THE CONTRIBUTION OF PROSTANOIDS TO ALLERGY AND LUNG INFLAMMATION

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ABSTRACT

RACHEL COTE': The Contribution of Prostanoids to Allergy and Lung Inflammation
(Under the direction of Beverly H. Koller)

Through respiration, the airway is exposed to foreign materials and relies on the immune system to interpret each novel antigen's pathogenicity. Failure to establish unresponsiveness to innocuous particles can initiate atopy, characterized by elevated immunoglobulin E (IgE) levels. In susceptible individuals, asthma can develop, causing airway inflammation, reversible airflow obstruction, and airway hyperresponsiveness (AHR). Leukocytes rely on information received in the form of cytokines, chemokines, and lipid mediators to determine if an immune response is warranted or, alternatively, if immune tolerance should be established.

Prostanoids are lipid mediators, produced by cyclooxygenase (COX) enzymes, which can both promote and limit inflammatory processes. Rodent models of atopic pulmonary allergy demonstrate that inhibition of prostanoid synthesis, by either COX isoform, augments disease parameters, assigning a protective role to these pathways. We evaluated the role of prostaglandin E_2 (PGE_2) to this process, utilizing mice lacking microsomal PGE_2 synthase 1 (mPGES1). Unlike a loss of COX activity, a deficiency in mPGES1 ameliorates airway inflammation at a step subsequent to sensitization, suggesting that this prostanoid augments the effector arm of pulmonary allergy. Further, synthesis of PGE_2 from the lung, itself, is implicated in our model.

PGI_2, another prostanoid, is thought to limit disease in multiple pulmonary afflictions. Utilizing IP -/- animals and COX-1 -/- animals, we demonstrate a contribution for this prostanoid in

COX-1-dependent protection during atopic pulmonary episodes, potentially by inhibiting inflammatory cytokine release. While mediation by PGI_2 occurs during both branches of an allergic response, the contribution is more substantial during allergy elicitation, perhaps indicating that local antigen exposure is a requirement for PGI_2-mediated airway protection. Further we show that this protection occurs through immune cells recruited to the lung.

Finally, we demonstrate that a loss of PGE_2 or its receptors does not impede the immune system's ability to establish tolerance in the airways when innocuous antigen is inhaled prior to sensitization. Instead a modest reduction in tolerance is observed when PGI_2 signaling through the IP receptor is lost. Taken together, the work presented in this dissertation suggests that PGI_2, rather than PGE_2, limits atopic immune responses in the respiratory tract.

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129	129S6/SvEv
AA	Arachidonic acid
AERD	Aspirin-exacerbated respiratory disease
Alum	Aluminum hydroxide
AM	Alveolar macrophage
AHR	Airway hyperresponsiveness
APC	Antigen presenting cell
ASM	Airway smooth muscle
B6	C57BL/6
BALF	Bronchoalveolar lavage fluid
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
$cPLA_2$	Cytosolic phospholipase A_2
COX	Cyclooxygenase
CRTH2	Chemoattractant receptor-homologous molecule expressed on T_H2 cells
DC	Dendritic cell
DO11.10	OVA-specific TCR (BALB/c)
EP	E prostanoid
EPO	Eosinophil peroxidase
FcεRI	High-affinity IgE receptor
FoxP3	Forkhead Box P 3
GATA3	GATA-binding protein 3

GI	Gastrointestinal
HDM	House dust mite
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IL	Interleukin
Ilo	Iloprost
i.p.	Intraperitoneal
IP	I prostanoid
i.t.	Intratracheal
IT	Immunotherapy
KO	Knockout
Lck	Lymphocyte-specific protein tyrosine kinase
LMC	Lung mononuclear cell
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mDC	Myeloid dendritic cell
mPGES1	Microsomal PGE_2 synthase 1
mPGES2	Microsomal PGE_2 synthase 2
MUC	Mucin
NSAID	Non-steroidal anti-inflammatory drug
OT-II	Ova-specific TCR (C57BL/6)
OVA	Ovalbumin
pDC	Plasmacytoid dendritic cell

PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PKA	Protein kinase A
PM	Peritoneal Macrophage
PRR	Pattern recognition receptor
RBC	Red blood cell
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T_H1	T helper 1
T_H2	T helper 2
T_H17	T helper 17
T_{reg}	T regulatory
TCR	T cell antigen receptor
TLR	Toll like receptor
TGF-β	Transforming growth factor beta
TXA_2	Thromboxane
Veh	Vehicle

WT   Wildtype

CHAPTER I

INTRODUCTION

Allergy and Asthma Manifestations and Significance

The term "allergy" refers to an inappropriate adaptive immune response mounted against an innocuous antigen. The most common form of allergy, atopic allergy, effects more than 25% of the worldwide population and results from an overproduction of immunoglobulin E (IgE) antibody against a specific antigen. The development and severity of an allergic response are shaped by many factors including the concentration and type of allergen exposure, additional mediators an exposure occurs with, and an individual's genetic makeup. (1) Allergies can be local, affecting only a specific rgan, or systemic, resulting in anaphylaxis (2). Pollen, pet dander/saliva, occupational substances nd dust mites represent common triggers of atopic allergy.

