 by flow cytometry.

3.6*B* and *C*. $CD4^+$ T cells were defined as $CD3^+$ $CD4^+$ cells, and basophils were defined as $DX5^+$ $Fc\varepsilon R1^+$ cells. The total numbers of $CD4^+$ T cells and basophils as well as IL-13^+Th2 cells and basophils were significantly increased in RSV-infected mice compared to mock-infected mice (**Figure 3.8**). GLP-1R agonist treatment in RSV-infected mice significantly decreased the numbers of $CD4^+$ cells and basophils as well as IL-13^+ Th2 cells and basophils compared to RSV-infected vehicle-treated mice (**Figure 3.8**). Together, these data indicate that inhibition of IL-13-producing ILC2, Th2 cells, and basophils collectively contribute to the decrease in total lung IL-13 seen with GLP-1R agonist treatment.

GLP-1R signaling inhibits the production of IL-33 and the number of IL-33-producing epithelial cells in the lungs

IL-33 activates type 2 cytokine-producing immune cells including ILC2, Th2, and basophils. To determine whether GLP-1R agonist treatment mediates IL-33 production during RSV infection, we measured the whole lung concentration of IL-33 protein 12 hours post-infection, at the height of lung IL-33 during RSV infection.[207] RSV infection significantly increased the total concentration of IL-33 in the lungs compared to mock-infected mice (**Figure 3.9*A***). RSV-infected GLP-1R agonist-treated mice had significantly decreased levels of lung IL-33 compared to RSV-infected vehicle-treated mice. To examine the effect of GLP-1R signaling on IL-33 expression on a per epithelial cell basis, we used *Il33*$^{Citrine/+}$ reporter mice. Epithelial cells were defined as $CD45^-$ $CD146^+$ $EpCAM^+$ cells (**Figure 3.6*E***). There were significant increases in the total number of cells in the lung and the percentage of epithelial cells that were IL-33^+ in the

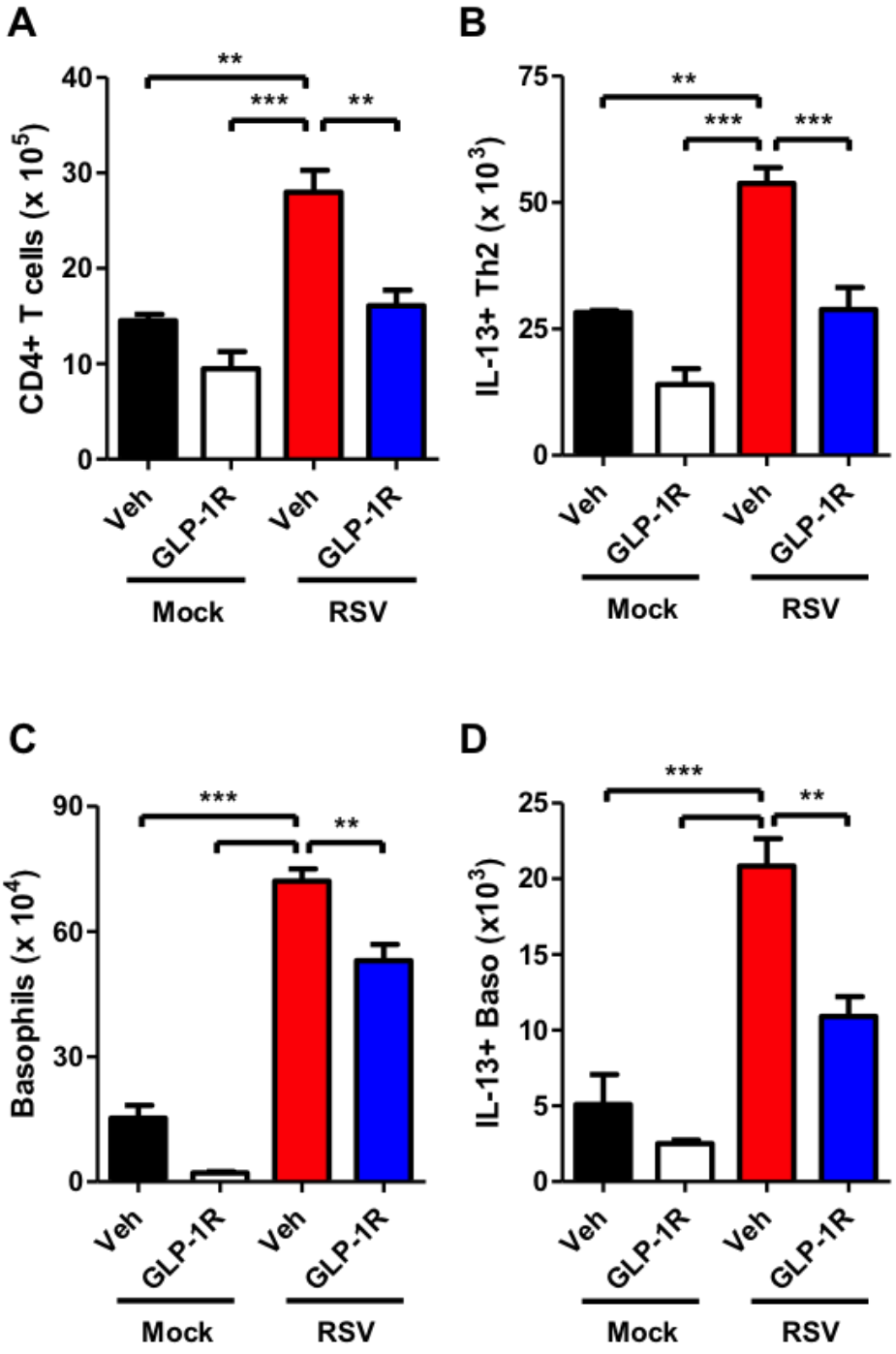

Figure 3.8. GLP-1R agonist decreases IL-13-producing Th2 cells and basophils 6 days after RSV infection. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. (**A**) Total number of $CD4^+$ Th2 cells, (**B**) IL-13^+ Th2 cells, (**C**) basophils, and (**D**) IL-13^+ basophils. Data plotted as mean + SEM. n = 3-6 mice per group representative of 2 independent experiments. **p < 0.01, ***p < 0.001 by one-way ANOVA.

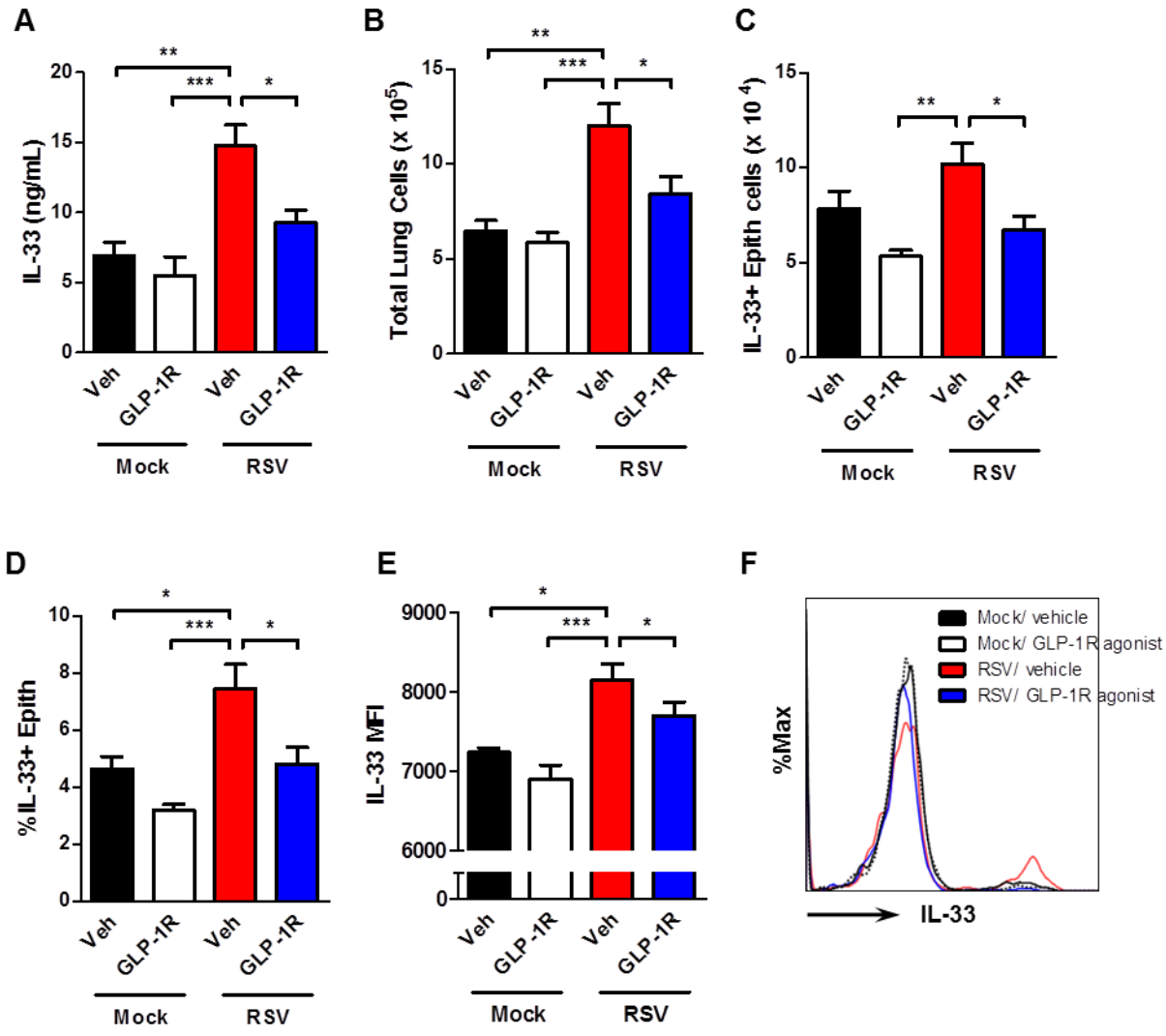

Figure 3.9. GLP-1R agonist decreases whole lung IL-33 accumulation and IL-33-producing epithelial cells 12 hours after RSV infection. *Il33*$^{Citrine/+}$ reporter mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. (**A**) ELISA for IL-33 in whole lung homogenate (left lung only). (**B**) Total number of live lung cells, (**C**) total number of IL-33$^+$ epithelial cells, and (**D**) percent of epithelial cells that are IL-33$^+$. MFI of (**E**) IL-33 expression in epithelial cells. (**F**) Representative IL-33 expression measured by flow cytometry in epithelial cells. Data plotted as mean + SEM. n = 3-6 mice per group representative of 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA.

RSV-infected group compared to the mock-infected group at day 6 post-infection (**Figure 3.9*B-D***). GLP-1R agonist treatment in RSV-infected mice significantly decreased the total number of cells in the lung, the total number of IL-33-producing epithelial cells, and the percentage of epithelial cells that were IL-33^{+} compared to RSV-infected vehicle-treated mice. There was significantly decreased MFI of IL-33 in the epithelial cells of GLP-1R agonist-treated mice compared to vehicle-treated mice, indicating decreased IL-33 production on a per epithelial cell basis with GLP-1R agonist treatment (**Figure 3.9*E-F***). These data indicate that GLP-1R agonist treatment inhibits IL-33-producing epithelial cells during RSV infection.

GLP-1R signaling does not impact weight loss, viral load, or anti-viral interferon responses during RSV infection

We next sought to determine the effect of GLP-1R signaling on anti-viral responses during RSV infection. To determine whether GLP-1R agonist treatment had a deleterious effect on viral-associated disease severity parameters, we evaluated weight loss after infection. Relative to the respective mock-infected groups, RSV-infected GLP-1R agonist-treated mice did not have increased weight loss compared to vehicle-treated mice (**Figure 3.10*A***). Next, we sought to determine whether GLP-1R agonist treatment altered viral load, an indicator of RSV disease severity.[225] There were no significant differences in the viral load at days 4 or 6 after RSV infection between RSV-infected GLP-1R agonist and vehicle-treated mice (**Figure 3.10*B***). Consistent with these data, we did not observe any significant differences in lung IFN-γ expression or IFN-γ^{+} Th1 and NK cells between RSV-infected GLP-1R agonist and vehicle-treated mice 6 days

post-infection (**Figure 3.10*C-E***). The gating strategy for IFN-γ^+ Th1 and NK cells are shown in **Figure 3.6*B* and *D***. NK cells were defined as $CD3^-$ $DX5^+$ cells. Further, there were no significant differences in lung IFN-α, IFN-β, or IL-27 production 12 hours post-infection (**Figure 3.11**).

GLP-1R signaling prevents airway inflammation and does not impact RSV F-protein specific antibody responses during secondary RSV infection

To determine whether GLP-1R agonist treatment during primary infection has an impact on the immune response to a later secondary infection, we infected mice with RSV a second time 30 days following primary RSV infection (**Figure 3.12**). The mice were assessed 6 days after secondary RSV infection. GLP-1R analog treatment during primary infection significantly decreased the number of RSV-induced total BAL cells and lymphocytes compared to vehicle treatment after secondary infection (**Figure 3.13*A***). The mice treated with GLP-1R agonist during primary infection did not exhibit altered lung IFN-γ expression compared to vehicle-treated mice during secondary RSV infection (**Figure 3.13*B***). This was consistent with the unaltered lung IFN-γ production between GLP-1R agonist and vehicle-treated mice during primary RSV infection (**Figure 3.10*C***). GLP-1R agonist treatment during primary infection did not alter RSV F-protein-specific antibody responses following secondary infection (**Figure 3.13*C-E***). This was consistent with the unaltered viral load between RSV-infected GLP-1R agonist and vehicle-treated mice (**Figure 3.10*B***). Collectively, these data demonstrate that GLP-1R agonist treatment does not exacerbate disease or impede anti-viral responses,

