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Investigation Into Regulatory Mechanisms That Limit The Th2 Response in a Mouse Model of Allergic Asthma

By

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Dissertation

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Investigation Into Regulatory Mechanisms That Limit The Th2 Response in a Mouse Model of Allergic Asthma

Chairperson: Kevan Roberts, Ph.D.

Abstract

The prevalence of asthma has markedly increased in recent decades. Asthma affects approximately ten percent of the population of the United States, and is the leading cause of childhood hospitalization. This epidemic has been attributed to air pollution, childhood immunizations and a more sanitary living environment. Allergic asthma is clinically characterized by airway hyperreactivity (AHR), increased mucus production and airway remodeling. On the cellular level, pulmonary eosinophilic infiltration and augmented levels of serum IgE arise as a consequence of a $CD4^+$ Th2 cell response in the airway following exposure to allergen. It has been proposed that the chronic inflammation and associated airway events evident in this disease stem from a failure to regulate the underlying immune response. How these events are regulated in the healthy lung is yet unclear. In studies to investigate the mechanisms underlying such regulation we found that firstly, co-transfer of expanded natural $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (nTregs) mediated regulation of $CD4^+$ Th17 effector cells as exemplified by diminished levels of IL-17 and decreased neutrophilic infiltration in the airways. In contrast, co-transfer of nTregs did not attenuate the lung inflammation elicited by $CD4^+$ Th2 or Th1 polarized cells. Interestingly, using the C129.IL4GFP mice we found that nTregs have the capacity to inhibit IL-4 production and Th2 differentiation in vitro. Secondly, mice with genetically disrupted receptors ($IP^{-/-}$) for the lipid-mediator prostacyclin (PGI_2), demonstrated increased airway inflammation, eosinophilic infiltration and airway hyperreactivity following immunization and repeated aerosol challenge with ovalbumin. Moreover these mice displayed reduced serum immunoglobulin levels. In summary, nTregs serve a specific function in controlling Th17 cell effector functions, but not Th1 or Th2 inflammation. Additionally, PGI_2-IP signaling is an important pathway for inhibiting allergic pulmonary inflammation by controlling $CD4^+$ Th2 cell effector functions.

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TABLE OF CONTENTS

TITLE PAGE *i*

ABSTRACT *ii*

ACKNOWLEDGEMENTS *iii*

TABLE OF CONTENTS *iv*

LIST OF FIGURES *viii*

1.0 CHAPTER ONE: INTRODUCTION AND RELEVANT BACKGROUND 1

1.1 Airway Inflammation and Asthma 2

1.1.1 Pathophysiology of Asthma 2
1.1.2 Airway Hyperresponsiveness 4

1.2 $CD4^+$ T Effector Cell Differentiation 5

1.2.1 $CD4^+$ Th2 Differentiation and Effector Functions 8
1.2.2 $CD4^+$ Th1 Differentiation and Effector Functions 10
1.2.3 $CD4^+$ Th17 Differentiation and Effector Functions 11

1.3 The Regulation of the Immune Response 13

1.3.1 Foxp3 Expressing Tregs 13
1.3.2 $CD4^+CD25^+Foxp3^+$ Natural Tregs 13
1.3.3 $CD4^+$ iTregs 15
1.3.4 The Expression of Foxp3 by Tregs 16
1.3.5 CD4+Th1/Th2/Th17 Effector Cells and Tregs: a Balancing act 18

1.4 Immunoglobulins 19

1.5 Cell Types Involved In Asthma Associated Airway Inflammation 21

1.5.1 Role for Eosinophils in Asthma 21
1.5.2 Dendritic Cells 24
1.5.3 Neutrophils 25
1.5.4 Mast Cells 26

1.6 Regulation of The Lung Inflammatory Process 27

1.6.1 Eicosanoids 28
1.6.2 Eicosanoid Pre-Resolution Molecules-Lipoxins, Resolvins 31

1.7 Animal Models 32

2.0 CHAPTER TWO: MATERIALS AND METHODS 35

2.0.1 Cell culture 35
2.0.2 Animals 35

2.0.3 Immunization of $IP^{-/-}$ and wt mice with OVA/Alum 35
2.0.4 Lung Histology 36
2.0.5 Measurement of Airway Inflammation 36
2.0.5.1 Cell differential Count 37
2.0.5.2 Eosinophil peroxidase assay 37

2.0.6 Measurement of Cytokines 38

2.0.6.1 IL-4, IL-5 and IFN-γ Measurements 38
2.0.6.2 IL-17 Measurements 38

2.0.7 Measurement of Immunoglobulins 37
2.0.8 Monitoring the phenotypic changes of the $IP^{-/-}$ $CD4^{+}$T cells in vitro 38
2.0.9 Isolation of Lung Mononuclear Cells 38
2.0.10 Preparation of CD4+ Th1, Th2 and Th17 cells 38
2.0.11 Preparation and Expansion of $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ Regulatory T cells in Culture 41

2.0.11.1 Quantification of Foxp3 Expression 42

2.0.12 Adoptive Transfer of CD4+T cells in to Mice and Ovalbumin Aerosol Challenge 43
2.0.13 Monitoring the Regulation of Th2 Differentiation of CD4+ T cells in vitro 44
2.0.14 FACS Analysis of Spleen, Lymph Node, and Lung Mononuclear cells from in vivo experiments with Th1, Th2 or Th17 co-transferred with nTregs 44
2.0.15 Statistical Analysis 45

3.0 **CHAPTER THREE:** A role for prostacyclin and its receptor, IP in regulating allergic inflammation and the humoral response 46

3.0 Abstract 47

3.1 Introduction 48

3.2 Results 50
3.2.1 Characterization of Allergic Pulmonary Responses in Mice Lacking PGI2 Receptors- $IP^{-/-}$ 50

3.2.1.1 Establishing a mouse model of antigen induced airway inflammation using CD57BL/6 and $IP^{-/-}$ mice 51
3.2.1.2 Histologic examination of lung inflammation in $IP^{-/-}$ and wt mice 53
3.2.1.3 Airway Hyperresponsiveness is increased in $IP^{-/-}$ mice
3.2.1.4 Elevated numbers of inflammatory cells present

in the BAL of IP-/- mice 55
3.2.1.5 Cell-associated eosinophil peroxidase activity in BAL of wt and $IP^{-/-}$ mice 56
3.2.1.6 Characterization of lymphocytes in the BALF that were recruited to the lung during Th2 mediated inflammation by BACS analysis 57
3.2.1.7 Characterization of lymphocyte populations found in the lung tissue spleen and peripheral lymphoid tissue by FACS analysis 60
3.2.1.8 Cytokine production by lung mononuclear cells 62
3.2.1.9 Comparison of Th2 differentiation in vitro by wt and $IP^{-/-}$ cells 63

3.2.2 Serum immunoglobulins in naïve unimmunized IP-/- mice differ from wt mice 65

3.2.2.1 $IP^{-/-}$ mice display higher IgE and IgG responses 67
3.2.2.2 $IP^{-/-}$ mice have altered surface immunoglobulin expression 70

3.2.3 Nonspecific cyclooxygenase inhibitor- Indomethacin augments airway inflammation in wt mice but not IP-R deficient mice 72
3.2.3.1 Indomethacin does enhance eosinophilia in the BALF of wt mice 73
3.2.3.2 Indomethacin treatment does not alter the lymphocyte population in the BALF of immunized wt or $IP^{-/-}$ mice that have inhaled OVA 75
3.2.3.3 Indomethacin treatment does not alter the cytokine production in the BALF of immunized wt of $IP^{-/-}$ mice that have inhaled OVA 76
3.2.3.4 The COX-2 selective inhibitor NS-398 did not increase airway inflammation in wt or $IP^{-/-}$ mice 77

3.2.4 $CD4^+$ T cell levels of L-selectin (CD62L) are regulated by PGI_2 78

3.3 Discussion

3.3.1 Asthma: Incidence and pathology 82
3.3.2 Prostacyclin—Role in modulating the immune response 83

3.3.2.1 $IP^{-/-}$ mice have increased pulmonary inflammation following immunization and exposure to aerosolized OVA 86
3.3.3 Immunoglobulin response in $IP^{-/-}$ mice 89

3.3.3.1 Levels of IgG1 and IgE are elevated in $IP^{-/-}$ mice 89

3.3.3.2 Immunoglobulin isotypes are altered in serum $IP^{-/-}$ mice 89
3.3.4 PGI_2 promotes L-selectin expression by CD4+ T cells 90
3.3.5 A role for PGI2 and its receptor IP in the augmentation of allergic lung inflammation elicited by inhibitors of COX enzymes 91
3.3.6 Summary 93

4.0 CHAPTER FOUR: Antigen-Specific CD4+Foxp3+ Natural Treg cells Limit Th17- but not Th1- or Th2- Mediated Lung Inflammation 95

4.0 Abstract 96

4.1 Introduction 97

4.2 Results 100

4.2.1 Identification and characterization of $CD4^+CD25^+Foxp3^+$ natural Tregs in naïve DO11.10 mice 100
4.2.2 Expanded nTregs did not suppress Th2 or Th1 mediated airway inflammation 102
4.2.3 Expanded nTregs suppress Th2 polarization and Il-4 expression by $CD4^+$T cells in vitro 105
4.2.4 Expanded nTreg cells suppress Th17 mediated airway inflammation 107
4.2.5 Expanded nTreg cells did not suppress Th17 responses in vitro 109

4.3 Discussion 111

5.0 CHAPTER FIVE Conclusions 115

REFERENCES 120

LIST OF FIGURES

Chapter 1 **Introduction**

Figure 1.1	Inflamed small airway	4
Figure 1.2	$CD4^+$ Differentiation	5
Figure 1.3	Th1/Th2 Paradigm	7
Figure 1.4	Foxp3 Signaling	17
Figure 1.5	The Structure of the Immunoglobulin protein	20
Figure 1.6	Mechanism of Airway Inflammation	27
Figure 1.7	Eicosanoid pathway	29

Chapter 3

Figure 3.1	OVA immunization and aerosolization protocol for the induction of airway inflammation in wt and $IP^{-/-}$ mice	51
Figure 3.2	The optimal number of immunizations required for discernment of phenotypic differences between $IP^{-/-}$ and wt C57BL/6 mouse strains	52
Figure 3.3	Increased inflammation in the airway of $IP^{-/-}$ mice following immunization and challenge with OVA	53
Figure 3.4	Mice deficient for the IP receptor demonstrate augmented AHR	55
Figure 3.5	Inflammatory cells are increased in the BAL of $IP^{-/-}$ mice	55
Figure 3.6	EPO levels are increased in IP receptor deficient mice	56
Figure 3.7	Delineation of lymphocyte populations in BAL	58-59
Figure 3.8	FACS analysis of the number of T and B lymphocytes present in the lung, lymph node and spleen immunized mice following OVA inhalation for 7 days	61
Figure 3.9	Th2 response in LMC	63
Figure 3.10	IL-4 production is elevated inTh2 polarized cells from $IP^{-/-}$ mice	64
Figure 3.11	Isotype serum immunoglobulin differences between $IP^{-/-}$ and wt mice	66
Figure 3.12	Immunoglobulin levels are altered in $IP^{-/-}$ mice	68-69
Figure 3.13	Cell surface expression of IgE and IgG1 on $CD19^+$ B cells	71
Figure 3.14	Indomethacin treatment of murine model of pulmonary inflammation	73
Figure 3.15	Increased numbers of eosinophils in OVA immunized/ challenged wt mice after treatment with indomethacin	74
Figure 3.16	EPO levels are increased in indomethacin treated wt mice	75
Figure 3.17	Treatment with indomethacin did not alter lymphocyte populations in the BALF of immunized and challenged mice	76

Figure 3.18	Treatment with non-specific COX inhibitor indomethacin does not significantly alter the expression of Th2 cytokines in LMC of wt mice	77
Figure 3.19	COX-2 selective inhibitor NS-398 scheme	77
Figure 3.20	COX-2 selective inhibitor NS-398 did not increase airway inflammation in wt or $IP^{-/-}$ mice	
Figure 3.21	L-selectin is retained on $CD4^{+}$Th2 cells in the presence of Iloprost	79
Figure 3.22	Treatment with indomethacin does not affect L-selectin expression by BAL lymphocytes on wt or $IP^{-/-}$ mice	79
Figure 3.23	Cell adhesion molecule L-selectin expression in $IP^{-/-}$ mice	81
Figure 3.24	Proposed mechanism for PGI_2-IP signaling in the regulation of allergic pulmonary inflammation	85
Figure 3.25	NSAID exacerbation of allergic inflammation	93

Chapter 4

Figure 4.1	Identification and expansion of OVA-specific $CD4^{+}$ T cells in DO11.10 mice	102
Figure 4.2	Expanded natural Tregs failed to suppress Th1 and Th2 mediated lung inflammation	104
Figure 4.3	Expanded natural Tregs did suppress the differentiation of $CD4^{+}$ Th2 cells	106
Figure 4.4	Expanded natural Tregs did suppress Th17-mediated lung inflammation, cytokine production but had no affect on airway function	109
Figure 4.5	Expanded $CD4^{+}$ Tregs failed to inhibit $CD4^{+}$ Th17 proliferation of cytokine production in vitro	110

CHAPTER ONE

Introduction and Relevant Background

1.0 Introduction

In the past two decades, the prevalence of asthma has almost doubled, reportedly affecting approximately 10% of the population of the United States. The severity of the problem is illustrated by the observation that asthma exacerbations are the leading cause of hospitalization among young children (1). This epidemic has been attributed to air pollution, childhood immunizations, and more hygienic living conditions, with no single cause identified as the chief cause. In the late 1980s the "hygiene hypothesis" proposed that a lack of early childhood exposure to infectious agents, symbiotic organisms and parasites augments susceptibility to allergic disease, by restricting development of the immune systems ability to self-regulate (2). Adaptive immunity is predominantly meditated by T and B cells, which possess an extensive diversity in antigen recognition, antigen specificity, potent effector activity and long lasting immunologic memory (3). The effectiveness of this response can pose a serious threat to the host by way of aberrant immune reactions. Such a response is exemplified in allergic asthma. A major challenge in immunology is to determine how unresponsiveness or tolerance of the adaptive immune system to self-antigens is established, maintained and controlled as to avoid damage to the host. In addition, non-steroidal anti-inflammatory drugs (NSAIDs) have been clinically associated with disease exacerbation in asthmatics (4). The objective of the work described in this manuscript is to better understand how regulation of these aberrant immune responses factor in the pathogenesis of allergic inflammation in the lung. The work outlined in this dissertation is divided into two separate aims. Aim one

addresses potential mechanisms by which NSAIDs exacerbate airway inflammation. The approach adopted was to investigate the role of prostacyclin (PGI_2) signaling, a prostanoid product of cyclooxygenase activity, that is inhibited by NSAID activity. The second aim was accomplished by investigating and proposing a potential mechanism for the role played by natural regulatory T cells (nTregs) in mediating suppression of the immune response in a murine model of pulmonary inflammation.

1.1 Airway Inflammation and Asthma

Human allergic asthma is a complex condition characterized by increased levels of IgE, airway eosinophilic inflammation, airway hyperreactivity (AHR), increased mucus production and airway remodeling (1, 5). Importantly, $CD4^+$ T cells orchestrate the inflammatory response in asthma. Leukocyte accumulation in the lung has the capacity to mediate many aspects of the pathophysiology of chronic inflammatory diseases including asthma and chronic obstructive pulmonary disease (COPD) (6).

1.1.1 Pathophysiology of Asthma

The inflammatory response in asthma is characterized by the infiltration of macrophages, eosinophils and lymphocytes into the airway wall, accompanied by mucus and the shedding of airway epithelial cells (7). Structural changes, "airway remodeling", occur as the epithelial and stromal cells attempt to repair the damage to the airway caused by local inflammation. Asthma is associated with airway remodeling and thickening of the airway wall by 10% to 300% (8). The small airways (2-4mm) are the primary pulmonary structures affected in moderate asthma, while severe to fatal disease

frequently involves all but the largest airway passages. The airway epithelium plays an important role as a physical barrier and, in its damaged state, is fundamental to asthma pathogenesis. Areas of epithelial metaplasia and damage, thickening of the subepithelial basal lamina, increased numbers of myofibroblasts and other evidence of airway remodeling are found in all but the mildest forms of asthma (9). The sub-epithelial layer, which is generally 4-5 microns thick in the normal pulmonary anatomy, ranges from 7-23 microns in the asthmatic resulting from the deposition of collagen (types I, III, and IV), fibronectin and tenascin (10, 11). Myofibroblasts, which produce collagens and have been linked to hyperplasia in asthmatic lungs, contribute to the increase in airway smooth muscle mass resulting in an increase of up to three times the normal area (12).

Airway inflammation is a multicellular process involving several distinct types of of inflammatory cells, which include eosinophils, neutrophils, $CD4^{+}$T lymphocytes and mast cells with eosinophilic infiltration being the predominant feature (13). The inflammatory process is largely limited to the conducting airways but with increased severity and chronicity the inflammatory infiltrate may extend to the small airways and adjacent alveoli (14). The inflammatory response in the small airways extends beyond the airway smooth muscle (Figure 1.1), whereas responses in the large airways are mainly in the submucosal tissue (15).

Figure 1.1 ***Inflamed small airway***

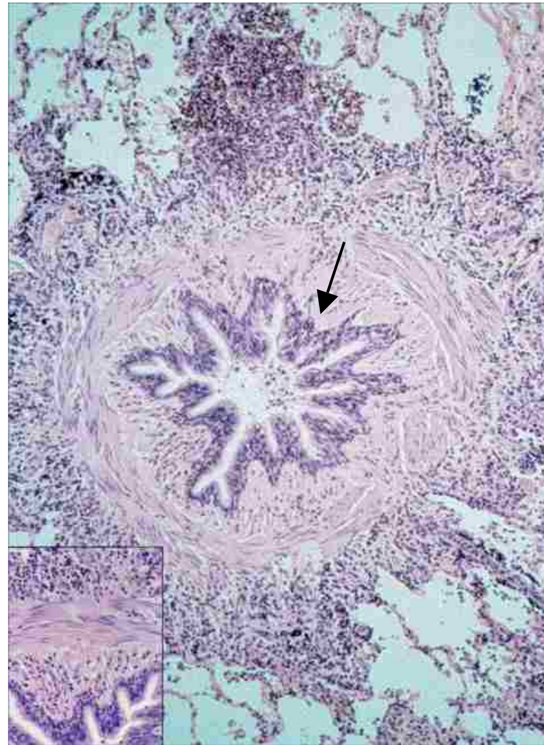

Figure 1.1 A severely inflamed airway from a asthmatic patient. The inset shows submucosal fibrosis and increased small muscle (16)

1.1.2 Airway Hyperresponsiveness (AHR)

AHR and airway obstruction are considered a consequence of the combined effects of airway remodeling and inflammation. AHR is defined as increased bronchoconstrictor response to a nonspecific stimulus (17). The precise mechanisms that control AHR are poorly understood. However, it is known that the magnitude of AHR closely correlates with the level of airway inflammation (18). Additional factors associated with AHR include: reduced airway diameter, increased smooth muscle contractility and degree of epithelial injury, dysfunctional neuronal regulation, increased microvascular permeability and many inflammatory mediators (19). In addition,

allergen-induced asthma in mouse has been associated with complement factor 5 (C-5) (20).

1.2 CD4$^+$ T Effector Cell Differentiation

Since CD4$^+$ Th2 cells drive the inflammatory response in asthma, it is important to understand the nature of how cells differentiate from naïve T cells into a polarized effector cells (Th1, Th2 and Th17). Figure 1.2 illustrates the three known types of CD4$^+$ pro-inflammatory effector cells, the factors that evoke their differentiation and the cytokines that they produce. Several different types of regulatory cells have also been reported which differ in phenotype and cytokine requirements. The regulatory cells depicted here will be discussed in depth in subsequent sections.

Figure 1.2 ***CD4$^+$ Differentiation***

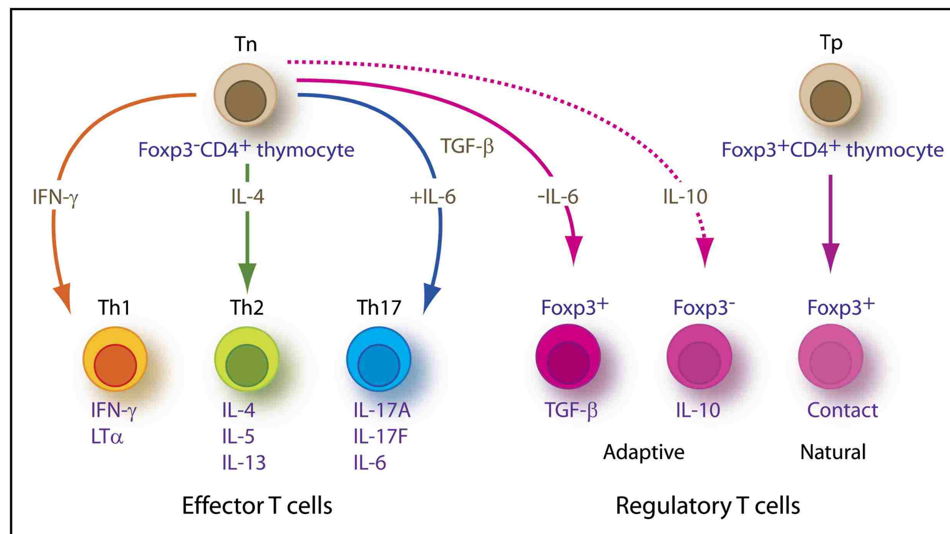

Figure 1.2 *Diversification of the CD4 T Cell Lineages*. The Th1/Th2 paradigm was described nearly twenty years ago. The number of distinct lineages has increased over the past decade. The arrows indicate dominant cytokines involved with specific lineages. The cytokines below the cell type indicate key effector or regulatory cytokines expressed by differentiated cells of that lineage (21).

CD4 effector T cells otherwise known as helper T cells (Th) play a pivotal role in engineering immune responses through their capacity to provide help to other cells of the adaptive or innate immune systems. A defining characteristic of adaptive immunity is the antigen-driven differentiation of clonally restricted lymphocyte precursors into effector cells of enhanced functional potential (Figure 1.2). Naïve T cells differentiate into effector T cells and as a consequence, possess an increased capability for orchestrating pathogen clearance. In part this is achieved by producing cytokines that activate the innate immune system and facilitate clearance of pathogens (Figure 1.2). The balancing of these immune responses is reliant on proper regulation of the differentiation and function of the Th cells. Dysregulation of Th cell function or proliferation may lead to inefficient clearance of pathogens or cause inflammatory diseases or autoimmunity. The dogma that Th cells are functionally heterogeneous and cytokines are important for Th cell function can be extended to distinguish the different classes of Th cells with distinct biological functions. Th1, Th2, Th17, and recently characterized-Tfh (T follicular helper), and Th9 cells are involved in inflammatory responses while regulatory T cell (Treg) including naturally occurring Treg (nTreg), induced Treg (iTreg) and class 1 Treg (Tr1) engage in immune suppression (3, 22).

Figure 1.3 ***Th1/Th2 Paradigm***

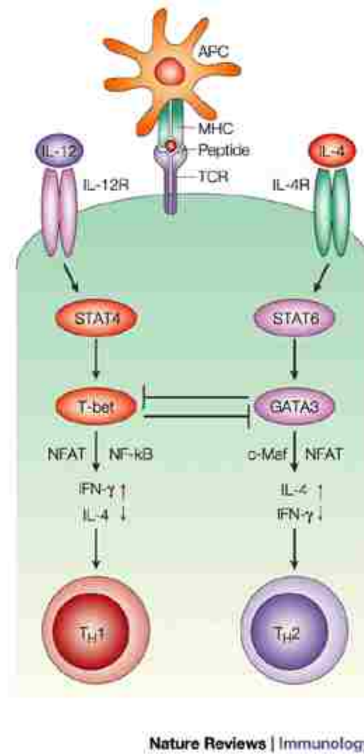

Figure 1.3 *The cross regulation of CD4+ Th1 and Th2 cell transcription factors T-bet and GATA3.* Coffman and Mossman proposed that in response to environmental cues, two different effector cell types derived from CD4+ T helper cells (23).

Many factors influence Th cell differentiation. These include TCR stimulation, co-stimulation, kinase cascades, transcriptional networks, with cytokine signaling being accepted as the predominant factor in driving Th differentiation. Initial studies of T cell biology defined two classes of $CD4^+$ T cells; those that help B cells for immunoglobulin class switching, or those who enhanced macrophage activation or cell mediated immunity (24, 25). These distinct cell populations correlated with the production of factors that either promoted or inhibited B cell class switching to IgE (26). Mossman and Coffman then proposed the T helper type 1 (Th1)-Th2 hypothesis, which postulated that subsets of $CD4^+$ T cells express reciprocal patterns of immunity via production of distinct profiles of cytokine secretion, either delayed-type hypersensitivity (cell-mediated immunity)

(Th1) or allergic (Th2) (27). Furthermore, each Th subset promotes its own development and proliferation via the cytokines they secrete (28, 29), such that the induction of one type of response suppresses the induction of the other (30), (figure 1.3).

Differentiation of naïve $CD4^+$ T cells to an effector phenotype requires antigen-ligation with the T cell receptor (TCR) from the antigen-presenting cell (APC) in the context of class II MHC. The nature and strength of the signal of the antigenic stimulus influences Th polarization (31, 32), and the final lineage commitment (33). An additional stimulatory signal mediated via the CD28 on T cells to the B7 molecules on the APC is also essential for efficient T cell activation (34).

1.2.1 $CD4^+$ Th2 Differentiation and Effector Functions

The hallmark features of allergic asthma, elevated serum IgE, mucus hypersecretion, eosinophilia, and enhanced AHR to nonspecific antigenic stimuli, have all been linked to the effector functions of Th2 cytokines. Therefore, it is useful recognize how these cells are derived. Th2 cells are characterized as cells that express IL-4, IL-5, IL-9, IL-10, and IL-13 and are often associated with the humoral responses during which high levels of pathogen specific immunoglobulin are generated to neutralize foreign organisms. IL-4 is the cytokine known to have the greatest influence in driving Th2 differentiation (35, 36). IL-4 when bound to IL-4Rα signals via the STAT6 pathway (37), inducing high levels of the transcription factor GATA-3 resulting in commitment to Th2 differentiation, therefore upregulating T cell production of IL-4 (38-41). Additionally, the Th2 immune response is important in the defense of extracellular pathogens such as helminths and nematodes (42, 43). $CD4^+$ Th2 lymphocytes contribute to the

inflammatory response and to airway remodeling by producing cytokines. Th2 cells promote IgG1 and IgE class switching and eosinophil recruitment (24, 25, 44, 45). Th2 cells are crucial for mucosal immunity (mucus hyper-secretion and increased contractility) in the lung (this will be discussed in detail in subsequent sections). IL-4 in combination with TGFβ has been shown to drive the differentiation of the newly identified Th9 cell lineage (46, 47).

In asthma, $CD4^+$ Th2 cells are thought to initiate and perpetuate disease. Lymphocytes make up a small percentage of the total leukocytes in the lung. However, CD4 T cells are increased in the airways of asthma patients. IL-4, IL-5, and IL-13 protein and mRNA levels are increased in bronchoalveolar lavage (BAL) fluid, BAL cells and airway biopsies of asthmatics (5). The Th2 differentiation transcription factor, GATA-3 is expressed at high levels in CD4 T cells from the airways of asthmatics (48).

IL-4 promotes eosinophilia indirectly via the promotion of autocrine Th2 development and murine eosinophils, themselves, produce IL-4 (49, 50), and the IL-4 receptor alpha (IL-4Rα) (51, 52). Importantly, IL-4 directs IgE synthesis and mast cell growth and activation in both human and mouse. IL-4 activates human vascular endothelial and respiratory epithelial cells to produced eosinophil chemoattractant cytokines. In the mouse, IL-4 preferentially induces antibody isotype switching to IgG1 and IgE (53).

IL-5 promotes eosinophil generation in the bone marrow and entry into the blood. In contrast, IL-13 induced the production of eotaxins 1 and 2 in the lung tissue and lung lumen macrophages, respectively, to recruit the eosinophils from the blood to the lung (54). As a part of a epithelial protection mechanism, IL-13 stimulated mucus production

and secretion (55), and also reduces the ciliary beat frequency (56), and the ciliated cells are sloughed. In asthma, a poorly functioning mucociliary escalator and increased mucus production result in mucus pooling, cough and increased airway obstruction.

1.2.2 CD4$^+$ Th1 Differentiation and Effector Functions in Asthma

Although Th1 cells are not the primary cell type involved in asthma, it is important to have an understanding of their role in inflammation. Th1 cells are defined by the capacity to produce the pro-inflammatory cytokine interferon gamma (IFNγ), and provide protection against intracellular pathogens and viruses. Th1 differentiation requires both TCR ligation and IFN-γR- signal transcription activator of T lymphocytes (STAT) 1 induction and the transcription factor T-bet (57, 58).

