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THE REGULATION OF MUCOSAL IMMUNITY BY INFLAMMATORY
MEDIATORS

By

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Bachelor of Science in Microbiology, Montana State University, Bozeman, MT 2009

Dissertation

presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in Toxicology

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The Regulation of Mucosal Immunity by Inflammatory Mediators

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Abstract

Despite massive efforts to develop effective therapeutic options for the treatment of asthma, disease prevalence continues to increase. Approximately 300 million people worldwide have been estimated to be affected by asthma, and it remains a leading cause of childhood hospitalization. While environmental as well as genetic causative agents have been identified, the heterogeneous nature of the disease has proven difficult to effectively treat. Asthma is characterized as chronic inflammation of the airway characterized by increased airway hyperreactivity, increased goblet cell hyperplasia, and airway remodeling. While these effects are traditionally regarded to be mediated through the actions of CD4⁺ Th2 cells, recent advances have suggested that underlying innate immune cell populations within the airway mucosa might exert a critical role in the development of asthma. However, the inflammatory mediators and respective mechanisms responsible for this role remain poorly understood. In this study, we attempt to elucidate the role of two mucosal-associated inflammatory mediators, S-nitrosoglutathione and prostaglandin I₂ in the pathogenesis of asthma in two distinct mouse models of allergic asthma. In our first study, we demonstrated that therapeutic treatment using SPL-334, a specific inhibitor of S-nitrosoglutathione reductase, significantly reduced the development of allergic airway inflammation. In our second study, we demonstrated that loss of prostaglandin I₂ signaling resulted in significant alterations in pulmonary NK cells. Using an *in vivo* depletion method, we demonstrated that pulmonary NK cells are responsible for attenuated development of house dust-mite induced allergic airway inflammation. In our third and final study, we demonstrated that loss of prostaglandin I₂ signaling leads to substantial reduction in the production of non-antigen-specific IgG2b and IgA via an undetermined mechanism. The results of this dissertation strongly suggest that regulation of mucosal immunity by inflammatory mediators such as S-nitrosoglutathione and prostaglandin I₂ can have profound effects on the development of mucosal-associated diseases, and offer novel insights into potential future therapeutic targets for the treatment of diseases such as asthma.

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CHAPTER ONE

Introduction and General Background

1.0 Introduction

In the past 40 years, the prevalence of asthma has continued to increase steadily, and current estimates indicate that approximately 300 million people worldwide are currently affected by asthma (Masoli, Fabian et al. 2004). The high cost associated with morbidity and mortality in patients suffering from asthma reflects the severity of the disease and the need for effective therapeutic approaches. In particular, the development of asthma in young children has been shown to result in significant numbers of hospitalizations and deaths, particularly in patients afflicted by poverty and inadequate access to medical care (Mitchell 1985; Evans, Mullally et al. 1987; Beasley 2002). The heterogeneous nature of the disease has made effective treatment of asthma extremely difficult, as a wide variety of both environmental and genetic factors contribute to asthma pathogenesis (Holloway, Yang et al. 2010). The most prevalent clinically recognized form of asthma is allergic, or atopic, asthma (Kim, DeKruyff et al. 2010). This form of asthma is predominantly mediated through adaptive immune responses, specifically through the actions of CD4⁺ Th2 cells and IgE-producing B cells. These immune cells are capable of extensive recognition and specificity towards a diverse array of antigens, resulting in potent effector function and long-lasting immunological memory (Sakaguchi, Yamaguchi et al. 2008). Recognition of environmental antigens, referred to as allergens, by immune cells resident in airway mucosal tissue results in extensive activation of the adaptive immune system that can pose a serious threat to the host. Animal models of asthma, particularly rodent models, have been used to great effect to elucidate the

underlying cellular and molecular mechanisms responsible for pathogenesis of asthma, and most of our current understanding of the Th2-associated nature of asthma is a result of extensive use of animal models (Bates, Rincon et al. 2009; Holmes, Solari et al. 2011). However, while the role of the adaptive immune system in the regulation of asthma pathogenesis has been widely studied, the role of the innate immune system remains underappreciated (Holtzman 2012). In particular, our understanding of the mediators responsible for regulation of innate immunity in response to allergen exposure remains unresolved. The objective of the studies described in this dissertation are to expand our current knowledge of the regulatory role of inflammatory mediators present in mucosal tissue, and how regulation of innate immunity by these inflammatory mediators alters the pathogenesis of asthma. The work presented herein can be divided into two distinct aims. Our first aim was to utilize a specific inhibitor of S-nitrosoglutathione reductase, SPL-334, to characterize the role of S-nitrosoglutathione in the regulation of an animal model of allergic asthma. Our second aim was to determine whether loss of prostaglandin I₂ signaling resulted in alteration of innate immune cells that had the capacity to affect the development of asthma pathogenesis in an animal model of allergic asthma.

1.1 Pathophysiology of Asthma

Allergic asthma is a complex, heterogeneous disease defined as chronic inflammation of the airway clinically associated with breathlessness, wheezing, and coughing following exposure to allergen. These clinical manifestations arise from the activation of the immune system resulting in increased airway levels of IgE, infiltration of the airways by inflammatory immune cells such as eosinophils, airway hyperreactivity (AHR), goblet cell hyperplasia resulting in increased airway mucus production, and

airway remodeling (Strachan 1989; Robinson, Hamid et al. 1992; Cohn, Elias et al. 2004; Kowalski 2007). Classically, CD4⁺ T cells are considered the primary orchestrator of asthma-associated inflammatory responses. However, in addition to CD4⁺ T cells, infiltration of the airway by macrophages, dendritic cells, eosinophils, and mast cells leads to exacerbation of the inflammatory response resulting in a milieu of cytokines, chemokines, and cytotoxic mediators that ultimately culminate in airway hyperreactivity, mucus production, and airway remodeling (Mukherjee and Zhang 2011).

1.1.1 Airway Hyperreactivity and Airway Remodeling

Airway hyperreactivity (AHR) is defined as increased bronchoconstriction in response to nonspecific stimulus of the airway (Boushey 1982). While the precise mechanism underlying AHR is poorly understood, it has been recognized that there is a close correlation between the degree of AHR and levels of airway inflammation (Jeffery, Wardlaw et al. 1989). The consequences of AHR include a reduction in airway diameter, increased smooth muscle contractility, increased microvascular permeability, and generation of inflammatory mediators that can result in epithelial injury (Skoogh 1984; Fryer, Lein et al. 2004). The damage to epithelial cells triggers enhanced mucus production and shedding of epithelial cells into the airway (Holgate 2008). Attempts by epithelial and stromal cells to repair the damage caused by inflammatory mediators results in remodeling of the airway through the deposition of collagen (type I, II, III) and fibronectin, and subsequent thickening of the airway epithelial layer from 10 to 300% (Roche, Beasley et al. 1989; Homer and Elias 2000).

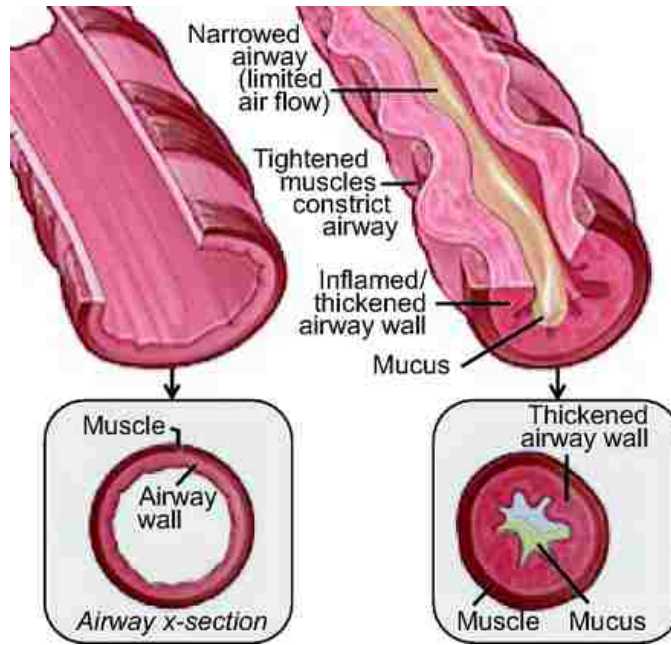


Figure 1.1 *Airway Remodeling.* Inflammatory mediators induced by allergen exposure result in smooth muscle contractility, epithelial shedding, goblet cell hyperplasia, and collagen deposition that severely restrict airflow (Doeing and Solway 2013).

1.2 Immune Cells Involved in Asthma-associated Inflammation

1.2.1 $CD4^+$ T Cells

As $CD4^+$ T cells are the hallmark immune cell responsible for mediating the inflammatory effects observed in allergic asthma, understanding the effector functions of this immune population is critical in understanding asthma pathogenesis. Th2 cells are characterized based on the expression of a specific cytokine profile which include IL-4, IL-5, and IL-13 that are capable of aiding the differentiation and activation of humoral responses in order to generate antigen-specific responses. IL-4 has been recognized to have the greatest influence in driving Th2 differentiation, as IL-4 signaling via the STAT6 pathway induces transcription factor GATA3 and subsequent commitment to the Th2 lineage, resulting in a cascade effect responsible for the expansion of a large

population of Th2 cells from naïve T cells (Swain, Weinberg et al. 1990; Zheng and Flavell 1997). The production of IL-4 by CD4⁺ T cells promotes direct and indirect recruitment of eosinophils into the airway, as IL-4 signaling has been shown to activate endothelial and epithelial cells that subsequently produce chemokines responsible for eosinophil recruitment (Dubois, Schweizer et al. 1998; Nakamura, Luster et al. 2001). Additionally, IL-4 has been shown to induce the synthesis of IgE by activated B cells, as well as promote IgG1 and IgE isotype switching (Hasbold, Hong et al. 1999). IL-13 shares a significant number of similarities in the signaling role involved in the pathogenesis of asthma. However, IL-13 has been shown to be a more potent inducer of AHR and chronic remodeling changes in the airway via smooth muscle hyperplasia and subepithelial fibrosis (Grunig, Warnock et al. 1998; Wills-Karp, Luyimbazi et al. 1998; Zhu, Homer et al. 1999). IL-5 is a highly effective and necessary eosinophil recruitment and survival factor during allergic lung inflammation, capable of driving eosinophils in the bone marrow to enter into the periphery (Huffnagle, Boyd et al. 1998).

Table I. *Cytokines that directly promote features of asthma*

Feature	Cytokines Implicated
Eosinophilia	IL-4, IL-5, IL-13
Goblet cell metaplasia	IL-4, IL-13
Airway hyperresponsiveness	IL-4, IL-13, IL-17A
IgE production	IL-4, IL-13
Mastocytosis	IL-3, IL-9
Alternative macrophage activation	IL-4, IL-13
Smooth muscle remodeling	IL-4, IL-13
Th2 induction/maintenance	IL-4, IL-9, IL-17E (IL-25), IL-33, TSLP
Subepithelial fibrosis	IL-4, IL-13, TGF-β

Figure 1.2 *The effector functions of Th2-associated cytokines in Allergic Asthma.* Th2-associated cytokines produce a wide range of effector functions that drive the inflammatory response observed following allergen exposure (Finkelman, Hogan et al. 2010).

1.2.2 Eosinophils

While CD4⁺ T cells retain the most influence in the orchestration of asthma pathogenesis, the most distinctive immune cells associated with allergic asthma are eosinophils. They are found not only in significantly increased numbers in the lung tissue of asthmatic patients, but found to be expanded out into the sputum and bronchoalveolar lavage fluid as well (Bousquet, Chanez et al. 1990; Lemiere, Ernst et al. 2006). The contribution of eosinophils to allergic airway inflammation is regarded as primarily a release of pro-inflammatory cytotoxic granule proteins such as EPO as well as through release of lipid mediators (Weller, Lim et al. 1996; Gleich 2000). The levels of eosinophils and eosinophil-derived products found in the airways remain the standard for indicating the severity of allergic asthma inflammation (Venge 2004).

Eosinophils display a prodigious capacity for the synthesis and secretion of inflammatory mediators, and approximately 35 inflammatory cytokines, chemokines, cytotoxic factors, and growth factors have been described, and the storage of these compounds in intracellular granules means activation of eosinophils results in immediate and indiscriminate inflammation directly at the site of activation (Lacy and Moqbel 2000; Hogan, Rosenberg et al. 2008).

1.2.3 Dendritic Cells

In order to induce a CD4⁺ T cell-mediated immune response following allergen exposure, the processing and presentation of allergen must occur. In the airway, three subsets of dendritic cells have been identified that are capable of sampling the airway for the presence of inhaled antigens. These subsets are derived from Ly-6C-expressing peripheral monocytes, and are characterized as CD11b⁻CD103⁺, CD11b^{hi}CD103⁻, and

CD11b^{lo}CD103⁻ (Masten and Lipscomb 1999; Sung, Fu et al. 2006; Condon, Sawyer et al. 2011). While all subsets are capable of the internalization and processing of antigen, differential pathogen receptor expression suggests that these dendritic cells are capable of inducing responses to highly specific antigens (Condon, Sawyer et al. 2011). Processing of antigen results in loading antigenic peptide onto the MHC class II or HLA (mouse and human, respectively) receptor, allowing for subsequent presentation to T cells (Riese and Chapman 2000). The presentation is accomplished via migration to the draining lymph nodes of the lung, where engagement of the MHC/antigen complex binds to the T cell receptor in addition to several additional co-receptors, resulting in allergen sensitization and subsequent memory response (Humrich, Humrich et al. 2006; Smit and Lukacs 2006)

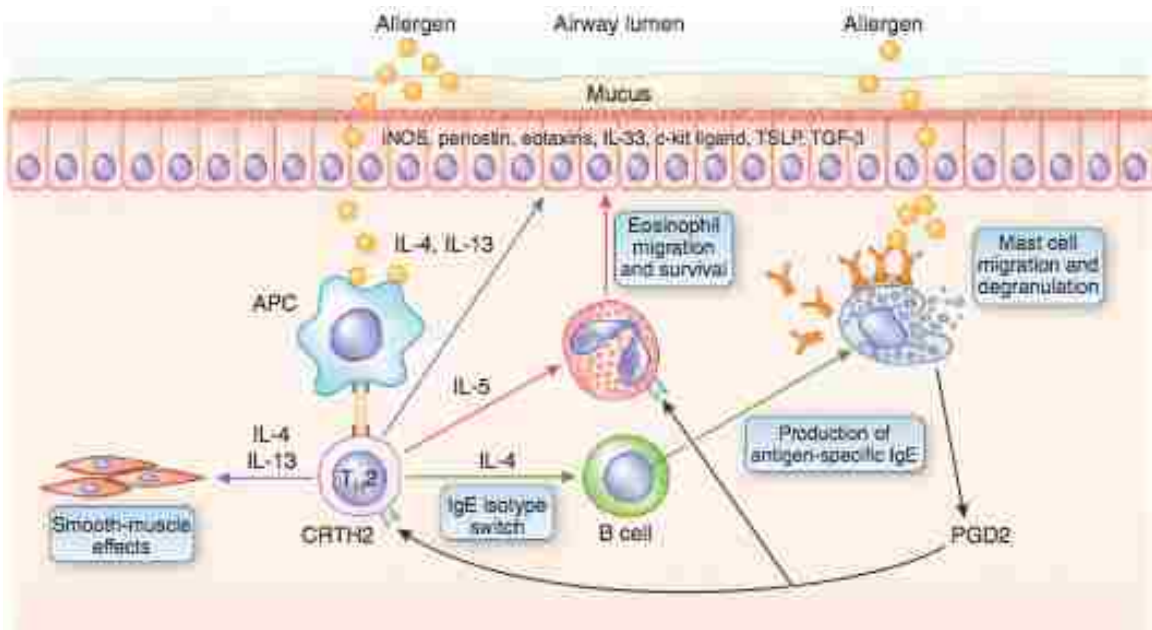


Figure 1.3 Overview of Th2-mediated development of allergic inflammation. Activation of Th2 cells following allergen presentation by dendritic cells results in cytokine production capable of regulating a wide variety of cell types. This regulation results in inflammation of the airway as a response to allergen exposure.

1.3 Animal Models of Asthma

Using animal models to further our understanding of human diseases is a universal practice in biomedical research. In regard to allergic asthma, animal models have a long history of use to elucidate the basic immunological biology and mechanisms responsible for pathogenesis (Karol, Cormier et al. 1994). Mouse models of allergic asthma are the most commonly utilized animal model, as genetic variability and ease of use create a powerful tool for deeper understanding of the role of the immune system in the development and progression of allergic asthma. This section highlights two highly utilized mouse models of allergic asthma, the traditional Ovalbumin-sensitized mouse model and the more recently adopted House dust mite-induced mouse model of allergic asthma.

1.3.1 Ovalbumin-induced Mouse Model

Ovalbumin (OVA) is a water-soluble protein that comprises the majority of egg white. Vaz et al demonstrated that intraperitoneal injection of low dose OVA was capable of inducing an antibody response in inbred mouse strains (Vaz, Vaz et al. 1971). A few years later, Van Hout & Johnson demonstrated that aerosolized OVA was capable of inducing IgE antibody production in rats following initial sensitization utilizing *Bordetella pertussis* heat-killed vaccine (Van Hout and Johnson 1972). The use of adjuvants such as *B. pertussis* in the induction of allergic reactions led to some dispute amongst researchers, and Ahlstedt et al demonstrated that aerosolized OVA alone was capable of producing a robust inflammatory response in the airways without the use of adjuvants (Ahlstedt and Bjorksten 1983). These early studies led to the development of

the OVA-induced protocols utilized today for the induction of allergic lung inflammation in mice.

Acute models of asthma typically involve *intraperitoneal* injection of OVA with or without the addition of an adjuvant such as alum or Al(OH)₃ over the course of two weeks followed by daily OVA aerosol challenge for 3 – 7 days (McMillan and Lloyd 2004). This model has been shown to reproduce several key characteristics of asthma, including elevated IgE, infiltration of the airways by inflammatory immune cells, goblet cell hyperplasia, as well as the development of airway-hyperreactivity and bronchoconstriction (Kumar, Herbert et al. 2008). However, it has been described previously that repeated OVA aerosol and *intravenous* challenged mice will develop tolerance rather than sensitization (Van Houten and Blake 1996; Pape, Merica et al. 1998).

For the study presented in Chapter 3 of this dissertation, in order to facilitate the analysis of the CD4⁺ T cell response to inhaled OVA, we utilized the DO11.10 adoptive transfer model similar to that described by Kearney et al (Kearney, Pape et al. 1994). Naïve CD4⁺ T cells from DO11.10 mice express a transgenic $\alpha\beta$ T cell receptor specific for OVA₃₂₃₋₃₃₉ peptide presented by the I-A^d class II MHC that can be detected using the anti-clonotypic antibody KJ1-26 (Haskins, Kubo et al. 1983). DO11.10 T cells have been shown to undergo clonal expansion following antigen exposure, as well as display resistance to the development of tolerance (Kearney, Pape et al. 1994). Adoptive transfer of DO11.10 CD4⁺ T cells into Balb/c mice and subsequent exposure to OVA has been shown to induce airway inflammation that closely mimics that seen in asthma (Lee, Jaffar et al. 1999). The availability of the anti-clonotypic antibody KJ1-26 provides a

method for the identification of OVA-specific donor T cells *in vivo*. The lack of development of T cell tolerance in response to OVA exposure, combined with the biologically relevant clonal expansion of an antigen-specific T cell population that results in the characteristic immune responses observed in asthma make the DO11.10 adoptive transfer model highly attractive for use in studying asthma pathogenesis.

1.3.2 House Dust Mite Allergen-induced Mouse Model

Although the traditional OVA-induced animal model of allergic asthma has provided an impressive amount of relevant data over the last several decades, there are concerns with the model that have led researchers to continue the search for models that more closely mimic clinical features and relevancy to human disease. Primary among these concerns is that the systemic delivery and immune activation of OVA and additional adjuvants does not mimic human exposure and sensitization. Additionally, the development of tolerance following prolonged exposure to OVA results in studies skewed towards acute development rather than chronic development of asthma. Recent efforts to address these concerns have resulted in the adoption of mouse models utilizing house dust mite allergen exposure to mimic asthma development.

House dust mites (HDM) are members of the family Pyroglyphidae, and two primary species have been the focus of intense interest in the development of asthma, the European house dust mite (*Dermatophagoides pteronyssinus*) and the American house dust mite (*D. farinae*). Several studies have indicated that asthmatic patients exhibit significantly elevated HDM-specific IgE in airways and serum compared to control patients, indicating a role for HDM in the development of asthma in humans (Shimojo, Hirano et al. 1992; Nahm, Kim et al. 2000). Additionally, exposure to HDM in asthmatic

patients resulted in development of airway hyperreactivity and increased airway inflammation (Roche, Chinnet et al. 1997). Analysis of HDM has revealed an impressive array of allergens, primarily proteases, present in the HDM gastrointestinal tract that have been subdivided into 24 major groups based on molecular category, immunological activity, and quantitative allergenicity (Calderon and Cox 2014). Of these, Group 1 and Group 2 (Der p/f 1 and Der p/f 2, respectively) have been generally accepted to be the most clinically relevant, and the development of recombinant protein reagents for these allergens has led to increased focus on the mechanisms involved in HDM-induced asthma pathogenesis. It is particularly noteworthy that mice can be sensitized by HDM extracts entering the airway and hence serve as a close approximation to the natural route of allergen sensitization. Although the ultimate outcome of sensitization to HDM is the development of a CD4⁺ Th2-driven IgE-dependent allergic response, research into the mechanisms of HDM-induced asthma has revealed an expanded role for the involvement of the innate immune system in the development of asthma. Specifically, it has been described that HDM has the capacity to directly activate alveolar macrophages, mast cells, and epithelial cells through PAR and TLR signaling and induce Th2-associated cytokine responses without previous sensitization (Jacquet 2013; Wang 2013).

For the study described in Chapter 4 of this dissertation, we utilized a chronic HDM-induced model of allergic asthma in order to address some of the concerns regarding the OVA-induced model of allergic asthma as well as to maintain relevance following wider adoption of HDM-induced models in asthma-related research. It has been described previously that continuous *intranasal* exposure to HDM in Balb/c mice elicited eosinophilic and CD4⁺ T cell airway infiltration, increased production of Th2-

associated cytokines by *in vitro* stimulated splenocytes, and airway remodeling not observed in OVA-exposed Balb/c mice (Johnson, Wiley et al. 2004). Additionally, the increased relevance of innate immunity in the development of HDM-induced asthma that has been observed in other studies has tantalizing potential as an area for further research interest that could lead to clinically relevant results.

CHAPTER 2

MATERIALS & METHODS

2.0 Materials & Methods

2.0.1 Cell Culture Media

Cells were cultured in Complete RPMI (CRPMI) media comprised of L-glutamine(-) RPMI 1640 media supplemented with 5% fetal bovine serum (FBS), L-glutamine (Life Technologies, Carlsbad CA), penicillin and streptomycin (Life Technologies), Hepes (Life Technologies), Sodium Pyruvate (Life Technologies), and 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO).

2.0.2 Animals

DO11.10, Balb/c, $IP^{-/-}$, and C57BL/6 mice were purchased from Jackson Labs, Bar Harbor ME (The Jackson Laboratory) and were bred and housed in pathogen-free conditions in the animal facilities at the University of Montana (Missoula, MT). $IP^{-/-}$ mice were originally a generous gift of Dr. Garret A. Fitzgerald (University of Pennsylvania, Philadelphia, PA). All experiments described in this dissertation were performed to the guidelines of the National Institutes of Health, Bethesda, NIH, IACUC.

2.0.3 Animal Sensitization

2.0.3.1 Preparation of DO11.10 $CD4^{+}$ Th2 Cells

Peripheral lymph nodes obtained from DO11.10 mice were depleted of $CD8^{+}$ using MACS beads (Miltenyi Biotech, Auburn CA) and incubated in CRPMI (5×10^5 cells/ml) in the presence of OVA₃₂₃₋₃₃₉ (1 μ g/ml; Mimotopes, San Diego, CA), IL-4 (2 μ g/ml; R&D Systems, Minneapolis, MN), and mAb anti-IFN- γ (5 μ g/ml clone R4-6A2; American Type Tissue Collection [ATTC], Manassas, VA) for 4 days at 37°C.

Following incubation, cells were restimulated in identical culture conditions with the addition of IL-2 (10 ng/ml; R&D Systems) for 4 days at 37°C. On day 8, cells were depleted of class II⁺ cells by treating with mAb anti-class II (5 µg/ml clone M5/114; ATTC) for 30 min, followed by incubation with 10 µg/ml plate-bound mouse anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) for 1 hr. Non-adherent (class II⁻ cells) CD4⁺ Th2 cells (>98% purity) were collected for analysis and transfer experiments.

2.0.3.2 Transfer of Polarized DO11.10 CD4⁺ Th2 Cells and OVA Aerosol Challenge

Eight-day polarized DO11.10 CD4⁺ Th2 cells generated as described in section 2.0.3.1 were adoptively transferred (6x10⁶ cells/mouse) into Balb/c animals via intravenous injection (100 µl of suspension). Mice (four to six per group) were then challenged by exposure in a chamber to aerosolized solutions of OVA (0.5%, Grade V; Sigma-Aldrich) for 20 min/day, over 7 consecutive days using a Wright's nebulizer (Buxco). Control mice were exposed to OVA aerosols but did not receive DO11.10 Th2 cells or received Th2 cells but were exposed to aerosolized PBS.

2.0.3.3 Intranasal Sensitization to Derp House Dust Mite Allergen

IP^{-/-} and WT mice (four to six per group) were intranasally sensitized with 30 µl whole Derp house dust mite (HDM) allergen (100 µg/mouse; Greer, Lenoir, NC) on day 0. Following sensitization, mice received subsequent challenge with HDM (50 µg/mouse in 30 µl volume PBS; Greer) on day 7 and day 14. Three days following final HDM challenge, mice were analyzed for development of allergic lung inflammation. Control mice received intranasal 30 µl PBS on day 0, day 7, and day 14.

2.0.4 Cell Purification

2.0.4.1 Isolation of Lung Mononuclear Cells

Lung tissue was mechanically dissociated into small fragments using a pair of scissors followed by chemical dissociation using 0.1% collagenase (Type IV, Sigma-Aldrich) for 60 min at 37°C in CRPMI. Fragments were vigorously shaken every 15 minutes. Following dissociation, lung digest was filtered through a 70 µm nylon cell strainer (Falcon) and washed in CRPMI by centrifugation at 1350 RPM at 4°C for 10 minutes. Viable mononuclear cells were isolated from the lung digest over a Percoll density step gradient (41.7% and 67.6%).

To prepare the Percoll density step gradient, the stock solution of Percoll (GE Healthcare, Piscataway, NJ) was made by adding 3.5 ml of 10X PBS into 35 ml Percoll. The heavy (67.6%) and light (41.7%) density Percoll were made by combining 20 ml stock Percoll with 9.6 ml CRPMI and 15 ml stock Percoll with 21 ml CRPMI, respectively. Lung digest was resuspended in 7 ml of light density Percoll and carefully layered on top of 7 ml of heavy density Percoll. Finally, 7 ml CRPMI was carefully layered on top of the resuspended lung digest. This Percoll density gradient was centrifuged at 1900 RPM at 4°C for 30 minutes with acceleration and brake set to the lowest available setting. The mononuclear cells were removed using Pasteur pipette (Alpha Laboratories, Hampshire, UK) and washed with HBSS (Life Technologies) at 1350 RPM at 4°C for 10 minutes, and resuspended in CRPMI.

2.0.4.2 Isolation of Peyer's Patch Mononuclear Cells

The small intestines of IP^{-/-} and WT mice were removed and the fecal content removed by flushing with 5 ml of CRPMI. Peyer's patches were removed from the

intestinal wall using scissors and chemically dissociated using 0.5 mg/ml collagenase (Type IV, Sigma-Aldrich) for 40 min at 37°C in CRPMI. After 40 min, 125 µl 0.5 M EDTA (Sigma) was added for 5 minutes at 37°C to halt collagenase activity. Following dissociation, Peyer's patch digest was filtered through a 70 µm nylon cell strainer (Falcon) and washed in CRPMI by centrifugation at 1350 RPM at 4°C for 10 minutes, and resuspended in CRPMI.

2.0.4.3 Isolation of Splenic Mononuclear Cells

The spleens of IP^{-/-} and WT mice were removed, mechanically dissociated by forcing through a 70 µm nylon cell strainer (Falcon), and washed in CRPMI by centrifugation at 1350 RPM at 4°C for 10 minutes. Splenic cells were resuspended in 7 ml media, layered on top of 7 ml Lympholyte Mouse Cell Separation Media (Cedarlane, Burlington, NC), and centrifuged at 2000 RPM for 20 min at 4°C with acceleration and brake set to the lowest available setting. The lymphocytes were removed, washed in HBSS at 1350 PRM for 10 minutes at 4°C, and resuspended in CRPMI.

2.0.4.4 Isolation of Lymph Node Mononuclear Cells

The brachial and mesenteric lymph nodes were removed from IP^{-/-} and WT mice, mechanically dissociated by forcing through a 70 µm nylon cell strainer (Falcon), and washed in CRPMI by centrifugation at 1350 RPM at 4°C for 10 minutes. Lymph node lymphocytes were resuspended in CRPMI.

2.0.4.5 Isolation of Bone Marrow Mononuclear Cells

The femurs of IP^{-/-} and WT mice were removed using scissor and the bone marrow flushed out with 5 ml CRPMI using a syringe (BD Biosciences, Franklin Lakes,

NJ). Bone marrow was then washed in CRPMI by centrifugation at 1350 RPM at 4°C for 10 minutes, then resuspended in CRPMI.

2.0.4.6 Collection of Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) was collected by cannulation of the trachea of euthanized mice (100 µl Euthasol) and washing the airways with 3 X 0.5 ml of PBS. BALF was centrifuged to yield pelleted cells, which were pooled. The soluble phase BALF was used for cytokine determination. BAL cells of four to six animals were used for flow cytometry analysis, cell differential counts, and determination of eosinophil peroxidase levels as described below.

2.0.4.7 Collection of Serum Samples for Immunoglobulin Quantification

Individual serum samples were collected from euthanized (100 µl Euthasol) IP^{-/-} and WT mice by cardiac puncture and placed in 1.5 ml Eppendorf tubes (VWR, Radnor, PA) containing 50 µl of EDTA (Sigma). Individual samples were allowed to sit on ice for 20 min, followed by centrifugation at 14000 RPM for 10 min at 4°C. Serum was carefully collected and frozen down at -20°C for immunoglobulin quantification as described below.

2.0.4.8 Collection of Fecal Content for sIgA Quantification

The small intestines of IP^{-/-} and WT mice were removed and flushed as described in Chapter 2 section 2.0.4.2. Flushed fecal contents were collected individually and centrifuged at 2000 RPM for 10 min at 4°C, and supernatant was collected for sIgA quantification as described below.

2.0.5 Natural Killer Cell Protocols

2.0.5.1 Natural Killer Cell Depletion Utilizing mAb anti-NK1.1

Systemic depletion of natural killer (NK) cells in IP^{-/-} and WT mice was accomplished by intraperitoneal (i.p.) injection of 150 µl anti-NK1.1 monoclonal antibody (mAb)(250 µg PK136, ATCC) one day prior (day -1) to HDM challenge (day 0) as described in Chapter 2 Methods section 2.0.3.3. Mice received subsequent i.p. injections of 150 µl mAb anti-NK1.1 (250 µg PK136, ATCC) on day 3, day 6, day 10, and day 13. Control mice received i.p. injection of 150 µl PBS on day -1, day 3, day 6, day 10, and day 13.

2.0.5.2 Oral-pharyngeal Adoptive Transfer of Natural Killer Cells

Splenic mononuclear cells were isolated from IP^{-/-} and WT mice as described in section 2.0.4.3, and NK cells purified by magnetic cell sorting (MagCelect, R&DSystems). Sorted NK cells were selected on the basis of being CD3⁻NK1.1⁺ and purity was determined by measuring the proportion of CD3⁻NK1.1⁺ cells (87-92% over three experiments). Sorted NK cells were resuspended (5×10^5 cells/ml) in RPMI lacking FBS but supplemented with 10 ng/ml IL-2, and 30 µl was instilled by oral-pharyngeal administration (four mice per group) 24 hours following HDM administration (100 µg/mouse; Greer).

2.0.6 Intranasal Administration of SPL-334

Prior to administration, SPL-334 (SAJE Pharma, Baltimore, MD) was dissolved in PBS by sonication for 5 min. DO11.10 Th2 cell-recipient mice (four to six per group) were lightly anesthetized with isoflurane to allow for intranasal administration of 30 µl SPL-334 (0.1 mg/kg or 1 mg/kg) for 7 consecutive days. Administration of SPL-334

occurred 5 hours prior to OVA aerosol exposure as detailed in Chapter 2 Methods section 2.0.3.2. Control animals received intranasal administration of 30 μ l PBS for 7 consecutive days, 5 hours prior to OVA aerosol exposure.

2.0.7 Cytokine & Chemokine Quantification

2.0.7.1 In vitro Stimulation of Cells Isolated from Dissociated Lung Tissue

Immune cells isolated from dissociated tissue as described in Chapter 2 Methods section 2.0.4 were stimulated *in vitro* with either plate-bound mAb or recombinant cytokines.

For plate-bound stimulation, mAb anti-CD3 (2 μ g/ml clone 2C11; ATCC) mAb anti-NK1.1 (20 μ g/ml clone PK136; ATCC), or mAb anti-NKp46 (10 μ g/ml clone 29A1.4; Biolegend) was added to 24 well cell culture plates (Greiner Bio-One, Monroe, NC) and incubated for 24 hours at 37°C. Following incubation, cells (1×10^6 cells/ml) were added and incubated for 24 hours at 37°C, with subsequent collection of supernatants. In the case of mAb anti-NK1.1 and mAb anti-NKp46, recombinant IL-2 (10 ng/ml; Biolegend) was added to wells in conjunction with addition of cells. Supernatants were frozen down at -20°C for further cytokine quantification

For recombinant protein stimulation, recombinant IL-2 (10 ng/ml; Biolegend), recombinant IL-23 (20 ng/ml; R&D Systems), OVA₃₂₃₋₃₃₉ peptide (1 μ g/ml; Mimotopes, San Diego, CA) or HDM (20 μ g/ml; Greer) were added to 24 well cell culture plates (Greiner Bio-One) in conjunction with cells from isolated tissue (1×10^6 cells/ml) and incubated for 24 hours at 37°C. Supernatants were frozen down at -20°C for further cytokine quantification.

2.0.7.2 ELISA Cytokine & Chemokine Quantification: CX3CL1, CCL2, CCL11, GM-CSF, IFN- γ , TNF- α , IL-22, IL-4, IL-5, IL-13, IL-17

To examine cytokine production, enzyme-linked immunosorbant assay (ELISA) was performed using commercial kits. CX3CL1, CCL2, CCL11, IL-4, IL-5, IL-13, and IL-22 were quantified using R&D systems kits. GM-CSF, IFN- γ , and TNF- α were quantified using Biolegend kits.

The general protocol for ELISA assay is as follows: 100 μ l of capture antibody diluted in coating buffer (either PBS or bicarbonate buffer) was added to 96 well plates (Greiner Bio-One) and stored at 4°C overnight followed by 3 washes on Thermo Electron Wellwash 5 MK2 Plate washer (Fisher Scientific) with ELISA wash buffer (WB) (0.05% Tween PBS). Following washing, 200 μ l of blocking buffer (1% bovine serum albumin (Sigma) in PBS) was added and incubated at room temperature for a minimum of 2 h. Blocking buffer was removed by washing WB prior to addition of sample or standard (50 μ l or 100 μ l depending on kit) diluted in blocking buffer. Samples were covered and incubated overnight at 4°C. Following overnight incubation, plates were washed 3X WB and 100 μ l of detection antibody diluted in blocking buffer was added and plates incubated for 90 minutes at room temperature. Plates were then washed 3X WB and 100 μ l strep-avidin horse-radish peroxidase (SA-HRP) diluted in blocking buffer was added to plates. Plates were washed 3X WB and 100 μ l TMB Single Solution (Invitrogen, Rockport, MD) was added to each well, incubated for up to 10 minutes avoiding direct light, and the reaction was stopped with 50 μ l 0.3 M H₂SO₄ (Sigma). The absorbance was read at 450 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.0.7.3 Bronchoalveolar Lavage Fluid Th2 Cytokine Quantification Using Meso Scale Discovery V-PLEX: IL-4, IL-5, IL-13

For the quantification of BALF Th2 cytokines IL-4, IL-5, and IL-13, a 10-Plex Mouse Proinflammatory Panel 1 (Meso Scale Discovery, Rockville, MD) was used according to manufacturer kit specifications. Plated BALF samples were analyzed using a QuickPlex SQ 120 (Meso Scale Discovery).

2.0.8 Immunoglobulin Detection & Quantification

2.0.8.1 ELISA for Quantification of IgG1, IgG2b, IgG2c, IgG3, IgM, IgA

For examining immunoglobulin production in serum, ELISA was performed using SBA Clonotyping System/HRP (Southern Biotech, Birmingham, AL). Briefly, capture antibody was diluted to a concentration of 5 µg/ml in PBS and added to 96 well plates (Greiner Bio-One) stored overnight at 4°C. Following overnight incubation, plates were washed using Thermo Electron Wellwash 5 MK2 Plate washer with ELISA wash buffer (WB) (0.05% Tween PBS). 200 µl of blocking buffer (1% bovine serum albumin (Sigma) in PBS) was added and plates were incubated at room temperature for a minimum of 2 h. Blocking buffer was removed by 3X WB prior to addition of 100 µl sample or standard diluted in blocking buffer, and plates were covered and stored overnight at 4°C. Following overnight storage, plates were washed WB and 100 µl detection antibody diluted 1:500 in blocking buffer was added to plates for 90 minutes. Plates were washed with 3X WB and 100 µl TMB Single Solution (Invitrogen) was added to each well, incubated for up to 10 minutes avoiding direct light, and the reaction was stopped with 50 µl 0.3 M H₂SO₄ (Sigma). The absorbance was read at 450 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.0.8.2 ELISA for Quantification of secretory IgA

For quantification of sIgA in BALF and fecal content, our laboratory had previously developed an ELISA protocol specifically designed for recognition of IgA coupled to secretory component (pIgR) (Jaffar, Ferrini et al. 2009). Briefly, 96 well plates (Greiner Bio-One) were coated with goat anti-pIgR (2 µg/ml; R&D Systems) overnight at 4°C. Following overnight incubation, plates were washed 3X WB, and incubated with BALF or fecal content samples diluted 1:50 overnight at 4°C. Following overnight incubation, plates were washed 3X WB and incubated with 1:500 diluted HRP-IgA detection antibody (Southern Biotech) at room temperature for 90 min followed by addition of 100 µl TMB (BD Biosciences). No mouse sIgA standard is commercially available; however, results were corrected per microgram BALF or fecal protein and expressed as fold increase from control values.

2.0.9 Airway Inflammation

2.0.9.1 Eosinophil Peroxidase Assay

This assay was specifically used for the detection of eosinophil peroxidase (EPO) produced by eosinophils in the BALF as determined by colorimetric assay. 100 µl of PBS was added to each well of a 96 well flat-bottom plate (Greiner Bio-One). The cells from BALF collected as described in Chapter 2 Methods section 2.0.4.6 were resuspended in PBS (pH 7.0) in a final volume of 300 µl. In triplicate, 100 µl of cell suspension was added to each well and serially diluted 1:2 through the 8th well. The substrate solution was prepared by crushing one tablet of orthophenylene diaminedihydrochloride (OPD)(Sigma)(final concentration of 0.1% OPD) in 50 µM Tris-HCL (Sigma) containing 0.1% Triton X-100 (Sigma) and 1 mM hydrogen peroxide

(Sigma). 100 μ l of substrate solution was added to each well and plates were incubated at room temperature for 30 min or until sufficient color development had occurred. 50 μ l of 0.3 M H₂SO₄ (Sigma) was added to stop the reaction, and the absorbance was measured at 495 nm using a SpectraMax 190 Microplate Reader (Molecular Devices). The measurements determined using this assay were total EPO, not released EPO, and are indicative of the number of eosinophils present in the BAL.

2.0.9.2 Airway Hyperreactivity: Respiratory Resistance and Dynamic Compliance

In order to assess pulmonary function and airway hyperreactivity, control and OVA-challenged (with or without SPL-334 treatments) Balb/c mice were anesthetized and tracheotomized. Respiratory resistance (R_L, cm H₂O.s/ml) and dynamic compliance (C_{Dyn}, ml/cm H₂O) to increasing concentrations of methacholine inhalation (1.5 – 24 mg/ml) were measured by mechanical ventilation using Buxco Resistance/Compliance equipment (Buxco Research Systems, Wilmington, NC) by Mary Buford in the Inhalation Core Facility (University of Montana).

2.0.10 Cell Staining

2.0.10.1 Cell Differential

Cytospin preparations were performed on 5x10⁴ cells followed by staining using a Wright-Giesma protocol (Hema 3 Staining Kit, Thermo Scientific). Cell differential percentages were determined by light microscopy evaluation and expressed as absolute cell numbers.

2.0.10.2 Lung Histology: Hematoxylin and Eosin Stain

The lower lobes of non-lavaged lungs were removed and fixed in 4% paraformaldehyde and embedded in paraffin using a Shandon Citadel tissue processor

(Thermo Scientific). Microtome sections were cut at 5- μ m thickness and stained with hematoxylin and eosin (H&E) staining using a Shandon Varistain 24 – 4 (Thermo Scientific).

2.0.10.3 Lung Histology: Periodic acid-Schiff Stain (PAS)

Schiff reagent was made by heating distilled water to boiling, removing water from heat and adding 4 g basic fuchsin (Sigma), and heating the solution again to boiling. The reagent solution was allowed to cool to 50°C and filtered. 80 mL of 1 N hydrochloric acid was added, the solution allowed to cool completely, and 4 g of sodium metabisulfite (Sigma) were added. Reagent solution was allowed to stand overnight in the dark, followed by addition with shaking of 2 g activated charcoal for 1 min. Schiff reagent was filtered and stored at 4°C for 2 to 4 months as needed.

For PAS staining, tissue sections generated as described in Chapter 2 Methods section 2.0.10.2 were deparaffinized and hydrated in water. These sections were then oxidized in 0.5% periodic acid solution (2.5 g periodic acid in 500 ml distilled water) for 5 min followed by rinsing in distilled water. Tissue sections were placed in Schiff reagent for 15 minutes followed by washing in lukewarm tap water for 5 min. Sections were counterstained in Mayer's hematoxylin (10 dips) followed by washing in tap water for 5 min. Tissue was dehydrated and coverslipped using a synthetic mounting medium. Slides were then imaged with an iCys CompuCyte Laser Scanning Cytometer (LSC, Westwood, MA) in order to determine the amount of PAS staining and bronchial wall thickness between samples.

2.0.10.4 Immunofluorescent Microscopy

For immunofluorescent staining, lung tissue was frozen in Sakura Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA). Cryosections were mounted on glass slides, blocked, and dual stained with 100 μ l mAb. Coverslips were mounted with FluorSave (EMD, Gibbstown, NJ) or Prolong gold (Life Technologies, Gand Island, NY) and sections were examined using an Olympus FV1000 IX81 inverted laser scanning confocal microscope (with spectral detection and TIRF Module)(Olympus Corporation of the America, New Orleans, LA).

2.0.10.5 Flow Cytometry: Surface Receptor Expression

Cells (1×10^6) isolated from BALF and dissociated tissue as described in Chapter 2 Methods section 2.0.4 were added to 5 ml polystyrene round bottom tubes (BD Biosciences) and centrifuged at 1350 RPM for 10 min at 4°C. After media was poured off and the cell pellet broken up, 100 μ l FC block (clone 2.4G2; ATCC) was added to each tube for 5 min. 1- 2 μ l of flurochrome-conjugated antibodies (Chapter 2 Methods section 2.0.10.8) were added to each tube and tubes were placed on ice avoiding direct light for 20 min. Cells were washed with 1 ml staining buffer and centrifuged at 1350 RPM for 10 min at 4°C, resuspended in 500 μ l staining buffer and transferred to filter-top 5 ml polystyrene round bottom tubes (BD Biosciences). Stained cells were analyzed using a BD FACSAria (BD Biosciences) utilizing FACSDiVa software for performing multi-parameter analysis of immune cells.

2.0.10.6 Flow Cytometry: Intracellular Staining

For intracellular staining of cytokines and transcription factors, we used a modified FOXP3 Fix/Perm Buffer Set protocol (Biolegend). Normal surface staining of

immune cells as described in Chapter 2 Methods section 2.9.10.5 was performed prior to intracellular staining. 1 ml of 1X Fix/Perm solution (Biolegend) was added to each tube, followed by incubation for 20 min at room temperature avoiding direct light. Cells were washed with 1 ml staining buffer by centrifugation for 10 min at 4°C. Cells were resuspended in 1 ml 1X FOXP3 Perm Buffer (Biolegend), followed by incubation for 15 min at room temperature avoiding direct light. Cells were centrifuged for 10 min at 4°C, and resuspended in 100 µl of 1X FOXP3 Perm Buffer (Biolegend). 1 – 2 µl of fluorochrome-conjugated antibodies (Chapter 2 Methods section 2.0.10.8) were added to each tube, followed by incubation for 20 min at room temperature avoiding direct light. Cells were washed with 1 ml staining buffer by centrifugation for 10 min at 4°C, resuspended in 500 µl staining buffer and transferred to filter-top 5 ml polystyrene round bottom tubes (BD Biosciences). Stained cells were analyzed using a BD FACSAria (BD Biosciences) utilizing FACSDiVa software for performing multi-parameter analysis of immune cells.