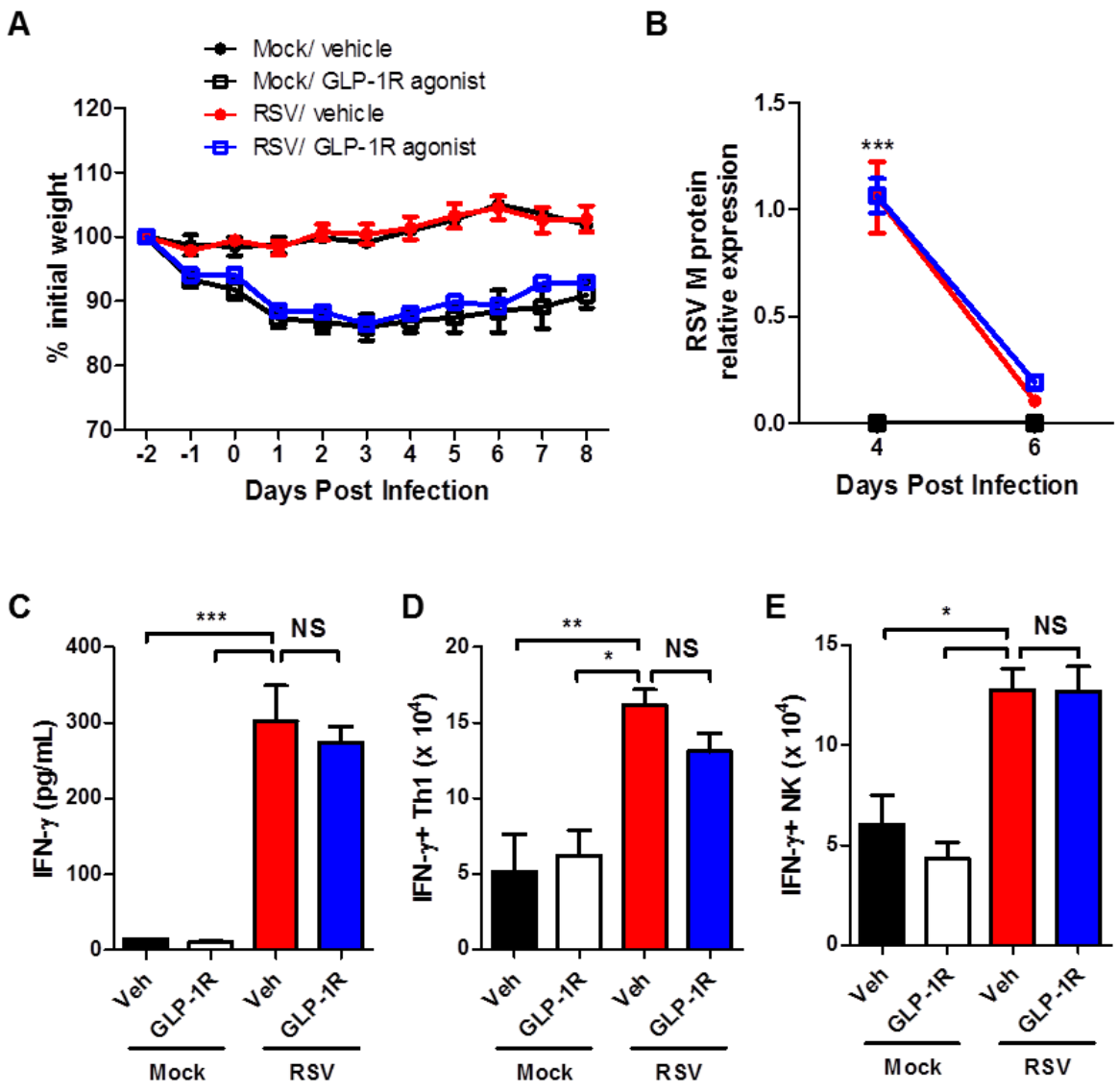

Figure 3.10. GLP-1R agonist does not have negative effects on weight loss, viral load, or IFN-γ production 6 days after RSV infection. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. (**A**) Percent of original body weight. (**B**) Lung mRNA levels for RSV M protein were determined on days 4 and 6 after infection and normalized to GAPDH. (**C**) ELISA for IFN-γ in whole lung homogenate (right lung only). (**D**) Total number of IFN-γ^+ Th1 and (E) IFN-γ^+ NK cells 6 days after infection. Data plotted as mean + SEM. n = 3-6 mice per group representative of 2 (**A, C-E**) or 3 (**B**) independent experiments. *$p < 0.05$ and **$p < 0.01$ by two-way ANOVA. NS = not significant.

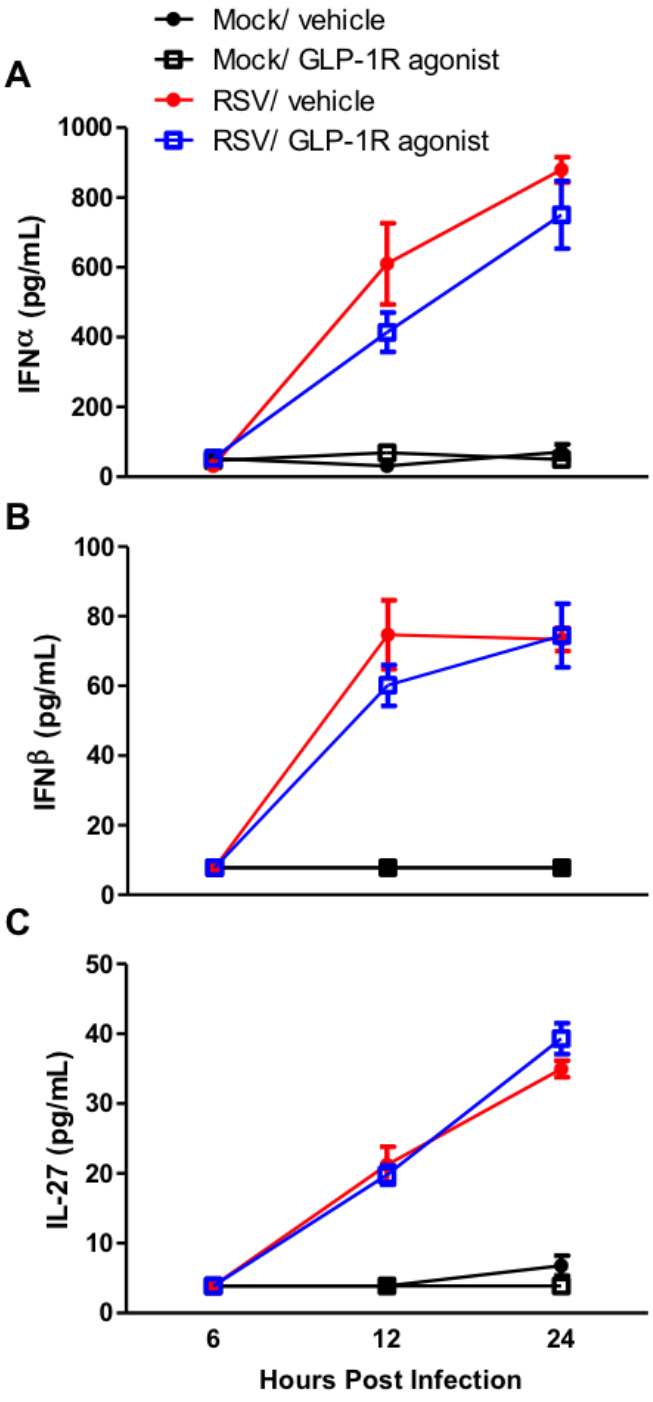

Figure 3.11. GLP-1R agonist does not have negative effects on early interferon responses during RSV infection. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 105 PFU of RSV strain 12/12-6 or mock inoculum. ELISAs for (**A**) IFN-α, (**B**) IFN-β, and (**C**) IL-27 in whole lung homogenate. Data plotted as mean + SEM. n = 3-6 mice per group representative of 3 experiments. Two-way ANOVA.

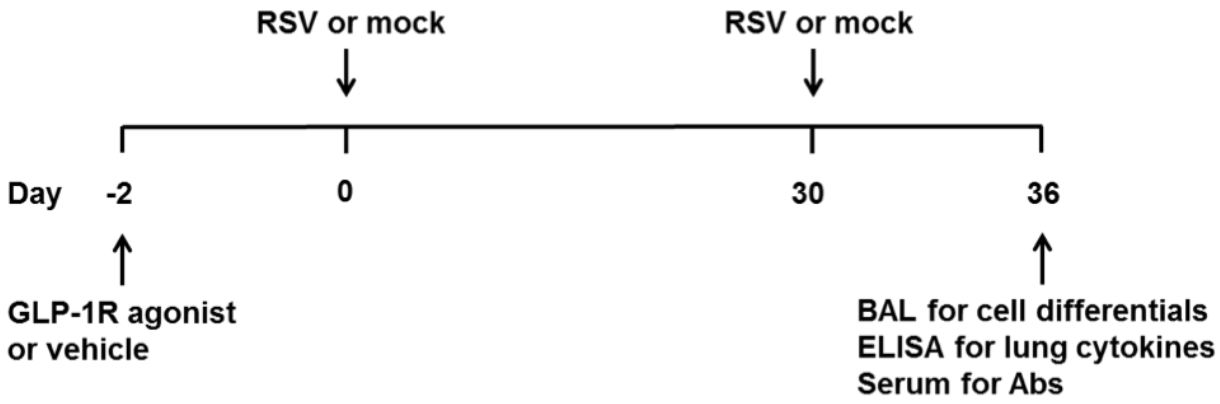

Figure 3.12. Protocol for administration of GLP-1R agonist or vehicle, primary RSV or mock infection, and subsequent secondary RSV or mock infection.
BALB/cJ mice were treated with GLP-1R agonist or vehicle beginning on day -2 and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum on day 0. Treatment was given twice daily until day 8. On day 30, mice were infected a second time with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum.

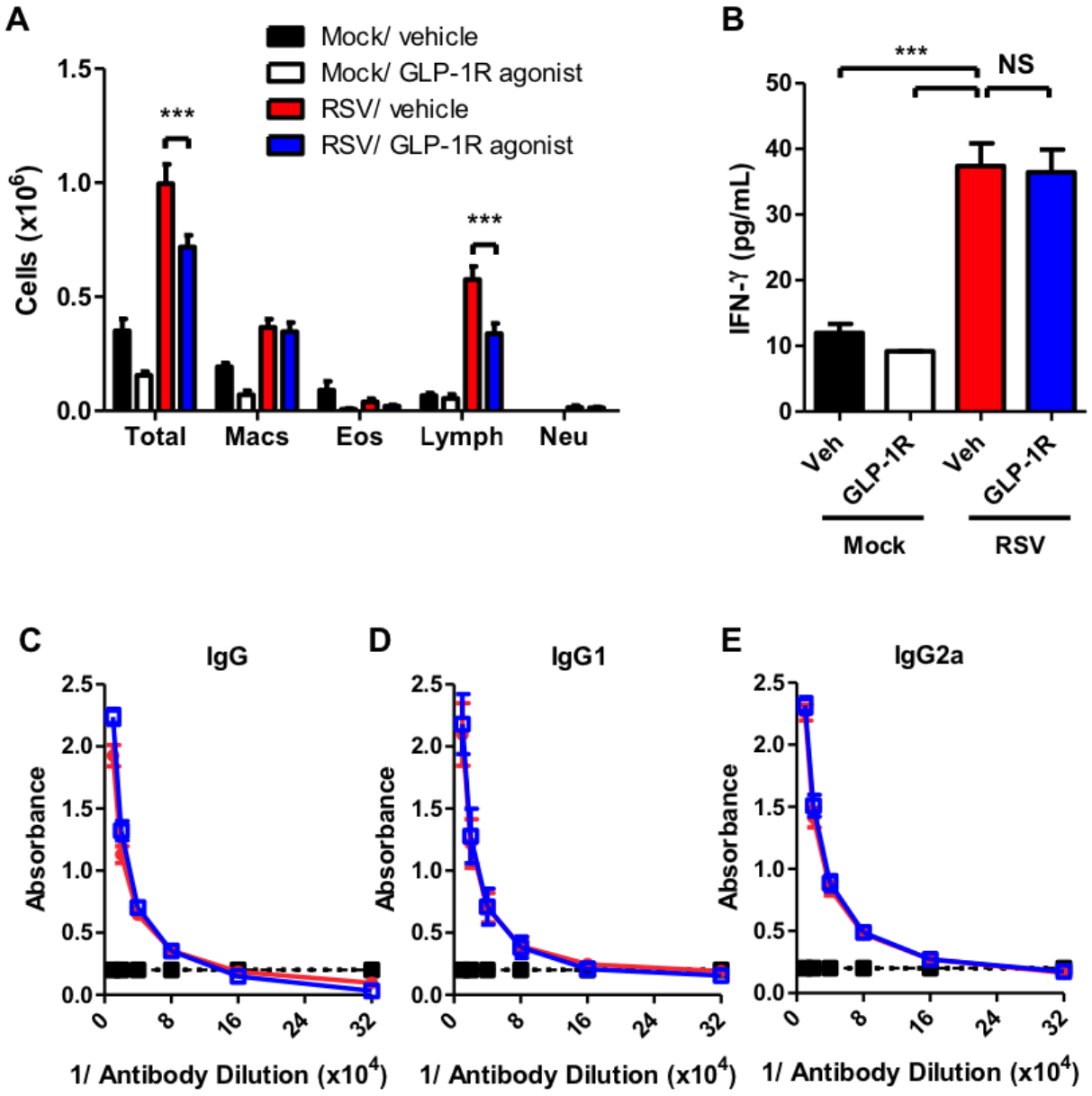

Figure 3.13. GLP-1R agonist treatment during primary infection prevents airway inflammation and does not have negative effects on antibody responses or IFN-γ production during secondary RSV infection. BALB/cJ mice were treated with GLP-1R agonist or vehicle and infected with 9 x 10^5 PFU of RSV strain 12/12-6 or mock inoculum. Mice were re-infected 30 days after primary infection, and serum was collected 6 days after secondary infection. (**A**) BAL cell counts 6 days post-infection. (**B**) ELISA for IFN-γ in whole lung homogenate (right lung only). ELISA for RSV F-protein-specific (**C**) IgG, (**D**) IgG1, and (**E**) IgG2a. Data plotted as mean + SEM. n = 6-12 mice per group combined from 2 independent experiments. One-way ANOVA. NS = not significant.

and implicate GLP-1R agonist as a potential therapeutic for attenuating type 2-associated immunopathology during RSV infection.

Human PheWAS analysis identifies an association between GLP-1 signaling and acute bronchitis and bronchiolitis

Based on the GLP-1R signaling-mediated attenuation of type 2-associated immunopathology during RSV infection, we next sought to identify associations between GLP-1R and human RSV disease. The rs7578597 variant of *THADA*, a loss-of-function single polymorphism nucleotide (SNP) encoding thyroid adenoma-associated protein, associated with lower beta-cell response to GLP-1.[220] We performed a phenome-wide association study on the *THADA* SNP rs7578597 (missense, T1187A) and 1,000 phenotypes in 29,713 individuals of European ancestry (EA) from the Vanderbilt BioVU biobank following previously validated methods.[226] The previously reported association with diabetes mellitus was confirmed (odds ratio [OR] = 0.88, $P = 1.4 \times 10^{-3}$; **Table 3.1**). A highly significant association of rs7578597 with acute bronchitis and bronchiolitis was identified (OR = 1.24, $P = 6.3 \times 10^{-3}$; **Figure 3.14** and **Table 3.1**). There were 1,223 acute bronchitis and bronchiolitis cases and 22,347 controls. Interestingly, rs7578597 also significantly associated with two other respiratory phenotypes: acute pharyngitis (OR = 1.31 $P = 5.1 \times 10^{-3}$) and postinflammatory pulmonary fibrosis (OR = 1.43, $P = 9.4 \times 10^{-3}$; Table 3.1).