IFN-γ is an important macrophage-activating factor, critical for the clearance of certain intracellular pathogens and viruses. It is also necessary for immunoglobulin class switching in B cells by promoting production of IgG2a. T cells express the heterodimeric IFN-γ receptor-1 and -2 and thus can be stimulated by IFN-γ though the Jak1/Jak2/STAT1 pathway (59, 60). In addition, STAT4 signaling is critical for IL-12 signaling and thus the full commitment of Th1 cells (61, 62). IL-12 is a soluble factor that potently induces Th1 differentiation (63-65). CD4$^+$ T cells only express the IL-12 receptor (IL-12R) upon TCR stimulation (66), and IL-12R is maintained only on Th1 cells and downregulated on Th2 cells (63, 64). IL-12 signaling is necessary for the expression of IL-18 receptor α (IL-18Rα). IL-18 serves as a cofactor with IL-12 in promoting IFN-γ production in CD4$^+$ T cells and Th1 differentiation (67). Because Th1 cells maintain IL-12 and IL-18 receptors even in the resting state, IL-12 and IL-18

stimulation can induce large amounts of IFN-γ by differentiated Th1 cells in the absence of TCR stimulation (68, 69).

1.2.3 CD4^{+} Th17 Differentiation and Effector Functions

Although the Th2 cell has been indicated as the primary cell type involved in the allergic immune response, likely responsible for contributing to the ongoing chronic inflammatory response, recent studies have demonstrated provocative data for a role for IL-17 producing Th17 cells.

It is likely that Th17 cells evolved as an extension of the adaptive immune system specialized for enhanced host protection against extracellular bacteria and some fungi, microbes not well covered by Th1 or Th2 immunity (70). Th17 cells, as indicated by the name, produce IL-17A (commonly known as IL-17), E and F (71-73). Recent studies have demonstrated Th17 cells also produce IL-21 and IL-22 (74-77). The Th17 response apparently shares commonality with both Th1 and Th2 responses where they contribute to the resistance to *Listeria, Salmonella, Toxoplasma, Cryptococcus, Leishmania* and *Francisella* (78, 79). Preferential production of IL-17 by T cells during infection with specific pathogens such as *Bacteriodes fragilis* (80), *Borrelia burgdoferi, Mycobacterium tuberculosis (81)* and some fungal species (82) suggesting that Th17 cells respond to specific pathogens and are required for their clearance. It also appears that Th17 cells play a pivotal role in the induction and propagation of some autoimmune conditions. IL-17 expression has been associated with autoimmune diseases such as Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), psoriasis, irritable bowel disease (IBD), as well as

allergic responses (73, 83). IL-25 (IL-17E) may serve a potentially important role in the mediation of dysregulated Th2 responses that cause asthma or other allergic disorders.

The retinoic acid-related orphan receptors (ROR) are the key transcription factors for Th17 cell differentiation. ROR-γt is upregulated in T cells in response to IL-23 and its expression highly associates with IL-17 expression (84). The genes targeted directly regulated by ROR-γt have yet to be identified. However, IL-17 is a good candidate because conserved ROR response elements are located in its promoter region (85). TGF-β and IL-6 cooperate in a non-redundant fashion to promote Th17 commitment (47, 86, 87).

It has been proposed that IL-17 or IL-17F act in cooperation with IL-22 to augment the expression of antimicrobial peptides by human epithelial cells that are associated with host defense, such as β-defensin 2 (75), suggesting that the Th17 lineage may have evolved to eliminate pathogens at mucosal surfaces. Recently, our laboratory has demonstrated that Th17 cells play a crucial function in lung mucosal immune defense by promoting the polymeric Ig receptor (pIgR)-mediated delivery of IgA and IgM into the airway lumen where they contribute to airway immunity. These observations show that pIgR expression in the airway epithelium is typically low but is rapidly up-regulated by IL-17 (88).

1.3 The Regulation of the Immune Response

1.3.1 Foxp3 Expressing Tregs

Two types of Tregs that express Foxp3 have been discovered to date. The first is generated in the thymus and bear a $CD4^+CD25^+$ phenotype. Since they are generated in the absence of specific priming, they are known as natural or nTregs. The second type of Treg cell is formed in the periphery in response to antigen stimulation the presence of TGF-β. These cells are known as induced Tregs (iTregs).

1.3.2 $CD4^+CD25^+Foxp3^+$ Natural Tregs

The immune system protects the host from an extensive range of pathogenic microorganisms while avoiding autoimmunity. Tregs play a central role in maintaining immunological unresponsiveness to self-antigens and a role in limiting inflammation also has been proposed (3). Disruption in the development of Tregs is a primary cause of autoimmune disease in humans and animals. Furthermore, adaptive immune response typically involves, not only recruitment and activation of effector T and B cells but also the induction and recruitment of Tregs (3). Depletion of $CD25^+CD4^+$ T cells augments effective tumor immunity in otherwise non-responding animals and augments microbial immunity in chronic infection (89, 90). Alternately, $CD25^+CD4^+$ T cells enriched from wild type mice inhibit allergy, and establish tolerance to organ grafts after bone marrow transplantation, and promote feto-maternal tolerance (91). nTregs are a subset of CD4+ T cells that develop in the thymus comprising approximately 5-10% of peripheral $CD4^+$T cells. The most prominent function of nTregs is to maintain self-tolerance and immune homeostasis. nTregs are anergic and do not actively secrete large amounts of effector

cytokines. They do, however, produce considerable levels of inhibitory cytokines IL-10 and the membrane bound form of TGF-β (92). nTregs constitutively express the IL-2 receptor α chain (CD25), high levels of the cytotoxic T lymphocyte antigen-4 (CTLA-4) and glucocorticoid induced TNF receptor (GITR) (3).

Differentiation from thymocytes into nTregs in the thymus is likely to occur due to precursor nTregs possessing a TCR with a higher affinity for thymic MHC/self-peptide ligands than other T cells, and these highly self-reactive T cells would be recruited to the Treg cell lineage during the course of thymic T cell selection (93-95). Also, mutations in the LAT (linker of activated T cell) gene, which encodes a TCR proximal transduction molecule, abolishes the generation of Foxp3^{+} T cells, indicating the requirement of a strong signal via the TCR for the development of nTregs in the thymus (96). Additionally, the intensity of the interaction between T cell accessory molecules and their ligands on thymic stromal cells factors in the generation of nTreg cells. An example of this is deficiency of CD28, CD40, CD11a/CD18 or the B7 molecules results in a substantial reduction of CD25+CD4+ Treg cell in the thymus and periphery (91). At the cellular level, both medullary thymic epithelial cells (mTECs) and DCs in the thymus contribute to Treg generation (97-99).

IL-2 is another molecule critical for the function of Tregs. Mice deficient in CD25 succumb to spontaneously developed T cell-mediated fatal lymphoproliferative and inflammatory diseases (100, 101). Humans with IL-2 deficiency syndrome manifest these symptoms along with severe autoimmunity and allergy, indistinguishable from immune dysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome (102). Although a recent study demonstrated that IL-2 is not absolutely required for

development (103). IL-2 is required for optimal Treg function, likely through facilitating their proliferation and survival (100, 101). The Treg surface marker, CD25 IL-2Rα) is a component of the high affinity IL-2 receptor and is functionally essential to Treg development (3). Binding of IL-2 to its receptor activates the JAK3-STAT5 pathway (104, 105). IL-2 is required for sustained expression of Foxp3 and CD25 in nTregs *in vitro* (103, 106). IL-2 inhibits TGF-β/IL-6-dependent differentiation of naïve T cells to inflammatory Th17 cells (107). Foxp3 in concert with other transcription factors and co-activators/co-repressors inhibits the transcription of IL-2 rendering them highly dependent on exogenous IL-2, produced chiefly by activated effector T cells.

Thymic stromal lymphopoietin is an IL-7 like cytokine that is known to induce the generation of thymic nTregs specifically through DC interactions *in vitro* (99). TGF-β is required for development and peripheral maintenance of nTregs in mice (108, 109).

1.3.3 CD4$^+$ iTregs

In addition to the naturally occurring Tregs, differentiation of regulatory T cells can be induced (iTregs) from naïve T cells following TCR engagement in the presence of TGF-β (110, 111) and IL-2 (107). These iTregs subsequently produce large amounts of IL-10 and TGF-β and act to suppress immune responses with minimal antigen specificity (112-114). TGF-β is obligatory for the *de novo* generation of iTreg and crucial for the accompanied increase in Foxp3 expression following TCR stimulation (111, 115, 116). IL-2 facilitates the differentiation of naïve CD4$^+$ cells into iTregs while inhibiting their differentiation to Th17 cells (107). In addition, in the presence of TGF-β, retinoic acid secreted by a certain subset of DCs, has been demonstrated to induce Foxp3 Tregs in the

gut (117-119). It is yet unclear as to the stability of iTregs in the periphery. In humans, unlike mice, naïve T cells readily express Foxp3 upon TCR stimulation, although the expression is generally much lower and more transient than in nTregs (120, 121).

1.3.4 The Expression of Foxp3 by Tregs

Tregs are produced in the thymus or can be induced from naïve T cells in the periphery. The transcription factor Foxp3 is considered to be the master regulator driving the differentiation and function of Tregs (122-124). Foxp3 is an X-linked transcription factor belonging to the Fork-head protein family, which is highly expressed specifically in nTregs and can be induced by TGF-β following antigenic stimulation in iTreg cells (125). The *FOXP*3 gene was first identified as the defective gene in the mouse strain scurfy. Scurfy is an X-linked recessive mutant that is lethal in hemizygous males within a month of birth, displaying hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines (126). In addition, mutations in the human *FOXP3* gene resulted in IPEX (127). Continuous expression of Foxp3 is critical for maintaining the suppressive activity of Tregs (128).

Recent searches for Foxp3 target genes have shown that Foxp3 directly or indirectly controls hundreds (~700) of genes and binds directly to 10% of them (129, 130). Known targets genes for Foxp3 include; *IL-2, CD25, CTLA-4* and *GITR (129)*. Foxp3 has been shown to control the cellular and molecular programs involved in Treg function as a homo-oligomer maintaining either direct or indirect interactions with the transcription factors NFAT (nuclear factor of activated T cells), AML1 (acute myeloid leukemia-1)/Runx1 (runt-related transcription factor-1)/HAT (histone acetyl transferase)/

HDAC (histone deacetyl transferase complex and possibly NFκB (3). NFAT forms a complex with AP-1 and NFκB promoting the expression of IL-2, IL-4, CTLA-4 and other genes associated with conventional T cell function (131), (Figure 1.4). It has recently been shown that the Foxp3 NFAT interaction is dependent on binding to DNA. Substitutions in the forkhead domain of Foxp3 that disrupt this interaction impair the ability of Foxp3 to repress IL-2, activate CTLA-4 and CD25, and confer suppressive activities when expressed in normal T cells. It is thought that AML1/Runx1 physically binding to Foxp3 at its N-terminus and NFAT at the C- terminus may result in repressing transcription of these cytokines essential for the activation and differentiation of T cells to the effector phenotype while inducing upregulation of CD25 and CTLA4 (132), (Figure 1.4).

Figure 1.4 *Foxp3 Signaling*

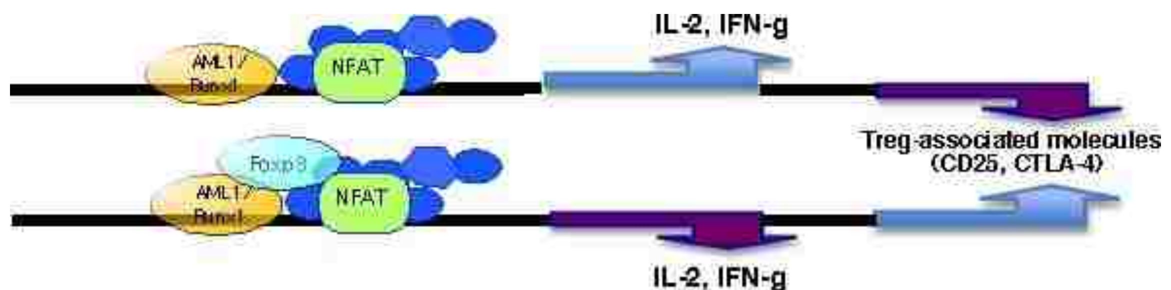

Figure 1.4 *Control of the function of CD4+CD25+ Tregs by Foxp3.* Foxp3 is required for the transcriptional complexes involving NFAT and AML1/Runx1 activate or repress the genes encoding cytokines and several cell-surface molecules in Tregs (3).

Additional forms of Tregs exist and these include Tr1 producing IL-10 and TGF-β in the periphery. These cells comprise a CD4+ cell lineage distinct from iTregs and nTregs since they do not express Foxp3 although they display repressor activities (111, 133).

1.3.5 CD4$^+$Th1/Th2/Th17 Effector Cells and Tregs: a Balancing Act

Effector T cell subsets are characterized by their unique cytokine profiles. A central principle of effector T cell development learned by the establishment of the Th1/Th2 paradigm was the counter-regulatory nature of the cytokine and transcription factor networks that induce, stabilize or inhibit the maturation of Th1 and Th2 effectors cells. The discovery of the third major effector lineage, Th17 effector cells has advanced this concept and fostered an appreciation of the developmental plasticity of CD4$^+$ T cells (134). In contrast to the Th1/Th2 cells, which are considered terminally differentiated insofar as their respective cytokine profiles, recent work has suggested that Th17 cells are less rigid. Th17 and Tregs share the requirement for TGF-β, (which inhibits both Th1 and Th2 cell development), for their respective differentiation and function. First considered as a branch of Th1 cell lineage, it is now clear Th17 cells arise as a distinct population, independent of the classical Th1 and Th2 programs (135). Furthermore, the Th1 and Th2 cytokines, IFN-γ and IL-4, respectively, are strong inhibitors of early Th17 development, consistent with the cross-inhibitory roles played by cytokines in effector T cell differentiation. However, the common requirement of TGF-β for both Th17 and iTreg cell development challenges the prevailing theory of the Th1/Th2 paradigm that distinct inductive cytokines are uniquely linked to a developmental lineage. The fate of Th17 or iTreg cell differentiation is coordinated via IL-6 signaling (47, 86, 87). IL-6 suppresses Treg function (136) and maturation. IL-6 is produced by dendritic cells (DCs) activated by microbial products or IL-21, and by IL-6 stimulated T cells to establish an autocrine loop (74, 76). IL-6 in the presence of TGF-β induces Th17 cell differentiation. Alternatively, in the absence of IL-6, iTreg maturation will proceed. Further, recent

studies have confirmed this observation by demonstrating that induction of the key transcription factors for Th17 and Tregs, RORγt and Foxp3, respectively (77, 84), share a common dependence on TGF-β signaling and that the transcription factors directly interact, establishing a competitive antagonism that determines Th17 versus iTreg lineage specification (137-139). It is noteworthy that both IL-6 and IL-21 signaling are mediated by STAT3, thereby providing a common intermediate for the ROR transcription, thus Th17 promoting cytokines may act through STAT3- dependent pathways to reverse the Foxp3-mediated repression of RORγt in developing Th17 cells (138). In addition, Th17 differentiated cells appear to have the capacity to deviate to a Th1 progeny in response to IL-12 signaling, demonstrating late developmental plasticity of committed Th17 precursors (140, 141). Similarly, Tregs also seem to retain substantial developmental plasticity. IL-17 and IFN-γ expression by induced or natural Tregs (in the absence of TGF-β has been reported (138, 142-144). Conversely, the reciprocal conversion of Th17 cells into Tregs has not yet been described.

1.4 Immunoglobulins

The lymphocytes of the adaptive immune system have evolved to recognize a great variety of different antigens from bacteria, viruses and other diseases-causing organisms. The immunoglobulins (Igs) are the antigen-recognizing molecules produced by B cells. Ig is found on the cell membrane of B cells as well as a secreted form. The B cell receptor (BCR) is the cell surface Ig and acts when bound to antigen to transmit a signal for B cell activation, leading to clonal expansion and specific antibody production. The secreted Ig (antibody) has several functions. These include binding specifically to

molecules pathogens or their toxic products in the extracellular spaces that elicited the immune response. As well as recruitment of cells and molecules to destroy the pathogen once the antibody is bound. Binding to antibody neutralizes viruses and marks pathogens for destruction by phagocytosis and complement.

Five different classes or isotypes of immunoglobulins are known: IgM, IgD, IgA, IgG and IgE. IgG is the most abundant antibody and has four subclasses: IgG1, IgG2, IgG3 and in humans-IgG4. The Th2 cytokine IL-4 induces IgG1 and IgE production; IFN-γ induces IgG2a, while TGF-β drives production of IgG2b and IgA. Furthermore, in mouse IgG2a and IgG2b have been described. The serum levels of the different Igs differ dramatically, The levels of the different immunoglobulin isotypes present in serum are hugely different with IgG1 levels being around 9mg/mL in contrast to IgE which is typically 50ng/mL.

Figure 1.5 ***The Structure of the Immunoglobulin protein***

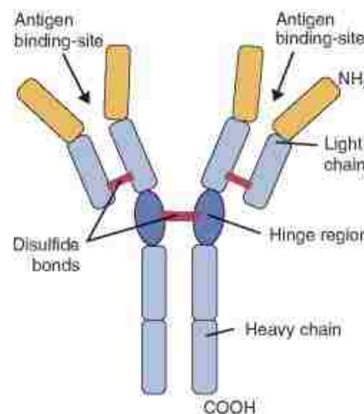

Figure 1.5 *The structure of the immunoglobulin protein*. The immunoglobulin molecule is composed of two heavy chains and two light chains joined by disulfide bonds so that each heavy chain is linked to a light chain and the two heavy chains are linked together (53).

Figure 1.5 shows how the antibody is designated into two parts; the variable (antigen binding) region (gold), which by definition, varies extensively between antibody molecules and the constant region (blue), which is similar across most antibodies and engages the effector functions of the immune system.

IgE isotype antibodies are typically present in low concentrations in the plasma and are mainly produced by plasma cells in the mucosal-associated lymphoid tissues (145). Asthma patients have elevated serum levels of both total and antigen specific IgE. Most of the IgE produced is bound by its high affinity Fc receptor (FcεRI), which is expressed by mast cells and basophils (145). Cross linking of IgE on tissue mast cells by specific antigen results in the local release of inflammatory mediators such as histamines and leukotrienes, enzymes and cytokines that mediate the symptoms of asthma. Various cell types, including B cells, express the low affinity IgE receptor FceRII. IgE bound to FcεRII facilitates allergen uptake by B cells, enhancing presentation to T cells and augmenting secondary immune responses.

1.5 Cell Types Involved In Asthma Associated Airway Inflammation

1.5.1 Role for Eosinophils in Asthma

Airway eosinophilia is the most distinctive characteristic in asthma and has been considered central in the pathogenesis of disease (1). Eosinophils are thought to contribute to allergic airway inflammation by release of their pro-inflammatory cytotoxic granule proteins, generation of lipid mediators such as the cysteinyl-leukotrienes, and their release of mediators that can induce lung remodeling (146, 147). The predominant cell type in allergic inflammation, eosinophils are not only found in the airway wall in the

asthmatic patient, but also abundantly in the sputum and bronchoalveolar lavage fluid (13, 148, 149). To date, levels of eosinophils and their granule proteins are among the most reliable indicators of disease severity in the lungs of asthmatic patients (150). Additionally, IL-5 is responsible for selective differentiation of eosinophils and stimulation of eosinophil release from the bone marrow into the peripheral circulation (151, 152).

Eosinophils are pleiotropic multifunctional leukocytes involved in the initiation and propagation of diverse inflammatory responses, as well as modulators of innate and adaptive immunity (153). The recruitment of eosinophils to the lung as the prominent cellular infiltrate has been well documented (154). Eosinophils traffic from the circulating blood into peripheral tissues byway of several processes involving rolling, tethering and firm adhesion to the vascular endothelium followed by trans-endothelial migration into the tissue (155, 156). These processes are orchestrated by the concerted efforts of chemokine and cytokine signaling, adhesion molecules and their receptors expressed on vascular endothelial cells (157). The initial steps of eosinophil rolling and tethering are regulated by selectins, single-chain transmembrane glycoproteins, on the surface of eosinophils and their ligands expressed on the endothelium (156, 158). Eosinophils have been shown to constitutively express L-selectin which regulates eosinophil rolling on the endothelium (159, 160). Eosinophils are recruited from the bone marrow as CD34 precursor cells. Following their release of prostaglandin D2 (PGD_2) cysteinyl leukotrienes, cytokines and chemokines, these airway eosinophils migrate through the microvasculature into the airway wall. Release of eosinophil secondary granule proteins (ESGP) by activated eosinophils are indicated as a primary

function executed by these cells. Eosin stained ESGPs contain toxic cationic proteins such as major basic protein (MBP)-1 and MBP-2, eosinophil peroxidase (EPO) and eosinophil-associated ribonucleases, including eosinophil cationic protein and eosinophil-derived neurototoxin (161). The effector functions mediated by ESGPs include cytotoxic activities potentially leading to airway damage and lung dysfunction (162-164), as well as activities that effect proximal lung cells such as ESGP release into the airway lumen with subsequent toxicity that compromises the integrity of cell membrane permeability that may result in the damage or death of lung epithelium (165).

Eosinophils are capable of synthesizing and secreting at least 35 important inflammatory and regulatory cytokines, chemokines, and growth factors (153). Many of these cytokines are potent inducers of immune responses in asthma and other inflammatory diseases. In some situations, eosinophils are the chief producers of cytokines such as TGF-β, which is linked with tissue remodeling in asthma (166). A major distinction in cytokine production between eosinophils and T cells, which generate much larger quantities of cytokines, is that eosinophils store their cytokines intracellularly as preformed mediators, whereas cytokine production is induced only after T cell activation (167).

EPO levels can be exploited to evaluate the severity of the eosinophilic infiltration. EPO concentration is readily calculated from cells collected by way of bronchoalveolar lavage in humans or total lung lavage from mice. EPO catalyzes the peroxidative oxidation of halides (such as bromide, chloride, and iodide) and pseudohalides (thiocyanate) present in the plasma together with hydrogen peroxide generated by dismutation of superoxide produced during respiratory burst (168-170).

This reaction leads to the formation of bactericidal hypohalous acids, particularly hypobromous acid, under physiologic conditions. Eosinophils are robust producers of extracellular superoxide due to expression of high levels of the enzyme complex that generates superoxide (NADPH oxidase) (171) and preferential assembly of the enzyme complex at the cell surface (172).

1.5.2 Dendritic Cells

A prominent characteristic of allergen sensitization is the uptake and processing of inhaled allergens/antigens by DCs in the airway epithelium and surrounding mucosal tissue, with their processes extended out to the airway surface (173-175). Antigen internalized by the DC, is processed and the antigen peptide is then loaded onto the HLA (human)/MHC Class II (mouse) molecule for subsequent presentation to T cells (176). IgE bound to high affinity receptor on the DC enhances uptake of the antigen (177). Once the DC engages the antigen, it is chemotactically signaled to migrate to the lung draining lymph nodes, where it makes contact with naïve T cells (178, 179). Engagement of the MHC/peptide complex to the TCR, accompanied by binding of costimulatory molecules, B7 on the DC to CD28, initiates sensitization and the subsequent immune response to the specific antigen (180). Inefficient interaction between these costimulatory molecules or engagement of CTLA-4 to either of the B7 receptors may lead to anergy (181-183). Once activated, T cells migrate to the airway under the influence of several chemokines interacting with their respective receptors (184, 185). The activated T cells then become potent producers of a variety of cytokines, primarily IL-3, IL-4, IL-5, IL-6, IL-9, IL-13 and granulocyte-macrophage colony stimulating factor (GM-CSF) (186).

1.5.3 Neutrophils

Although the neutrophil is not a predominant cell known in the pathophysiology of asthma, they are understood to play a role in more sever disease states. The upper tract of the respiratory system is colonized with commensal bacteria, whereas the lower tract is sterile. A critical component of the initial innate immune response in the lung is the vigorous recruitment of neutrophils, which peaks within the first 6 hours of an inflammatory response. Neutrophils have the capacity to sense pathogens and migrate across a chemotactic gradient through the epithelium. Recruitment of these cells is a multi step process; Initially, in response to mediators of acute inflammation, vascular endothelial cells upregulate expression of E and P selectin (CD62E and CD62P). The circulating neutrophils express mucins and tetrasaccharides, which are capable of binding to the selectin that tethers them to the vascular endothelium, allowing neutrophilic rolling in the direction of the circulation (187). During circulatory migration, cytokines and chemoattractants act on the neutrophils. These molecules initiate G-protein mediated activating signal that causes conformation modification of the integrin adhesion molecules resulting in neutrophil adhesion followed by transendothelial migration (187). Once in the tissues, the activated neutrophils upregulate chemokine receptors and subsequently migrate up a gradient of the chemoattractant. In addition, the activating signal also stimulates neutrophilic metabolic pathways to a respiratory burst, producing reactive oxygen and nitrogen species (ROS and NOS), which upon release along with mediators of neutrophil primary and secondary granules (proteases, phospholipases, elastases, and collagenases) play a crucial role in the killing of viral pathogens and also contribute to tissue damage subsequent to an inflammatory response (187). Investigations

have shown that efficient neutrophil accumulation is important to induce an adaptive immune response via expression of IL-12 induction of Th1 differentiation of $CD4^+$ T cells (188). In addition, Th17 cells are generated in response to bacterial or fungal infection and subsequent IL-17 production is associated with increases in neutrophil influx and neutrophil-derived products such as metalloproeinases, elastases, and again, ROS and can have detrimental roles that contribute to the pathogenesis of severe lung inflammatory diseases (189).

1.5.4 Mast Cells

Mast cells have been long associated with the early asthmatic reaction subsequent to allergen exposure (190). Two different localized populations of mast cells have been described in asthma: mast cells found in the epithelium and submucosa are fundamental to disease pathogenesis and are highly responsive to inhaled allergens contributing to bronchoconstriction (191), and more recently, mast cells located in the peripheral and deeper in the airways are associated with some of the chronic inflammatory responses in asthma (192). Mast cells are markedly increased in association with airway smooth muscle in both the large and small airway through the action of autacoid mediators such as leukotriene D_4 (LT)$D_{4,}$ prostaglandin D2 (PGD_2) and histamine, and also contributing to the remodeling response (193, 194).

Upon activation, human mast cells release stored histamine, tryptase, heparin and cytokines as well as newly expressed eicosanoids, including; PGD_2 and LTD_4, which interact with cell-surface receptors on eosinophils, macrophages, basophils and mast cells, serving as chemoattractant as well as priming agents (195).

1.6 Regulation of the Lung Inflammatory Process

In the healthy individual, mucosal surfaces encounter nontoxic proteins continuously and vigorous immune responses are not usually induced. The respiratory tract is required to maintain its essential gas exchange function and as a result, has evolved to limit access of pathogen to the immune system with barriers such as the mucosal layer and tight junctions (Figure 1.6).

Figure 1.6 ***Mechanism of Airway Inflammation***

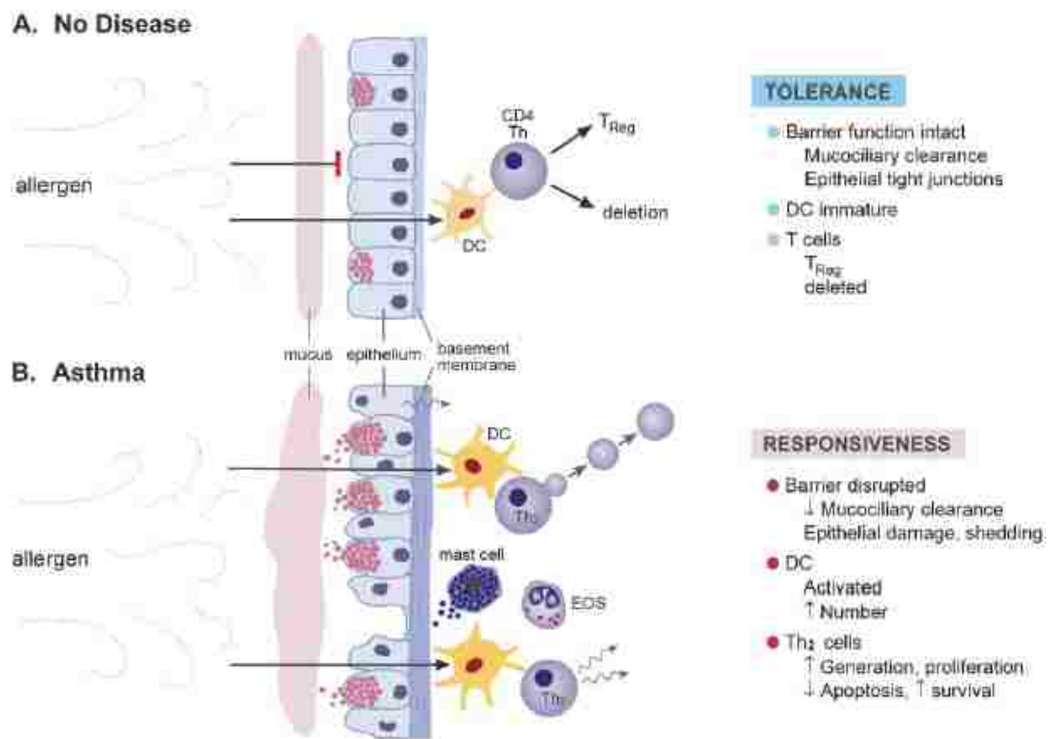

Figure 1.6 *Inhaled allergen exposure to the airway.* A. In non-inflamed tissue, inhaled allergen does not induce an immune response due to intact protective features of the respiratory tract support immune tolerance. B. The asthmatic response includes inflamed airways promoting immune responsiveness, including stimulation of Th2 cells and activation of inflammatory cells such as eosinophils and subsequent release of their inflammatory mediators (1).