2.0.10.7 Intracellular Staining Using PMA and Ionomycin

For intracellular staining of Granzyme A, Granzyme B, and IFN- γ , immune cells isolated from lung tissue as described in Chapter 2 Methods section 2.0.4.1 required prior stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin to initiate production. Cells were resuspended at a concentration of 2×10^6 cells/ml and 500 µl of cells were added to 5 ml polystyrene round bottom tubes (BD Biosciences). 50 ng PMA (Sigma), 500 ng ionomycin (Sigma), and 1 µl GolgiPlug (Brefeldin A)(BD Biosciences) were added to each tube and then incubated 4 h (IFN- γ) or 24 h (Granzyme A and Granzyme B) at 37°C. Following incubation, cells were washed 2X with staining buffer

by centrifugation for 10 min at 4°C, and the intracellular staining protocol described in Chapter 2 Methods section 2.0.10.6 was used to complete intracellular staining.

2.0.10.8 Flow Cytometry Antibodies

	Specificity	Clone	Conjugated	Company
Chapter 3	Anti-CD4	GK1.5	APC-Cy7	BD Biosciences
	Anti-CD8	53-6.7	PE	Biologend
	Anti-DO11.10 TCR	KJ1-26	APC	eBioscience
	Anti-Gr-1 (Ly-6G/Ly-6C)	RB6-8C5	APC-Cy7	Biologend
	Anti-CD11b	M1/70	APC	Biologend
	Anti-F4/80	BM8	AF647	Biologend
	Anti-Siglec-F	E50-2440	PE	BD Biosciences
	Anti-IL-4	11B11	APC	Biologend
	Anti-IFN- γ	XMG1.2	FITC	Biologend
	Anti-NK1.1	PK136	APC, FITC, PE	Biologend
Chapter 4	Anti-CD3	145-2C11	PE, FITC, APC	Biologend
	Anti-CD19	6D5	APC-Cy7	Biologend
	Anti-Dx5 (CD49b)	Dx5	PE	Biologend
	Anti-CD27	LG.3A10	APC	Biologend
	Anti-CD127	A7R34	APC	Biologend
	Anti-Ly49A	YE1/48.10.6	FITC	Biologend
	Anti-Ly49C	14B11	FITC	Biologend
	Anti-CD117 (c-kit)	2B8	APC	Biologend
	Anti-NKp46	29A1.4	PE, APC	Biologend

Chapter 5	Anti-NKG2D	CX5	PE	eBioscience
	Anti-Granzyme A	GzA-3G8.5	APC	eBioscience
	Anti-Granzyme B	GB11	FITC	Biologend
	Anti-IFN- γ	XMG1.2	APC	Biologend
	Anti-CD11b	M1/70	FITC	Biologend
	Anti-F4/80	BM8	APC	Biologend
	Anti-Siglec-F	E50-2440	PE	BD Biosciences
	Anti Gr-1(Ly-6G/Ly-6C)	RB6-8C5	APC-Cy7	Biologend
	Anti-CD4	GK1.5	APC-Cy7, FITC	Biologend
	Anti-CD8	53-6.7	PE	Biologend
	Anti-CD45R/B220	RA3-6B2	APC	Biologend
	Anti-IgA	C10-3	FITC	BD Biosciences
	Anti-IgM	RMM-1	PE	Biologend
	Anti-CD3	145-2C11, 17A2	FITC, APC, AF647	Biologend
	Anti-CD4	GK1.5	FITC	Biologend
	Anti-CD8	53-6.7	FITC	Biologend
	Anti-NKp46	29A1.4	PE, APC	Biologend
	Anti-ROR γ t	B2D	APC	eBiosciences
	Anti-IL-23r	O78-1208	PE	BD Biosciences
	Anti-CD127	A7R34	APC	Biologend

2.0.11 Statistics

Unless otherwise described, the data presented in this dissertation are summarized as mean \pm SEM. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA) using unpaired student's t test (Welch's correction was utilized where F test revealed unequal variance between groups). Statistical significance was attributed to data achieving $p < 0.05$.

Chapter 3

S-Nitrosoglutathione Reductase Inhibition Regulates Allergen-Induced Lung Inflammation and Airway Hyperreactivity

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3.0 ABSTRACT

Allergic asthma is characterized by Th2 type inflammation, leading to airway hyperresponsiveness, mucus hypersecretion, and tissue remodeling.

S-Nitrosogluthathione reductase (GSNOR) is an alcohol dehydrogenase involved in the regulation of intracellular levels of S-nitrosothiols. GSNOR activity has been shown to be elevated in human asthmatic lungs, resulting in diminished S-nitrosothiols and thus contributing to increased airway hyperreactivity. Using a mouse model of allergic airway inflammation, we report that intranasal administration of a new selective inhibitor of GSNOR, SPL-334, caused a marked reduction in airway hyperreactivity, allergen-specific T cells and eosinophil accumulation, and mucus production in the lungs in response to allergen inhalation. Moreover, SPL-334 treatment resulted in a significant decrease in the production of the Th2 cytokines IL-5 and IL-13 and the level of the chemokine CCL11 (eotaxin-1) in the airways. Collectively, these observations reveal that GSNOR inhibitors are effective not only in reducing airway hyperresponsiveness but also in limiting lung inflammatory responses mediated by CD4⁺ Th2 cells. These findings suggest that the inhibition of GSNOR may provide a novel therapeutic approach for the treatment of allergic airway inflammation.

3.1 Introduction

3.1.1 Biochemical Alterations Identified in Asthma

While the principle effort to delineate processes that drive the pathogenesis of asthma have rightly centered on the immunological events occurring in the airway, these immunological events occur in conjunction with specific biochemical processes as well. These reactions have received intense scrutiny as a consequence of the regulatory capacity they can exert on immune function. Several key biomarkers that occur downstream of these potential biochemical alterations have been identified that have the potential to influence immunological as well as respiratory function.

Superoxide dismutases (SOD) are a family of metalloprotein enzymes responsible for the scavenging and removal of reactive oxygen species, in particular superoxide radical following production of nitric oxide (NO), through the conversion of superoxide radical into hydrogen peroxide and oxygen (McCord and Fridovich 1988). They can be divided into three types based on the reactive metal ion present in the active site of the enzyme: (i) Copper/Zinc (Cu/Zn) (ii) Manganese (Mn) (iii) or Iron (Fe) (Mruk, Silvestrini et al. 2002). Of these types, eukaryotes primarily express the Cu/Zn isoform of SOD in the cytosol and Mn isoform of SOD in mitochondria. Additionally, an extracellular Cu/Zn isoform of SOD (SOD_{EX}) has been described that resides in the extracellular matrix of several tissues including the brain, lungs, and extracellular fluid such as plasma (Marklund 1984). Superoxide radicals can induce widespread and substantial damage in the airway through direct interaction with cells to create a ‘cascade’ of reactive oxygen species, as well as through interaction with nitric oxide radicals to form peroxynitrite (Jain, Kannan et al. 1998). Several studies have shown that

serum activity of SOD is low in patients with severe asthma, suggesting a potential role of reactive oxygen species such as superoxide radicals in inducing airway obstruction and hypersensitivity (De Raeve, Thunnissen et al. 1997; Comhair, Ricci et al. 2005). However, attempts to treat asthma utilizing antioxidant therapy have ultimately proven unsuccessful (Fogarty, Lewis et al. 2003).

Arginase is a metalloprotein enzyme containing Mn in its active site that is responsible for the final enzymatic reaction of the urea cycle via conversion of L-arginine into L-ornithine and urea (Wu, Lee et al. 1998). Increased activity of arginase has been observed in asthmatic patients with decreased lung function, in addition to the identification of single nucleotide polymorphisms (SNPs) in populations with asthma (Lara, Khatri et al. 2008; Litonjua, Lasky-Su et al. 2008). The role of arginase in decreased lung function is not entirely clear, although it has been proposed that competition with nitric oxide synthases (NOSs) for L-arginine results in decreased production of NO, a smooth muscle relaxant, in the airway leading to bronchoconstriction and airway hyperreactivity (Strapkova and Antosova 2011).

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NOS that is released following degradation of methylated proteins (Boger 2004). The lung has been shown to be a major source of ADMA, and in mice it has been shown that elevated ADMA levels in the airway are responsible for the inhibition of NOS activity, resulting in increased bronchoconstriction and airway hyperreactivity (Bulau, Zakrzewicz et al. 2007; Klein, Weigel et al. 2010).

Eosinophil peroxidase (EPO) is a haloperoxidase critical to the cytolytic function of eosinophils. In the presence of hydrogen peroxide and either bromide (preferred) or

chloride, EPO generates hypobromite or hypobromous acid, which is stored in intracellular granules until needed for the removal of multicellular parasites as well as a limited repertoire of bacteria (Mayeno, Curran et al. 1989). As one of the hallmark features of allergic asthma is accumulation of eosinophils in the airway, it should be little surprise that elevated levels of EPO exist in the airways of asthmatic patients, and urinary bromotyrosine levels have been used to track increased airway eosinophilia (Wedes, Wu et al. 2011). As hypobromous acid is highly cytotoxic, an increased level of EPO in the airway is highly undesirable and likely leads to increased injury of the airway.

3.1.2 Nitric Oxide Signaling

When analyzing the biochemical alterations described in Chapter 3 Section 3.1.1 that have been implicated to exert a role in asthma pathogenesis, a noticeable characteristic becomes apparent. Specifically, all the biochemical pathways associated with these biomarkers are dependent to varying degrees on the involvement of the NO signaling pathway. A deeper understanding of this pathway is helpful in understanding how alterations in NO signaling can lead to profound effects on the airway.

3.1.2.1 Synthesis of Nitric Oxide

Due to the importance of NO in both cell signaling and as an effector molecule for immune-regulated defense against pathogens, synthesis of NO occurs widely in a wide variety of tissues. While several isoforms of nitric oxide synthases (NOSs) exist, all isoforms utilize the substrate L-arginine in conjunction with co-substrates reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) and O₂ for the production of NO and L-citrulline utilizing electron transfer via the cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH₄), and heme

(Forstermann and Sessa 2012). Additionally, binding of calmodulin to NOS enzymes is a critical step in the facilitation of electron transport, and intracellular increases in Ca^{2+} are therefore critical in controlling NO synthesis (Hemmens and Mayer 1998).

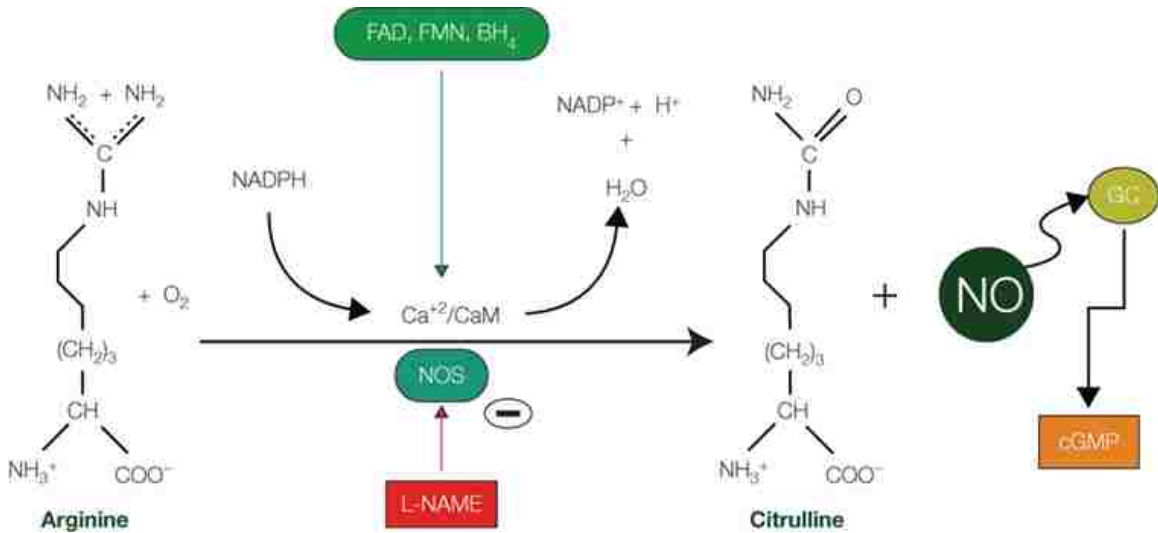


Figure 3.1 *Nitric Oxide Synthesis.* Metabolism of arginine resulting in NO and citrulline is mediated by several nitric oxide synthase isoforms in conjunction with co-substrates NADPH and O₂ as well as co-factors including FAD, FMN, and BH₄. Binding of CA²⁺ to NOS via calmodulin (CaM) is a critical step in NO synthesis. Inhibition of NO production occurs when compounds such as NG-nitro-L-arginine methyl ester (L-NAME) bind to NOS (Freire, Guimaraes et al. 2009).

Neuronal NOS (nNOS; NOS-1) is constitutively expressed in immature and mature neurons of both the central and peripheral nervous systems, localized to synaptic spines, as well as being present in skeletal muscle smooth muscle, and cardiac muscle myocytes (Chen, Tu et al. 2004; Zhou and Zhu 2009). The production of NO via nNOS has been shown to play an important role in long term potentiation, a form of synaptic plasticity critical in the process of learning and memory, and inhibition of nNOS leads to impairment of this process (Bohme, Bon et al. 1993; Bon and Garthwaite 2003). Binding of NO to proteins that have accessible metal-containing amino acids such as cysteine residues in muscle, a process termed S-nitrosylation discussed more fully in Chapter 3

section 3.1.3, can affect muscle contractility (Xu, Eu et al. 1998). Additionally, the expression of nNOS and subsequent synthesis of NO by nitrergic nerves has been shown to alter smooth muscle tone (Forstermann and Sessa 2012). However, NO has been implicated to play a deleterious role in excitotoxicity and ischemia, as overstimulation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate results in increased intracellular Ca^{2+} levels capable of inducing high levels of NO synthesis which can lead to subsequent cytotoxicity in surrounding neurons (Eliasson, Huang et al. 1999).

Endothelial NOS (eNOS; NOS-3) is also constitutively expressed, primarily in endothelial cells, in a membrane-anchored form, but has been shown in cardiac myocytes, neurons, syncytiotrophoblasts, and kidney tubule epithelial cells (Forstermann and Sessa 2012). The association of eNOS and endothelial cells results in several critical functions in controlling cardiovascular function. NO synthesized from eNOS has been shown to cause dilation of blood vessels through stimulation of soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (Forstermann, Mulch et al. 1986). Originally identified as endothelial-derived growth factor (EDGF), NO plays an integral role in the proliferation of both vascular smooth muscle and in the stimulation of angiogenesis (Garg and Hassid 1989; Han and Stewart 2006). eNOS-derived NO has also been shown to have an effect on the immune system and vascular inflammation by decreasing the production of chemoattractant protein MCP-1, also known as CCL2, by endothelial cells in addition to suppressing the expression of adhesion molecules CD11/CD18 on leukocytes resulting in decreased extravasation of leukocytes into tissue (Kubes, Suzuki et al. 1991; Zeiher, Fisslthaler et al. 1995).

The final isoform of NOS is inducible NOS (iNOS; NOS-2). Unlike nNOS and eNOS, iNOS is not constitutively expressed in cells, and altered amino acid structure at the calmodulin-binding site results in high affinity binding of calmodulin by iNOS independently of intracellular Ca^{2+} concentration (Cho, Xie et al. 1992). Instead, iNOS is transcriptionally regulated via activation of transcription factors such as $\text{NK-}\kappa\text{B}$, AP-1, signal transducer and activator of transcription (STAT)-1 α , interferon-regulatory factor-1 (IRF-1), and nuclear factor interleukin-6 (NF-IL-6) (Kleinert, Euchenhofer et al. 1998; Dlaska and Weiss 1999; Ganster, Taylor et al. 2001). Activation of these signaling pathways have been shown following cellular stimulation utilizing bacterial lipopolysaccharide, $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, IL-1 β , and IL-12, while IL-4, IL-13, and $\text{TGF-}\beta$ has been implicated to inhibit iNOS expression (Green, Scheller et al. 1994; MacMicking, Xie et al. 1997; Bogdan 2001). iNOS expression has been identified in a wide variety of immune cells, especially innate immune cells such as macrophages, dendritic cells, natural killer cells, neutrophils, and even in non-immune populations such as epithelial and endothelial cells (Bogdan 2001; Ricciardolo, Timmers et al. 2003). The widespread adoption of NO by the immune system, and even non-immune cells, as a mechanism for the initial early-stage clearance of infection should be an indication of the effectiveness and importance of NO in defense against pathogens.

3.1.2.2 Mechanism of Action of Nitric Oxide

NO is a membrane-permeable, gaseous free radical and the smallest known signaling molecule. Capable of rapid diffusion following synthesis, NO has the capacity to interact with intracellular molecular sites in an autocrine or paracrine manner. The best-characterized site of action involving NO is the iron-containing heme component of

guanylate cyclase where stimulation of the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP), a key component in the activation of a protein kinases involved in cell signaling pathways, occurs (Ignarro and Kadowitz 1985). Additionally, NO has been found to regulate gene transcription of vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin (CD62E) by endothelial cells, regulate mRNA translation of ferritin, and induce post-translational modification in proteins via ADP ribosylation (Pozdnyakov, Lloyd et al. 1993; Pantopoulos and Hentze 1995; Khan, Harrison et al. 1996).

The concentration of NO that is synthesized has a direct impact on the mechanism of action of NO. For example, high concentrations produced by iNOS in activated macrophages have potent cytotoxic capabilities including the killing of tumor cells and elimination of various viral, bacterial, fungal, and parasitic pathogens (Denis 1991; Karupiah, Xie et al. 1993). The ability of NO and its reactive nitrogen intermediates to induce mutation of DNA, inhibit DNA repair and protein synthesis, alter protein function via S-nitrosylation, inactivate enzymes through binding and disruption of metal-containing active sites, or induce lipid peroxidation can lead to highly effective control of pathogens by the innate immune system. However, high concentrations of NO can have deleterious effects on the host tissue as well, as the mechanisms utilized to destroy pathogens are not pathogen-specific. As a free radical, NO has the capacity to induce cell and tissue damage itself, or the interaction of NO with superoxide radical can result in the formation of peroxynitrite, a highly cytotoxic molecule (Radi, Beckman et al. 1991). NO has been implicated to be the initial factor involved in the initiation of septic shock following bacterial endotoxin exposure, leading to massive vasodilation, hypotension, and capillary

damage (MacMicking, Nathan et al. 1995). Therefore, when attempting to understand the role that NO is exerting during a pathological event, it is critical to consider that small changes in NO synthesis and signaling could lead to vastly differing outcomes.

3.1.2.3 Role of Nitric Oxide in the Airway

The capability of NO to induce vasodilation and smooth muscle relaxation, in addition to its potent antimicrobial effects, lead to extensive investigation of the role of NO in a location where all three facets are in close proximity, namely the airway. The capacity of NO-containing compounds to induce bronchodilation through activation of guanylate cyclase and increased cGMP was identified in 1989 and raised further interest in the airway regulatory abilities of NO (Gruetter, Childers et al. 1989). In addition to the activation of guanylate cyclase, modification of proteins through addition of NO to metal-containing amino acids, discussed more fully in section 3.1.3, has been identified as an additional mechanism via which NO can regulate bronchodilation that is independent of cGMP (Gaston, Drazen et al. 1994). The use of NOS inhibitors such as N^ω-nitro-L-arginine methyl ester (L-NAME) or N⁹-monomethyl-L-arginine (L-NMMA) have been shown in guinea pigs to result in significant increases in airway resistance following bronchoconstriction-inducing stimuli, including allergens (Nijkamp, van der Linde et al. 1993; Persson, Friberg et al. 1993; Ricciardolo, Nadel et al. 1994). Utilizing NOS knockout-mice, recent work by Bratt et al revealed that the production of NO by iNOS during allergen-induced airway inflammation is essential for the reduction of lung inflammation, and that upregulation of iNOS during inflammation required eNOS, but not nNOS, activation (Bratt, Williams et al. 2010). The role of eNOS in controlling bronchoconstriction through iNOS-synthesized NO is interesting when considering that

eNOS-derived NO has been shown to play a critical role in regulating pulmonary vasoconstriction and vascular permeability in patients with pulmonary hypertension, chronic obstructive pulmonary disease, and cystic fibrosis (Dinh-Xuan, Higenbottam et al. 1991). While the source of NO in the airway appears to be of little consequence to overall affect on the lungs, it is clear that the presence of NO in the airway is capable of having a dramatic influence on airway function.

3.1.2.4 Regulation of Nitric Oxide in the Airway

Due to the importance of NO as a key cell signaling molecule, a critical consideration when considering the biological efficacy of NO lies not only in its rate of synthesis following stimulation, but also in the rate of decomposition. While the rate of decomposition is reliant on factors such as initial concentration, systemic location, or presence of other reactive gasses, the half-life of NO in blood has been estimated to range between 0.05 – 1.8 ms (Liu, Miller et al. 1998). In order to facilitate long-term signaling in tissue, storage of NO is therefore necessitated.

3.1.2.4.1 Nitrosylation

As a free radical, NO can be rapidly oxidized into NO-derived intermediates. Early work by Stamler et al described the stabilization of NO via binding to proteins containing sulfhydryl-groups such as thiols (Stamler, Jaraki et al. 1992). This redox reaction, known as S-nitrosylation, falls under the broader category of nitrosylation reactions, which also includes the binding of NO to transition metals as well as tyrosine, known as metal nitrosylation and tyrosine nitration, respectively (Mengel, Chaki et al. 2013). Over 100 protein substrates have been identified to undergo nitrosylation, primarily through S-nitrosylation, indicating the critical importance of nitrosylation in

controlling signal transduction (Stamler, Lamas et al. 2001). A critical early example of the role of S-nitrosylation in controlling receptor signaling was the discovery that the N-methyl-D-aspartate (NMDA) receptor was regulated via S-nitrosylation (Lipton, Choi et al. 1993). While nitrosylation can occur spontaneously, enzymatic control of the reaction has been recognized to play a critical role in maintain the levels of nitrosylated proteins in tissue. Enzymes such as ceruloplasmin, superoxide dismutase, and even NOS have been identified to induce nitrosylation, while enzymes such as thioredoxin and S-nitrosoglutathione reductase have been identified to lead to the removal of NO from nitrosylated proteins, or denitrosylation (Hess, Matsumoto et al. 2005). It is critical to recognize that S-nitrosylation of proteins does not remove the biological activity of NO, as S-nitrosylated proteins have been shown to induce vasodilation via activation of guanylate cyclase in a more controlled manner than pure NO (Stamler, Jaraki et al. 1992). This enhanced activity of NO in tissue means that regulation of nitrosylated proteins in tissue takes on critical importance.

3.1.2.4.2 S-Nitrosoglutathione & S-nitrosoglutathione Reductase

Proteins that have undergone S-nitrosylation are referred to as S-nitrothiols (SNOs). In the airway, the reaction of NO with the sulfhydryl group present on glutathione results in the formation of S-nitrosoglutathione (GSNO). In humans, GSNO has been identified to be the most abundant SNO present in both cells as well as extracellular spaces, such as the airways, and addition of exogenous NO into the airway was shown to lead to increased formation of GSNO (Gaston, Reilly et al. 1993). Liu et al described a glutathione-dependent formaldehyde dehydrogenase that has been highly evolutionarily conserved termed S-nitrosoglutathione reductase (GSNOR) capable of

regulating GSNO levels on both a tissue and intracellular level (Liu, Hausladen et al. 2001).

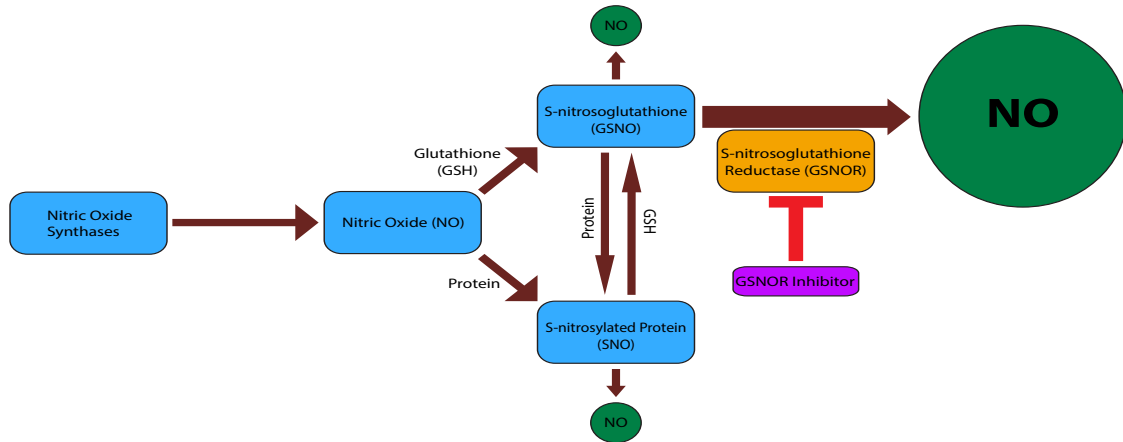


Figure 3.2 *Regulation of Nitric Oxide Signaling.* Nitric oxide has the capacity to bind to thiol containing proteins such as glutathione (GSH), via S-nitrosylation, resulting in stabilization of NO signaling in both cells and tissues. The release of protein-bound NO in the airways is regulated by S-nitrosoglutathione reductase (GSNOR), and this down-regulation in NO signaling via destabilization has been implicated to have dramatic effects on airway responses. It has been suggested that inhibition of GSNOR could be an effective potential therapeutic option for airway disorders such as asthma.

GSNO has been shown to have potent and long-lasting biological activity similar to that of native NO, including bronchodilatory capabilities (Gaston, Reilly et al. 1993). Treatment of tracheal smooth muscle cells *in vitro* utilizing GSNO revealed that exogenous GSNO has a 100-fold greater potency in inducing bronchodilatory activity than theophylline, a drug commonly used for the treatment of respiratory diseases including asthma (Gaston, Drazen et al. 1994). This has led to recognition of its role in potentially regulating the development and severity of diseases characterized by bronchoconstriction and airway hyperreactivity such as asthma. Examination of airway GSNO levels in asthmatic children revealed that severe asthma is associated with decreased levels of airway GSNO (Gaston, Sears et al. 1998). Examination of GSNO in

animal models of allergic asthma has revealed that degradation of GSNO occurs resulting in bronchoconstriction of the airways (Fang, Johns et al. 2000). In an attempt to understand how degradation of GSNO is occurring in asthmatic airways, researchers have focused on whether GSNOR dysregulation could be a potential underlying cause. GSNOR^{-/-} mice exhibit increased levels of SNOs in the airways, leading to protection from the development of methacholine-induced airway hyperreactivity following OVA-sensitization (Que, Yang et al. 2009). Additionally, supplementation of the airway with GSNO of OVA-sensitized and –challenged mice resulted in amelioration of airway hyperreactivity, but did not alter the infiltration of the airway by inflammatory immune cells (Que, Yang et al. 2009). These studies suggest that pharmacological intervention with the goal of controlling levels of GSNO in the airway could provide a unique and powerful therapeutic approach to combat respiratory diseases such as asthma.

3.2 RESULTS

3.2.1 Characterization of Allergic Pulmonary Responses in Mice Treated with S-nitrosoglutathione Reductase Inhibitor SPL-334

The GSNO/GSNOR system exerts significant influence on airway responsiveness and asthma pathogenesis (see Chapter 3 Background section 3.1.2.4.2). In this study, we utilized the specific GSNOR inhibitor SPL-334 to establish whether therapeutic inhibition of GSNOR could lead to alteration in the development of allergic asthma. Sanghani et al identified SPL-334 (C3) as a potent and selective inhibitor of GSNOR activity capable of inducing increased intracellular SNO levels in RAW 264.7 cells following treatment (Sanghani, Davis et al. 2009). Additionally, SPL-334 was demonstrated to result in suppression of NF- κ B activation due to the inability to reverse nitrosylation of IKK β (Sanghani, Davis et al. 2009). These characteristics strongly

suggested that inhibition of GSNOR utilizing SPL-334 could be a potential therapeutic approach to the treatment of allergic asthma.

3.2.1.1 *Reduced Infiltration of Airways by Inflammatory Immune Cell Populations*

Infiltration of the airway by inflammatory immune cell populations, specifically eosinophils and CD4⁺ Th2 cells, is considered one of the defining characterizations of allergic asthma. Following adoptive transfer of OVA-specific Th2 cells and subsequent exposure to aerosolized OVA (detailed in Chapter 2 Methods), Th2 recipient mice, but not control animals (no Th2 adoptive transfer), developed a pronounced influx of lymphocytes and eosinophils (Figure 3.3) as determined by total cell number and measurement of EPO levels in the BALF. Intranasal SPL-334 treatment resulted in a significant reduction in both infiltrating lymphocytes and eosinophils into the airway (Figure 3.3).

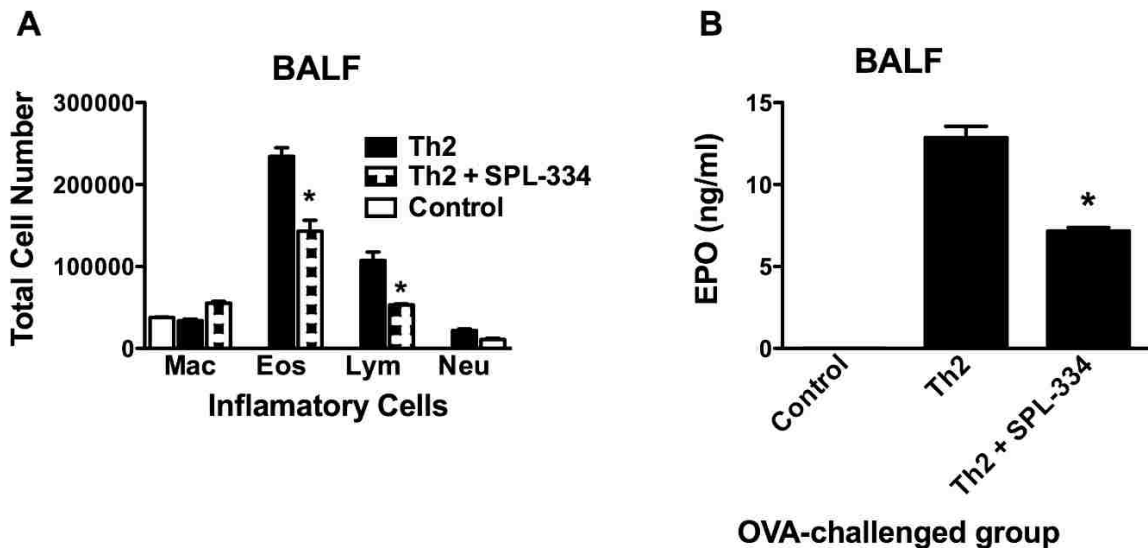


Figure 3.3 Treatment with the GSNOR inhibitor SPL33-4 Caused a Reduction in Allergic Lung Inflammation. DO11.10 CD4⁺ Th2 cells were adoptively transferred into Balb/c recipient mice that were then exposed to aerosolized OVA for 7 consecutive days. Mice were treated intranasally with either the GSNOR inhibitor SPL-334 (Th2 + SPL-334, 0.1 or 1 mg/kg daily) or vehicle (Th2). Control mice did not receive Th2 cells but did receive inhaled OVA aerosols. (A) Cell differential counts in the BALF were determined by light microscopic evaluation of stained cytopsin preparations. Results are expressed as absolute numbers (per mouse) of lymphocytes (Lym), macrophages (Mac), eosinophils (Eos), and neutrophils (Neu). (B) EPO levels in the BALF from Th2 recipient or control mice were assessed by colorimetric analysis. Results are mean \pm SEM (n= 4 – 6) in triplicate and are representative of four independent experiments. *p < 0.05, compared with Th2 group.

Consistent with the reduction in inflammation, FACS analysis showed a reduction in the proportion of CD4⁺KJ-126⁺ T cells as well as CD11b⁺Siglec-F⁺ eosinophils present in the BALF of mice receiving GSNOR inhibitor as compared to the Th2 untreated group (Figure 3.4). There was no alteration observed in the number or proportion of CD11b⁺GR-1⁺ neutrophils or CD11c⁺F4/80⁺ alveolar macrophages in the airway (Figure 3.4).

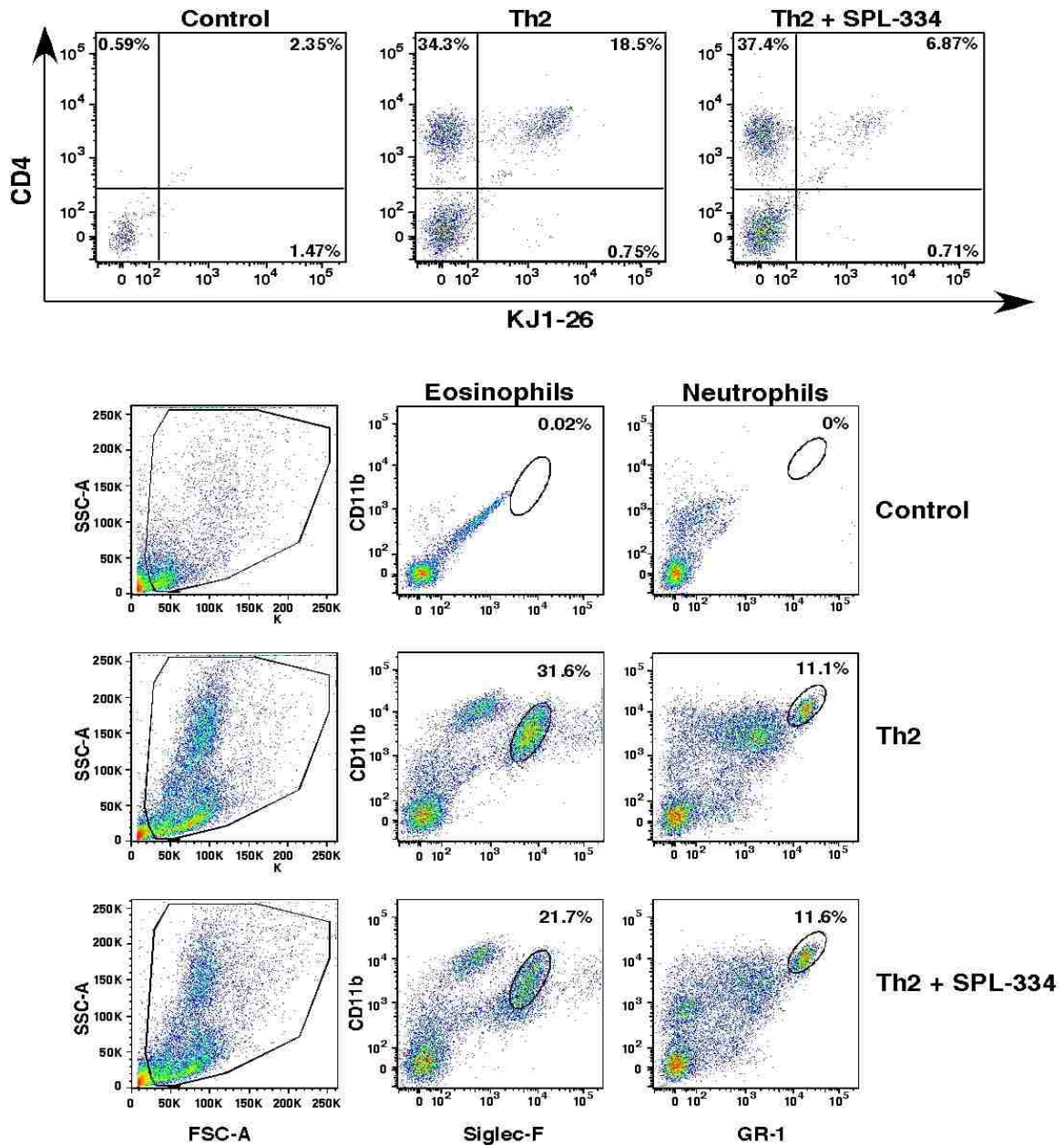


Figure 3.4 GSNOR Inhibitor SPL-334 Reduced the Number of OVA-specific T cells and Eosinophils During Allergic Airway Inflammation. DO11.10 CD4⁺ Th2 cells were transferred into Balb/c mice that were then challenged with aerosolized OVA for 7 consecutive days. Mice were treated with either SPL-334 (Th2 + SPL-334) or vehicle (Th2). Control mice did not receive Th2 cells but did receive inhaled OVA aerosols. (A) The number of CD4⁺ KJ-126⁺ T cells in the BALF following OVA inhalation as determined using flow cytometry. (B) The number of CD11b⁺Siglec-F⁺ eosinophils or CD11b⁺Gr-1⁺ neutrophils in the BALF following OVA inhalation as determined using flow cytometry. Data are representative of two independent experiments.

3.2.1.2 *Reduced Peribronchial Inflammation, Mucus Production, & Airway Hyperreactivity*

Analysis of lung histology utilizing H&E staining revealed that Th2 recipient mice had markedly increased peribronchial and perivascular inflammation compared to control mice (Figure 3.5). In addition, a substantial increase in mucus production was observed and characterized utilizing %PAS staining per bronchiole (Figure 3.5) following OVA exposure. Treatment with SPL-334 resulted in a significant reduction in both mucus secretion and bronchial inflammation when compared to Th2 recipient mice (Figure 3.5). Control mice did not develop any pulmonary inflammation or mucus secretion following aerosolized OVA exposure.

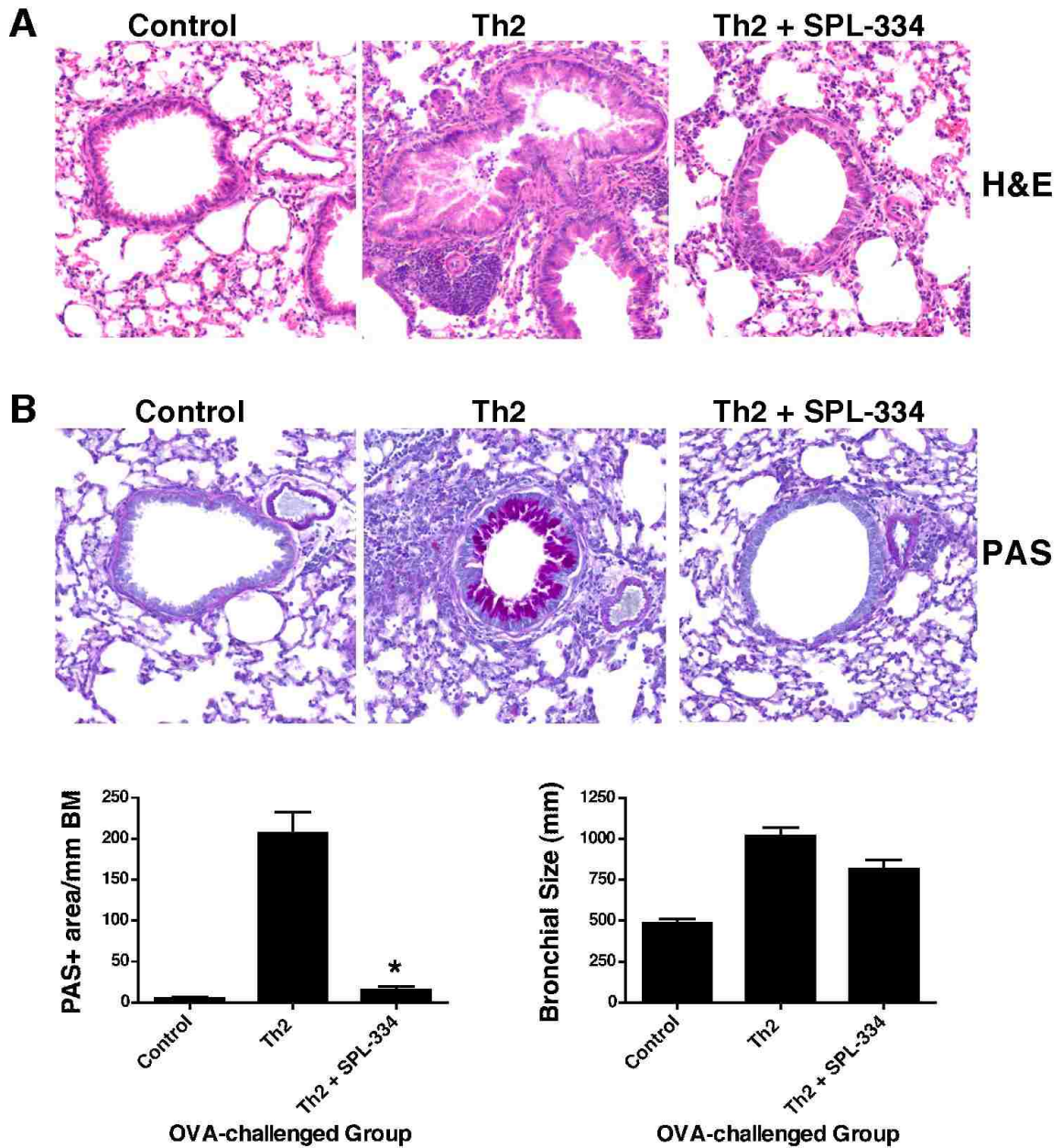


Figure 3.5 GSNOR Inhibitor SPL-334 Caused a Reduction in Peribronchial Inflammation and Mucus Secretion During Airway Inflammation. DO11.10 CD4⁺ Th2 cells were transferred into Balb/c mice that were then challenged with aerosolized OVA for 7 consecutive days. Mice were treated with either SPL-334 (Th2 + SPL-334) or vehicle (Th2). Control mice did not receive Th2 cells but did receive inhaled OVA aerosols. (A) Peribronchial inflammation determined by histological analysis by staining lung tissue section with H&E (20X). (B) Mucus production determined by histological analysis by staining lung tissue sections with PAS (20X). PAS staining was expressed as % PAS+ area per bronchiole, and bronchial wall thickness expressed in μm . Data are representative of two independent experiments. * $p < 0.05$, compared with Th2 group.

The pulmonary inflammation observed following histological analysis was associated with an increase in airway hyperreactivity, as measured by airway resistance (R_L) and dynamic compliance (C_{Dym}), in Th2 recipient mice when compared to control mice (Figure 3.6). Treatment with SPL-334 resulted in reduced airway resistance and improved dynamic compliance when compared to Th2 recipient mice (Figure 3.6).

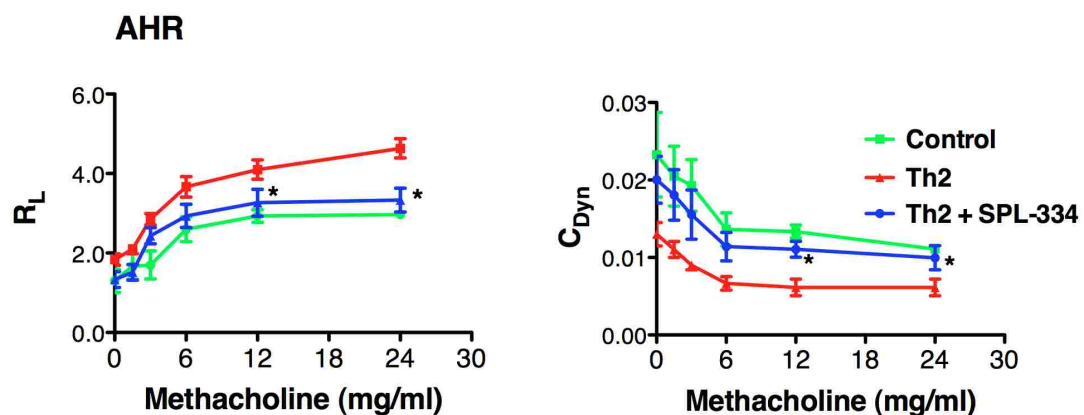


Figure 3.6 Treatment with the GSNOR Inhibitor SPL334 Caused a Reduction in AHR. DO11.10 CD4⁺ Th2 cells were transferred into Balb/c mice that were then challenged with aerosolized OVA for 7 consecutive days. Mice were treated with either SPL-334 (Th2 + SPL-334) or vehicle (Th2). Control mice did not receive Th2 cells but did receive inhaled OVA aerosols. Lung Resistance (R_L) and dynamic compliance (C_{Dym}) was assessed in anesthetized and tracheotomized mice that were mechanically ventilated in response to increasing concentrations of methacholine inhalation. Data are mean \pm SEM (n=10) *p < 0.05, compared with Th2 group.

3.2.1.3 Reduced Production of Th2-associated Cytokines

Following observation that the GSNOR inhibitor SPL-334 affected the severity of lung inflammation following aerosolized OVA exposure, we characterized the effect of SPL-334 on Th2-associated cytokine production during OVA-induced airway inflammation. Stimulation of isolated lung mononuclear cells from Th2 recipient mice using OVA₃₂₃₋₃₃₉ peptide or anti-CD3 showed production of high levels of IL-4, IL-5, and

IL-13 (Figure 3.7). Control mice displayed low production of all three cytokines following similar stimulation (Figure 3.7). However, a significant reduction when compared to Th2 recipient mice in the levels of IL-5 and IL13, though not IL-4, was observed following SPL-334 treatment (Figure 3.7).