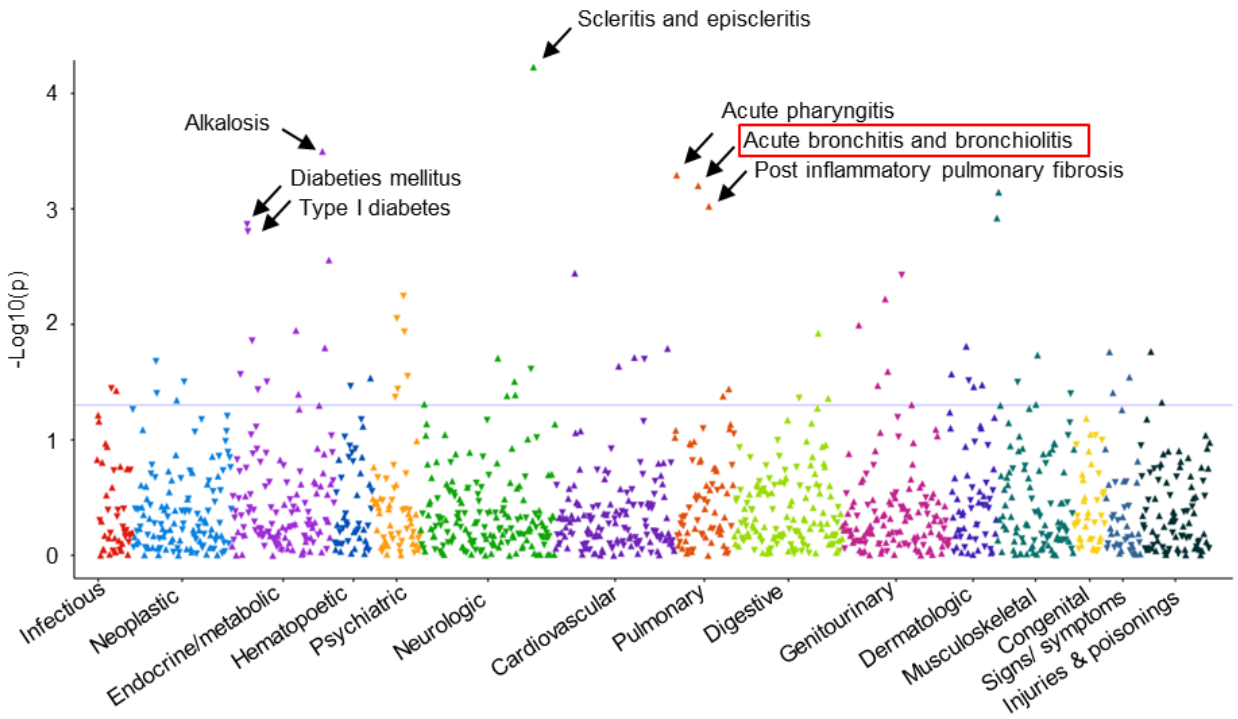

Figure 3.14. Phenome-wide association study (PheWAS) plot for *THADA* rs7578597 shows significant association between THADA and acute bronchitis and bronchiolitis in humans. Each panel represents 1,000 phenotypes tested for association with THADA rs7578597, using logistic regression assuming an additive genetic model adjusted for age, sex, study site, and the first 3 principal components. Phenotypes were grouped along the x axis by categorization within the PheWAS code hierarchy. rs7578597 associated with acute bronchitis and bronchiolitis associated (OR = 1.24, P = 6.3×10^{-3}).

Phenotype	Cases	Controls	Odds ratio	p
Scleritis and episcleritis	67	25573	2.33	0.00006
Alkalosis	187	18873	1.67	0.00032
Acute pharyngitis	798	22135	1.31	0.00051
Acute bronchitis and bronchiolitis	1223	22347	1.24	0.00063
Postinflammatory pulmonary fibrosis	349	19926	1.43	0.00094
Diabetes mellitus	5032	21287	0.88	0.00136
Type 1 diabetes	468	21287	0.67	0.00157
Morbid obesity	972	24650	1.23	0.00276
Other forms of chronic heart disease	1095	21791	1.22	0.00359
Other disorders of male genital organs	243	9538	0.58	0.00374
Personality disorders	107	20065	0.41	0.00566
Other symptoms/disorders or the urinary system	4228	21638	1.11	0.00600
Anxiety, phobic and dissociative disorders	3229	20065	0.89	0.00884

Table 3.1. PheWAS results for THADA rs7578597 in humans. All phenotypes with $p<0.01$ are reported below.

Discussion

RSV infection is the leading cause of hospitalization in infants and an important cause of morbidity and mortality in elderly and high-risk adults. Moreover, severe RSV bronchiolitis is a significant risk factor for childhood asthma,[87,227,228] and RSV infection is a major cause of asthma exacerbation in both children and adults.[229] Current therapeutic options are limited. Ribavirin, a nucleoside analog that inhibits viral replication, demonstrated poor effectiveness in treating RSV-induced disease.[230] Corticosteroids are also ineffective during viral-induced bronchiolitis, failing to reduce the number of hospital admissions, length of stay, or disease severity.[113,231,232] The majority of treatment strategies are thus supportive, focusing on fluid and respiratory maintenance. The only FDA-approved preventative therapy is palivizumab, an antibody directed against the surface-exposed RSV fusion protein, which given as prophylaxis decreases the number of RSV-associated hospitalizations by 55%.[114,115] While effective, palivizumab is currently only recommended for infants in the first year of life with chronic lung disease, with hemodynamically significant congenital heart disease, or born significantly premature (< 29 weeks).[233] Moreover, decades of research have failed to yield a safe and effective RSV vaccine. Recent reports indicate that FDA-approved GLP-1R agonists exhibit immunoregulatory properties, which has led to the first clinical trials studying their effect as anti-inflammatory compounds. Our study demonstrates for the first time that GLP-1R signaling is efficacious in inhibiting RSV-induced immunopathology, including IL-33 and IL-13 production as well as airway inflammation, responsiveness, and mucus production.

The effect of GLP-1R signaling on the function of cells that produce type 2 cytokines during inflammation has not been described. Here we report that GLP-1R agonist treatment dramatically inhibited the function of ILC2. ILC2 are important producers of IL-13 and mediators of mucus production, and smooth muscle responsiveness during inflammatory disease states including viral and helminth infection, asthma, chronic rhinosinusitis, and atopic dermatitis.[15–19,24,26,207,234–241] GLP-1R signaling decreased the total number of ILC2 and the percentage of ILC that were IL-13^{+} during RSV infection. IL-7 signaling through CD127, the IL-7R, is required for ILC progenitors and ILC2 cells.[241] GLP-1R signaling during RSV infection decreased CD127 expression in ILC2, concurrent with a decreased IL-13 production on a per ILC2 basis. GLP-1R signaling also decreased the total numbers of IL-13-producing CD4^{+} Th2 cells and basophils. The concerted actions of innate and adaptive cellular mediators potentiate type 2-associated inflammation.[23] ILC2 are critical for generating type 2 inflammation because they recruit dendritic cells into the draining lymph node to promote Th2 cell differentiation locally in the airway wall.[21] ILC2 also help establish the adaptive type 2 response by expressing MHC and costimulatory signals that trigger type 2 cytokine production.[22] CD4+Th2 cell and basophils concomitantly promote ILC2 responses.[22,23] Future studies will be required to precisely delineate the network of cellular interactions that modulate GLP-1 attenuation of RSV-induced IL-13 production. In this study, we show that GLP-1 collectively attenuates ILC2, Th2 cell, and basophil function during RSV infection. These findings are consistent with reports showing that the GLP-1R agonist liraglutide inhibits IL-4 production from activated invariant natural killer T (iNKT) cells *in vitro,* which was associated with reduced numbers of circulating

iNKT cells in patients with T2D.[173] GLP-1R agonists also modulate the function of lymphocytes, neutrophils, and macrophages, decrease the production of cytokines and proinflammatory mediators, and inhibit the recruitment of hematopoietic cells.[172–174,176,242] The secondary messengers cAMP and PKA are associated with the anti-inflammatory actions of GLP-1R agonists on thymocytes and macrophages.[171,243] Future studies will determine whether GLP-1R stimulation of these signaling molecules also mediate the inhibition of type 2 immune cells.

IL-33 is a primary therapeutic target for inhibiting inflammatory lung disease given its central role in mediating both innate and adaptive immunity during lung inflammation.[29] There have been no reports identifying pharmacologic agents approved for human use which also inhibit lung IL-33 production. Herein we show that GLP-1R signaling inhibited lung IL-33 production during RSV infection. IL-33 reduction represents one possible mechanism by which GLP-1R signaling inhibited type 2 cytokine-producing cells. This is the first report of an FDA-approved pharmacologic agent inhibiting lung IL-33 production, and this finding has significant implications as it may provide an alternative to biologic therapies such as monoclonal antibodies or receptor antagonists that target IL-33-mediated diseases.

Two recent trials of antiviral RSV therapies have shown early results of more rapid viral clearance and partial attenuation of disease severity.[118,119] These promising results highlight the importance of developing anti-inflammatory therapies that can be used in conjunction with anti-virals to achieve complete disease abrogation. GLP-1R agonists are attractive anti-inflammatory therapeutic candidates, since we found that GLP-1R signaling does not have deleterious effects on protective antiviral responses

during infection. Our data demonstrate that GLP-1R signaling is unlikely to have negative effects on viral load or anti-viral interferon and antibody production. The lack of an effect by GLP-1R signaling on interferon production differs from what has been observed in other inflammatory diseases. The lack of an effect by GLP-1R signaling on interferon production differs from what has been observed in other inflammatory diseases. These data suggest that GLP-1R signaling may have unique effects on the type 1 response in different disease states. Determining the effect of GLP-1R signaling on distinct arms of the immune response in a given disease model is essential when developing therapeutics. Our results indicate that targeting the GLP-1 signaling axis is unlikely to exacerbate virally-associated disease metrics while still providing relief to type 2-associated immunopathology in the airways.

A recent randomized control trial evaluated the effectiveness of liraglutide in reducing rates of first occurrence of death from cardiovascular disease, nonfatal myocardial infarction, or nonfatal stroke in patients with Type II diabetes presenting with a high risk for cardiovascular disease.[178] This was associated with reduced rates of anti-inflammatory effects independent of changes in insulin levels.[177] A recent prospective cohort study evaluated the effectiveness of liraglutide in reducing disease severity in patients with psoriasis and Type II diabetes.[180] Patients on liraglutide therapy had decreased Psoriasis Area and Severity Index (PASI) scores. GLP-1R signaling also decreased inflammatory cell infiltration and goblet cell hyperplasia in models of chronic lung inflammation such as allergic airway inflammation and bleomycin-induced fibrosis.[181,182] Systemic and localized tissue inflammatory responses play a major role in the pathophysiology of cardiovascular disease, psoriasis, and chronic lung disease.

Given that dysregulated immune responses also drive severe RSV illness, it is intriguing to consider the therapeutic potential of GLP-1 for treating severe RSV infection. Our studies demonstrate that a GLP-1R agonist was capable of attenuating IL-33, IL-13, and type 2-associated immunopathology during primary RSV infection and preventing airway inflammation during secondary RSV infection, providing a small animal model proof of concept for this therapeutic approach. GLP-1R is expressed on human immune cells,[173] supporting the translational significance of using GLP-1R to inhibit immune cells during RSV infection. Importantly, we administered liraglutide at the same dose that is used in patients with T2D. This dose does not stimulate insulin secretion or decrease glucose levels in people without diabetes under euglycemic conditions, carries little risk for hypoglycemic events, and is administered at doses that do not induce weight loss, an important safety consideration for use in infants and elderly patients.[210] PheWAS is a new, validated reverse genetics approach that associates genetic variants of interest with phenotypes by linking a database of de-identified genotyping to a broad range of electronic medical record (EMR)-derived clinical phenotypes.[222] The EMR phenotypes are derived from clusters of common *International Classification of Diseases, Ninth Revision*, codes. The loss-of-function *THADA* SNP rs7578597 associated with lower beta-cell response to GLP-1.[220] Whether rs7578597 directly or indirectly affects GLP-1 function remains to be determined. In this study, the identification of an association between a *THADA* sequence variant (rs7578597) and acute bronchitis and bronchiolitis in humans points to a potential role of GLP-1R signaling in RSV disease in humans.

This study is the first investigation of GLP-1R signaling during viral infection. These data demonstrate the therapeutic potential of using GLP-1R agonist in a

therapeutically relevant manner (systemic administration) during RSV infection. We used the clinically relevant RSV strain 12/12-6, a virus that that was isolated from an infant with severe bronchiolitis and potentiated significant type 2 cytokine and mucus production in mice. We show that administration of a GLP-1R agonist attenuates type 2-associated immunopathology during RSV infection. Together, these data highlight a novel potential therapeutic for RSV infection, a disease for which there currently is no treatment after infection has occurred.

CHAPTER IV

PGI_2 SIGNALING ORCHESTRATES REGULATORY AND EFFECTOR T CELL RESPONSES DURING ALLERGIC AIRWAY INFLAMMATION

Introduction

Regulatory T cells (Tregs) are pivotal in suppressing immune responses and maintaining tolerance. Tregs are characterized by expression of the forkhead domain-containing transcription factor Foxp3, the master regulator that is essential for Treg differentiation, function, and maintenance.[47,244] Mutations in the Foxp3 locus result in Treg deficiency associated with the development of fatal multiorgan autoimmune diseases and allergies.[245–247] Tregs constitute 5-10% of all CD4+ T cells. Natural Tregs (nTregs) differentiate in the thymus before their exit into the peripheral circulation. Inducible Tregs (iTregs) are generated following exposure to cognate antigen in the context of transforming growth factor (TGF)-β and interleukin (IL)-2. Following insult or challenge, activated Tregs accumulate and proliferate in local lymph nodes and tissues, subsequently inhibiting effector T cell (Teff) responses. nTregs and iTregs synergistically exert immunosuppressive activity via multiple mechanisms including cell-cell contacts or secretion of immunomodulatory cytokines such as IL-10 and TGF-β. Since failure to maintain appropriate Treg numbers or function leads to autoimmune, malignant, and immunodeficient conditions, there is extensive interest in modulating Tregs as a therapeutic strategy for transplant, cancer, autoimmune disease, and allergy.[248–250]

Over the last four decades, the prevalence of allergic rhinitis, asthma, and atopic eczema increased markedly in developed countries.[1,2] Mounting evidence suggests that drugs inhibiting cyclooxygenase (COX) enzymes in the arachidonic acid metabolic pathway may contribute to the increased allergy prevalence.[251–253] Animal studies confirmed that COX inhibition increased allergic sensitization; augmented allergic airway inflammation; and enhanced lung expression of IL-4, IL-5, and IL-13 cytokines expressed by $CD4^+$ Th2 cells, supporting a role for the COX pathway in regulating allergic inflammation.[254–261] Because COX inhibition increases allergic inflammation in humans and experimental animal models, this suggests that a COX product restrains allergen-induced inflammatory responses and may be a novel treatment strategy for allergic diseases.