However, the respiratory mucosa is not impenetrable. Many aeroallergens possess protease activity that may allow them access through the mucosal barrier consequently, activating innate immune responses by mast cells, γ/δ T cells and NK-T cells thereby promoting Th2 cell development and the ensuing secretion of IL-4 and IL-13 (196-198). Alternately, inhaled antigens activate mechanisms of suppression to induce immune unresponsiveness to naïve T cells. These tolerogenic effects of inhaled antigens have been shown to be both antigen specific and nonspecific (199-202), and can render CD4+ T cells anergic to antigen (203) $CD4^+$, $CD8^+$ and γ/δ T cells can be induced by inhaled antigens to a regulatory phenotype (200, 204, 205). APCs at mucosal sites have unique features that stimulate the development of tolerance. The generation of iTregs appears to be primarily under the control of mucosal DCs that produce IL-10 and promote the generation of Tr1/Th3 cells and Th2 cells (201). When an effector/ suppressor population is established in the lymphoid tissue, the ensuing cytokine environment will influence the subsequent cellular interactions and lead to immune tolerance. Also, downregulation of Class II MHC and costimulatory molecules on DCs at mucosal sites play important roles in the divergence of mucosal immune responses toward T cell differentiation to a regulatory phenotype or anergy of the effector cells (206, 207). Conversely, a feedback mechanism has been described, illustrating that Tregs have the capacity to induce tolerogenic DCs that have decreased costimulatory activity and can induce anergy in CD4+ T cells, thus enhancing immune unresponsiveness (208, 209). In addition to the Treg/DC paradigm in the lung, alveolar macrophages are extremely potent in suppressing immune responses whereby imposing a regulatory effect on the DC (210).

1.6.1 Eicosanoids

In addition to cell mediated immune regulation, lipid mediators are also crucial in the signaling pathways associated with both pro and anti-inflammatory immune responses. Eicosanoids are the general class of signaling molecules derived from the product of oxygenation omega-3 (ω-3) or omega-6 (ω -6) essential fatty acids (EFAs).

Figure 1.4 *Eicosanoid pathway*

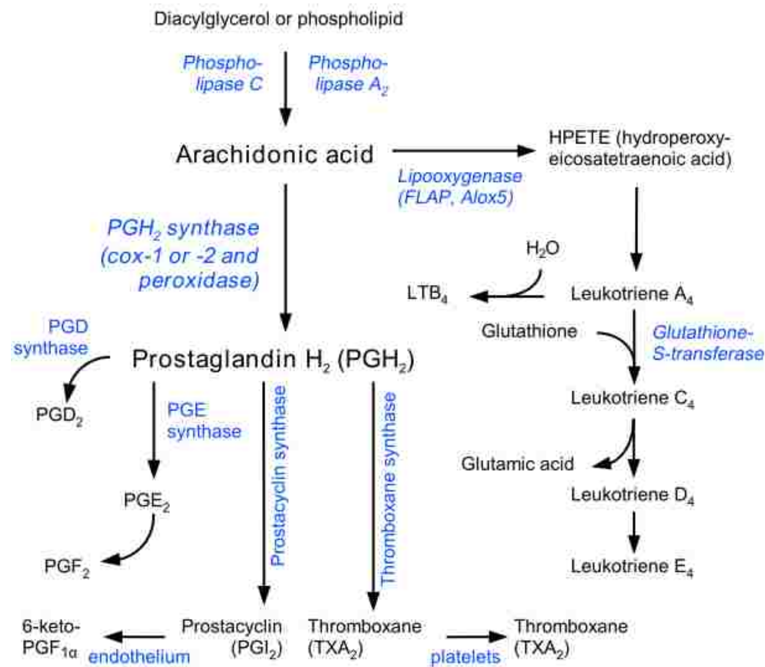

Figure 1.4 *Metabolism of Arachidonic acid to PGI2. Membrane bound diacylglycerol or phospholipids are generated via the cyclooxygenase pathway* (211).

The eicosanoids – prostaglandins (PG) and thromboxanes (TX) mediate local immune responses such as inflammation, vasoconstriction or vasodilatation, coagulation, pain and fever. Prostanoids are products of arachidonic acid metabolism synthesized via

the cyclooxygenase (COX) pathway (Figure 1.4) (181). The two isoforms of this enzyme, COX-1 and COX-2, metabolize arachidonic acid to PGH_2, which is subsequently processed by specific enzymes to generate a series of products, most notably PGD_2, PGE_2, $PGF_2\alpha$, prostacyclin (PGI_2), and thromboxanes (Tx). COX-1 is constitutively expressed in most cell types, and is the predominant form present in the gastrointestinal tract, kidney, and platelets (212-214). COX-2 is expressed at inflammatory sites by macrophages, neutrophils, and activated mesenchymal cells and is though to produce prostanoids that contribute to inflammatory swelling, pain, and fever (212-214). Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, are nonselective COX inhibitors and inhibit these inflammatory processes. The inhibition of COX-1, however, underlies the gastrointestinal toxicity of NSAIDs in humans, and this has prompted the development of COX-2-selective inhibitors such as NS-398 and celecoxib, which circumvent such detrimental effects (215). The spectrum of prostanoids catalyzed by COX-2 differs from that synthesized by COX-1. Specifically, COX-2 is thought to be important for the production of PGE_2 and PGI_2 (216). PGI_2 has anti-thrombotic effects in vivo and plays an important role in inflammation and pain perception (217). Despite these potent actions, the effects of PGI_2 appear to be very localized, since the prostanoid is highly unstable, with $t_{1/2}$ of ~30 seconds under physiological conditions (212).

Leukotrienes also play a vital role in mediating inflammation. The leukotriene LTB4 is involved in cell adhesion and chemotaxis of leukocytes and stimulates aggregation, enzyme release, and generation of superoxide in neutrophils (218). Commercially available pharmaceuticals, drugs such as montelukast and zafirlukast that

block leukotriene receptor signaling have been shown to reduce symptoms associated with allergic inflammation such as asthma, psoriasis, and rheumatoid arthritis.

1.6.2 Eicosanoid Pro-Resolution Molecules – Lipoxins, Resolvins

Lipoxins (LXs) are metabolites of arachidonic acid converted via lipoxygenase (LO), and were the first lipid mediators identified as having inflammation resolving properties. These eicosanoids are structurally and functionally distinct from pro-inflammatory prostaglandins and leukotrienes (219). Produced at the site of inflammation, these molecules are act via cell-cell interactions of activated epithelial cells followed by recruitment of eosinophils or neutrophils (219). LXs signal through specific interaction with the high affinity LXA4 (ALX) receptor, which is expressed on the surface of human airway epithelial cells, PMNs, monocytes, enterocytes and synovial fibroblasts (220-224). Airway inflammation or injury induces upregulation of the ALX receptor on both proximal airway and distal alveolar cells (220, 225).

LXs are produced in many human inflammatory lung diseases, including asthma. In fact, a characteristic of severe asthma is diminished LX expression (226, 227). LXs provide stop signals for PMN chemotaxis, trans-endothelial and trans-epithelial migration, production of reactive oxygen species (ROS) and neutrophilic granule release (228-230). Conversely, LXs promote monocytes locomotion (231), macrophage mediated clearance of apoptotic PMN and microbial products by phagocytosis (232). LX and their stable analogs have been shown to prevent PMN mediated tissue injury, enhance bronchial epithelial cell proliferation in response to injury and block release of

pro-inflammatory cytokines from epithelial cells (233). Furthermore, LXs are associated with increased epithelial cell anti-infectious activities (234).

Resolvins are also pro-resolving lipid mediators derived from omega-3 fatty acids via either COX-2 or Cytochrome P450 enzymes and subsequentially modified by PMN LOs to the bioactive form (235). Receptors for resolvins have been identified on monocytes, macrophages and DCs (236). Although resolvins have yet to be identified in lung disease, they likely play a role in resolving allergic airway responses as they have anti-inflammatory effects in models of peritonitis and ischemia-reperfusion injury Resolvin E1 inhibits PMN transmigration and promotes the phagocytosis of apoptotic PMN by macrophages (237-239). Recent studies have suggested Resolvin E1 as a potential therapeutic for the treatment of asthma (240).

1.7 Mouse models of asthma

Asthma is a complex disease with multifaceted etiologies and complicated underlying mechanisms. Along with most human diseases, studies using laboratory animals have provided much of the current understanding of the mechanisms driving asthma. The ability to produced accurate animal equivalents of the disease in the animal is amid the top concerns of the investigator when crafting the experimental design. The development of these animal models is a continued work in progress. Although various aspects of asthma have been persuasively demonstrated in animals, invariably every animal model misses some critical feature of the human disease. In addition, very few animals are known to develop any condition similar to asthma, with the exception to an allergic syndrome in cats (241). The knowledge that asthma involves interactions

between the innate and adaptive immune systems can be in large part credited to studies done in animal model (242). Much like most animal-based biomedical research, mice are, in general, are the species of choice to study asthma, because of the immunological and molecular tools available to study them as well as the obvious practical advantages in terms of cost and simplicity of breeding protocols.

Only a very limited literature database in terms of PGI_2 and asthma is available at the present as well as currently no commercially available markers specific to the IP, makes study of this signaling pathway very challenging. The experimental model used to study the role of PGI_2 signaling in allergic asthma, employed mice with a dysfunctional receptor for PGI_2 – $IP^{-/-}$. This model system was able to reliably and reproducibly induce pulmonary inflammation in both wt and knockout animals by immunizing and exposing the primed animals to aerosolized whole OVA. Using this model system, we were able to show that loss of the ability to signal via the IP, manifested in increased AHR, augmented pulmonary lymphocyte an eosinophilic influx, as well as elevated serum IgE, therefore we deemed this model could potentially translated to human asthma like symptoms and was sufficient for this research.

The DO11.10 mouse is genetically engineered that the T cell receptor specifically recognizes the 323-339 amino acid sequence of the OVA peptide. The investigation described in chapter four, exploited this property, where effector and regulatory T cells from the DO11.10 mouse were expanded in culture, to provide cells used for adoptive transfer studies in the wt (BALB/c) mice. The recipient animals were subsequently repeatedly exposed to aerosolized whole OVA to induce a quantifiable lung inflammation which reliably demonstrated characteristics similar to those in detected in human asthma

(243). In addition, the KJ1-26 antibody which is specific for the transgenic TCR could be used to evaluate the activities of the transferred cells apart from the host response.

2.0 Materials and Methods

2.0.1 Cell culture

Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, L-glutamine (Gibco, Carlsbad CA), penicillin and streptomycin (Gibco), Hepes (Gibco), Sodium Pyruvate (Gibco) and 2-mercaptoethanol (Sigma).

2.0.2 Animals

DO11.10, BALB/c, C57BL/6 and C129-IL4 mice were purchased from Jackson Labs, Bar Harbor ME (The Jackson Laboratory) were bred and housed in pathogen-free conditions in the animal facility at the (University of Montana, Missoula, MT). $IP^{-/-}$ mice were the generous gift of Dr. Garret A. Fitzgerald (University of Pennsylvania, Philadelphia, PA). All experiments were performed to the guidelines of the National Institutes of Health, Bethesda, NIH. IACUC

2.0.3 Immunization of $IP^{-/-}$ and wt mice with OVA/Alum

OVA/Alum antigen inoculant [1mg/mL] was prepared by mixing together 2 mL of 2.0 mg/mL Ovalbumin (Sigma, St. Louis, MO) in PBS and 2 mL of Imject®Alum (aluminum hydroxide, magnesium hydroxide and inactive stabilizers (Pierce, Rockford, IL) followed by a 30 min incubation at 37°c. The mixture was then centrifuged at 1200 RPM x 10 min. The pellet was resuspended in 4 mL of PBS. The $IP^{-/-}$ and wt mice, in groups of 4, received 100 μl of the OVA/Alum by intra-peritoneal (ip) injection (100 μg). The mice were then rested for 10 days, before initiating treatment and or exposure to aerosolized OVA in PBS.

2.0.4 Lung histology

Non-lavaged lungs were obtained and one part of the lung tissue was fixed in 10% Formalin, embedded in paraffin, and then (2 μm) sections stained with H&E, which was performed by Lou Herritt in the Confocal Microscopy and Image Analysis Laboratory. The samples were imaged on an Olympus fluorescent microscope.

2.0.5 Measurement of Airway Inflammation

BAL was performed by cannulation of the trachea of each euthanized (Euthasol 100μl) animal and washing the airways with 3 x 0.5 mL of PBS to collect BAL fluid. BAL fluid of four animals was pooled and EPO levels present in BAL cells were determined (method described below).

2.0.5.1 Cell differential

Cytospin preparations were performed on $5x10^4$ cells followed by staining the cells using a Wright-Giemsa-protocol (Hema 3 Staining kit, Fisher Scientific, Houston, TX). Cell differential percentages were determined by light microscopic evaluation of stained and expressed as absolute cell numbers. Levels of cytokines IL-4, IL-5 or IL-17 in the BAL were measured by ELISA as described.

2.0.5.2 Eosinophil peroxidase (EPO) assay

This assay is specifically used for detecting eosinophil peroxidase (EPO), which is contained within eosinophils. The EPO activity in BAL cells was determined by colorimetric assay. 100 μl of PBS is added to each well in a 96 well flat bottomed plate

(Falcon). The cells from the BAL fluid are resuspended in PBS pH 7.0 in a final volume of 300 μl. In triplicate for each sample, 100 μl of the cell suspension is added to the top well and serial diluted through the 8th well. The substrate solution is prepared by crushing one tablet of orthophenylene diaminedihydrochloride, (OPD, Sigma) (final concentration of 0.1%) in 50 μM Tris-HCL (Sigma) containing 0.1% Triton X-100 (Sigma) and 1 mM hydrogen peroxide (Sigma). 100 μl of the substrate solution is added to each well and the plates are then incubated at room temperature for 30 min or until sufficient color development has occurred at which time 50 μl or 0.3 M sulfuric acid (Sigma) is added to stop the reaction. The absorbance is measured using an automatic plate reader (Molecular Devices VersaMax microplate reader) at 495 nm. The measurements taken were total and not released EPO, and were indicative of the number of eosinophils present in the BAL.

2.0.6 Measurement of Cytokines

2.0.6.1 IL-4, IL-5 and IFN−γ Measurements

To examine cytokine production, cells from digested lung tissue, BAL or in vitro (4 and 8-day polarized cells) (5 x 10^5/mL) were stimulated with immobilized anti-CD3 (2 μg/ mL) for 24 h, and the supernatants were harvested for measurement of IL-4, IL-5 and IFN-γ by enzyme linked immunosorbant assay (ELISA) were performed. The ELISA is a technique is used to detect an unknown amount of cytokine in a sample.

The protocol used to assay cytokines IL-4, IL-5 and IFN-γ are as follows: 50 μl of anti-IL4 (clone 11B11 from hybridoma cultivated in our laboratory), IL-5 (Pharmingen), IFN-γ (R4-6A2, American Type Culture Collection (ATCC), Manassas, VA) capture antibodies [5.0 μg/mL final], diluted in 1 mM carbonate/bicarbonate buffer (pH 9.6), are

pipetted into a 96 well plate and stored at 4°C overnight followed by 2 washes on an automated plate washer (Thermo Electron Wellwash 5 MK2 Plate washer, Fisher Scientific) with ELISA wash buffer (WB) (PBS & 0.5%Tween). 200 μl of blocking buffer (1% bovine serum albumin (BSA) (Sigma) in 1 mM carbonate/bicarbonate buffer pH 9.6) was added and incubated at room temperature for a minimum of 2 h; the excess was then washed off with WB x 5. Standards and samples were added (50 μl/well) and incubated over night at 4°C. The plates were washed with WB x 5. Biotin-conjugated detection antibodies (anti-IL-4 (Pharmingen), IL-5 (Pharmingen) and IFN-γ (Pharmingen) were diluted to 2.5 μg/mL in blocking buffer and 50 μl was added to each well and allowed to incubate at room temperature for 45 min followed by washing with WB x 5. Streptavidin-conjugated horseradish-peroxidase (SA-HRP) (Jackson ImmunoResearch Laboratories Westgrove ,PA) was diluted 1:4000 in blocking buffer. 100 μl of SA-HRP was added to each well and incubated for 30 min at room temperature. The plates were washed with WB x 5 and blotted dry. 100μl of 3,3',5,5' Tetramethylbenzidine (TMB) (Sigma) substrate was added to each well, incubated up to 30 min at room temperature at which point the reaction is stopped with 100 μl of 0.3 M H_2SO_4 (Sigma). The absorbance was read at 495 nm.

2.0.6.2 IL-17 Measurements

The R&D systems (Minneapolis, MN) IL-17 ELISA kit was used to quantify this cytokine. The protocol was similar to the previously described with the exception of the following: The capture antibody was diluted to 2.0 μg/ml in PBS and 100 mL added per

well in a 96 well plate. The plate was sealed and incubated at room temperature overnight. The plate was washed with WB x 3. The plate was blocked by adding 300 μl of blocking buffer (1% BSA in PBS) to each well and incubating the plate for a minimum of 1 h at room temperature. The plate was washed with WB x 3. 100 μl of samples or standards (diluted in blocking buffer) were added to the appropriate well and incubated 2 hours at room temperature. The plates were washed with WB x 3. 100 μl of detection antibody [200 ng/mL] was added per well and incubated for 2 h at room temperature. The plates were washed with WB x 3. 100 μl of SA-HRP (1:200) was added to each well and incubated for 20 min at room temperature in the dark. The plate was washed with WB x 3. 100 μl of TMB was added to each well, the plate was incubated avoiding direct light for up to 20 min and the reaction was stopped with 0.3 M H_2SO_4. The absorbance is measured at 450 nm on the plate reader.

2.0.7 Measurement of Immunoglobulins

$IP^{-/-}$ and wt mice were euthanized by injection of 100 μl Euthasol (Virbac, Fort Worth TX), IP. Blood was collected via cardiac puncture and the serum fraction was stored at -20°C. Total IgE, IgG1 and IgG2a were assayed using a protocol similar to that used to measure IL-4. The following antibody pairs were used; IgE capture Ab (Pharmingen clone R35-93), detection Ab (Pharmingen clone R35-72); IgG1 capture Ab (Pharmingen clone A85-3), IgG1detection Ab (Pharmingen clone A85-1); IgG2a capture Ab (Pharmingen clone R11-89), IgG2a detection Ab (Pharmingen R19-15). IgEκ chain isotype control (anti-TMP). IgA, IgG2b, IgG3 and IgM were assayed using Southern Biotech immunoglobulin ELISA kit (Catalog # 5300-05)

2.0.8 Monitoring the phenotypic changes of the IP$^{-/-}$ CD4+ T cells in vitro

To monitor the effect of PGI_2 on Th2 polarization in vitro, PLN cells from IP$^{-/-}$ and wt mice were first depleted of CD8$^+$ cells by incubation of the cells with 2 μl anti-CD8 PE (Pharmingen) for 20 min in the dark then washing with 1ml staining buffer, centrifuging for 5 min at 1200 RPM. The pellet was then incubated with 20 μl anti-PE microbeads for 20 min in the dark followed by another washing. The pellet is resuspended in MACS buffer and the CD8$^+$ cells are depleted by magnetic column (Miltenyi Biotec Inc). The flow through was collected and the CD8 depleted cells (1x10^6 cells/mL) were cultured on immobilized anti-CD3 (2 μg/mL) in the presence of IL-4 (2 ng/mL) and anti-IFN-γ (5μg/ml) for 4 days. On day five the cells were harvested and counted. FACS was performed to analyze the expression of CD4 (APCCy7, Pharmingen), CD8a (PE Biolegend, San Diego, CA) CD62L (FITC, Pharmingen) and ICOS (PE, Pharmingen)

2.0.9 Isolation of Lung Mononuclear Cells

Lung tissue was dispersed by collagenase (Type IV, Sigma-Aldrich) and fractionated using Percoll gradient (GE Healthcare, Piscataway, NJ) as detailed previously (244) and the resultant lung mononuclear cells (LMCs) were stimulated with OVA peptide or anti-CD3 for 24 h.

2.0.10 Preparation of CD4$^+$ Th1, Th2 and Th17 cells

Peripheral lymph node cells (PLN) from DO11.10 mice (5 x10^5/mL) were induced to differentiate into a Th2 effector phenotype by incubation for 4 d in the presence of $OVA_{323–339}$ peptide (1μg/ml, Mimotopes, San Diego, CA) and murine IL-4 (2

ng/ml; R&D Systems,) plus anti-IFN-γ Ab (5 μg/mL, R4-6A2; American Type Culture Collection (ATCC), Manassas, VA). After 4 d of incubation, the cells were restimulated as before for another 4 d, but this time also in the presence of IL-2 (10 ng/mL R&D Systems). To drive T cell differentiation into a Th1 phenotype, PLN cells from DO11.10 mice ($5x10^5$cells/ml) were incubated in the presence of $OVA_{323–339}$ peptide (1 μg/mL), and mouse IL-12 (1ng/ml; R&D Systems) plus anti-IL-4 Ab (5 μg/ml, 11B11; ATCC). After 4 days of culture, cells were restimulated as before for another 4 d but this time also in the presence of IL-2 (10 ng/mL). To generate Th17 effector cells, PLN cells ($5x10^5$/mL) were incubated in complete RPMI with mitomicin C (Sigma, St. Louis, MO)-treated APCs ($1x10^6$/mL) in the presence of TGF-β (2 ng/mL; eBioscience, San Diego, CA), IL-6 (10 ng/mL; R&D Systems), anti- IFN-γ (5μg/mL HB-170), and anti-IL-4 Ab (5μg/mL 11B11). After 4 days of culture, the cells were restimulated as before, in addition to the presence of IL-23 (10ng/mL; R&D Systems).

2.0.11 Preparation and Expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ Regulatory T cells in Culture

$CD4^+CD25^+$ T regulatory cells were purified from DO11.10 PLN cells using MACS magnetic cell sorting beads. (Miltenyi Biotec Inc., Auburn CA). The homogenized lymph nodes were first depleted of the unwanted cells by staining with $CD4^+CD25^+$ Regulatory T cell biotin-antibody cocktail (anti-mouse abs: CD8a, CD11b, CD45R, CD49b and Ter-119, incubated for 10 min. Adding anti-biotin microbeads and CD25-PE, incubated followed by washing the cells with MACS buffer. The resuspended cells were then separated via magnetic column, the flow through fraction was collected

and incubated with anti-PE microbeads, again washed and resuspended in MACS buffer and the PE positive CD25 cells were depleted by capture on magnetic column. The $CD25^+$ cells were eluted when the magnetic column was removed from the magnet and flushed through with MACS buffer. The eluted cells were then put through a second series of magnetic selection using a fresh magnetic column. FACS was used to evaluate the purity of the $CD4^+CD25^+$ cell population from the isolated T cells and the efficiency of the isolation from the $CD4^+CD25^-$ fraction of cells.

The purified $CD4^+CD25^+$ and $CD25^-$ populations ($5x10^5$ cells/mL) were cultured in complete RPMI media in the presence of $OVA_{323-339}$ peptide (1 μg/mL), IL-2 (10 ng/mL), IL-4 (2 ng/mL) + anti-IFN-γ antibody (5 μg/mL), and mitomycin C-treated antigen-presenting cells (APC) ($2x10^6$/mL BALB/c spleen cells). After 4 d of culture, the cells were re-stimulated as stated above without adding further APCs but with the addition of exogenous IL-2 (10 ng/mL), for 4 additional d. The expansion of $CD4^+CD25^+$ T cells in the presence of exogenous IL-2 was limited and was typically around a 30-fold increase in cell numbers over 8 d. Foxp3 expression was verified as described below. The $CD4^+CD25^+$ T cells expanded in IL-2 plus IL-4 were used for experiments to examine suppressor function.

2.0.11.1 Quantification of Foxp3 Expression

$CD4^+CD25^+$ and $CD4^+CD25^-$ cells that were expanded under Th2 polarizing conditions for 8 d were intracellular stained using anti-Foxp3 Ab. To determine the purity of the culture, the Biolegend Mouse Treg Flow™ Kit (FOXP3 Alexa Fluor ® 488/CD4

APC/CD25 PE) was used. First, 20 μl of CD4 APC/CD25 PE cocktail was added to$1x10^6$ cells/ tube. The tubes were vortexed and incubated at room temperature in the dark for 20 min, followed by a wash with 1 mL of stain buffer (5 min at 1200 RPM). Working solutions of FOXP3 Fix/Perm buffer (4X) and FOXP3 Perm buffer (10X) were prepared. The cells were resuspended in 1 mL of FOXP3 Fix/Perm buffer (1X) and incubated at room temperature in the dark for 20 min. The cells were again washed in 1 mL of stain buffer and again with 1 mL FOXP3 perm buffer. The cells were then resuspended in 1 mL FOXP3 perm buffer and incubated at room temperature in the dark for 15 minutes followed by centrifugation (5 minutes at 1200 RPM). The cell pellet was resuspended in 100μl of FOXP3 perm buffer. 5 mL of either Alexa Fluor® 488- anti-mouse FOXP3 antibody or Alexa Fluor ® 488 mouse IgG1, k isotype control was added to the cells and incubated at room temperature in the dark for 30 min. The cells are washed twice with cell staining buffer and resuspended in 0.5 mL staining buffer and analyzed on the FACSaria.

2.0.12 Adoptive Transfer of CD4+ T cells into Mice and Ovalbumin Aerosol Challenge

$3x10^6$ expanded Tregs were adoptively transferred into BALB/c mice at the same time as $8x10^6$ DO11.10 $CD4^+$ Th1, Th2 or Th17 cells. Mice (four per group) were then intra-nasally challenged by exposure to aerosolized solutions of OVA (0.5%, Grade V; Sigma-Aldrich, Poole, U.K.) for 20 min/d over 7 consecutive d using a Wright's nebulizer (Buxco). Control mice were mice that received either Treg, Th1, Th2 or Th17 cells alone or no cells but were exposed to OVA aerosols.

2.0.13 Monitoring the Regulation of Th2 Differentiation of $CD4^+$ T cells in vitro

To monitor the effect of Treg cells on Th2 polarization, IL-4 reporter mice were used, designated IL-4/GFP mice (IL-4 green fluorescent protein enhanced transcript, C129.IL-4. PLN cells ($1x10^6$), collected from naïve C129.IL-4 mice, were co-cultured with DO11.10 Tregs ($2x10^5$), in the presence of plate-bound anti-CD3 (2C11 2 μg/mL) (ATCC), IL-4 (2 ng/mL) and anti-IFN-γ (5 μg/mL) for 4 d. FACS was used to examine GFP expression. FACS analysis of endogenous GFP/IL-4 expression (FITC) vs. $CD4^+$ (CD4 APC-Cy7, Pharmingen, San Diego, CA). Anti-clonotype KJ1-26-APC-Cy7 (Pharmingen) staining was used to identify and subtract the DO11.10 Treg cells from the analysis, which was performed on a FACSAria (Becton Dickinson, by DIVA software, Franklin Lakes, NJ).

2.0.14 Flow cytometric analysis of spleen, lymph node and lung mononuclear cells from in vivo experiments with Th1, Th2 or Th17 co-transferred with nTregs

Cells were stained and analyzed on a FACSAria (BD Biosciences, San Diego, CA) using FACSDiVa software for performing three-color analysis to enumerate $CD4^+$ T cells (APC-Cy7), OVA-specific T cells (KJ1-26-FITC) and $CD25^+$ T cells (PE). Flow cytometric analysis of endogenous GFP/IL-4 expression (FITC) vs $CD4^+$ (anti-CD4 APC Cy7) was performed. The DO11.10 Tregs are enumerated using PE conjugated anticlonotype antibody KJ-126. The same staining scheme was used in addition to granulocyte identification by anti-Gr1 (Pharmingen) Biotin/Streptavidin-APC (Pharmingen) and anti-CD11b FITC (Pharmingen) to for neutrophils in LMNC.

2.0.15 Statistical Analysis

Unless otherwise described, data are summarized as means $\pm$ SEM. Data obtained from adoptive transfer experiments comparing two variables, were analyzed using the students t test, and differences were considered statistically significant with $p < 0.05$. To determine statistical significance of groups, one or two way ANOVA tests were used for analysis. The Prism software package was used in all cases.

Chapter 3

A Role for Prostacyclin and its Receptor, IP in Regulating Allergic Inflammation and the Humoral Response

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Running Title: PGI_2-IP in Regulates Allergic Inflammation and the Humoral Response

Keywords: Lung inflammation, Th2, PGI_2, IgE

Abbreviations: AHR, airway hyperreactivity; Treg, regulatory T cell; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; LMC, lung mononuclear cell; PLN, peripheral lymph node cell.

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3.0 Abstract

In order to investigate the mechanism underlying the anti-inflammatory properties of PGI_2 a murine model of allergic lung inflammation was developed in which PGI_2 receptor deficient mice, $IP^{-/-}$, were immunized with OVA and exposed to repeated ovalbumin (OVA) aerosols. We observed that lack of expression of the PGI_2 receptor resulted in a marked increase in pulmonary inflammation demonstrated by augmented airway hyperreactivity, and eosinophilic infiltration into the lungs of $IP^{-/-}$ mice. $IP^{-/-}$ mice, following immunization and aerosol inhalation, displayed augmented levels of IL-4 production by LMC and elevated serum levels of IgE and IgG1. Interestingly, in $IP^{-/-}$ control animals not exposed to OVA, the level of serum antibodies of several isotypes was profoundly decreased. FACS analysis revealed that the number of $CD19^+$ B cells, $CD4^+$ and $CD8^+$T cells was elevated in the lungs of $IP^{-/-}$ compared to wt mice following OVA inhalation. Moreover, in OVA-immunized $IP^{-/-}$ mice, treatment with non-selective COX inhibitor indomethacin failed to increase the level of pulmonary eosinophilia. In contrast, the eosinophilic inflammation in wt mice was markedly increased by indomethacin treatment. Finally, in vitro studies showed that CD4+Th2 cells treated with the stable PGI_2 analog Iloprost express high levels of L-selectin (CD62-L). Our results identify PGI_2-IP as an important pathway for inhibiting allergic pulmonary inflammation that appears to play a part in NSAID-induced exacerbations of allergic inflammation. The humoral response is also markedly influenced by expression of the PGI_2 receptor, IP.