Atopy is a risk factor for asthma and 90% of asthmatics are considered atopic. However, asthma is a heterogeneous disease and asthmatic attacks can result from multiple factors beyond allergens including: air pollution, aspirin, and excercise (3). Asthma is defined as a chronic inflammatory disease of the airways accompanied by reversible airway obstruction and airway hyperresponsiveness (AHR). Clinical manifestations of an acute asthmatic response include the presence of a cough, chest tightness, wheezing and loss of breath. The severity of an attack varies and can range from a mild cough to respiratory arrest and death. Frequent or prolonged exposures to an allergen may result in chronic asthma associated with airway remodeling. A diagnosis of asthma can be confirmed by the administration of a pulmonary function test. This test is designed to identify AHR following challenge of the airways with a common bronchoconstrictor, such as methacholine,

which is reversible upon treatment with a bronchodilator. (1) β_2-agonists (bronchodilating agents) and glucocorticoids (drugs that ameliorate inflammation by inhibiting key transcription factors) are the two most common forms of treatment for asthmatic patients. These treatments are effective in 90-95% of patients; however, they do not provide long term prevention of lung function decline. (4)

The prevalence of allergy and asthma has escalated dramatically in the past decades, particularly in westernized countries, affecting an estimated 300 million individuals worldwide (5). In the United States alone, approximately 30 million adults and 10 million children report that they've experienced an asthmatic episode (6). In 2005, a survey exploring the burden of asthma in adults in the U.S reported that, on average, asthmatics were more likely to be unemployed, spend illness-related days away from work bedridden, have greater limitations on activities, and accrue almost $2000 more in medical expenditures, compared to healthy individuals. The estimated national medical expenditure for adult-related asthma was $18 billion dollars annually. (7) In 2007, this affliction resulted in 1.75 million emergency room visits and 3,447 deaths (8).

Speculation into the source of the inflated prevalence of disease occurring over recent decades in westernized countries has led to the theory that urbanization contributes significantly to asthma and related allergies. Proponents for this school of thought site epidemiological studies as evidence. For instance, in Africa, the prevalence of asthma is the highest in South Africa (8.1%), the nation considered to be the most developed on the continent (5).

The Hygiene Hypothesis

Ninety percent of asthmatics are diagnosed by the age of 6, suggesting that events occurring early in life influence allergen sensitization (9). The "hygiene hypothesis," originally proposed in 1989 (10), postulates that exposure to particles ubiquitous in rural environments, such as lipopolysaccharide (LPS) or animal dander, during youth reduces one's risk of developing allergy

later in life. This idea is substantiated by large epidemiological studies reporting that children raised on farmland are less prone to allergies than children brought up in urban environments (11-13). Additionally, older siblings (14) and early day care attendance (15), factors thought to elevate antigen exposure in youth, have been inversely correlated with the development of allergy.

Mechanistically, the hygiene hypothesis asserts that activation of the innate immune system through stimulation of pattern recognition receptors (PRRs), such as the toll like receptor (TLR) family, by ubiquitous environmental antigens during early childhood may cause the adaptive immune system to favor T helper 1 (T_H1) or T regulatory (T_{reg}) cell responses when encountering a novel antigen. Thus, exposure to unrecognized innocuous allergens at later time points does not prime the immune system to produce IgE, typical of T helper 2 (T_H2) cell-mediated responses. Multiple human studies have noted a correlation between allergy incidence and certain TLR polymorphisms (16, 17) and *in vivo* animal studies have demonstrated that activation of the innate immune system can inhibit subsequent allergic responses to novel antigens (18-20). Blumer and colleagues reported that mice exposed prenatally to LPS produced elevated levels of interferon-γ (IFN-γ), a characteristic T_H1-cytokine, at birth and exhibited an attenuated allergic response to ovalbumin (OVA) antigen (21). The hygiene hypothesis suggests that as countries become more westernized, their aseptic practices improve and early exposure to ubiquitous factors that prime the innate immune system declines. As a result, antigen exposure results in T_H2 responses in susceptible individuals.

Environmental Pollutants

The hygiene hypothesis cannot account for the disparities observed in allergy prevalence existing between socioeconomic classes and racial/ethnic groups. Studies show that poor inner-city minorities have an elevated risk for developing allergy and asthma (22-24). For instance, compared to Caucasians, one report observed that African-Americans were 3 times more likely to require

hospitalization for asthma-related illness in 2005 (25). This trend may arise as a consequence of elevated levels of air pollutants found in inner-city areas (26-29). Diesel fuel (30), nitric dioxide (31), ozone (31, 32), and cigarette smoke (33, 34) are linked to elevated allergy incidence in children. Additionally, proximity to a major road, where concentrations of diesel fuel and nitrogen dioxide are high, is viewed as a risk factor for childhood asthma (35-38). Experimental evidence suggests that inhalation of these pollutants, on their own, is sufficient to alter lung function in humans and rodents (39-41). These toxins can also function as adjuvants to initiate adverse immune responses to innocuous particles (42-45).

Air pollutants can both augment IgE production from B cells (46, 47) and enhance the expression of T_H2 cytokines (42, 48). Further, these irritants are potent inducers of reactive oxygen species (ROS) (49, 50), created only at minimal levels during homeostasis, which can react with proteins, DNA, and lipids to cause cellular damage and oxidative stress (51). Evidence suggests that the creation of ROS by air pollutants may, in part, mediate both their independent actions and adjuvant properties on lung function. For instance, the adverse actions of pollutants in the airways can be ameliorated by the administration of antioxidants (38, 52).

Genetics

While the environment and living conditions a person is exposed to can clearly shape allergic and asthmatic reactions, these responses are additionally influenced by an individual's genetic makeup. This is highlighted by studies showing that asthma concordance is higher among monozygotic twins than dizygotic twins (53, 54). Linkage analysis studies represent an early approach for the identification of causative genes. These studies rely on information generated from families in which multiple members are affected. DNA from these subjects is analyzed at markers spanning the genome to identify regions shared by affected individuals at a higher than expected rate.