Prostaglandin I_2 (PGI_2) is one of the lipid products formed in the COX pathway of arachidonic metabolism. The other lipid molecules produced in the COX pathway are prostaglandin E_2 (PGE_2), prostaglandin D_2, prostaglandin $F_{2\alpha}$, and thromboxane A_2. PGI_2 binds to the G protein–coupled receptor IP and exerts its biological activities through autocrine and paracrine mechanisms. We have previously described the immune suppressive function of PGI_2, and PGI_2 analogs inhibited Th1 and Th2 effector cytokine production *in vitro*.[154,196] Endothelial cell-derived PGI_2 inhibited the recruitment of Th2 cells in the lung in an adoptive T-cell transfer model.[262] In contrast, IP knockout (KO) mice have augmented Th2 immune responses, increased airway inflammation and hyper-responsiveness to OVA.[156] Intranasal administration of the PGI_2 analog iloprost protected against Th2-mediated cardinal features of asthma, including the development of allergic lung inflammation and methacholine-induced airway responsiveness (AR).[159]

Finally, absence of PGI_2 signaling during sensitization broke immune tolerance elicited by prior mucosal allergen exposure.[160] Therefore, PGI_2 modulates immune responses and immune tolerance as well.

We hypothesized that PGI_2 signaling through IP protects against dysregulated Treg and Teff responses. Endogenous PGI_2 signaling protected against autoimmune manifestations including splenomegaly and epicarditis that developed in aged IP KO mice. PGI_2 promoted Treg stability and suppressive function as well as iTreg differentiation. PGI_2 simultaneously promoted Teff proliferation and susceptibility to Treg- mediated suppression. There is extensive interest in the application of Tregs autoimmune and allergic diseases. Our findings demonstrate that PGI_2 signaling promotes both Tregs and may represent a novel treatment strategy for Treg-mediated diseases.

Materials and Methods

Mice

8 week old female WT BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). IP KO mice were generated by homologous recombination in embryonic stem cells and were backcrossed to a BALB/c background for 10 generations. *Rag2-/-*, Foxp3-GFP, DO11.10 TCR-transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Foxp3-GFP and DO11.10 mice were crossed with IP KO mice to produce Foxp3-GFP x IP KO and DO11.10 x IP KO mice. Age-matched mice were used at 8 to 12 or 36 to 40 (for aging experiments) weeks old. Mice were maintained under specific pathogen free conditions and used in compliance

with the revised 2011 Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.[214]

Treg Suppression Assay

$CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ nTregs were isolated from WT or IP KO spleens by using a $CD4^+CD25^+$ Treg isolation kit (Miltenyi, San Diego, CA). Teff were labeled with 2.5 μM CFSE (Invitrogen) for 10 minutes at 37°C in PBS and 0.1% BSA and then co-cultured with an increasing ratio of Tregs in the presence of anti-CD3 (1 μg/mL, 145-2C11; BD) and anti-CD28 (4 μg/mL, 37.51; BD). Cells were incubated for 3 days and then harvested for flow cytometric analysis of CFSE dilution. Proliferation in Treg:Teff wells was compared to that in Teff only wells to determine the percent inhibition of proliferation at each ratio. The percent inhibition was calculated as follows: [(proliferation at ratio/ proliferation Teff only)*100].

iTreg Differentiation

Naïve $CD4^+$ T cells (1×10^5 cells/well) from the spleens of Foxp3-GFP or Foxp3-GFP x IP KO mice were isolated using a $CD4^+CD62L^+$ T cell isolation kit (Miltenyi). T cells were activated with anti-CD3 (0.5 μg/mL) and differentiated into iTregs by adding rmIL-2 (0.25 μg/mL; Peprotech, Rocky Hill, NJ) and rhTGF-β (1 μg/mL; Peprotech). Cells were cultured for 6 days and then harvested for flow cytometric analysis of iTreg differentiation.

Flow Cytometry

T cells were harvested and cells were blocked with anti-FcR antibody (BD Biosciences) and in select experiments stained with surface markers against CD3 (17A2) from BioLegend (San Diego); CD4 (H129.19), or CD25 (PC61.5) from BD Biosciences (San Jose, CA).

Cytokine Measurements

Lungs were snap-frozen in liquid nitrogen at the time of harvest. Lungs were mechanically disrupted using 1 mL of MEM media and homogenized via BeadBeater (BioSpec Products, Bartlesville, OK). Protein measurements were performed using Quantikine (IL-13, and IL-5) enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer instructions (R&D Systems, Minneapolis, MN). The levels of 8 cytokines (IL-10, TGF-β, IFN-γ, IL-2, IL-4, IL-5, IL-13, and IL-17) in 25 uL of culture supernatant were assayed simultaneously, in duplicate, using a murine cytokine 8-plex antibody bead kit (EMD Millipore, Billerica, MA) and a Luminex-100 array assay reader (Luminex Corp., Austin, TX).

OVA-Induced Airway Inflammation

Naïve $CD4^+$ Teff and $CD4^+CD25^+$ Tregs were isolated from DO11.10 spleens by using $CD4^+CD62L^+$ and $CD4^+CD25^+$ Treg isolation kits (Miltenyi). To facilitate homing of T cells to the lungs, *Rag-2/-* mice were challenged with OVA protein (50 μg in 50 μL of saline, intranasal (i.n.) or an equal volume of saline; Sigma-Aldrich, St. Louis, MO) on day −1. On day 0, 2.5×10^6 Teff from DO11.10 mice and 1, 0.5, or 2×10^6 Treg from

DO11.10 mice were adoptively transferred by retro-orbital injection. Mice were challenged with OVA for 3 consecutive days after cell transfer (50 µg/mouse/day, i.n.). Animals were sacrificed one day after the last airway challenge.

RNA-Seq

nTregs from the spleen of IP KO and WT mice were isolated with a FACSAria II (BD) for mRNA sequencing analysis. RNA from nTregs was isolated with a Total RNA Purification kit (Norgen, Thorold, ON). RNA was quantified with a Qubit 2.0 fluorometer (Invitrogen) and RNA quality was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA) with an RNA nano chip (Agilent). Samples with an RNA integrity number of >8 were used for library preparation. 50 ng of total RNA derived from splenic nTregs was used for low-input, stranded mRNA library preparation (NEB, Ipswich, MA). Library size distribution was assessed with a High Sensitivity DNA chip (Agilent) on a Bioanalyzer 2100. Paired-end 75-base pair sequencing, targeting at least 7×10^6 reads per sample, was performed on a HiSeq300 (Illumina) following the manufacturer's protocol. Sequencing data were analyzed with GeneSpring (Version 13; Agilent).

Quantitative PCR

Total RNA was isolated using a Qiagen RNAesy micro kit (Valencia, CA) and cDNA was generated. Commercially available primers and probes for Pglyrp1, Ccdc61, and GAPDH were used (Applied Biosystems). Gene expression was normalized to GAPDH before the fold change was calculated. The fold change in gene expression was calculated via the comparison of gene expression to that of nTregs from WT mice.

Statistical Analysis

Groups were compared using unpaired t-test, one-way analysis of variance (ANOVA) with Bonferroni post test, or two-way ANOVA with Dunn's multiple comparison test, as appropriate with GraphPad Prism (Version 5; GraphPad Software, San Diego, CA). Measurements below the limit of detection were assigned half of the value of the limit of detection for statistical comparisons.

Results

IP deletion causes autoimmune manifestations and disrupts Treg homeostasis and stability

We hypothesized that PGI_2 signaling through IP protects against dysregulated Treg and Teff responses and subsequent autoimmune manifestations. Whole animal histopathology was performed on 36 week-old IP KO and WT mice. Aged IP KO mice exhibited splenomegaly and right ventricular free wall epicarditis (**Figure 4.1*A-C***). HIstopathological evaluation of diseased mice showed infiltration in the spleen and heart, whereas WT mice did not show any noticeable pathology.

To identify the underlying mechanisms responsible for the autoimmune manifestations observed in the aged IP KO mice, we first sought to examine whether T cell homeostasis and Treg stability were impaired in the periphery of IP KO mice. Comparison of IP KO mice with WT mice demonstrated unaltered spleen cellularity (**Figure 4.1*D***). Flow cytometric analysis revealed increased numbers of $CD3^+$ cells as well as $CD3^+CD4^+$ T cells in the spleens of IP KO mice compared to WT mice (**Figure 4.1*E-G***). The aforementioned difference in autoimmune manifestations between aged

IP KO mice and WT mice could be due to a numerical decrease in the Tregs in the absence of PGI_2 signaling. While there was no significant difference in the proportion of $CD4^+$ T cells that expressed CD25 and Foxp3 between IP KO and WT mice (**Figure 4.1*H***), the absolute number of Tregs was unexpectedly increased in IP KO mice compared to WT mice (**Figure 4.1*I***). However, there was a statistically significant 2-fold decreased MFI of Foxp3 in the nTreg of IP KO mice compared to WT mice, indicating decreased Foxp3 protein expression on a per-Treg basis in IP KO mice (**Figure 4.1*J***). There was no difference in CD25 MFI in Treg from IP KO and WT mice (**Figure 4.1*K***).

IP-deficient Treg are less suppressive and IP-deficient Teff are resistant to nTreg-mediated suppression

Despite the increased absolute number of Tregs, Teff numbers persisted in IP KO mice. This led us to hypothesize that a lack of endogenous PGI_2 signaling results in general impairment of Treg suppressive function. To test this hypothesis, we isolated $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^-$ Teff from IP KO and WT mice using commercially available magnetic sorting kits (Miltenyi, San Diego, CA) and performed *in vitro* suppression assays. Teff were labeled with CFSE and then co-cultured with an increasing ratio of Tregs in the presence of anti-CD3 and anti-CD28 for 3 days. Suppressive function was determined as the ability of Treg to suppress Teff proliferation. Tregs from IP KO mice were less suppressive compared to Tregs from WT mice (**Figure 4.2*A*-*C***). We also evaluated production of the canonical Treg, Th1, Th2, and Th17 cytokines. Analysis of cytokine production showed decreased production of the immunoregulatory cytokine IL-10 as well as increased production of the Th2

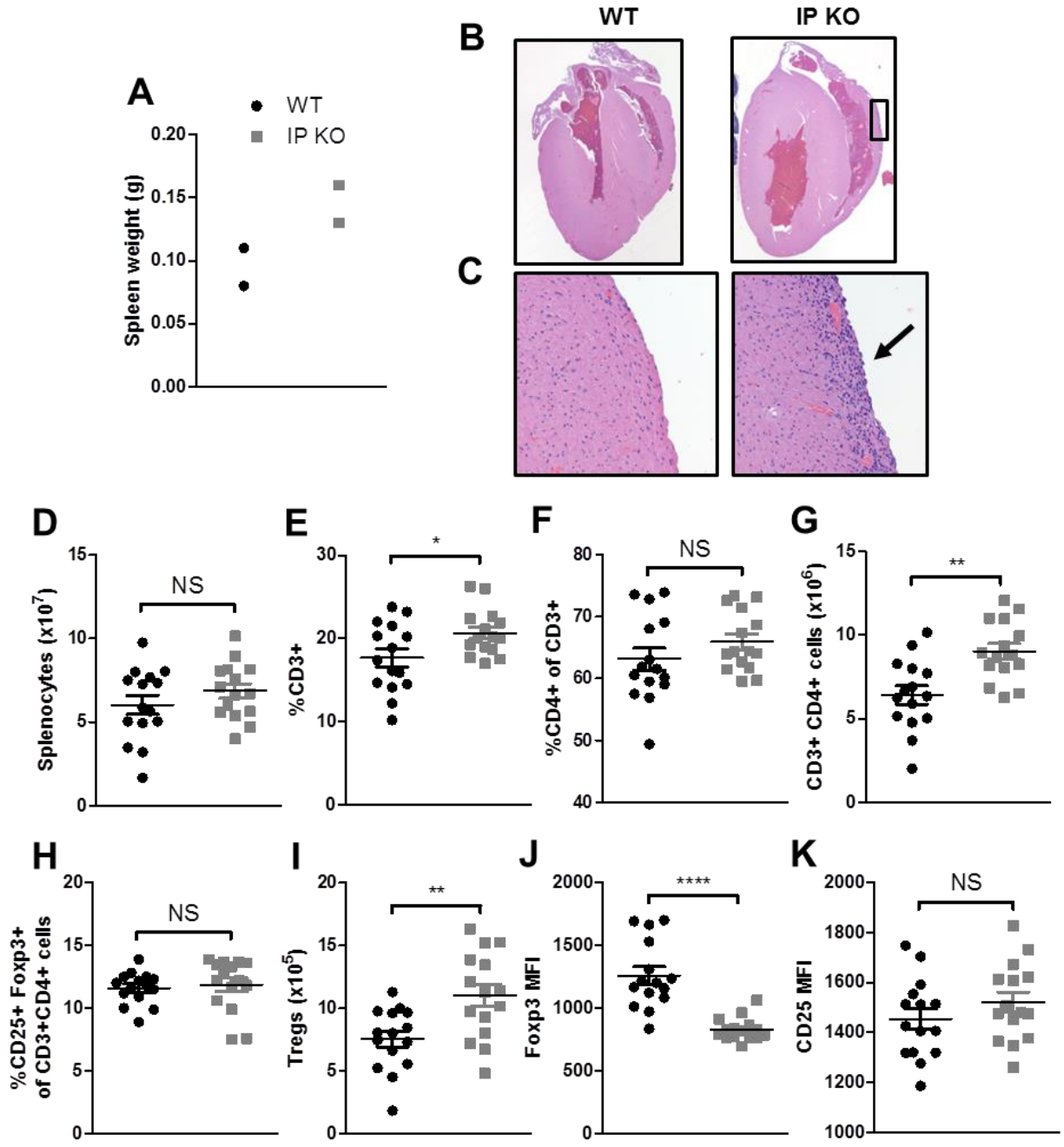

Figure 4.1. IP deletion causes autoimmune manifestations and disrupts Treg homeostasis. (**A**) Splenomegaly and representative sections of (**B**) heart (20x magnification) and (**C**) epicarditis (400x magnification) in IP KO mice at the age of 36 weeks. (**D**) Number of cells in the spleens of IP KO and WT mice. (**E**) Splenic $CD3^+$, (**F-G**) $CD3^+$ $CD4^+$, (**H-I**) $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in IP KO and WT mice. (**J**) Foxp3 MFI and (**H**) CD25 MFI of splenic $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs IP KO and WT mice. Data plotted as mean + SEM. n = 2 mice per group (**A-C**). n = 15 mice per group combined from 3 independent experiments (**D-J**). *p < 0.05, **p < 0.01, ***p < 0.001 by unpaired T-test (**D-J**).