3.1 Introduction

The prevalence of asthma has dramatically increased worldwide in the past two decades. Allergic asthma is characterized by airway hyperreactivity (AHR) and chronic bronchial inflammation in response to a range of environmental stimuli such as allergens. The hallmarks of allergic asthma include airway infiltration by $CD4^+$ T cells and eosinophils, thickening of the airway tissues and increased mucus production and elevated levels of serum IgE (1, 5). It has been proposed that allergic asthma is associated with dysregulation of the Th2 type inflammatory response. Subsequent to allergen exposure Th2 cytokines IL-4, IL-5, IL-9 and IL-13 are key in driving pulmonary inflammation and AHR. IL-4 is particularly crucial in $CD4^+$ T cell commitment to a Th2 phenotype and IL-9 and IL-13 are important in AHR and mucus production. IL-5 mediates eosinophil development, activation and recruitment to the affected tissues (30, 245, 246). While the Th2 phenotype is linked to asthma and clearance of extracellular organisms, Th1 cells and their production of IFN-γ have been linked to viral clearance. IFN-γ has been identified with inhibition of antigen-induced infiltration of T cells and eosinophils in to the airways of mice (247). Currently, both nTregs and PGI_2 have been shown to play important roles in regulating allergic inflammation in asthma (243, 244).

PGI_2 is a major product of cyclooxygenase (COX) metabolism. In this process, arachidonic acid is cleaved from the membrane phospholipids by phospholipase A2 and converted by COX to PGH_2. PGH_2 is then converted, via the isomerase- PGI_2 synthase (PGI_2S), to PGI_2.

PGI_2 is a potent vasodilator and inhibitor of platelet aggregation (248). PGI_2 is very unstable at physiological pH and is readily hydrolyzed to form the inactive 6-keto-

$PGF_{1\alpha}$ (249). PGI_2 exerts its biological effect by binding to a cell surface G protein-coupled IP receptor (250). IP receptor activation by an agonist leads to augmented production of intracellular cAMP via stimulation of adenylate cyclase (250). PGI_2-IP receptor signaling is important in preventing thrombosis, inhibiting injury-induced vascular proliferation (251), modulating allergic airway responses and mediating inflammatory swelling and pain (217, 252).

It has been shown in our laboratory previously that PGI_2 production is increased during Th2 but not Th1-mediated pulmonary inflammation and plays a key role in regulating allergic responses (244, 253). The aim of this study was to determine the mechanism by which PGI_2-IP signaling mediates regulation of allergic airway inflammation.

3.2 Results

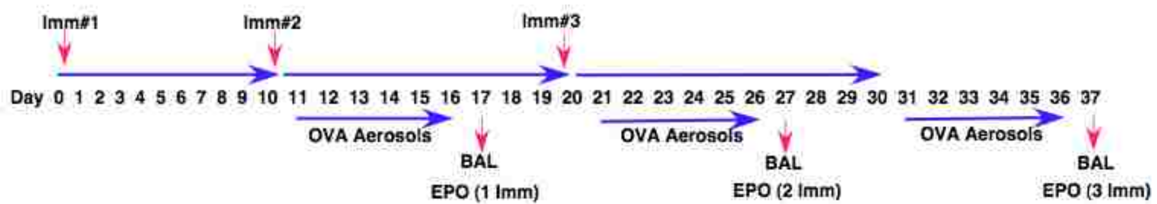
3.2.1 Characterization of Allergic Pulmonary Responses in Mice Lacking PGI_2 Receptors- $IP^{-/-}$

Since the receptor for PGI_2 (IP) is upregulated by IL-4 and COX-2 inhibitors exacerbate allergic inflammation (244, 253), we examined the role of PGI_2 in modulating allergic lung inflammation (244, 253). To elucidate the role of PGI_2 signaling in the modulation of the inflammatory response occurring in the asthmatic airway, we assessed the difference in the inflammatory response in wild type C57BL/6 mice and animals that have genetically altered non-functional prostacyclin receptor ($IP^{-/-}$), using a murine model of allergic asthma. It has previously been shown by Nagai et al (2003), that these mice primed with antigen and exposed to repeated aerosol challenges will elicit an eosinophilic response that is more severe than that observed in wt mice. However, the underlying mechanism as to how this exaggerated response occurs is unknown. The work illustrated in this study demonstrates that PGI_2 signaling plays a role in the regulation of the allergen induced inflammatory response produced in the lung. Since Th2 cells preferentially express IP, we examined how PGI_2 influences Th2 responses both in vivo and in vitro. We demonstrate phenotypic differences between the $IP^{-/-}$ and wt mice, in relation to the Th2 response and exploit these differences to shed light on the underlying mechanism of action utilized by PGI_2 signaling in the regulation of pulmonary inflammation and AHR.

3.2.1.1 *Establishing a mouse model of antigen induced airway inflammation using C57BL/6 and IP$^{-/-}$ mice.*

We developed a model of murine immunization and antigen aerosol exposure to study how PGI_2 signaling impacts allergen induced airway inflammation. In deriving a protocol to optimize the phenotypic differences between the wt and the PGI_2 IP$^{-/-}$ mouse strains, we performed a series of experiments in which the antigen dose was varied. Figure 3.1 illustrates that wt and IP$^{-/-}$ strains were immunized with 1, 2 or 3 doses of a PBS suspension of 100μg of whole OVA protein complexed with Alum adjuvant. The animals were rested for 10 days between doses. Subsequent to last immunization and rest period, the animals were then exposed to aerosolized OVA for 20 minutes a day for 6 days. Twenty-four hours following the final challenge, the animals were euthanized and bronchoalveolar lavage (BAL) was performed to collect BAL fluid (BALF).

Figure 3.1 ***OVA immunization and aerosolization protocol for the induction of airway inflammation in wt and IP$^{-/-}$ C57Bl/6 mice.***



Eosinophil infiltration to the airways is a defining characteristic of asthma and a gauge of the severity of the inflammatory response. Eosinophil peroxidase (EPO) is an easily quantified product of eosinophils and indicator of airway inflammation. Cell-

associated EPO levels were present in the BAL collected from immunized and challenged wt and $IP^{-/-}$ animals. A single OVA immunization was sufficient to elicit an eosinophilic response on subsequent OVA aerosol challenge. Figure 3.1 demonstrates that in $IP^{-/-}$ mice, a 2.5-fold increase in the level of EPO activity, compared to the wt mice, was induced in the mice that received one dose of OVA/Alum and exposure to OVA aerosols. Animals immunized with either 2 or 3 doses resulted in a pronounced pulmonary inflammation, whereby the difference in eosinophilic infiltration between the $IP^{-/-}$ and wt could not be discerned. Based on this data, the protocol requiring one dose of OVA/Alum and the time course outlined in figure one was sufficient to induce a pulmonary eosinophilia and was chosen for the investigation of allergen induced airway inflammation, described in this chapter.

Figure 3.2

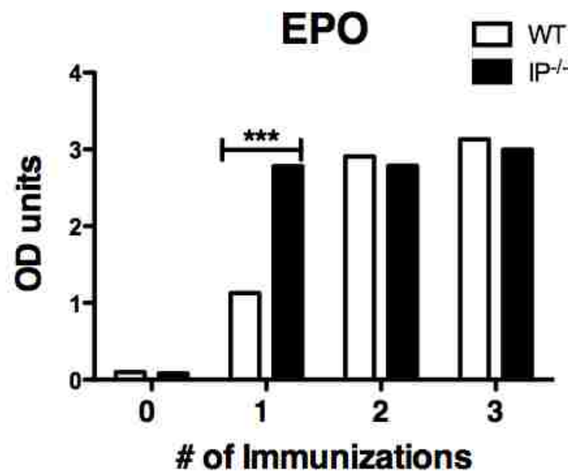


Figure 3.2 ***The optimal number of immunizations required for discernment of phenotypic differences between $IP^{-/-}$ and wt C57BL/6 mouse strains***. All mice received 100μl of 1mg/mL OVA/Alum in PBS and received one, two or three doses and repeated OVA aerosols following a ten-day rest period. EPO levels were determined by colometric analysis. Data represents means ± SEM (n=3) and represents two separate experiments. *** = p <0.001

3.2.1.2 *Histologic examination of lung inflammation in $IP^{-/-}$ and wt mice*

To examine whether the deficiency of the IP receptor would influence the development of pulmonary inflammation, the lungs of sensitized (immunized) mice subsequent to repeated exposure to OVA aerosol were examined for structural changes. Histological evaluation using H&E stained sections, revealed a pronounced peribronchial and perivascular eosinophilic inflammation in the lung parenchyma of the OVA challenged $IP^{-/-}$ mice (Figure 3.3). This was in marked contrast to the moderate levels of inflammation observed in the in the airways of challenged wt mice. In both mouse strains, inflammatory cells were restricted to sites that were juxtaposed to airways and blood vessels. Inflammatory cells did not infiltrate into the alveolar spaces. Normal pulmonary architecture and vasculature was observed in the control unchallenged mice.

Figure 3.3

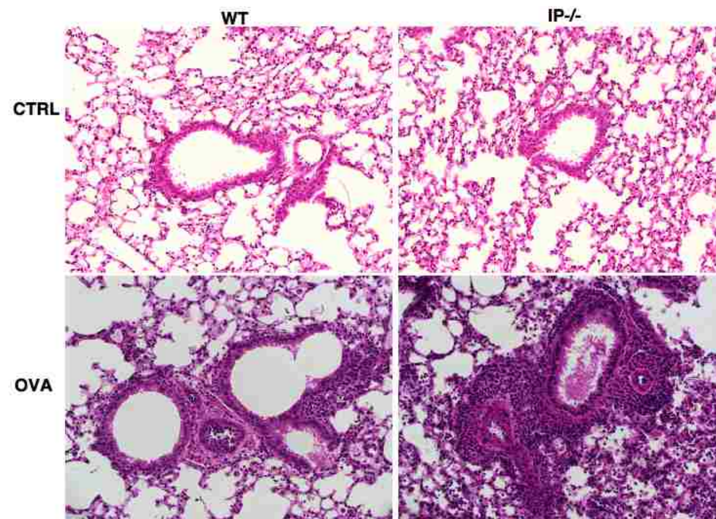


Figure 3.3 ***Increased inflammation in the airway of $IP^{-/-}$ mice following immunization and aerosol challenge with OVA*** Lung tissue histology stained with H&E (X25). Primed WT mice were exposed to OVA aerosols for 7 days displayed peribronchial and perivascular eosinophilic inflammation that was markedly increased in the mice deficient for the gene encoding the IP receptor ($IP^{-/-}$). Control mice did not receive OVA/Alum immunization nor exposure to OVA aerosols.

3.2.1.3 *Airway hyperresponsiveness is increased in $IP^{-/-}$ mice*

Increased airway responsiveness is an exaggerated airway narrowing in response to many stimuli and is a defining characteristic of the allergic asthmatic response. AHR was evaluated using the drug methacholine to provoke bronchoconstriction in the sensitized mice that had received repeated aerosol challenge. Lung resistance (R_L) is the opposition to airflow caused by the constricted, inflamed airways; and the distensibility, or dynamic compliance (C_{Dyn}) of the lungs, the two physiological parameters used to quantify the severity of AHR.

Augmented AHR was observed in the $IP^{-/-}$ animals demonstrated by a marked increase in R_L and diminished C_{Dyn} (69% and 72%, respectively) (Figure 3.4). Conversely, subsequent to OVA exposure, wt mice responded in the normal range under these experimental conditions, whereby moderate increase in AHR was observed as demonstrated by slight increase in R_L and decrease in C_{Dyn} (27% and 51%, respectively) from baseline (Figure 3.4).

Figure 3.4

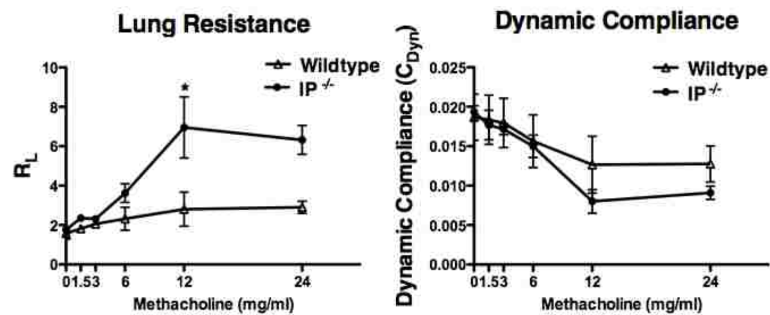


Figure 3.4 Mice deficient for the IP receptor demonstrate augmented AHR. WT C57Bl/6 and $IP^{-/-}$ mice were immunized with OVA/alum and exposed to aerosolized OVA for 20 minutes daily for 7 days. AHR was measured 24 hours after the last challenge. Changes in airway function are reported as response to inhaled methacholine. Exaggerated increases in airway resistance following exposure to OVA aerosols indicated AHR. One tailed students t test were performed to demonstrated statistical significance. $*p \leq 0.05$

3.2.1.4 *Elevated numbers of inflammatory cells present in the BAL of IP$^{-/-}$ mice*

To elucidate the cell types involved the pulmonary inflammation resulting from sensitization and challenge, BAL was performed on wt and IP$^{-/-}$ mice 24 hours after the final exposure to OVA aerosol and the accumulated inflammatory leukocytes in the fluid were quantified by light microscopy (Figure 3.5). The level of airway inflammation was elevated in the IP$^{-/-}$ mice, which was displayed by a two-fold increase in eosinophilic and lymphocytic infiltration when compared to the wt. Differential cell counts of control mice revealed that although low numbers of lymphocytes were present in the BAL of wt animals that were immunized and exposed to ova aerosols, the number of lymphocytes in the BAL of IP$^{-/-}$ animals was markedly higher than observe in wt mice. In contrast to the unchallenged control animals, high levels of eosinophilic infiltration occurred in the lungs of the wt mice receiving aerosol treatment. Negligible accumulation of macrophage and neutrophil were detected in the lungs of either the wt or IP$^{-/-}$ animals under either control (no immunization-no challenge) or primed/OVA challenged conditions.

Figure 3.5

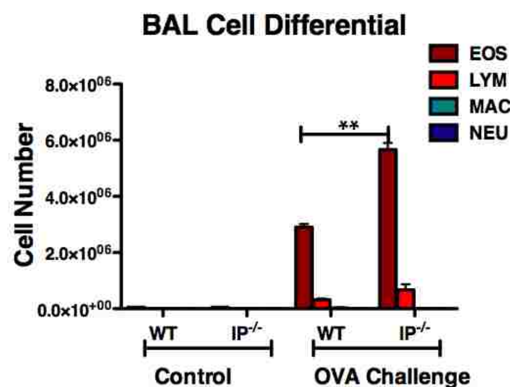


Figure 3.5 ***Inflammatory cells are increased in the BAL of IP$^{-/-}$ mice.*** BAL fluid was collected and cell differential counts were determined by light microscopic evaluation of cytospin preparations. Results are expressed as absolute numbers of macrophages (MAC), lymphocytes (LYM), eosinophils (EOS), and Neutrophils (NEU). One tailed students t tests were performed to determine significant differences between animal groups ($p = ^{**} < 0.002$).

3.2.1.5 Cell-associated eosinophil peroxidase activity in BAL of wt and $IP^{-/-}$ mice.

Eosinophil infiltration to the airways is a defining characteristic of atopic asthma and a gauge of the severity of the inflammatory response. Eosinophil peroxidase (EPO) is an easily quantified product of eosinophils and indicator of airway inflammation. Cell associated EPO levels were determined from BAL collected from sensitized and challenged wt and $IP^{-/-}$ animals (Figure 3.6). Minimal concentrations of EPO were detected in the BAL of the control (not immunized/challenged) animals. In contrast, a marked increase in the concentrations of EPO was detected in the sensitized/challenged wt mice. Interestingly, the level of EPO present in the BAL from sensitized/challenged $IP^{-/-}$ mice was much higher (2.2 fold increase) than that observed in the wt mice. These results are consistent with the observations made using BAL differential counts.

Figure 3.6

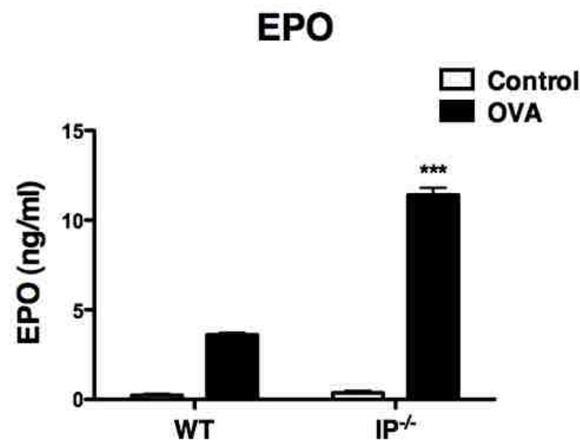


Figure 3.6 EPO levels are increased in IP receptor deficient mice. Airway inflammation is augmented in immunized wt and $IP^{-/-}$ mice subsequent to repeated OVA aerosols. EPO levels were measured from BAL taken from animals sacrificed 24 hours post final OVA challenge. EPO levels were determined by colometric analysis. Data represents means $\pm$ SEM (n=3) and represents three separate experiments.*** p= <0.01 (Challenged $IP^{-/-}$ compared to wt mice)

3.2.1.6 Characterization of lymphocytes in the BALF that were recruited to the lung during Th2 mediated inflammation by FACS analysis

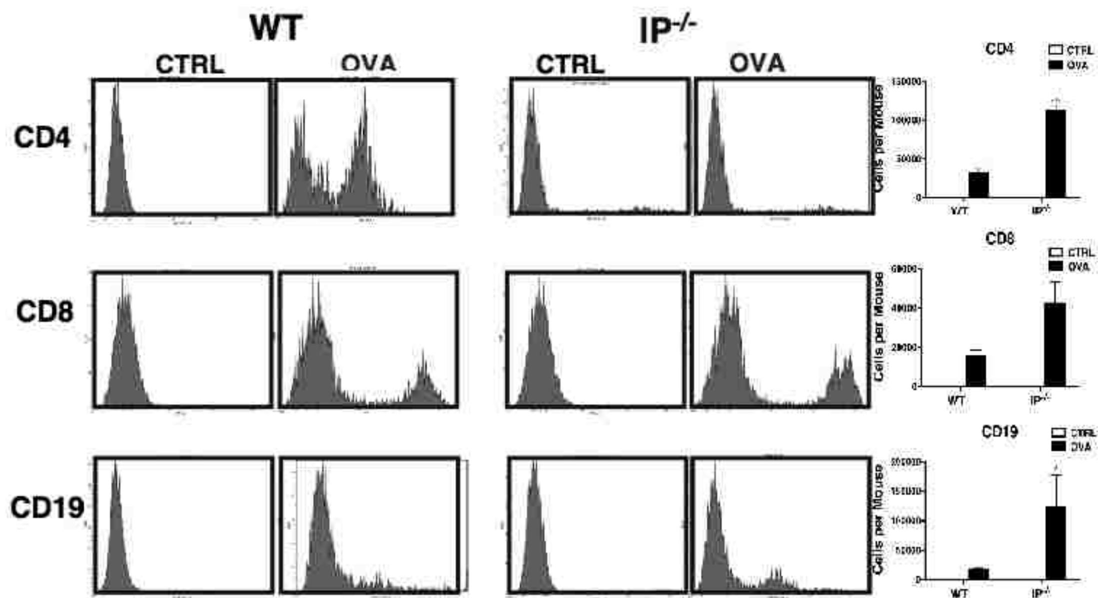
Given that $IP^{-/-}$ mice displayed a heightened level of pulmonary inflammation, it was important to resolve the underlying mechanisms that caused this difference in phenotype. If PGI_2 was operative at the level of a specific cell type, differences in the range of cell lineages presumably may change in $IP^{-/-}$ mice. Flow cytometry was used to identify cell types that were recruited to the lungs in both wt and $IP^{-/-}$ mice. To further examine the role of PGI_2 signaling via the IP receptor on regulating distinct pulmonary lymphocyte populations in the allergic inflammatory response, wt and $IP^{-/-}$ mice were immunized and exposed to repeat OVA aerosols and the characteristics of lymphocytes recruited to the lungs were analyzed by FACS. OVA aerosol challenge of wt mice previously immunized with OVA resulted in the recruitment of CD4, CD8 T cells and B cells to the airway. Figure 3.7 shows that delineation of the cells present in the BALF collected from the untreated and primed/challenged animals demonstrated that comparison to the wt, $IP^{-/-}$ mice had increased numbers of CD4, CD8 T cells and CD19 B cells. Indeed, elevated numbers of lymphocytes were detected in the BAL of $IP^{-/-}$ mice in contrast to the wt mice, in particular a 2.5, 1.8, and 5.9 fold increase in $CD4^+$, $CD8^+$ T cells and $CD19^+$ B cells, respectively (Figure 3.7A). Negligible lymphocytes were detected in the BAL of the wt or $IP^{-/-}$ mice control (no immunization nor OVA aerosols). The BAL from the wt mice contained increased numbers of lymphocytes in contrast to the unchallenged control mice.

To further determine the type of T cells appearing with greater abundance in the BALF of the $IP^{-/-}$ mice, the presence of αβ and γδ TCR expressing T cells was examined.

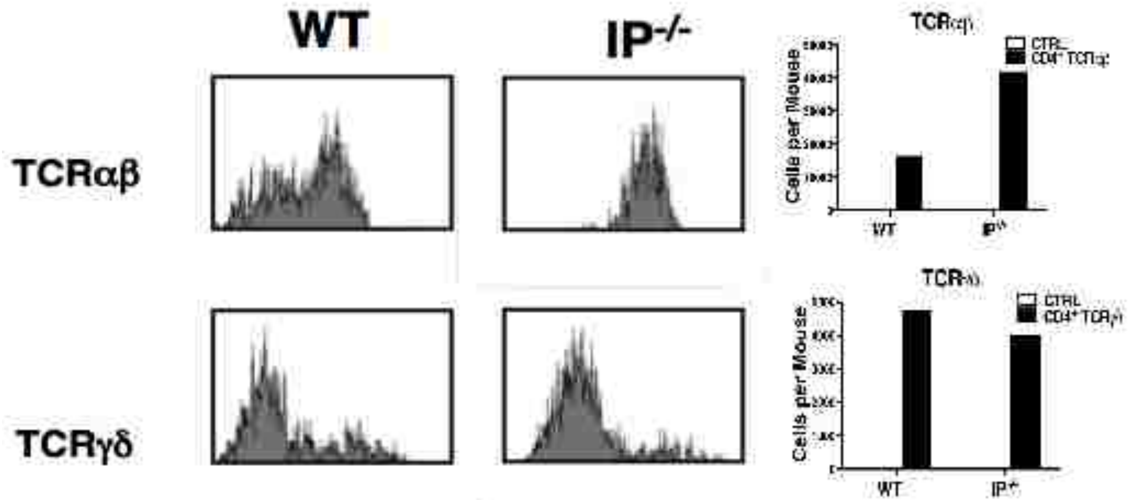
The priming and challenge protocol produced elevated numbers of BAL $\alpha\beta$·TCR^{+} T cells that were evident both the IP$^{-/-}$ and the wt mice as with CD4^{+} numbers the $\alpha\beta^{+}$ T cells were most numerous in the IP$^{-/-}$ BAL. Conversely, only low levels of $\gamma\delta^{+}$ TCR expression in the BALF of either the wt or IP$^{-/-}$ mice (Figure 3.7B). Since NK-T cells have been shown to play an important role in promoting AHR in mouse models of asthma (196), using the NK1.1 antibody it was observed that NK1.1- NK-T cell expression was slightly, but not statistically significantly, increased in the BAL of the treatment group of IP$^{-/-}$ mice in contrast to the otherwise small number of cells present in the wt mice. Macrophage staining using anti-CD11b antibody showed no difference between wt and IP$^{-/-}$ mouse groups (Fig 3.7C).

Figure 3.7

A . T & B Lymphocytes in BAL fluid



B CD4$^+$ αβ / γδ TCR



C. NKT and Macrophage

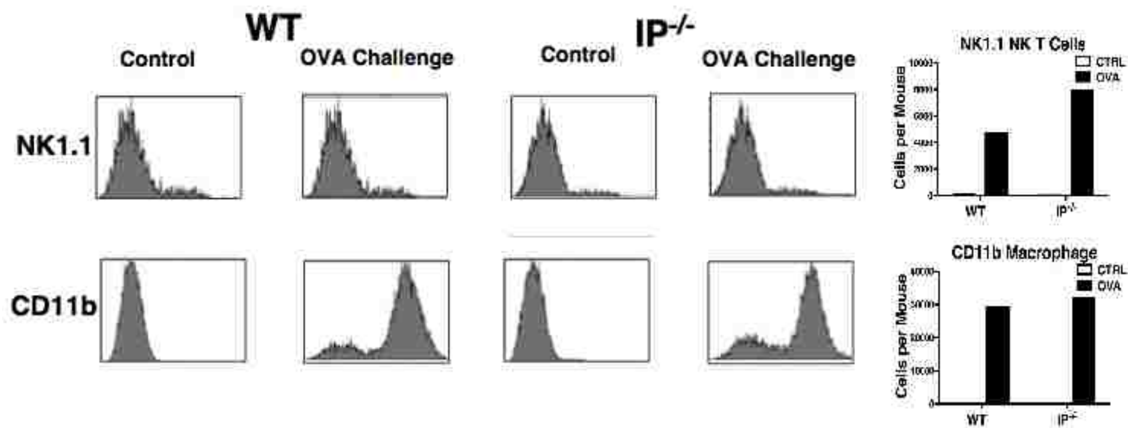


FIGURE 3.7 (a-c). ***Delineation of lymphocyte populations in BAL.*** **a.** BAL was performed on sensitized wt and IP$^{-/-}$ mice that received OVA aerosols for 20 minutes a day for 7 consecutive days. Cells were stained with anti-CD4 APCcy7 Ab, anti-CD8 PE and anti-CD19 APCcy7 antibodies and 30K events per condition were analyzed. Flow cytometry was also performed to analyze CD4$^+$ stained cells. CD4+ T cells were stained with anti-CD4-APC-Cy7 and anti-TCRαβ FITC or anti-TCRγδ-FITC b. Anti-NKT-PE and anti-CD11b-FITC were used to evaluate the NKT and Macrophage populations present in the BAL of OVA immunized and aerosolized mice. b. Representative histograms are example from one of 3 separate experiments. Bar graphs demonstrate means$\pm$ SEM (n=3) and are representative of three separate experiments. One tailed students t tests were performed to determine statistical significance.

3.2.1.7 Characterization of lymphocyte populations found in the lung tissue spleen and peripheral lymphoid tissue by FACS Analysis

The preceding data has documented that an augmented inflammatory response occurred in the airways of the $IP^{-/-}$ mouse in contrast to the wt. To study potential differences in systemic cellular responses in wt and $IP^{-/-}$ mice the lung tissue and a range of lymphoid tissues were analyzed. Cells found in the lungs, lung draining, brachial and mesenteric lymph nodes (LN) and spleen mice that were immunized and exposed to repeated OVA aerosol challenge, were evaluated by FACS analysis (Figure 3.8). The low numbers of cells that were obtained from lung draining LN, were insufficient to perform a complete analysis. Therefore these cells in addition to the brachial and mesenteric LN populations were typically pooled for analysis. Increased numbers of $CD19^{+}$B cells were found in the spleen of the $IP^{-/-}$ mouse in contrast to the wt only after immunization/challenge and not observed in the naïve or unchallenged immunized control animals. No significant differences between the wt and $IP^{-/-}$ groups were found in CD4 and CD8 T cell populations in lung mononuclear cells (LMC), LN or spleen. This data indicates that the principal inflammatory response elicited in this model is located in the airways of these mice and thus best understood by addressing the tissue of the lung and the cells collected in the lung lavage. It is important to note that although CD4, CD8 and CD19 positive $CD4^{+}$ T cells are increased in BAL of $IP^{-/-}$ mice, there was no clear difference in LMC of $IP^{-/-}$, possibly pointing to differences in recruitment to separate compartments in the lung.