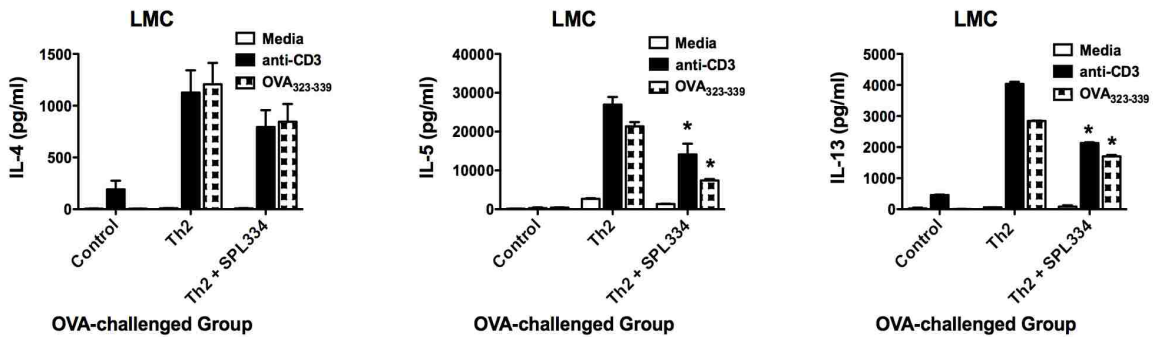


Figure 3.7 Treatment with the GSNOR Inhibitor SPL334 Reduced Th2 Cytokine Production During Allergic Airway Inflammation. DO11.10 CD4⁺ Th2 cells were transferred into Balb/c mice that were then challenged with aerosolized OVA for 7 consecutive days. Mice were treated with either SPL-334 (Th2 + SPL-334) or vehicle (Th2). Control mice did not receive Th2 cells but did receive inhaled OVA aerosols. LMCs were obtained from control mice and Th2 recipient mice that were treated with either SPL-334 or vehicle. LMCs were stimulated with anti-CD3 (2 µg/ml) or OVA₃₂₃₋₃₃₉ peptide (1 µg/ml) for 24 h and supernatant analyzed for IL-4, IL-5, or IL-13 production by ELISA. Data are mean ± SEM (n=4) in triplicate and are representative of two independent experiments. *p < 0.05, compared with Th2 group.

3.2.1.4 Reduced Production of Asthma-associated Chemokine CCL11

CCL11, also known as eotaxin-1, plays a critical role in the chemotaxis of eosinophils into the airway during allergic asthma. Therefore, we examined whether treatment with GSNOR inhibitor had an effect on airway CCL11 production. Similar to the increased production of Th2-associated cytokines described above, Th2 recipient mice exposed to aerosolized OVA displayed an increased production of CCL11 in both BALF and following *in vitro* stimulation of lung mononuclear cells (Figure 3.8). However, increased *in vitro* CCL11 production was only observed following stimulation with OVA₃₂₃₋₃₃₉ peptide, not anti-CD3 (Figure 3.8). Treatment with SPL-334 resulted in

a significant reduction in CCL11 production in both BALF and following *in vitro* stimulation of lung mononuclear cells when compared to Th2 recipient mice (Figure 3.8). The decrease in *in vitro* CCL11 production was observed in both OVA₃₂₃₋₃₃₉ peptide and anti-CD3 stimulated lung mononuclear cells (Figure 3.8).

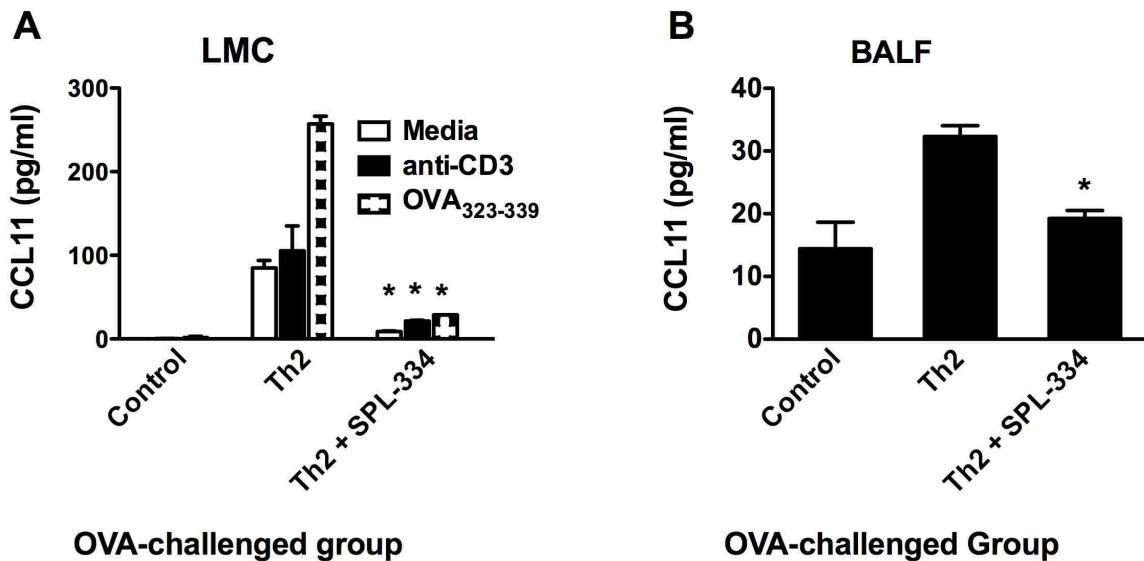


Figure 3.8 Treatment with the GSNOR Inhibitor SPL334 Reduced CCL11 Production During Allergic Airway Inflammation. DO11.10 CD4⁺ Th2 cells were transferred into Balb/c mice that were then challenged with aerosolized OVA for 7 consecutive days. Mice were treated with either SPL-334 (Th2 + SPL-334) or vehicle (Th2). Control mice did not receive Th2 cells but did receive inhaled OVA aerosols. LMCs were obtained from control mice and Th2 recipient mice that were treated with either SPL-334 or vehicle. LMCs were stimulated with anti-CD3 (2 µg/ml) or OVA₃₂₃₋₃₃₉ peptide (1 µg/ml) for 24 h and supernatant analyzed for CCL11 (eotaxin) by ELISA. Data are mean ± SEM (n=4 – 6) in triplicate and are representative of two independent experiments. *p < 0.05, compared with Th2 group.

3.3 DISCUSSION

Work performed by Que et al utilizing transgenic mouse models deficient in expression of GSNOR have suggested it plays a critical role in the pathogenesis of asthma (Que, Liu et al. 2005). Examination of the airways revealed that airway epithelial cells were the primary GSNOR-expressing cell population, but was expression was also observed in macrophages and infiltrating leukocytes (Que, Liu et al. 2005). Exposure of

WT mice to ovalbumin resulted in increased GSNOR activity in the airway lining fluid, possibly as a consequence of epithelial or inflammatory cell lysis (Que, Liu et al. 2005). This elevation in GSNOR activity following allergen challenge has been suggested to result in increased breakdown of endogenous airway SNOs in human asthmatic airway lining fluid (Gaston, Reilly et al. 1993; Que, Liu et al. 2005). GSNOR^{-/-} transgenic mice displayed attenuated development of bronchoconstriction in response to metacholine challenge that was concurrent with elevated levels of airway SNOs, suggesting strongly that GSNOR-mediated regulation of endogenous airway SNOs plays a critical role in protection from AHR (Que, Liu et al. 2005). The accumulating evidence for the role of GSNOR in asthma pathogenesis makes the investigation of potential inhibitors of GSNOR a tantalizing target for the treatment of asthma (Henderson and Gaston 2005; Wu, Romieu et al. 2007; Que, Yang et al. 2009).

In this study, we elucidated the effects SPL-334, a selective inhibitor of GSNOR, on allergic airway inflammation. SPL-334 was identified by Sanghani et al as compound C3, and shown to exclude GSNO from its binding site and preferentially inhibit GSNOR when compared to other alcohol dehydrogenase isoforms (Sanghani, Davis et al. 2009). The regulation of GSNOR activity led to increased protein S-nitrosylation in C3-treated RAW 264.7 cells and the subsequent accumulation of intracellular SNOs, indicating a role for GSNOR in the active regulation of SNO turnover and metabolism within the cells (Sanghani, Davis et al. 2009). Our study was the first to demonstrate that intranasal administration of SPL-334 results in significantly reduced Th2-mediated airway inflammation, mucus production, and AHR in a mouse model of asthma. Specifically, SPL-334 treatment led to a marked decrease in the number of OVA-specific T cells and

eosinophils present in the BALF of mice that received adoptive transfer of DO11.10 Th2 cells and subsequent inhalation exposure to aerosolized OVA. Consistent with the observed decrease in BALF Th2 cells, there was a significant reduction in the production of the Th2 cytokines IL-5 and IL-13 by LMCs of SPL-334-treated mice following stimulation with OVA₃₂₃₋₃₃₉ peptide or KJ-126 mAb, consistent with the decrease in the infiltration of OVA-specific Th2 cells into the airways observed in this group. It is likely this phenomena was responsible for the reduced level of inflammatory cell infiltrate evident in H&E stained tissues and mucus secretion observed in the airway. Additionally, a significant decrease in CCL11 (eotaxin) production by stimulated LMCs, as well as in CCL11 levels in the BALF, were observed in SPL-334-treated mice compared to vehicle-treated animals. Administration of SPL-334 during allergen-induced inflammation also led to a reduction in spontaneous production of CCL11 by cultured LMCs, suggesting that lung stromal cells may be a source of CCL11 in the airway sensitive to GSNOR inhibition.

As CCL11 is a potent chemokine for recruitment of eosinophils into the airway during allergic inflammation, the observed reduction of airway infiltration by eosinophils in this study are likely to be a result of GSNOR-mediated reduction in the production of CCL11. It is possible the inhibition of GSNOR by SPL-334 led to the suppression of NF- κ B activation by airway epithelial and stromal cells, which have been shown to express CCL11 by a NF- κ B- & STAT6-dependent mechanism following stimulation with TNF- α and IL-4 (Matsukura, Stellato et al. 1999). As NF- κ B is thought to have a pivotal role in inflammatory responses through transcriptional regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and

inducible enzymes such as iNOS and cyclooxygenase 2. Consequently, its regulation has the potential to affect many aspects of the allergic response (Li and Verma 2002). In the present study, intranasal treatment with SPL-334 resulted in a reduction in the number of Th2 cells accumulating in the lungs, in addition to reduced levels of IL-5, IL-13, and CCL11 present in the airways, suggesting that inhibition of GSNOR induced suppression of NF- κ B activation. Consistent with this hypothesis, SPL-334 treatment has been shown to cause smooth muscle relaxation and suppression of NF- κ B activation (Sanghani, Davis et al. 2009). Concordant with our findings, Sun et al have also generated a novel GSNOR inhibitor that has proven effective at reducing airway eosinophilic inflammation as well as improved metacholine-induced bronchoconstriction (AHR) in OVA-exposed mice (Sun, Qiu et al. 2012). Taken together, these studies suggest that GSNOR inhibition could have enticing potential as a therapeutic option for the treatment of asthma.

In summary, this study demonstrated that intranasal administration of SPL-334, a selective inhibitor of GSNOR, limits eosinophilic and Th2 cell inflammation, mucus production, and airway hyperreactivity in a mouse model of allergic asthma. Although further studies are needed, continued investigation of the effects of GSNOR inhibition in experimental asthma are needed, and this study suggest that increasing airway GSNO levels through the selective inhibition of GSNOR may provide a novel therapeutic option for the treatment of allergic airway inflammation in asthma and related lung diseases.

Chapter 4

Loss of Prostacyclin Signaling in the Regulation of Asthma

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Working Title: Role of PGI₂ in the Programming of Pulmonary NK Cells that Limit Allergic Inflammation

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4.0 ABSTRACT

The increasing prevalence of asthma represents a clear public health concern. The heterogeneous nature of asthma requires continuous improvement in our understanding of the mechanisms regulating asthma pathogenesis. In particular, the role of the innate immune system and the inflammatory mediators regulating their activation, represent a novel area for developing new therapeutic approaches. In this study, we developed a mouse model of house dust mite-induced allergic asthma, and utilized transgenic knockout mice deficient in the prostacyclin (PGI₂) receptor (IP) to understand the role of PGI₂ in the regulation of innate immunity during the development of allergic airway inflammation. Our results revealed that loss of PGI₂ signaling caused significant alteration in naïve pulmonary NK cell phenotype, specifically up-regulation in activating receptor NKp46 expression. *In vitro* stimulation with either α -NK1.1 or α -NKp46 mAb resulted in significantly increased production of IFN- γ compared to WT mice. Interestingly, evaluation of allergic airway inflammation in IP^{-/-} mice following HDM exposure revealed an attenuated allergic response, characterized by decreased infiltration of the airway by inflammatory immune cells, decreased peribronchial inflammation and goblet cell mucus production, and reduced production of Th2-associated cytokines. *In vivo* depletion of NK cells using α -NK1.1 mAb PK136 in IP^{-/-} mice prior to HDM sensitization resulted in restoration of allergic airway inflammation. These results suggest a previously unrecognized role for PGI₂ signaling in the regulation of NK cell function, as well as indicating a critical role for NK cells in the pathogenesis of asthma following HDM exposure.

4.1 Background

4.1.1 Prostaglandins

While protein-based signaling molecules such as cytokines and chemokines are the primary focus in immunological cell signaling, other cell-derived signaling molecules have an equally critical role in the regulation of immune cells. Lipid-based signaling molecules are capable of exerting both activating, as well as inhibitory, immunoregulatory effects on a wide variety of immune cell types. Eicosanoids are endogenous lipid-based signaling molecules derived from arachidonic acid that have varied and potent effects under physiological and pathophysiological conditions (Funk 2001). Eicosanoids encompass a broad array of compounds that includes prostaglandins, leukotrienes, and lipoxins (Hirata and Narumiya 2012). While the role of prostaglandins in regulating immune responses has been recognized for 40 years, recent advances have resulted in renewed interest on their immunoregulatory role (Plescia, Smith et al. 1975; Goodwin, Bankhurst et al. 1977).

4.1.1.1 Prostaglandin Synthesis

Following cellular activation, increases in intracellular Ca^{2+} result in phospholipase A_2 -mediated release of arachidonic acid from membrane phospholipids into the cytoplasm (Gijon and Leslie 1999). The production of prostaglandins is initiated by the action of two cyclooxygenase (COX) isoforms, the constitutively expressed COX-1 and the inducible COX-2, both of which catalyze the conversion of free arachidonic acid into prostaglandin H_2 (PGH_2) in a two-step reaction (Smith and Langenbach 2001). Further conversion of PGH_2 into the five primary bioactive prostaglandins is catalyzed by cell-specific prostaglandin synthases, responsible for the production of prostaglandin D_2

(PGD₂), prostaglandin E₂ (PGE₂), prostaglandin F_{2α} (PGF_{2α}), prostaglandin I₂ (PGI₂), and thromboxane A₂ (TXA₂) (Hirata and Narumiya 2012). COX-1 expression occurs in almost all cell types, particularly at high levels in cells of the gastrointestinal tract, kidneys, and platelets, while COX-2 is expressed under inflammatory conditions by macrophages, neutrophils, dendritic cells, and activated mesenchymal cells (Davies, Bailey et al. 1984; Vane, Bakhle et al. 1998; Garavito and DeWitt 1999). Of particular importance, non-steroidal anti-inflammatory drugs (NSAIDs) are typically capable of nonspecifically inhibiting COX-1 and COX-2. This inhibition plays a critical role in the beneficial anti-inflammatory and analgesic effects of NSAIDs, as well as in deleterious NSAID-associated gastrointestinal toxicity (Vane 1976; Rao and Knaus 2008).

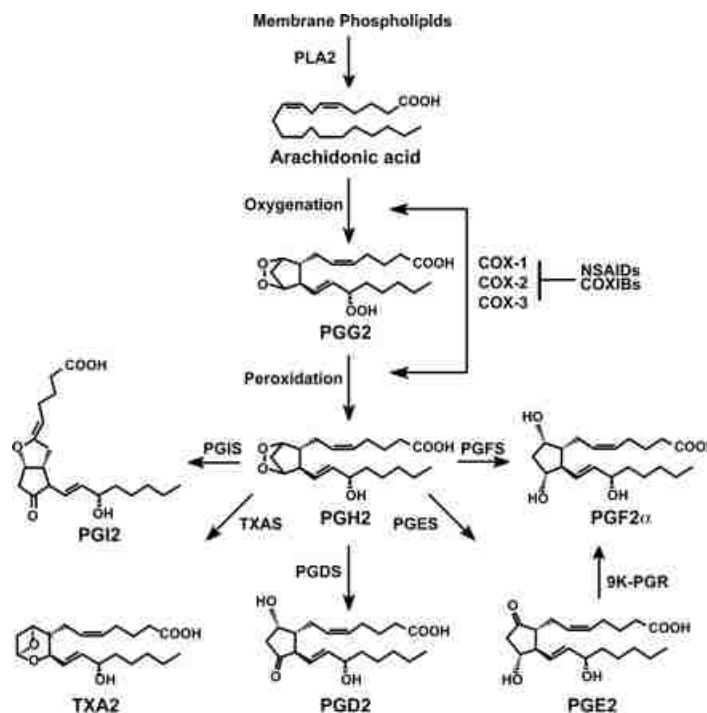


Figure 4.1 *Prostanoid Biosynthesis.* Membrane phospholipids are metabolized to arachidonic acid via PLA2. Cyclooxygenase isoforms further metabolize the formation of prostaglandin H₂ (PGH₂) which is converted into prostaglandins by prostaglandin-specific synthases. Prostaglandin synthesis can be disrupted through the inhibition of COX isoforms such as NSAIDs (Sirois, Sayasith et al. 2004).

4.1.1.2 Prostaglandin Signaling

Prostaglandin signaling is mediated by several specific receptors. The first prostaglandin receptor identified, TXA₂ receptor (TP), was revealed to be a G-protein coupled receptor expressed by platelets (Hirata, Hayashi et al. 1991). Subsequent analysis led to the identification of eight additional prostaglandin receptors with a high degree of homology, that include two isoforms of prostaglandin D₂ receptor (DP1 & DP2), four isoforms of prostaglandin E₂ receptor (EP1 – 4), as well as single receptors of prostaglandin I₂ (IP) and prostaglandin F_{2α} (FP) (Sugimoto, Namba et al. 1992; Honda, Sugimoto et al. 1993; Watabe, Sugimoto et al. 1993; Hirata, Kakizuka et al. 1994; Namba, Oida et al. 1994). The nine prostaglandin receptors are phylogenetically classified into three subfamilies based on structural and functional similarities based on signal transduction pathways (Toh, Ichikawa et al. 1995). The DP1-EP2-EP4-IP subfamily couple to G_s resulting in increased cAMP production, and subsequent cellular activation, the EP1-FP-TP subfamily couple to G_q resulting in increased cytosolic Ca²⁺ mobilization, while the EP3-DP2 subfamily couples to G_i resulting in decreased cAMP production and Ca²⁺ mobilization (Katoh, Watabe et al. 1995; Hirai, Tanaka et al. 2001; Sands and Palmer 2008). While prostaglandin receptors display preferentially high affinity for their respective prostaglandins, it has been observed that some cross-reactivity exists in certain receptors (Kiryama, Ushikubi et al. 1997).

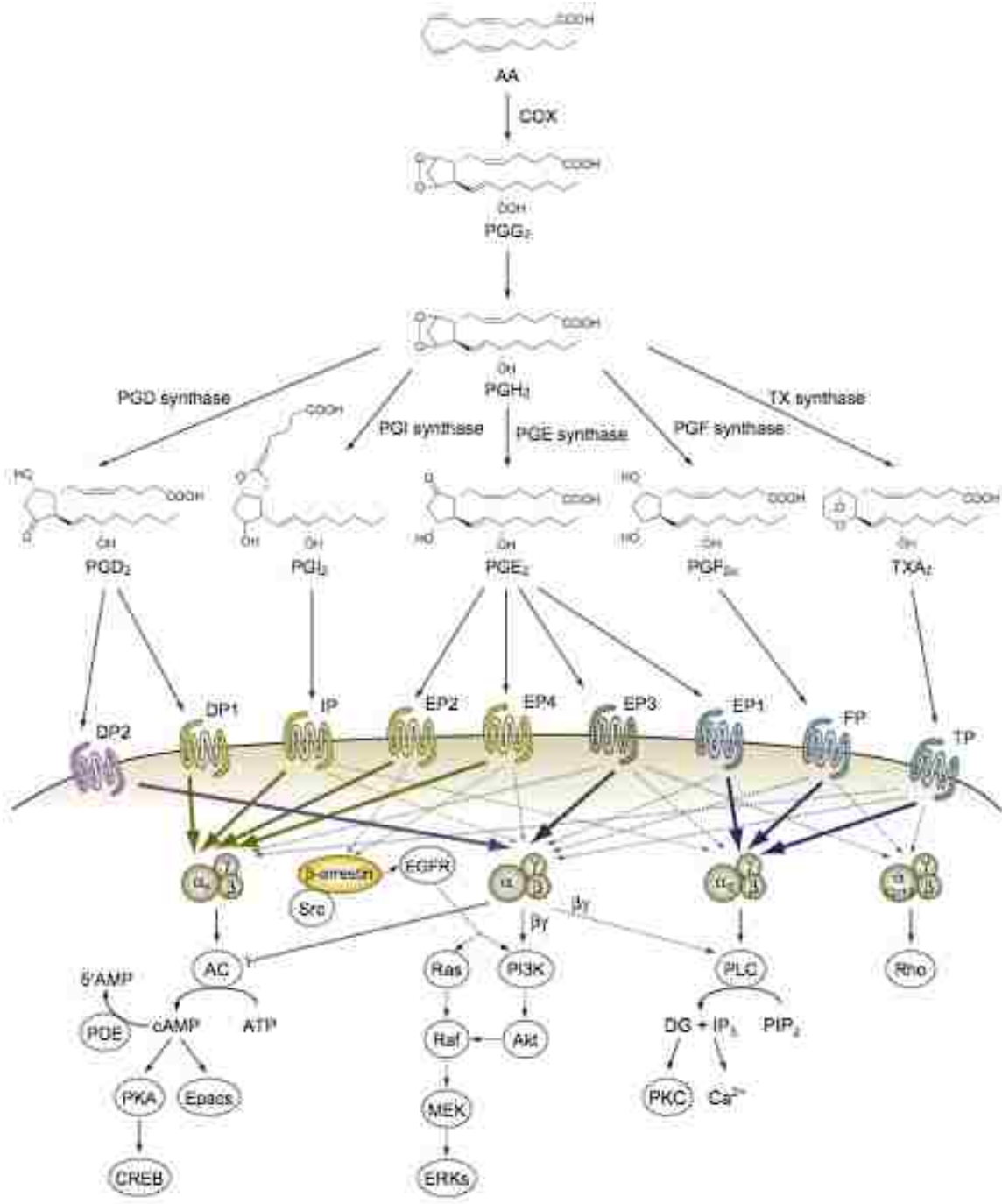


Figure 4.2 *Prostanoid Signaling.* Prostaglandins bind to prostanoid-specific G-protein coupled receptors. Signaling through these receptors leads to the activation of several diverse signal transduction pathways capable of regulating activation and inhibition of cellular function (Hirata and Narumiya 2012).

4.1.2 Prostaglandin-associated Regulation of Respiratory Immune Responses

While prostaglandins play a crucial role in the regulation of responses in a diverse array of tissues such as the gastrointestinal tract, cardiovascular system, respiratory tract, and uterine tissue, the role of prostaglandins in regulating innate and adaptive immune responses has received the most recent attention. In the respiratory tract, alteration in prostaglandin signaling has been associated with both beneficial and deleterious effects on the pathogenesis of asthma. It is critical to consider the inflammatory environment where prostaglandin signaling occurs in order to fully understand the regulatory role of prostaglandins in controlling immune cell function.

4.1.2.1 Adaptive Immune Cells

Recognition of bacterial lipopolysaccharide (LPS) and IL1- β results in potent NF- κ B-induced COX-2 expression in macrophages and dendritic cells that is followed by subsequent production of prostaglandins capable of regulating immune cell function in an autocrine or paracrine manner (Rocca and FitzGerald 2002). Binding of PGE₂ has been implicated to inhibit MHC class II expression and TNF- α production in macrophages and dendritic cells, while leading to increased production of IL-10 in dendritic cells (Nataraj, Thomas et al. 2001; Vassiliou, Jing et al. 2003). PGI₂ and PGD₂ have been shown to inhibit the activation and maturation of monocyte-derived dendritic cells, resulting in reduced capacity to induce T cell-associated responses (Gosset, Bureau et al. 2003; Zhou, Hashimoto et al. 2007). PGD₂-mediated inhibition of migration to draining lymph nodes by pathogen-activated peripheral dendritic cells plays a critical role in the inhibition of T cell activation (Hammad, de Heer et al. 2003; Angeli, Staumont et al. 2004). In addition

to these indirect methods of inhibiting T cell activation, TXA₂ has been shown to inhibit direct DC-T cell interaction (Kabashima, Murata et al. 2003).

In mice and humans, PGE₂ has been shown to enhance both Th1 and Th17 differentiation *in vitro*, while *in vivo* administration of EP inhibitors resulted in decreased accumulation of both Th1 and Th17 cell migration into draining lymph nodes (Yao, Sakata et al. 2009; Esaki, Li et al. 2010). In particular, PGE₂ regulation of IL-23 production by dendritic cells results in increased expansion and activation of Th17 cells that have been implicated to increase the progression and severity of several diseases, including asthma (Chizzolini, Chicheportiche et al. 2008; Boniface, Bak-Jensen et al. 2009). Treatment of T cells *in vitro* utilizing an IP agonist revealed that PGI₂ signaling drives Th1 differentiation and subsequent decreases in contact hypersensitivity responses (Nakajima, Honda et al. 2010). However, PGI₂ has also been shown to inhibit the production of cytokines by both Th1 and Th2 cells following activation, indicating that IP signaling contributes to both pro- and anti-inflammatory regulation of cellular function (Zhou, Blackwell et al. 2007). The role of prostaglandin signaling in B cells has remained more complicated than that of T cells. While PGE₂ has been shown to inhibit B cell proliferation, it has been observed that it also has the capacity to induce IgG and IgE class switching (Fedyk and Phipps 1996; Roper, Graf et al. 2002). Additionally, in germinal centers, dendritic cell-derived IL-21 in conjunction with PGE₂ readily induced costimulatory B cell apoptosis (Magari, Nishikawa et al. 2011).

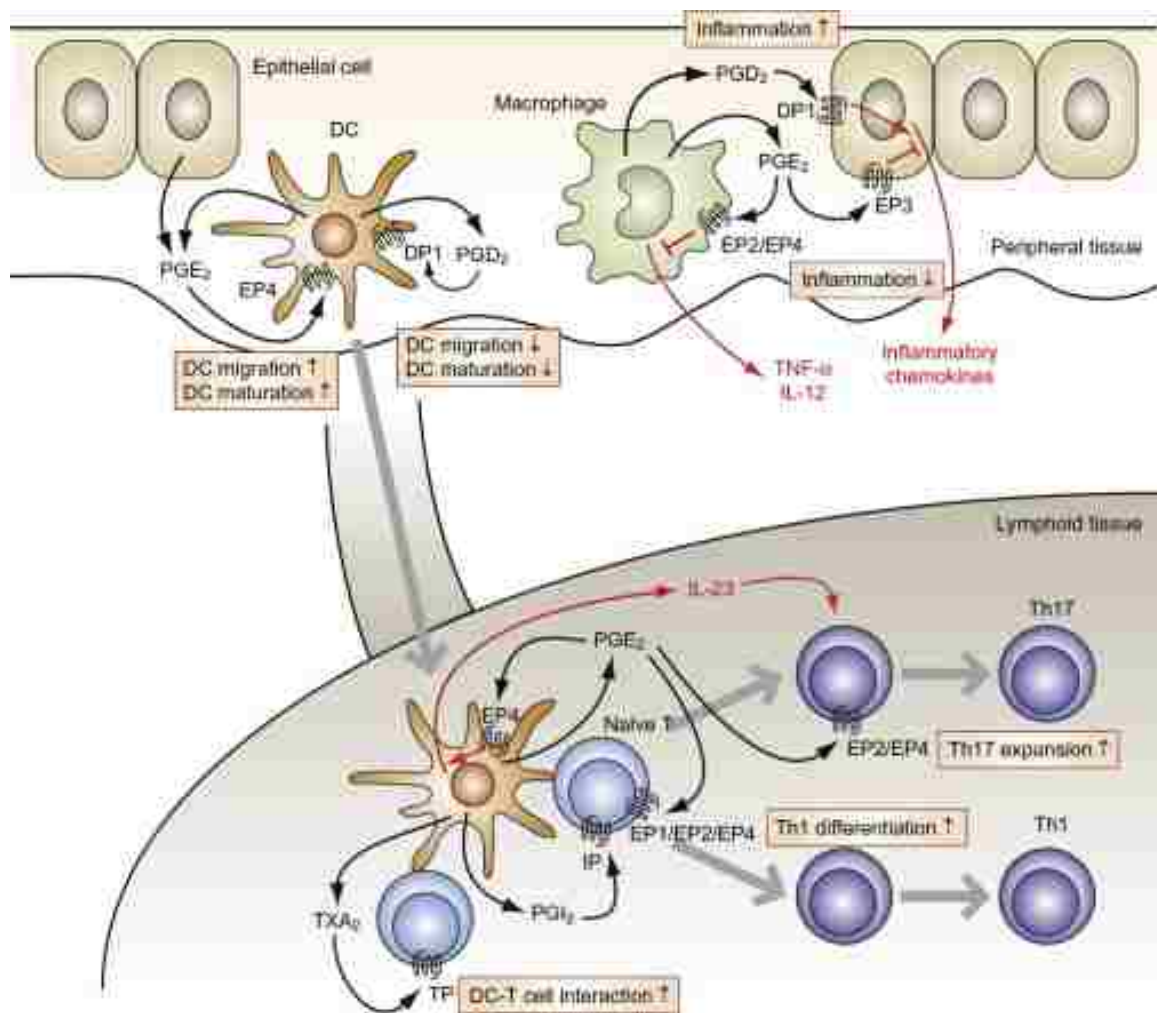


Figure 4.3 *Regulation of Adaptive Immunity by Prostaglandins.* Autocrine and paracrine signaling by prostanoids has the capacity to lead to the activation and inhibition of dendritic cells. The resulting changes in dendritic cell populations result in alterations in Th cell maturation and differentiation (Hirata and Narumiya 2012).

4.1.2.2 Innate Immune Cells

PGE₂ signaling in neutrophils has been associated with reduced inflammatory responses, while autocrine signaling of PGD₂ by eosinophils results in eosinophil activation (Wright, Moots et al. 2010; Luna-Gomes, Magalhaes et al. 2011). PGE₂ has been shown to result in suppression of NK cell effector function directly, while

production of PGE₂ by dendritic cells leads to decreased NK cell production of IFN- γ as well as suppression of cytotoxicity (Van Elssen, Vanderlocht et al. 2011; Holt, Ma et al. 2012). NK cell-derived PGD₂ has also been shown to inhibit NK cell cytotoxicity, migration, and cytokine production in an autocrine manner (Chen, Perussia et al. 2007). Alteration of epithelial cell function in the airway and subsequent skewing of the T cell response has been associated with prostaglandin signaling, as PGD₂ signaling has been shown to induce the secretion of Th2-associated chemokines, while PGE₂ has been shown to inhibit this secretion resulting in decreased airway inflammation (Matsuoka, Hirata et al. 2000; Kunikata, Yamane et al. 2005). Prostaglandins exhibit duality in controlling innate $\gamma\delta$ T cells responses as well, as PGE₂ has been demonstrated to reduce $\gamma\delta$ T cell cytotoxicity, while work we have previously performed has indicated that PGI₂ promotes the development of IL-17-producing $\gamma\delta$ T cells that exacerbate allergic airway inflammation in mice (Martinet, Jean et al. 2010; Jaffar, Ferrini et al. 2011).

4.1.3 Natural Killer Cell Effector Functions

A key component of the innate immune system, natural killer (NK) cells were originally described as large, granular cells of the lymphoid lineage with natural affinity (i.e. no priming required) towards tumor-specific cytotoxicity both through direct interaction as well as through the production of cytokines (Trinchieri 1989). In addition to their well-documented targeting of tumor cells, NK cells are also capable of recognizing and killing virus-infected cells (Biron, Byron et al. 1989). While most studies have focused on the cytotoxicity mediated by NK cells, recent advances have begun to spur interest in the regulatory role of NK and NK-related immune cells (Spits and Di Santo 2011; Bernink, Peters et al. 2013). The increased focus on the regulatory

capabilities of NK cells offers new opportunities to explore the role that NK cells have in the regulation of disease pathogenesis, especially in capacities not related to direct defense against invading pathogens or tumors.

4.1.3.1 Regulation of Natural Killer Cell Function

While NK cell-mediated regulation of the immune system has become the most recent focus of NK cell-related research, cytotoxicity still plays a vital role in understanding how NK cells interact with their environment, including other immune cells. One of the hallmark features of NK cells is their innate capacity to mediate cytotoxicity without any apparent need to be primed via MHC-presented antigen, and thus providing a mechanism to rapidly respond to encountered pathogens or virus-infected cells. With no regulation, this constitutive state of cytotoxicity would have devastating consequences from autoreactivity to unchecked inflammatory responses. Fortunately, an elaborate system of activating and inhibiting receptors has evolved in order to highly regulate the destructive capabilities of NK cells.

4.1.3.1.1 Inhibitory Receptors

In 1986, Karre et al published a seminal study that would influence all further understanding of NK cell effector function (Karre, Ljunggren et al. 1986). In this study, Karre et al described that NK cells have the capability of targeting lymphoma variants that were deficient in MHC Class I while ignoring lymphoma variants that maintained MHC class I expression. In essence, this allowed the NK cells to recognize the difference between self and non-self cells within tissue, allowing NK cells to target cells that down-regulate MHC class I expression in order to avoid the attention of cytotoxic CD8⁺ T cells. This initial look at the regulation of NK cell-mediated cytotoxicity

revealed the existence of inhibitory receptors controlling NK cell effector function. Further studies have identified a vast array of inhibitory receptors in both mice and humans, including killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), and C-type lectin type II glycoproteins (Ravetch and Lanier 2000). Some of these inhibitory receptors, such as Ly49a & Ly49c in mice and various KIR (CD158) isotypes in humans, are capable of recognizing specific classical MHC class I ligands (Lanier 2008). The inhibitory receptor CD94/NKG2A is conserved in both rodents and primates, and binds to the widely expressed nonclassical MHC class I molecule Qa-1^b leading to inhibition of NK-mediated effector functions (Colmenero, Zhang et al. 2007).

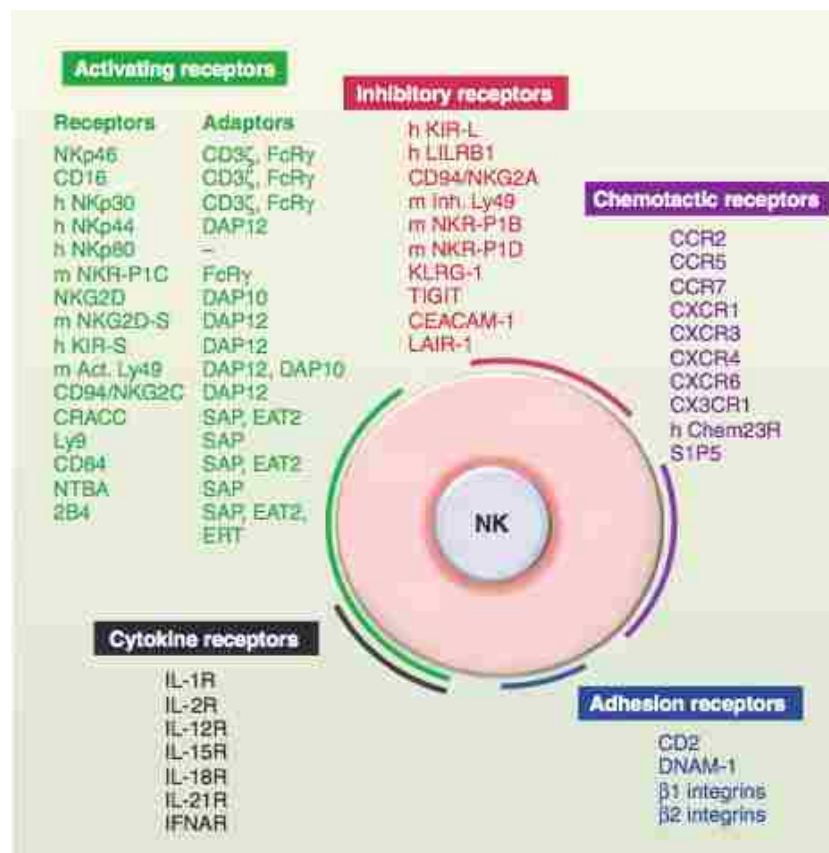


Figure 4.4 *Natural Killer Cell Receptor Expression.* NK cells express a wide variety of surface receptors. Binding of ligands to activating and inhibitory receptors leads to regulation of NK cell cytotoxicity (Vivier, Raulet et al. 2011).

4.1.3.1.2 *Activating Receptors*

While inhibitory receptors play a crucial role in the regulation of NK cell effector function, inhibitory signals alone are not the sole arbiter of NK-cell mediated cytotoxicity. In addition to the loss of inhibitory signaling, recognition of ligands via activating receptors on the surface of NK cells are necessary, in most cases, to fully engage NK cell effector function. These activating receptors thus allow for the targeting of tumor or virus-infected cells that do not down-regulate expression of MHC class I (Correa, Corral et al. 1994). The prototypical NK cell activating receptor is NKG2D, a C-type lectin surface receptor expressed by NK cells, $\gamma\delta$ T cells, CD8⁺ T cells, and activated macrophages (Jamieson, Diefenbach et al. 2002). While serving as a co-stimulatory receptor for T cells, NK cells and activated macrophages respond directly to NKG2D cross-linking following association with the adaptor proteins DAP10 and DAP12 (Diefenbach, Tomasello et al. 2002; Jamieson, Diefenbach et al. 2002). Similarly to NKG2D, the lectin-like type II membrane proteins Ly49H have also been shown to induce activation of NK cells in conjunction with DAP12 to induce cytotoxicity in addition to cytokine production (Lanier 1998). In addition to these lectin receptors, identification of immunoglobulin-like type I membrane proteins termed natural cytotoxicity receptors (NCRs) which include NKp30, NKp44, and NKp46 has greatly expanded the repertoire of activating receptors responsible for regulating NK cell function (Koch, Steinle et al. 2013). NKp46 (CD335; NCR-1) has been identified on both murine and human NK cells as a highly specific marker of NK cells, and it has been suggested that it represents an evolutionally conserved method for the removal of tumors and viral pathogens as blocking of NKp46 with monoclonal antibody resulted in

significantly reduced cytotoxicity of certain tumors (Pessino, Sivori et al. 1998; Moretta, Bottino et al. 2001). This removal has been shown to be mediated via recognition of several different ligands, including virally-associated ligands like Hemagglutinin as well as endogenous ligands such vimentin or tumor-specific antigens (Harris, Kapur et al. 1992; Mandelboim, Lieberman et al. 2001; Garg, Barnes et al. 2006; Halfteck, Elboim et al. 2009).

4.1.3.2 Natural Killer Cell-Mediated Cytotoxicity

Activation of NK cells following tumor or pathogen recognition results in the prototypical NK cell effector response of cytotoxicity. NK cell-mediated cytotoxicity requires direct contact between NK cells and target cells, and occurs via three possible mechanisms.

The primary mechanism of NK cell-mediated cytotoxicity utilizes cytoplasmic granule toxins that lead to apoptosis of target cells via exocytosis, similarly to cytotoxic T lymphocytes. Within these cytoplasmic granules, NK cells package a potent combination of perforin, a membrane-disrupting protein, and a family of structurally related serine proteases known as granzymes (Grz) (Smyth, O'Connor et al. 1996). The use of perforin-deficient knockout mice has clearly demonstrated the critical role that perforin has in NK cell-mediated cytotoxicity, but this role extends beyond allowing Grz access to target cell cytoplasm, as it has been demonstrated that Grz uptake into target cells can occur efficiently and rapidly following receptor-mediated endocytosis (Kagi, Ledermann et al. 1994; Smyth, Browne et al. 1995; Motyka, Korbitt et al. 2000). It has been suggested that the role of perforin in NK-cell mediated cytotoxicity leads to disruption of endosomal trafficking following Grz uptake in target cells (Browne, Blink et al. 1999).

There are eleven different Grz that have been described to date, but granzyme A (GrzA) and granzyme B (GrzB), which are conserved between mice and humans, have received the most focus in attempts to understand the mechanism of NK-cell mediated cytotoxicity (Smyth, O'Connor et al. 1996). In combination with perforin, GrzB induces apoptosis of target cells via caspase-dependent oligonucleosomal fragmentation of DNA, while GrzA has been demonstrated to generate single-stranded DNA nicks that induce apoptosis in target cells via a caspase-independent mechanism (Heusel, Wesselschmidt et al. 1994; Beresford, Xia et al. 1999). Mouse models deficient in GrzA/GrzB have provided interesting insights into the role of Grz in NK cell-mediated cytotoxicity. Specifically, while the role of Grz in NK-mediated cytotoxicity cannot be denied, functional redundancy in individual Grz leads to only moderate decreases in Grz-induced apoptosis, suggesting that individual Grz may have distinct cell signaling effects beyond inducing cytotoxicity (Heusel, Wesselschmidt et al. 1994; Ebnet, Hausmann et al. 1995).

The secondary mechanism utilized during NK-cell mediated cytotoxicity relies on the activation of tumor necrosis family (TNF)-related death receptor pathways on target cells. The two most highly characterized TNF death receptor pathways involved in NK cell-mediated cytotoxicity utilize NK expression of the TNF-related apoptosis-inducing ligand (TRAIL) or Apo2 ligand, or the Fas ligand (FasL). While TRAIL is constitutively expressed by a small subset of liver NK cells, TRAIL expression is typically induced in peripheral NK cells following stimulation of NK cells with IL-2, IL-12-induced IFN- γ , or IL-15 (Smyth, Cretney et al. 2001; Takeda, Hayakawa et al. 2001; Takeda, Smyth et al. 2001). TRAIL receptors such as TRAIL-R2 (DR5) are widely expressed by both murine and human cells, and activation following TRAIL binding results in the activation of

caspase 8-dependent apoptosis pathway (Wiley, Schooley et al. 1995; Chaudhary, Eby et al. 1997). In addition to TRAIL, NK cells have also been shown to express the homologous FasL protein. The most intensely studied death receptor, Fas (CD95) binding of FasL ultimately results in caspase-8 activation and subsequent cellular apoptosis. While several tumor lineages down-regulate Fas expression, it has been observed that NK cell production of IFN- γ induces Fas expression on tumor cells resulting in Fas-dependent apoptosis (Screpanti, Wallin et al. 2001).

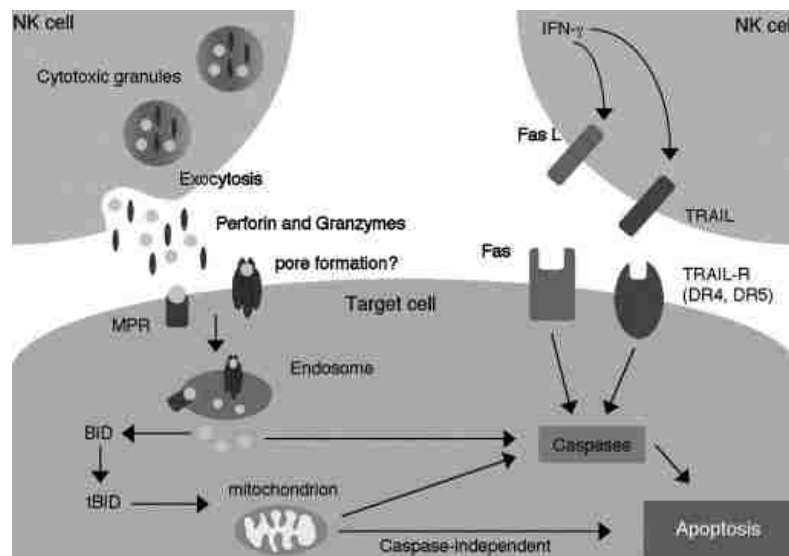


Figure 4.5 *Mechanisms of Natural Killer Cell Cytotoxicity.* Activation of NK cells results in NK-mediated cytotoxicity by two distinct mechanisms. (1) The release of reactive granule products such as granzymes and perforin from NK cells results in caspase-dependent and caspase-independent target cell apoptosis. (2) IFN- γ signaling induces death receptor ligand expression such as FasL or TRAIL on the surface of NK cells which upon binding to the appropriate TNF-family death receptor induces caspase-dependent target cell apoptosis (Smyth, Cretney et al. 2005).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is the final mechanism of NK cell-mediated cytotoxicity. NK cells have been shown to express multiple isoforms of Fc receptor, including Fc γ RIIIa/ Fc γ RIIc (CD16/CD32), Fc μ R, and Fc α R that bind the Fc regions of IgG, IgM, and IgA, respectively (Pricop, Rabinowich et al. 1993; Trinchieri and Valiante 1993; Mota, Manciuola et al. 2003). Activation of NK cells by CD16 cross-

linking results in potent activating signals capable of overriding inhibitory KIR signals and subsequent lysis of target cells utilizing cytokine production and granule-mediated cytotoxicity (Chan, Kung Sutherland et al. 2012).

4.1.3.3 Natural Killer Cell-Derived Cytokines: IFN- γ

While cytotoxicity has long been recognized as the primary method by which NK cells interact with pathogens and non-pathogens, the production of cytokines by NK cells results in extensive regulation of both innate and adaptive immunity. NK cells have been observed to produce several cytokines following activation, none are as critical as IFN- γ in NK cell effector function.