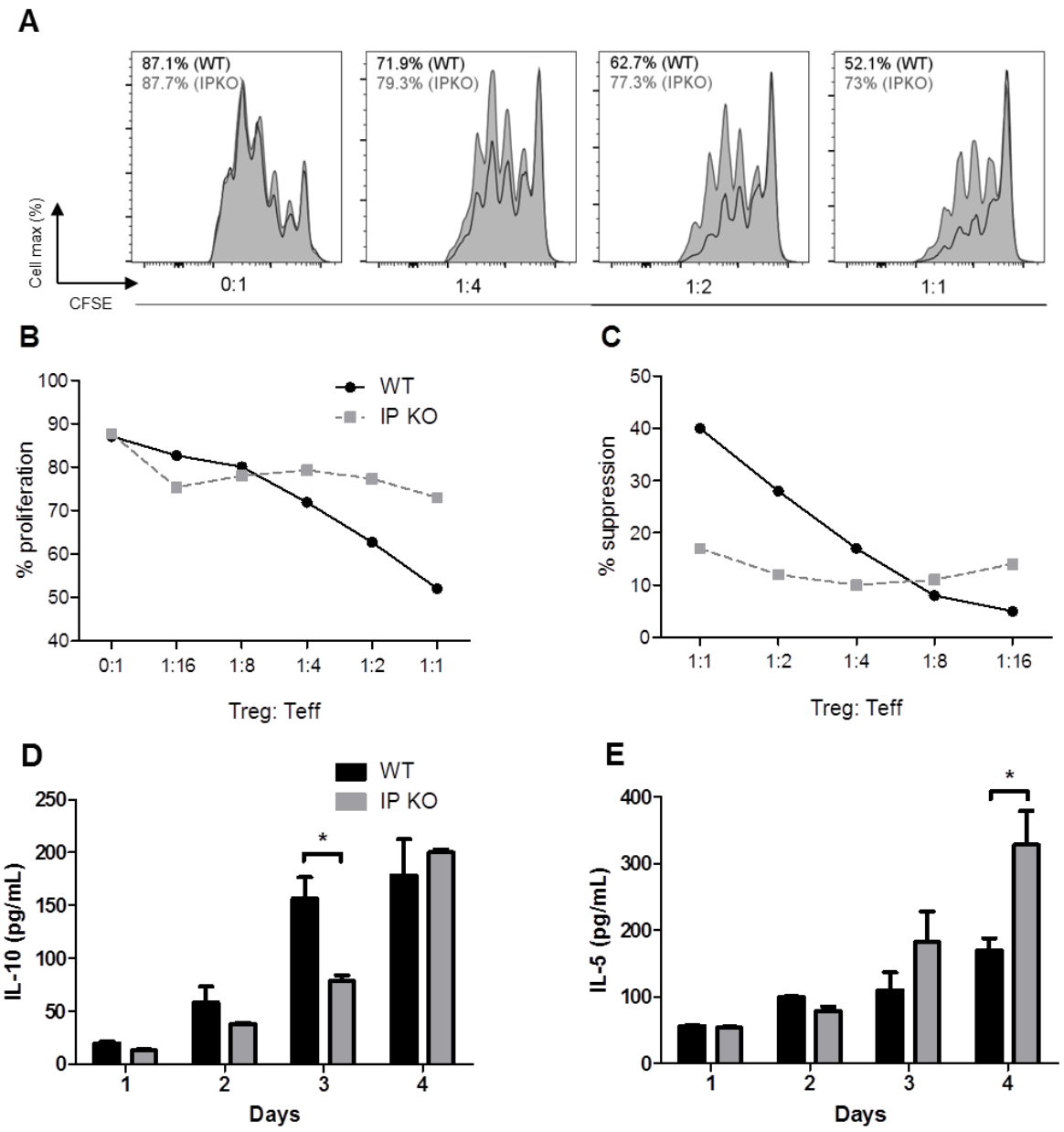

Figure 4.2. IP-deficient Tregs are less suppressive *in vitro*. Suppression assays were performed using purified $CD4^+CD25^-$ Teff and $CD4^+CD25^+$ Tregs. (**A**) Comparison of inhibition at varying ratios of Tregs to Teff. Percent (**B**) suppression and (**C**) proliferation of Teff from WT mice in co-culture with Tregs from IP KO or WT mice. IL-10 (**D**) and IL-5 (**E**) production in cultured supernatants of Tregs stimulated with anti-CD3 and anti-CD28. Data plotted as mean + SEM. n = 3 representative of 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA (**A-C**) or unpaired T-test (**D-E**).

cytokine IL-5 in the supernatants of stimulated Treg cultures from IP KO mice compared to supernatants of stimulated Treg cultures from WT mice (**Figure 4.2*D* & *E***). Stimulated Tregs from IP KO mice also produced less IFN-γ and IL-17 compared to stimulated Tregs from WT mice (**Figure 4.3*A* & *B***). We did not find statistically significant differences in production of TGF-β, IL-2, IL-4, and IL-13 (not detected) between stimulated Tregs from IP KO and WT mice *in vitro* (**Figure 4.3*C-E***). Increased Th2 cytokine production was also observed in supernatants of stimulated naïve $CD4^+$ T cells from IP KO mice compared to supernatants of stimulated naïve $CD4^+$ T cells from WT mice.[263] Thus, Th2 responses are broadly and selectively dysregulated in mice lacking PGI_2 signaling.

To determine whether Teff from IP KO mice are also resistant to Treg-mediated suppression, we incubated both WT and IP KO Teff with WT Tregs. The ability of WT Tregs to suppress IP KO Teff proliferation was reduced compared with their inhibition of WT Teff (**Figure 4.4**). Together, these data suggest that PGI_2 enhances both Treg function and Teff responsiveness to Treg-mediated suppression.

IP deletion inhibits iTreg differentiation

As iTregs act in concert with other Treg subpopulations to maintain peripheral tolerance, we further hypothesized that endogenous PGI_2 signaling may modulate *in vitro* iTreg differentiation in addition to Treg stability and function. To test this, we isolated naïve $CD4^+$ $CD62L^+$ T cells from Foxp3-GFP x IP KO and Foxp3-GFP mice using commercially available magnetic sorting kits (Miltenyi). As optimal iTreg differentiation from naïve $CD4^+$ T cells

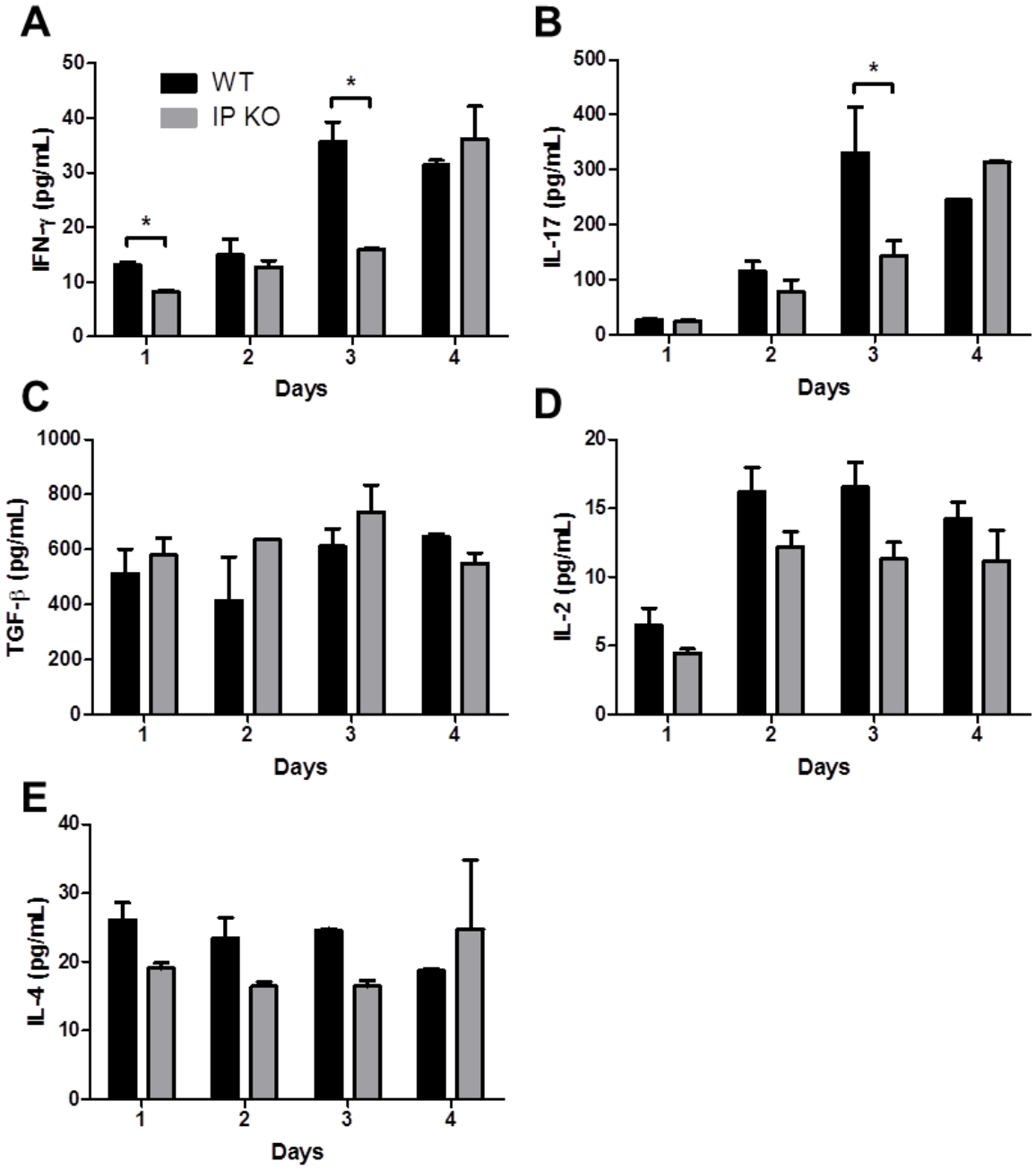

Figure 4.3. Stimulated Tregs from IP KO mice produce less IFN-γ and IL-17 and similar amounts of TGF-β, IL-2, and IL-4 compared to Tregs from WT mice. IFN-γ (**A**) IL-17 (**B**), TGF-β (**C**), IL-2 (**D**), and IL-4 (**E**) production in cultured supernatants of Tregs stimulated with anti-CD3 and anti-CD28. Data plotted as mean + SEM. n = 3 representative of 2 independent experiments.

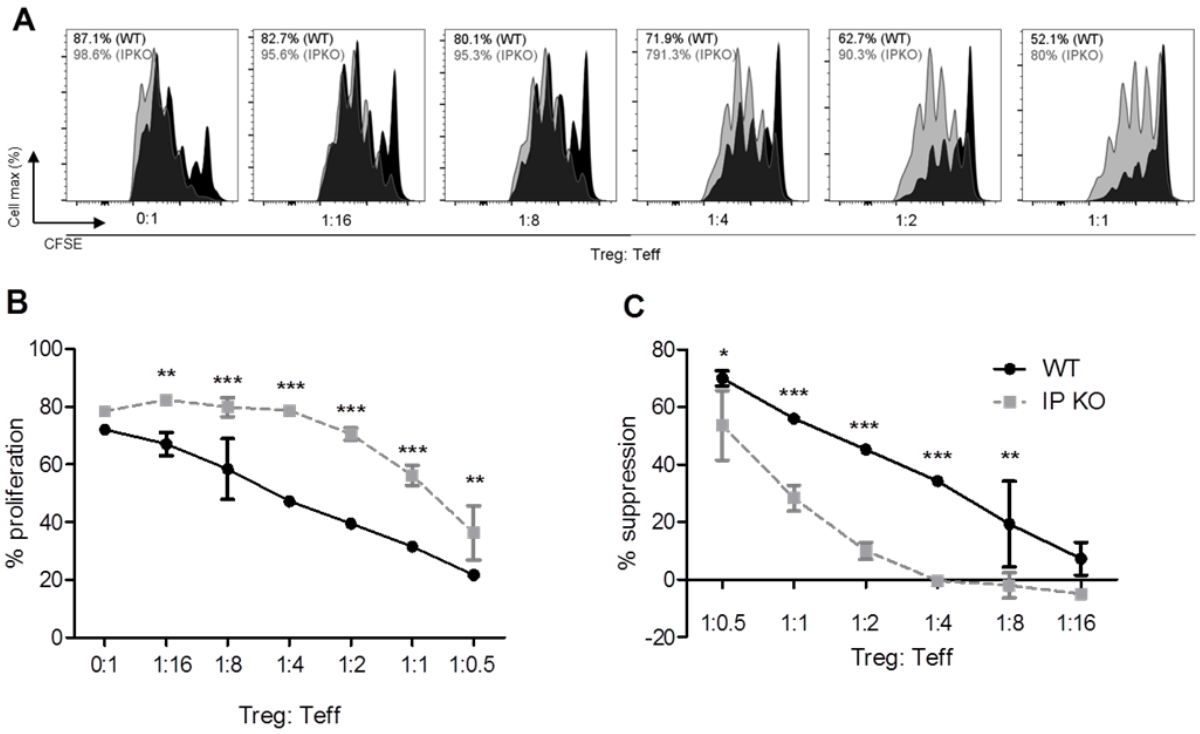

Figure 4.4. IP-deficient Teff are resistant to Treg-mediated suppression. Suppression assays were performed using purified $CD4^{+}CD25^{-}$ Teff and $CD4^{+}CD25^{+}$ nTregs. (**A**) Comparison of inhibition at varying ratios of nTregs to Teff. Percent (**B**) proliferation and (**C**) suppression of Teff from IP KO or WT mice in co-culture with nTregs from WT mice. Data plotted as mean + SEM. n = 3 representative of 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA.

results from weak anti-CD28 costimulation,[264] naïve $CD4^+$ T cells were stimulated with anti-CD3 and cultured in the presence of IL-2 (0.25 µg/mL) and TGF-β (1 µg/mL) for 6 days. IP deletion significantly increased the proliferative competence of naïve CD4+ T cells from IP KO mice compared to naïve $CD4^+$ T cells from WT mice (**Figure 4.5*A* & *B***). However, IP deletion significantly decreased the percentage of Foxp3-expressing iTregs that differentiated from naïve CD4+ T cells compared to WT mice (**Figure 4.5*C* & *D***). Thus, our data indicate that PGI_2 is a novel cofactor in TGF-β-mediated iTreg generation.

Tregs attenuate Th2 responses in an OVA-induced airway inflammation model

As PGI_2 has been implicated in Th2-mediated immune diseases, we developed an antigen-induced airway inflammation model of asthma to assess the *in vivo* suppressive function of nTregs (**Figure 4.6**). Teff and Tregs were adoptively transferred into *Rag2-/-* mice that had been challenged with OVA protein intranasally (i.n.) or saline one day before cell transfer to promote cell migration to the airways.[42] Following cell transfer, mice were challenged with OVA (i.n.) or saline for three consecutive days and sacrificed one day after the last OVA challenge to measure lung homogenate levels of IL-13, which mediates airway mucus production. Reconstitution with Teff resulted in appreciable lung homogenate levels IL-13. Mice that received Teff without OVA challenge or mice challenged with OVA without Teff transfer had lung homogenate levels of IL-5 and IL-13 that were <50 pg/ml (data not shown). Transfer of WT Tregs inhibited the lung levels of IL-13 (**Figure 4.7**).