One advantage to this technique is that it has the potential to reveal novel candidate genes, given that no prior hypothesis is necessary to generate a linkage analysis report. For instance, this method identified a link to the gene, a disintegrin and metalloproteinase 33 (*ADAM33*) (55), uncovering a previously unrecognized pathway in allergy.

The complex nature of asthma and allergies limits the usefulness of linkage analysis. Studies utilizing this approach typically identify large regions containing multiple susceptibility loci, each with only minimal impact on the risk for disease. For instance, studies have consistently shown asthma and allergy linkage to the region 5q31-33, a stretch of DNA containing the genetic information for multiple genes associated with allergy and asthma including interleukin (IL)-4 and IL-13. (56)

Another method for identification of genetic loci linked to atopic disease is the candidate-gene association approach. This methodology relies on DNA from unrelated asthmatics and healthy controls to characterize allele variants of a hypothesized susceptibility locus linked to disease. These studies allow for a larger sample population and therefore can identify variants that may cause only modest phenotypic alterations. However, because unrelated individuals will have a smaller percentage of shared markers at any particular region, this type of scan requires higher resolution than family-based studies. Hypothesis-driven studies which analyze single nucleotide polymorphism (SNP) variants of genes involved in allergy pathways have been a useful technique in confirming most of the genes currently known to affect susceptibility. (56)

Genome wide association studies are widely being used for linkage identification in allergy and asthma (57). Utilization of this approach has the advantage of analyzing SNPs located across the entire genome to identify the variants most often inherited by individuals symptomatic for disease. The availability of the HapMap resource, which identifies common haplotype variations in 4 large

populations, the density of SNPs now available across the human genome, and the availability of

DNA from large populations makes these studies feasible for complex disorders such as asthma (58).

Respiratory Tract Mucosal Tolerance

Normal respiration exposes the mucosal surface of the human airways to 10,000 liters of air

every day. This air carries within it a broad range of foreign particles, the majority of which are

innocuous plant and animal-derived antigens ubiquitous in the environment. With each breath, the

immune system must accurately interpret the pathogenicity of the material it is exposed to in order to

combat and expel particulates which pose a risk to the host while avoiding unwarranted immune

responses directed against harmless environmental constituents. (59) Some of the innocuous particles

that the immune system encounters, including pet dander, mold, and pollen, provoke allergic

responses in susceptible individuals when the immune system mistakes them as dangerous and

sensitizes the adaptive immune system. The majority of individuals, however, experience no adverse

response to the innocuous particles present in the air, instead developing a state of immune

unresponsiveness, or tolerance, when exposure to these environmental agents occurs. Research

indicates that a complex system of checks and balances exists at mucosal surfaces to expel particles,

sample antigens, and accurately decipher harmful from innocuous antigens. These mechanisms

involve the coordinated actions of multiple cell types and mediators. Rodents have been used to

explore this phenomenon. As an example, allergic responses resulting from immunization and

challenge are significantly attenuated when antigen is introduced to the airways prior to sensitization

(60-62).

Epithelial Cells: Physical Barrier Protection and Immune Activation

The airway mucosa is covered by an epithelial barrier consisting of ciliated cells and

secretory cells that contribute to mucociliary clearance in the lumen. Secretory cells, comprised of

goblet, clara, and serous cells, release mucus and surfactant into the airways. Submucosal glands also contribute to these secretions in the large airways. Water is the main constituent of mucus, making up 97% of this gelatinous substance. The remaining 3% consists of mucins, proteins, salts, lipids, and cellular debris. Mucins are complex glycoproteins responsible for the viscoelastic properties of mucous secretions. In the airways, the predominant mucins (MUCs) are MUC5AC and MUC5B. These agents enhance trapping of airborne particulates entering the respiratory tract. Mucus is propelled proximally by the actions of cilia to remove inhaled particles. Independent of ciliary-mediated clearance, expulsion of particulates from the respiratory tract can be accomplished by a cough reflex (63). In the distal airways, clara cells release surfactant which creates a film barrier over the airways, further separating allergens from epithelial cells and aiding ciliary movement (64). Additionally, epithelial cells release peptidases, protease inhibitors, and antimicrobial products into the lumen that can directly lyse pathogens. Among these factors, lysozyme, lactoferrin, and secretory leukocyte proteinase inhibitor (SLPI) are present in the highest concentrations (65)

Based on molecular weight, adjacent epithelial cells form tight junctions which serve to exclude particles evading mucociliary clearance mechanisms. These junctions, made up of interacting proteins and receptors, including zona occludens 1-3, occludins, and claudins, provide a physical barrier between cells designed to regulate the passage of material through the paracellular space. Proteases are capable of breaching the epithelium by cleaving the proteins contributing to tight junctions (66, 67)

Beyond representing a physical barrier utilized to separate the external environment from one's internal organs, epithelial cells are proficient in identifying and responding to particulates. Cells of the epithelium can recognize patterns common to many pathogens through PRRs including: TLRs, nucleotide-binding oligomerization domain (NOD) protein like receptors (NLRs), and protease

activated receptors (PARs) which activate the innate immune system. In response to PRR binding, epithelial cells can release chemokines that attract the appropriate leukocytes to their surface, in addition to releasing cytokines, lipid mediators, reactive oxygen and nitrogen species, and growth factors. The combination of factors released from epithelial cells shapes the microenvironment of responding leukocytes and mediates their response. Supernatant from both human and murine derived airway epithelial cells are capable of inhibiting the maturation of dendritic cells (68); however, some studies emphasize a necessity for physical contact with epithelial cells for immune mediation (69). While the default signals released by epithelial cells may inhibit inflammatory immune reactions during homeostasis, activated epithelial cells can mobilize effector cells (70). For instance, evidence suggests that TLR4 signaling on structural cells is critical for the priming of T_H2 responses to the house dust mite (HDM) antigen (71)