Figure 3.8

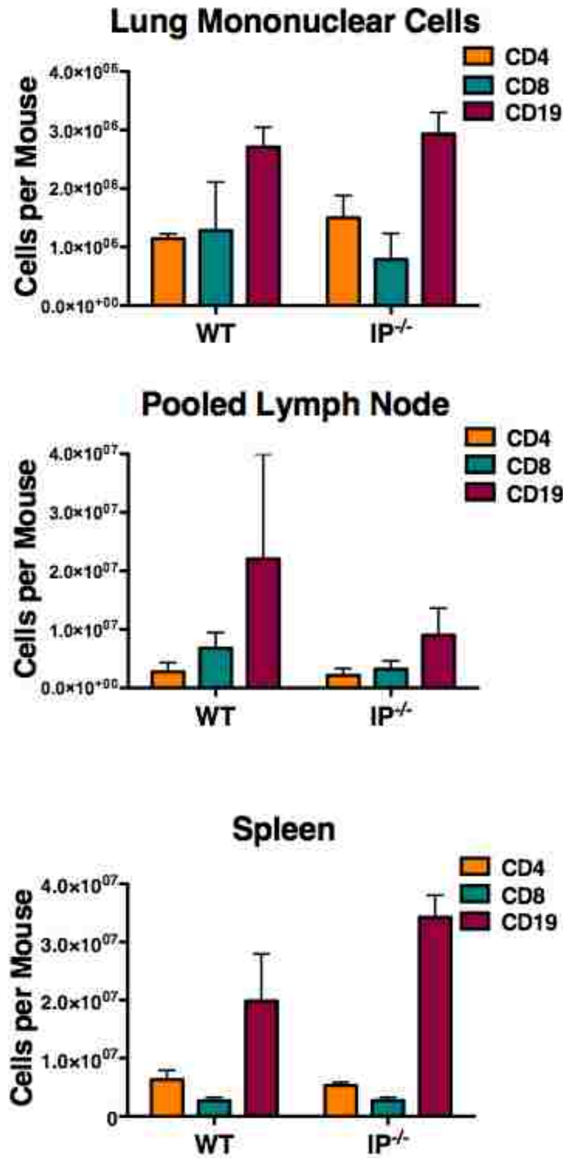


Figure 3.8 ***FACS analysis of the number of T and B lymphocytes present in the lung, lymph node and spleen immunized mice following OVA inhalation for 7 days.*** LMC were isolated from the collagenase digested lung tissue. Spleen MNC were isolated from tissue after mechanical homogenization and centrifugation in a gradient matrix. Because of low numbers of lung draining and brachial LN, cells were pooled for analysis. Flow cytometry was used to detect the presence of the anti-CD4-APCcy7, anti-CD8-PE and anti-CD19-APCcy7 positively stained cells. Unpaired t test with Welch's correction was performed to find these data did not reach statistical significance.

3.2.1.8 Cytokine production by lung mononuclear cells (LMC)

Since Th2 cells preferentially express IP we examined the inflammatory response in mice deficient for the IP receptor, by monitoring cytokine production subsequent to sensitization and repeated exposure to inhaled OVA. To assess the inflammatory response in the airways, the cytokine concentration in the BAL fluid was assayed to evaluate the level of the Th2 response. However, in the BALF, neither IL-4, IL-5 nor IFN-γ were found at levels above the detectable limit (<5 pg/mL), for the sensitivity of our assay (results not shown), in either wt or $IP^{-/-}$ mice. Mononuclear cells extracted from the lungs of the same control and animals exposed to OVA aerosols were restimulated overnight on plate bound anti-CD3 and the supernatant was collected and assayed for IL-4, IL-5 and IFN-γ (Figure 3.9). Consistent with our previous findings that PGI_2 is responsible for inhibiting levels of Th2 cytokines (253), increased levels of IL-4 and IL-5 were produced by LMC from immunized and challenged $IP^{-/-}$ mice when compared to the wt mice (Figure 3.9). Importantly, the levels of Th1 cytokine IFN-γ were not found to display any difference between the wt and the $IP^{-/-}$ controls or treatment groups (Figure 3.9). This suggests that OVA-specific Th2 cells were recruited to the lungs following OVA inhalation. Interestingly, LMC prepared from both wt and $IP^{-/-}$ control mice that had not been immunized or challenged produced low levels of IL-4, IL-5 and IFN-γ.

Figure 3.9

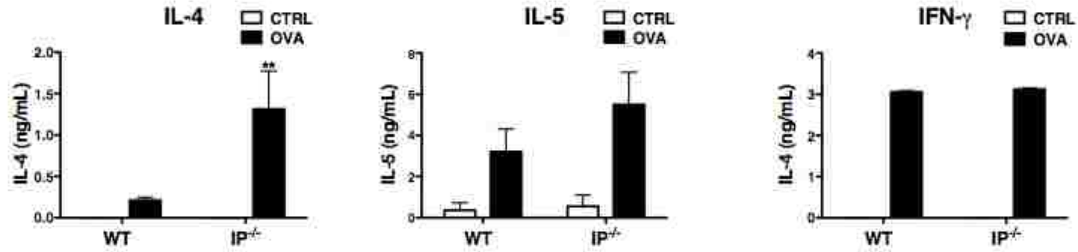


Figure 3.9 Th2 response in LMC. Exposure to aerosolized OVA augments Th2 cytokines in the airways of $IP^{-/-}$ mice. The lungs of wt and $IP^{-/-}$ mice were collagenase digested and the residing mononuclear cell populations were restimulated on plate bound anti-CD3. Levels of IL-4, IL-5, and IFN-γ from the supernatant were determined by ELISA. Naïve control mice were not exposed to aerosolized OVA. The data shown here represent the mean and SEM from three separate experiments. *= $p < 0.05$ challenged $IP^{-/-}$ mice compared with challenged wt mice.

3.2.1.9 Comparison of Th2 differentiation in vitro by wt and $IP^{-/-}$ cells

Since Th2-mediated inflammation was augmented in $IP^{-/-}$ mice, it was important to determine whether $IP^{-/-}$ cells showed any difference in their capacity to undergo Th2 polarization in vitro. PGI_2 is very labile and rapidly hydrolyzed at physiological pH to form the inactive 6-keto-PGF (249). In vivo studies have shown that the majority of injected PGI_2 disappears from the circulation within minutes (254). Therefore, the stable analog of PGI_2 – Iloprost, was used in these experiments. Based on the profound augmentation of IL-4 levels produced in the $IP^{-/-}$ mice, and the findings- that Iloprost inhibits the production of IL-4 in BALB/c mice (unpublished data), we evaluated the production of cytokines produced in the wt and $IP^{-/-}$ mice, in the presence of Iloprost, in order to determine the impact of PGI_2 signaling on Th2 polarization and their subsequent production of cytokines. $CD4^+$ cells from both wt and $IP^{-/-}$ mice were cultured under Th2

polarizing conditions. The 4 day polarized cells were stimulated with anti-CD3 and their cytokine profiles were determined. Interestingly, IL-4 production by the IP$^{-/-}$ cells was much higher than that observed in the wt culture (Figure 3.10). Only low concentrations of IL-4 were produced by the wt culture and that was reduced in the presence of Iloprost. Unexpectedly, the Iloprost treatment also reduced the production of IL-4 from the IP$^{-/-}$ culture, suggesting alternate pathways, or non-PGI_2-prostaglandin receptors were activated by Iloprost. The day-4 polarized wt and IP$^{-/-}$ cells still produced IFN-γ, (abundance of IFN-γ is a characteristic of the C57Bl/6 strain), with the latter expressing significantly higher levels. IFN-γ production was again reduced by Iloprost in both wt and IP$^{-/-}$ cultures.

Figure 3.10

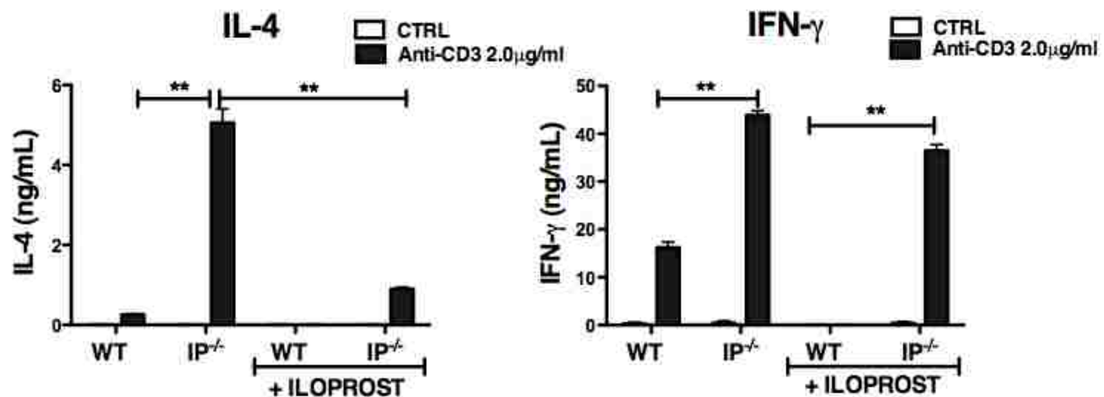


Figure 3.10 ***IL-4 production is elevated in Th2 polarized cells from IP$^{-/-}$ mice***. The brachial and mesenteric lymph nodes were homogenized and the remaining cells were depleted of $CD8^+$ T cells, and expanded in IL-4 and anti-IFNγ on stationary anti-CD3 (1mg/μL) for 4 days. The cells were counted and restimulated overnight on anti-CD3 as described. The supernatant was collected after 24 hours and stored at -20. Levels of IL-4 and IFNg were determined in triplicate by ELISA. Data is representative of two experiments. Two way ANOVA tests were performed to show statistical significance
** = $p < 0.001$

3.2.2 *Serum immunoglobulins in naive unimmunized $IP^{-/-}$ mice differ from wt mice*

One additional phenotypic parameter that was addressed was to measure serum antibody levels. Seven different isotypes from 6 wt and 6 $IP^{-/-}$ mice were analyzed. Interestingly, the serum levels of IgG2b and IgA were markedly lower in naive $IP^{-/-}$, in comparison to the wt mice by 93% and 70%, respectively (Figure 3.11). In addition, serum IgE, IgG1, IgG2a and IgG3 levels were also decreased by 43%, 41%, 60% and 38% in $IP^{-/-}$, respectively. Serum IgM levels were, however, similar and reflected less than 10% difference between the two mouse strains. Since, with the exception of IgM, all Ig expression requires isotype switching, these results suggest PGI_2 signaling plays a role in the mechanism of isotype switching.

Figure 3.11

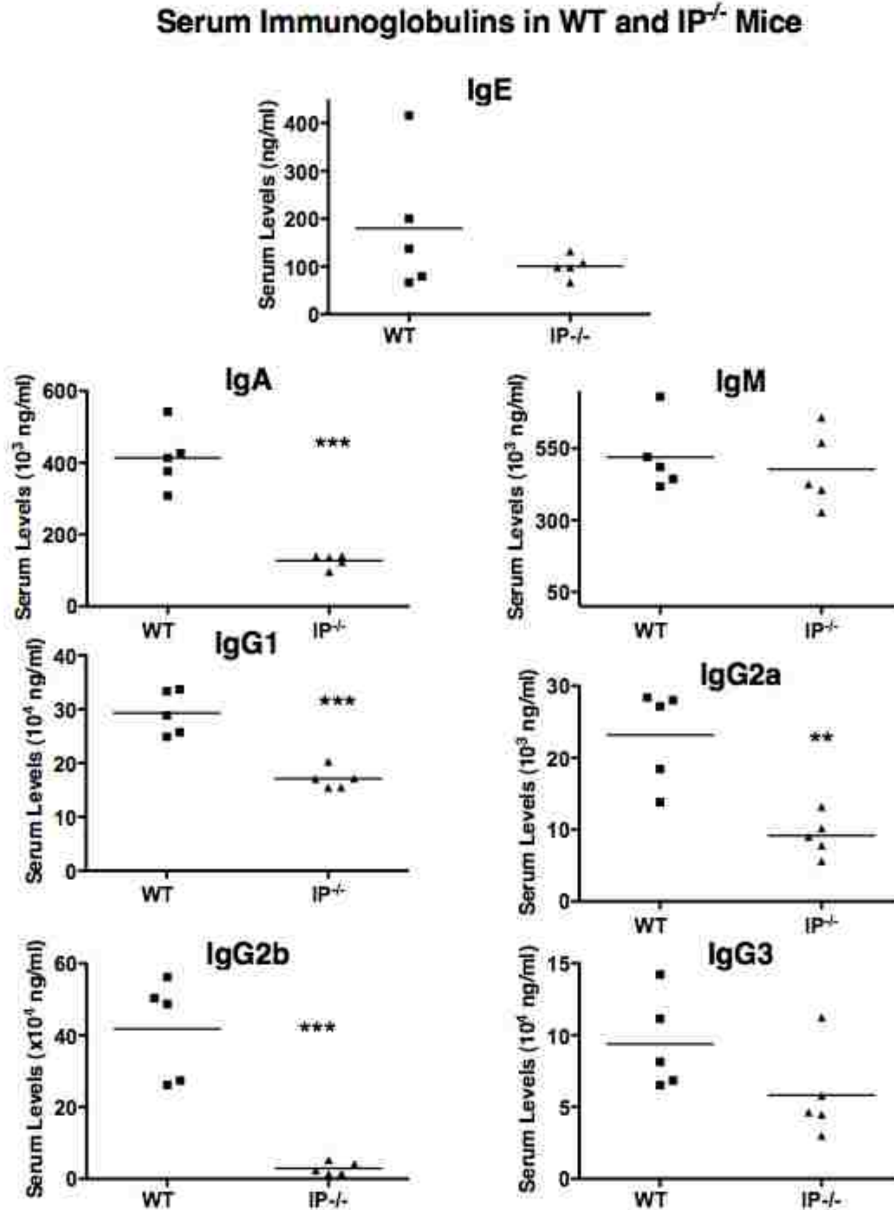


Figure 3.11 ***Isotype serum immunoglobulin differences between $IP^{-/-}$ and wt mice.*** Serum was collected from naïve wt and $IP^{-/-}$ mice. IgM, IgA, IgG1, IgG2a, IgG2b, and IgG2c levels were determined by ELISA. Statistical significance was determined by one tailed students t test **=$p < 0.001$, ***=$p < 0.0005$

3.2.2.1 IP$^{-/-}$ mice display higher IgE and IgG1 responses

Allergy is caused by a number of inhaled or otherwise ingested small-protein allergens that elicit IgE production in susceptible individuals. These experiments have shown that the IP$^{-/-}$ mouse displays increased levels of pulmonary eosinophilia. Given that Th2 cells promote IgE and IgG1 isotype switching in addition to driving allergic inflammation, we investigated antibody responses in the IP$^{-/-}$ mouse. IgE levels in mouse serum are typically less than 1 ng/ml, compared to IgG1, which is typically more than 9 mg/mL in serum (53). Interestingly, repeated exposure of OVA immunized animals to OVA aerosols resulted in the dramatic increase in serum total IgE levels. Serum IgE levels in IP$^{-/-}$ mice were 2 fold higher in (2687 $\pm$ 81.9ng/ml) than in wt mice (1317 $\pm$ 60.5ng/mL), The total serum IgE of IP$^{-/-}$ control (immunized only) mice had a two-fold increase over the wt mice (284.5 $\pm$ 131.5 ng/m and 193 $\pm$ 61.6 ng/ml), respectively (Figure 3.12A-1). In contrast to the elevated basal levels of serum IgE found in naïve wt mice, the increased production of IgE in the immunized and immunized/challenged IP$^{-/-}$ mice suggest involvement of PGI_2 regulation of isotype switching to IgE. Conceivably, PGI_2 may be one of several prostaglandins contributing to this form of regulation.

In contrast to the serum, the concentration of IgE in the BAL fluid (BALF), under any condition, was below the level of detection for the sensitivity of our assay (Figure 3.12A-2).

Figure 3.12A-3 depicts OVA specific IgE present under these circumstances in the serum and the BAL. Neither naive wt or IP$^{-/-}$ mice, or mice that were immunized but were not exposed to OVA aerosols, produced detectable concentrations of OVA specific IgE (data not shown). After 7 days of exposure of wt or IP$^{-/-}$ to aerosolized OVA, a trend

to increase in serum levels of OVA specific IgE was noted. Interestingly, after aerosol exposure, $IP^{-/-}$ mice, OVA specific serum IgE levels in serum were 3.5 fold higher than wt.

Figure 3.12

A (1-3) ***IgE production- Total and OVA specific is elevated in $IP^{-/-}$ mice***

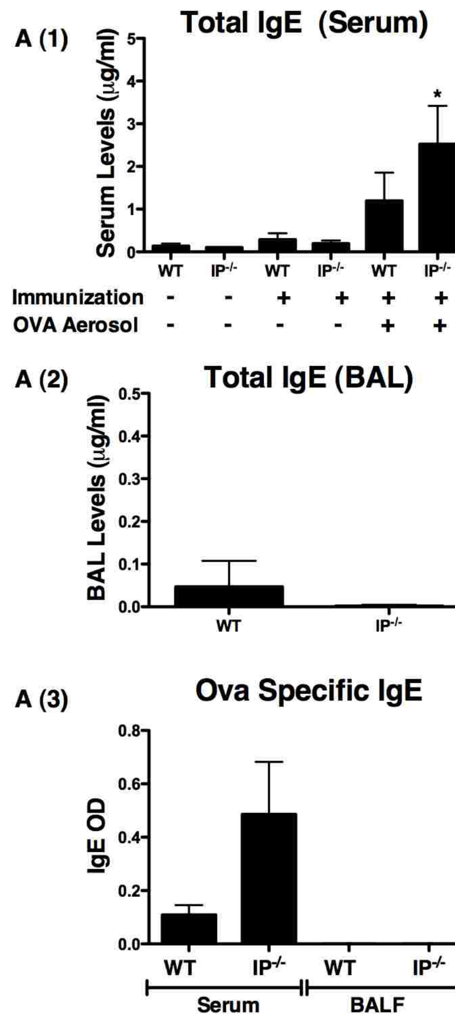
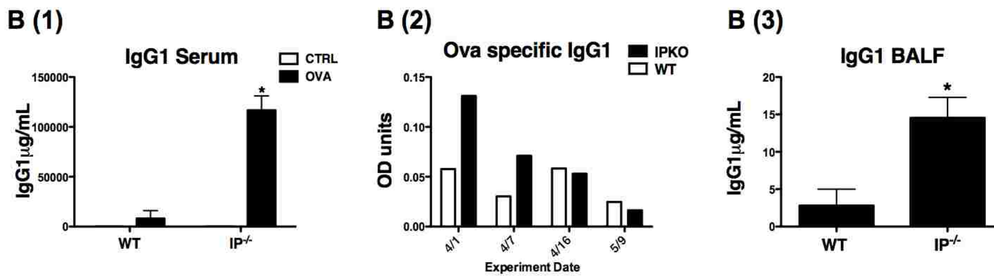


Figure 3.12 (a, 1-3) ***Immunoglobulin levels are altered in $IP^{-/-}$ mice***. Ig production by wt compared to $IP^{-/-}$ mice was determined by ELISA following immunization and OVA inhalations. a. Total IgE and OVA specific IgE Data shown are the mean and SEM of 3 separate experiments. Students t test was used to determining significance *=p<0.05, ***=p< .001 (Challenged $IP^{-/-}$ compared to wt)

Not surprisingly, serum IgG1 levels in both mouse strains were much higher than those for IgE. Consistent with the trend observed for IgE responses, serum IgG1 levels from naïve wt mice have roughly twice the basal level of the $IP^{-/-}$ mice (Figure 3.12B-1). In the $IP^{-/-}$ mouse, following OVA inhalation the levels of IgG1 were markedly higher than wt values (14 and 4.2 fold) in the serum and the BALF, respectively, following OVA challenge Figure 3.12B-1,3). Evaluation of the concentration of OVA specific IgG1 proved in general, inconsistent. However the serum from $IP^{-/-}$ mouse produced slightly higher levels of the antigen specific IgG1 antibody Figure 3.12B-2). The IgG2 serum levels of naïve $IP^{-/-}$ mice were half that of the wt mice. Following sensitization and repeated OVA inhalations the serum IgG2a levels were 2 fold higher than wt mice (Figure 3.12C).

Figure 3.12 B (1-3) ***IgG1 production- Total and OVA specific is augmented in $IP^{-/-}$ mice***



C. ***Total IgG2a is augmented in the serum and BAL of IP-/-mice***

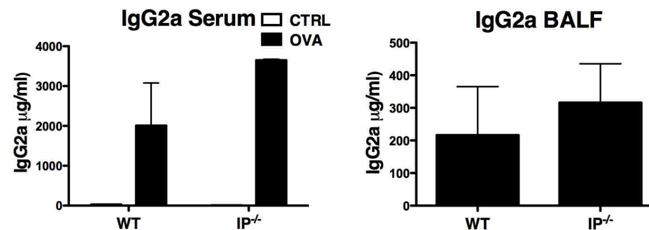


Figure 3.12 (b-c) ***Immunoglobulin levels are altered in $IP^{-/-}$ mice***. Ig production by wt compared to $IP^{-/-}$ mice was determined by ELISA following immunization and OVA inhalations. b. Total IgG1. c. Total IgG2a. Data shown are the mean and SEM of separate experiments. Students t test were performed to determine a difference between the IP-/- and wt mice. *=p<0.05, ***=p< .001 (Challenged $IP^{-/-}$ compared to wt)

3.2.2.2 IP$^{-/-}$ mice have slightly elevated numbers of IgE^{+} B cells

It has been proposed that IgE isotype switching expression can occur in the lung mucosa (255). As far as the mouse model of pulmonary inflammation, it has been presumed that most IgE production takes place in the proximal lymph nodes and is transported in by the lymph. To monitor whether the B cells recruited to the airways contributed to the IgE and or the IgG1 response, BAL cells were stained for surface expression of these immunoglobulin isotypes. The IP$^{-/-}$ mice, following either immunization only or immunization and aerosol challenge, demonstrated elevated numbers of lymphocytes in the BAL (156.6% and 22.7% increases, respectively), in comparison to the wt mice (Figure 3.13). Figure 3.13 illustrates that surface IgE expression was slightly increased on wt CD19^{+}B cells following inhalation of OVA aerosols for 7 days. In comparison to the wt, IgE expression was elevated by 7.8% in the IP$^{-/-}$ B cells. Low numbers of B cells were identified in the BALF of control mice for either wt or IP$^{-/-}$ mice. Surface IgG1 was slightly elevated in the wt mice following sensitization and challenge and again, further augmentation of expression by 5.6% was observed in the IP$^{-/-}$ in relation to the wt B cells.

Figure 3.13

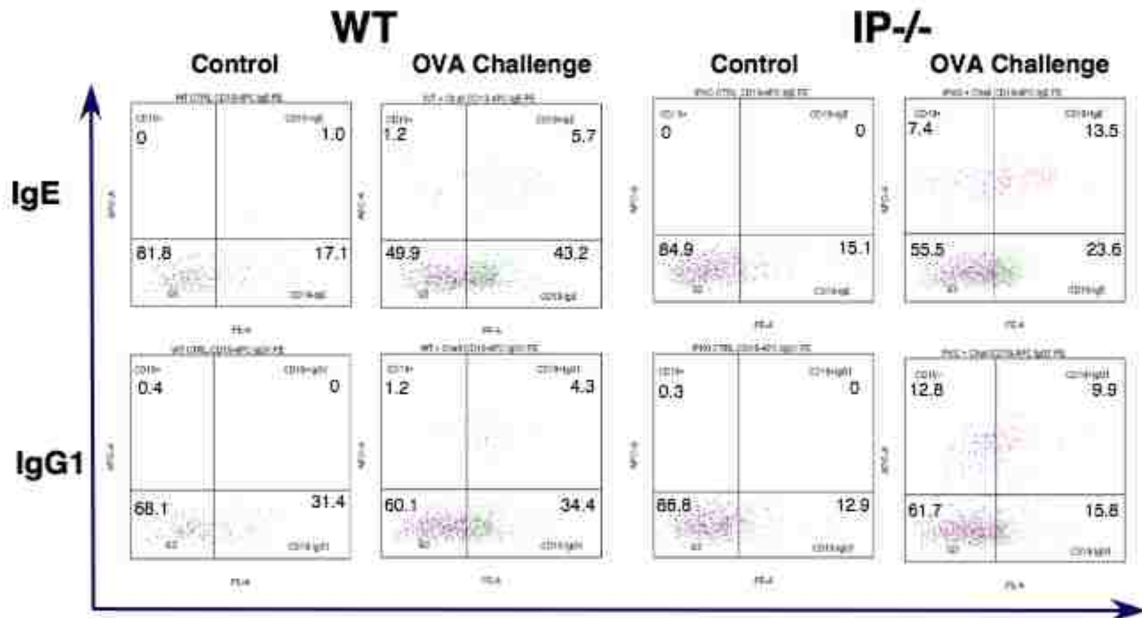
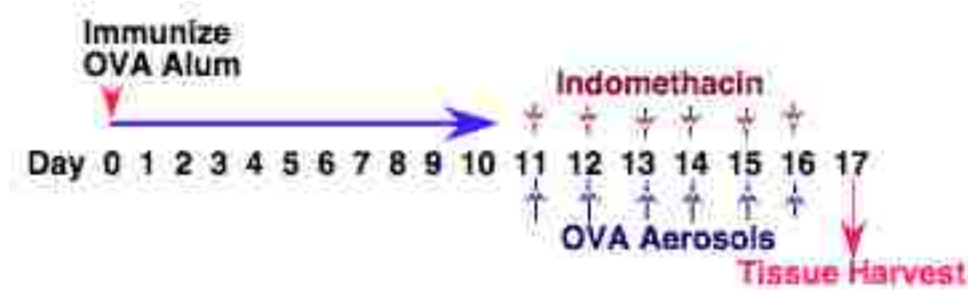


Figure 3.13 ***Cell surface expression of IgE and IgG1 on CD19⁺ B lymphocytes***. Flow cytometry analysis was performed on the BAL of sensitized and OVA aerosolized wt and $IP^{-/-}$ mice 24 hours after final challenge. (a) Flow cytometry analysis of relative numbers of cells from wt and $IP^{-/-}$ groups in the lymphocyte gate (per 10k total cells counted) found in BALF. (b) The B cells were identified by gating on APC conjugated to anti-CD19. Each of the individual Ig's (IgE and IgG) were detected by PE.

3.2.3 Nonspecific Cyclooxygenase Inhibitor- Indomethacin augments airway inflammation in wt mice, but not IP-R deficient mice

PGI_2 is a product of arachidonic acid metabolism via the cyclooxygenase (COX) pathway synthesized by the action of prostacyclin synthase. The two isoforms of the cyclooxygenase enzyme, COX-1 and COX-2, metabolize arachidonic acid to PGH_2, which is subsequently processed by prostacyclin synthase to form PGI_2. Atopic asthmatics are typically advised to avoid using NSAIDs since they can exacerbate an asthmatic response (256). It has been proposed that this effect in part is a consequence of shunting arachidonic acid metabolism towards leukotriene biosynthesis (257). Our laboratory has previously demonstrated that selective inhibition of COX-2 in vivo specifically reduced PGI_2 synthesis and resulted in a marked increase in Th2-mediated, but not Th1-mediated, lung inflammation (244). In support of this conclusion, the data presented in section 3.2.1 clearly demonstrates that the type 2 inflammatory response is augmented when PGI_2 signaling is blocked, by way of disruption of IP receptor. The range of prostanoids whose production requires COX-2 differs from those that are dependent on COX-1. COX-2 is thought to be critical for the production for PGE_2 and PGI_2. NSAIDs, such as aspirin and indomethacin, are nonselective COX inhibitors and suppress the effects contributed to by prostanoids such as inflammatory swelling, pain and fever (212-214). Revealing the role played by PGI_2 in limiting allergic inflammation required comparison of the biological functions of other prostanoids such as PGD_2, PGE_2, $PGF_2\alpha$, and thromboxanes which are also downstream metabolites the COX pathway.

Figure 3.14 ***Indomethacin treatment of murine model of pulmonary inflammation***



We used the murine model of pulmonary inflammation illustrated in Figure 3.14 to investigate whether PGI_2 signaling influences lung mucosal Th2 responses. Wt C57BL/6 and IP receptor deficient, $IP^{-/-}$,mice were sensitized with OVA and received repeated exposure to aerosolized OVA. In addition, the $IP^{-/-}$ mouse was used to determine the contribution of PGI_2 to exacerbations of allergic inflammation elicited by a COX-2 selective inhibitor; NS-398 and non-selective COX 1 and 2 inhibitor, indomethacin.

3.2.3.1 Indomethacin does enhance eosinophilia in the BALFof wt mice

BAL performed 24 hours after the final OVA challenge, revealed that treatment or immunized wt animals with indomethacin during the 6 day aerosol exposure, resulted in a dramatic increase in pulmonary eosinophilic inflammation in wt mice (Figure 3.15, 3.16). Conversely, indomethacin treatment of $IP^{-/-}$ mice did not increase the level of eosinophilia over that observed in animals that had not received the drug (Figure 3.15). In addition, although BALF lymphocyte numbers were slightly elevated following OVA inhalation, negligible numbers of macrophages and neutrophils were present in the BALF of either wt or $IP^{-/-}$ mice (Figure 3.15).