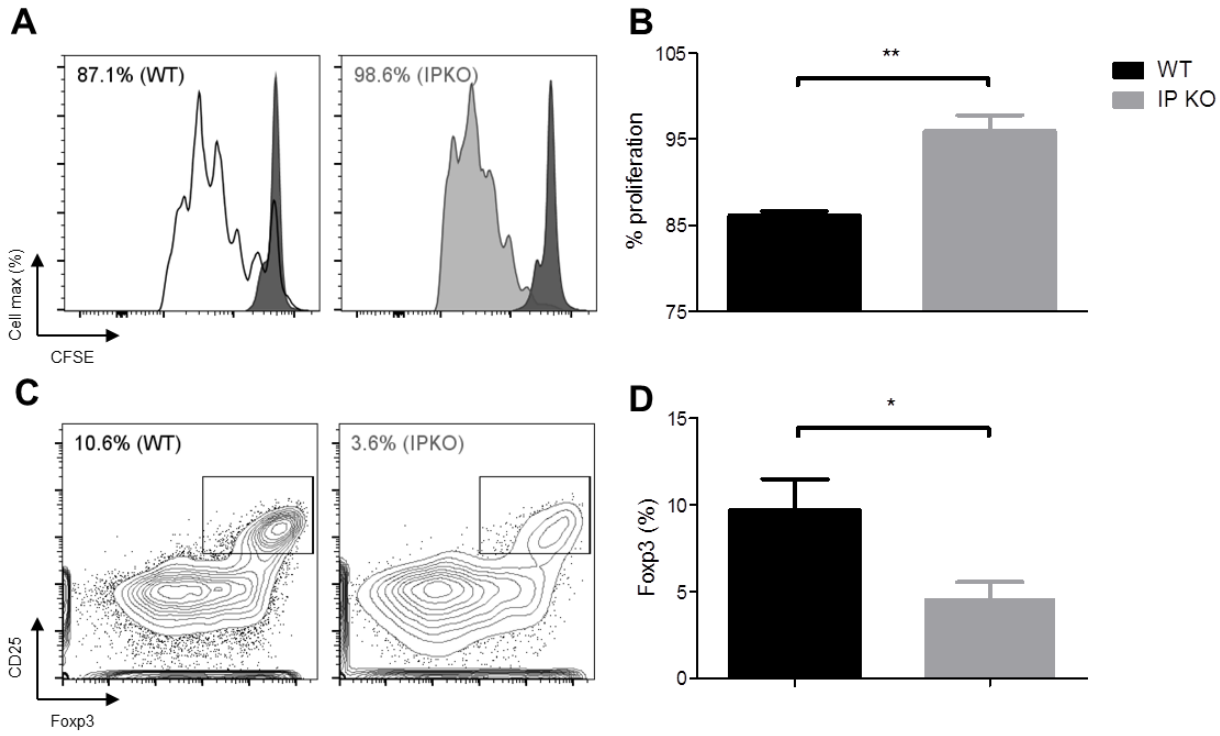

Figure 4.5. IP deletion inhibits iTreg development from naïve CD4$^+$ T cells. Naïve CD4$^+$ T cells from IP KO and WT mice were activated with anti-CD3 and differentiated into iTregs by adding IL-2 and TGF-β. (**A**) Representative CFSE dilution measured by flow cytometry in activated naïve CD4$^+$ T cells. (**B**) Percent proliferation of activated naïve CD4$^+$ T cells. (**C**) Representative Foxp3 expression measured by flow cytometry in differentiated iTreg. (**D**) Percent of CD4$^+$ T cells that are CD25$^+$ Foxp3$^+$. Data plotted as mean + SEM. n = 3 representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$ by one-way ANOVA.

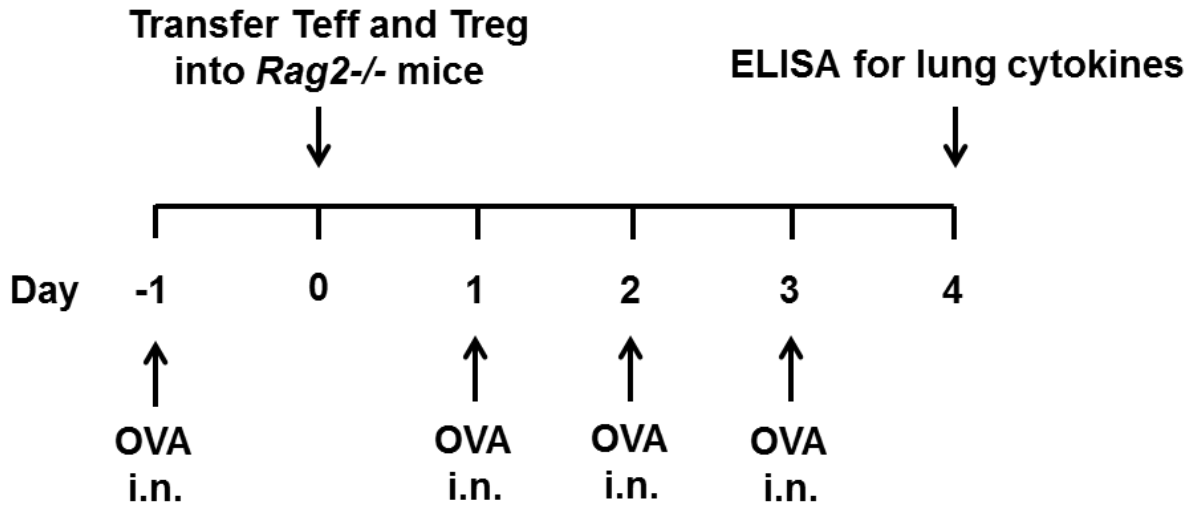

Figure 4.6. Protocol for *in vivo* model of OVA-induced airway inflammation. *Rag2-/-* mice were challenged with OVA protein (50 µg) i.n. on day -1. On day 0, Teff from DO11.10 mice (2.5 x 10^6) and nTregs from DO11.10 mice (0, 0.5, or 2 x 10^6) were transferred into the recipient mice. Mice were subsequently challenged with 50 µg OVA for 3 consecutive days. Mice were sacrificed on day 4, one day after the last airway challenge. Cytokine and chemokine levels in the BAL fluid and lung homogenate were determined by ELISA.

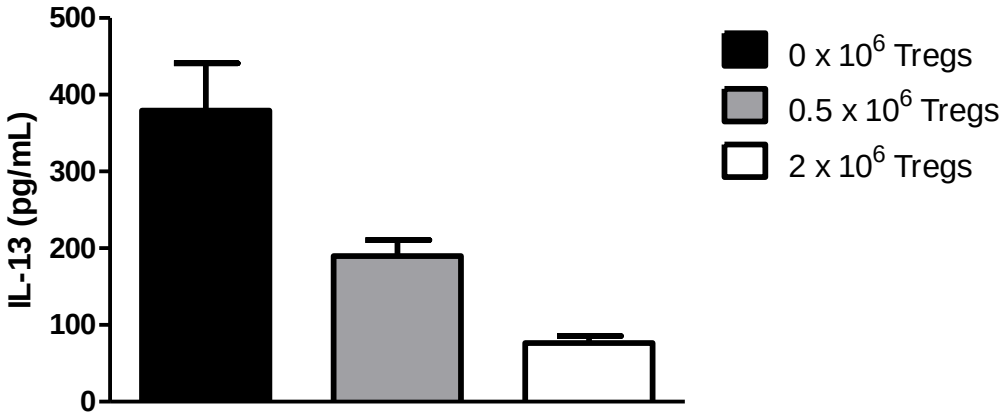

Figure 4.7: Tregs attenuate Th2 responses in an OVA-induced airway inflammation model. *Rag2-/-* mice received Teff and Tregs from DO11.10 mice in a model of OVA-induced airway inflammation. ELISA for IL-13 in whole lung homogenate. Data plotted as mean + SEM. n = 3 mice per group.

Pglyrp1 and Ccdc61 *are decreased in IP-deficient Tregs*

To further investigate the molecular mechanisms by which PGI_2 regulates the function of Tregs, we isolated Tregs from IP KO and WT mice and comparatively analyzed the transcriptomes of these cells by next-generation sequencing-based RNA sequencing (RNA-seq). IP-deficient and IP-sufficient Tregs exhibited similar expression of many molecules with a presumed role in the suppressive properties of Tregs. We confirmed that result on the protein level by flow cytometry for the immunomodulatory receptor CTLA-4, the ATP ectonucleotidase CD39, and the IL-2 receptor complex (data not shown). However, mRNA expression of *Pglyrp1* and *Ccdc61* was significantly decreased in IP-deficient Tregs compared to WT nTregs (**Figure 4.8*A***). Subsequent independent confirmation by qPCR showed statistically significant 5-fold decreases in *Pglyrp1* and *Ccdc61* expression in IP-deficient Tregs compared to WT Tregs (**Figure 4.8*B*** & **C**).

Discussion

Allergic diseases affect up to 30% of the population worldwide, with asthma being one of the most common chronic diseases in which affected individuals may suffer considerable mortality.[1,2] Although corticosteroids are effective at managing most disease, 10% of patients do not respond to the treatment, and they are associated with severe long-term side effects. Whereas healthy individuals have more Tregs than Th2 effector cells, allergic patients have a defect in either the number of regulatory cells, their suppressive activity, or both.[265,266] Various approaches to redress the balance between regulatory and effector cells, such as allergic-specific immunotherapy and

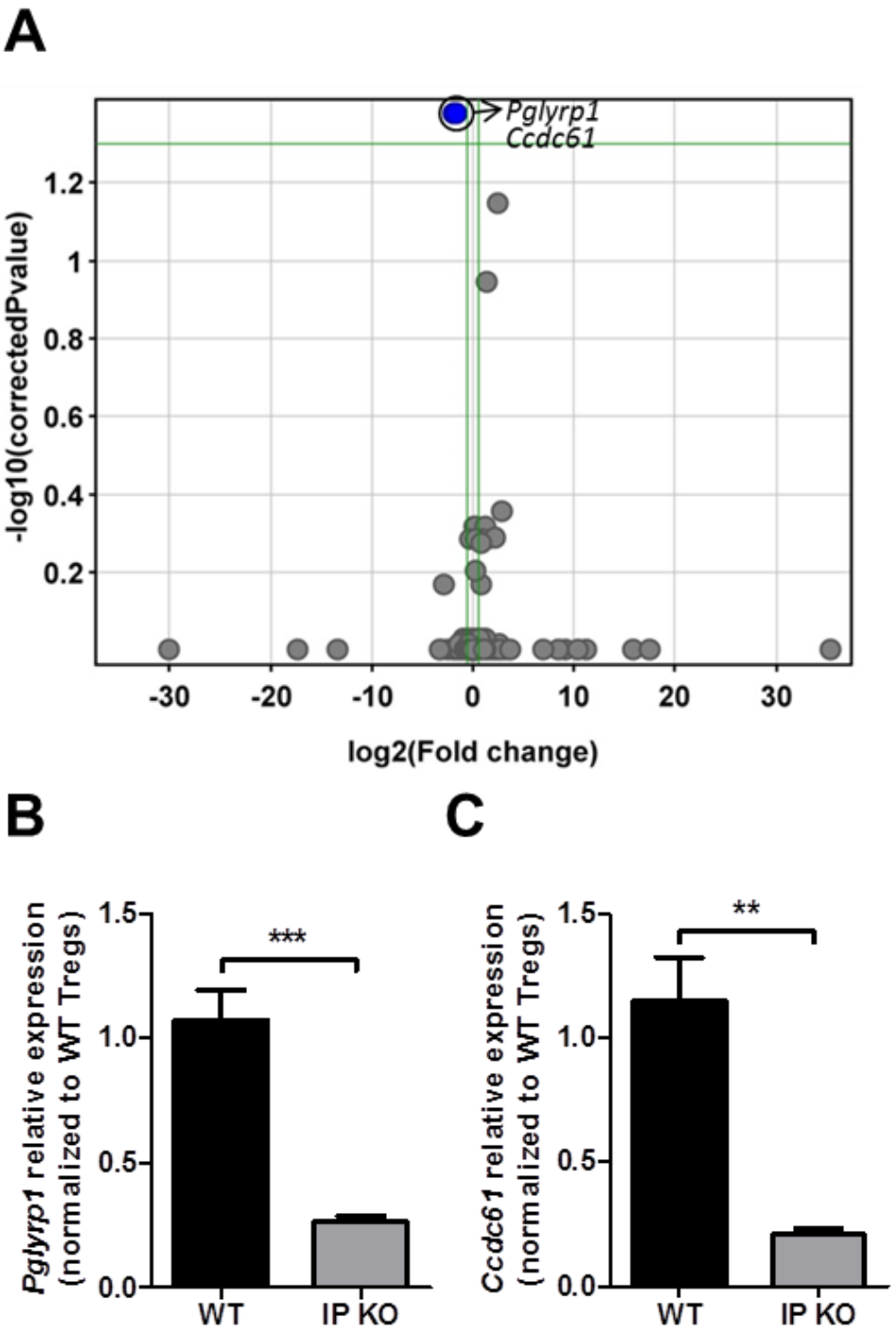

Figure 4.8. *Pglyrp1* and *Ccdc61* are decreased in IP-deficient Tregs. RNA-seq was performed on RNA isolated from splenic nTregs of WT and IP KO mice. (**A**) Volcano plot of genes downregulated in IP KO nTregs relative to their expression in IP-sufficient Treg cells. (**B**) Plygrp1 and (**C**) Ccdc61 mRNA expression by qPCR. Each sample is normalized to GAPDH and relative expression is compared to that of nTregs from WT mice. Data plotted as mean + SEM. n= 5-6 mice per group representative of 2 independent experiments. *p < 0.05 by unpaired T-test (B).

biologics targeting cytokines or immunoglobulin E (IgE), have been tested, but each has limitations. Administration of Tregs to restore immune tolerance has been explored as a potential therapeutic approach to asthma as well as transplantation and autoimmune diseases. [248–250] The present study describes PGI_2 as a new modulator of Treg function.

The effect of PGI_2 on Treg stability has not been described. Our group has previously shown that IP KO mice have greater numbers of Tregs during aeroallergen-induced immune tolerance.[267] Here we similarly report that IP KO mice had increased numbers of Tregs compared to WT mice under homeostatic conditions. However, IP-deficient Tregs demonstrated decreased Foxp3 protein expression on a per Treg basis. This finding suggests that the increased number of Treg cells in IP KO mice is an effort to maintain inflammatory homeostasis in these mice compared with WT mice. Currently, there are insufficient animals to make a valid statistical analysis to determine whether the findings of autoimmune manifestations in the aged IP KO mice are due to chance. However, we will have the opportunity to determine if there is a significant difference in autoimmune manifestations in aged IP KO versus WT mice in the future. The IP-deficient Treg population might show enrichment for cells with unstable Foxp3 expression. A population of $CD25^{-}$ $Foxp3^{+}$ Tregs with lower Foxp3 expression was recently described.[268] Future studies will determine whether PGI_2 signaling leads to enrichment of this less stable Treg population. IP-deficient Tregs were less suppressive in *in vitro* suppression assays. Moreover, IP-deficient Teff were resistant to Treg-mediated suppression *in vitro*. The Th2 response mediates allergic airway inflammation and is correlated with disease severity. Some studies suggest that Teff resistance to Treg-mediated suppression contributes to asthma pathology in addition to Treg

dysfunction.[269] The stability of Tregs during inflammation has been called into question with multiple reports demonstrating evidence of Treg plasticity, whereby immunoregulatory Tregs revert into pathogenic Teff during inflammation.[270–274] We found evidence of altered stability of IP-deficient Tregs in that *ex vivo* stimulation of Tregs from IP KO mice induced delayed IL-10 production and greater IL-5 production compared to Tregs from WT mice. These results are consistent the finding that iloprost-treated dendritic cell (DC)s induced the generation of IL-10-producing Tregs.[275] PGI_2 may enable the reprogramming of Tregs into effector Th2 cells. Further, Treg dysregulation may contribute to the enhanced Th2 immune response and airway inflammation observed in IP KO mice.