IFN- γ is the canonical Th1-associated cytokine, involved in removal of intracellular bacterial and viral pathogens as well as tumors (Kaplan, Shankaran et al. 1998; Jouanguy, Doffinger et al. 1999; Ikeda, Old et al. 2002; Filipe-Santos, Bustamante et al. 2006). Signaling via IFN- γ can lead to increased expression of MHC class I and class II on mononuclear cells such as macrophages as well as epithelial cells (Basham and Merigan 1983; King and Jones 1983). Activation of macrophages by IFN- γ results in increased phagocytosis and production of antimicrobials such as superoxide radicals, nitric oxide, and hydrogen peroxide (Boehm, Klamp et al. 1997). IFN- γ plays a critical role in the determination of T helper cell lineage, as IFN- γ has been observed to lead to permanent commitment to the Th1 lineage in addition to suppressing Th2 proliferation and cytokine production (Grogan and Locksley 2002; Ho and Glimcher 2002; Murphy and Reiner 2002). NK cell-derived IFN- γ in particular has been shown to enhance CD4⁺ Th1 cell polarization in secondary lymphoid tissues as well as promote dendritic cell maturation (Gerosa, Baldani-Guerra et al. 2002; Morandi, Bougras et al. 2006). Similarly

to their ability to rapidly induce cytotoxicity following activation, NK cells are capable of rapid and prolific production of IFN- γ immediately following activation. Stimulation of NK cells with IL-12, IL-18, IL-15, IL-2, or type I Interferons (IFN- α/β) results in significant production of IFN- γ (Lieberman and Hunter 2002; Walzer, Dalod et al. 2005). In particular, IL-12, originally identified as NK cell stimulatory factor, synergistically increases production of IFN- γ by NK cells following co-stimulation with IL-18 (D'Andrea, Rengaraju et al. 1992). In contrast, TGF- β has been identified as a potent negative regulator of NK cell IFN- γ production, and is an important component in preventing uncontrolled NK cell-mediated inflammation (Li, Wan et al. 2006).

4.2 RESULTS

4.2.1 Characterization of Pulmonary Natural Killer Cells in the Airways of Naïve IP^{-/-} Mice

Previous work done in our laboratory by Jaffar et al displayed how alterations in prostacyclin signaling had a critical role on the development of asthma through the action of innate $\gamma\delta$ T cells (Jaffar, Ferrini et al. 2011). The biological effects of prostacyclin action *in vivo* were examined using mice lacking the prostacyclin receptor (IP). Results from this study suggested that prostacyclin signaling could be involved in the regulation of other innate immune cell populations as well. Examination of the lung tissue of naïve IP^{-/-} mice revealed that there was a significantly increased number of NK1.1⁺ cells present in the airway compared to wild-type C57BL/6 (WT) mice (Figure 4.6). NK1.1, also known as CD161 or Nkrp1c, is a C-type lectin receptor expressed on the cell surface of NK cells as well as NKT cells and Th17 cells (Bartel, Bauer et al. 2013). Further characterization of this NK1.1⁺ population revealed a lack of CD3 expression, identifying

the population as NK cells (Figure 4.6). Little is known of the characteristics of NK cells that reside in the lung tissue. Consequently, we found the doubling in the number of pulmonary NK cells resulting from lack of prostacyclin signaling in the $IP^{-/-}$ mouse intriguing. The ability of prostacyclin to inhibit NK cell cytotoxicity has been previously described in purified, pharmaceutically treated NK cells (Lanefelt, Ullberg et al. 1983), but the role that prostacyclin signaling exerts on *in vivo* NK cell development and function has been poorly examined. Utilizing the $IP^{-/-}$ mouse allows us a unique opportunity to examine the modulatory role of prostacyclin signaling on NK cell function *in vivo*.

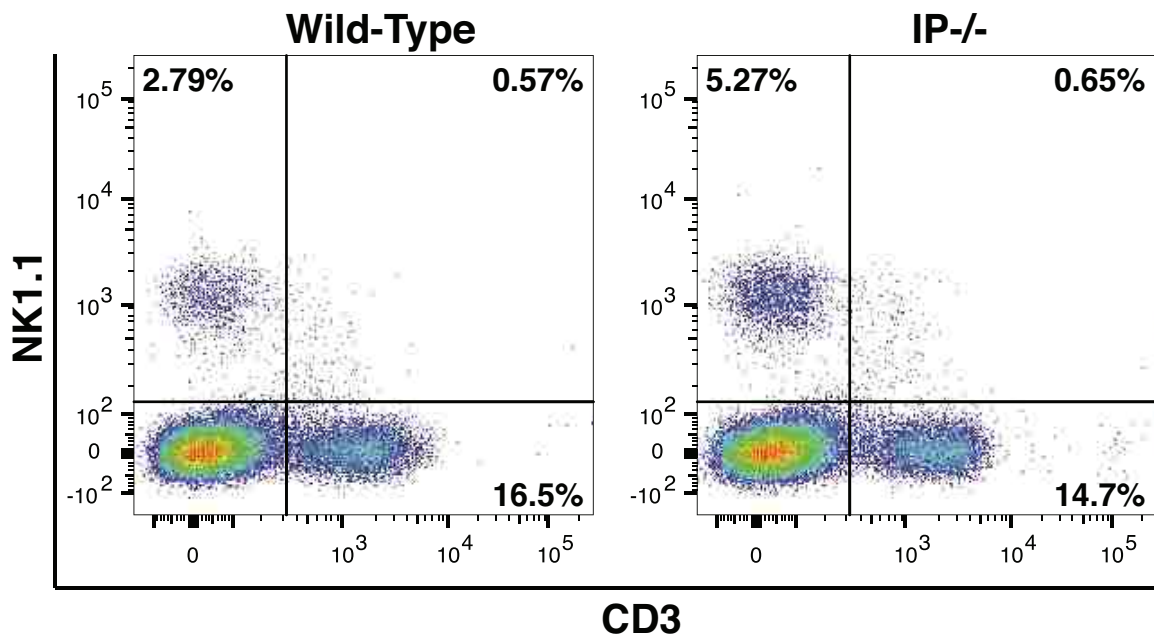


Figure 4.6 Naïve $IP^{-/-}$ Mice Display Increased Numbers of Pulmonary Natural Killer Cells. Lung mononuclear cells were obtained from dissociated lung tissue from naïve $IP^{-/-}$ and WT mice. LMCs were stained with anti-CD3 (FITC) and anti-NK1.1 (APC) and analyzed for receptor expression using a BD FACS Aria Flow Cytometer. Natural killer cells were classified as $CD3^{-}NK1.1^{+}$.

4.2.1.1 Increased Expression of the Activating Receptor NKp46 By Natural Killer Cells in IP^{-/-} Mice

The term ‘NK cell’ actually encompasses a broad array of specific subtypes based on factors such as tissue residency, stage of maturity, and effector function. Classification of these specific subtypes is generally based on surface expression of several distinct markers.

Recently, differences in marker expression between ‘conventional’ (cNK) and ‘tissue resident’ (trNK) NK cells have been described based on expression of CD49b, also known as Dx5, and CD49a (Yokoyama, Sojka et al. 2013), respectively. cNKs are found in the spleen and periphery, whereas trNKs have been described primarily in the liver, although in the skin and uterine lining as well (Sojka, Plougastel-Douglas et al. 2014). In addition to differences in surface receptor expression, differences in effector function reveal that cNKs and trNKs have distinct immunological roles (Sojka, Plougastel-Douglas et al. 2014). Initial characterization of NK cells present in the airways revealed that NK cells (defined as CD3⁻CD19⁻NK1.1⁺) exclusively expressed CD49b in both IP^{-/-} and WT mice (Figure 4.7).

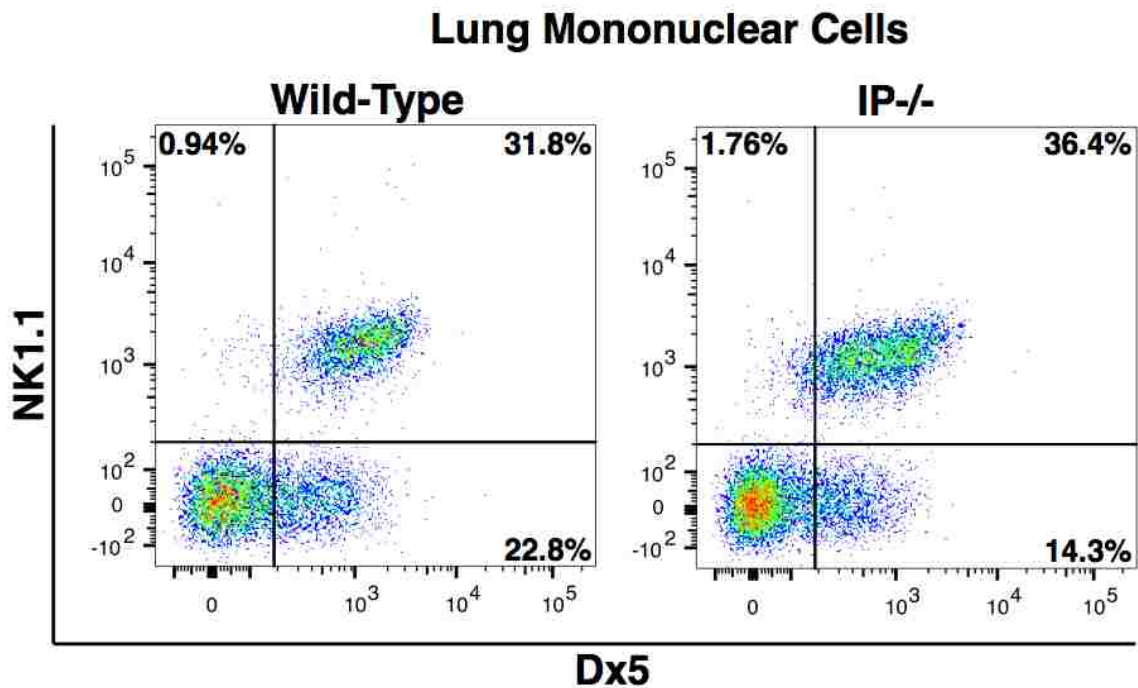


Figure 4.7 Naïve IP^{-/-} and WT Pulmonary Natural Killer Cells Express Dx5 (CD49b). Lung mononuclear cells were obtained from dissociated lung tissue from naïve IP^{-/-} and WT mice. LMCs were stained with anti-CD3 (FITC), anti-CD19 (APCCy7), anti-NK1.1 (APC), and anti-Dx5 (CD49b)(PE) and analyzed for receptor expression using a BD FACSARIA Flow Cytometer. Natural killer cells were classified as CD3⁻CD19⁻NK1.1⁺.

NK cell development can be divided into several stages of maturity characterized by expression of specific surface receptors (Di Santo 2006). We compared IP^{-/-} and WT mouse pulmonary NK cells for a selection of these receptors and found minimal differences in expression profiles. Flow cytometry analysis revealed that both IP^{-/-} and WT mouse pulmonary NK cells had essentially identical phenotypic profiles of CD27, Ly49a, Ly49c, CD117, and CD127 expression (Figure 4.8). Expression of CD27, LY49a, and Ly49c was observed on approximately 20% (20% to 23%, 17% to 18%, & 15% to 21%, respectively) of NK1.1⁺ cells in both IP^{-/-} and WT mice, while no

expression of CD127 (IL-7r) or CD117 (c-kit) was observed on NK1.1⁺ cells of either strain (Figure 4.8).

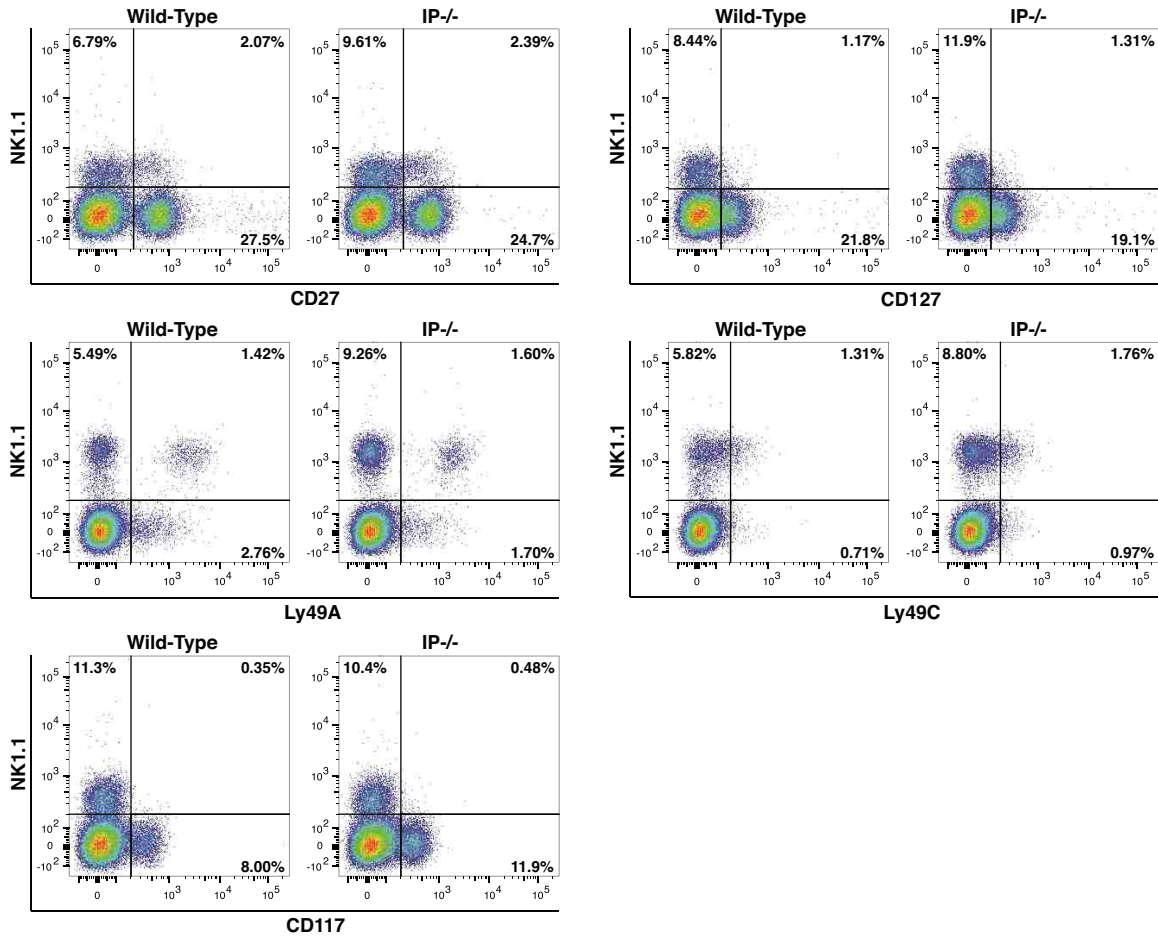


Figure 4.8 Naïve IP^{-/-} and WT Pulmonary Natural Killer Cell Phenotyping. Lung mononuclear cells were obtained from dissociated lung tissue from naïve IP^{-/-} and WT mice. LMCs were stained with anti-CD3 (FITC, PE, APC), anti-CD19 (APCCy7), anti-NK1.1 (APC, FITC), anti-CD27 (APC), anti-CD127 (APC), anti-Ly49A (FITC), anti-Ly49C (FITC), and anti-CD117 (c-kit)(APC) and analyzed for receptor expression using a BD FACSARIA Flow Cytometer. Natural killer cells were classified as CD3⁻CD19⁻NK1.1⁺.

The effector functions of NK cells are controlled by a complex system of activating and inhibitory receptors expressed on the NK cellular surface (Vivier, Raulet et al. 2011). Although less recognized than classic NK activation receptors such as NKG2D and aKIRs, natural cytotoxicity receptors (NCRs) are a family of activating receptors that upon direct binding to both exogenous and endogenous ligands lead to NK cell activation (Hudspeth, Silva-Santos et al. 2013). NCR1, more commonly referred to as NKp46, is expressed by several species, including humans and mice, and is considered the primary NCR involved in pathogen and tumor recognition due to evolutionary conservation (Koch, Steinle et al. 2013). Expression of NKp46 on the cellular surface of NK cells is one of the key markers of NK cell maturity in mice, as well as providing an indication of the activation state of NK cells (Di Santo 2006). Therefore, we examined the expression of NKp46 on pulmonary NK cells in $IP^{-/-}$ mice. We found that NK cells present in the airway of $IP^{-/-}$ mice had significantly increased expression of activating receptor NKp46 when compared to WT mice (Figure 4.9). Approximately 82% of the $IP^{-/-}$ mouse pulmonary NK cells expressed NKp46, as opposed to 35% of WT mouse pulmonary NK cells. Further comparison of NKp46 expression by $IP^{-/-}$ mouse NK cells at several other key immunological sites, including the spleen (93% compared to 50%), brachial (82% to 37%) & mesenteric lymph nodes (77% to 36%), Peyer's patches (59% to 36%), and bone marrow (54% to 23%), revealed that NK cells present in all of those sites had significantly increased expression of NKp46 in $IP^{-/-}$ mice when compared to WT mice, suggesting a systemic shift in $IP^{-/-}$ mouse NK cell expression of NKp46 (Figure 4.9).

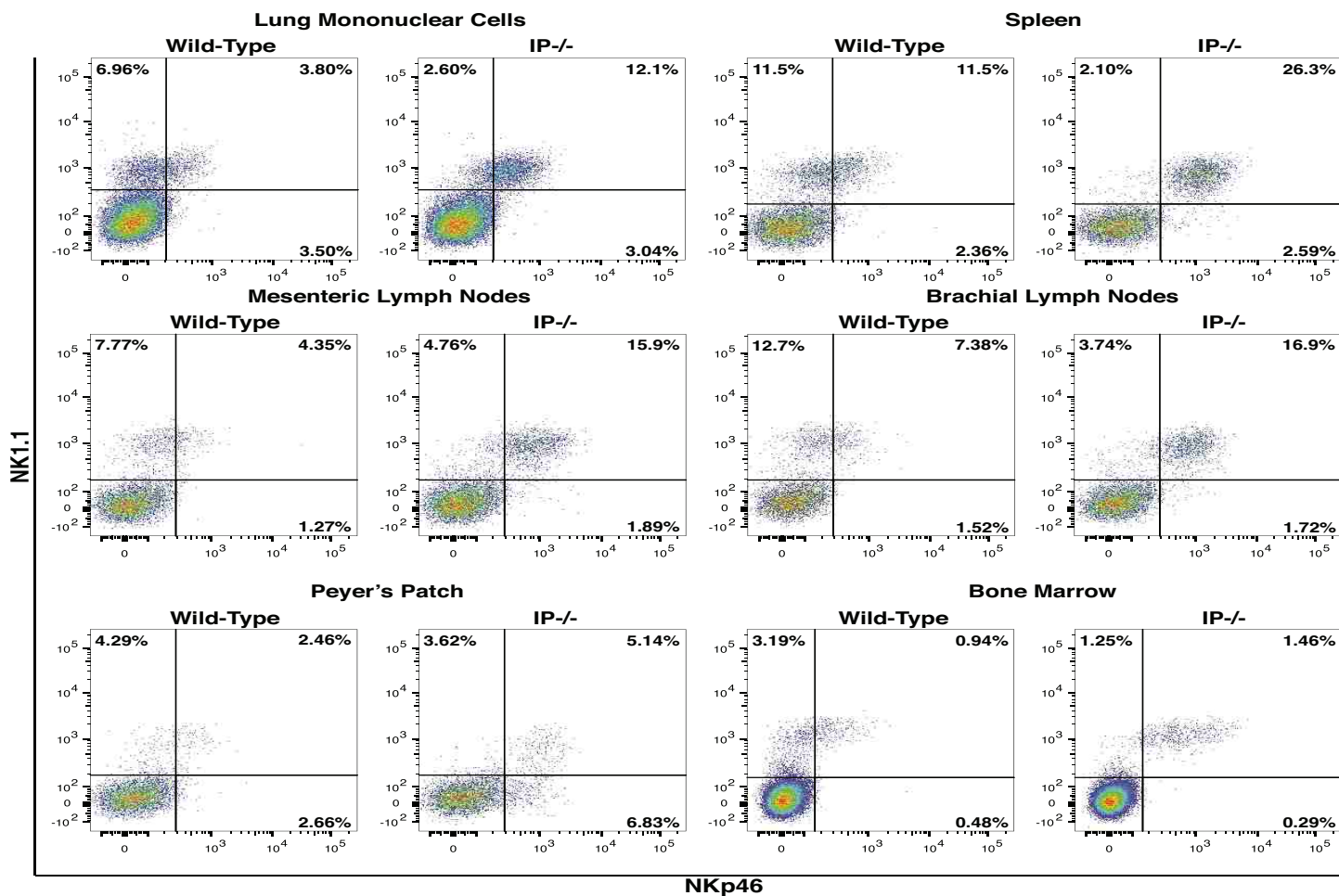


Figure 4.9 Naïve IP^{-/-} Natural Killer Cells Display Increased Systemic Expression of Activating Receptor NKp46. Mononuclear cells were obtained from dissociated lung tissue, spleen, mesenteric and brachial lymph nodes, peyer's patches, and bone marrow from native IP^{-/-} and WT mice. Mononuclear cells were stained with anti-CD3 (FITC), anti-CD19 (APCCy7), anti-NK1.1 (PE), and anti-NKp46 (APC) and analyzed for receptor expression using a BD FACSaria Flow Cytometer. Natural killer cells classified as CD3⁻CD19⁻NK1.1⁺.

NKG2D is recognized as one of the primary activating receptors responsible for initiation of NK cell effector functions. Although we attempted to examine NKG2D expression by splenic NK cells in both $IP^{-/-}$ and WT mice, flow cytometry analysis was unable to detect any level of NKG2D expression on splenic NK cells of either strain (Figure 4.10).

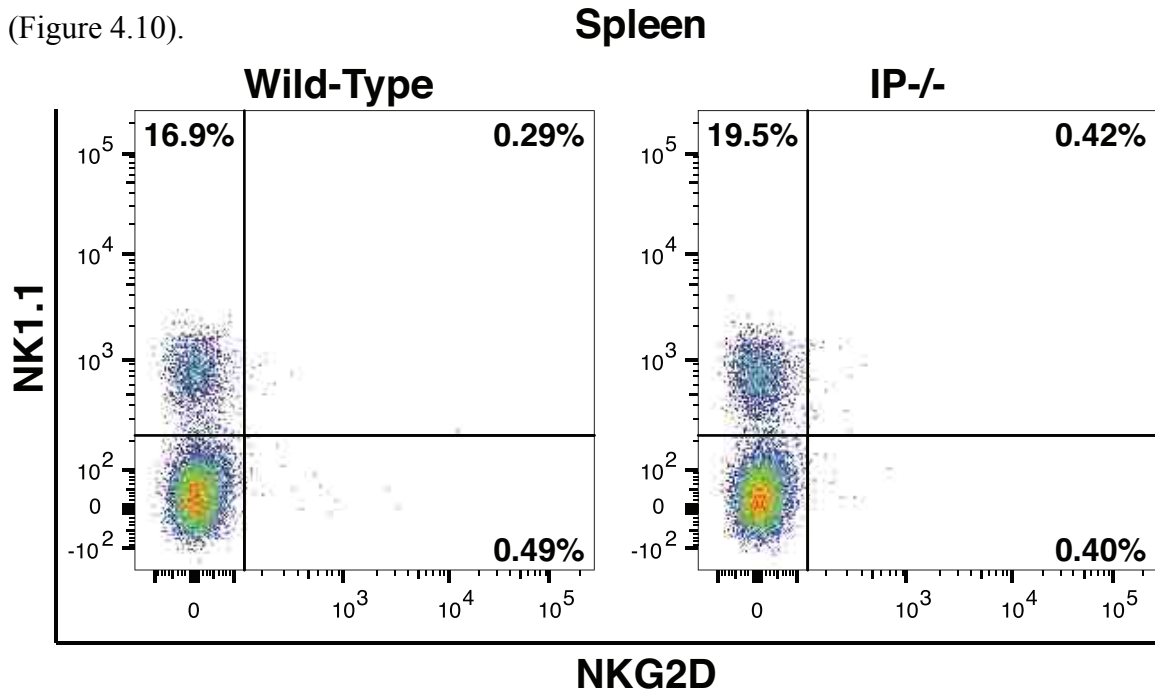


Figure 4.10 Naïve $IP^{-/-}$ and WT Splenic Natural Killer Cells Lack Expression of NKG2D. Splenic mononuclear cells were obtained from dissociated spleen tissue from naïve $IP^{-/-}$ and WT mice. Mononuclear cells were stained with anti-NK1.1 (APC) and anti-NKG2D (PE) and analyzed for receptor expression using a BD FACSAria Flow Cytometer.

4.2.1.2 Increased Intracellular Expression of Granzyme A by Natural Killer Cells in $IP^{-/-}$ Mice

The significant increase in systemic expression of activating receptor NKp46 by $IP^{-/-}$ mouse NK cells described in section 4.2.1.1 could lead to potential ramifications for $IP^{-/-}$ mouse NK cell cytotoxicity. As described more fully in Chapter 4 Background

section 4.1.3.2, excretory lysosomal exocytosis is a primary method of NK cell-mediated cytotoxicity, specifically through the release of cytotoxic proteins such as perforin and granzymes. Granzymes A & B are the most abundant granzymes, and recognition of the cytotoxic capabilities of these molecules has been known since the early 1990s (Hayes, Berrebi et al. 1989) (Shi, Kam et al. 1992). Induction of granzyme production in NK cells was accomplished by 24-hour *in vitro* treatment utilizing PMA/ionomycin as described in Chapter 2 Methods Section 2.0.10.7. Following treatment, FACS analysis utilizing intracellular staining revealed that IP^{-/-} mouse NK cells displayed markedly increased intracellular (IC) levels of granzyme A when compared to WT mice (Figure 4.11). Approximately 78% of IP^{-/-} pulmonary NK cells displayed production of IC granzyme A following PMA/ionomycin treatment, as opposed to 65% of WT pulmonary NK cells. Flow cytometry analysis of IC granzyme B revealed that there were minimal differences in production in IP^{-/-} pulmonary NK cells compared to WT pulmonary NK cells, with approximately 16% of IP^{-/-} pulmonary NK cells compared to 20% of WT pulmonary NK cells expressing production of IC granzyme B following PMA/ionomycin treatment (Figure 4.11). This increase in intracellular granzyme A was only observed in pulmonary NK cells, with no difference observed between IP^{-/-} and WT splenic NK cells (Figure 4.11). Consequently, the majority of pulmonary NK cells present in the IP^{-/-} mouse are granzyme A⁺ and likely to exhibit increased cytotoxicity.

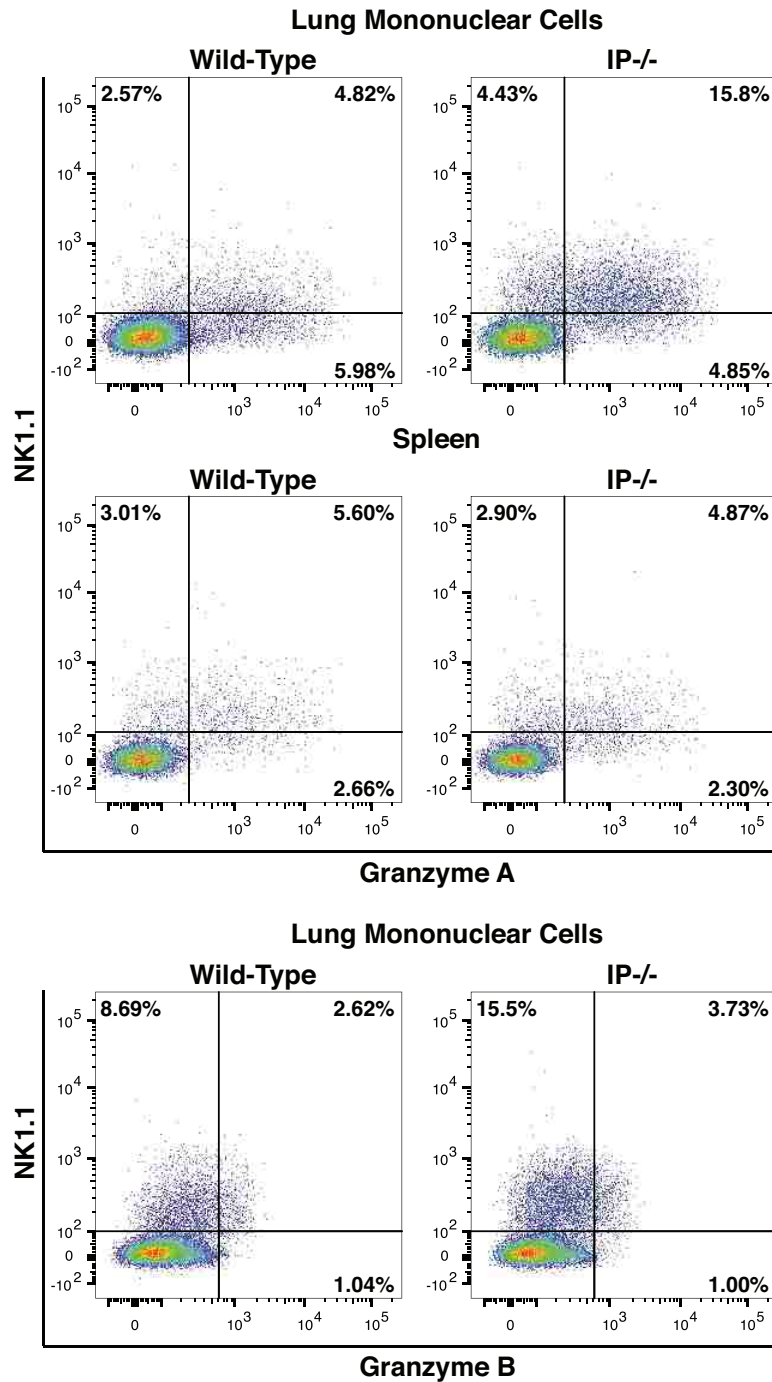


Figure 4.11 Naïve IP^{-/-} Pulmonary Natural Killer Cells Display Increased Production of Granzyme A. Mononuclear cells were obtained from dissociated spleen and lung tissue of naïve IP^{-/-} and WT mice. Mononuclear cells were treated with PMA/ionomycin for 24 hours prior to intracellular staining. Mononuclear cells were stained with anti-CD3 (PE), anti-CD19 (APCCy7), anti-NK1.1 (FITC, APC), anti-Granzyme A (APC), and anti-Granzyme B (FITC) and analyzed for receptor and intracellular cytokine expression using a BD FACSARIA Flow Cytometer. Natural killer cells were classified as CD3⁺CD19⁻NK1.1⁺.

4.2.1.3 Increased Intracellular and in vitro Production of IFN- γ by Natural Killer Cells in IP^{-/-} Mice

In addition to production of cytotoxic lysosomal proteins such as perforin and granzymes, NK cells are recognized to produce several pro-inflammatory cytokines in response to activation, particularly IFN- γ and TNF- α (Perussia 1996). The increased expression of activating receptor NKp46 as well as increased production of cytotoxic protein granzyme A, described in section 4.2.1.1 and 4.2.1.2 respectively, seen in naïve IP^{-/-} mouse pulmonary NK cells could also be associated with the increased production of pro-inflammatory cytokines. Induction of pro-inflammatory cytokine production in NK cells was accomplished by 24-hour *in vitro* treatment utilizing PMA/ionomycin as described in Chapter 2 Methods Section 2.0.10.7. Flow cytometry analysis utilizing IC staining revealed that IP^{-/-} pulmonary NK cells had slightly decreased production of IC IFN- γ when compared to WT pulmonary NK cells (Figure 4.12). Approximately 61% of IP^{-/-} pulmonary NK cells produced IC IFN- γ following PMA/ionomycin treatment, as opposed to 70% of WT pulmonary NK cells.

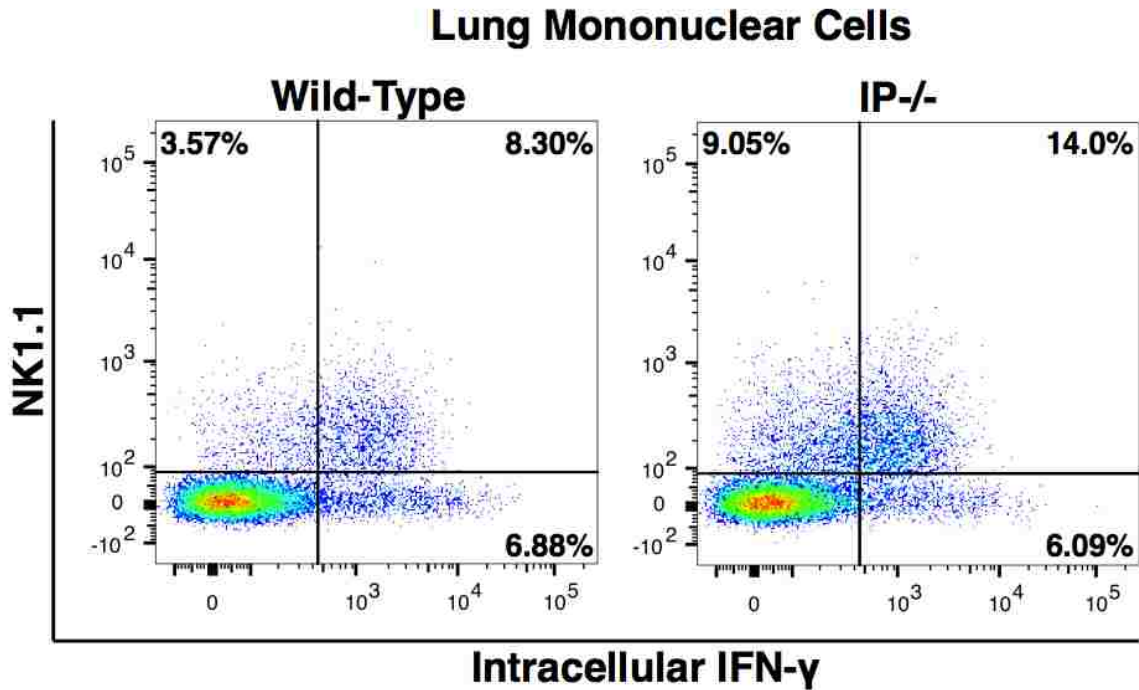


Figure 4.12 Naïve IP^{-/-} Pulmonary Natural Killer Cells Display Reduced Intracellular IFN-γ Production. Lung mononuclear cells were obtained from dissociated lung tissue from naïve IP^{-/-} and WT mice. LMCs were treated with PMA/ionomycin for 24 hours prior to intracellular staining. LMCs were stained with anti-CD3 (FITC), anti-CD19 (APCCy7), anti-NK1.1 (PE), and anti-IFN-γ (APC) and analyzed for receptor and intracellular cytokine expression using a BD FACSaria Flow Cytometer. Natural killer cells were classified as CD3⁺CD19⁻NK1.1⁺.

As mentioned in section 4.2.1, NK1.1 is a primary surface marker for recognizing NK cells, and it has also been shown to be a signaling molecule involved in the activation of NK cells (Arase, Arase et al. 1996). Specifically, Arase et al. described that NK1.1 receptor cross-linking with co-stimulation utilizing IL-2 led to significant production of IFN-γ in both NK cells and NK1.1⁺ iNKT cells. Unexpectedly, IP^{-/-} pulmonary NK cells had slightly decreased production of pro-inflammatory cytokine IFN-γ as determined by intracellular (IC) flow cytometry (Figure 4.12). To examine these results further, we performed *in vitro* stimulation of isolated lung mononuclear cells utilizing anti-NK1.1 mAb PK136 in the presence or absence of IL-2 as described in Chapter 2 Methods

Section 2.0.7.1. Stimulation of NK1.1 in the presence of IL-2 resulted in significantly increased production of IFN- γ by IP^{-/-} lung mononuclear cells compared to IFN- γ produced by WT lung mononuclear cells (930 pg/ml compared to 414 pg/ml), while stimulation of NK1.1 alone was not sufficient to induce significant cytokine production in either strain (Figure 4.13). Although IFN- γ production was induced in both strains following *in vitro* NK1.1 stimulation, we observed consistently low levels of less than 50 pg/ml TNF- α production by pulmonary NK cells in either strain following *in vitro* NK1.1 stimulation in the presence or absence of IL-2 (Figure 4.13).

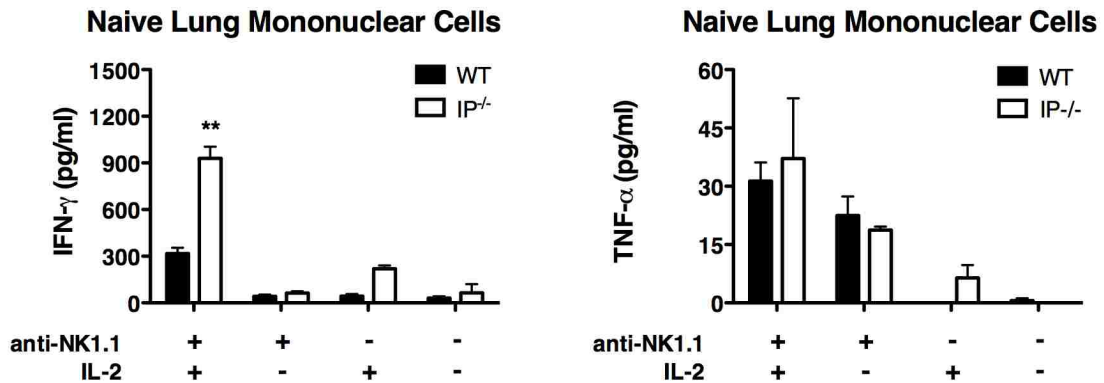


Figure 4.13 Naïve IP^{-/-} Pulmonary Natural Killer Cells Display Significantly Increased *in vitro* IFN- γ Production Following NK1.1 Stimulation. Lung mononuclear cells were obtained from dissociated lung tissue from naïve IP^{-/-} and WT mice. LMCs were stimulated *in vitro* with anti-NK1.1 mAb (20 μ g/ml) \pm IL-2 (10 ng/ml) for 24 h at 37°C. Supernatants were analyzed for IFN- γ and TNF- α production by ELISA. Data are mean \pm SEM (n = 3 – 6) in triplicate and are representative of three independent experiments. **p < 0.01 compared to WT mice

Since NK-like cell populations such as iNKT and Th17 cells have been found to both express NK1.1 (Bartel, Bauer et al. 2013) and respond to NK1.1 stimulation (Arase, Arase et al. 1996), it is possible that the increase in IFN- γ levels by stimulated IP^{-/-} lung mononuclear cells described in Figure 4.13 could be attributed to these alternative

populations. Therefore, a more specific NK cell activation is necessary to truly establish pulmonary NK cell involvement. Walzer et al. described that 98% of NKp46 expression occurs on mouse NK cells, while the remaining 2% is expressed on a minute fraction of non-invariant NKT cells, making it a highly selective marker for recognition of NK cell populations (Walzer, Blery et al. 2007). In addition, they also demonstrated that stimulation utilizing anti-NKp46 mAb resulted in IC IFN- γ production by stimulated splenic NK cells (Walzer, Blery et al. 2007). To clearly establish pulmonary NK cell IFN- γ production, we performed *in vitro* stimulation of isolated lung mononuclear cells utilizing anti-NKp46 mAb in the presence or absence of IL-2 as described in Chapter 2 Methods Section 2.0.7.1. Stimulation of lung mononuclear cells utilizing NKp46 in the presence of IL-2 resulted in the production of IFN- γ that was significantly elevated in IP^{-/-} lung mononuclear cells when compared to IFN- γ produced by WT lung mononuclear cells (753 pg/ml compared to 177 pg/ml)(Figure 4.14). In comparison to anti-NK1.1 + IL-2 stimulation, IP^{-/-} lung mononuclear cells stimulated with anti-NKp46 + IL-2 produced slightly lower levels of IFN- γ compared to IFN- γ produced in anti-NK1.1 stimulated IP^{-/-} lung mononuclear cells (753 pg/ml compared to 930 pg/ml), a difference which was found to be non-significant. We believe that this similarity in IFN- γ production following both NK1.1 and NKp46 stimulation provides strong evidence that pulmonary NK cells are specifically responsible for the observed increase in IFN- γ levels observed in stimulated IP^{-/-} mouse lung mononuclear cells.

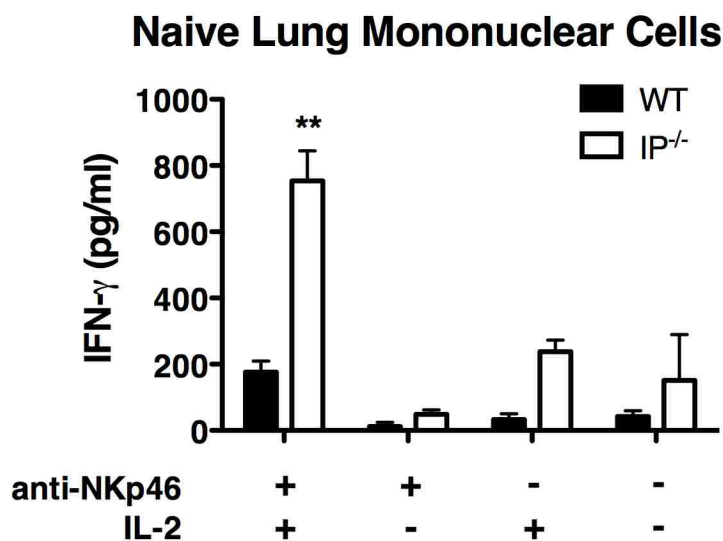


Figure 4.14 Naïve IP^{-/-} Pulmonary Natural Killer Cells Display Significantly Increased *in vitro* IFN- γ Production Following NKp46 Stimulation. Lung mononuclear cells were obtained from dissociated lung tissue from naïve IP^{-/-} and WT mice. LMCs were stimulated *in vitro* with anti-NKp46 mAb (20 μ g/ml) \pm IL-2 (10 ng/ml) for 24 h at 37°C. Supernatants were analyzed for IFN- γ production by ELISA. Data are mean \pm SEM (n = 3) in triplicate and are representative of two independent experiments. **p < 0.01 compared to WT mice

4.2.2 IP^{-/-} Mice Display an Attenuated Development of Allergic Lung

Inflammation Elicited Following House Dust Mite Exposure

Given the marked increase in pulmonary NK cells present in lung tissue of IP^{-/-} mice that was coincident with elevated expression of activating receptor NKp46 as described in Chapter 4 Methods section 4.2.1, it was important to determine if lung inflammatory responses had been affected by this increased NK cell population.

Although it has been recognized for almost two decades that one of the defining features of the asthmatic phenotype in humans is elevated serum levels of house dust mite (HDM)-specific IgE (Shibasaki, Noguchi et al. 1997) (Ohshima, Yamada et al. 2002) (Kimura, Tsuruta et al. 2000), animal models of allergic asthma have been slow to adapt. The earliest study we were able to identify suggesting the efficacy of an HDM-induced

animal model of allergic asthma was submitted by Cates et al. back in 2004 (Cates, Fattouh et al. 2004). Despite the attempts of this and other studies that demonstrate the viability of HDM as a more clinically relevant animal model as compared to the traditional ovalbumin (OVA)-induced animal models, it is only in the last few years that a dramatic shift has seemed to occur in preference of the HDM model when seeking asthma-related funding opportunities. In part, this reflects recently available HDM preparations that are endotoxin-free from commercially available sources.

In order to maintain pace with advancements in the field, our laboratory adopted and developed a protocol for the HDM-induced mouse model of allergic asthma (see Chapter 2 Methods Section 2.0.3.3). One of the key advantages of the HDM-induced model lies in the direct sensitization of the airway to the allergen via intranasal delivery. Although new advancements in the field have led to the development of commercially available purified recombinant HDM-associated proteins, the use of whole dust mite extract has been predominantly used due to lack of more sophisticated reagents. However, contamination of whole dust mite extract with other potential antigens, in particular LPS which is a potent activator of the immune system, are a concern when analyzing the capacity of whole dust mite extract to induce an immune response. Therefore, we were careful to utilize batches of whole dust mite extract with undetectable levels of LPS in order to remove a potential confounding factor in subsequent exposure-related immune responses.

Although previous work in our laboratory has shown that prostacyclin signaling exerts a crucial role in the development of ovalbumin-induced allergic lung inflammation, the role of prostacyclin signaling in HDM-induced allergic lung

inflammation remains relatively unknown, although an increase in prostacyclin present in the airway has been observed in mice following HDM exposure (Herrerias, Torres et al. 2009). In this study, we performed a short-term sensitization of the murine airway via intranasal exposure to whole dust mite extract obtained from Greer followed by two further challenges one week apart (see Chapter 2 Methods Section 2.0.3.3 for specific details). Following allergen sensitization and challenge, analysis of HDM-induced pulmonary inflammation in $IP^{-/-}$ and WT mice was determined.

4.2.2.1 Reduced Infiltration of the Airway by Inflammatory Cells in $IP^{-/-}$ Mice

As mentioned in Chapter 3, infiltration of the airway by inflammatory immune cells including Th2 lymphocytes, mast cells, alveolar macrophages, and eosinophils is a defining characteristic of allergic asthma. The observation of increased tissue and circulatory eosinophilia has been noted since the earliest characterization of the disease, although the exact role in driving the asthmatic phenotype was contested between the 1970s and 1980s (Wardlaw, Brightling et al. 2000). Continued demonstration that eosinophil-derived proteins are capable of causing substantial injury to the airway cemented the role of the eosinophil as the principal effector cell of asthma (Frigas, Motojima et al. 1991). This focus on the eosinophil has meant that any relevant animal model of asthma has been primarily characterized by the induction of eosinophilia in the airway. Thus, several techniques have been utilized to quantify the presence of eosinophils in the airway to study the development and treatment of allergic asthma. The use of cell differential counts and flow cytometry analysis has been universally used to visualize and quantify increases in eosinophilia. Additionally, the release of eosinophil-derived proteins such as eosinophil peroxidase (EPO) into the airway has been

recognized since the mid-1990s as biomarkers of increased airway eosinophilia (Cheng, Pillar et al. 1993) (Bjornsson, Janson et al. 1996).