Dendritic Cells

Dendritic cells (DCs), given their name based on their long branching arms which resemble dendrites of the nervous system, uptake antigen and present it to pools of naïve T cells residing in local lymph nodes. DCs exist in a dense network beneath the epithelium of the airways, making them an important antigen presenting cell (APC) of the respiratory tract. In rats, research demonstrates that several hundred DCs reside per millimeter squared in the upper airways; however these cells become less frequent in the lower airways. (72) Beyond internalizing antigen that breaches the epithelial barrier, evidence indicates that DCs can additionally form tight junctions with epithelial cells, extending their dendrites into the lumen to directly sample inhaled particulates (73). How mucosal DCs are able to discriminate between pathogenic and non-pathogenic antigens is unclear; however, like epithelial cells, DCs express several classes of PRRs and costimulation of these receptors in the context of the mediators released by the epithelial cells may determine how an antigen is received (74).

DCs surveying the antigenic content of the airways exist in an immature state (75). This implies that these cells are highly specialized to sample and process antigen but are inefficient at presenting these particles to naïve T cells. Once a particle is taken up and processed by a DC, this APC no longer acquires new antigen, instead undergoing a maturation process involving the upregulation of molecules which aide in presentation including major histocompatibility complex (MHC) II and co-stimulatory molecules, such as cluster of differentiation (CD) 80 and CD86. As part of this maturation, DCs become attracted to specific chemokines that promote their migration to local lymph nodes, rich in naïve T cells (76). The cytokines and mediators released by DCs when they encounter these target T cells shape the progression of the immune response. T cells utilize their T cell antigen receptor (TCR) to interact with DCs and sample antigen expressed as MHC-peptide complexes. Whereas antigen bound to MHC I attracts $CD8^+$ cells, allergen presented by MCH II promotes the differentiation and expansion of the $CD4^+$ T cell subset. $CD4^+$ cells can differentiate into at least 4 types of effector cells depending on the context under which they are stimulated **(Fig 1.1)**.

T-cell priming by APCs appears to be a necessary step in the establishment of airway tolerance. Transfer of pulmonary DCs exposed to inhaled antigen confers immune unresponsiveness in recipients, however when ICOS-ligand, necessary for T cell interactions, is neutralized on transferred DCs, they lose their ability to induce tolerance (77, 78). Further, neutralizing CD86 at the time of antigen inhalation also abrogates immune tolerance (79). DCs may promote this process by stimulating naïve cells to become T regulatory (T_{reg}) cells, cells which release the anti-inflammatory cytokines transforming growth factor-beta (TGF-β) or IL-10 (78, 80). It is not clear what differences exist in DCs that allow them to establish tolerance rather than immunogenicity. One theory asserts that when an antigen is internalized, DCs require a second stimulation, through PRR activation, that alerts them to danger. Without this costimulation, DC maturation is incomplete and interactions with

T cells result in immune unresponsiveness. One group observed that although tolerogenic antigen-loaded DCs had normal expression of costimulatory molecules, MHC II levels were reduced on these APCs (77). The ability to promote tolerance may also depend on the class of DC responsible for antigen presentation. Plasmacytoid (p)DCs may promote airway tolerance while presentation by myeloid (m)DCs often elicits allergy (81).

Alveolar Macrophages

Alveolar macrophages (AMs) dominate the alveolar space and conducting airways, constituting 90% of the leukocytes present during homeostasis (82). The main function of AMs during homeostasis appears to involve phagocytosis of innocuous particles in order to prevent the uptake and presentation of these antigens to the adaptive immune system by DCs (83). This theory arises from the observation that despite the fact that AMs internalize the majority of inhaled antigen and inherently possess the ability to present it, they do not migrate to local lymph nodes following uptake. In addition, elimination of AMs results in elevated response to antigens in the airways and increased APC presence in draining lymph nodes (84, 85). Beyond sequestering antigen, AMs may promote airway tolerance by suppressing DC maturation and inhibiting the localization of DCs to the airway (85, 86). AMs can also release cytokines with anti-inflammatory properties including IL-10 (87).

Regulatory T cells

While prevention of autoimmune diseases involves control of autoreactive T cells by several mechanisms including anergy and deletion, research exploring the generation and maintenance of respiratory tolerance to foreign innocuous antigens focuses on the generation of suppressor T_{reg} cell populations. These cells actively inhibit responses by effector T cells. T_{reg} cells constitutively

express CD25 and are characterized as either naturally occurring or adaptive. Naturally occurring T_{reg} cells, produced in the thymus, constitute 5-10% of the $CD4^+$ T cells present during homeostasis in humans and mice. *In vitro* work reveals that these cells can inhibit the proliferation of effector T cells (88). The significance of naturally occurring T_{reg} cells to immune unresponsiveness is highlighted by the discovery that neonatal thymectomy causes organ-specific autoimmune pathology in mice, a condition preventable by adoptive transfer of $CD4^+ CD25^+$ T cells (89). Unlike naturally occurring T_{reg} cells which are produced as a normal branch of the T cell repertoire, adaptive T_{reg} cells originate from $CD4^+ CD25^-$ T cells that are converted to $CD25^+$ suppressive cells as a result of signals received in the periphery.