An emerging concept of Treg modulation is the acquisition of other Th-specific transcription factors, which renders Tregs capable of specifically controlling the respective Th-mediated immune responses. For example, Treg-specific suppression of Th1 , Th2, and Th17 cells was demonstrated through specific transcription factors expressed in Tregs including T-bet, IRF4, and STAT3.[51,276,277] Of relevance to this study, the initiation of Th2 cell-driven immune responses was dependent on expression of the transcription factor IRF4 which endowed Tregs with the ability to suppress Th2 responses *in vivo*.[51] Recent studies demonstrated that Bcl-6 and Csnk2b also promote Th2 responses and modulate Treg function.[50,278] IP-deficient Tregs did not show altered expression of IRF4, Bcl-6, or Csnk2b (data not shown). Recent studies have identified a number of potential mechanisms involved in Treg suppression that may be useful for enhancement of suppression and/ or Treg numbers including CD39 signaling and CTLA-4. However, data from the current study demonstrate that expression of these

molecules were unaltered between Tregs from IP KO versus WT mice. Thus, the present study suggests that Tregs use unique PGI_2-controlled mechanisms to protect against inflammation.

Pglyrp1 and *Ccdc61* expression was decreased in IP deficient versus IP-sufficient Tregs. While less is known about *Ccdc61*, *Pglyrp1* - originally identified for its role in antibacterial innate immunity-[279] was identified as having a role in asthma in a genome-wide profiling of the lung transcriptome of mice with human dust mite (HDM)-induced experimental asthma.[280] *Pglyrp1* has demonstrated pro-inflammatory effects in three mouse models of inflammatory skin diseases, including atopic dermatitis, contact dermatitis, and psoriasis.[281,282] Moreover, Treg activity and recruitment to the skin was decreased in mice that lacked *Pglyrp1* in a mouse model of chemically induced, psoriasis-like inflammation.[281] A subsequent study by the same group found higher numbers of Tregs in the lungs of mice that lacked *Plygrp1* in a mouse model of HDM-induced experimental asthma.[283] The authors posit that their disparate findings regarding the effect of *Pglyrp1* on Tregs could be explained by an underlying role for *Pglyrp1* in inhibiting plasmacytoid (p)DCs that activate Tregs in their second study. In addition to possibly *Pglyrp1*, a transcriptional module downstream of PGI_2 signaling is likely modified to facilitate efficient Teff suppression by Tregs. Treg populations may be heterogeneous in this regard, if signaling-dependent post-translational modifications of PGI_2 signaling or recruitment of other nuclear factors are needed for Treg-mediated suppression. We propose that PGI_2 signaling might alter certain components of transcriptional machinery promoting Treg stability and function to efficiently control the present immune response. It is likely that changes in expression of a combination of

genes, but not a single gene, account for the impaired suppressor capacity of IP-deficient Tregs. Additionally, PGI_2 may coordinate with several stabilizing factors to induce Foxp3 protein expression in $CD4^+$ T cells given the diverse proinflammatory stimuli Tregs may encounter in their lifespan. Identification of these stabilizing factors and further characterization of the molecular mechanism underlying the Foxp3-stabilizing effect of PGI_2 will help provide a full molecular picture on the physiological regulation of Tregs, as well as establish the basis for the application of Tregs in immunotherapy.

The Food and Drug Administration (FDA) has approved PGI_2 for treatment of primary pulmonary hypertension. PGI_2 has also been used safely and effectively in patients with other causes of pulmonary hypertension, including the inflammatory conditions scleroderma and systemic lupus erythematosus.[151] The attenuation of Treg-mediated suppression by drugs is a promising therapeutic strategy that has mediated the attenuation of some conditions such as organ rejection and Crohn's disease.[284,285] However, whether PGI_2 exerts anti-inflammatory effects in *vivo* in part by promoting Treg function and thereby breaching immunological unresponsiveness to allergens remains to be elucidated. Our results identify a new function for PGI_2 as an important link between anti-inflammation and the local Treg response. The ability of PGI_2 to promote regulatory networks in response to inflammation may represent a more general mechanism by which PGI_2 and other anti-inflammatory prostaglandins such as PGE_2 limit immune-mediated damage to self at barrier tissues.[286,287]

Together, these data suggest that PGI_2 creates a protolerogenic environment that is potent enough to promote Treg and suppress Teff responses. PGI_2 has appeal

for a broad range of inflammatory diseases that are driven by an imbalance between pro- and anti-inflammatory T cell responses and have reached alarming prevalence in industrialized nations.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of findings

Patients with asthma have innate immune defects with pathogens, which may partially explain why these individuals are at increased risk for invasive bacterial infections and bacterial pneumonia. γδ T cells reside at the interface of epithelial-environmental interfaces such as the respiratory and gastrointestinal tracts, and are a critical first line of defense against bacterial and fungal pathogens. A subset of γδ T cells are the γδ17 cells which produce large quantities of IL-17, a cytokine crucial to the anti-bacterial and anti-fungal function of these cells. IL-4 is produced at high levels in the airway of asthma patients. I found that γδ T cell expression of the type I IL-4R is functional, as IL-4 induces STAT6 phosphorylation in γδ T cells. IL-4 inhibits γδ17 cell production of IL-17. IL-4 also decreases Sgk1 expression, which may lead to destabilized IL-23R expression on γδ17 cells. Endogenous IL-4 reduces lung γδ17 cell numbers during acute *KP* infection. IL-4 inhibition of γδ17 cells provides a possible explanation for why asthmatic patients are at greater risk for invasive bacterial disease (**Figure 5.1** & **Chapter I**).

GLP-1R agonists are a well-accepted and safe treatment for T2D. Recent evidence suggests that GLP-1R signaling also has anti-inflammatory effects. Severe RSV-associated illness is in part caused by type 2-associated immunopathology. GLP-1R agonist treatment attenuated immunopathology during RSV infection. The GLP-1R agonist decreased total lung IL-13 and IL-33 levels, with concurrent decreases in IL-13^{+}

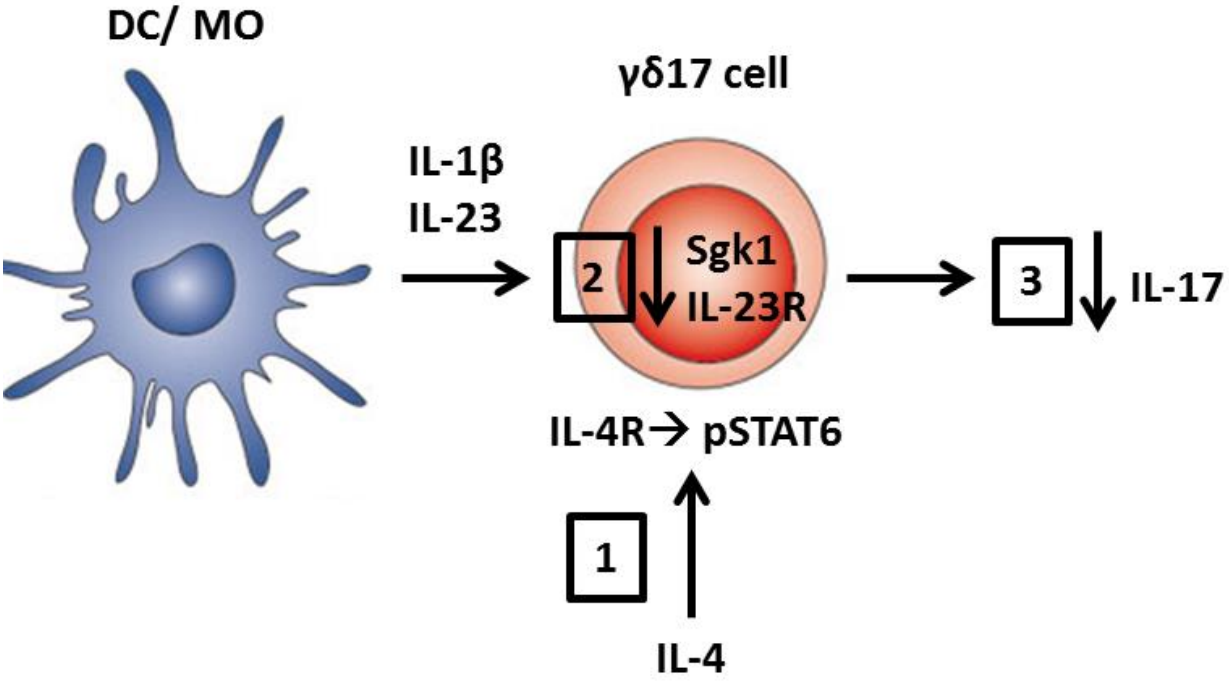

Figure 5.1. STAT6 attenuates γδ 17 cells.

group 2 innate lymphoid cell (ILC2), $CD4^+$ Th2 cells, and basophils as well as IL-33^+ epithelial cells. GLP-1R agonist treatment prevented airway inflammation, and did not impact viral load, anti-viral interferon and antibody production during secondary RSV infection. Relative to the respective mock-infected groups, RSV-infected GLP-1R agonist-treated mice did not have increased weight loss compared to vehicle-treated mice. A phenome-wide association study (PheWAS) identified a link between GLP-1R signaling and acute bronchitis and bronchiolitis in humans. GLP-1R agonists are the first known FDA-approved agents to inhibit IL-33 and may represent a novel treatment strategy for RSV bronchiolitis (**Figure 5.2** & **Chapter II**).

Tregs suppress immune responses and maintain tolerance by inhibiting T cell activation. There is extensive interest in the application of Tregs in allergic diseases. PGI_2 signaling through the IP receptor inhibits inflammation and promotes tolerance during allergic airway inflammation. I found that PGI_2 protects against autoimmunity and promotes nTreg stability and suppressive function as well as iTreg differentiation. PGI_2 simultaneously promotes Teff proliferation and susceptibility to nTreg-mediated suppression. Thus, PGI_2 may represent a novel treatment strategy for diseases that result from Treg dysregulation (**Figure 5.3** & **Chapter III**).

Future directions

Targeting single components of the immune system versus the network as a whole

There is considerable interest in targeting multiple components of the immune system to attenuate airway inflammation. Corticosteroids, the most widely prescribed therapeutic for allergic asthma, function by broadly suppressing immune responses.

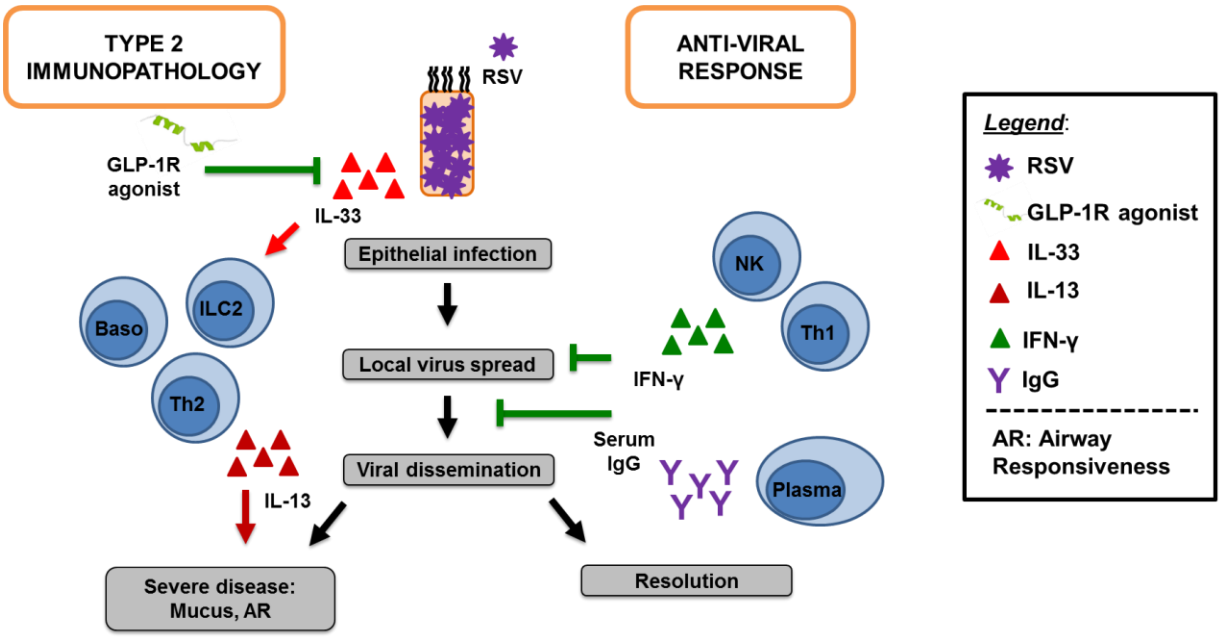

Figure 5.2. GLP-1 attenuates RSV-induced immunopathology.

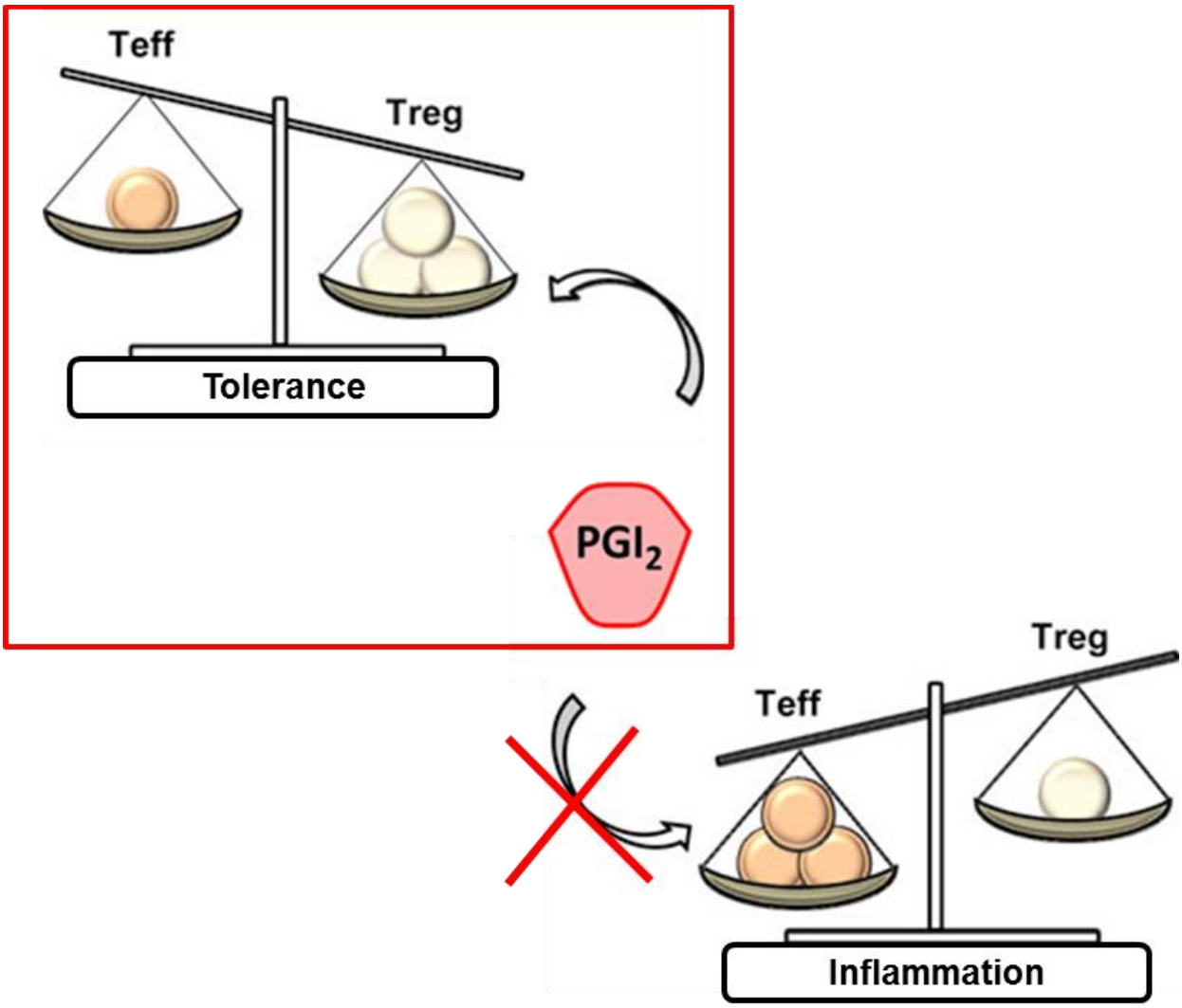

Figure 5.3. PGI_2 orchestrates Treg and Teff responses.