Inflammatory cell infiltration into the bronchoalveolar lavage fluid (BALF) is an effective indicator of the inflammatory process in the lung. Therefore, following the short-term intranasal HDM exposure described in Chapter 2 Methods Section 2.0.3.3, examination of the BALF was performed in order to determine the effects of HDM exposure on infiltration by inflammatory immune cells into the airway of IP^{-/-} mice. We revealed that HDM-exposed IP^{-/-} mice displayed significantly reduced numbers of both infiltrating eosinophils and lymphocytes present in the BALF when compared to HDM-exposed WT mice as determined by both total cell number as well as measurement of BALF EPO levels (Figure 4.15). Although both eosinophil and lymphocyte infiltration into the airway was affected by intranasal HDM exposure, we observed no significant differences in the number of neutrophils or macrophages present in the BALF of HDM-exposed IP^{-/-} mice when compared to HDM-exposed WT mice as determined by total cell number, although there was a non-significant reduction in the numbers of macrophages present in BALF of both control and HDM-exposed IP^{-/-} mice compared to control and HDM-exposed WT mice (Figure 4.15). Control mice that had not been exposed to intranasal HDM had essentially undetectable numbers of eosinophils, lymphocytes, and neutrophils in both IP^{-/-} and WT BALF as determined by total cell number, while macrophage numbers remained essentially identical when comparing HDM-exposed and control animals of their respective strains (Figure 4.15).

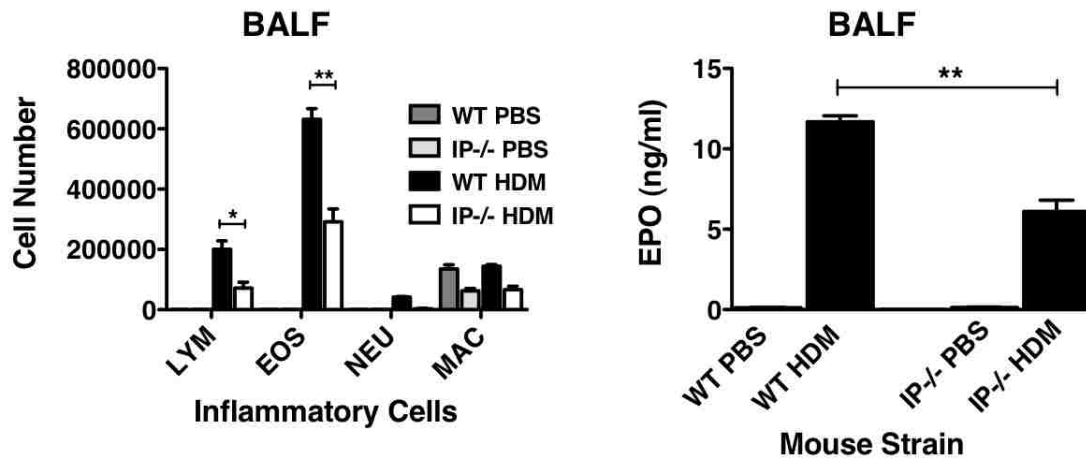


Figure 4.15 *IP*^{-/-} Mice Display Attenuated Infiltration of the Airway by Inflammatory Cells Following Intranasal Exposure to HDM. *IP*^{-/-} and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to determine development of allergic lung inflammation. Control mice received intranasal PBS. (A) Cell differential counts in the BALF were determined by light microscopic evaluation of stained cytospin preparations. Results are expressed as absolute numbers (per mouse) of lymphocytes (Lym), macrophages (Mac), eosinophils (Eos), and neutrophils (Neu). (B) EPO levels in the BALF of HDM-sensitized or control mice were assessed by colorimetric analysis. Results are \pm SEM mean (n = 3) and are representative of two independent experiments. * p < 0.05, compared to WT HDM mice ** p < 0.01, compared to WT HDM mice

The identification of granulocyte populations in the BALF using FACS analysis is a multi-step process. All granulocytes, including eosinophils, neutrophils, and basophils, can be differentiated from other immune cells based on the light scatter properties of the cell. These scatter properties, which include forward scatter and side scatter, are a relative analysis of the size and the cytoplasmic contents of a cell, respectively. Plotting these scatter property values against each other leads to specific grouping of granulocytes due to their large size and dense granule content in the cytoplasm. However, to differentiate granulocyte populations, specific surface receptor expression must be

analyzed. Although all granulocytes express particular markers in common, such as CD11b and F4/80, eosinophils and neutrophils can be individually identified by the expression of Siglec-F and Gr-1, also known as Ly-6G, respectively (Zhang, Angata et al. 2007) (Noffz, Qin et al. 1998). Consistent with the reduction in infiltrating immune cell populations observed by cell differential in the BALF, flow cytometry analysis of the airway revealed that HDM-exposed IP^{-/-} mice displayed a reduction in the proportion of CD11b⁺Siglec-F⁺ eosinophils as well as CD4⁺ T lymphocytes present in the airway when compared to HDM-exposed WT mice (Figure 4.16). There was no alteration observed in the number or proportion of CD11b⁺Gr-1⁺ neutrophils in the airway of HDM-exposed IP^{-/-} mice compared to HDM-exposed WT mice (Figure 4.16). Control mice that received no exposure to HDM displayed low numbers of CD11b⁺Siglec-F⁺ eosinophils, CD4⁺ lymphocytes, and CD11b⁺Gr-1⁺ neutrophils present in the airway with no noticeable differences between strains (Figure 4.16).

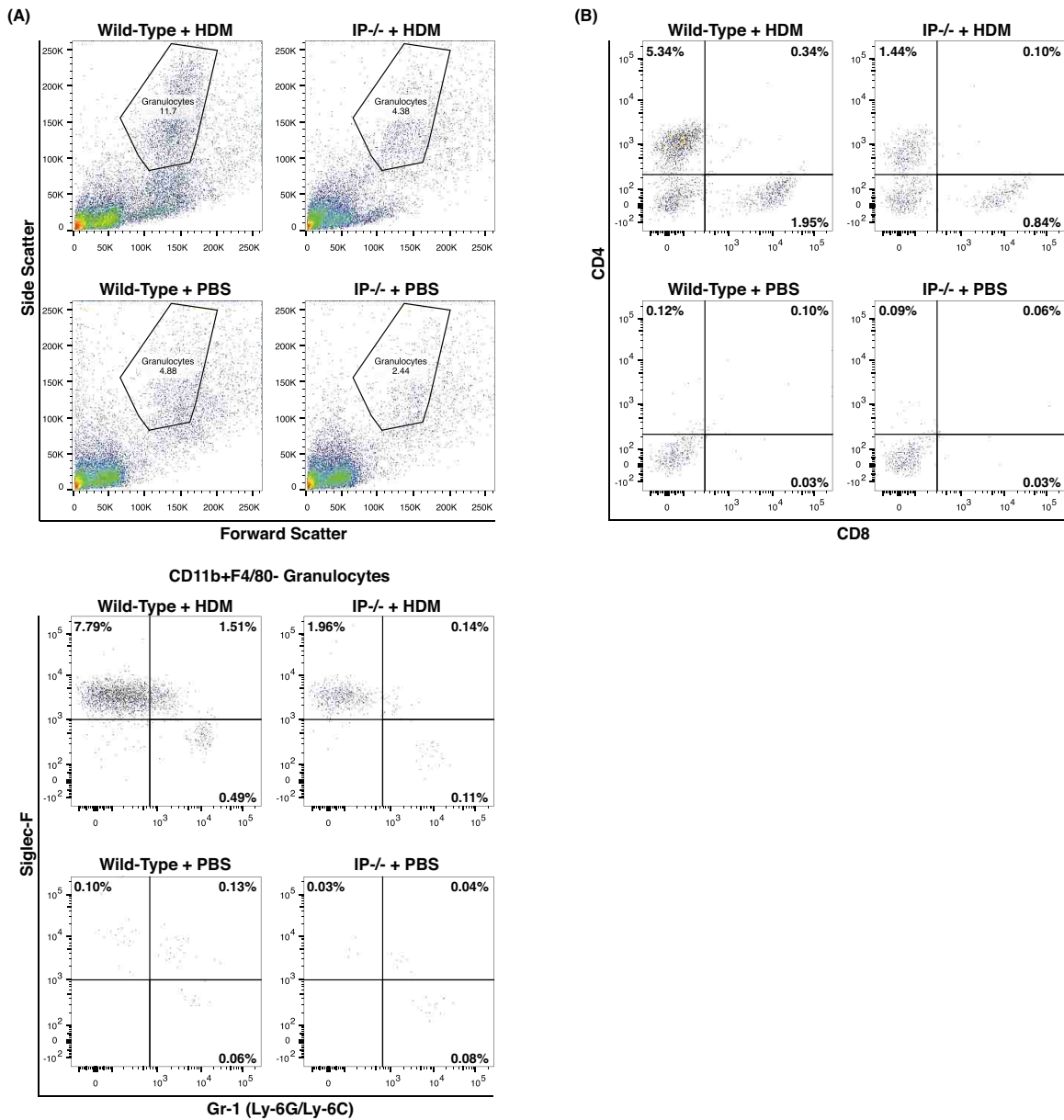


Figure 4.16 Flow Cytometry Analysis of Infiltrating Inflammatory Immune Cells into the Airway of IP^{-/-} and WT Mice Following Intranasal Exposure to HDM. IP^{-/-} and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to determine development of allergic lung inflammation. Control mice received intranasal PBS. BALF cells were stained with anti-CD3 (APC), anti-CD4 (APCCy7), anti-CD8 (PE), anti-CD11b (FITC), anti-F4/80 (APC), anti-Siglec-F (PE), and anti-Gr-1 (Ly-6G/Ly-6C) and analyzed for receptor expression using a BD FACSaria Flow Cytometer.

4.2.2.2 Histologic Examination of Lung Tissue

In addition to infiltration of the airway by inflammatory immune cells, one of the primary clinical pathologies of allergic asthma is airway obstruction and remodeling due to increased peribronchial inflammation, mucus secretion, and airway hyperreactivity (Shifren, Witt et al. 2012). We therefore utilized H&E staining to characterize the effects of HDM exposure on peribronchial inflammation in the airway of mice following exposure to HDM. We found that HDM-exposed IP^{-/-} mice had markedly decreased peribronchial inflammation when compared to HDM-exposed WT mice (Figure 4.17). Examination of control mice that received no exposure to HDM revealed that HDM-exposed WT mice had substantially increased peribronchial inflammation when compared to control WT mice, while HDM-exposed IP^{-/-} mice had a slight increase in peribronchial inflammation compared to control IP^{-/-} mice (Figure 4.17).

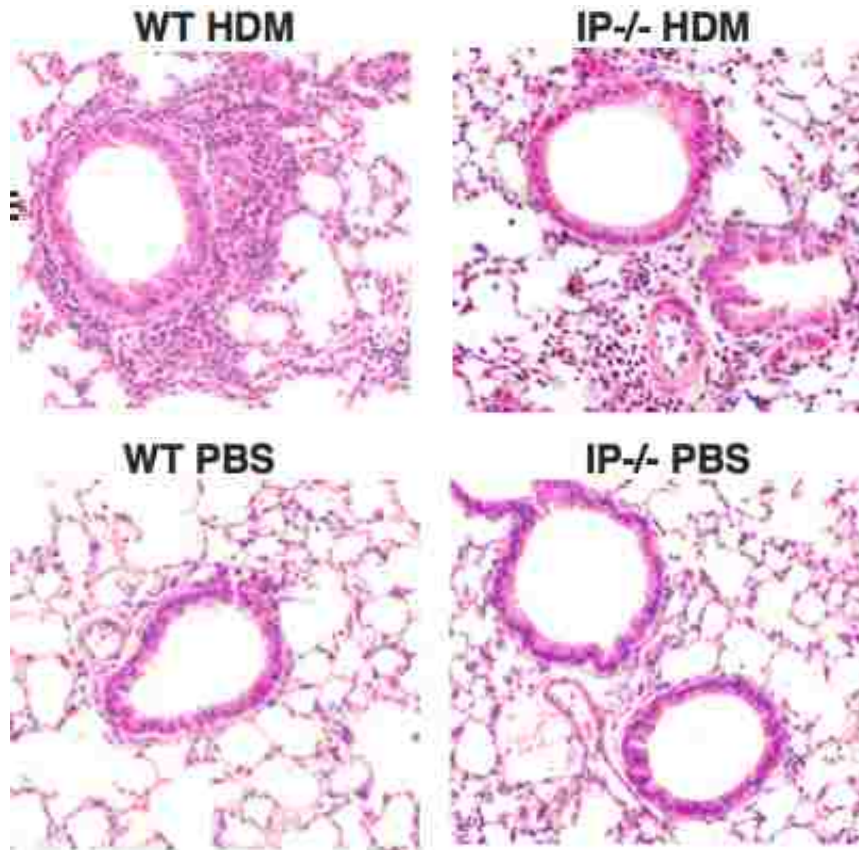


Figure 4.17 $IP^{-/-}$ Mice Display Decreased Peribronchial Inflammation Following Intranasal Exposure to HDM . $IP^{-/-}$ and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, peribronchial inflammation was determined by histological analysis by staining lung sections with H&E (20X).

Additionally, analysis of mucus secretion was performed utilizing % Periodic acid-Schiff (PAS) staining per bronchiole. We found that HDM-exposed $IP^{-/-}$ mice had significantly decreased mucus secretion in the airway when compared to HDM-exposed WT mice (Figure 4.18). Similarly to the H&E analysis of peribronchial inflammation, we found that HDM-exposed WT mice had substantially increased airway mucus production when compared to control WT mice, while HDM-exposed $IP^{-/-}$ mice had a

slight increase in airway mucus production when compared to control $IP^{-/-}$ mice (Figure 4.18).

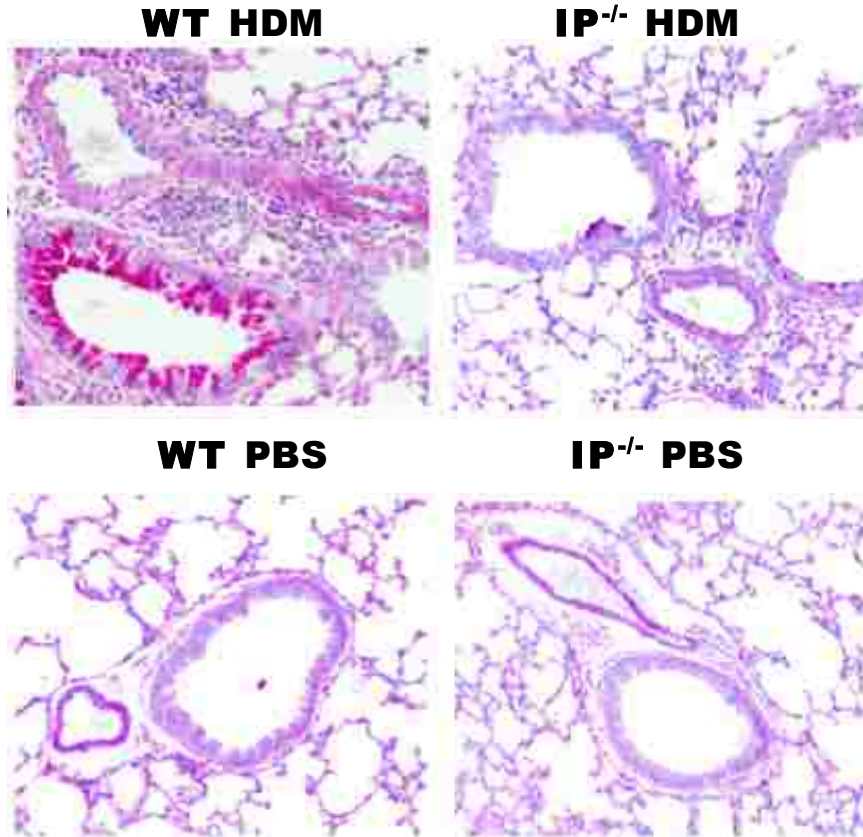


Figure 4.18 $IP^{-/-}$ Mice Display Decreased Airway Mucus Production Following Intranasal Exposure to HDM . $IP^{-/-}$ and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, mucus production was determined by histological analysis by staining lung sections with PAS (20X).

4.2.2.3 Reduced Production of Th2-associated Cytokines in $IP^{-/-}$ Mice

The role of Th2-associated cytokine as an initiating factor during allergic asthma has been widely accepted for over two decades (Robinson, Hamid et al. 1992). The initiation of a Th2 response and subsequent role of Th2-associated cytokines IL-4, IL-5, and IL-13 in the recruitment and activation of B cells, mast cells, and eosinophils has been studied extensively and described in more detail in Chapter 1 Introduction. As

demonstrated in Chapter 4 section 4.2.2.1 and 4.2.2.2, loss of prostacyclin signaling results in a reduction in infiltration of the airway by inflammatory effector cells as well as peribronchial inflammation and mucus secretion associated with HDM-induced allergic lung inflammation. Due to the regulation of both these outcomes by Th2-associated cytokines, we examined whether loss of prostacyclin signaling altered the levels of Th2-associated cytokines in the airway.

In order to characterize Th2 cytokine levels in the airway, we analyzed BALF of $IP^{-/-}$ and WT mice following intranasal HDM exposure. We found that HDM-exposed $IP^{-/-}$ mice had reduced levels of IL-4 (< 5 pg/ml to ~30 pg/ml), IL-5 (< 2 pg/ml to ~5 pg/ml), and IL-13 (< 50 pg/ml to ~300 pg/ml) when compared to HDM-exposed WT mice (Figure 4.14). HDM-exposed WT mice had increased levels of IL4, IL-5, and IL-13 when compared to control WT mice, while the levels of these cytokines were not significantly increased in HDM-exposed $IP^{-/-}$ mice when compared to control $IP^{-/-}$ mice (Figure 4.19). Both control $IP^{-/-}$ and WT mice displayed levels of these cytokines in the BALF below the threshold of detection (Figure 4.19).

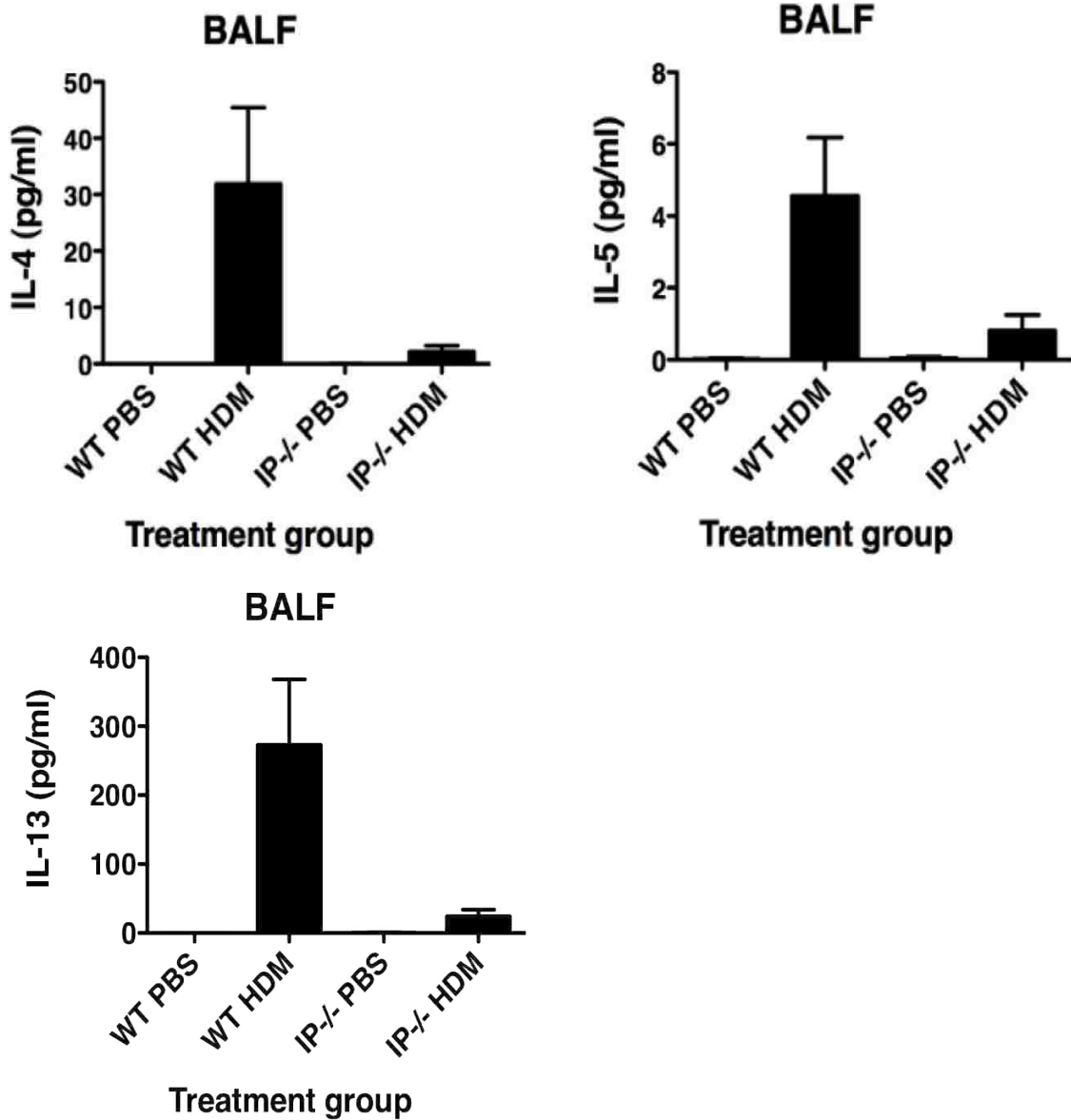


Figure 4.19 **IP^{-/-} Mice Display Attenuated Th2 Cytokine Production Following Intranasal Exposure to HDM.** IP^{-/-} and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to determine development of allergic lung inflammation. Control mice received intranasal PBS. BALF was assessed using Meso Scale Discovery V-PLEX Mouse Proinflammatory Panel to determine IL-4, IL-5, or IL-13 production. Results are \pm SEM mean (n = 3) and are representative of two independent experiments.

4.2.2.4 Altered Production of GM-CSF, CX3CL1, and CCL2 in the Airways of IP^{-/-} Mice

Once immune cells responsible for airway surveillance have recognized an allergen capable of eliciting an immune response, recruitment of effector cells to the site of allergen challenge typically follows. Although dendritic cells and alveolar macrophages are often regarded as the principal immune cells responsible for airway surveillance, it has been well documented that bronchoepithelial cells have a critical role in the innate recognition of airway antigens. Upon antigen recognition by a wide variety of pathogen recognition receptors (PRRs), bronchoepithelial cells release a wide variety of cytokines and chemokines responsible for the activation and recruitment of inflammatory immune cells (Salazar and Ghaemmaghami 2013). These signaling proteins capable of recruitment and activation of inflammatory immune cells produced by bronchoepithelial cells, as well as dendritic cells and macrophages, are critical to understanding the underlying mechanisms responsible for the development of allergic asthma.

One of these signaling proteins, CX3CL1, also known as fractalkine, is a novel chemokine recognized to exist as a membrane-bound adhesion protein in addition to being cleaved to function as a soluble chemotactic signaling protein (Bazan, Bacon et al. 1997). Expression of CX3CR1, the receptor for CX3CL1, had been shown to occur on monocytes, NK cells, and CD4⁺ & CD8⁺ T cells, and treatment of those immune cells with soluble CX3CL1 was capable of inducing recruitment and adhesion of NK cells and T cells, but not monocytes (Imai, Hieshima et al. 1997). The ability of CX3CL1 to recruit CD4⁺ T cells led to subsequent studies on the role of CX3CL1 in allergic asthma,

and it was shown that atopic asthma patients displayed significantly increased production of CX3CL1 in the BALF following allergen challenge in addition to increased plasma concentrations before allergen challenge compared to non-asthmatic controls (Rimaniol, Till et al. 2003).

Another signaling protein, CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), was the first CC chemokine identified in humans (Yoshimura, Yuhki et al. 1989). The receptor for CCL2 is separated into two isoforms, CCR2A and CCR2B, which are restrictively expressed on specific cell types, with CCR2B being expressed by monocytes and activated NK cells (Deshmane, Kremlev et al. 2009). Elevated levels of MCP-1 present in the BALF of patients suffering from atopic asthma compared to healthy controls has been observed (Jahnz-Rozyk, Kuna et al. 1997), and more recently it was reported that primary airway smooth muscle cultures from biopsies of asthmatic patients displayed increased secretion of CCL2 when compared to healthy controls (Singh, Sutcliffe et al. 2014).

Finally, granulocyte-macrophage colony-stimulating factor (GM-CSF), a member of the cytokine family that also includes IL-3 and IL-5, has been recognized to control the recruitment and activation, as well as be produced by, monocytes, macrophages, dendritic cells, and T lymphocytes (Yamashita, Tashimo et al. 2002). In particular, GM-CSF has been shown to be essential for eosinophil survival, chemotaxis, and activation in regard to degranulation as well cytokine production (Owen, Rothenberg et al. 1987). With the capacity to widely affect many immune cells attributed to the pathogenesis of asthma, the role of GM-CSF in allergic asthma has received much research attention. An increase in GM-CSF levels in the BALF of atopic asthma patients compared to healthy controls has

been observed (Robinson, Hamid et al. 1992), and airway epithelial cells of asthmatics patients have also been shown to produce more GM-CSF than those of healthy controls (Sousa, Poston et al. 1993). Treatment of mice with neutralizing anti-GM-CSF antibodies resulted in decreased AHR in addition to airway inflammation (Yamashita, Tashimo et al. 2002). More recently, the facilitation of allergic airway inflammation following sub-threshold HDM exposure has been attributed to a GM-CSF/IL-33 controlled pathway (Llop-Guevara, Chu et al. 2014).

The lack of infiltration of the airway by inflammatory immune cells in the IP^{-/-} mouse following HDM exposure described in section 4.2.2.1 could be attributed to the alterations in chemokines such as CX3CL1, CCL2, or GM-CSF. Therefore, we examined the airway of control and HDM-exposed IP^{-/-} mice to determine whether the levels of these chemokines in the airway were significantly altered. We found that non-HDM-exposed control IP^{-/-} mice had 1387 pg/ml CX3CL1 present in BALF, which was significantly elevated compared to the 808 pg/ml CX3CL1 observed in control WT mice (Figure 4.20). Since CX3CL1 has been demonstrated to be a potent chemokine for the recruitment of NK cells, this raises the possibility that the observed increase in CX3CL1 level is responsible for the elevated number of pulmonary NK cells in naïve IP^{-/-} mice. Exposure to HDM resulted in a significant decrease in CX3CL1 levels to 616 pg/ml present in the BALF of IP^{-/-} mice compared to 1387 pg/ml in control IP^{-/-} mice, as well as a significant reduction to 450 pg/ml in HDM-exposed WT mice compared to 808 pg/ml CX3CL1 in control WT mice (Figure 4.20). Comparison of CXCL1 levels between HDM-exposed IP^{-/-} and HDM-exposed WT mice revealed no significant differences (Figure 4.20).

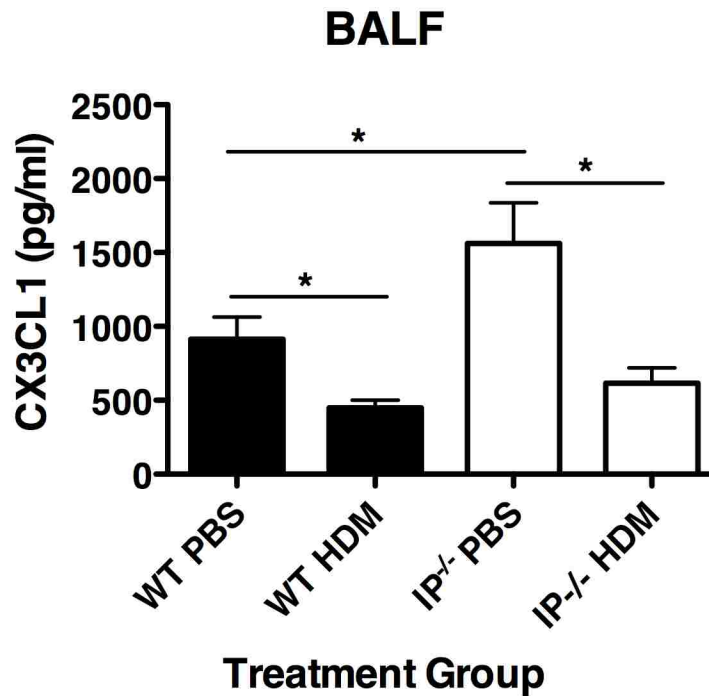


Figure 4.20 Naïve IP^{-/-} Mice Display Increased Levels of CX3CL1 in the Airway. IP^{-/-} and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to determine CX3CL1 (fractalkine) levels by ELISA. Control mice received intranasal PBS. Results are \pm SEM mean (n = 3) and are representative of three independent experiments. *p < 0.05

Examination of CCL2 levels in the airway revealed that production of CCL2 in control IP^{-/-} and WT mouse BALF was very low, with less than 10 pg/ml CCL2 present in the BALF of either strain (Figure 4.21). Following exposure to HDM, IP^{-/-} mice displayed a non-significant elevation in BALF CCL2 production to 21 pg/ml compared to < 10 pg/ml present in the BALF of control IP^{-/-} mice (Figure 4.21). This was directly contrasted by the results observed in WT mice, as HDM-exposed WT mice displayed a significant increase in CCL2 production to 154 pg/ml CCL2 present in the BALF compared to < 10 pg/ml CCL2 found in control WT mice (Figure 4.21). Comparison of BALF CCL2 levels between HDM-exposed IP^{-/-} and WT mice revealed that the 21 pg/ml

CCL2 present in the BALF of HDM-exposed $IP^{-/-}$ mice was significantly reduced compared to the 154 pg/ml CCL2 observed in the BALF of HDM-exposed WT mice (Figure 4.21). These results suggest that following HDM exposure, production of CCL2 in the airways of $IP^{-/-}$ mice is markedly decreased. As CCL2 is a critical component for the recruitment of inflammatory monocytes that mature to dendritic cell and macrophage populations that have been associated with driving allergic lung inflammation (Chen, Liu et al. 2013) (Lee, Jeong et al. 2014), the inability of $IP^{-/-}$ mice to induce CCL2 production following HDM exposure could be a possible mechanism in the attenuated development of allergic lung inflammation.

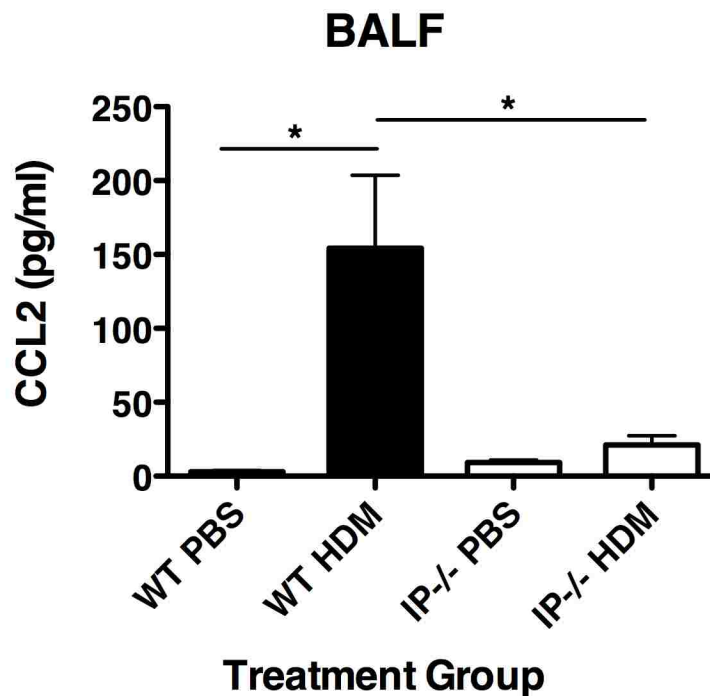


Figure 4.21 $IP^{-/-}$ Mice Display Attenuated Production of CCL2 in the Airway Following Intranasal Exposure to HDM. $IP^{-/-}$ and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to assess CCL2 (MCP-1) production by ELISA. Control mice received intranasal PBS. Results are \pm SEM mean (n = 3) and are representative of three independent experiments. * p < 0.05

Finally, we examined the levels of GM-CSF present in the airway of control and HDM-exposed IP^{-/-} and WT mice. We attempted to measure levels of GM-CSF present in the BALF, but we found that they were below the level of detection for the ELISA kit used in this study. This is in agreement with previous publications suggesting that this cytokine has an affinity for the extracellular matrix and is not freely released into the airway. Therefore, we plated dissociated LMCs from naïve and HDM-exposed IP^{-/-} and WT mice *in vitro* as described in Chapter 2 Methods Section 2.0.7.1. We found that control IP^{-/-} mice had similar levels of production of GM-CSF when compared to GM-CSF produced by control WT mice (161 pg/ml compared to 121 pg/ml)(Figure 4.22). However, following HDM exposure, LMCs from both IP^{-/-} and WT mice displayed significant increases in GM-CSF production when compared to control mice. HDM-exposed IP^{-/-} LMCs displayed significantly increased production of GM-CSF compared to non-exposed control IP^{-/-} LMCs (291 pg/ml compared to 161 pg/ml) (Figure 4.22). HDM-exposed WT LMCs displayed significantly increased production of GM-CSF compared to non-exposed control WT LMCs (981 pg/ml compared to 121 pg/ml)(Figure 4.22). Despite the significantly increased production of GM-CSF by HDM-exposed IP^{-/-} LMCs compared to control IP^{-/-} LMCs, comparison of IP^{-/-} and WT LMC production of GM-CSF revealed that HDM-exposed IP^{-/-} LMCs had significantly reduced production of GM-CSF compared to HDM-exposed WT LMCs (291 pg/ml compared to 981 pg/ml) (Figure 4.22). Although the source of the GM-CSF in the lungs was not determined, these results suggest that IP^{-/-} mice have a deficiency in GM-CSF production in the lungs following HDM exposure compared to WT mice. As GM-CSF is critical for the survival of eosinophils in tissue following release from the bone marrow (Park and Bochner

2010), as well as in the development and activation of monocyte-derived dendritic cells necessary to drive the Th2 response (van de Laar, Coffler et al. 2012), the reduction in GM-CSF production observed in HDM-exposed IP^{-/-} mice could be a critical component in the attenuated development of allergic lung inflammation.

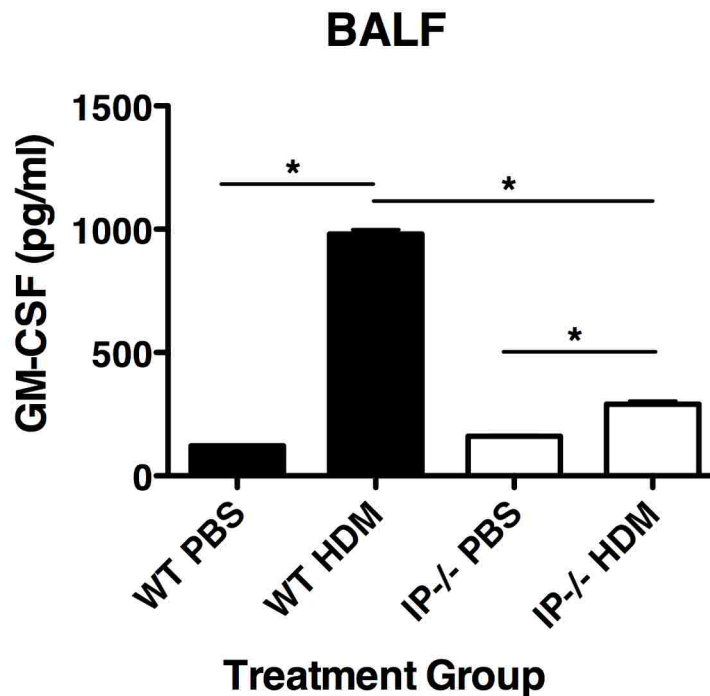


Figure 4.22 IP^{-/-} Mice Display Attenuated Production of GM-CSF in the Airway Following Intranasal Exposure to HDM. IP^{-/-} and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, lung mononuclear cells were isolated and incubated for 24 hours at 37°C. Supernatants were subsequently harvested to assess GM-CSF production by ELISA. Results are \pm SEM mean (n = 3) and are representative of two independent experiments. *p < 0.05

4.2.2.5 Increased Production of IFN- γ and TNF- α by Pulmonary NK Cells Stimulated *in vitro*

As described in Chapter 4 section 4.2.1, naïve IP^{-/-} mice display significantly increased numbers of pulmonary NK cells (identified as CD3⁻CD19⁻NK1.1⁺). These pulmonary NK cells appear to be associated with a highly cytotoxic phenotype with

increased expression of activating receptor NKp46, increased intracellular production of granzyme A, and significantly increased production of IFN- γ , but not TNF- α , following *in vitro* stimulation with either NK1.1 or NKp46. In order to determine the effect of HDM exposure on this altered pulmonary NK cell population, we isolated LMCs from HDM-exposed IP^{-/-} and WT mice and stimulated them *in vitro* utilizing anti-NK1.1 mAb PK136 in the presence or absence of IL-2 as described in Chapter 2 Methods Section 2.0.7.1. Following exposure to HDM, IP^{-/-} mouse NK cells had significantly increased production of IFN- γ compared to HDM-exposed WT mice (1210 pg/ml compared to 241 pg/ml)(Figure 4.23). However, comparison of HDM-exposed IP^{-/-} mice to unexposed control IP^{-/-} mice revealed that exposure to HDM did not appear to have any effect on production of IFN- γ , as levels were nearly identical (1210 pg/ml compared to 1086 pg/ml)(Figure 4.23). Similarly, comparison of HDM-exposed WT mice to unexposed control WT mice revealed that exposure to HDM did not appear to have any effect on production of IFN- γ (241 pg/ml compared to 261 pg/ml)(Figure 4.23). However, we found that exposure to HDM resulted in significantly increased production of TNF- α from HDM-exposed IP^{-/-} mouse pulmonary NK cells compared to HDM-exposed WT mouse pulmonary NK cells (544 pg/ml compared to 129 pg/ml), while TNF- α production was consistently low (<100 pg/ml) from both control IP^{-/-} and WT mouse pulmonary NK cells (Figure 4.23). In marked contrast, attempts to analyze IFN- γ and TNF- α levels in the BALF were unsuccessful as levels were found to be below the threshold of detection. The ability of HDM to induce TNF- α , but not IFN- γ , production in IP^{-/-} mouse pulmonary NK cells is an intriguing development that will require further investigation.

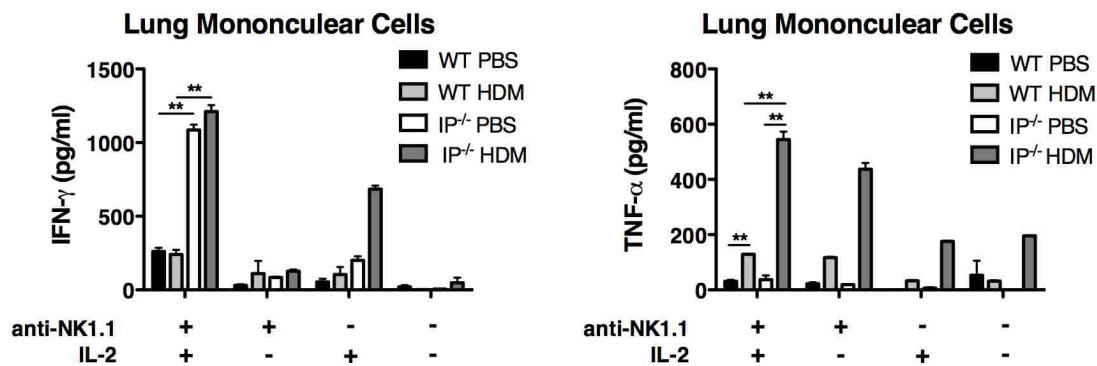


Figure 4.23 **IP^{-/-} Pulmonary Natural Killer Cells Display Significantly Increased *in vitro* IFN- γ and TNF- α Production Following NK1.1 Stimulation.** IP^{-/-} and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, lung mononuclear cells were obtained from dissociated lung tissue from IP^{-/-} and WT mice. LMCs were stimulated *in vitro* with anti-NK1.1 mAb (20 μ g/ml) \pm IL-2 (10 ng/ml) for 24 h at 37°C. Supernatants were analyzed for IFN- γ and TNF- α production by ELISA. Data are mean \pm SEM (n = 3) in triplicate and are representative of three independent experiments. **p < 0.01 *p < 0.05

4.2.3 Depletion of Natural Killer Cells Restores Allergic Lung Inflammation in IP^{-/-} Mice

Although NK cells have a critical role in the modulation of infection and inflammation in the airway in a variety of different diseases understanding the regulation of pulmonary NK cells during pathogenesis of asthma is still severely lacking (Culley 2009). The question of whether NK cells exert a beneficial or deleterious role in the development of asthma has been confused by conflicting reports. Marcenaro et al demonstrated that NK cells activated with IL-4 were incapable of driving immature DC maturation responsible for Th1 cell priming, resulting in an environment favorable to the development of a Th2-associated response (Marcenaro, Della Chiesa et al. 2005). Korsgren et al had demonstrated almost five years earlier that depletion of NK cells prior to allergic sensitization resulted in significantly reduced development of eosinophilia and Th2-associated cytokines production in the airway, indicating that NK cells were critical

for the development of allergic lung inflammation (Korsgren, Persson et al. 1999). However, recent work has also demonstrated that resolution of allergic lung inflammation by resolvin E1 is accomplished through NK cell-mediated clearance of eosinophils and T lymphocytes (Haworth, Cernadas et al. 2011). Understanding the mediators that regulate the function of pulmonary NK cells offer us a unique and vital insight into finally resolving the role of pulmonary NK cells in the development of allergic lung inflammation.

The attenuated development of allergic asthma observed in $IP^{-/-}$ mice following HDM exposure described in section 4.2.2 was very unexpected based on our previous experience with the ovalbumin-induced mouse model of allergic asthma. Understanding the underlying mechanism responsible for this attenuated response could be of great interest in developing future therapeutic targets for allergic lung inflammation. The results presented in Chapter 4 section 4.2.1 demonstrated that loss of prostacyclin signaling leads to an alteration in the phenotype of the naïve pulmonary NK cells found in $IP^{-/-}$ mice. Specifically, pulmonary NK cells of $IP^{-/-}$ mice appear to exhibit characteristics indicative of increased cytotoxicity, including increased expression of activating receptor NKp46, increased baseline production of granzyme A & IFN- γ , and increased production of TNF- α following HDM exposure. The question then arises, does this altered NK cell population in $IP^{-/-}$ mice exert influence in the attenuated development of allergic lung inflammation following HDM exposure as described in Chapter 4 section 4.2.2? To answer that question, a critical aspect was the depletion of NK cells prior to exposure to HDM. Therefore, we performed *in vivo* depletion of NK cells utilizing anti-

NK1.1 mAb PK136 prior to HDM exposure with subsequent analysis of the development of allergic lung inflammation.

4.2.3.1 Depletion of Natural Killer Cells Utilizing anti-NK1.1 Monoclonal Antibody PK136

In order to determine the physiologic role of NKp46⁺ pulmonary NK cells in the attenuation of HDM-induced allergic lung inflammation, it is first necessary to devise a method for their removal from the tissue. One of the most commonly used immunological techniques for removal of a cell population of interest is *in vivo* treatment utilizing a monoclonal antibody (mAb) recognizing a cell-specific receptor. In the case of NK cells, markers such as NK1.1 (Bartel, Bauer et al. 2013) have been used very successfully as a method of *in vivo* NK cell depletion since the development of PK136, a mAb specific to NK1.1, in 1984 (Koo and Peppard 1984). Since the development of PK136, it has been shown to induce the depletion of NK cells *in vivo* in a highly specific and selective manner (Seaman, Sleisenger et al. 1987) (Kulesza, Hoser et al. 2006) (Miller, Andres et al. 2003). The strong supporting background for the use of PK136 as an excellent method for the *in vivo* depletion of NK cells led us to utilize PK136 for our own depletion strategy.

Following *in vivo* treatment of IP^{-/-} and WT mice with PK136 as outlined in Chapter 2 Methods Section 2.0.5.1, flow cytometry analysis was performed in order to determine whether treatment utilizing anti-NK1.1 mAb PK136 in WT and IP^{-/-} mice resulted in depletion of natural killer cell populations in the airway. As described in Chapter 4 section 4.2.2.1, NK cells were defined as a CD3⁻CD19⁻NK1.1⁺ cell population. Treatment of both IP^{-/-} and WT mice utilizing PK136 resulted in complete depletion of

NK1.1⁺ mononuclear cells in the airway when compared to untreated controls of either strain (Figure 4.24). In order to verify specificity of PK136 in removing NK cells, *in vivo* depletion utilizing control rat IgG was performed as described in Chapter 2 Methods Section 2.0.5.1. No alteration in NK1.1⁺ mononuclear cells (NK cells) was observed in the airway when compared to untreated controls of either strain (Figure 4.24). This data indicates that the *in vivo* depletion strategy for NK cells described in Chapter 2 Methods Section 2.0.5.1 used in this study leads to highly selective and successful *in vivo* depletion of NK cells in the airway.