The generation of most T_{reg} cells, both naturally occurring and adaptive, appears to rely on actions of the transcription factor forkhead box P3 (Foxp3). The significance of Foxp3 is underscored by the discovery that rodents lacking a functional copy of this transcription factor develop a lethal immune syndrome that is inhibited by adoptive transfer of $CD4^+ CD25^+$ T_{reg} cells (90, 91). Additionally, mutations in the human gene coding Foxp3 are responsible for immunodysregulation, polyendocrinopathy enteropathy, X-linked, a disease associated with autoimmune endocrine pathology and allergic manifestations (92). Foxp3 can also convert effector T cells into suppressive T_{reg} cells. Addition of this factor to cultures of $CD25^-$ effector cells converts them into T_{reg} cells (91) and conversion of T_{reg} cells in the presence of TGF-β involves activation of Foxp3 (93). However, not all T_{reg} cells are reliant on Foxp3 expression. A subset of T_{reg} cells which produce high levels of IL-10,Tr1 cells, do not appear to express Foxp3, suggesting that at least one alternate pathway can be utilized to generate these cells (94).

While a genetic loss of T_{reg} cells clearly establishes a link between this cell type and the prevention of autoimmune diseases, the importance of these cells to the generation and maintenance

of immune tolerance in the respiratory tract is also documented. For instance, adoptive transfer of antigen-specific $CD4^+ CD25^+$ cells ameliorates acute allergic lung allergy in recipient animals (95) and prevents airway remodeling in a chronic allergy model (96). Conversely, depletion of these cells prior to allergen challenge results in exacerbated lung inflammation (97). The mechanism utilized in this system to promote airway tolerance remains unclear. Multiple studies have emphasized a dependency on cell to cell contact with effector T cells for T_{reg}-mediated suppression (98, 99).

Alternatively, T_{reg} cells may induce immunosuppression through IL-10-dependent actions. Indeed, the significance of IL-10 in this process is suggested by multiple works modeling respiratory tract tolerance (100-102) although it is unclear whether the important source of this cytokine is the T_{reg} cells or the effector T cells. Recipients transferred with T cells depleted of $CD25^+$ populations experience exacerbated pulmonary allergy that is surprisingly accompanied by a decrease in bronchoalveolar lavage fluid (BALF) T_H2 cytokine levels, including IL-10. This data suggests that T_{reg} cells may not downregulate the actions of T_H2 effector cells but rather enhance production of anti-inflammatory cytokines from these cells (103). In agreement with this, Kearley and colleagues reported that transfer of immune suppression by T_{reg} cells was dependent on production of IL-10 from effector $CD4^+$ T cells (95). TGF-β, another anti-inflammatory cytokine, may also contribute to T_{reg}-mediated airway tolerance. However, although production of this factor has been identified as an essential step in some models (80), it is generally believed to be more critical to the establishment of tolerance in the gastrointestinal (GI) tract (104).

Immunotherapy

Exploitation of the mechanisms promoting immune tolerance represents a promising approach for the treatment and prevention of allergic responses. Antigen-specific immunotherapy (IT) utilizes incremental delivery, typically through subcutaneous administration, of a specific antigen

in an attempt to suppress allergy symptoms. The benefits of this system are highlighted by studies

showing recipients have long-term remission upon allergen challenge (105), reduced sensitivity to

novel antigens (106), and prevention of disease progression to asthma (107). T_{reg} cell generation is

thought to account for the success of this treatment regimen. Indeed, studies have measured elevated

$CD4^+ CD25^+$ levels and enhanced expression of IL-10 and TGF-β in individuals receiving IT (108,

109). Further, a murine model utilizing this system shows long-term abatement of allergen-specific

IgE production and T_H2 responses, potentiated by IL-10-producing T_{reg} cells (110). This therapy is

not without its caveats. The efficacy of this treatment is not as pervasive in asthma as it is in allergy

(111) and widespread use of subcutaneous treatment is limited by concerns that such a system can

trigger anaphylaxis. Research is currently underway to develop a safer mode for IT delivery and may

involve the use of adjuvants to magnify tolerogenic properties of APCs. Studies evaluating the safety

and efficacy of sublingual delivery (112) and peptide IT (113) are being conducted.

Atopic Allergic Sensitization Phase

Allergic responses occur in two phases, a sensitization phase and an effector phase. The early

events precipitating an allergic reaction occur in much the same manner as those which precede

respiratory tract immune tolerance. However, under these circumstances, the microenvironment

established at the airway epithelium and the signals received by DCs support antigen sensitization

rather than tolerance **(Fig 1.2)**. Following antigen uptake, DCs become activated, maturing fully, and

presentation of antigen to naïve T cells occurs in the presence of IL-4 and IL-2 rather than IL-10.

Expression of IL-4 promotes the expansion of T_H2 cells from naïve $CD4^+$ populations (114, 115). IL-

4 upregulates expression of GATA-binding protein 3 (GATA3), the master regulator of T_H2 cell

differentiation, by inducing phosphorylation of signal transducer and activator of transcription

(STAT) 6 (116). STAT6 expression, which also appears to be essential for T_H2 cell differentiation

(117), can be activated *in vivo* by IL-2, IL-7 or thymic stromal lymphopoietin (118). Costimulatory

signals, including interaction between CD28 on T cells and CD86 on DCs, enhance this differentiation (119).

The differentiation and expansion of antigen-specific T_H2 cells is followed by the activation of naive B cells into mature IgE producing plasma cells. This process involves interactions with CD40L and CD23 on T cells with their respective receptors on B cells. Following these interactions, B cells undergo class switch recombination to halt production of IgM in favor of antigen specific IgE. IL-4 and IL-13, cytokines released from activated T_H2 cells, enhance the efficiency of this process (120, 121). After its production, IgE diffuses out of the cell and circulates through the blood, eventually binding to the carboxylic fragment of surface high-affinity IgE receptors (FcεRI) expressed at high levels predominately on mast cells and basophils.