However, in 5-10% of patients, the disease is refractory to corticosteroid treatment and often leads to hospital admissions caused by respiratory viral and/ or bacterial infections. Corticosteroid use for RSV disease is similarly limited, with studies examining the effect of corticosteroid, bronchodilator, and anti-viral ribavirin therapy routinely showing no improvement compared to placebo for RSV bronchiolitis. Palivizumab, the only FDA-approved preventative therapy, is currently only recommended for infants in the first year of life with underlying conditions. The development of novel preventative and therapeutic strategies is imperative given the prevalence of the poorly-controlled asthma patient population and the lack of interventions that improve outcomes for RSV bronchiolitis once infection has occurred.

This need formed the basis of my investigations in which I explored whether γδ17 cell attenuation could provide an explanation for why asthmatic patients have significantly greater risk for invasive bacterial disease than nonasthmatic subjects (**Chapter I**), whether type 2 immune response inhibition could result in decreased immunopathology during RSV infection (**Chapter II**), and whether an anti-inflammatory agent could enhance the function of Tregs, an important cellular mediator of inflammation (**Chapter III**).

Heterologous immunity as a barrier to regulating airway inflammation

Multifaceted immune responses during inflammation present a significant challenge to attenuating pathogenic inflammatory responses while preserving protective host immune mediators during airway disease. *In vivo* small animal models of disease

allow the study of separate components of the immune response when examining the downstream consequences of potential therapeutic agents in preclinical studies.

In **Chapter I**, I found that endogenous IL-4 reduces lung γδ17 cell numbers during acute *KP* infection. Whether STAT6 signaling attenuates γδ17 cells and subsequent protective neutrophilic infiltration and bacterial burden in the context of allergic airway inflammation remains to be determined. Additional studies using mouse models of allergic airway inflammation with subsequent bacterial infection would shed further light on the impact of the Th2 cytokine-rich milieu in the asthmatic lung on the subsequent development of bacterial pneumonia. In **Chapter II**, I found that GLP-1 signaling has no effect on the production of the Th1 cytokine IFN-γ when administered therapeutically during RSV infection. However, a study by another group reported that GLP-1R agonist administration inhibited invariant (i)NKT cells secretion of IFN-γ *in vitro*. The effect of GLP-1 signaling on cellular IFN-γ production likely depends on the unique combination of secondary signaling pathway(s) activated by GLP-1 in distinct cell types. Future studies elucidating the signaling pathways utilized by GLP-1 in different immune cells will enhance our understanding of GLP-1 immunobiology. In **Chapter III**, I found that PGI_2 signaling promotes nTreg suppressive function. Although PGI_2 exhibits anti-inflammatory effects in most disease models studied to date, PGI_2 has been described to have a pro-inflammatory role in the context of rheumatoid arthritis. Thus, PGI_2's overall effect on inflammation is likely governed by the timing, location, and context of immune responses. Additional studies are currently underway in our group to investigate the role of PGI_2 signaling on nTregs in a Th2-driven mouse model of allergic airway inflammation as well as in Th1-driven mouse models of colitis and lupus. These

studies will reveal whether PGI_2 signaling consistently enhances Treg function in Th1 versus Th2, inflammatory versus autoimmune, and localized versus systemic disease conditions.

Precision medicine for airway inflammation

The rapid increase in scientific and bioinformatics tools that can be used to generate and search vast amounts of experimental, clinical, genetic, phenotypic, environmental, and demographic data to yield new knowledge about various disorders has yielded a new era of precision medicine. The driving vision behind the precision medicine initiative aims to develop an approach for disease treatment and prevention that takes into account individual variability for each person.

In the field of asthma, recent studies suggest that identifying distinct subsets of patients leads to more favorable responses to biologics that impair the functioning of the unique pathways involved in airway pathology. [288] For example, patients whose asthma is associated with increased levels of biomarkers of a Th2 phenotype have demonstrably benefitted more from therapy targeting elements of the Th2 response compared to patients with low levels of Th2 biomarkers.[131,289,290] My findings in **Chapter I** suggest that drugs targeting the IL-4 pathway could have detrimental effects on anti-bacterial host immunity in asthma patients. Conversely, my findings in **Chapter III** suggest that PGI_2 creates a protolerogenic environment that is potent enough to promote nTreg suppressive function and suppress Teff responsiveness to Treg-mediated suppression. These findings will contribute to the growing body of data our

scientific community will use to construct our initial working models of precision medicine for asthma treatment.

In the field of RSV disease, delineating the roles of viral and host factors in RSV immunopathology will better inform therapeutic strategies. Our group has been the first to sequence and identify RSV strains significantly associated with significantly increased risk of recurrent wheezing outcomes in infants as well as differential immune responses in mice. In **Chapter II**, we demonstrated that GLP-1R signaling attenuated RSV-induced immunopathology using a strain of RSV that was isolated from a hospitalized infant with severe lower respiratory tract infection and bronchiolitis from these studies. This strain contained a previously described mutation of 2 tandem stop codons for the RSV attachment glycoprotein (G) gene (2stop-A4G), which was associated with enhanced bronchiolitis severity in infants. The continuation of these studies will reveal whether RSV strains containing the 2stop-A4G mutation also associate with the development of RSV immunopathology and later childhood asthma. To this end, our group has recreated several recombinant viruses with the G and F proteins of clinical isolates containing 2stop-A4G or WT sequences using a reverse genetics approach. Our hypotheses for these future studies are that infection with RSV strains that contain the 2stop-A4G mutation enhance innate and adaptive Th2 immune responses, and augment subsequent allergic airway inflammation while exacerbating existing allergen- induced airway inflammation. Additional future work should be done to examine whether our finding that GLP-1R signaling attenuated RSV-induced immunopathology is also observed using additional pathogenic and/ or 2stop-A4G clinical strains of RSV.

RSV predominantly infects infants, with age as the principal risk factor for severe disease. Differences in the immune response to RSV in the very young are postulated to be critical in determining the clinical outcome of RSV infections. Thus, the effect of host age on the development of immunopathology and severe disease following RSV infection remains a critical question in the field. The neonatal immune response to RSV infection has been characterized by observed patterns of skewing towards Th2 and Th17 polarization and away from protective Th1 responses. However, no studies to date have compared differential responses of primary bronchial epithelial cells, the host cell for RSV, between infants and older populations during infection. Differentiating primary epithelial cells from humans in air-liquid interface (ALI) cultures represents a valuable tool for recapitulating the *in vivo* human bronchial epithelium. Given our group's unique access to difficult-to-attain specimens, a future study should analyze the differentially expressed transcriptome of primary infant versus toddler bronchial epithelial cells in ALI cultures infected with pathogenic RSV strains collected from infants with severe bronchiolitis. Such a study would provide clinically-relevant insight into which factors critical to initiating the protective immune response to RSV infection may be lacking in the neonatal bronchial epithelium.

The PheWAS algorithm represents a recently developed tool that allows for the linkage of subject-level deidentified genotyping information with electronic medical record-derived clinical phenotypes. In **Chapter II**, a PheWAS identified a link between GLP-1R signaling and acute bronchitis and bronchiolitis, corroborating our findings demonstrating the significance of GLP-1R signaling during RSV infection and expanding the relevance of these findings to humans. Additional studies are currently underway in

our group to examine whether genetic variants that affect GLP-1 and PGI_2 signaling also associate with allergic disease phenotypes.

Towards reliably controlling immune responses during airway inflammation

Although controlling aberrant immune responses during various states of airway inflammation has been demonstrably achieved in small animal models of disease, a significant poorly-controlled asthma patient population and a lack of interventions that improve outcomes for RSV bronchiolitis once infection has occurred still remain.

In **Chapter I**, I found that IL-4 also attenuates γδ17 production of IL-17 in a STAT6-independent manner. Further studies elucidating the molecular determinants of this finding could help circumvent the potential detrimental effects of anti-IL-4 therapies on anti-bacterial host immunity in asthma patients. In **Chapter II**, I found that treatment with a GLP-1R agonist attenuated IL-33 and immunopathology during RSV infection. GLP-1R agonists are the first known FDA-approved agents to inhibit IL-33, an important therapeutic target in inhibiting airway disease given its central role in mediating both innate and adaptive immunity-regulated lung inflammation. Thus, our group is currently working on elucidating the mechanisms by which GLP-1 signaling inhibits IL-33 production in human bronchial epithelial cells. Aerosolized and pathway-specific GLP-1R agonists are needed for more localized and potent as well as less off-target effects for treatment of airway inflammation. Dr. Niswender, our collaborator and GLP-1 expert, is actively working with Dr. Craig Lindsley to develop such new agents for clinical use.

There are many barriers currently precluding the effective use of clinical Treg-based therapies. Such barriers include Treg plasticity, whereby therapeutic,

immunoregulatory cells revert into pathogenic effectors of the immune system; concomitant use of immunosuppressants that interfere with Tregs; and obtaining sufficient numbers of Tregs from a single donor. In **Chapter III**, we found that PGI_2 promotes nTreg function and may represent a new treatment strategy for Treg-mediated diseases. Future studies further exploring this finding would greatly benefit from the generation of a mouse model in which IP deletion is inducible and specifically limited to Foxp3-expressing Tregs. Such a model would allow greater resolution in studying the effect of PGI_2 on Tregs, enabling the collection of data looking into the role of PGI_2 on Tregs during development and in tissue-specific regions. The generation of a mouse model in which IP deletion is combined with fate-mapping of Foxp3-expressing Tregs would also allow further study of the effect of PGI_2 on Treg plasticity and the generation of ex-Tregs. The findings in this work contribute to our understanding of how to enhance Treg function, alongside current efforts to delineate more active Treg subsets and more potent antigen-specific Tregs. Future efforts in Treg research should also be directed towards overcoming the clinical barriers to the use of Treg-based therapies.

As discussed in **Chapter I**, recent therapeutic strategies for treating allergic asthma have targeted downstream IgE and Th2 cytokines such as IL-4, IL-5, IL-13, and IL-17. The most recent emerging therapies for attenuating type 2-related airway inflammation are targeting upstream mediators such as TSLP, IL-33, and GATA3. Interestingly, these upstream-acting therapies have shown efficacy in a broader range of diseases- including atopic dermatitis and colitis- in which Th2 responses play a role.

There is much to learn from other fields that have successfully tailored immune responses for more effective disease resolution. For example, chimeric antigen receptor

(CAR) T cells specifically target cancer cells and significantly increased survival rates in some forms of cancer. One could imagine extending this approach to generate chimeric antigen receptor Tregs or inhibitory IgG-secreting plasma cells specific for certain allergens. Immune checkpoint inhibitors re-activate cytotoxic T cells and also demonstrated efficacy in reducing tumor size. Activation and ligation of the cell surface receptor programmed cell death protein 1 (PD-1) results in apoptosis. PD-1 was also recently found on iNKT cells and ILC2. Blocking PD-1 with an anti-PD-1 antibody reduced IL-13-producing ILC2 during influenza infection as well as IL-5-producing ILC2 and lung inflammation during airway challenge with the protease allergen papain. Anti-PD-1 antibody also prevented $CD8^+$ T cell impairment and reduced viral titers during acute viral lower respiratory infection with human metapneumovirus, suggesting that immune checkpoint inhibition could dually attenuate type 2 immunopathology while enhancing anti-viral host immune responses.

Numerous pathogenic versus protective bacterial species have been identified as modulators of airway inflammation. For example, dietary *Lactobacillus johnsonii* supplementation protected against both airway allergen challenge and RSV infection. I contrast, nasal *Streptococcus* and *Haemophilus* colonization have been linked to increased RSV disease severity. Similar to fecal transplantation for the treatment of *Clostridium difficile* infection, the future of treatment for airway inflammation could very well entail cocktails enriched in protective bacterial species engineered to outcompete pathogenic microbes. Future characterization of the human microvirome will undoubtedly uncover new players in airway inflammation.

Engineered microbes have additionally been used as delivery vehicles for therapeutic agents such as cytokines, antigens, antibodies, or genetic materials. Inoculation with innocuous lung commensals engineered to release immunoregulatory cytokines or anti-inflammatory antibodies could represent a valuable therapeutic strategy for combating airway disease. Many groups are also designing synthetic materials to optimize the spatial and temporal delivery of drugs. Specifically, an array of nanoparticles, engineered cell-nanoparticle combinations, and cytokine-releasing microspheres are in development to target the correct tissue site, control entry into the cell, and moderate the kinetics of drug signal delivery. Similarly, engineered exosomes are emerging as promising drug delivery vehicles given their cell membrane composition which include key adhesive proteins.

Identifying new therapeutic targets, developing new therapies and modes of drug delivery, determining optimal combinations of new drugs with previous-line therapies, and continually fine-tuning the application of therapeutic regimens in line with our understanding of disease endotypes and host pharmacogenomics will all be critical towards realizing the goal of controlling immune responses during airway inflammation.

Conclusions

The striking rise in the prevalence of allergic diseases has been accompanied by the rapid growth of technology and resources devoted to developing treatments for these disorders. Effectively controlling immune responses during various states of airway inflammation will require targeting multiple components of the immune system with great precision. The work presented here reveal new insights regarding the

regulation of specific immune modulators of allergic airway inflammation as well as acute pulmonary bacterial and viral infection. As we learn to control the various mediators of airway inflammation, our greatest challenge moving forward is translating these findings into the clinic. In the long run, our ability to integrate and translate our findings into meaningful, clinical therapies will dictate the next phase of progress in the field of asthma and immunology, hopefully making infectious asthma exacerbations, poorly-controlled asthma, and RSV-related morbidity and mortality things of the past.

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