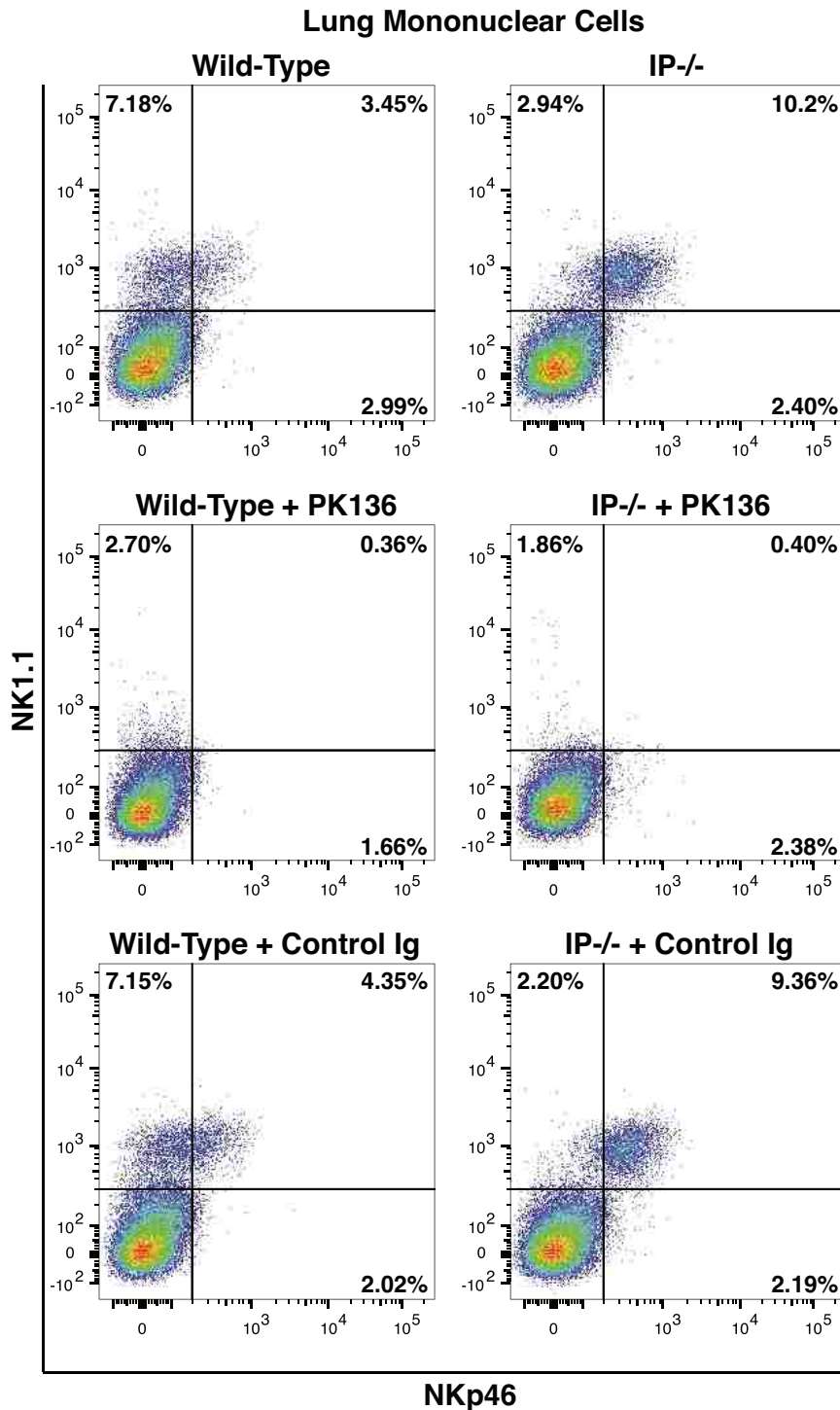


Figure 4.24 *In Vivo* Depletion of Pulmonary NK Cells Utilizing anti-NK1.1 mAb PK136. Naïve IP^{-/-} and WT mice received intraperitoneal (i.p.) injection of PK136 (250 µg) or rat IgG (control Ig) on day 0, followed by subsequent i.p. injection on day 3, 6, 10, and 13. Lung mononuclear cells were collected on day 14 to determine depletion of pulmonary NK1.1⁺ cells. LMCs were stained with anti-CD3 (PE), anti-CD19 (APCCy7), anti-NK1.1 (FITC), and anti-NKp46 (APC) and analyzed for receptor expression using a BD FACSAria Flow Cytometer. Natural killer cells were classified as CD3⁻ CD19⁺ NK1.1⁺.

4.2.3.2 Increased Infiltration by Inflammatory Immune Cell Populations Following NK Cell Depletion in IP^{-/-} Mice

As described in more detail in section 4.2.2.1, infiltration of the airway by inflammatory immune cell such as eosinophils and T lymphocytes is critical in the development of allergic lung inflammation. Following depletion of NK cells with PK136 and subsequent exposure to HDM as described in Chapter 2 Methods Section 2.0.5.1, examination of the BALF was performed in order to determine the role of NK cells in modulating infiltration of the airway by inflammatory immune cells following HDM exposure. We observed that PK136-treated IP^{-/-} mice displayed increased infiltration by eosinophils into the BALF when compared to untreated IP^{-/-} mice as determined by total cell number in addition to measurement of EPO levels in the BALF (Figure 4.25). Interestingly, we also observed increased infiltration by eosinophils into the airway of PK136-treated WT mice when compared to untreated, HDM-exposed WT mice (Figure 4.25). Treatment of IP^{-/-} and WT mice utilizing control rat IgG did not significantly impact eosinophilic infiltration of the airway (Figure 4.25) as determined by total cell number in addition to measurement of EPO levels in the BALF. Although a slight increase in airway infiltration was observed in PK136-treated IP^{-/-} mice, this infiltration was not significantly different from that of untreated, HDM-exposed WT mice (Figure 4.25). These results suggest that the NKp46⁺ NK cell population observed in naïve IP^{-/-} mice has the capacity to suppress infiltration of the airway by inflammatory immune cells such as eosinophils following exposure to HDM.

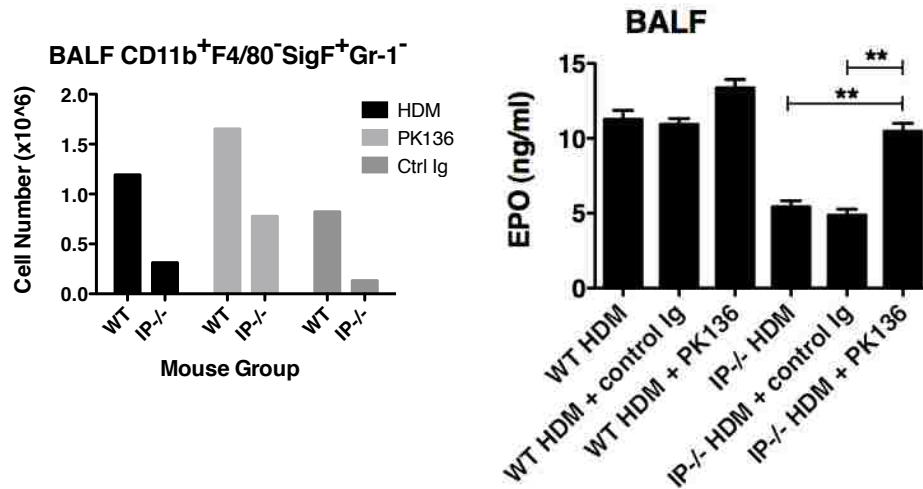


Figure 4.25 Depletion of Pulmonary NK Cells in IP^{-/-} Mice Leads to Increased Eosinophilic Infiltration of the Airway Following Exposure to HDM. Naïve IP^{-/-} and WT mice received intraperitoneal (i.p.) injection of PK136 (250 µg) or rat IgG (control Ig) on day -1, followed by subsequent i.p. injection on day 3, 6, 10, and 13. IP^{-/-} and WT mice were intranasally sensitized with 30 µl HDM (100 µg) on d 0 with subsequent intranasal challenge with HDM (50 µg) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to determine development of allergic lung inflammation. (A) Cell differential counts in the BALF were determined by light microscopic evaluation of stained cytopsin preparations. Results are expressed as absolute numbers (per mouse) of eosinophils (CD11b⁺F4/80⁺SigF⁺Gr-1⁻) (B) EPO levels in the BALF of HDM-sensitized mice receiving i.p. injection of PK136, control Ig, or PBS were assessed by colorimetric analysis. Results are ± SEM mean (n = 3) and are representative of two independent experiments. **p < 0.01

4.2.3.3 Increased Production of IL-13 Following NK Cell Depletion in IP^{-/-} Mice

Decreased infiltration of inflammatory immune cells into the airway and decreased peribronchial inflammation & mucus secretion was evident in IP^{-/-} mice following HDM exposure. In addition, decreased production of the Th2-associated cytokines IL-4, IL-5, and IL-13 characterized the attenuated allergic lung inflammation in IP^{-/-} mice following HDM exposure described in section 4.2. In order to characterize the effects of pulmonary NK cells on Th2-associated cytokine production in the airway, we analyzed BALF of IP^{-/-} and WT mice following depletion of NK cells. We observed that

PK136-treated $IP^{-/-}$ mice had significantly increased levels of IL-13 (122 pg/ml to 28 pg/ml) when compared to untreated, HDM-exposed $IP^{-/-}$ mice (Figure 4.26). While PK136 treatment was effective in increasing production of IL-13 following HDM exposure, we observed no significant changes in IL-13 production in the BALF following treatment of $IP^{-/-}$ mice with control rat IgG (Figure 4.26). Comparison of the levels of IL-13 present in the BALF of HDM-exposed $IP^{-/-}$ mice following PK136 treatment and untreated HDM-exposed WT mice revealed that depletion of NK cells prior to HDM exposure led to restoration IL-13 (~100 pg/ml) production in the airway (Figure 4.26). The levels of IL-13 in the BALF were not significantly altered following PK136 or control rat IgG treatment in WT mice, as the level of IL-13 (~100 pg/ml) remained constant between all three groups (Figure 4.26). This data is consistent with the increased infiltration of T lymphocytes in the airway following NK depletion and subsequent HDM exposure described in Chapter 4 section 4.2.3.2, and suggests that the altered $NKp46^{+}$ NK cells found in $IP^{-/-}$ mice suppress the development of a Th2 response in the airway, and this effect contributes to the attenuated allergic lung inflammation observed in HDM-exposed $IP^{-/-}$ mice.

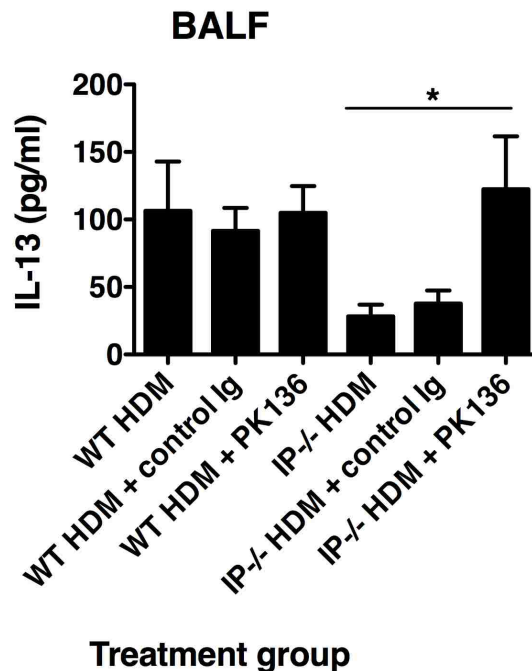


Figure 4.26 Depletion of Pulmonary NK Cells in $IP^{-/-}$ Mice Restores IL-13 Production in the Airway Following Intranasal Exposure to HDM. Naïve $IP^{-/-}$ and WT mice received intraperitoneal (i.p.) injection of PK136 (250 μ g) or rat IgG (control Ig) on day -1, followed by subsequent i.p. injection on day 3, 6, 10, and 13. $IP^{-/-}$ and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to determine production of IL-13 by ELISA. Results are \pm SEM mean (n = 3) and are representative of two independent experiments. *p < 0.05

4.2.3.4 Altered Production of GM-CSF, CX3CL1, and CCL2 in the Airway Following NK Cell Depletion in $IP^{-/-}$ Mice

As described more fully in Chapter 4 section 4.2.2.4, we examined the airway for alterations in the production of several asthma-associated chemotactic and maturation factors, including GM-CSF, CX3CL1, and CCL2. We observed that $IP^{-/-}$ mice had significantly reduced production of GM-CSF and CCL2 following HDM exposure compared to WT mice, while naïve $IP^{-/-}$ mice displayed significantly increased CX3CL1 levels in the airway compared to naïve WT mice (Figure 4.20 – 4.22). While the

increased CX3CL1 levels likely contribute to the increased numbers of pulmonary NK cells present in the lungs of naïve IP^{-/-} mice the reduction in GM-CSF and CCL2 production could contribute to the attenuated allergic lung inflammation observed in IP^{-/-} mice. Therefore, we examined whether restoration in GM-CSF or CCL2 production occurred following depletion of NK cells in HDM-exposed IP^{-/-} mice.

Examination of CCL2 revealed that depletion of NK cells utilizing PK136 in HDM-exposed IP^{-/-} mice resulted in a non-significant increase in CCL2 production (63 pg/ml CCL2) in the BALF compared to CCL2 production (21 pg/ml) in the BALF of untreated HDM-exposed IP^{-/-} mice (Figure 4.27). In WT mice, depletion of NK cells resulted in a non-significant decrease in CCL2 production in the BALF of untreated HDM-exposed WT mice when compared to PK136-treated HDM-exposed WT mice (154 pg/ml compared to 54 pg/ml)(Figure 4.27). Although CCL2 production in BALF of HDM-exposed IP^{-/-} mice (63 pg/ml) treated with PK136 was reduced compared to untreated HDM-exposed WT mice (154 pg/ml), this difference was found to be non-significant (Figure 4.27). These results suggest that depletion of NK cells in IP^{-/-} mice does result in partial restoration of CCL2 production in the airways following HDM exposure.

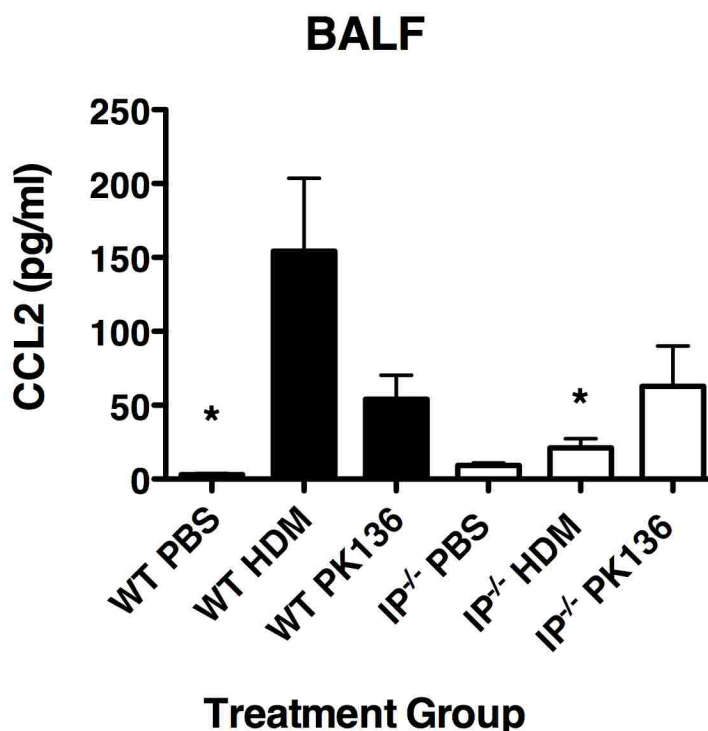


Figure 4.27 Depletion of NK Cells in IP^{-/-} Mice Leads to a Non-significant Increase in CCL2 Production in the Airway. IP^{-/-} and WT mice were given intraperitoneal injection of anti-NK1.1 mAb (PK136)(250 µg) 1 d prior to intranasal HDM exposure, and received twice weekly i.p. injection of PK136 (250 µg) for the duration of the experiment. IP^{-/-} and WT mice were intranasally sensitized with 30 µl HDM (100 µg) on d 0 with subsequent intranasal challenge with HDM (50 µg) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to assess CCL2 (MCP-1) production by ELISA. Control mice received intranasal PBS. Results are ± SEM mean (n = 4) and are representative of two independent experiments. *p < 0.05 compared to WT HDM mice

Depletion of NK cells utilizing PK136 in HDM-exposed IP^{-/-} mice resulted in a significant increase in GM-CSF production by LMCs (1035 pg/ml) when compared to GM-CSF produced by LMCs of untreated HDM-exposed IP^{-/-} mice (291 pg/ml) as well as untreated control IP^{-/-} mice (161 pg/ml) (Figure 4.28). In WT mice, depletion of NK cells also resulted in a significant increase in GM-CSF production by LMCs compared to LMCs from control WT mice (611 pg/ml compared to 121 pg/ml)(Figure 4.28). Interestingly, depletion of NK cells in HDM-exposed WT mice resulted in a significant

decrease in GM-CSF production when compared to untreated HDM-exposed WT mice (611 pg/ml compared to 981 pg/ml)(Figure 4.28). This decrease in GM-CSF production following NK cell depletion in WT mice could be explained by the loss of NK cells in the airway, which produce GM-CSF upon activation and contribute to overall airway GM-CSF production. Comparison of the GM-CSF produced by LMCs of PK136-treated IP^{-/-} mice (1035 pg/ml) to the GM-CSF produced by LMCs of untreated HDM-exposed WT mice (981 pg/ml), no significant differences were observed (Figure 4.28). These results suggest that the NKp46⁺ NK cells present in the lungs of naïve IP^{-/-} mice are capable of suppressing GM-CSF production in the lung following exposure to HDM, and depletion of this NK cell population results in complete restoration of GM-CSF production in the lung in response to HDM exposure.

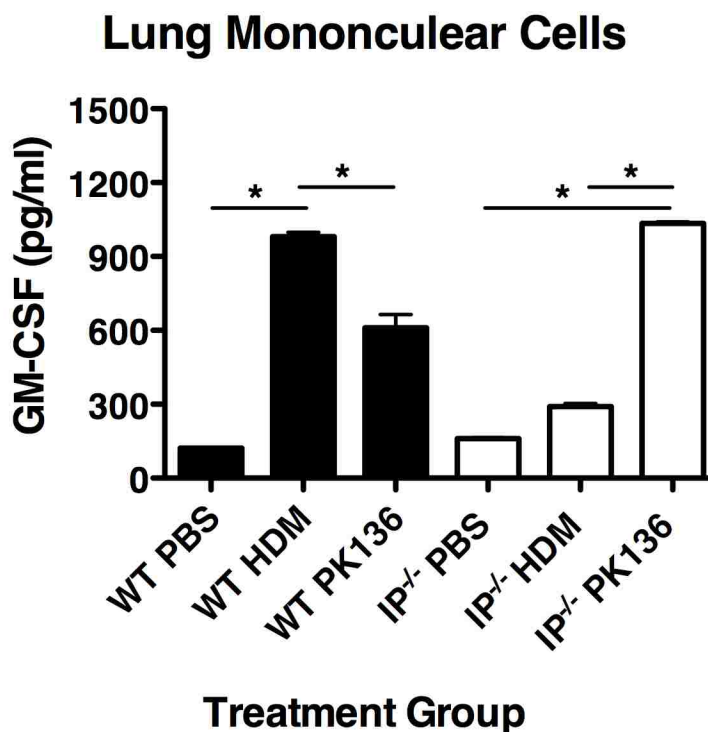


Figure 4.28 Depletion of NK Cells in IP^{-/-} Mice Results in Significantly Increased GM-CSF Production in the Airway Following Intranasal Exposure to HDM. IP^{-/-} and WT mice were given intraperitoneal injection of anti-NK1.1 mAb (PK136)(250 µg) 1 d prior to intranasal HDM exposure, and received twice weekly i.p. injection of PK136 (250 µg) for the duration of the experiment. IP^{-/-} and WT mice were intranasally sensitized with 30 µl HDM (100 µg) on d 0 with subsequent intranasal challenge with HDM (50 µg) on d 7 and d 14. 3 d following final HDM challenge, lung mononuclear cells were isolated and incubated for 24 hours at 37°C. Supernatants were subsequently harvested to assess GM-CSF production by ELISA. Control mice received intranasal PBS. Results are ± SEM mean (n = 4) and are representative of two independent experiments. *p < 0.05

Finally, examination of CX3CL1 revealed that depletion of NK cells in IP^{-/-} and WT mice had almost no effect on CX3CL1 production in the airway following HDM exposure, as non-significant difference were observed when comparing PK136-treated and untreated BALF of both strains (Figure 4.29). We found that PK136 treatment and subsequent HDM exposure in IP^{-/-} and WT mice resulted in a non-significant decrease in CX3CL1 levels in the BALF compared to unexposed IP^{-/-} and WT control mice (765

pg/ml to 1387 pg/ml and 615 pg/ml to 809 pg/ml CX3CL, respectively)(Figure 4.29).

These results lend support to the concept that, while CX3CL1 would seem to exert a critical role in the recruitment of NK cells to the lungs of naïve $IP^{-/-}$ mice, the role of CX3CL1 in direct response to HDM exposure in the attenuation of allergic lung inflammation observed in $IP^{-/-}$ mice is likely minimal.

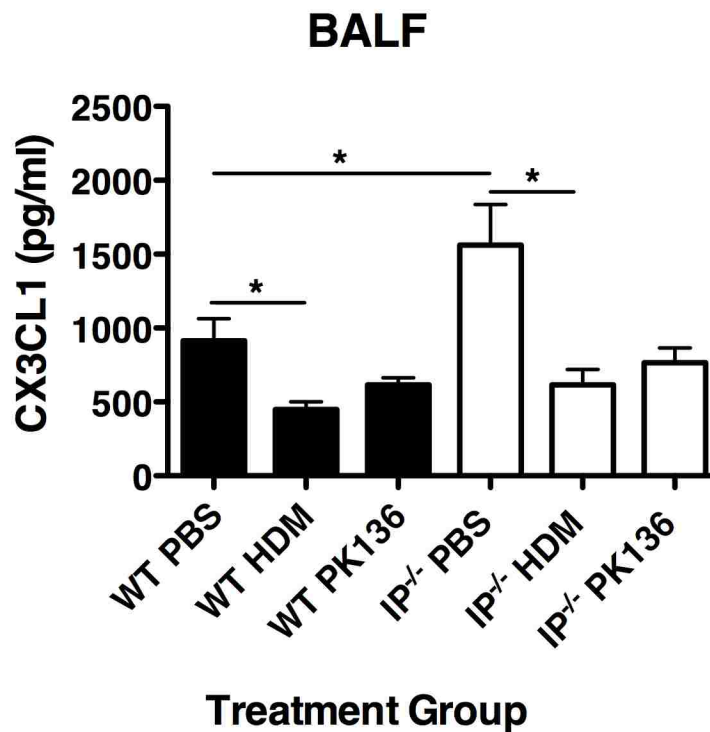


Figure 4.29 Depletion of NK Cells in $IP^{-/-}$ Mice Does Not Alter CX3CL1 Production in the Airway. $IP^{-/-}$ and WT mice were given intraperitoneal injection of anti-NK1.1 mAb (PK136)(250 μ g) 1 d prior to intranasal HDM exposure, and received twice weekly i.p. injection of PK136 (250 μ g) for the duration of the experiment. $IP^{-/-}$ and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. 3 d following final HDM challenge, BALF was collected to assess CX3CL1 (fractalkine) production by ELISA. Control mice received intranasal PBS. Results are \pm SEM mean (n = 4) and are representative of two independent experiments. *p < 0.05

4.2.4 Adoptive Transfer of IP^{-/-} Mouse Natural Killer Cells Results in Attenuated Response in Wild-type Mice

As described in Chapter 4 section 4.2.3, the use of anti-NK1.1 mAb PK136 to deplete NK cells in the lungs strongly implies that NK1.1⁺ cells are responsible for the attenuated development of allergic lung inflammation following exposure to HDM observed in IP^{-/-} mice. However, treatment with PK136 does have an important limitation when determining the specific role of NK cells, as most invariant NKT cells express NK1.1 and have been shown to be depleted following treatment with PK136 (Teige, Bockermann et al. 2010). Thus, in order to definitively state that NK cells are responsible for the attenuated development of allergic lung inflammation observed in IP^{-/-} mice, we needed to find a solution that clearly established NK cell, not iNKT cell, involvement. A confounding factor in resolving this issue is that, while examination of responses in Rag-1^{-/-} mice would facilitate analysis of NK cell involvement in isolation, the eosinophilic infiltration associated with allergic lung inflammation would be largely lost in the absence of adaptive immunity. Therefore, given these limitations, we examined whether adoptive transfer of purified NK cells directly into the airway of WT mice was sufficient to inhibit the development of allergic inflammation elicited following exposure to HDM as described in Chapter 2 Methods Section 2.0.5.2. The decision to utilize oral-pharyngeal administration as the route of adoptive transfer was based on the possibility that the pulmonary NK cells from IP^{-/-} mice regulate antigen-presenting immune cells such as alveolar macrophages involved in the recognition of HDM.

Following adoptive transfer of WT and IP^{-/-} mouse splenic NK cells and subsequent exposure to HDM as described in Chapter 2 Methods Section 2.0.5.2, we

examined the infiltration of eosinophils into the airway of WT mice. Adoptive transfer of both $IP^{-/-}$ and WT splenic NK cells resulted in significantly reduced eosinophilic infiltration into the airway when compared to HDM-exposed control WT mice as determined by measurement of BALF EPO levels (Figure 4.30). However, the suppression of eosinophil infiltration into the airway was most effective following adoptive transfer of $IP^{-/-}$ splenic NK cells (Figure 4.30). These results suggest that NK cells, and not other NK1.1-expressing immune cells such as iNKTs, are specifically responsible for the attenuated development of allergic lung inflammation observed in $IP^{-/-}$ mice, and that it is possible to induce in WT mice the attenuated response observed in the airways of $IP^{-/-}$ mice following HDM exposure through depletion of pulmonary NK cells.

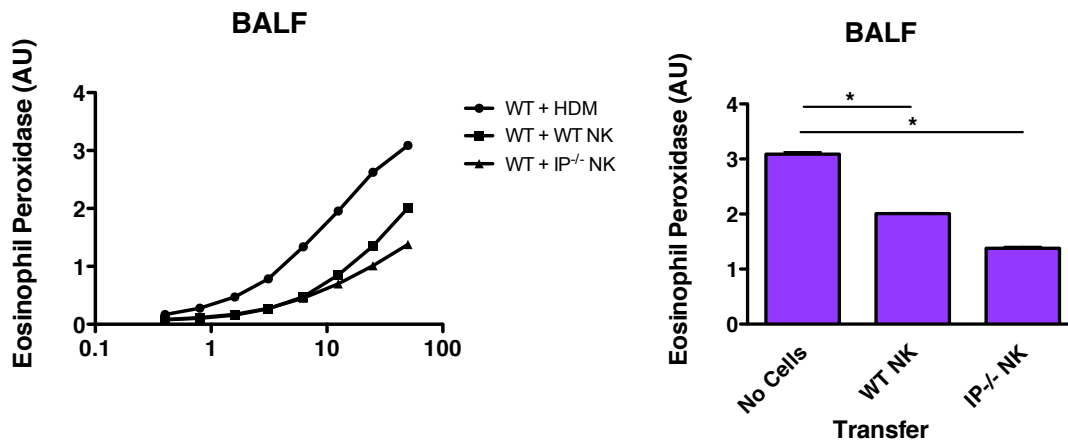


Figure 4.30 Adoptive Transfer of Splenic WT and $IP^{-/-}$ NK Cells Leads to Significantly Reduced EPO Production in the Airway Following Intranasal Exposure to HDM. $IP^{-/-}$ and WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. Splenic NK cells were purified from $IP^{-/-}$ and WT mice using MagCelect magnetic cell sorting. Purified NK cells were resuspended (5×10^5 cells/ml) in RPMI supplemented with 10 ng/ml IL-2 (no FBS), and 30 μ l was instilled by oral-pharyngeal administration 24 hours following initial HDM sensitization. Control mice received oral-pharyngeal PBS. BALF was collected and assessed by colorimetric analysis. Results are \pm SEM mean ($n = 4$) and are representative of two independent experiments. * $p < 0.05$

Understanding that $IP^{-/-}$ and WT mice exhibit significant differences in airway chemokine levels while naïve as well as following HDM exposure (see Figure 4.20 – 4.22 & Figure 4.27 – 4.29), we attempted to examine airway chemokine levels in WT mice exposed to HDM following oral-pharyngeal adoptive transfer of $IP^{-/-}$ splenic NK cells. WT mice receiving $IP^{-/-}$ splenic NK cells displayed significantly increased production of CX3CL1 (525 pg/ml) compared to WT mice receiving no adoptively transferred cells (254 pg/ml)(Figure 4.31). However, no significant differences were observed in CCL2 levels (Figure 4.31). These results suggest that adoptive transfer of NK cells into the airway initiates either the induction or release of CX3CL1 into the airway, and could play a vital role in the regulation of airway responsiveness to allergens such as HDM.

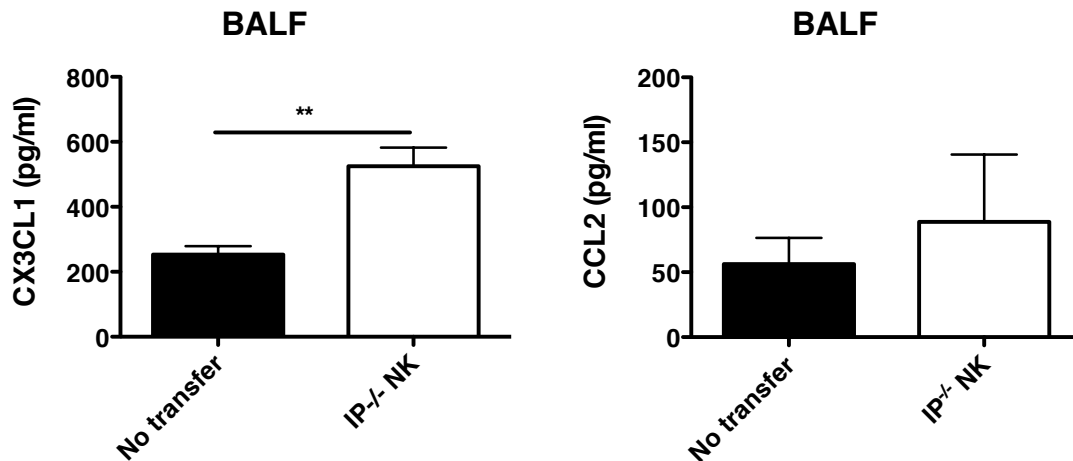


Figure 4.31 Adoptive Transfer of Splenic $IP^{-/-}$ NK Cells Leads to Significantly Levels of CX3CL1 in the Airway Following Intranasal Exposure to HDM. WT mice were intranasally sensitized with 30 μ l HDM (100 μ g) on d 0 with subsequent intranasal challenge with HDM (50 μ g) on d 7 and d 14. Splenic NK cells were purified from $IP^{-/-}$ mice using MagCelect magnetic cell sorting. Purified NK cells were resuspended (5×10^5 cells/ml) in RPMI supplemented with 10 ng/ml IL-2 (no FBS), and 30 μ l was instilled by oral-pharyngeal administration 24 hours following initial HDM sensitization. Control mice received oral-pharyngeal PBS. BALF was collected and assessed ELISA. Results are \pm SEM mean (n = 4) and are representative of two independent experiments. *p < 0.01

4.2.5 Naïve IP^{-/-} Mice Display Reduced Numbers of Vimentin⁺ Alveolar Macrophages in the Airway

While alterations in airway production of chemokines, particularly CX3CL1, offer a putative mechanism for the attenuated development of allergic airway inflammation in IP^{-/-} mice following HDM exposure, the direct role of NK cells remained unresolved. The observed increase in NKp46 expression by pulmonary NK cells offered another potential direction for understanding the underlying mechanism. In addition to recognizing exogenous bacterial and viral ligands, NKp46 also has the capacity to recognize endogenous ligands. The most well-described of these ligands is vimentin, a universally expressed structural protein that has been shown to activate NK cells resulting in cytotoxicity (Harris, Kapur et al. 1992; Garg, Barnes et al. 2006; Chong, Zhou et al. 2010). A wide variety of cell types have been shown to express surface vimentin, including macrophages, dendritic cells, monocytes, and T cells (Benes, Maceckova et al. 2006; Chen, Tseng et al. 2009; Chong, Zhou et al. 2010). It is possible that NK-mediated targeting of vimentin-expressing pulmonary immune cells could result in the attenuated development of allergic airway inflammation. Examination of BALF in naïve IP^{-/-} mice revealed a significant decrease in vimentin⁺ alveolar macrophages present in the airway when compared to WT mice (Figure 4.32). Recent studies have identified a pathogen recognition receptor, dectin-2, that is critical for promoting the HDM-induced immune response (Clarke, Davis et al. 2014; Norimoto, Hirose et al. 2014; Parsons, Li et al. 2014). Examination of dectin-2 expression in conjunction with vimentin expression in naïve IP^{-/-} mice revealed that dectin-2⁺vimentin⁺ alveolar macrophages are markedly decreased when compared to WT mice. It is possible that NK-mediated targeting of

vimentin-expressing alveolar macrophages in the airways results in loss of the immune cells responsible for the initial recognition and presentation of HDM antigen, and could be a possible mechanism for the attenuated development of allergic airway inflammation observed in $IP^{-/-}$ mice.

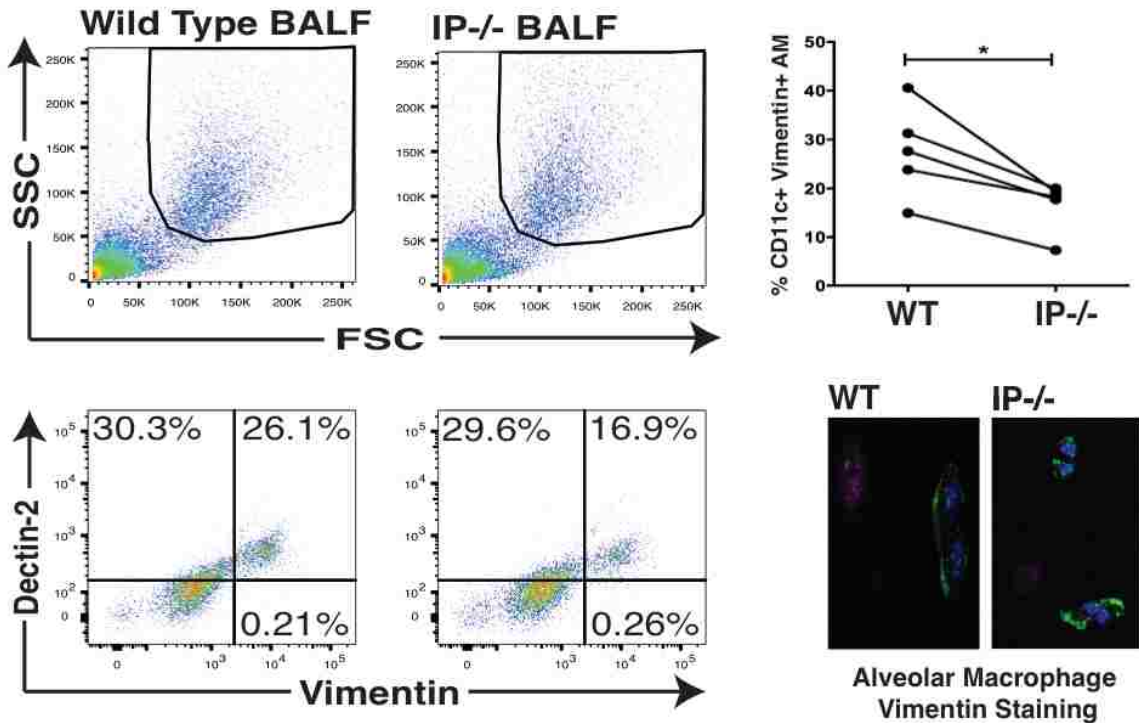


Figure 4.32 Airways of Naïve $IP^{-/-}$ Mice Display Reduced Numbers of Vimentin⁺ Alveolar Macrophages. BALF was collected from naïve $IP^{-/-}$ and WT mice and stained with anti-CD11c, anti-Dectin-2, and anti-vimentin, and analyzed for receptor expression using a BD FACSaria Flow Cytometer.

4.3 DISCUSSION

Asthma is classically considered a disease in which the adaptive immune system, specifically Th2 lymphocytes, plays a central role. However, the innate immune system likely contributes to the pathogenesis of asthma and its role should not be overlooked. As front-line sentinels in virtually every tissue, innate immune cells have the capacity for recognition and responsiveness to environmental allergen long before the adaptive

immune system develops the capacity to respond. Innate immune cells such as NK cells have been implicated to exert a role in asthma pathogenesis, as they have been demonstrated to regulate the development of T cell-mediated allergic inflammation of the airway (Korsgren, Persson et al. 1999; Wingett and Nielson 2003; Ple, Barrier et al. 2010). Surprisingly, it has been shown that IgE stimulation can activate murine NK cells via Fc γ R resulting in the production of a variety of cytokines including IFN- γ , TNF- α , and GM-CSF as well as direct cytotoxicity targeting IgE-coated cells (Arase, Arase et al. 2003). Clinical observations of asthmatic patients have revealed enhanced NK activity in peripheral blood, and suggested that NK cells migrate into the airways following antigen exposure (Timonen and Stenius-Aarniala 1985; Jira, Antosova et al. 1988). Previous reports have investigated the effect of NK cells on allergic lung inflammation with contradictory findings, in some instances NK cells inhibited allergic lung inflammation and in others they promoted eosinophilic inflammation (Walker, Checkel et al. 1998; Han, Fan et al. 2008; Haworth, Cernadas et al. 2011; Farhadi, Lambert et al. 2014). However, none of these reports investigated the properties of tissue NK cells in the absence of specific eicosanoids. This is particularly relevant given that NK cells are responsive to a range of endogenously produced eicosanoids which include resolvin-E1, lipoxin-A4, PGD₂, PGE₂, and leukotrienes (Zielinski, Gisinger et al. 1984; Bray and Brahmi 1986; Chen, Perussia et al. 2007; Barnig, Cernadas et al. 2013). Nevertheless, attempts to understand the underlying regulatory signals responsible for controlling NK cell function during allergic inflammation remain unresolved, and represent a highly viable avenue of research for understanding asthma pathogenesis.

In this study, we attempted to expand our understanding of the role that prostaglandins, specifically PGI₂, exert on NK cell function during allergic lung inflammation in a model using mice lacking expression of the PGI₂ receptor (IP). A previous study published from our laboratory has demonstrated the potent immunoregulatory role of PGI₂ on innate immune cells in the airway and the subsequent impact on the regulation of asthma pathogenesis (Jaffar, Ferrini et al. 2011). Previous studies have demonstrated that NK cell effector function is effectively down-regulated via direct and indirect signaling via PGE₂ and PGD₂ (Linnemeyer and Pollack 1993; Yakar, Melamed et al. 2003; Chen, Perussia et al. 2007). However, relatively few studies have looked at PGI₂-mediated regulation of NK cell effector function, although stable analogues of PGI₂ have been suggested to regulate the activation and inhibition of NK cells (Constantini, Kornowski et al. 1991).

4.3.1 Loss of PGI₂ Signaling Causes Significant Phenotypic and Functional Alterations in Pulmonary Natural Killer Cells

Understanding the role of PGI₂ signaling on pulmonary NK cell effector function began with characterization of these immune cells in the airway of naïve IP^{-/-} mice. Interestingly, examination of the lungs revealed significantly increased numbers of pulmonary NK cells (identified as CD3⁻CD19⁻NK1.1⁺ cells) in naïve IP^{-/-} compared to C57BL/6 (WT) mice. NK1.1 is an activating receptor universally expressed by NK cells, and stimulation of this receptor in conjunction with IL-2 has been shown to result in active proliferation and cytokine production by NK cells (Karlhofer and Yokoyama 1991; Reichlin and Yokoyama 1998). In addition to increased numbers of CD3⁻CD19⁻ cells expressing NK1.1, IP^{-/-} pulmonary NK cells exhibited markedly increased expression of

another activating receptor, NKp46 (CD335). While this increased expression of NKp46 was observed most strongly in the airways, evaluation of systemic NKp46 expression revealed a virtually universal up-regulation of this receptor in IP^{-/-} mouse NK cells indicating that prostacyclin signaling may play a critical role in the regulation of NKp46 expression. NKp46 is universally and specifically expressed by NK cells, and the evolutionary conservation of NKp46 across species has led to the suggestion that identification of NK cells based on NKp46 expression may be the most highly accurate method to identify NK cells classification, rather than NK1.1 (Narni-Mancinelli, Chaix et al. 2011). NKp46 is a critically important activating receptor for NK cells, responsible for the recognition of a wide variety of exogenous ligands, including viral ligands such as hemagglutinin (Mandelboim, Lieberman et al. 2001). Activation of NK cells via NKp46 has been shown to result in potent cytotoxicity and cytokine production in order to resolve infections, particularly following viral recognition (Vankayalapati, Wizel et al. 2002; Bozzano, Picciotto et al. 2011; Pembroke, Christian et al. 2014).

Increased expression of NKp46 by IP^{-/-} pulmonary NK cells could potentially result in NK cells programmed for increased pro-inflammatory responses and increased NK-cell mediated cytotoxicity. Indeed, our analysis of naïve IP^{-/-} pulmonary NK cells revealed increased numbers of cells producing Granzyme A when compared to WT mice. Granzyme B staining was found to be typically weak. Curiously, analysis of intracellular IFN- γ seemed to suggest that naïve IP^{-/-} pulmonary NK cells similar capability for cytokine production. However, *in vitro* stimulation of naïve IP^{-/-} pulmonary NK cells utilizing either NK1.1 and IL-2 or NKp46 and IL-2 costimulation resulted in significantly increased production of IFN- γ , but not TNF- α , presumably reflecting the elevated number

of NK cells present in the lungs of $IP^{-/-}$ mice. As these results are suggestive of increased NK cell cytotoxicity, we attempted to determine whether $IP^{-/-}$ pulmonary cells were capable of increased lysis of tumor cell lines. However, the results of these initial studies were inconclusive (data not shown), and further analysis of $IP^{-/-}$ pulmonary NK cells in order to characterize alterations in cytotoxicity will be required to definitively address this question. However, these results still indicate that following ligand binding by NK1.1 or NKp46, $IP^{-/-}$ pulmonary NK cells have the capacity for significantly increased effector function when compared to WT pulmonary NK cells.

Our results suggest that the expression of NKp46 by pulmonary and systemic NK cells may be regulated by prostacyclin signaling, and that PGI_2 potentially inhibits NK cell effector function in WT mice through the regulation of NK-derived cytotoxic mediators and activating receptor expression. This regulation of NK cell effector function by PGI_2 has the capacity to dramatically alter the role of NK cells in the pathogenesis of a wide variety of potential diseases, including asthma.

4.3.2 Attenuation of House Dust Mite-Induced Allergic Airway Inflammation Following the Loss of PGI_2 Signaling

Almost a decade ago, Cates et al described a novel HDM-induced animal model for the study of allergic asthma (Cates, Fattouh et al. 2004). While exposure to OVA has been widely accepted as the standard animal model in the study of allergic asthma, the advantages of HDM as a biologically relevant animal model of allergic asthma have recently led to wide spread adoption of HDM-induced models. HDM-specific allergy is the most prevalent cause of sensitization associated with asthma, and almost 85% of asthmatics in highly populated area of North and South America are HDM allergic

(Pollart, Chapman et al. 1989; Thomas, Hales et al. 2010). Although previous work done in our lab has focused on understanding the role of PGI₂ in the regulation of OVA-induced allergic airway inflammation, maintaining consistency with advancements in the field required the development and adoption of a model of allergic asthma involving HDM-induced short-term intranasal sensitization of murine airways. Unexpectedly, while WT mice developed the predicted levels of allergic airway inflammation following HDM exposure, IP^{-/-} mice exhibited an attenuated immune response characterized by lack of infiltrating inflammatory immune cells, reduced peribronchial inflammation and goblet cell mucous secretion, and reduced production of Th2-associated cytokines in the airway. While an increase in airway levels of PGI₂ following HDM exposure has been described, understanding whether airway PGI₂ production in response to HDM exposure exerts pro- or anti-inflammatory effects has so far remained unclear (Herrerias, Torres et al. 2009). These results suggest that PGI₂ signaling is a critical requirement for the promotion of HDM-induced allergic airway inflammation. However, the mechanism underlying this observed decrease in infiltrating immune cells and subsequent reduction in allergic airway inflammation remained unclear.

In an attempt to elucidate a mechanism, we examined the airway for alterations in the production of chemotactic factors involved in the recruitment of asthma-associated immune cells. Specifically, airway levels of CX3CL1 (fractalkine), CCL2 (MCP-1), and GM-CSF were chosen based on their well-studied capacity for inducing recruitment of adaptive and innate immune cells into the airway as well as their association with asthma (Owen, Rothenberg et al. 1987; Imai, Hieshima et al. 1997; Deshmane, Kremlev et al. 2009; Singh, Sutcliffe et al. 2014). Analysis of these chemokines revealed substantial

alterations in all 3 chemokines, both pre- and post-HDM exposure when comparing IP^{-/-} and WT mice. Naïve IP^{-/-} mice displayed significantly increased production of airway CX3CL1, although treatment of both IP^{-/-} and WT mice with HDM resulted in significant reduction in CX3CL1 production when compared to naïve mice. This increase in CX3CL1 observed in IP^{-/-} mice is particularly interesting when considering the marked increase in pulmonary NK cells present in the airway. NK cells have been well-described to express the receptor for CX3CL1 (CX3CR1), and treatment of NK cells with CX3CL1 has been shown to induce their recruitment (Sechler, Barlic et al. 2004; Huang, Shi et al. 2006; Hamann, Unterwalder et al. 2011; El-Shazly, Doloriert et al. 2013). It is possible that this constitutive increase in airway CX3CL1 observed in naïve IP^{-/-} mice results in the recruitment and increased tissue residence described earlier in this dissertation. However, the source of CX3CL1 production remains unclear, and further studies will be needed to definitively resolve the potential sources. Unlike CX3CL1, examination of CCL2 and GM-CSF revealed that naïve IP^{-/-} and WT mice did not display constitutive production of either cytokine. However, unlike WT mice, HDM-exposure failed to induce production of CCL2, and only minor, though significant, increases in GM-CSF were observed in the airways of IP^{-/-} mice following HDM exposure. As CCL2 and GM-CSF are critical in recruiting inflammatory monocytes capable of maturing into antigen-presenting dendritic cells, the loss of these signaling molecules could result in an inability to initiate the differentiation and activation of the adaptive immune system responsible for allergic airway inflammation (van de Laar, Coffey et al. 2012; Chen, Liu et al. 2013; Lee, Jeong et al. 2014). Alterations in any of these chemokines could be a putative mechanism underlying the attenuated development of allergic asthma observed in IP^{-/-}

mice, and further analysis will be required to determine the respective contributions of each of these chemokines.