Allergic Effector Phase

T_H2 Type Cytokines

The effector phase of an allergic airway response is initialized upon exposure to an antigen in a sensitized organism **(Fig 1.3)**. Antigen taken up by DCs in sensitized hosts can be presented to antigen-specific T cells circulating the mucosal system directly at the airway surface (122). Once activated, these effector T_H2 cells release a unique profile of cytokines transcribed from a region on human chromosome 5. This profile includes the production of IL-4, IL-13, IL-5, and IL-9. Although T_H2 cells are believed to be the main source of these cytokines during allergic responses, additional cell types, including mast cells and eosinophils contribute to their expression (123). Adoptive transfer of allergen-specific T_H2 cells into naïve animals prior to antigen challenge can initiate eosinophil recruitment, increased mucus production, and AHR (124, 125), underscoring the critical contribution of these cells and their cytokines to lung allergy.

IL-4 and IL-13 share the IL-4R-α receptor, providing these cytokines with overlapping functions. Polymorphisms in this shared receptor are linked to atopy and asthma in humans (126, 127) While each of these factors contributes to both the sensitization and effector arms of allergy (128), the actions of IL-4 appear to dominate during antigen sensitization. Using a neutralizing antibody, Coyle et al. demonstrated that loss of IL-4 during atopic sensitization prevents a subsequent allergic response from occurring. However, when the same antibody is administered to sensitized animals prior to challenge, blockade of IL-4 does not prevent allergy (129). While not critical, IL-4 does contribute to the effector phase of allergy in several ways. IL-4 enhances the expression of adherence factors for leukocytes on endothelial cells at sites of inflammation (130) and may contribute to eosinophilia (131).

Conversely, the contributions of IL-13 to allergy are more prolific during the effector phase of an allergic reaction. Neutralizing IL-13 during allergen challenge is sufficient to prevent many allergic symptoms, especially mucus production and AHR in immunized mice and animals with a genetic loss of IL-13 do not develop mucus production or AHR (131). Conversely, administration of IL-13, exogenously or through transgenic overexpression, induces AHR, eosinophilia, IgE production, mucus secretions, and subepithelial fibrosis (132-134). IL-13, like IL-4, is also implicated in the recruitment of inflammatory cells to the site of inflammation (135, 136).

In vitro and *ex vivo* experimentation has highlighted the role of IL-5 in both the differentiation and survival of eosinophils (137-140). Further, a role for this factor in eosinophil recruitment is demonstrated by an *in vivo* study in which administration of IL-5 reduces eosinophil numbers in the bone marrow while concomitantly increasing levels of circulating eosinophils (141). Additionally, neutralization of IL-5 in rodents results in a significant attenuation of eosinophilia (142-144) and a complete loss of eosinophilia is observed in IL-5 deficient rodents (145) Moreover,

transgenic expression of IL-5 causes spontaneous development of eosinophilia (146, 147). In asthmatic individuals, administration of a single dose of neutralizing antibody against IL-5 effectively reduces blood eosinophil levels for up to 16 weeks (148).

IL-9 was initially recognized as a T_H2 cytokine that contributes to T cell and mast cell proliferation (149, 150); however, further analysis suggests that IL-9 serves as a growth factor for B cells, as well (151). Interest in the contribution of this cytokine to lung allergy arose following the discovery that a polymorphism present in certain breeds of mice, which conveys airway hyporesponsiveness, maps to a locus containing the IL-9 gene. Measurements of this cytokine are reduced in these hyporesponsive animals (152). However, the results of *in vivo* analysis are conflicted regarding the extent to which IL-9 alters lung allergy. Systemic transgenic expression causes mastocytosis in multiple compartments, including in the airways (153) and mice with lung-specific expression exhibit eosinophilia, mastocytosis, mucus accumulation, subepithelial fibrosis, and AHR (154, 155). However, while IL-9-deficient mice have significantly attenuated goblet cell hyperplasia and mastocytosis, in a pulmonary granuloma model, the endogenous development of T, B, or mast cell population are unaffected (156). Further, IL-9-deficient rodents had no significant reductions in AHR, eosinophilia or mucus secretion following sensitization with OVA/alum and subsequent challenge (157). Mast cells do not serve a critical function in OVA/alum sensitization models of allergy (158) which may explain why no phenotype was observed in these IL-9 -/- mice. Collectively, these results may indicate that the major function of IL-9 is to mediate mast cell dependent pathology.

Mast Cells

In addition to the IL-9 released by T_H2 cells, stem cell factor released by epithelial cells can recruit mast cells to the site of inflammation (159). In the airways, antigen binding to IgE/FcεRI

complexes aggravates these structures, triggering mast cell degranulation. This process can transpire within minutes of antigen recognition (123). Degranulation involves the release of preformed mediators from mast cell granules, a process involving fusion of the granule's cytoplasm membrane with the plasma membrane of the mast cell (160). The factors released from mast cells during degranulation include vasoactive amines, serine proteases, proteoglycans, and some cytokines. This degranulation exacerbates and contributes to the allergic responses mediated by T_H2 cell cytokines. Additionally, liberation of these mediators can stimulate sensory nerves and induce coughing (161). Beyond the release of these preformed factors, mast cells can de novo synthesize lipid mediators and transcribe cytokines and chemokines; however, this process does not occur as rapidly as degranulation (162).