Figure 3.15

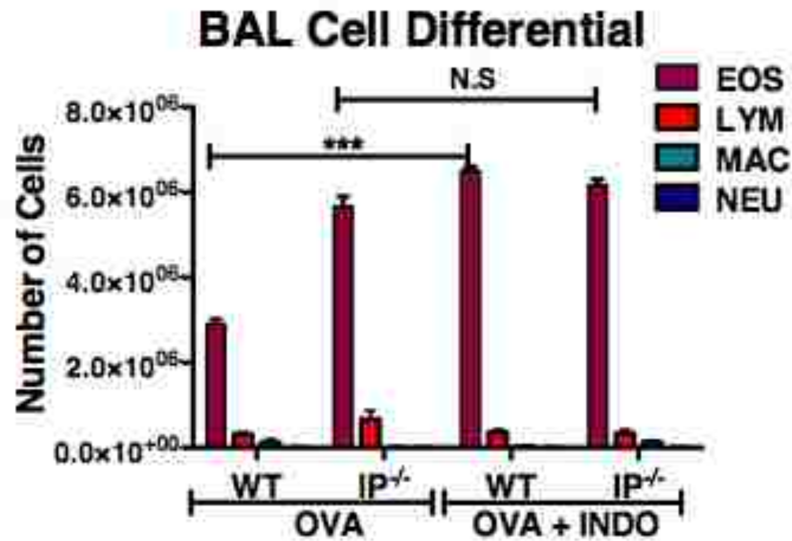


Figure 3.15 ***Increased numbers of eosinophils in OVA immunized/challenged wt mice after treatment with indomethacin.*** BAL fluid was collected from immunized wt or $IP^{-/-}$ mice following repeated OVA aerosols and receiving treatment with non-specific COX inhibitor indomethacin, and cell differential counts were determined by light microscopic evaluation of cytospin preparations. Results are expressed as absolute numbers of macrophages (MAC), lymphocytes (LYM), eosinophils (EOS), and Neutrophils (NEU). One tailed students t test were performed to determine significant differences between animal groups (p *** < 0.001).

The striking increase in the total number of eosinophils in the indomethacin-treated wt animals was also reflected by the elevated level of EPO activity it the BALF (Figure 3.16). In contrast, indomethacin treatment of $IP^{-/-}$ mice did not increase the levels of cell-associated EPO.

FIGURE 3.16

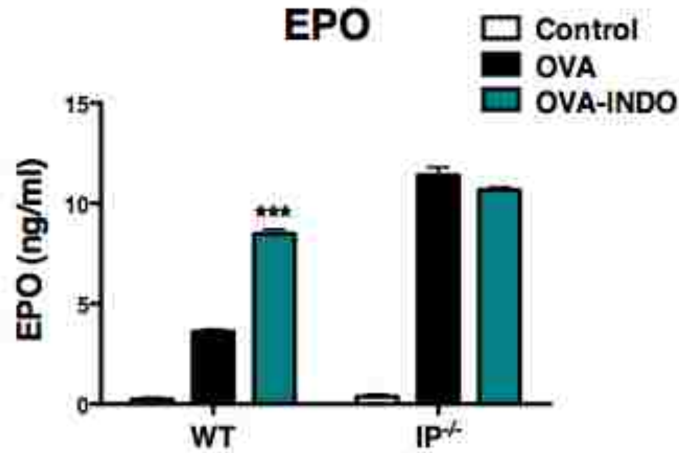


Figure 3.16 ***EPO levels are increased in indomethacin treated wt mice***. Airway inflammation in immunized wt and IP$^{-/-}$ mice subsequent to repeated OVA aerosols. EPO levels were measured from BAL taken from animals 24 hours post final OVA challenge. EPO levels were determined by colometric analysis. Data represents means $\pm$ SEM (n=3) and represents three separate experiments. Students t test was performed to determine ***p= <0.001

3.2.3.2 ***Indomethacin treatment does not alter the lymphocyte population in the BALF of immunized wt or IP$^{-/-}$ mice that have inhaled OVA***

In contrast to the dramatic eosinophilic response that developed in the airways of wt mice following indomethacin treatment, the recruitment of CD4^{+}, CD8^{+} or CD19^{+} cells was not significantly increased by indomethacin treatment (Figure 3.17). Total numbers of each lymphocyte population present in the BALF of wt or IP$^{-/-}$ mice remained unchanged following indomethacin treatment when compared to the control mice.

Figure 3.17

A

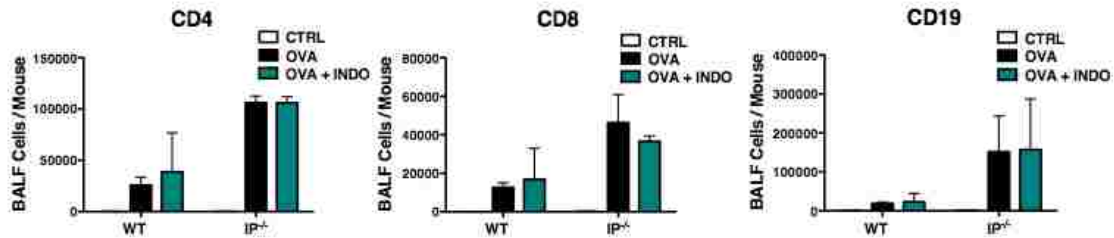


Figure 3.17 ***Treatment with indomethacin did not alter lymphocyte populations in the BALF of immunized and challenged mice.*** BAL was performed on sensitized wt and $IP^{-/-}$ mice that received OVA aerosols 20 minutes a day for 7 consecutive days. $1x10^6$ cells were stained with anti-CD4 APC-Cy7 ab, anti-CD8 PE and anti-CD19 APC-Cy7 antibodies and $3X10^4$ events per condition were analyzed. Although this data did not reach statistical significance, the graphs represent means$\pm$ SEM (n=3) and are representative of three separate experiments. One tailed students t tests were performed to determine statistical significance whereby the data did not demonstrate variations between the control and treatment groups.

3.2.3.3 Indomethacin treatment does not alter the cytokine production in the BALF of immunized wt or $IP^{-/-}$ mice that have inhaled OVA.

In accordance with the lymphocyte data from the BALF, measurements of IL-4 and IL-5 from anti-CD3 restimulated LMC revealed that treatment with indomethacin did not significantly impact levels of Th2 type cytokine production by LMC from either the wt or $IP^{-/-}$ mice following OVA inhalation (Figure 3.18). Diminished levels of the Th1 cytokine−IFN-γ in both wt and $IP^{-/-}$ along with the lack of variation of production of this cytokine between control groups suggest that although non-specific COX inhibition restricts IFN-γ production, the Th1 response is unaffected by PGI_2 signaling.

Figure 3.18

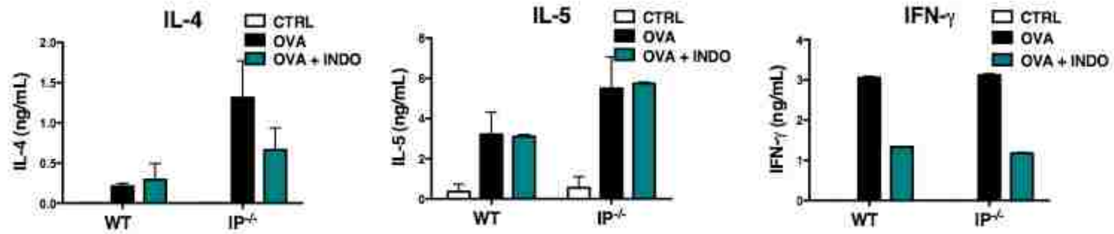
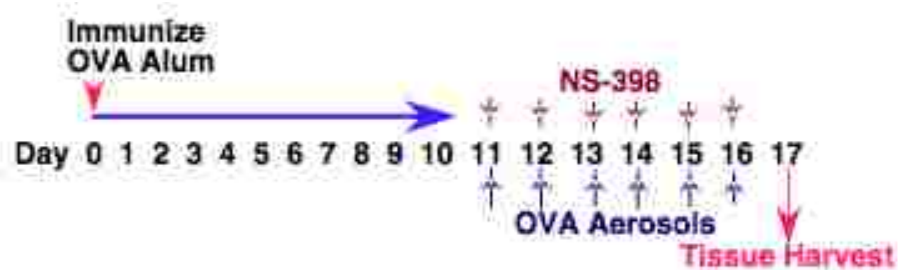


Figure 3.18 ***Treatment with non-specific COX inhibitor indomethacin, does not significantly alter the expression of Th2 cytokines in LMC of wt mice***. The lungs of wt and IP$^{-/-}$ mice were collagenase digested and the residing mononuclear cell populations were restimulated on immobilized anti-CD3. Levels of IL-4, IL-5, and IFN-γ from the supernatant, was determined by ELISA. Naïve control mice were not exposed to aerosolized OVA. Data represent the mean and SEM from three separate experiments.

3.2.3.4 The COX-2 selective inhibitor NS-398 did not increase airway inflammation in wt or IP$^{-/-}$ mice.

Figure 3.19



In comparison to the sensitized and challenged control groups, treatment of mice with NS-398 (Figure 3.19) resulted in no significant modification of pulmonary inflammation in the wt mice, however, unexpectedly, significantly reduced infiltration of eosinophils to the airways was observed in the IP$^{-/-}$ NS-398 treatment groups (Figure 3.20). This event may be indicative of alternate prostanoid pathways operative in the

different mouse strains. Again, only small, populations of lymphocytes and macrophages, and no neutrophils, were present in the BALF of either wt or IP$^{-/-}$ groups.

Figure 3.20

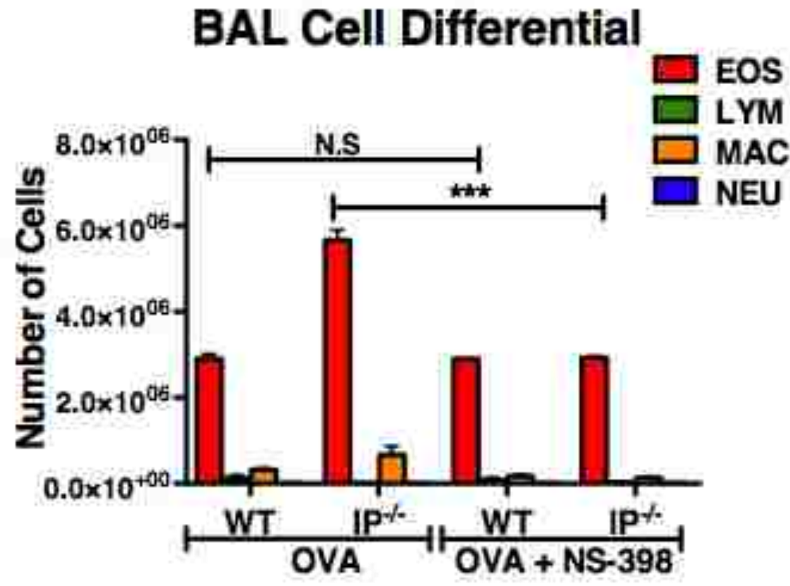


Figure 3.20 ***The COX-2 selective inhibitor NS-398 did not increase airway inflammation in wt or IP$^{-/-}$ mice*** BAL fluid was collected from immunized wt or IP$^{-/-}$ mice following OVA aerosols NS-398, and cell differential counts were determined by light microscopic evaluation of cytospin preparations. Results are expressed as absolute numbers of macrophages (MAC), lymphocytes (LYM), eosinophils (EOS), and Neutrophils (NEU). One tailed students t test were performed to determine significant differences between animal groups (p *** < 0.001)

3.2.4 CD4$^+$ T cell levels of L-selectin (CD62L) are regulated by PGI_2

L-selectin is expressed by naïve CD4+ T cells and is progressively lost following activation. We have observed that Th2 polarized CD4+ T cells that have been exposed to PGI_2 retain CD62L (L-Selectin) expression on the cell surface in vitro (Figure 3.21). Whether this occurrence is due to increased expression of CD62L or inhibition of receptor cleavage is unknown. Given that in addition to CD62L, the IP receptor is also highly expressed on Th2 cells, we considered whether Th2 cells from the IP$^{-/-}$ mouse

would demonstrate a contrary effect. Interestingly, no observable differences were detected between immunized wt or $IP^{-/-}$ mice receiving indomethacin treatment during exposure to OVA aerosols and animals not receiving the drug (Figure 3.21).

Figure 3.21

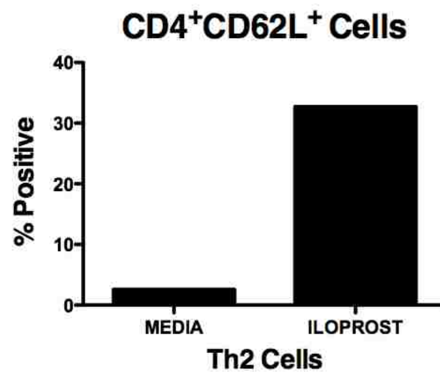


Figure 3.21 ***L-Selectin (CD62L) is retained on CD4+Th2 cells in the presence of Iloprost.*** FACS analysis was performed on peripheral LN cells from DO11.10 mice cultured under Th2 polarizing conditions in the presence or absence of Iloprost. FACS analysis was performed and the gate was set on the lymphocyte population and the APCCy7 positive CD4 cells were selected. Staining was done using anti-CD62L conjugated to FITC. The data is representative of three separate experiments.

Figure 3.22

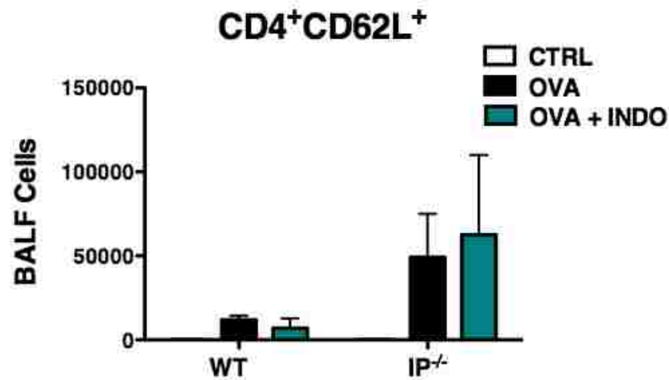


Figure 3.22 ***Treatment with Indomethacin does not affect L-selectin expression by BAL lymphocytes on wt or $IP^{-/-}$ mice***. Wt and $IP^{-/-}$ mice were immunized and received daily I.P. injections of Indomethacin (5mg/kg) immediately prior to inhaling OVA for six consecutive days. BAL was performed and the cells were analyzed by FACS. $CD4^+$ and $CD62L^+$ cells were stained with APCcy7 and FITC antibodies, respectively. The data represents three independent experiments. One tailed students t tests were performed and reflected no differences between treatment and control groups.

It was important to investigate whether CD62L expression is compromised in the IP$^{-/-}$ mouse during Th2 polarization and allergic inflammation. To address this, we cultured naïve wt and IP$^{-/-}$ cells for five days under Th2 polarizing conditions, in the presence or absence of non-selective COX-2 inhibitor indomethacin. In addition to L-selectin, inducible T cell co stimulator (ICOS), a T cell surface molecule that is expressed after cell activation, was evaluated. After 120 hours in culture, other than a reduction in the numbers of B cells present in the culture of the IP$^{-/-}$ cells not receiving indomethacin, no other differences in L-selectin expression were observed between wt and IP$^{-/-}$ mice. Surprisingly, ICOS expression was diminished in the IP$^{-/-}$ mice after 120 hours, which was rescued with indomethacin treatment (Figure 3.23).

Figure 3.23

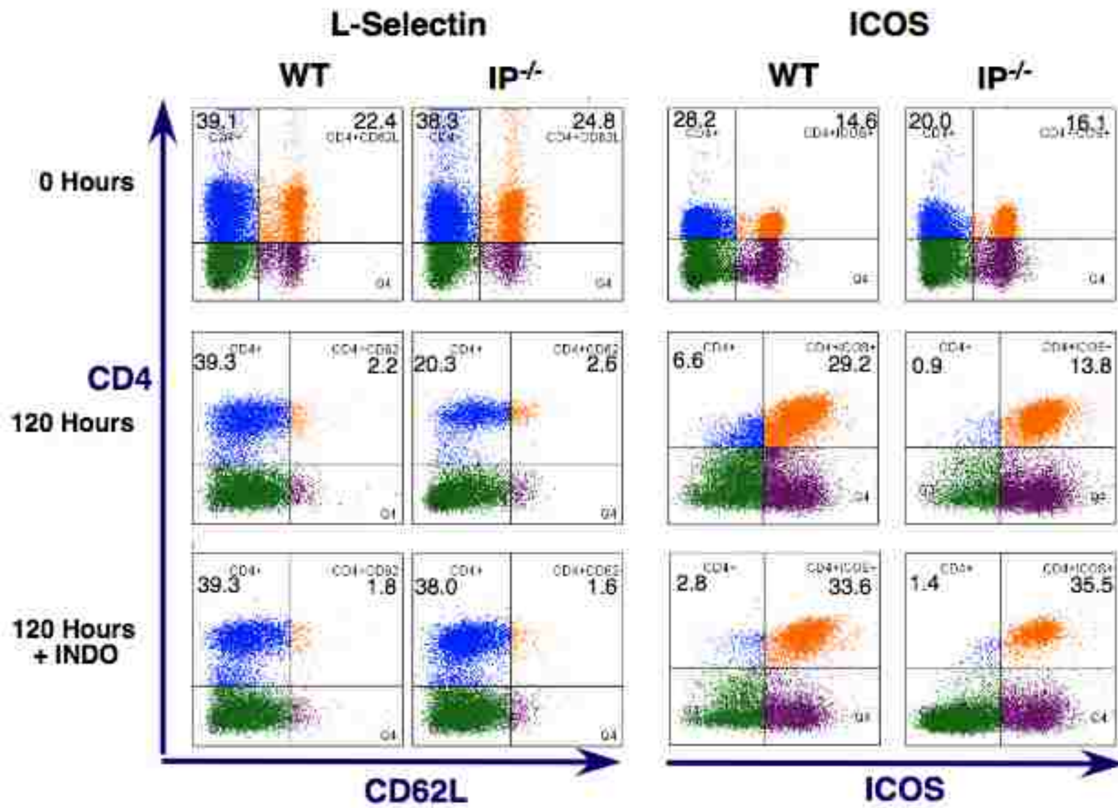


Figure 3.23 ***Cell adhesion molecule L-Selectin (CD62L) expression in IP$^{-/-}$ mice.*** FACS was performed on naïve wt and IP$^{-/-}$ cells that were cultured for five days in the under Th2 polarizing conditions, in the presence or absence of non-selective COX-2 inhibitor indomethacin. CD4+ (APCcy7) cells were gated and CD62L and ICOS, (FITC anti-PE, respectively) expression was evaluated.

3.3 Discussion

3.3.1 Asthma: Incidence and pathology

The prevalence of asthma has markedly increased worldwide in recent decades. An estimated 20 million Americans suffer from asthma and approximately half of those cases are allergy-associated asthma. The incidence of asthma has been increasing since the early 1980s across all age, sex and ethnic groups (258). Asthma is the most common chronic childhood disease, affecting more than one child in 20. Nearly 5 million asthma sufferers are under 18 years of age (259). It is more prevalent among African Americans than Caucasians and these ethnic differences in prevalence, morbidity and mortality are highly associated with poverty, urban air quality, indoor allergens, and lack of patient education and inadequate medical care (260). Although treatments are available that alleviate the symptoms associated with asthma, there is no treatment that cures this disease.

Asthma is a chronic inflammatory disorder, which is characterized by attacks of wheezing and shortness of breath due to bronchoconstriction, mucus secretion, airway hyperresponsiveness to non-specific stimuli and airway wall thickening (261). In allergic (atopic) asthma, airway inflammation is triggered by specific allergens (dust mites, pollen, animal dander), or non-specific triggers (air pollutants or viral infection). Two-thirds of all asthma patients have atopic asthma, which is indicated by elevated levels of serum IgE and positive skin prick test to common allergens (262, 263).

Allergic asthma is an immune disorder that is dominated by Th2 lymphocytes, IgE, mast cells, eosinophils, macrophages, and cytokines, which drives airway inflammation. The asthmatic inflammatory response also involves local epithelial,

mesenchymal, vascular and neurological events (153). It has been proposed that asthma arises from the dysfunction in events that would typically mediate the resolution of an underlying T cell response (5, 181, 264).

3.3.2 Prostacyclin – Role in modulating the immune response

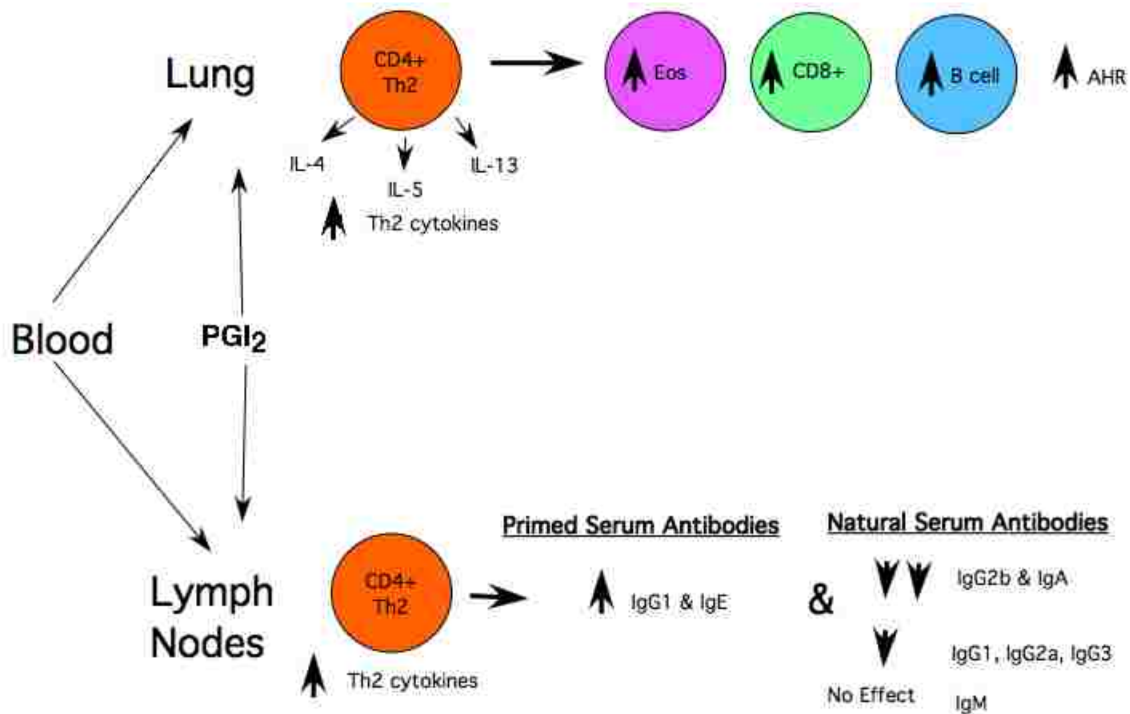
T cells at mucosal sites, such as the lung, are subject to immune regulation, partially from the actions of COX- derived prostanoids (265). Prostanoids are lipid mediators generated from the oxidative metabolism of arachidonic acid by COX-1 and COX-2 enzymes and specific synthases (213, 214). It is well recognized that prostaglandins (PG), play an important homeostatic function in the lung, particularly as regulators of cell proliferation, differentiation and apoptosis (266). High concentrations of PG are produced in the normal lung, with typically large amounts of PGE_2 and PGF_{1a} found in the BAL (244). However, in this respect, PGI_2 biosynthesis in the lung is different than these other prostaglandins, since its production was concurrent with the onset of a Th2-mediated pulmonary inflammatory reaction and was highly dependent on COX-2. PGI_2 was originally discovered as a lipid mediator expressed by vascular tissue that both inhibits platelet aggregation and is a potent vasodilator (267, 268). Endothelial cells, human follicular dendritic cells, thymic nurse cells, and human fibroblast are some of the cell types express COX-2 and prostacyclin synthase, both of which are requirements for the production of PGI_2 (269-272). Using synthetic analogs, PGI_2 signaling has been associated with regulating innate immunity including the inhibition of phagocytosis and bacterial killing activities in peritoneal macrophages (273). In addition, human follicular dendritic cells have been shown to inhibit T cells in the germinal center

by controlling PGI_2 production via expression of PGI_2 synthase (PGIS) (270, 274, 275). Treatment with Iloprost was found to interfere with the action of lung myeloid DCs by inhibiting their maturation and migration to the mediastinal lymph node, thereby abolishing the induction of allergen specific Th2 responses (276).

The prostacyclin receptor (IP) is expressed in mature thymocytes and splenic lymphocytes. In addition, IP mRNA has been found in neurons, megakaryocytes, and smooth muscle cell of the aorta, coronary and pulmonary arteries, in mouse (277). Using mice in which the gene encoding the IP cannot be expressed ($IP^{-/-}$), the biological significance of PGI_2-IP signaling was shown to have crucial functions in preventing thrombosis, inhibiting injury-induced vascular proliferation (251), regulating allergic airway responses (252), and mediating inflammatory swelling and pain (217). Endogenously produced PGI_2 has also been shown to act on the intracellular level via the nuclear peroxisome proliferator-activated receptor (PPAR-γ) (278, 279).

Previous studies from our laboratory reported that the PGI_2 receptor (IP), is expressed by T cells and this expression is augmented by IL-4. Although T cells do not produce PGI_2, $CD4^+$ effector Th2 cells, preferentially express the IP receptor (253). Yet the underlying processes by which PGI_2-IP signaling regulates airway inflammation, remains unclear. Our data demonstrate that disruption of PGI_2-IP signaling results in heighted allergic immune responses in the lung. This effect is shown in $IP^{-/-}$ mice by augmented Th2 cytokines, lymphocytes, eosinophil recruitment, IgE and IgG1 levels subsequent to OVA immunization and aerosol challenge. Decreased serum immunoglobulin levels in naïve $IP^{-/-}$ mice suggests that PGI_2 signaling may serve an important regulatory function in immunoglobulin isotype switching.

Figure 3.24 Proposed mechanism for PGI_2-IP signaling in the regulation of allergic pulmonary inflammation



To obtain a better understanding of the anti-inflammatory effects of PGI_2, using a murine model of allergic asthma, we assessed the differences in lung inflammation and antibody response in wild type C57BL/6 mice and $IP^{-/-}$ mice. Nagai and coworkers previously showed, using an immunized/challenged model in different $IP^{-/-}$ mice, an eosinophilic response that is more sever than that observed in wt mice. These $IP^{-/-}$ mice also had increases in both, numbers of Th2 cytokines, and levels OVA specific serum IgE and IgG1 (252, 280). Although compelling, these data were cursory and the underlying mechanism employed by PGI_2 signaling, was not resolved.

3.3.2.1 $IP^{-/-}$ mice have increased pulmonary inflammation following immunization and exposure to aerosolized OVA

Given that the receptor for PGI_2 is induced by IL-4, we established an in vivo murine model to investigate the role of PGI_2 signaling in allergic airway inflammation. This model was based on a protocol of antigen immunization and aerosol challenge would induce eosinophilic inflammation in these mice to enable observation of the phenotypic changes that occur as a result of deficient PGI_2-IP signaling. A single OVA immunization was sufficient to induce an eosinophilic response upon subsequent OVA aerosol challenge. Obtaining the optimal antigen exposure was crucial for this investigation. To maximize any differences, the search for phenotypic differences between $IP^{-/-}$ and wt mice was especially challenging due to the overlapping functions of prostaglandins which may mask the more subtle events specific to PGI_2 signaling.

Since PGI_2 production and its receptor are upregulated during Th2-mediated pulmonary inflammation, we investigated the effect imposed by antigen priming and repeated exposure to OVA aerosols on the lung tissue of $IP^{-/-}$ mice. Our findings showed a profound perivascular and peribronchial eosinophilic inflammation in the histological examination of the $IP^{-/-}$ mice. Increased numbers of $CD4^+$, $CD8^+$ T and $CD19^+$ B lymphocytes, in particular eosinophils, were present in the BAL of $IP^{-/-}$ mice. In addition, augmented levels of cell associated EPO in the BAL and the heightened AHR response of the $IP^{-/-}$ mice; suggest PGI_2 signaling inhibits antigen-induced bronchoconstriction in asthmatic lungs.

The heightened pulmonary inflammatory response in $IP^{-/-}$ mice required that we assess if PGI_2 signaling was operating at the level of a specific cell type. As expected,

Flow cytometric analysis confirmed that $IP^{-/-}$ mice had increased numbers of $CD4^+$, $CD8^+$ T and $CD19^+$ B cells in the BAL. Given that these heightened inflammatory responses occurred in the airways of the $IP^{-/-}$ mouse, it was important to detail the potential differences in systemic cellular responses in the wt and the $IP^{-/-}$ mice. Interestingly, elevated numbers of $CD19^+$ B cells were isolated to the spleen of the $IP^{-/-}$ mice, and the response was dependant on OVA sensitization and challenge. In addition, these inflammatory cells appear to localize to the airways of these mice. That this difference in infiltrating lymphocyte populations between $IP^{-/-}$ and wt mice is not observed in the LMC suggests that PGI_2 signaling influences cell recruitment to the lung compartment. It is important to also consider that the lung tissue is populated with large numbers of resident lymphocytes and that although inflammation is occurring in this space, the ability to discern variances between the strains is diminished because of these background cells.

It has previously been shown that CD4+ NK1.1 NK-T cells play a critical role in eliciting airway eosinophilic inflammation (196). Using this model, we found no obvious NK T cell differences in the number of NK-T cells in $IP^{-/-}$ mice in this model of airway inflammation.

The IP receptor is preferentially expressed on Th2 polarized CD4 cells (253), therefore it was crucial to evaluate the production of the Th2 cytokines IL-4 and IL-5. Since cytokine levels in BAL were below the threshold of sensitivity for detection, we assessed the cytokine concentration in LMN cells. It is very interesting that, although no quantifiable differences were reflected in the lymphocyte populations between $IP^{-/-}$ and wt mice after immunization and OVA aerosol challenges, the level of IL-4 production was markedly and specifically increased in LMN from the $IP^{-/-}$ mice, suggesting that

OVA specific Th2 cells were recruited to the lungs following OVA inhalations. The level of IL-5 production was elevated in $IP^{-/-}$ mice but did not reach significance. In accordance with previous reports, levels of Th1 cytokine IFN-γ were unaffected by the lack of functional IP receptor. Collectively these findings suggest that PGI_2 plays an important immunoregulatory function by limiting lung mucosal Th2 responses. These observations underscore IL-4 as not only as essential for the differentiation of $CD4^+$ Th2 cells, but also in promoting a mechanism that limits the progression of allergic inflammation.