Our results demonstrate that loss of PGI₂ signaling results in attenuation in the development of allergic airway inflammation, suggesting that production of PGI₂ in the airway following HDM exposure promotes the development of allergic airway inflammation. Understanding the mechanism responsible for the attenuated inflammation observed in this study could potentially lead to novel therapeutic approaches responsible for inhibiting asthma pathogenesis through the regulation of PGI₂ signaling in the airway.

4.3.3 Natural Killer Cells Are Responsible For The Attenuated Development of House Dust Mite-Induced Allergic Airway Inflammation

While our results clearly indicated a role for PGI₂ signaling in the development of HDM-induced allergic airway inflammation, the mechanism responsible for attenuation remained unclear. While alterations in the production of chemokines including CX3CL1, CCL2, and GM-CSF in the airway of IP^{-/-} mice were observed, the underlying cause of these alterations remained in question. Fortunately, the substantial changes in the NK cell populations we observed in IP^{-/-} mice presented a clear target as a potential mediator of the attenuated response to HDM. This potential was further strengthened by the examination of pulmonary NK cells following HDM exposure, as IP^{-/-} pulmonary NK cells displayed significantly increased production of IFN- γ and TNF- α compared to WT mice. While we had observed increased IFN- γ in naïve IP^{-/-} pulmonary NK cells, TNF- α production had previously remained unaltered. The changes in TNF- α production we observed following HDM exposure suggested further alterations in the IP^{-/-} pulmonary NK cells.

In this study, we utilized an *in vivo* depletion method using anti-NK1.1 mAb PK136 in order to systemically deplete NK cells prior to HDM exposure in IP^{-/-} and WT mice. Depletion of NK cells in IP^{-/-} mice and subsequent intranasal HDM exposure resulted in restoration of allergic airway inflammation as characterized by increased eosinophilic airway infiltration and significantly increased production of Th2-associated cytokines, specifically IL-13. As *in vivo* depletion of NK1.1 has the potential to lead to depletion of NKT cells, we also utilized oral-pharyngeal adoptive transfer of purified NK cells to definitively show that NK cells are responsible for the attenuated allergic airway inflammation observed in IP^{-/-} mice. Adoptive transfer of IP^{-/-} splenic NK cells resulted in significant inhibition of eosinophilic infiltration of WT mouse airways. These results suggest a previously unrecognized role of NK cell-mediated suppression of airway inflammation that appears to be regulated by PGI₂ signaling. However, the specific mechanism utilized by NK cells that could suppress allergic airway inflammation remains unresolved. The observed phenotype of IP^{-/-} pulmonary NK cells suggests increased cytotoxicity, and it's possible that these NK cells are responsible for the removal of immature antigen-presenting cells, as NK-mediated cytotoxicity of immature dendritic cells has been well described (Carbone, Terrazzano et al. 1999; Della Chiesa, Vitale et al. 2003; Walzer, Dalod et al. 2005; Poggi and Zocchi 2007). Alternatively, NK cells could conceivably modulate the vascular endothelium, resulting in reduced leukocyte recruitment into the airway in response to HDM as CX3CL1-mediated NK cytotoxicity against endothelial cells has been observed previously (Yoneda, Imai et al. 2000). Finally, NKp46 has been shown to recognize endogenous ligands such as vimentin, and binding of vimentin by NKp46 results in up-regulation of NKp46

expression and NK-mediated cytotoxicity (Chong, Zhou et al. 2010). Examination of vimentin-expression in the airway of IP^{-/-} mice revealed markedly reduced expression of vimentin compared to WT mice, and its expression was mainly associated with alveolar macrophages. Although further studies are needed to determine whether cells expressing increased vimentin interact with NK cells in the airways of IP^{-/-} mice.

In addition to the restoration of allergic airway inflammation in IP^{-/-} mice following depletion, we also examined whether depletion of NK cells resulted in any alteration in the levels of the chemokines CX3CL1, CCL2, and GM-CSF in the airway. While we did not observe any changes in the production of CCL2 or CX3CL1, we detected significant changes in airway production of GM-CSF. Depletion of NK cells in IP^{-/-} mice resulted in a significant increase in GM-CSF production in the airway resulting in levels similar to WT mice following HDM exposure. However, further studies will be needed to determine whether the increased production of GM-CSF is a consequence of increased airway infiltration by inflammatory immune cells, or a mediator of recruitment into the airway. Interestingly, although we observed no change in CX3CL1 levels in the airway of NK-depleted IP^{-/-} and WT mice following HDM exposure, adoptive transfer of IP^{-/-} splenic NK cells into the airway of WT mice resulted in a significant increase in airway CX3CL1 levels. While it remains to be seen whether these NK cells are responsible for the induction or release of CX3CL1 into the airway, the results are suggestive that CX3CL1 plays a critical role in the regulation of NK cells responsible for the attenuated development of allergic lung inflammation observed in IP^{-/-} mice.

Our results suggest that PGI₂ signaling is responsible for the regulation of NK cell effector functions that have the capacity to control the development of allergic airway

inflammation. While we have attempted to identify a putative mechanism for how this NK-mediated control is accomplished, further studies will be required to expand our understanding of the role of NK cells in the development of allergic lung inflammation.

4.3.4 Summary

Collectively, the results of this study represent a previously unrecognized role for the regulation of allergic airway inflammation involving PGI₂ signaling. PGI₂ appears to have an inhibitory effect on pulmonary NK cells, and removal of this inhibition through loss of prostacyclin signaling results in significant alterations in systemic NK phenotype. These altered NK cells are capable of mediating the attenuated development of allergic airway inflammation in response to HDM exposure. Although we have not yet identified a definitive mechanism behind this regulation, this study suggests that the inhibition of PGI₂ signaling in the airways could be a potentially new target for the development of novel therapeutic approaches that could eventually lead to inhibition the development of allergic asthma.

Chapter 5

Loss of Prostacyclin Signaling in the Regulation of Intestinal Immunity

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Working Title: Role of PGI₂ in the Regulation of Natural Antibody Production

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5.0 ABSTRACT

The intestinal mucosa represents a critical staging point for the initiation of humoral immune responses capable of defending against a wide array of potential pathogens. The regulation of adaptive and innate immune responses by endogenous cell-derived lipid mediators known as prostaglandins has long been acknowledged. While prostaglandins such as PGE₂ and PGD₂ have an extensive history of study involved in the activation and inhibition of a diverse assortment of immune responses in many tissues, the role of prostacyclin (PGI₂) as an immunoregulatory molecule has received far less focus. Utilizing a transgenic mouse model deficient in expression of the PGI₂ receptor (IP^{-/-}), we report that naïve IP^{-/-} mice display attenuated production of natural immunoglobulins IgG1, IgG2b, and IgA in serum, as well as secretory IgA in the bronchoalveolar lavage fluid and intestinal fecal contents. Examination of the intestinal mucosa of naïve IP^{-/-} mice revealed marked reduction in IgA⁺ B cells present in both the lamina propria and Peyer's patches when compared to C57BL/6 (WT) mice, although no alterations in Peyer's patch formation or germinal center structure were observed. Interestingly, stimulation of Peyer's patch lymphocytes using recombinant IL-23 resulted in significantly reduced production of IL-22. While further studies are needed to understand the mechanism underlying the attenuated production of natural antibodies in naïve IP^{-/-} mice, the results of this study suggest a previously unrecognized critical role for PGI₂ signaling in the regulation of humoral immune responses.

5.1 Background

5.1.1 Intestinal Immunity

5.1.1.1 Mucosal Immunobiology

When considering routes of potential environmental exposure that could lead to activation of the immune system, the mucous membrane comprising the lining of the digestive, respiratory, and urogenital tract should be of primary focus. With a combined surface area of approximately 400 m², no other site in the body offers more potential for successful entry of toxic elements and infectious pathogens (Peterson and Artis 2014). The physical mucosal barrier is comprised of a layer of columnar epithelial cells sealed by tight junctions with an underlying lamina propria and musculature which provides a physical separation between the interior of the body and the external environment (Turner 2009). In order to halt invasion by environmental pathogens, an intricate and extensive network of innate and adaptive immune cells has developed to successfully repel and resolve potential invaders. Immune cells, including macrophages, dendritic cells, plasma cells, and lymphocytes, reside beneath the epithelial barrier in the lamina propria.

Far from being a disorganized accumulation of cells spread haphazardly throughout the lamina propria, defense of the mucosal barrier by these immune cells is orchestrated by carefully organized lymphoid tissues known as mucosal-associated lymphoid tissues (MALTs) (Croitoru, Bienenstock et al. 1994). MALTs can be further subdivided into organ-specific MALTs and include gastrointestinal-lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT), nasopharyngeal-associated lymphoid tissue (NALT), and several other minor MALTs (Croitoru, Bienenstock et al.

1994). Functionally, MALT can be divided into two primary sites: (i) inductive sites (ii) and effector sites (Cesta 2006). Inductive sites are areas where presentation of antigen by antigen-presenting cells to T lymphocytes occurs in order to drive primary T cell responses. Activated T cells, mainly CD4⁺, are responsible for initiating immunoglobulin class switching and clonal expansion of IgA⁺ B cells that provide protection against invading pathogens. Once an immune response has been initiated at an inductive site, migration and relocalization of both antigen-specific T and B cells to effector sites occurs (Cesta 2006). This migration is driven by expression of a variety of chemokine receptors and adhesion molecules including CXCR4, α 4 β 1, P-selectin ligands, CD22, CCR10, and CCR9 that control site-specific migration throughout the mucosa, for example CCR9 is required for the homing of activated B cells to the small intestine (Pabst, Ohl et al. 2004; Mora and von Andrian 2008). While anatomically separated, MALT sites can be considered functionally connected, as induction of activated IgA⁺ B cells at an initial site of antigenic recognition can lead to IgA secretion at effector sites in different organs, and has been shown to occur independently of systemic immunity. Due to this critical capacity in the induction of mucosal immune responses, alterations in MALT function can have dramatic consequences for immune function, and evaluation of MALT remains a crucial aspect in studying the response of the immune system following antigenic insult.

5.1.1.2 Structure of the Gastrointestinal Tract: Gut - associated lymphoid tissue

The gastrointestinal (GI) tract is critical for the digestion and absorption of nutrients, in addition to serving as a primary barrier to harmful external pathogens as well as beneficial microorganisms of the GI microbiome. These two crucial roles result in essentially constant exposure to dietary, bacterial, fungal, and viral components. The

importance of robust and diligent immune surveillance in the GI tract cannot be understated. However, uninhibited, constant activation of immune cells would potentially lead to a rampant, continuous inflammatory state that would be highly detrimental to proper GI tract function. In order to maintain levels of activation that are effective, but not deleterious, the GI tract is stratified into several distinct layers that serve to maintain a state of homeostasis in the GI tract. As mentioned in Chapter 5 Section 5.1.1.1, these areas of the GI tract are known as gut-associated lymphoid tissue (GALT). GALT can be broken down into three layers: (i) an epithelial layer comprised of intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs) (ii) lamina propria containing lamina propria lymphocytes (LPLs) (iii) and a submucosal layer comprised of organized lymphoid aggregates known as Peyer's patches (PP), as well as minor aggregates such as colonic patches (CP) and isolated lymphoid follicles (ILFs). Each layer plays an important role in maintaining GALT functionality, and will be described in greater detail.

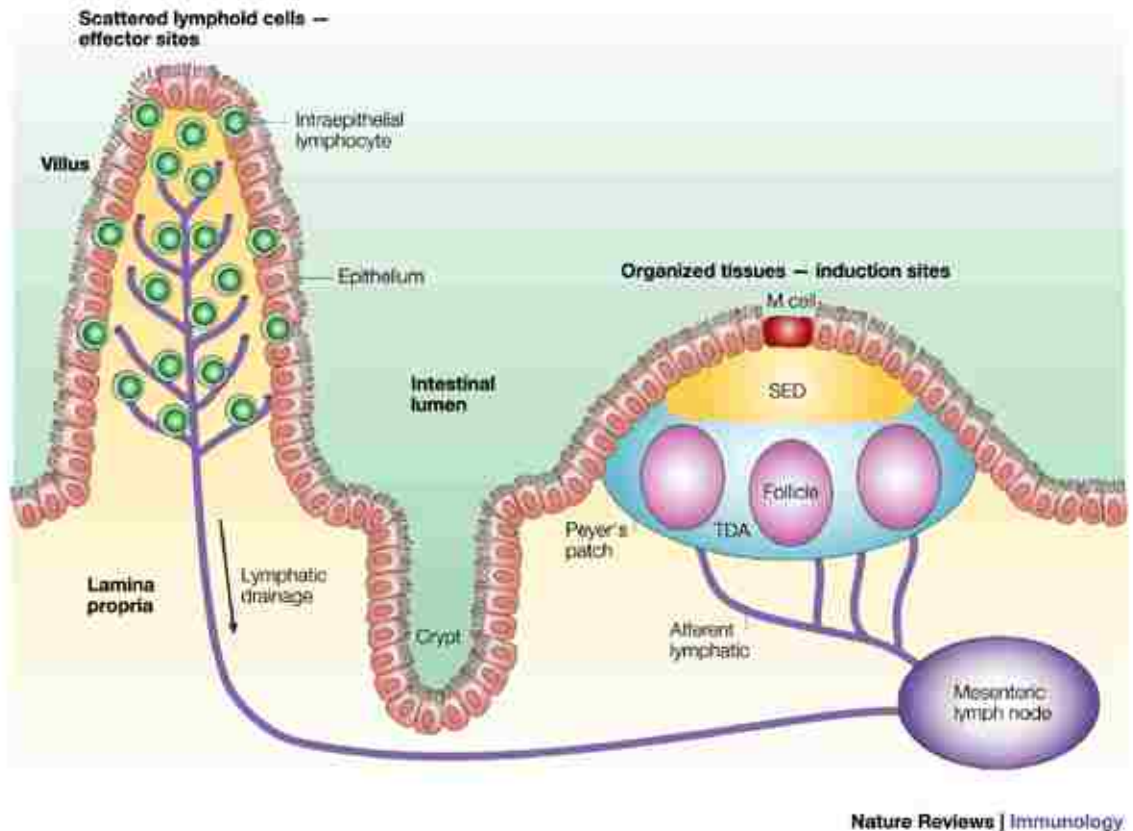


Figure 5.1 *Organization of Gut-associated Lymphoid Tissue (GALT).* The lymphoid tissue responsible for the initiation of gut-associated immune responses is a highly organized system capable of producing robust responses to antigens derived from both pathogens as well as commensal bacteria present in the intestinal lumen (Mowat 2003).

5.1.1.2.1 *Intestinal Epithelial Cells & Intraepithelial Lymphocytes*

The intestinal epithelium is a single cell layer comprised mainly of absorptive enterocytes. However, specialized lineages of intestinal epithelial cells (IECs) are also present throughout the epithelial layer. These IECs are poorly phagocytic, but are capable of producing large quantities of secretory molecules such as mucin 2 (MUC2) and antimicrobial proteins such as C-type lectins that establish and maintain a biochemical barrier to complement the physical barrier (Johansson, Phillipson et al. 2008; Gallo and Hooper 2012). In addition to direct production of secretory components, IECs are capable of directly transporting secretory IgA, a process more fully detailed in

Chapter 5 Section 5.1.2. In addition to secreting luminal products, IECs are responsible for maintenance of GALT function through regulation of immune cells as well. Production of cytokines such as thymic stromal lymphoietin (TSLP), transforming growth-factor β (TGF- β), and IL-25 as well as B cell-regulating factors such as B-cell activating factor (BAFF) and proliferation-inducing ligand (APRIL) has been shown to occur in IECs following signaling through a variety of pathogen-recognition receptors (Zaph, Troy et al. 2007; Zaph, Du et al. 2008; Zeuthen, Fink et al. 2008). These factors promote the development of tolerogenic dendritic cells and macrophages that in combination with BAFF and APRIL have been shown to drive T cell-independent IgA class-switching in naive B cells (Xu, 2007 #280).

In addition to IECs, the epithelial layer of the GALT is comprised of large numbers of intraepithelial lymphocytes (IELs). This highly heterogenous population is located at the basolateral surface of the epithelial layer of the GI tract as well as in the intercellular space between epithelial cells (Cheroutre, Lambolez et al. 2011). Murine IELs are CD3⁺ and express either the $\gamma\delta$ (45-65%) or $\alpha\beta$ (35-45%) T cell receptor with 80% of all IELs expressing CD8 (Lefrancois 1991; Maloy, Mowat et al. 1991). The $\alpha\beta$ T cell receptor of CD8⁺ IELs is predominantly found to be expressed as a CD8 $\alpha\alpha$ ⁺ homodimer (Kunisawa, Takahashi et al. 2007). Upon activation, IELs display potent cytolytic capacity, and both $\alpha\beta$ and $\gamma\delta$ IELs are capable of producing IL-2, IL-6, IFN- γ , TNF- α , or TGF- β (James, Kwan et al. 1990; Taguchi, McGhee et al. 1990). The activation of $\gamma\delta$ IELs can result in expression of Fas ligand that been shown to have the capacity to induce lysis in target cells during *in vitro* assays, suggesting the possibility that IELs are capable of removing damaged IECs present in the epithelial layer (Sakai,

Kimura et al. 1997). It's important to note that the cytolytic capabilities of IELs can exert both a protective role through removal of pathogens and maintenance of epithelial barrier function, as well as a deleterious role through inflammation leading to epithelial barrier disruption, and that further investigation into the role of IELs in mucosal immunity remains a potentially lucrative research direction (Cheroutre, Lambolez et al. 2011).

5.1.1.2.2 *Lamina Propria Lymphocytes*

The lamina propria (LP) is a loose layer of connective tissue found beneath the GI epithelial layer. This tissue has a high population of immune cells including lymphocytes, plasma cells, and macrophages. Unlike IELs, LP T cells almost exclusively express the $\alpha\beta$ T cell receptor (approximately 95%), and maintain a $CD4^+$ to $CD8^+$ ratio that shares greater similarities with peripheral T cells than IELs (James, Fiocchi et al. 1986; Fujihashi, Yamamoto et al. 1994). However, unlike peripheral T cells, LP T cells express a greater frequency of surface markers recognized to indicate activation or memory phenotypes, such as MHC Class II, $\alpha E\beta 7$, IL-2R, and CD45RO (Schieferdecker, Ullrich et al. 1992). While antigenic stimulation results in minimal proliferative capacity from LP T cells, production of high levels of IL-2, IFN- γ , IL-4, and IL-5 have been observed, suggesting that LP T cells are uniquely suited for enhancing B cell function in order to maintain mucosal immunity (Qiao, Schurmann et al. 1991). Indeed, the majority of LP B cells are plasma cells predisposed toward the production of IgA rather than IgM or IgG.

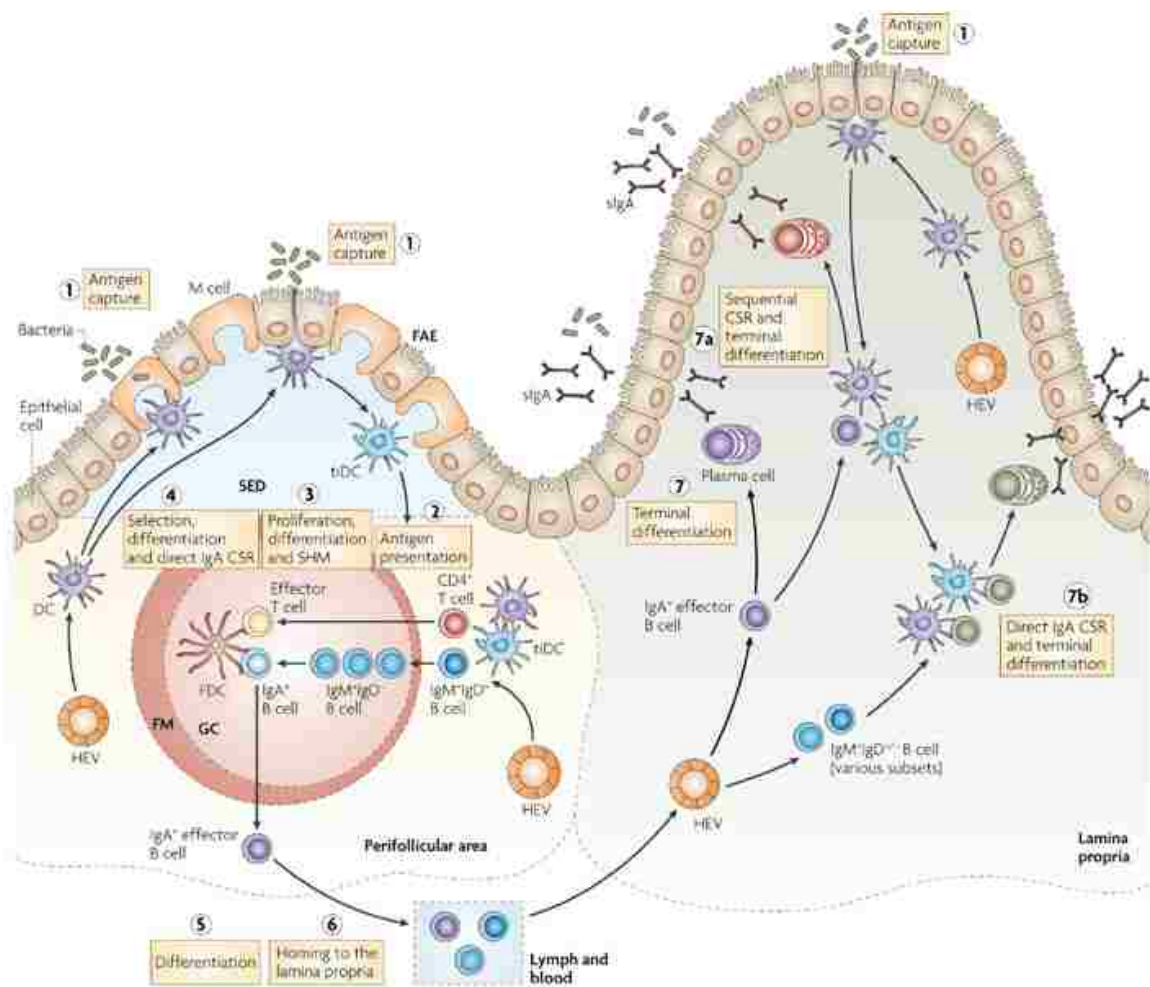
5.1.1.2.3 *Peyer's patches*

The final layer of GALT is located in the submucosa beneath the lamina propria. This layer is comprised of organized lymphoid aggregates known as Peyer's patches

(PP). Unlike the intestinal epithelium and lamina propria which are primarily effector sites, PP are the primary inductive site for mucosal immune responses. The unique nature of PP as an inductive site leads to a complex organized structure comprised of three regions, (i) follicle-associated epithelium (ii) the interfollicular region as denoted by subepithelial dome (iii) and the germinal center.

In order to induce mucosal immune responses, antigen processing and presentation must occur. In order to bypass the tight junctions sealing the epithelial layer against the intestinal lumen, specialized epithelial cells, known as M cells, are found periodically throughout the epithelial layer, and are found exclusively in epithelia localized at MALT sites identified as follicle-associated epithelium (FAE) (Neutra 1999). The apical surface of the M cell has been identified to lack surface microvilli while the basolateral surface is deeply invaginated to create a large extracellular space, often containing leukocytes, and thought to be involved in the formation of germinal centers. M cell differentiation has been observed to occur under the influence of membrane bound $LT\alpha_1\beta_2$ present primarily on B cells and lymphoid tissue-inducer cells (Debard, Sierro et al. 2001; Schmutz, Bosco et al. 2009). M cells utilize transepithelial transport in order to sample antigens from the intestinal lumen and transport them to lymphocytes on the basolateral membrane of the FAE, consisting of the interfollicular region or subepithelial dome. The interfollicular region of PP consists of heterogenous population of cells including B cells, T cells, dendritic cells, and macrophages where antigen presentation by dendritic cells and macrophages can take place.

Once antigen-presenting cells have processed antigen, presentation of antigen to T and B cells can occur. PP are connected to the systemic immune system by high endothelial venules, which allow for continuous migration of naive lymphocytes into the PP. Once recognition of the processed antigen occurs, the formation of germinal centers takes place underneath the subepithelial dome. In mice, these germinal centers are comprised of approximately 60% B cells, 25% T cells (45% CD4⁺, 35% CD8⁺, 20% CD4⁻CD8⁻), 10% dendritic cells, and 5% macrophages or neutrophils (Jung, Hugot et al. 2010). Several distinct dendritic cell subsets, including CD11c⁺CD8 α ⁺CD11b⁻ lymphoid dendritic cells, CD11c⁺CD8 α ⁻CD11b⁺ myeloid dendritic cells, and CD11c⁺CD8 α ⁻CD11b⁻ ‘double negative’ dendritic cells can be found throughout the FAE, subepithelial dome, and germinal center (Iwasaki and Kelsall 2000). Dendritic cells from PP exhibit significantly altered functional differences when compared to splenic dendritic cells, including increased potency in stimulation of allogeneic T cell proliferation, production of IL-4 and IL-10, and priming of T cells for lower production of Th1-associated cytokine IFN- γ (Iwasaki and Kelsall 1999). The skew in Th2-associated phenotype within the germinal center allows for expansive production of immunoglobulin (Ig) by B cells, predominantly IgA (approximately 70%) as opposed to the majority IgG observed in peripheral lymph nodes (Butcher, Rouse et al. 1982). Once activated in the PP, IgA⁺ B cells are capable of homing to a wide variety of effector MALT sites, contributing largely to the systemic IgA response from an initial intestinal pathogen or commensal exposure (Mora and von Andrian 2008).



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Figure 5.2 *Induction of Intestinal Immune Responses in Peyer's Patches.* Peyer's patches are the primary inductive site of intestinal immune responses. M cells sample intestinal lumen for antigens, which are then presented by dendritic cells to T and B cells present in germinal centers beneath the subepithelial dome (SED). Once activated, effector B cells migrate to inductive sites such as the lamina propria, where large amounts of IgA are produced (Cerutti 2008).

5.1.2 Immunoglobulins

In order to protect against the diverse array of pathogens that are encountered every day, lymphocytes of the adaptive immune system must have the capacity to recognize and respond in a highly specific and efficient manner. Immunoglobulins (Ig) are heterodimeric proteins that contain a variable binding region that is capable of

recognizing and binding antigens that can differ by single amino acids, allowing for highly specific responses to an antigen. Ig are produced by both naïve and activated mature B cells, and are found as both soluble, secreted proteins in addition to acting as B cell receptors when bound to the B cell membrane (Klein, Rajewsky et al. 1998).

Secreted Ig are capable of binding to specific antigens or toxic products, induce recruitment and activation of other immune cells to remove recognized pathogens, and neutralize viral pathogens and mark them for destruction via phagocytosis. Antigen that is recognized and bound by membrane-bound Ig results in several distinct changes in the B cell as activation occurs that allows for a robust and antigen-specific response.

Several distinct isotypes of Ig exist, and exert different roles in signaling and effector response to antigens. The initial Ig produced by mature, naïve B cells are IgM and IgD. IgM is a low specificity Ig that is found in both secreted and membrane-bound forms. IgD is found primarily as a membrane-bound Ig, and is found alongside surface IgM and is capable of transducing activating and inhibitory signals in B cells (Lutz, Ledermann et al. 1998). Following antigen recognition by surface IgM or IgD and concurrent stimulation by signaling via accessory cells such as dendritic cells or T cells, the naïve B cell undergoes a process known as isotype class switching where chromosomal alterations result in the production of the more highly specific Ig including IgG, IgA, and IgE (Stavnezer, Guikema et al. 2008). IgG is the predominant isotype in the blood (approximately 70 – 75%), and four subclasses have been identified IgG1, IgG2 (IgG2a and IgG2b in mice), IgG3, and IgG4 (humans only). Considered the hallmark of immunological memory for humoral immune responses, IgG is capable of activating the complement system, and has been shown to induce activation of immune

cells via binding to FcR-expressing cells such as macrophages, natural killers cells, B cells, neutrophils, dendritic cells, and eosinophils (Bruhns 2012; Karsten and Kohl 2012). IgE is found at the lowest concentration of any Ig isotype in the blood, but has been the focus of intense scrutiny due to the close association of IgE with hypersensitivity reactions, in addition to host defense against parasitic infections.

IgA is the final Ig isotype, and while it is found to contribute only 10 – 15% of the total Ig in blood, IgA is the predominant and primary antibody present at mucosal sites. Mucosal IgA is primarily found as a secreted dimer, and this dimerization occurs via a polypeptide known as the J chain, which is highly expressed by B cells present in mucosal sites and is critical for IgA function. B cells on the basolateral surface of the epithelial layer of mucosal surfaces are sequestered from environmental pathogens. In order for IgA from activated B cells to reach pathogens for neutralization, transport across the epithelial barrier must occur. When IECs recognize pathogen in the intestinal lumen via toll-like receptor 4, signaling mediated by the intracellular adaptor protein MyD88 occurs leading to increased production of the polymeric Ig receptor (pIgR) on the basolateral surface of the IEC (Bruno, Rogier et al. 2010). While epithelial cells constitutively express pIgR, use of targeted deletion of *Myd88* in transgenic mice has demonstrated that loss of MyD88 severely impacts expression of pIgR on the basolateral surface of IECs, leading to subsequently reduced IgA transcytosis (Frantz, Rogier et al. 2012). Binding of J chain present on dimeric IgA to pIgR results in a calcium-dependent transcytosis of the IgA-pIgR complex through the IEC cytosol (Giffroy, Courtoy et al. 2001). Once the complex reaches the apical surface of the IEC, proteolytic cleavage occurs, resulting in the release dimerized IgA containing a portion of the pIgR, known as

the secretory component, resulting in a complex identified as secretory IgA (sIgA) (Johansen and Kaetzel 2011). sIgA interacts with a wide range of viral, bacterial, and parasitic pathogens that are present at the mucosal surface. Unlike IgG, sIgA does not induce activation of inflammatory immune cells, acting primarily through neutralization by binding microbial adhesins, agglutination, and mucus trapping leading to exclusion of pathogens from the mucosa (Woof and Kerr 2006).

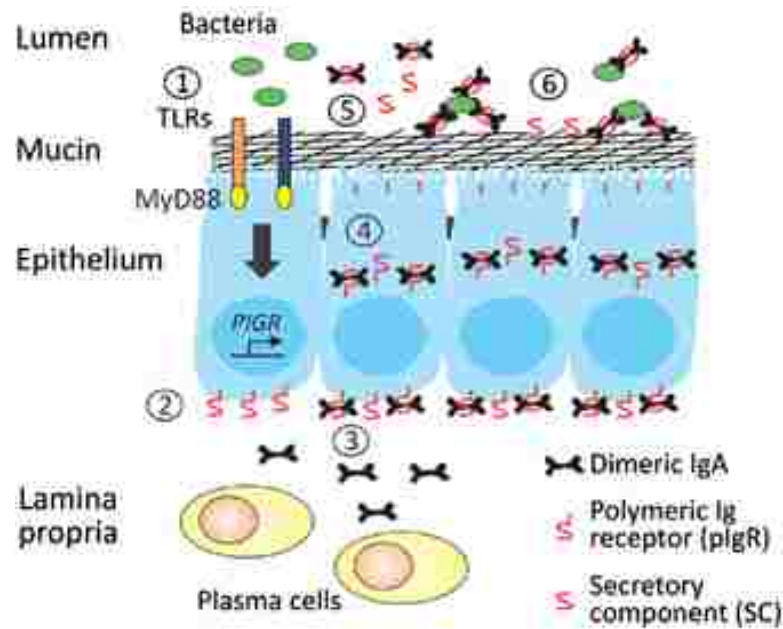


Figure 5.3 *IgA Transcytosis*. Transport of IgA across epithelium is a complex, highly regulated process. Recognition of antigens by toll-like receptors (TLRs) expressed by epithelial cells results in myD88-mediated transcription of *PIGR*, resulting in expression of polymeric Ig receptor (pIgR) on the basolateral surface of the epithelial cell. Dimeric IgA binds to pIgR, and undergoes transcytosis through the epithelial cell. Once on the luminal surface of the epithelial cell, dimeric IgA is cleaved while still carrying the secretory component (SC) (Johansen and Kaetzel 2011).

While the initiation of immunoglobulin-mediated immune responses typically occurs following antigen recognition, the existence of ‘natural’ antibodies that are produced by B-1 B cells have been identified (Lutz, Binder et al. 2009). These antibodies, which are mostly IgM but also include IgG and IgA, are characterized by germline-encoded genes in the variable region leading to stable, partially-restricted

reactivity pattern (Cukrowska, Sinkora et al. 1996). Production of these antibodies has been shown to be induced following recognition of IL-4 produced by natural killer T cells, but appears to be non-responsive to T cell major histocompatibility complex-mediated signaling (Kamijuku, Nagata et al. 2008). The role of natural antibodies in the recognition of self, altered self, and external antigens is an important first-response to external invasion, but also in maintaining systemic homeostasis (Simon and Spath 2003). While some studies have started to unravel the regulatory mechanism behind natural antibody production, there remain several uncertainties providing areas of exploration for further research.

5.2 RESULTS

5.2.1 Serum & Fecal Immunoglobulins in Naïve $IP^{-/-}$ Mice

Studies performed in our laboratory have shown that $IP^{-/-}$ mice have altered inflammatory responses (Jaffar, Wan et al. 2002; Jaffar, Ferrini et al. 2007; Jaffar, Ferrini et al. 2011). Further experiments were performed to determine if these effects were a consequence of specific immunological defects in the $IP^{-/-}$ mouse. Previously unpublished work done in our laboratory suggested that naïve $IP^{-/-}$ mice displayed decreased production of immunoglobulins of several isotypes (Teri Girtsman, Ph.D. Thesis). Specifically, naïve $IP^{-/-}$ mice displayed significantly reduced serum levels of IgG2b (93% reduction), IgA (70%), IgG2a (60%), and IgG1 (41%), in addition to a non-significant reduction in serum levels of IgE (43%) and IgG3 (38%) when compared to naïve C57BL/6 (WT) mice. No differences in serum levels of IgM were described when comparing $IP^{-/-}$ and WT mice. Since these immunoglobulin levels were analyzed in naïve

mice and were not elicited by any overt immunization, they most likely represent innate or ‘natural’ antibodies (Ochsenbein and Zinkernagel 2000).

A hallmark feature of mucosal immunity is the induction of a humoral immune response and subsequent production of various classes of immunoglobulins. In terms of secretory immunoglobulins, IgA has been previously described to comprise the majority of secretory immunoglobulins produced in humans, followed by the various IgG subclasses as well as IgM (Mestecky J 2005). The results described in Dr. Girtsman’s thesis suggested that mice lacking prostacyclin signaling had significantly reduced serum levels of ‘natural’ immunoglobulins that are specifically associated with mucosal immunity. Therefore, we attempted to characterize more fully the role of prostacyclin signaling in the regulation of mucosal-associated antibody production.

5.2.1.1 Naïve $IP^{-/-}$ Mice Display Decreased Systemic Production of IgA & IgG2b

In an attempt to replicate the results described in Dr. Girtsman’s thesis, we measured the serum antibody levels in $IP^{-/-}$ and WT mice as described in Chapter 2 Methods Section 2.0.8.1. Measurement of serum antibody levels revealed that IgG2b, IgA, and IgG1 were significantly decreased when compared to WT mice (26 $\mu\text{g/ml}$ compared to 414 $\mu\text{g/ml}$, 126 $\mu\text{g/ml}$ compared to 413 $\mu\text{g/ml}$, 447 $\mu\text{g/ml}$ compared to 800 $\mu\text{g/ml}$, respectively), as well as a non-significant decrease in IgG2c (449 $\mu\text{g/ml}$ compared to 734 $\mu\text{g/ml}$)(Figure 5.4). In contrast to Dr. Girtsman’s data, we observed a non-significant increase in serum IgM and IgG3 levels in $IP^{-/-}$ mice when compared to WT mice (953 $\mu\text{g/ml}$ compared to 567 $\mu\text{g/ml}$ and 615 $\mu\text{g/ml}$ to 219 $\mu\text{g/ml}$, respectively)(Figure 5.4). While there are some discrepancies in the serum antibody levels observed in naïve $IP^{-/-}$ mice between this and Dr. Girtsman’s work, comparison of

the data suggests that naïve IP^{-/-} mice consistently display decreased serum antibody levels of IgG2b and IgA when compared to WT mice.

Serum Immunoglobulins in IP^{-/-} and WT Mice

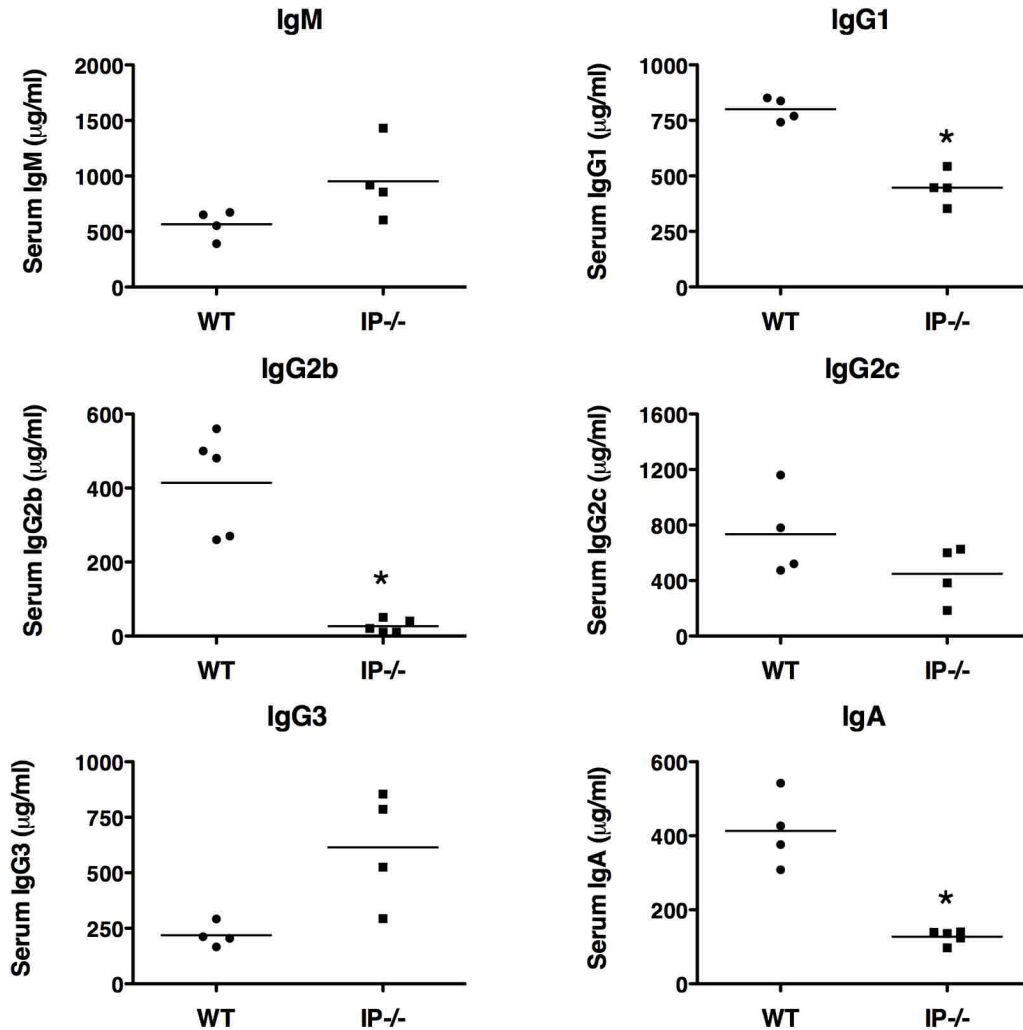


Figure 5.4 Naïve IP^{-/-} Mice Display Reduced Serum Levels of IgG1, IgG2b, and IgA. Serum was collected via cardiac puncture from naïve IP^{-/-} and WT mice and IgM, IgG1, IgG2b, IgG2c, IgG3, and IgA levels were determined by ELISA. Data are mean (n= 4 – 5) and are representative of two independent experiments. *p < 0.05, compared with WT group.

5.2.1.2 Naive IP^{-/-} Mice Display Decreased Bronchoalveolar and Fecal Secretion of IgA

As described in section 5.2.1.1, IP^{-/-} mice display significantly decreased serum levels of IgA. However, IgA is recognized to be the primary immunoglobulin present at mucosal locations including lymph nodes, small & large intestines, salivary glands, and bronchus- & nasal-associated lymphoid tissue (BALT/NALT)(Macpherson, McCoy et al. 2008). This close association with mucosal surfaces directly correlates with the high levels of IgA present in external secretions including nasal fluid, saliva, bronchoalveolar lavage fluid (BALF), and fecal excretions (Mestecky J 2005). While systemic IgA is found primarily in a monomeric form, IgA present at mucosal sites is primarily in a dimeric form consisting of two monomeric IgA subunits linked via a polypeptide referred to as the J chain (Koshland 1985). Recognition and binding of J chain by the polymeric immunoglobulin receptor (pIgR), which is expressed on the basolateral surface of epithelial cells, leads to transportation of dimeric IgA across the mucosal barrier (Vaerman, Langendries et al. 1998). Following transport to the apical surface of the epithelial cell, the IgA-J chain-pIgR complex is cleaved from the epithelial cell and maintains a portion of the pIgR remains attached and is known as the secretory component (Woof and Mestecky 2005). The majority (50-90%) of IgA present at mucosal surfaces and in external secretions displays this secretory component and is classified as secretory IgA (sIgA) (Woof and Mestecky 2005). In order to quantify sIgA, our lab had previously developed a specific ELISA described in Chapter 2 Methods

Section 2.0.8.2 in which anti-secretory component antibody was used for sample capture and anti-IgA was used for sample detection (Jaffar, Ferrini et al. 2009).

For this study, we focused on the production of sIgA at two mucosal sites, specifically the airways and gastrointestinal tract, by measurement of sIgA levels in the BALF and fecal extract, respectively. Measurement of sIgA present in BALF revealed that naïve $IP^{-/-}$ mice had markedly decreased BALF when compared to naïve WT mice (Figure 5.5). Similarly, measurement of fecal sIgA revealed that naïve $IP^{-/-}$ mice had significantly decreased fecal sIgA when compared to WT mice (Figure 5.5). These results suggest that loss of prostacyclin signaling has a critical effect on sIgA production at two distinct mucosal locations.

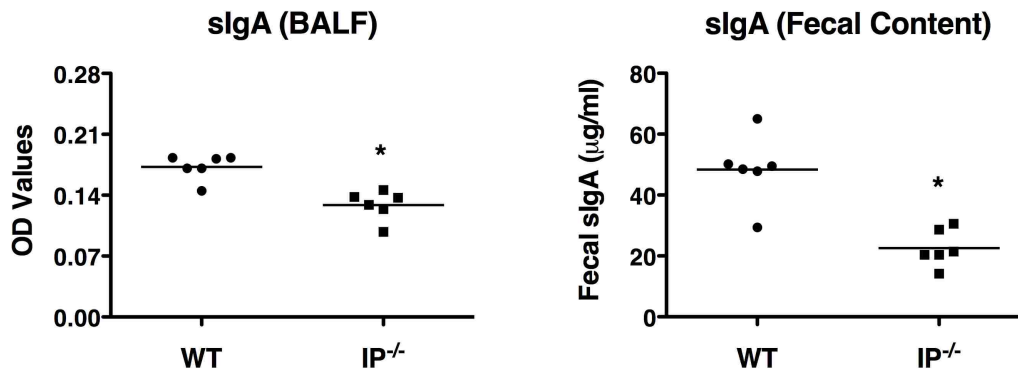


Figure 5.5 Naïve $IP^{-/-}$ Mice Display Reduced sIgA Production. Bronchoalveolar lavage fluid and fecal content was collected from naïve $IP^{-/-}$ and WT mice and sIgA levels were determined by ELISA. Data are mean (n= 6) and are representative of three independent experiments. * $p < 0.05$, compared with WT group.

5.2.1.3 Immunohistological Examination of IgA^{+} B Cells in Naïve $IP^{-/-}$ Mouse

Intestinal Lamina Propria & Peyer's Patches

The development of an IgA-mediated immune response can be divided into two distinct phases occurring in similar, yet distinct locations within the mucosa. The induction phase occurs primarily in mucosa-associated lymphoid tissues (MALT) and

mucosa-draining lymph nodes, while the effector phase occurs primarily in the lamina propria and surface epithelia of mucosal surfaces (Brandtzaeg, Kiyono et al. 2008). It has been described since the 1970s that Peyer's patches (PP) are MALT structures present in the small intestine that are the main site of sIgA induction in humans, rodents, and rabbits, and that stimulation of IgA⁺ plasmablasts in PP leads to recirculation and homing to other mucosal sites outside the gastrointestinal system (Craig and Cebra 1971; Pierce and Gowans 1975).