Eosinophils

Eosinophils are thought to be influential in mediating the late phase events of an allergic response. These events are similar in nature to those observed during the early phase; however late phase reactions typically do not occur until several hours after allergen exposure, peaking 6-9 hours after challenge and resolving 1-2 days post-exposure (162). Eosinophils represent bone-marrow derived cells recruited to the lung by chemokines released by mast cells, T_H2 cells, epithelial cells, and endothelial cells. In addition to IL-5, eotaxin family members produced mainly by epithelial cells (163) have been identified as eosinophil-specific chemoattractants. The importance of eotaxin to this process is highlighted by data demonstrating that administration of eotaxin blocking antibody to immunized rodents significantly attenuates eosinophil accumulation in the BALF and lung following antigen challenge (164, 165).

Similar to mast cells, eosinophils are granular cells that release preformed factors which contribute to allergy manifestations. Like mast cells, these cells can also de novo synthesize and

release lipid mediators and cytokines. The granular content of eosinophils is comprised of 4 major cationic proteins: major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase (EPO), all of which have toxic effects on cells (166). The exact function of eosinophils in lung inflammation has not fully elucidated. Two murine models utilizing targeted ablation of eosinophils provide evidence that eosinophils contribute to epithelial cell hypertrophy, mucus accumulation and AHR in episodes of acute allergy (167, 168). However, data generated using a third method of targeted ablation argues that these features can occur independently of eosinophilia (169). Additional studies suggest that eosinophil granular proteins can damage epithelial cells, reducing their barrier function, (170, 171) and can create ROS and nitrogen species (172-174).

When exposure to an antigen is frequent or prolonged, chronic inflammation and airway remodeling can occur, causing structural changes at the site of inflammation resulting from an imbalance between tissue repair and regeneration mechanisms. Airway remodeling is associated with deposition of extracellular matrix proteins, goblet cell hyperplasia, alterations in fibroblasts, proliferation of airway smooth muscle (ASM) cells, and vascular changes within the parenchyma (162). Studies utilizing targeted ablation of eosinophils suggest that eosinophilia can contribute to this process by augmenting subepithelial fibrosis and ASM hyperplasia (169, 175), however studies are conflicted on the extent to which TGF-β production, known to enhance airway remodeling, by eosinophils contributes to this phenotype. In addition to TGF-β, eosinophils also release multiple growth mediators which may promote airway remodeling (176). The importance of eosinophils to this phenomenon is supported by clinical data demonstrating that administration of anti-IL-5 reduces extracellular matrix protein deposition (177).

Prostanoid Synthesis

Prostanoids represent an important class of lipid mediator de novo synthesized and released by both leukocytes and structural cells involved in lung allergy. The production of these bioactive oxygenated C_{20} fatty acid mediators occurs through the metabolism of arachidonic acid (AA) (**Fig 1.3**). In response to a broad range of stimuli, cytosolic phospholipase A_2 ($cPLA_2$) and other phospholipases release AA, an unsaturated fatty acid, from membrane phospholipids (178). AA is initially processed into two intermediate forms, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by the actions of prostaglandin-endoperoxide synthases (cyclooxygenase, COX), colloquially known as COX-1 and COX-2. COXs are bifunctional enzymes that perform peroxidase activities in addition to their cyclooxygenase capacity. These isoforms share 60-65% sequence identity but are the products of unique genes. While enzymatic activity by COX isoforms is typically exerted through the formation of homodimers, the formation of COX-1/COX-2 heterodimers has also been described in cells where coexpression occurs (179). COX-1 is generally believed to produce most basal level prostanoids, given that it is constitutively expressed in many cell types. COX-2 expression, conversely, is absent under homeostatic conditions in most cells and instead, is induced by a diverse range of stimuli including inflammatory mediators (180).

The concept that COX-1 synthesizes all basal level prostanoids while induced expression relies solely on COX-2 is an oversimplified, antiquated view. Instead, these enzymes can each contribute to the production of autoregulatory, as well as inflammatory prostanoids. Constitutive expression of COX-2 is observed in multiple tissues of both human and rodent origin (181-186) and COX-2 deficient animals spontaneously develop severe renal nephropathy (187). Conversely, COX-1 expression is elevated in the lactating murine mammary gland and in the inflamed guinea pig gall bladder (188, 189). Further, while a genetic loss of COX-2 has no consequence on ear edema induced by AA administration, inflammation is attenuated in the absence of COX-1, implicating this

isoform in prostanoid synthesis in this model (187, 190). Experimental evidence suggests that COX-2 actions dominate when levels of AA are below 2.5uM, while levels of AA reaching 10uM and higher result in predominately COX-1 enzymatic actions (191). These findings may indicate that immediate responses to inflammatory signals are mediated by COX-1, prior to COX-2 upregulation. This idea is supported by a human-based study reporting that early prostanoid production following LPS administration is dependent on COX-1 while expression of COX-2 is not measured until an hour and a half after endotoxin exposure (192).

Following the conversion of AA by COX enzymes to PGH_2, product-specific synthases complete the synthesis into five bioactive prostanoids consisting of prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane (TXA_2). These prostanoids have autocrine/paracrine functions and mediate their actions through the selective binding of distinct but cognate rhodopsin-like 7-transmembrane-spanning G-protein coupled receptors. These receptors activate a range of secondary intracellular signaling pathways to exert their downstream affects.