The observation that IL-4 levels are increased in the lungs $IP^{-/-}$ mice, prompted investigation into whether this was an effect of PGI_2 signaling influencing the polarization of naïve $CD4^+$ or, alternatively inhibiting fully differentiated $CD4^+$ T cells. The production of IL-4 and IFN-γ was markedly higher in Th2 polarized $IP^{-/-}$ cells compared to wt cells. Wt cells produced low levels of IL-4, which was abolished in the presence of Iloprost. This observation strongly suggests that the anti-inflammatory effects of PGI_2 arise predominantly as a consequence of its action in blocking differentiation of $CD4^+$ Th2 cells. In $IP^{-/-}$ cells, IL-4 production was markedly diminished in the presence of Iloprost after 4 days of culture under Th2 polarizing conditions. This was unexpected and may either arise from Iloprost binding to the alternative PGI_2 receptor, PPARγ.

3.3.3 *Immunoglobulin response in IP$^{-/-}$ mice*

3.3.3.1 Levels of IgG1 and IgE are elevated in IP$^{-/-}$ mice

The Ig measured in the BAL fluid is used as a means to monitor local production of immunoglobulin in the lung and enable comparisons to be made with antibodies detected in the serum. Our experiment revealed that in the BAL, IgE levels remained low/undetectable in both the IP$^{-/-}$ and wt mice, whereas IgE levels were markedly increased in the serum of IP$^{-/-}$ mice. In contrast, IgG1 was elevated in both BAL and serum. It is important to consider that concentrations in mouse serum are on the order of milligrams for IgG1, and nanograms for IgE, and that a percentage of the IgG1 detected in the BAL may have leached from the serum into the airspace during the lavage procedure. Indicative of an OVA specific immune response and in concordance with previous reports (252, 280), serum levels of OVA specific IgE and IgG1 were markedly elevated in the immunized IP$^{-/-}$ mice following OVA aerosols. Collectively, these data suggest that augmented antibody production in the IP$^{-/-}$ mouse is occurring primarily outside the lung tissue and is a consequence of loss of regulation provided by PGI_2-IP signaling.

3.3.3.2 Immunoglobulin isotypes are altered in serum IP$^{-/-}$ mice

IgM is the first antibody produced in an immune response and does not require isotype switching. Strikingly, with the exception of IgM, all the other isotype levels assayed were decreased in the serum of naive IP$^{-/-}$ mice. Most pronounced were the IgG2b and IgA, with concentrations less than 10% and 30% of the wt mice, and both of which require TGF-β for expression. The reduced natural serum immunoglobulins in the IP$^{-/-}$ mouse could be a consequence of loss of PGI_2 signaling in the process of isotype

switching or subsequent B cell expansion. In this context human follicular dendritic cells have been shown to express PGI_2 synthase (270), thus proposing the interaction between follicular DC and Th2 cells in the follicle as an important location of immune regulation in the lymphoid tissue. Collectively, these observations suggest that PGI_2 acts to inhibit production of IgG1 and IgE associated with allergic inflammation in mice.

3.3.4 ***PGI_2 promotes L-Selectin expression by $CD4^+$ T cells***

Allergic inflammation is characterized by recruitment of specific leukocyte subpopulations from blood into tissue and requires a series of cell adhesion-molecule-mediated interactions between postcapillary vascular endothelium and the leukocyte cell surface. L-selectin is a lymphocyte specific adhesion molecule expressed on the surface of naïve T cells and is progressively shed following activation by antigen. Expression of L-selectin is required for activated lymphocytes to enter the lymph node. Interestingly, in the presence of PGI_2, $CD4^+$ T cells retain expression of L-selectin in vitro. Surprisingly, we found no correlation between lack of functional IP receptor and expression of L-selectin following OVA immunization and aerosol inhalations. This may be a consequence of the low frequency of antigen specific CD4+ T cells in OVA immunized mice. The source of PGI_2 in the lungs of OVA-challenged animals is likely to include fibroblasts and macrophages (243). However endothelial cells are also known producers of large amounts of this prostanoid (281). The unstable nature of PGI_2 suggests its immunomodulatory action is likely to be localized to the site of inflammation. It is likely that L-selectin upregulation by PGI_2 would serve a role in promoting migration of the $CD4^+$ Th2 cells from the blood to the lymph node where the antibody response will

develop. It is possible that this phenomenon may contribute to the reduced levels of native antibodies, indicative of decreased T cell entry to the lymph nodes a consequence of diminished L-selectin expression on $IP^{-/-}$ $CD4^{+}$ T cells.

3.3.5 *A role for PGI_2 and its receptor in the augmentation of allergic lung inflammation elicited by inhibitors of COX Enzymes*

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin are nonselective COX inhibitors and suppress the inflammatory processes initiated via PGI_2 and other PG signaling. The inhibition of COX-1 has been associated with gastrointestinal toxicity in humans and thus provoked the development of COX-2 selective inhibitors such as NS-398 and celecoxib, which circumvent such damaging effects (215).

Aspirin and most NSAIDs that inhibit COX enzymes precipitate asthma (282). Aspirin-induced asthma affects 5-10% of adult asthmatics (283). It has been proposed that aspirin, by inhibiting COX enzymes forces arachidonic acid metabolism down the leukotriene biosynthesis pathway. Aspirin-induced asthma is characterized by a chronic over production of cysteinyl leukotrienes (Cys-LT). LTC4 synthase the key enzyme in the generation of Cys-LT, is found overexpressed in the bronchi, and its mRNA is upregulated in peripheral blood eosinophils, of asthmatics. In addition, the gene encoding LTC4 synthase exist in two alleles, one of which is associated with severe, steroid dependent type of aspirin induced asthma (284).

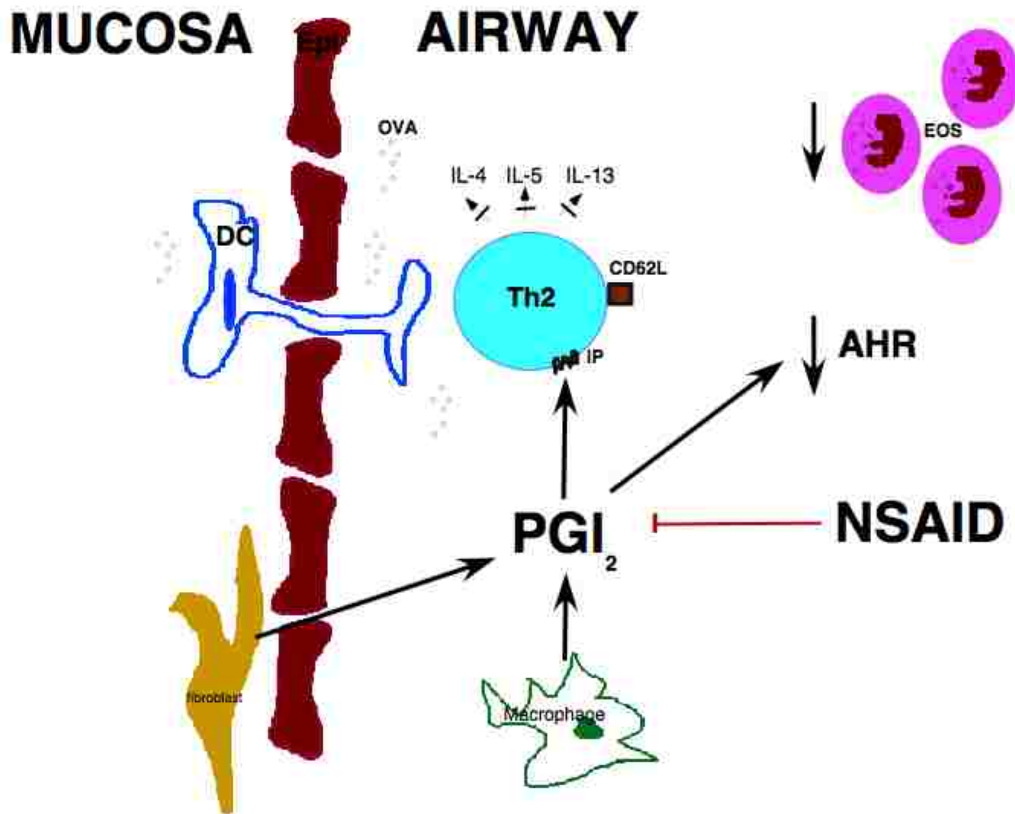
Our previous studies have demonstrated that inhibitors of both COX-1 and COX-2 (aspirin and indomethacin) or COX-2 alone (NS-398), augmented allergic lung

inflammation. To address the role of PGI_2 and its receptor in NSAID induced exacerbations of allergic inflammation we examined the effect of indomethacin on the levels of lung Th2-inflammation in $IP^{-/-}$ and wt animals.

During an inflammatory response multiple prostanoids are generated from the oxidative metabolism of arachidonic acid by COX-1 and COX-2 enzymes that act on a variety of cell types (271). Using a mouse model of pulmonary inflammation, we have shown that following immunization and inhalation with OVA, that $IP^{-/-}$ mice developed pronounced pulmonary inflammation shown by elevated AHR, EPO, and eosinophil and lymphocyte recruitment to the airway. Having developed a mouse model of asthma in wt and $IP^{-/-}$ mice afforded an opportunity to evaluate the roles of PGI_2 in NSAID-induced exacerbation. Treatment with indomethacin markedly augmented the intensity of the eosinophilia in the wt mice, but did not affect the levels of inflammation in the $IP^{-/-}$ mice. The number of $CD4^+$ T cells and $CD19^+$ B cells in the LMC of the $IP^{-/-}$ mice was higher than present in the wt mice following OVA immunization and aerosol challenge. Interestingly, the indomethacin treatment did not increase these levels in the $IP^{-/-}$ mice. Since the proinflammatory effects of indomethacin are evident in wt but lost in the $IP^{-/-}$ mice it appears that PGI_2 signaling is a significant component of indomethacin-induced exacerbations of allergic inflammation.

Treatment with selective COX-2 inhibitor NS-398 resulted in no significant modification of pulmonary inflammation in wt mice. Unexpectedly the $IP^{-/-}$ mice that received treatment with NS-398 had decreased presence of eosinophils in the airway after OVA immunization and aerosol challenges.

Figure 3.25 NSAID Exacerbation of Allergic Inflammation



3.3.6 Summary

Collectively, this data demonstrate that inflammatory events that arise in the lung invoke T and B cell interactions and subsequent antibody production are elevated in the $IP^{-/-}$ mouse. We propose here that PGI_2 dampens allergic inflammation in the tissues and inhibits the IgG1 and IgE response developing in the lymph nodes. Nevertheless, the mediator displays a major effect on the levels of natural antibodies found in the serum of animals.

These observations reveal a key role for PGI_2-IP signaling in regulating allergic responses in the lung, possibly by acting to inhibit T and B cell interactions resulting in downregulation of IgE production and diminished granulocyte activation. This study provides important insight into the regulatory processes that limit the severity of Th2-

mediated inflammatory reactions. There is mounting evidence of the importance of PGs as regulators of immunity, and thus an improved understanding of the diverse activities of these mediators is crucial for the design of novel approaches aimed at immune regulation.

Our experiments suggest that:

1. Allergic lung inflammation is elevated in $IP^{-/-}$ mice.
2. The $IP^{-/-}$ mouse develops a heightened IgG1 and IgE response.
3. Native Antibody levels of $IP^{-/-}$ mice are perturbed with reduced levels of IgA and IgG2b
4. Indomethacin treatment did not augment allergic inflammation in $IP^{-/-}$ mice suggesting that PGI_2 contributes to NSAID induced exacerbations of lung inflammation.

Chapter 4

Antigen-Specific CD4+Foxp3+ Natural Treg cells Limit Th17- but not Th1- or Th2-Mediated Lung Inflammation

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Running Title: Treg Cells Suppress Th17 – Mediated Lung Inflammation

Keywords:

Abbreviations: AHR, airway hyperreactivity; Treg, regulatory T cell; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; LMC, lung mononuclear cell; PLN, peripheral lymph node cell.

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4.0 Abstract

We have examined the ability of nTreg cells to suppress lung inflammatory responses mediated by $CD4^+$ Th1, Th2 and Th17 effector cells. OVA–specific nTreg cells isolated from DO11.10 mice were expanded in the presence of $OVA_{323-339}$ peptide, IL-2 and IL-4. The expanded cells retained Foxp3 expression and their ability to suppress lung mucosal inflammation was examined. Mice that had inhaled OVA and had received $CD4^+$ Th2 cells developed a pulmonary eosinophilia, and increased airway resistance upon challenge with methacholine. Cotransfer of $CD4^+$ nTregs failed to suppress the pulmonary eosinophilia mediated by $CD4^+$ Th2 cells. Nevertheless, Tregs were effective at inhibiting the Th2 polarization of naïve $CD4^+$ T cells. Similarly nTreg cells failed to inhibit murine lung inflammation elicited by $CD4^+$ Th1 cells. In contrast, Treg cells markedly inhibited the inflammatory response elicited by $CD4^+$ Th17 cells. This was evident from a marked reduction in of recruitment of neutrophils and the level IL-17 present in the BAL. Interestingly, $CD4^+$ Treg cell failed to inhibit the response of $CD4^+$ Th17 to anti-CD3 in vitro either in terms of proliferation or IL-17 production. Our results demonstrate that the immunoregulatory properties of Treg cells do extend to Th17 responses. Specifically, Treg cells play a key role in modulating Th17-mediated pulmonary inflammation by suppressing the development of airway neutrophilia. Conceivably, this is partly a consequence of inhibiting IL-17 production.

4.1 Introduction

Allergic asthma is characterized by airway hyperreactivity (AHR) and chronic mucosal inflammation that is associated with pulmonary eosinophilia, mucous hypersecretion and airway remodeling (285, 286). The involvement of $CD4^+$ Th2 cells in driving the inflammatory response in this disease has been proposed by several laboratories (286). More recently it has been suggested that $CD4^+$ Th17 cells may also contribute to the airway inflammation particularly in cases of severe disease (287-289). That chronic allergic pulmonary inflammation in atopic asthma results from a failure to regulate the lung mucosal Th2 immune responses normally evident has been proposed (6). However, delineating the nature and relative importance of specific regulatory pathways in liming different Th2 responses remains to be defined. Regulatory roles for anti-inflammatory cytokines (TGF-β or IL-10), prostaglandins (290, 291) and action of nTreg and iTreg cells have been reported (292-294). It has also been suggested that regulatory T cells (Treg cells) play an important role in preventing inflammatory processes by eliciting immune suppression to inhaled antigens (293). With respect to the latter, in recent years, multiple Treg phenotypes have been described (106). However, the two major types are the natural Treg cells (nTreg) which are generated in the thymus (91, 295) and induced Treg cells (iTreg) which are generated in the periphery (125). Both nTreg and iTreg express a key regulatory transcription factor, Foxp3, required for the development of the T regulatory phenotype (296, 297). nTregs are $CD4+CD25^+$ T cells that are pivotal for the maintenance of peripheral tolerance and preventing the onset of autoimmune disease (298-300). They achieve this by suppressing the activation and proliferation of $CD4^+$ and $CD8^+$ T cells (301-305). nTreg cells have been shown to inhibit

the development of allergic lung inflammation (293, 294, 306). $CD4^+Foxp3^+$ Tregs can also be induced from $CD4^+CD25^-$ precursors (iTregs) when encountering antigen in the presence of IL-2 and TGF-β (307, 308). iTreg cells although similar to nTreg cells in function, differ in principal antigen specificities and co-stimulatory molecules required for their generation (308). nTreg and iTreg cells mediate the suppression of T cell effector function through several mechanisms that require either direct cell contact (309) or the production of immunosuppressive cytokines such as IL-10 (310) and TGF-β (311). A thorough analysis demonstrating the sensitivity of naïve $CD4^+$ T cells and effector $CD4^+$ cells to suppression by nTregs has not been reported. A major limitation in using Treg cells to limit inflammatory responses is the low frequency of Treg cells present in lymphoid tissues. To circumvent this problem several investigators have expanded nTreg cells in vitro prior to transfer into animals or patients (312). Human $CD4^+CD25^+$ Treg cells from peripheral blood specific for human leukocyte antigen A2 (HLA-A2) have been successfully purified and expanded by TCR stimulation in the presence of high-doses of IL-2 (313). In addition, antigen specific murine nTreg cells have been expanded in culture in the presence of anti-CD3 and CD28 and IL-2 remaining phenotypically and functionally pure (314). Although nTreg and iTreg cells are functionally similar their response to IL-6 is markedly different since this cytokine can convert nTreg to IL-17 producing Th17 cells in contrast to iTregs which are resistant to conversion (308). Conversely, the induction of iTreg cells is inhibited by IL-4, which promotes the differentiation of a $Foxp3^-$ IL-9 and IL-10 expressing effector phenotype (315).

In this study, we used an adoptive transfer model to investigate the effectiveness of antigen-specific nTreg in limiting the inflammatory responses elicited by Th2 and Th17 cells to inhaled antigens. This approach required purifying, expanding, and characterizing nTreg cells prior to monitoring their effect on fully differentiated $CD4^+$ Th2 and Th17 cell responses and cytokine production. Comparisons were made with recipients of $CD4^+$ Th1 cells. Treg cells isolated from DO11.10 mice cultured in the presence of OVA peptide and IL-2 and IL-4 were highly effective at limiting Th17-mediated inflammation but failed to inhibit Th2-mediated lung inflammation. Nevertheless, Treg cells were effective at inhibiting the polarization of naïve $CD4^+$ T cells. To monitor Th2 polarization in vitro, the reduction of GFP expression by $CD4^+$ T cells from IL-4 reporter mice (4get mice), was monitored.

4.2 Results

4.2.1 Identification and characterization of $CD4^+CD25^+Foxp3^+$ natural Tregs in naïve DO11.10 mice

$CD4^+CD25^+$ regulatory T cells have been demonstrated to perform a central function in preventing organ-specific autoimmune diseases (300) and limiting inflammatory responses. Given that depletion of $CD4^+CD25^+$ cells was associated with heightened Th2-mediated pulmonary inflammation (294) we examined the ability of nTreg cells to suppress pulmonary inflammation. We used the OVA-specific TCR transgenic mouse DO11.10 as a source of Ag-specific $CD4^+CD25^+Foxp3^+$ T cells and determined their ability to suppress Th1, Th2 and Th17-mediated lung inflammation. Tregs were identified in DO11.10 mice by staining with the antibodies to CD25 and Foxp3 (Figure 4.1A). Flow analysis of peripheral lymph nodes demonstrated that 4-6% of naive DO11.10 $CD4^+$ T lymphocytes constitutively express CD25 (Figure 4.1A) (compared to 0.6% of the cells that stained positive for IgG isotype control). Memory $CD4^+$ T cells (316) and nTregs (312) have been identified in TCR transgenic mice previously. nTregs in DO11.10 mice expressed the OVA-specific transgenic TCR as evidenced by staining with the anti-clonotypic antibody KJ1-26 (Figure 4.1A). Flow analysis revealed that intracellular Foxp3 was expressed by 4.7% of peripheral lymph node $CD4^+$T cells. To verify that $CD4^+CD25^+$ cells were Tregs we used three color staining of the LN cells using anti-CD4, CD25, and Foxp3 antibodies. This approach revealed that 85-95% of the $CD4+CD25^+$ cells and 2% of $CD4^+CD25^-$ cells expressed Foxp3 (Figure 4.1A). Magnetic bead sorting of natural $CD4^+CD25^+$ Tregs from the lymph nodes of DO11.10 mice, yielded an average of $2x10^5$ viable cells per mouse.

Importantly we found that 69.6% of $Foxp3^+$ cells stained with the anti-clonotypic antibody KJ1-26 and would be expected to respond to $OVA_{323-339}$ peptide (Figure 4.1A).

The limited numbers of nTregs in DO11.10 mice made adoptive transfer experiments of these cells difficult. To circumvent this problem natural Tregs purified from DO11.10 mice were expanded in culture in the presence of $OVA_{323-339}$ peptide and exogenous IL-2 and IL-4. We have shown previously that $CD4^+CD25^+Foxp3^+$ T cells proliferate in the presence of IL-2 and IL-4 with retention of Foxp3 expression and suppressive function (294). Importantly, IL-4 has been shown to inhibit the generation of induced-Tregs, iTregs (315). Three $x10^6$ purified $CD4^+CD25^+$ cells typically yielded 20-$30x10^6$ cells after eight days. Intracellular Foxp3 staining demonstrated that the majority of $CD4^+CD25^+$ expanded continued to express Foxp3 (86%) and were clonotype specific (figure 4.1B,C). In contrast, activated $CD4^+CD25^-$ cells expanded under identical conditions expressed minimal levels of Foxp3 protein (0.1%) (Figure 4.1B). The expanded $CD4^+CD25^+$ cells provided a source of Tregs for use in experiments.

Figure 4.1 (a-c)

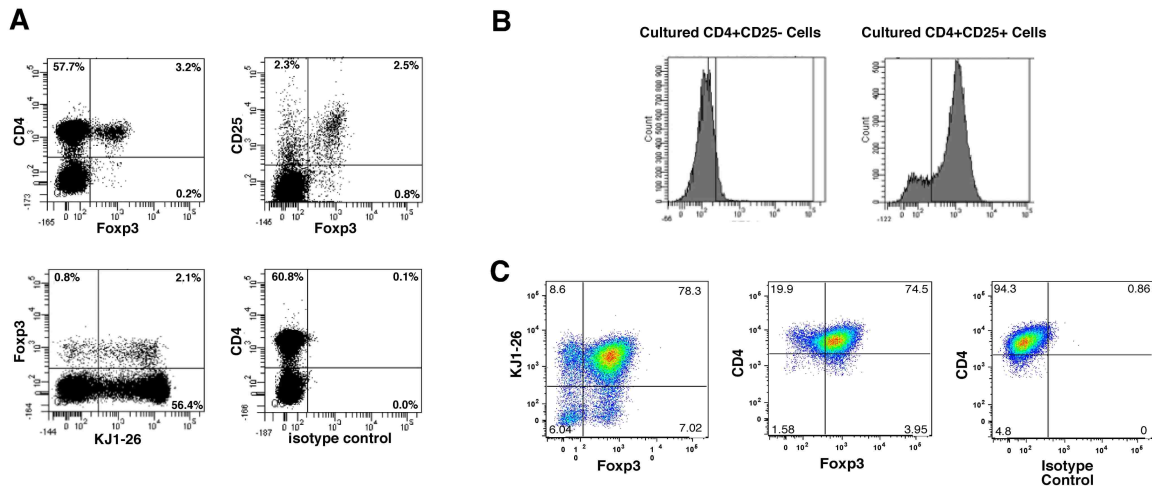


Figure 4.1 ***Identification and expansion of OVA-specific CD4$^+$ T cells in DO11.10 mice***. Peripheral lymph nodes were prepared from DO11.10 mice and stained using anti-CD4, CD25, Foxp3 antibodies and the anti-clonotypic antibody KJ1-26. **A.** The frequency of CD4$^+$, CD25$^+$ and KJ1-26$^+$ cells that expressed Foxp3 was determined. The proportion of Foxp3$^+$ cells were assessed by intracellular staining using Alexafluor 488-conjugated anti-Foxp3. **B.** CD4$^+$CD25$^+$ T cells were cultured in the presence of OVA$_{323-339}$ peptide (1μg/ml), IL-2 (10 ng/ml) and IL-4 (2ng/ml). After 8 days the cells were stained for Foxp3, CD25, CD4 expression as detailed previously. **C.** To determine whether the expanded Foxp3+ cells expressed the transgenic TCR the cultured cells were stained with KJ1-26, Foxp3, CD4 and isotype control antibodies.

4.2.2 Expanded nTregs did not suppress Th2 or Th1 mediated airway inflammation

We examined whether pulmonary inflammation mediated by fully polarized CD4$^+$ Th1 or Th2 polarized cells would be suppressed by co-transfer of expanded Tregs with the effector cells. CD4$^+$ cells from DO11.10 mice were cultured for 8 days in Th1 or Th2 polarizing conditions. These cells (10^7 cells/animal) were adoptively transferred alone or simultaneously with expanded Tregs into BALB/c mice followed by inhalation

challenges with aerosolized OVA. Effector CD4+ Th1 or Th2 cells elicited a mucosal inflammatory response that was characterized by the inflammatory cells that entered the lung tissue and BAL. nTregs were co-transferred into mice at the same time as $CD4^+$ Th1 or Th2 polarized effector cells at a ratio of 1 to 2, respectively. Consistent with previous findings (294), following exposure to OVA aerosols, a pronounced peribronchial and perivascular neutrophilic or eosinophilic inflammation and an increase in KJ1-26^+ T cells were observed in the lung parenchyma of recipients of the Th1 or Th2 cells, respectively (Figure 4.2A,B). Animals receiving Th2 cells developed a pronounced pulmonary eosinophilia evidenced by cytochemical staining and EPO activity in contrast to control animals that inhaled OVA but did not receive $CD4^+$ Th2 cells (Figure 4.2A,C). The mice receiving only nTregs showed no evidence of lung inflammation. The cotransfer of Tregs with Th2 cells failed to suppress the inflammatory response and reproducibly resulted in increased numbers of lymphocytes, macrophages and eosinophils in the BAL (Figure 4.2A) and higher levels of EPO (Figure 4.2C). Conversely, Th1-induced inflammation resulted in the recruitment of neutrophils (CD11b/GR-1^+ cells) and activated macrophages to the airway (Figure 4.2B,D). Cotransfer of nTregs failed to reduce the level of CD11b/Gr-1^+ neutrophils present in the BAL of Th1 recipient mice. nTregs did not reduce the number of OVA-specific Th1 or Th2 cells in the lungs since the number of $CD4^+$KJ1-26^+ present in the lungs was unaffected by cotransfer of nTregs (Figure 4.2E,F) while recipients of nTregs contained few $CD4^+$KJ1-26^+ T cells. It is clear from these results, that under the described conditions, Tregs when co-transferred with fully polarized T effector cells, were not only incapable of regulation, but may contribute to the enhancement of pulmonary inflammation.

Figure 4.2 (A-F)

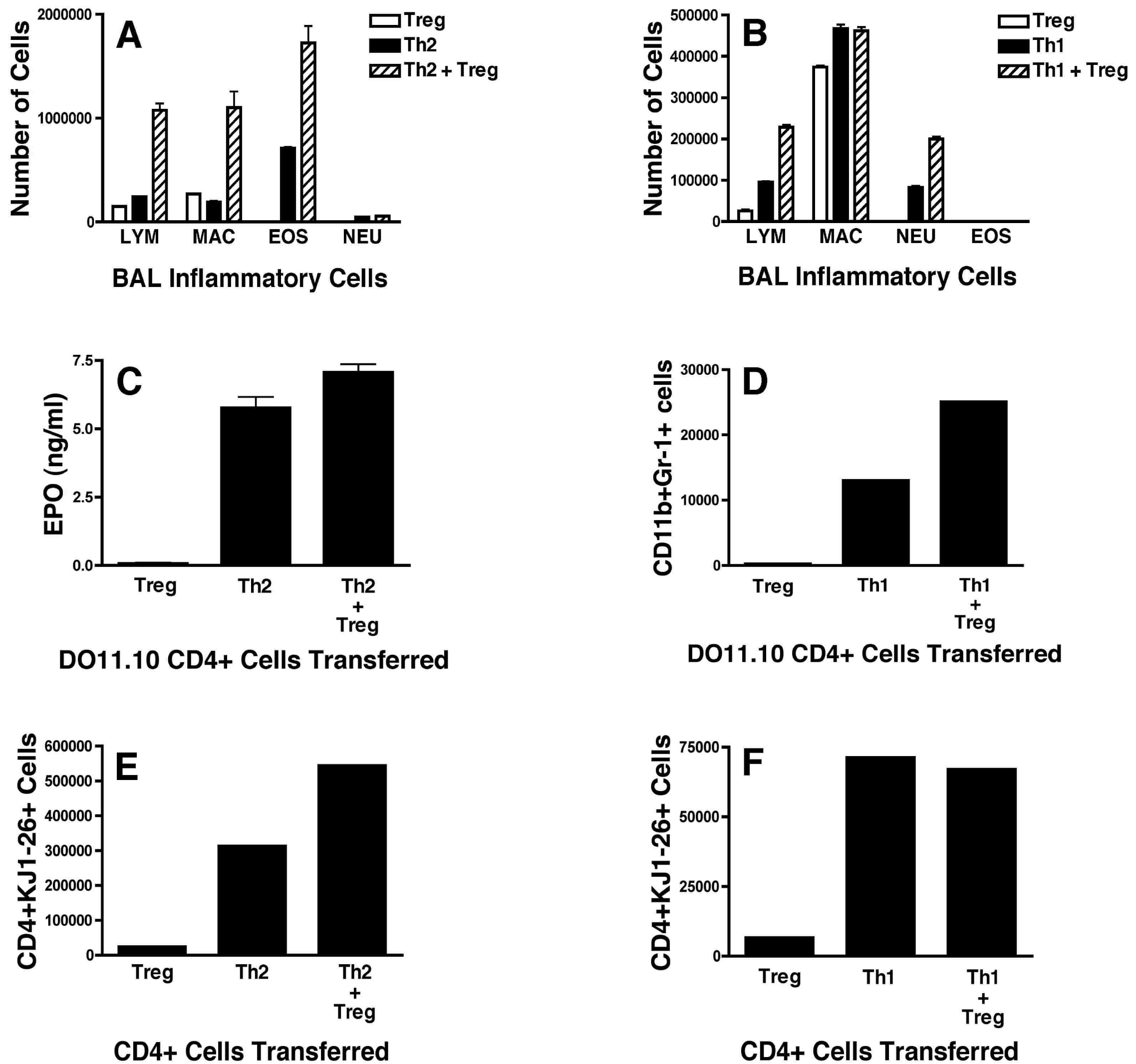


Figure 4.2 ***Expanded natural Tregs failed to suppress Th1 and Th2 – mediated lung inflammation.*** CD4+Th1 or Th2 differentiated cells and expanded nTreg cells were injected (10^7 cells /animal) into BALB/c mice that were then exposed to OVA aerosols for 7 consecutive days. Control mice did not receive the effector cells. **A.** BAL fluid was collected to evaluate the levels pulmonary inflammation elicited by CD4+ Th2 cells. The cell differential counts for recipients of Tregs, CD4+ Th2 cells and Th2 + Treg cells were determined by light microscopic evaluation of cytochemically stained cytospin preparations. Results are expressed as absolute numbers of lymphocytes (Lym), macrophages (Mac), eosinophils (Eos), and neutrophils (Neu). **B.** The cell differential counts for recipients of Treg, CD4+ Th1 cells and Th1 + Treg cells were determined and expressed as absolute numbers as previously. **C.** The level of EPO activity was determined by colorimetric analysis. **D.** The number of CD11b+Gr-1+ cell present in BAL of mice that received Th1 cells +/- Tregs was determined by flow cytometry. **E.**

Lung mononuclear cells were collected from the collagenase-digested lungs of Th2 recipient mice that had inhaled OVA. Anti-KJ1-26 clonotypic and anti-CD4 antibody staining was used to identify the ova-specific T cells and the cells were analyzed by flow cytometry. **F.** Lung mononuclear cells were prepared from Th1 recipient mice that had inhaled OVA and the number of CD4+KJ1-26+ cells determined as detailed previously.