With the knowledge that naïve IP^{-/-} mice appear to have significantly decreased production of fecal IgA as described in Chapter 5 section 5.2.1.2, we examined the intestinal mucosa to further analyze the underlying cause of IgA deficiency. Histologic examination of the small intestinal tissue from naïve IP^{-/-} and WT mice revealed that IP^{-/-} mice displayed a severely reduced presence of IgA⁺ B cells in the lamina propria when compared to WT mice (Figure 5.6). Additional histologic examination of PP from naïve IP^{-/-} and WT mice revealed a similarly marked reduction in the presence of IgA⁺ B cells when compared to WT mice (Figure 5.6).

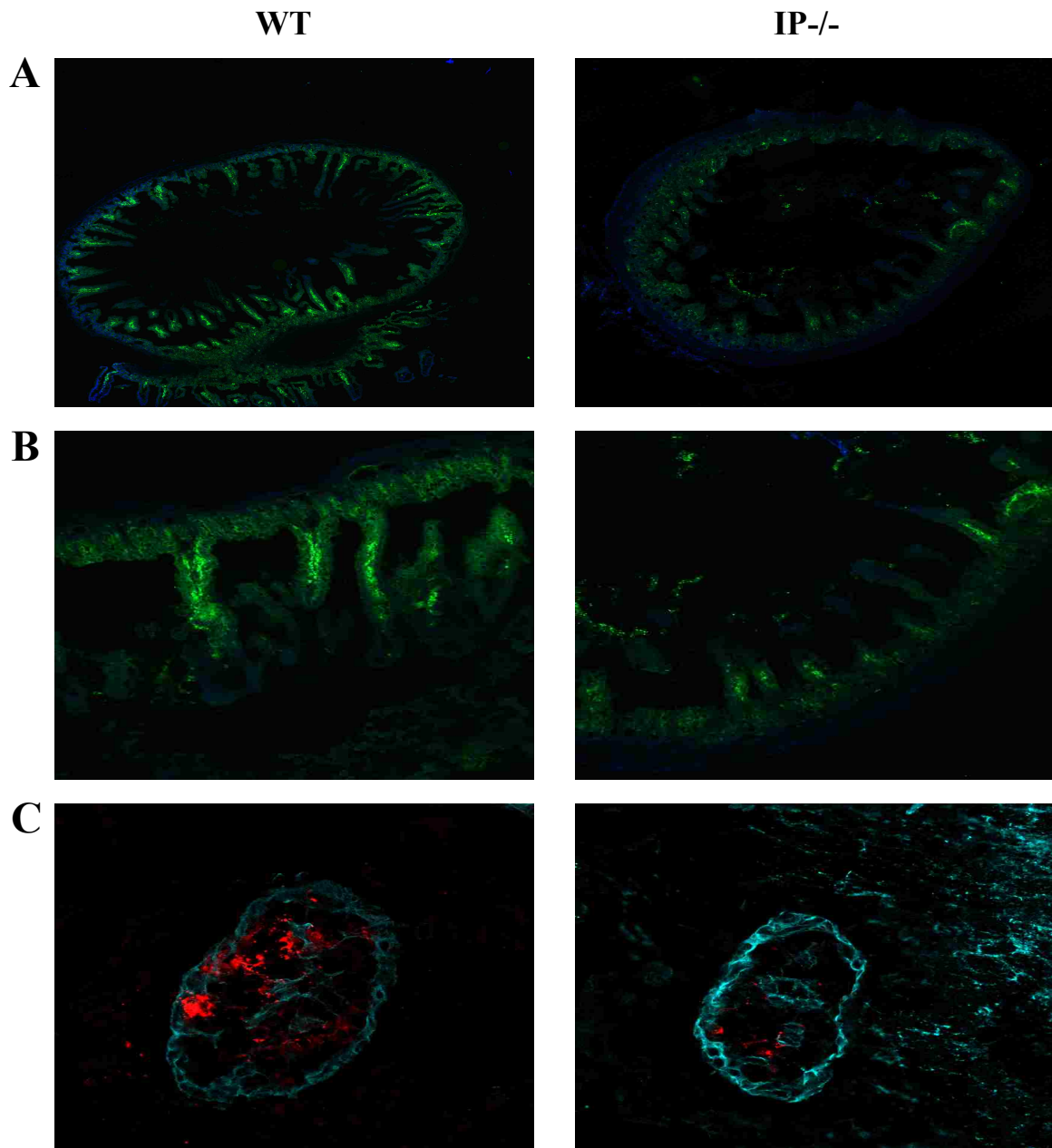


Figure 5.6 Naïve IP^{-/-} Mice Display Reduced IgA⁺ B Cells in the Lamina Propria and Peyer's Patches. Histological examination of the lamina propria and Peyer's patches of naïve IP^{-/-} and WT mice using immunofluorescence microscopy. (A) Intestinal cross-section of the small intestine lamina propria. IgA⁺ B cells were stained with anti-IgA (green). (B) Intestinal cross-section of the small intestine lamina propria (20X). (C) Peyer's patches of the small intestine (60X). Sections were stained with anti-CD45R/B220 (blue) and anti-IgA (red).

Flow cytometry analysis of naïve IP^{-/-} and WT PP revealed a marked reduction in B220⁺IgA⁺ cells in naïve IP^{-/-} mice compared to WT mice (7.6% compared to 16%)(Figure 5.7). No differences were observed when examining B220⁺IgM⁺ cells in naïve IP^{-/-} mice compared to WT mice (~11% in both strains)(Figure 5.7). These results suggest that loss of prostacyclin signaling affects not only the presence of IgA⁺ B cells at effector sites such as the intestinal lamina propria, but that the primary cause of the IgA deficiency observed in naïve IP^{-/-} mice could be a result of the reduced presence of IgA⁺ B cells present in PP resulting from a decreased inductive capability.

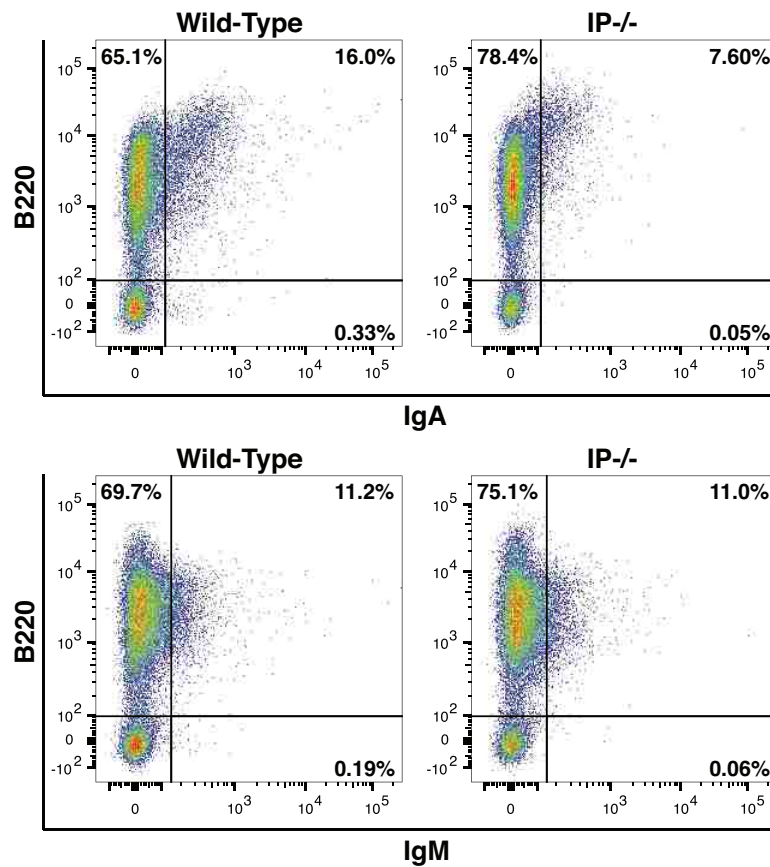


Figure 5.7 Flow Cytometry Analysis of Naïve IP^{-/-} Mice Display Reduced IgA⁺ B Cells in Peyer's Patches. Peyer's patches from naïve IP^{-/-} and WT mice were excised from the small intestine and mechanically and chemically dissociated. Peyer's patch cells were stained with anti-B220 (APC), anti-IgA (FITC), and anti-IgM (PE) and analyzed for receptor and immunoglobulin expression using a BD FACSAria flow cytometer.

5.2.2 Characterization of Altered Intestinal Secretory IgA Production in Naïve $IP^{-/-}$ Mice

5.2.2.1 No Alteration in Peyer's Patch Development or Structure is Observed in Naïve $IP^{-/-}$ Mice

Development of PP in the gastrointestinal tract occurs during embryogenesis, after approximately 16 days in mice, and is controlled by $IL7R^{+}CD3^{-}CD4^{+}CD45^{+}$ cells known as lymphoid tissue inducer cells (LTIs)(Yoshida, Honda et al. 1999). These LTIs are responsible for the recruitment and attachment of $VCAM-1^{+}$ and $ICAM-1^{+}$ stromal cells in the intestinal wall as a structural base for the formation of PP (Lamichhane, Azegami et al. 2014). Studies utilizing a variety of transgenic knockout-mice have demonstrated that deficiency in LTI-produced lymphotoxin- α & - β , as well as loss of LTI-expressed lymphotoxin- β receptor and IL-7R results in lack of PP development (Alimzhanov, Kuprash et al. 1997; Futterer, Mink et al. 1998; Yoshida, Honda et al. 1999). Similar to other secondary lymphoid tissues such as the spleen and lymph nodes, PP are organized into follicles comprised of B-cell rich germinal centers and T-cell rich intrafollicular regions, although unlike lymph nodes and spleen, the PP germinal centers are found even under homeostatic conditions due to the constant stimulation by commensal bacteria present in the intestinal lumen (Kunisawa, Kurashima et al. 2012). As PP are the main inductive sites for IgA-mediated immune responses, a reduction in the number of PP present in the small intestine or alteration in the gross follicle structure could be responsible for the marked reduction of IgA^{+} B cells present in the lamina propria of the intestines of naïve $IP^{-/-}$ mice described in Chapter 5 section 5.2.1.3. However, examination of PP in the small intestines of naïve $IP^{-/-}$ mice revealed no

significant differences in the total number of PP when compared to WT mice (data not shown). The binding of peanut agglutinin (PNA) by B cells to characterize PP germinal centers has been described previously (Butcher, Rouse et al. 1982), and immunofluorescence microscopy utilizing PNA staining revealed that germinal center formation was unaffected when comparing $IP^{-/-}$ and WT mice (Figure 5.8). The failure to generate intestinal lymphoid tissues such as PP might have been a consequence of alterations in LTI cells, but these results suggest loss of prostacyclin signaling does not adversely affect the ability of LTI cells to induce generation of intestinal lymphoid tissues. Additionally, these results suggest that loss of prostacyclin signaling does not result in reduced formation or gross structural defects of PP in the small intestine that could be responsible for the reduced IgA observed in the fecal secretions of naïve $IP^{-/-}$ mice.

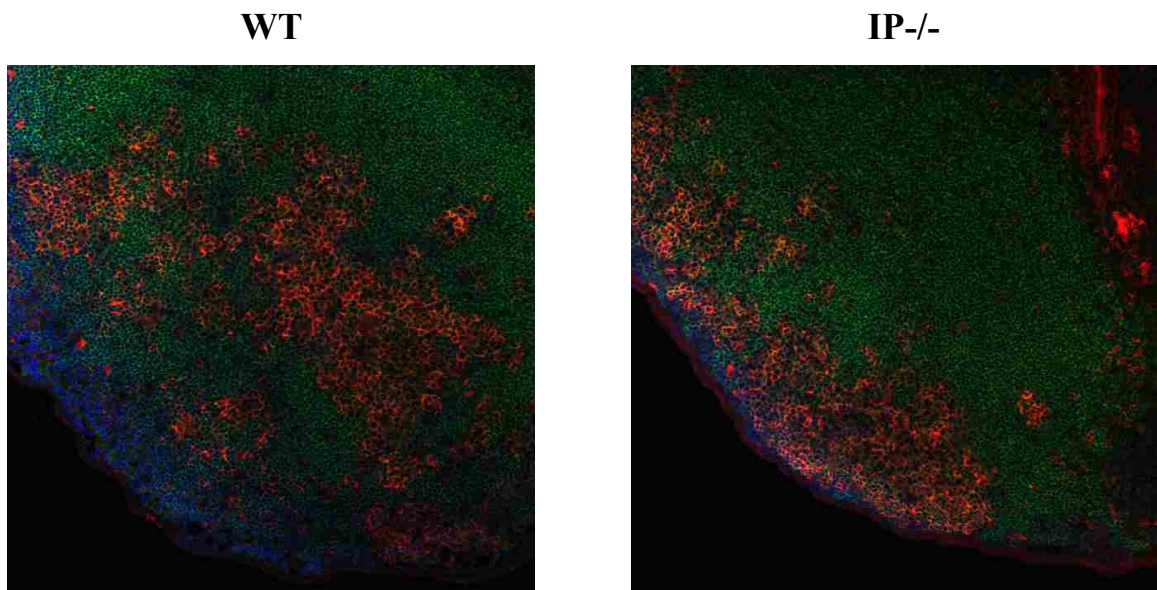


Figure 5.8 Naïve $IP^{-/-}$ Mice Display No Alteration in Peyer's Patch Germinal Center Formation. Histological examination of Peyer's patch germinal centers in $IP^{-/-}$ and WT mice using immunofluorescent microscopy. Sections were stained with peanut agglutinin (PNA)(red), anti-CD45R/B220 (green), and DAPI (blue)(40X)

5.2.2.2 Naïve IP^{-/-} Mice Display Decreased Intrafollicular CD3⁺ Cells in Peyer's Patches

Although there were no observable differences in the total number or gross structural organization of naïve IP^{-/-} mouse PP as described in Chapter 5 section 5.2.2.1, alterations in the presence of specific immune cell populations required for the initiation of an IgA-associated immune response could be responsible for the reduction in IgA observed in naïve IP^{-/-} mice. In addition to the T and B cell populations that comprise approximately 95% of the immune cells present in the PP, dendritic cells are found in the subepithelial dome region in addition to the intrafollicular region and are involved in the recognition and presentation of intestinal antigens to T and B cells present in the PP (Milling, Yrlid et al. 2010). Utilizing immunofluorescent microscopy, we found that the PP of naïve IP^{-/-} mice displayed a marked reduction in CD3⁺ cells in the inter-follicular region when compared to WT mice, while no noticeable differences in B220⁺ cells were observed in the germinal centers (Figure 5.9).

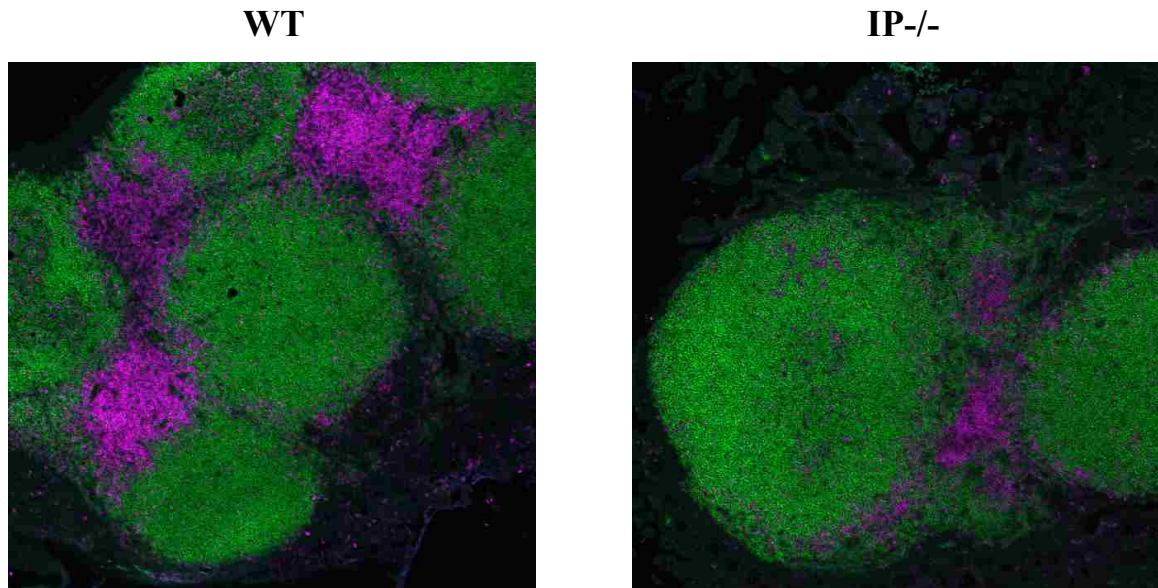


Figure 5.9 Naïve $IP^{-/-}$ Mice Have Decreased Numbers of $CD3^{+}$ cells in Peyer's Patch Intrafollicular Regions. Histological examination of Peyer's patch germinal centers in $IP^{-/-}$ and WT mice using immunofluorescent microscopy. Sections were stained with anti-CD3 (pink) and anti-CD45R/B220 (green)(20X)

Interestingly, specific analysis of $CD3^{+}$ cells present in the PP utilizing FACS indicated that while there was a slight decrease in the number of $CD3^{+}CD4^{+}$ cells present in the PP, a more substantial decrease was observed in the number of $CD3^{+}CD8^{+}$ cells present in the PP of naïve $IP^{-/-}$ mice when compared to naïve WT mice (Figure 5.10). Although $CD8^{+}$ T cells are traditionally recognized to suppress Ig production in B cells (van Vlasselaer, Gascan et al. 1992), more recent work has shown that $IFN-\gamma$ produced by $CD8^{+}$ T cells can preferentially induce B cell class switching from IgG1 to IgG2a and IgG2b (Mohr, Cunningham et al. 2010). These results suggest that alterations in the $CD3^{+}$ cells in the PP could be a potential cause of the IgA deficiency observed in naïve $IP^{-/-}$ mice.

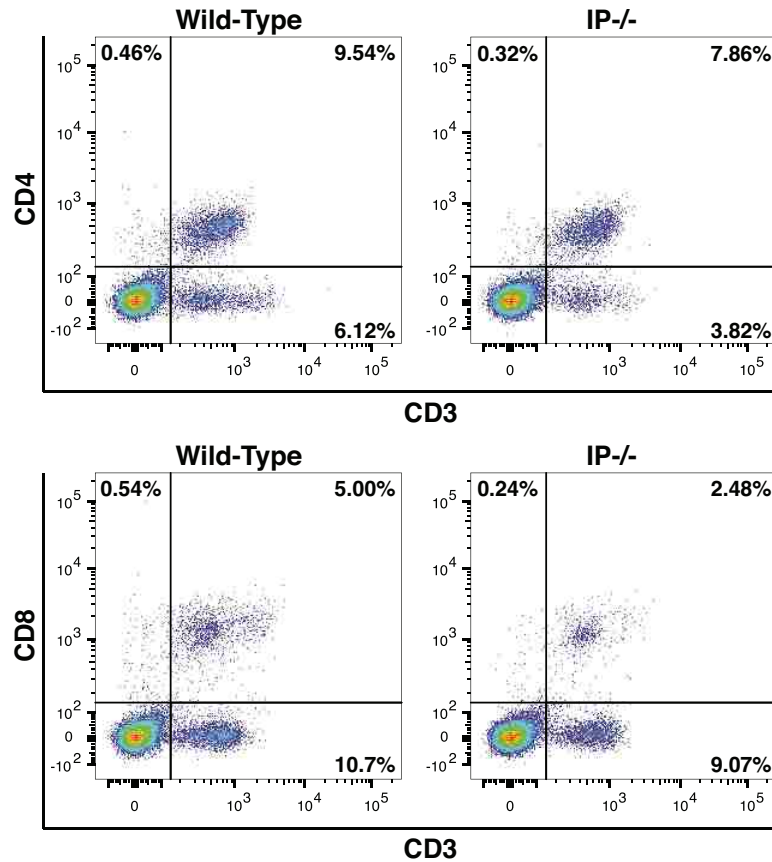


Figure 5.10 FACS Analysis of Naïve IP^{-/-} Mice CD3⁺ Cells in Peyer's Patches. Peyer's patches from naïve IP^{-/-} and WT mice were excised from the small intestine and mechanically and chemically dissociated. Peyer's patch cells were stained with anti-CD3 (AF647), anti-CD4 (FITC), and anti-CD8 (FITC) and analyzed for receptor expression using a BD FACSAria flow cytometer.

5.2.2.3 Naïve IP^{-/-} Mice Display No Alteration in the Number of ILC3 Cells Present in Peyer's Patches

As described in more detail in Chapter 4 section 4.2.1.1, naïve IP^{-/-} mice display increased systemic expression of activating receptor NKp46 (Figure 4.9). While NKp46 is primarily recognized as a natural killer cell activating receptor, in the last few years much attention has been focused on describing, characterizing, and classifying new populations of NK-related innate immune cells (Walker, Barlow et al. 2013) (Spits, Artis et al. 2013). These cells, known as innate lymphoid cells (ILCs), play a critical role in

the regulation of several mucosal inflammatory responses, including asthma and Crohn's disease (Geremia, Arancibia-Carcamo et al. 2011; Halim, Krauss et al. 2012). The current classification system divides ILCs into 3 distinct groups based on cytokine production, surface receptor expression, and transcription factor expression (Spits, Artis et al. 2013). Several well-described innate lymphocyte populations, such as natural killer cells and LTIs, have been recognized to share several distinctive characteristics of ILCs and have been included in the ILC classification.

In terms of intestinal immunity, Group 3 ILCs (ILC3s) have been shown to be the most prominently involved ILC population (Luci, Reynders et al. 2009). The Group 3 ILC family is comprised of NKp46⁺ ILC3s, NKp46⁻ ILC3s, and LTIs, classified primarily on the requirement of IL-7 for development, expression of transcription factor ROR γ t, and production & responsiveness to Th17-associated cytokines (Walker, Barlow et al. 2013). Of particular interest, splenic NKp46⁺ ILC3s have been demonstrated to reside in the splenic marginal zone and produce IgA-promoting factors APRIL and BAFF (Magri, Miyajima et al. 2014). The ability to influence IgA production, when taken into consideration with the alterations in NK cell populations described in Chapter 4, make NKp46⁺ ILC3s an intriguing possibility for involvement in the IgA deficiency observed in naïve IP^{-/-} mice.

In order to determine the possible role of NKp46⁺ ILC3s, we analyzed Peyer's patches of naïve WT and IP^{-/-} mice utilizing FACS for alterations in cellular markers typically associated with Type 3 ILCs. As observed in Chapter 4 Results section 4.2.1.1, naïve IP^{-/-} mice displayed a markedly increased number of NKp46⁺ cells in the PP when compared to naïve WT mice, which were evenly distributed between NK1.1⁺NKp46⁺ and

NK1.1⁻NKp46⁺ groups (Figure 4.9). Further examination of NKp46⁺ cells from naïve IP^{-/-} and WT mice PP revealed that there was no expression of ILC3-associated surface markers IL23r, CD127, or intracellular expression of transcription factor ROR γ t observed in either mouse strain (Figure 5.11). These results suggest that, while NKp46⁺ cells are increased in the PP of naïve IP^{-/-} mice, these cells are likely to be NK cells, not ILC3s, based on expression of ILC3 cellular markers. The role of these NK cells in the IgA deficiency observed in naïve IP^{-/-} mice remains to be determined.

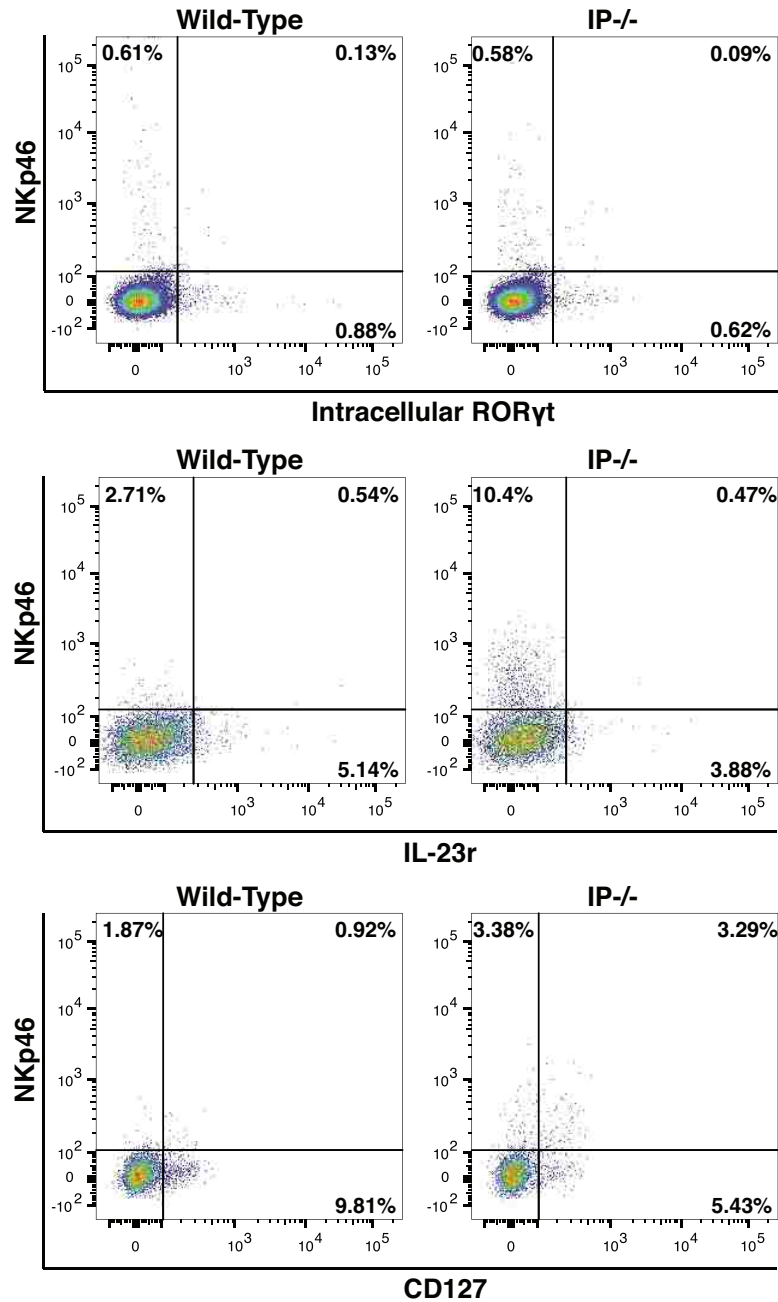


Figure 5.11 FACS Analysis of Naïve IP $^{-/-}$ ILC3 Cells in Peyer's Patches. Peyer's patches from naïve IP $^{-/-}$ and WT mice were excised from the small intestine and mechanically and chemically dissociated. Peyer's patch cells were stained with anti-NKp46 (PE, APC), intracellular anti-ROR γ t (APC), anti-IL-23r (PE), and anti-CD127 (IL-7r)(APC) and analyzed for receptor and transcription factor expression using a BD FACSAria flow cytometer.

5.2.2.4 *In vitro* Stimulation of Peyer's Patches from Naïve $IP^{-/-}$ Mice with IL-23

Results in Decreased Production of IL-22

The main effector cytokine of group 3 ILCs is recognized as IL-17 (Spits, Artis et al. 2013). However, NKp46⁺ ILC3s have been shown to produce IL-22, not IL-17, following stimulation with IL-23 (Cella, Fuchs et al. 2009). IL-22 is a member of the IL-10 family of cytokines, and exerts an important role in intestinal defense and epithelial barrier integrity (Tumanov, Koroleva et al. 2011). In order to determine whether IL-22 production in the PP was impacted by loss of prostacyclin signaling, we performed *in vitro* stimulation of dissociated PP with IL-23 as described in Chapter 2 Methods Section 2.0.7.1. Stimulation of PP with IL-23 resulted in significantly decreased production of IL-22 in naïve $IP^{-/-}$ mice when compared to WT mice (125 pg/ml compared to 325 pg/ml)(Figure 5.12). Additionally, we attempted to measure IL-17 production in dissociated PP following IL-23 stimulation, but IL-17 levels were found to be below the threshold of detection. These results suggest that loss of prostacyclin signaling leads to a reduction of IL-23-derived IL-22 production in the PP that could have potential deleterious effects on intestinal immunity.

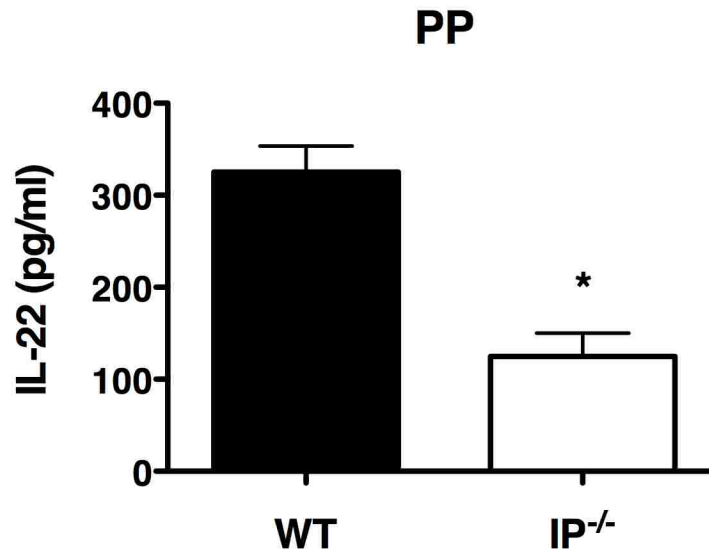


Figure 5.12 Peyer's Patches from Naïve IP^{-/-} Mice Display Reduced Production of IL-22 in Response to IL-23 Stimulation. Peyer's patches from naïve IP^{-/-} and WT mice were excised from the small intestine and mechanically and chemically dissociated. Peyer's patch cells were stimulated with recombinant IL-23 (20 ng/ml) for 24 h and supernatants analyzed for IL-22 production by ELISA. Data are mean ± SEM (n=6) in triplicate and are representative of two independent experiments. *p < 0.05, compared with WT mice

5.2.3 Depletion of Natural Killer Cells Does Not Affect Natural IgA or IgG2b in Naïve IP^{-/-} Mice

As described in Chapter 4, the altered natural killer cell population found in naïve IP^{-/-} mice demonstrated an effective ability to regulate the development of asthma, a classically defined Th2-associated disease. Regulation of Th2 effector function by this altered lymphocyte population could therefore be a possible mechanism underlying the immunoglobulin deficiency described in Chapter 5 section 5.2.1.1. In order to examine this hypothesis, depletion of NK cells utilizing anti-NK1.1 mAb PK136 was performed in naïve WT and IP^{-/-} mice as described in Chapter 2 Methods Section 2.0.5.1. ELISA analysis of serum and fecal IgA levels in both IP^{-/-} and WT mice revealed that depletion

of NK1.1⁺ cells did not significantly alter sIgA production when compared to non-depleted control mice of either strain (Figure 5.13). These results suggest that NK cells are not responsible for the sIgA deficiency observed in naïve IP^{-/-} mice.

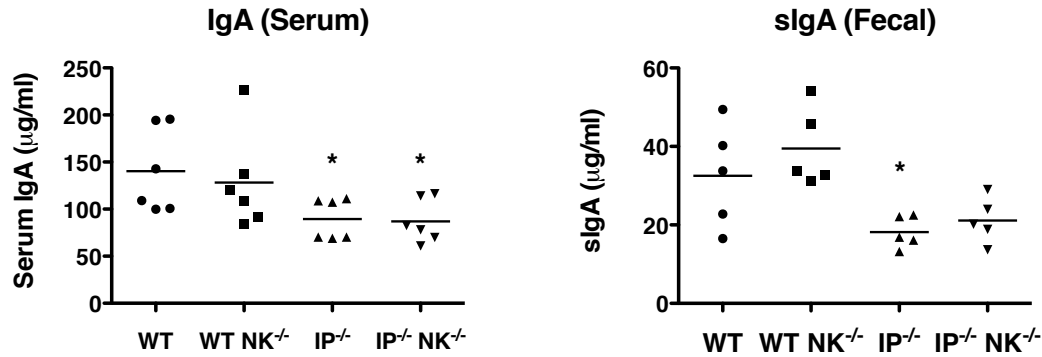


Figure 5.13 Systemic Depletion of NK Cells Does Not Alter Serum or Fecal IgA Levels. Naïve IP^{-/-} and WT mice received intraperitoneal (i.p.) injection of PK136 (250 µg) on day 0, followed by subsequent i.p. injection on day 3, 6, 10, and 13. Serum and fecal samples were collected on day 14 and analyzed by ELISA. Data are mean (n=6; n=5) and are representative of three independent experiments. *p < 0.05, compared with WT mice

5.3 DISCUSSION

Early characterization of IP^{-/-} mice revealed that total serum immunoglobulin levels were markedly reduced compared to WT mice. It is important to note in this study that both IP^{-/-} and WT mice were maintained germ-free in techniplast isolators, and should be considered specific pathogen free, however they are likely to retain undefined commensal intestinal microflora. Consequently, serum immunoglobulin levels of naïve mice are likely to reflect the release of natural antibodies as well as antibodies elicited by residual microflora. Natural antibodies have been shown to play a critical role in the protection of mucosal surfaces through neutralization, maintenance of B cell homeostasis, binding and clearance of bacterial pathogens, and recognition of self-antigens

(Ochsenbein, Fehr et al. 1999; Ehrenstein and Notley 2010; Panda, Zhang et al. 2013). Consequently, understanding the regulatory processes underpinning the production of natural antibodies has potential to greatly expand potential health outcomes. While the role of prostaglandins in controlling antigen-specific B cell responses has been well characterized, the role of prostaglandins in the regulation of non-specific natural antibody production remains unresolved.

In this study, we attempted to elucidate the effects of prostacyclin on the serum antibody levels. Natural antibodies have been previously described as non-specific immunoglobulins which are predominantly IgM, but may also include IgG or IgA, that are constitutively produced by B cells in the absence of antigenic stimulation (Cukrowska, Sinkora et al. 1996). Consistent with previously unpublished work performed in our lab, $IP^{-/-}$ mice displayed attenuated production of natural antibodies. Analysis of serum antibody levels in naïve $IP^{-/-}$ mice revealed that IgG2b, IgG2a, and IgA were dramatically reduced in comparison to WT mice. Additionally, secretory IgA levels in fecal contents were also found to be significantly reduced in $IP^{-/-}$ mice. The production of IgA by IgA^{+} plasmablasts located in the PP and LP of the intestinal mucosa is likely a major contribution to serum IgA levels (Craig and Cebra 1971; Pierce and Gowans 1975). Consistent with the findings that intestinal production of IgA was decreased in $IP^{-/-}$ mice, a reduced presence of IgA^{+} B cells in both the LP and PP was observed. As total B cell numbers remained unchanged between strains, these results are suggestive of alterations in the capacity of intestinal B cells to undergo class-switching from IgM, putatively as a result of deficiencies in prostacyclin signaling. Indeed, work performed by Roper et al indicated that prostaglandins, specifically PGE_2 , have the

capacity to induce B cell class-switching in the production of IgG1, although the mechanism by which this occurs is still under scrutiny (Roper, Graf et al. 2002). Supporting this hypothesis, we observed no differences in intestinal IgM⁺ B cells, suggesting that naïve B cells are not undergoing differentiation into IgA or IgG2b-producing B cells. In this context, it is interesting to note that both IgA and IgG2b isotype switching is dependent on TGF- β and both isotypes are absent in mice lacking TGF- β (Borsutzky, Cazac et al. 2004; Park, Seo et al. 2005).

Since several different mechanisms could be responsible for the regulation of B cell responses in the intestinal mucosa, we attempted to elucidate specific alterations in the IP^{-/-} mouse that could lead to altered antibody production. While the formation of PP in the intestinal epithelium is not a strict requirement for the induction of mucosal IgA responses to oral antigens, loss of PP has been demonstrated to have deleterious effects on the intestinal IgA response to bacterial pathogens (Yamamoto, Rennert et al. 2000; Hashizume, Togawa et al. 2008). Examination of the gross structure of PP revealed that loss of prostacyclin signaling does not lead to any alterations in overall development of PP formation or number present in the intestinal mucosa. Similarly, structural formation of PP in IP^{-/-} mice appeared to be unaffected since the number and size of germinal centers within the PP was normal when visualized using peanut agglutinin. However, determination of the cellular population comprising PP revealed that IP^{-/-} mice have reduced numbers of intrafollicular CD3⁺ lymphocytes. As CD4⁺ T cells are critical for the activation and development of B cell responses, alterations in this population could result in attenuated intestinal IgA production. Interestingly, analysis of T cell populations within IP^{-/-} PP revealed that the observed decrease in CD3⁺ T cells was CD8⁺ T cells, not

CD4⁺ T cells. While CD8⁺ T cells are typically recognized to suppress immunoglobulin production, recent work has demonstrated that CD8⁺ T cell-derived IFN- γ can preferentially induce the production of IgG2a and IgG2b, and the results of our study suggest a potential role for prostacyclin signaling in regulating CD8⁺ T cell responses responsible for the induction of B cell class-switching (Mohr, Cunningham et al. 2010). It is unclear how our finding of altered CX3CL1 production in IP^{-/-} mice described in Chapter 4 (Figure 4.20 and Figure 4.32) impacts the intestinal mucosa. However, it is interesting to note that CX3CL1 is a chemotactic factor for both CD8⁺ T cells and NK cells (Nishimura, Umehara et al. 2002). Conceivably, these types of cells are depleted from the PP as a consequence of CX3CL1-mediated migration to the intestinal lumen. However, further analysis of intestinal CD8⁺ T cell populations in the intestinal mucosa of IP^{-/-} mice will be needed to clearly establish the regulatory capabilities of prostacyclin signaling in this regard.

While Th2-associated responses are classically involved in the differentiation and development of B cell responses, factors derived from other immune cell populations also can influence intestinal IgA responses. In the past few years, the field of immunology has expanded greatly through the publication of studies involving the classification, characterization, and immunoregulatory role of innate lymphoid cells (ILCs) (Walker, Barlow et al. 2013) (Spits, Artis et al. 2013). Group 3 ILCs (ILC3s) have been implicated to prominently regulate intestinal immune responses, and splenic ILC3s expressing NKp46 have been demonstrated to produce IgA-promoting factors APRIL and BAFF, which are responsible for T-cell independent B cell activation and differentiation (Luci, Reynders et al. 2009; Magri, Miyajima et al. 2014). In Chapter 4 of this

dissertation, we demonstrated that $IP^{-/-}$ mice have systemically increased expression of NKp46, including in the PP (Figure 4.9). Although these cells were identified as NK cells in the airway, the presence of increased NK1.1⁻NKp46⁺ cells in the PP could indicate potential alterations in intestinal ILC3s. However, further characterization of this NKp46⁺ population based on several ILC3-specific surface and transcriptional markers revealed that these immune cells were unlikely to be ILC3s. It is possible that alterations in the ILC3 population exist in the $IP^{-/-}$ mouse, as a high density of the intestinal ILC3 population is present in the intestines exist in the LP and as IELs. However, attempts to isolate cells from these locations proved to be unsuccessful, and further studies involving these sites do represent an area of potential further development. The regulation of asthma, a classically defined Th2-associated disease, by the altered NK cells described in Chapter 4 of this dissertation, raises the interesting potential that NK cells present in the airway and intestinal mucosa could be responsible for the decreased production of natural antibodies through an as-of-yet undefined mechanism. However, depletion of NK cells in both $IP^{-/-}$ and WT mice failed to lead to any significant alterations in serum or fecal IgA levels.

Stimulation of dissociated $IP^{-/-}$ mouse PP with recombinant IL-23 resulted in significantly decreased production of IL-22. As IL-22 exerts an important role in the maintenance of intestinal defense and epithelial barrier integrity, deficiencies in IL-22 production have the capacity to greatly influence the intestinal mucosa (Tumanov, Koroleva et al. 2011). However, IL-23-induced production of IL-22 is displayed by a wide variety of immune cells in the intestinal mucosa, including Th17, Th22, $\gamma\delta$ T cells, NK cells, and ILC3s including LTIs (Zenewicz, Abraham et al. 2010). Prostaglandins,

specifically PGE₂, have been implicated to play a role in enhancing IL-22-mediated signaling in the intestines, and PGI₂ has been shown to promote the development of $\gamma\delta$ T cells in the airway that have the potential to produce IL-22 (Jaffar, Ferrini et al. 2011; Martin, Beriou et al. 2014). Further studies will need to be completed in order to identify the specific immune cell populations that are affected by loss of prostacyclin signaling resulting in attenuated IL-22 production, and the specific mechanism in which prostacyclin mediates regulation.

In summary, this study demonstrated that IP^{-/-} mice display attenuated production of antibodies in both the serum as well as the intestinal mucosa, representing a novel look at the role of prostacyclin signaling on antibody production in the absence of a known pathogen. Although further studies are needed to determine the exact mechanism underlying this attenuation, this study suggests a previously unrecognized role for prostaglandin signaling in the regulation of mucosal and systemic humoral immune responses.

Chapter 6

Conclusions

Mucosal barriers represent the most critical interface for the interaction between the immune system and environmental pathogens. The regulation of immunological function at these sites, through the actions of both exogenous and endogenous mediators, should therefore be considered key to understanding how responses to environmental pathogens are sequestered. In this dissertation, we attempted to elucidate the role of two specific inflammatory mediators, S-nitrosoglutathione and prostaglandin I₂, in the regulation of immunity in the airways and gastrointestinal tract. Strong functional parallels exist between PGI₂ and nitric oxide (and by inference SNOs) since both are potent inhibitors of platelet aggregation and vasodilation (Mitchell, Ali et al. 2008). Our findings offer novel insights into how these vital inflammatory mediators regulate immunological responses, and pave the way for new opportunities for therapeutic approaches targeting mucosal-associated diseases.

The steady increase in the prevalence of asthma in developed and developing countries over the past several decades represents a substantial public health crisis. The heterogeneous nature of the disease has created difficulties in our ability to fully focus our therapeutic options to lessen the impact and incidence of disease. Thus, a need has been predicated for a deeper understanding of the basal mechanisms responsible for the pathogenesis and resolution of asthma. Allergic asthma has been classically characterized as a chronic inflammatory disorder driven by predominant activation of a Th2-mediated adaptive immune response. However, attempts to regulate the Th2-associated immune response have not proven effective at reversing the disease process as

expected. Our data attempts to bridge our understanding of both the adaptive and innate immune responses in the pathogenesis of asthma by expanding our knowledge of the regulatory mediators S-nitrosoglutathione and prostaglandin I₂.

Inhibition of S-nitrosoglutathione reductase with the specific inhibitor, SPL-334, significantly reduced the development of allergic airway inflammation in a mouse model of allergic asthma. This was the first demonstration that this class of inhibitor can limit allergic airway inflammation. We speculate that this reduction is a consequence of increased airway concentrations of S-nitrosoglutathione, which has been shown to reverse allergic airway inflammation in response to allergen challenge. Limitations of our study arise from the fact that we did not measure the total levels of SNOs in the airway, or determine the cellular prevalence of S-nitrosoglutathione. Additionally, SPL-334 was found to be relatively insoluble, making administration of the drug difficult, and lack of access to GSNOR^{-/-} mice as a comparative tool to analyze the efficacy of SPL-334. Undoubtedly, resolution of these issues would be key to a more detailed understanding of the molecular mechanism underpinning the attenuated inflammation seen following SPL-334-mediated inhibition of S-nitrosoglutathione reductase.

Examination of the immunological properties of IP^{-/-} mice revealed that the PGI₂ receptor plays an important role in regulating the expression of NKp46 by NK cells, as well as controlling the number of NK cells residing in the lung. This is the first time that it has been shown that PGI₂ signaling plays an important role in the immunobiology of NK cells. In part, this may result from dysregulated expression or shedding of CX3CL1, possibly as a consequence of either dendritic cells or endothelial cells lacking IP expression. The resolution of the role of CX3CL1 signaling on the properties of

pulmonary NK cells and allergic inflammation could be accomplished through the use of CX3CL1-mCherry and CX3CR1-gfp reporter mice. Certainly, pulmonary NK cells in IP^{-/-} mice were shown to be responsible for attenuated development of allergic airway inflammation following exposure to house dust mite allergen, although the mechanism responsible remains to be resolved. These results suggest a previously unknown role for prostaglandin I₂ in the regulation of NK cell effector function, and also suggest a previously underappreciated capacity for NK cell regulation of asthma pathogenesis. Determining the effectiveness of specific IP antagonists in suppression of allergic inflammation, in addition to the impact of CX3CL1 release in the airway is a high priority and may form the basis for novel therapeutic approaches.

Our third study addressed how intestinal humoral immune responses are regulated through the actions of prostaglandin I₂. The observation that IP^{-/-} mice displayed a significant reduction in immunoglobulin production in the periphery and gastrointestinal tract was unexpected. While we were unable to resolve the mechanism underlying this effect, the potential role of CX3CL1 in modulating the reduced intestinal IgA response is worthy of future investigation, as CX3CL1 has been shown to play a critical role in the sampling of intestinal luminal antigens by dendritic cells (Niess, Brand et al. 2005).

In summary, the three complementary studies described in this dissertation provide insight as to events that underpin mucosal inflammatory processes. Future studies will focus on the examination of the relative role of NK cells and CX3CL1 in the immunological defects described in the airway and gastrointestinal tract of IP^{-/-} mice, leading to greater understanding of the molecular and cellular processes responsible for protection against environmental pathogens.

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