4.2.3 Expanded nTregs suppress Th2 polarization and IL-4 expression by $CD4^+$ T cells in vitro

We next evaluated whether expanded nTregs could inhibit the initial Th2 polarization of CD4+ cells. The differentiation of naïve $CD4^+$ T cells into fully polarized Th2 cells is characterized by the expression of the transcription factors GATA-3 and STAT6 and the cytokines IL-4 and IL-13. To monitor $CD4^+$ Th2 polarization in vitro we used the C129.IL4GFP (4GET) mouse (317) (Jackson Laboratory). C129.IL4GFP peripheral lymph node cells were co-cultured with the expanded Tregs on anti-CD3 coated plates (ratio of 1:4) in the presence of exogenous IL-4 and IL-2 in order to drive Th2 maturation. After 4 or 8 days in culture we determined the number of GFP expressing $CD4^+$ T cells by flow cytometry (figure 4.3A). Importantly, the KJ1-26 clonotypic antibody specifically recognized DO11.10 nTregs but not C129.IL4GFP $CD4^+$ cells which we exploited to gate Tregs and remove them from the analysis. Interestingly, nTregs from DO11.10 mice inhibited Th2 polarization, using GFP production as a measure of IL-4 expression in comparison to C129.IL4 alone control cells. After 4 days in culture the nTregs inhibited expression of GFP by the C129.IL4 cells by 50.6% (13.7% positive GFP staining compared to 64.3% positive for the cultures grown in the absence of nTregs) (Figure 4.3B). The reduction in IL-4 production by nTregs was not associated with an increase in the level of IFN-γ as observed by other reports (318). The

nTreg suppression was reversed by inclusion of an antibody to GITR (Figure 4.3C), which has been shown to reverse the action of Tregs (319). In contrast, the addition of a soluble form of OX40L failed to reverse the Treg mediated suppression of Th2 polarization (Figure 4.3C)

Figure 4.3

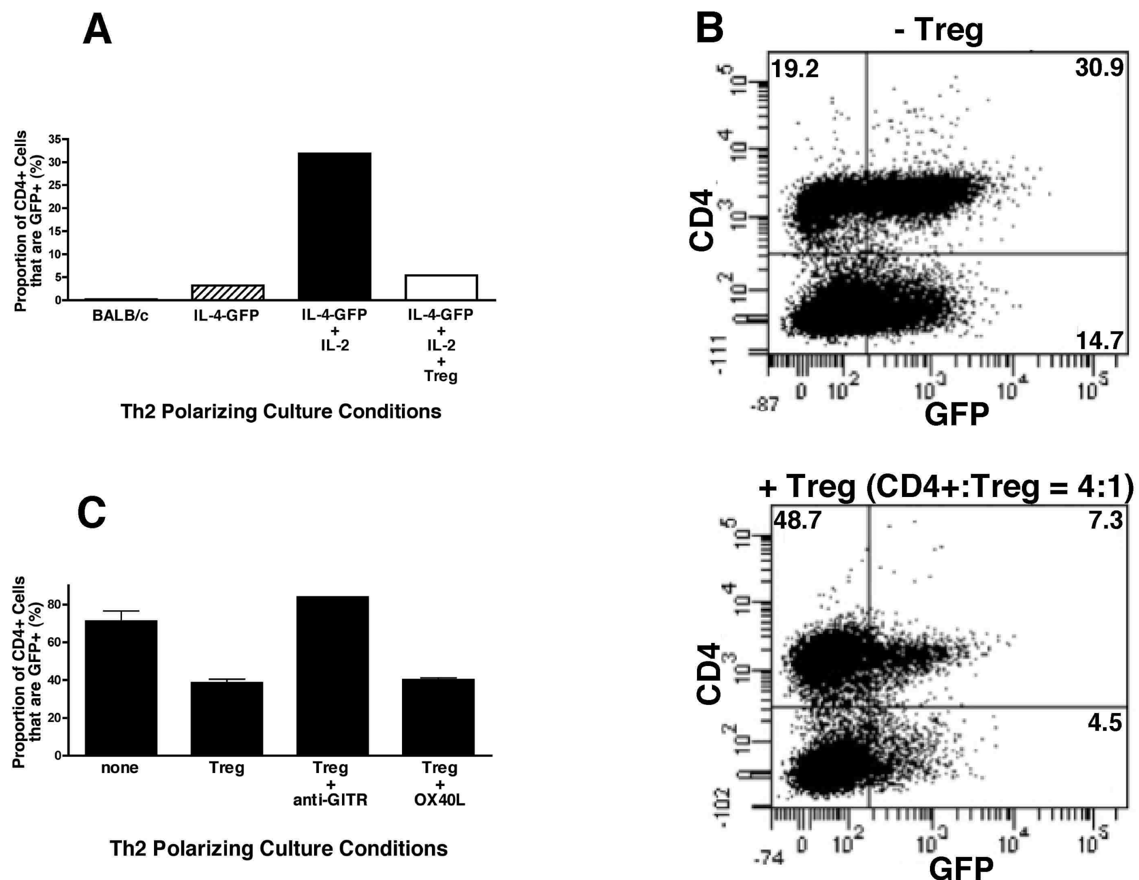


Figure 4.3 ***Expanded natural Tregs did suppress the differentiation of $CD4^+$Th2 cells***. C129.IL4 peripheral lymph node cells and expanded Tregs were co-cultured for 4 days on anti- CD3 coated plates (ratio 1:4) in the presence of exogenous IL-4. FACS analysis of GFP was performed after removing $CD4^+$KJ1-26^+ Tregs from the analysis by gating out cells staining with APCcy7 conjugated anti-KJ1-26. **A**. Examine the effect of exogenous IL-2 and Tregs on the GFP expression of C129.IL4 lymphocytes. C129.IL4 cells were cultured in the presence of immobilized anti-CD3 (2μg/ml) and IL-4 (2ng/ml) for 4 days. The effect of adding IL-2 (10ng/ml) or Treg (ratio 4$CD4^+$ cells : 1 Treg cell) on GFP production was determined **B**. Examine the suppression of GFP expression by C129.IL4 lymphocytes by Tregs. $CD4^+$ C129.IL-4 cells were cultured in the presence of

absence of Tregs using anti-CD3 a, IL-2 + IL-4 as previously. **C.** C129.IL-4 cells were stimulated in the presence of anti- CD3 and IL-4 and IL-2 and GFP expression determined after 4 days. Tregs were added to the culture in the presence of anti- GITR antibody (20μg/ml) of solubilized OX40L (20μg/ml).

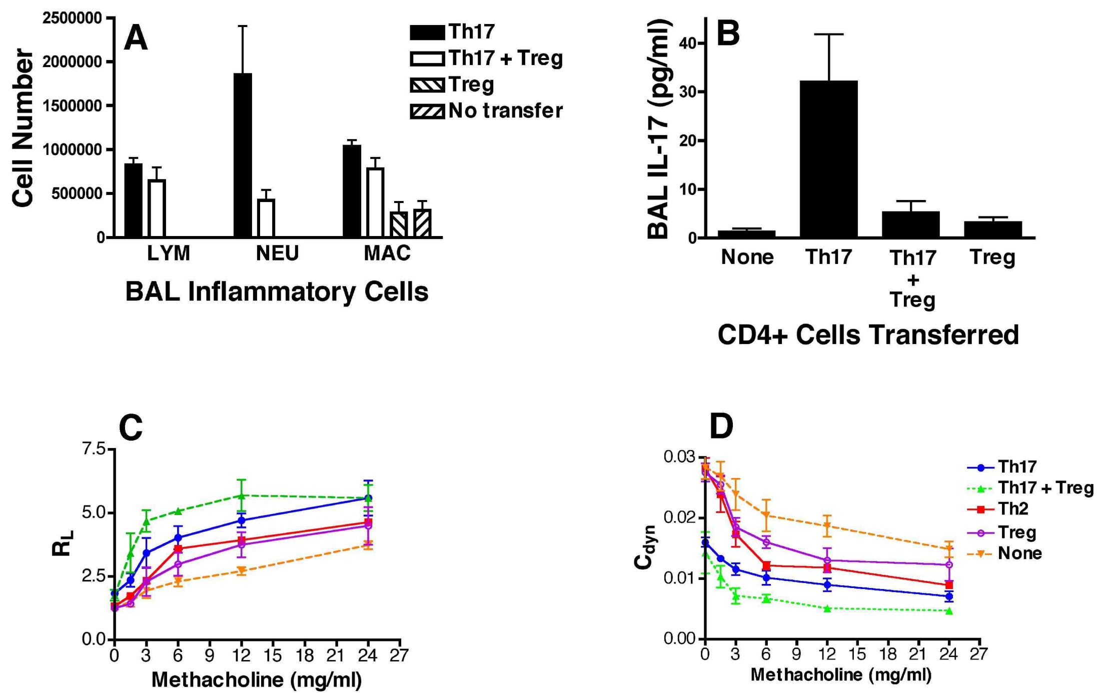
4.2.4 Expanded nTregs suppress Th17 mediated airway inflammation

The ability of nTregs to suppress lung mucosal Th17 responses was also examined. The adoptive transfer of DO11.10 $CD4^+$ Th17 into BALB/c mice and subsequent exposure to aerosolized OVA resulted in a pronounced airway neutrophilia (Figure 4.4A) that was associated with the presence of IL-17 in the BAL (Figure 4.4B). Interestingly, the cotransfer of nTregs with $CD4^+$ Th17 resulted in the marked reduction in Th17-mediated inflammatory responses as defined by the number of neutrophils present in the airways and levels of IL-17 present in the BAL (Figure 4.4B).

The reduction in Th17-mediated inflammatory processes in the airways following cotransfer of Tregs was also associated with the reduction in $KJ1\text{-}26^+$ cell in the BAL from (3.52-3.65)% to (1.72-1.77)%. In mice that received both $CD4^+$Th17 and Tregs it was important to discriminate between $KJ1\text{-}26^+$ effector Th17 and Tregs present in the lung. Intra-cellular staining of IL-17 revealed that cotransfer of Tregs markedly reduced the proportion of IL-17 expressing $KJ1\text{-}26^+$ cells present in LMC (27.4% to 11.3%). Th17 recipient mice displayed increased levels of airway resistance and reduced dynamic compliance compared to control mice and were significantly worse than recipients of $CD4^+$ Th2 cells alone (Figure 4.4C). Interestingly, this trend was not reversed by the cotransfer of nTregs which was not surprising given that transfer of Tregs alone resulted in an increase in airway resistance and reduced compliance. Interestingly, the increase in

airway resistance and reduction in dynamic compliance elicited by Th17 and nTregs appeared additive (Figure 4.4D). These findings confirm that although the Th17-mediated inflammatory response was markedly reversed by antigen specific nTregs this was not accompanied by an improvement in airway function (Figure 4.4C,D). A marked peribronchial and perivascular inflammation was observed in Th17 recipient mice, which was reversed with the co-transfer of Tregs (Figure 4.4E).

Figure 4.4



E

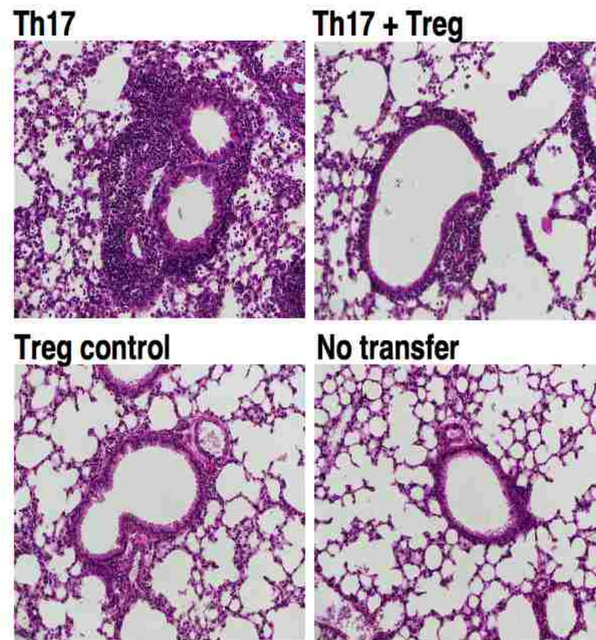


Figure 4.4 ***Expanded natural Tregs did suppress Th17- mediated lung inflammation, cytokine production but had no effect on airway function.*** **A.** To assess the effects of expanded nTregs on the neutrophilic response induced by Th17 cells, BAL cell differential counts were determined after samples were centrifuged using a Shandon Cytospin followed by staining with Hema3. Results are reported in absolute numbers of cells collected. B. IL-17 levels in the BAL fluid from different experimental groups (n=3) of animals were measured by commercially available ELISA kit (e-Bioscience). **C. ,D** Airway resistance and dynamic compliance were measured over a range of methacholine concentrations **E**. Histological analysis of lung tissue was performed using H&E staining.

4.2.5 Expanded nTregs did not suppress Th17 responses in vitro

The possibility that the Tregs suppressed Th17 function in vitro was also examined Th17 cells were stimulated with immobilized anti-CD3 in the presence of different numbers of Tregs forming Th17:nTreg ratios of 4:1, 8:1 and 16:1. The addition of nTregs to $CD4^+$ Th17 cells did not inhibit IL-17 production by the stimulated Th17 cells over a range of ratios (Figure 4.5A). Similarly, the proliferation of the cells in response to anti-CD3 was not affected by the presence of nTregs (Figure 4.5B). These

data strongly suggest that nTregs do not act directly on $CD4^+$ Th17 cells and raises the possibility that Tregs may act indirectly by modifying dendritic cell function.

Figure 4.5

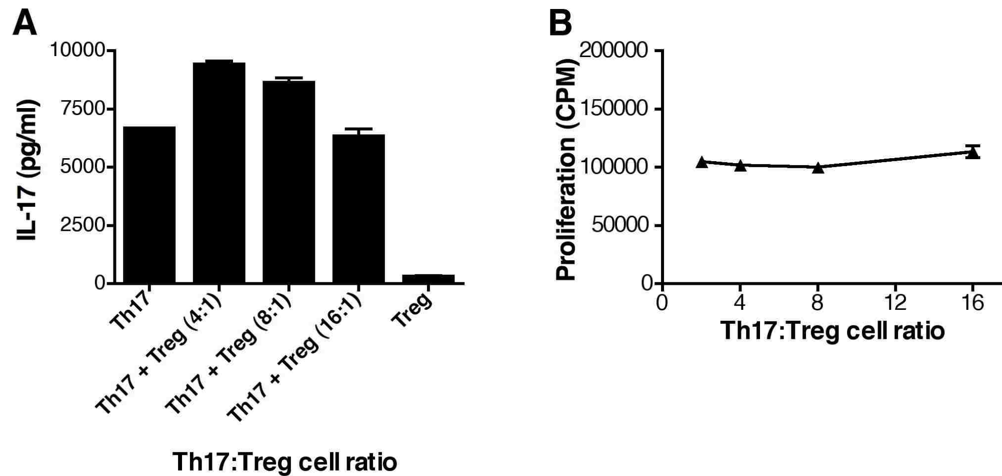


Figure 4.5 ***Expanded $CD4^+$ Tregs failed to inhibit $CD4^+$ Th17 proliferation of cytokine production in vitro.*** CD4+ Th17 cells were added to 24 well plates pre-coated with 2µg of anti-CD3 (2C11). CD4+ expanded Tregs were added to the wells at different concentrations. Controls comprised of Th17 cells or expanded Tregs alone. Culture supernates were harvested after 18h and assayed for IL-17 by ELISA. Similarly the levels of proliferation were determined by adding 1µCi of 3H-TdR to the cells and the levels of incorporation determined after 18h by scintillation counting.

4.3 Discussion

Allergic asthma is characterized by production of Th2 cytokines, IgE antibodies, eosinophilic lung inflammation, airway hyperresponsiveness, airway remodeling and mucus hyperproduction (320, 321). A role for $CD4^+$ T cells and NK-T cells in driving the inflammatory response has been proposed (322). The inhalation of soluble antigens typically results in the onset of sustained tolerance that reverses the progression of the inflammatory process (323). However the mechanisms underlying the resolution of airway mucosal Th2 mediated inflammation remain poorly understood. Moreover, the regulatory mechanisms operative during the early differentiation of $CD4^+$ cells may differ from fully differentiated effector cells in part because of the different cytokine dependence. $CD4^+CD25^+Foxp3^+$ (Treg) cells are considered an important regulator of the immune system, and have been shown to prevent autoimmunity (300) and the development of *Helicobacter hepaticus* induced colitis (324, 325). The therapeutic potential of transferring exogenous Treg cells was first demonstrated in a murine model of colitis, showing that in SCID mice disease progression could be reversed, by transferring $CD4^+CD25^+$ Tregs. Furthermore, successful treatment of severe colitis with Tregs could be reversed upon treatment of the animals with anti-IL-10 monoclonal antibody (mAb) (326), implicating IL-10 as a requirement in Treg mediated regulation of inflammation. However, regulation was evident in the absence of IL-10 or TGF-β (327). In addition, $CD4^+CD25^+Foxp3^+$ Tregs are capable of regulation of both $CD4^+$ and $CD8^+$ T cell responses, in part by inhibiting IL-2 production and restricting clonal T cell expansion and development of memory (303). In this study, we used the DO11.10 TCR-transgenic mouse to model antigen induced lung inflammation and examine the influence

of Ag-specific regulatory T cells in the suppression of these responses. Consistent with previous reports, 4-6% of the $CD4^+$ T cells from DO11.10 mice constitutively express CD25 (294, 318, 328). In addition, these $CD4^+CD25^+$ T cells expressed the Foxp3 protein and the transgenic TCR as determined by staining with the anti-clonotypic antibody, KJ1-26. The principal difficulty encountered when delineating the anti-inflammatory properties of $CD4^+CD25^+Foxp3^+$ Treg cells are obtaining sufficient Tregs with a known antigen specificity. To circumvent this limitation we expanded natural-Tregs from the OVA-specific TCR transgenic mouse DO11.10 in vitro in the presence of $OVA_{323-339}$, IL-2 and IL-4. Our results demonstrated that freshly isolated and expanded $CD4^+CD25^+$ DO11.10 Tregs expressed the Foxp3 protein and mediated suppressive activity.

We investigated the capacity of nTregs in limiting the onset of inflammation mediated by fully differentiated $CD4^+$ Th1, Th2 and Th17 cells. Interestingly, $CD4^+$ Th17, but not Th1 or Th2, mediated lung inflammation was suppressed by the cotransfer of expanded nTregs. The $CD4^+$ Th17 cells following transfer into mice retained a Th17 phenotype insofar as they produced IL-17 but not IFN-γ following OVA inhalation so demonstrating that immune deviation of the transferred $CD4^+$ T cells away from a Th17 phenotype was not evident. Conceivably, the expansion of the $CD4^+$ Th17 cells was inhibited by the transferred $CD4^+$ Tregs. In this context adoptively transferred $CD4^+$ Th17 cells did proliferate in vivo in response to inhaled antigen and this proliferation was dependent on the IL-2R. Inhibiting Th17 cell expansion could arise from direct regulation of the $CD4^+$ Th17 cells or act via inhibiting antigen presentation by DCs. It has been previously been reported that Tregs can suppress immune responses by suppressing DC maturation and/or CD80/CD86 expression (329). This may explain why nTregs failed to

suppress Th17 responses in vitro. Our experiments prove that nTregs were capable of suppressing the Th2 polarization of naïve $CD4^+$ T cells but render little effect over fully polarized Th2 cells. Although IL-2 is known to suppress Th17 cells, IL-2 played a significant role in the expansion of the $CD4^+$ Th17 cells prior to transfer into hosts and after aerosol challenge. It is conceivable, that the regulation observed arose from the suppression of IL-2 production and Th17 expansion in vivo.

The effective regulation could arise as a consequence of preventing expansion and/or cytokine production by $CD4^+$ Th17 cells by either directly interacting with the T cells or dendritic cells that present inhaled antigens in the lung. It unclear why effector $CD4^+$ Th17 cells, but not Th1 or Th2 cells, are susceptible to regulation by nTregs. This may arise from differences in intrinsic properties of effector T cells. Also the lung mucosal environment may facilitate Treg function during Th17-mediated inflammation. In this context, it has been demonstrated that nTregs purified from DO11.10 mice only marginally suppressed Th2-mediated airway inflammation (330). Interestingly, Treg mediated suppression of Th2-mediated inflammation cells was considerably enhanced by the phosphodiesterase 4 inhibitor, rolipram (330). Possibly a consequence of promoting suppression of the cell-contact dependent transfer of cAMP to Th2 cells, that has been proposed to contribute to regulatory function (330, 331).

In summary $CD4^+CD25^+Foxp3^+$ Tregs play a key role in regulating airway inflammation mediated by $CD4^+$ Th17 cells. In addition, our results demonstrate that Tregs can inhibit Th2 polarization. IL-2 and progression of the $CD4^+$ cells through several cell cycles has been shown to be essential for effective Th2 polarization. This

suppression was evident in the presence of exogenous IL-2 implying that that action of Tregs was irrespective of IL-2-dependent proliferation. A better understanding of the mechanism underlying the immunomodulatory qualities exerted by Tregs could provide important information leading to novel approaches to control the airway inflammatory response manifest in bronchial asthma.

The regulatory events leading to the limitation of lung inflammation remain unclear. Further elucidation of the role of nTregs is at this time necessary and critical in order to broaden our understanding of mechanisms underlying diseases such as asthma.

Chapter 5

Conclusions

An increase in the prevalence of asthma in past decades coupled with the observation that presently no treatment is available that impacts on the incidence of this disease, has predicated the need for a deeper understanding of the mechanisms underlying this disease. Allergic asthma is chronic inflammatory disorder in which a type 2 response by $CD4^+$ T cells in the airways predominates in driving the inflammation. It is however, becoming increasingly apparent that the disease is heterogeneous with respect to immunopathology and clinical phenotypes (332). Although the current thought is that asthma stems predominately from Th2-type lymphocytes, IgE, mast cells eosinophils, and cytokines, that respond to antigen in the airways, it is becoming more evident that mesenchymal, vascular and neurological events are also involved in directing the Th2 phenotype to the lung via aberrant injury-repair mechanisms (6). It has been proposed that allergic inflammation arises from the dysfunction of events that normally lead to the resolution of the underlying T cell response (6). To date it is assumed that Th2 driven inflammation and the IgE response are under the same mechanism of regulation. However, it is conceivable that during inflammation, the mucosal pro-inflammatory response is regulated by events that do not affect the IgE response, and vice versa. Our data suggests the possibility that PGI_2 more effectively regulates the IgE response that the other aspects of $CD4^+$ Th2 driven inflammation, potentially involving a novel role in mediating isotype switching. Moreover, NSAIDs, such as aspirin, have been implicated in the exacerbation of these effects.

Collectively, the findings described in this text outline cellular and molecular actions with potential roles in the regulation of these inflammatory events. The first set of experiments addressed the role of the eicosanoid-PGI_2. PGI_2 has previously described roles as an anti-thrombotic agent, as well as a messenger of signaling events leading to inflammation and pain (217). The rationale behind these experiments is such that since the IP receptor is selectively expressed on $CD4^+$ Th2 cells, (but not Th1 differentiated cells) and that prostanoids typically suppress immune responses, we proposed that inhibition of PGI_2-IP signaling would lead to elevated pulmonary inflammation. Indeed disruption to the receptor for PGI_2, IP in C57BL/6 mice resulted in a phenotype with an augmented Th2 type pulmonary inflammation, heightened AHR and increases in eosinophilic infiltration and IgE and IgG1 in the airways of these animals following OVA immunization and aerosol challenge. Interestingly, the unimmunized and unchallenged $IP^{-/-}$ mice appeared to have a defect in antibody production connected with isotype switching, since all of the serum Ig levels were markedly decreased, with the exception of IgM--which doesn't require isotype switching. Even more interesting is that both of the two most dramatically diminished natural antibodies were IgA and IgG2b, which require TGF-β for their production.

Remembering that $CD4^+CD25^+$ cells expanded in IL-4 express the IP receptor and consequentially having noticed the immunomodulatory effects of PGI_2 on Th2 type inflammation, it was logical to examine the potential involvement of nTregs on suppressing the allergic inflammation. Surprisingly, co-transfer of nTregs inhibited Th17 mediated lung inflammation, but not Th1 or Th2 in terms of reduced neutrophilia and IL-17 production in vivo. A question we are left with is why Th17 cells are apparently more

susceptible to regulation by Tregs than Th1 or Th2 cells. One possible explanation for this phenomenon is that transferred Th17 cells may undergo a higher rate of expansion than do the Th1 or Th2 transferred cells, Consequently, suppression of the cell cycle may have a more pronounced effect on Th17 compared to Th1 or Th2 mediated inflammation. It would be useful to labeling the transferred Th2 or Th17 cells with CFSE and examine the relative expansion of the effector cells in vivo. Alternatively the regulation of the Th17 response may be occurring via Treg-APC-effector interactions through mechanisms involving reduction in the effectiveness of antigen presentation in the airway itself.

Future Directions

Investigation into the role of PGI_2-IP signaling pathway in immune responses

1) Investigate the role prostacyclin plays in the IgA and IgG2b response. Both isotypes require TGF-β and our preliminary data shows that Iloprost induces TGF-β protein production by lymph nodes. The cellular source of TGF-β is currently unknown, but from the available data, it appears be produced from a non-T cell.

Following immunization with OVA/alum the defect in IgA and IgG2b production is lost and no longer detectable in the serum of $IP^{-/-}$ mice. This procedure uses an alum adjuvant via intra-peritoneal route of immunization. Consequently, using this protocol, antibody production is likely to occur primarily in the spleen and mask the defect present in other lymphoid tissue. It is possible that the observed defect in unimmunized mice may arise a mechanism operative in the lymph nodes, or gut associated lymphoid tissues which results in lower concentrations of serum antibody therefore masking the effect. Therefore it would be of interest to investigate the effect of priming with alternate

adjuvants, doses or routes of immunization to explore the underlying mechanisms responsible for the abnormal in antibody production. Moreover, a series of adoptive transfer experiments where IP expressing T or B cells are transferred into $IP^{-/-}$ mice in an attempt to fix the defect may prove informative.

Experiments to further understand the roles played by Tregs in allergic asthma

1) It is important to characterize of the molecular mechanism by which nTregs influence $CD4^{+}$ Th17-mediated inflammation. Is it by an IL-10 dependent mechanism requiring a reduction in airway APC function? Or alternatively is it by regulating the $CD4^{+}$ cells directly?

2) Investigate the differences in the regulatory effects between nTreg and iTreg in the capacity to inhibit Th2 and Th17-mediated lung inflammation.

3) Isolate Tregs from lung tissue directly and monitor their ability to suppress inflammatory processes. Evaluate antigen presentation and migratory mechanisms from the lung localized Tregs.

4) Characterize the phenotype of $Foxp3^{+}$ Tregs in the lungs using the Foxp3-GFP mouse. Further elaboration is needed of the differences between Treg inhibition of Th2 and Th17-mediated lung inflammation. This

5) TGF-β is required for the production of IgA and IgG2b. Preliminary data from our laboratory has demonstrated this cytokine is altered in the $IP^{-/-}$ mouse. Since TGF-β production is one of the regulatory actions of Treg cells, experiments designed to disclose a Treg population in the $IP^{-/-}$ mouse that may be involved and the mechanism

underlying in this immunoglobulin defect may provide important information to better understand of nTreg inhibition of the inflammatory response.

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