The important role of COX-generated mediators in the regulation of inflammation, fever and pain, is highlighted by the clinical efficacy of non-steroidal anti-inflammatory drugs (NSAIDs), common analgesics that prevent COX enzymatic activity through competitive inhibition of active-site binding (193). However, widespread use of these drugs can potentiate gastric maladies, further illustrating the significant role of prostanoids in tissue maintenance, as well. These toxic side effects are attributed to a loss of gastroprotective COX-1 derived prostanoids and resulted in the development of COX-2 specific inhibitors, coxibs. While utilization of these products reduces GI tract toxicities, coxib usage is linked to adverse cardiovascular events, emphasizing that both isoforms of the COX enzyme participate in maintaining homeostasis. (194)

Prostaglandin E$_2$

PGE$_2$ is the most ubiquitously expressed prostanoid in the body. Initially, the isomerization of this enzyme from PGH$_2$ was attributed to three PGE$_2$ specific synthases: microsomal PGE$_2$ synthases (mPGES) 1 and 2, as well as cytosolic PGE$_2$ synthase (cPGES). The first synthase to be described, mPGES1, was originally isolated from bovine and sheep vesicular glands and found to be dependent on glutathione as a cofactor (195, 196). The human form of this synthase was later purified in1999 (197). Expression of mPGES1 has been demonstrated at varying levels in many tissues and cell types (197). Similar to COX-2, expression of mPGES1 increases dramatically in response to inflammatory stimuli including LPS and IL-1β, however coupling of mPGES1 with COX-1 in the production of PGE$_2$ has also been observed (189, 198). The *in vivo* contribution of this synthase was originally described by Trebino et al. who demonstrated that mice lacking mPGES1 synthase have reduced arthritis related inflammation and have attenuated pain responses resulting from deficits in PGE$_2$ production (199). Mice deficient in mPGES1 also show reduced angiogenesis (200).

A second proposed glutathione-dependent PGE$_2$ synthase, cPGES, was purified from the rat brain in 2000 and is identical to p23, a chaperone that binds heat shock protein-90. Initial *in vitro* analysis of this factor suggested that cPGES couples with COX-1 to produce PGE$_2$. In support of this, constitutive expression of cPGES was observed in multiple tissues although elevated expression was also measured in brain tissue following LPS exposure (198). However, the generation of cPGES-deficient mice does not support a role for this synthase in the *in vivo* production of PGE$_2$ (201). While levels of this prostanoid are attenuated in null animals, COX levels and the levels of additional prostanoids are also reduced, suggesting a more pleiotropic function for this factor in the prostanoid pathway (201).

A third synthase, mPGES2, was isolated from bovine heart tissue and shown *in vitro* to convert PGH_2 to PGE_2 independently of glutathione (202). Although the name of this synthase arose from its membrane location at discovery, further analysis identified that mPGES2 is golgi-associated at synthesis and the proteolytic removal of its N-terminal hydrophobic domain results in a mature cytosolic mPGES2 with expression in many organs (203). *In vitro*, coupling of mPGES2 is observed with both COX-1 and COX-2, however mPGES2 demonstrates a preference for COX-2 (203). In spite of this *in vitro* data, no phenotype or alterations in PGE_2 production are observed in genetically-engineered mPGES2- deficient animals, arguing that mPGES2 does not contribute to the *in vivo* production of this prostanoid (204).

Following synthesis, PGE_2 exerts pleiotropic autocrine and paracrine functions (205). Actions by this mediator occur through selective binding to four G-protein-coupled E prostanoid (EP) receptors: EP1-4. Binding of the G_q coupled EP_1 receptor results in an increase of intracellular Ca^{2+} levels while actions by the G_s coupled EP_2 and EP_4 receptors occur through elevation of intracellular cyclic adenosine monophosphate (cAMP). EP_3 is unique in that alternative splicing allows this receptor to bind to multiple G-coupled receptors and subsequently activate distinct downstream pathways. Depending on the variant, EP_3 can couple with the G_i, G_s, and G_q-protein receptors to reduce or enhance cAMP levels or elevate intracellular calcium, respectively, although coupling with G_i appears to predominate (206). PGE_2 is de novo synthesized, rather than stored, and is rapidly metabolized within minutes of its synthesis by the actions of 15-hydroxyprostaglandin dehydrogenase (PGDH) (207).

Owning to the fact that PGE_2 synthesis can be initialized by 2 cyclooxygenase isoforms and can then bind with high affinity to four distinct receptors, each with unique expression patterns throughout the body, it is not surprising that this prostanoid is implicated in many, sometimes

seemingly opposing functions in the body. While release of PGE_2 is a critical step in the maintenance of many biological processes, it is also implicated in inflammation: Indeed high levels of this prostanoid have been measured at sights of inflammation (208, 209) and injection of PGE_2 is sufficient to cause many of the cardinal symptoms associated with an acute inflammatory response (210). This mediator has been demonstrated to enhance vascular permeability and vasodilation, which give rise to the redness and swelling associated with inflammation. In a model of ear edema, mice lacking the EP_3 receptor have significantly reduced levels of edema and protein extravasation (211). Production of this prostanoid also increases hyperalgesia; animals missing key components of the PGE_2 pathway have attenuated pain responses (199, 212). PGE_2 is additionally implicated in the fever response (213).

PGE$_2$ plays a significant role in normal gastrointestinal processes where it is thought to promote a cytoprotective environment, emphasized by the fact that gastric toxicities associated with chronic NSAID usage are attributed to loss of mucosal surface integrity maintained by this mediator. Production of PGE_2 can reduce gastric acid and pepsin secretions (214), stimulate mucus (215) and bicarbonate secretions (216), enhance mucosal blood flow, and promote renewal of mucosal progenitor cells (217-219). Additionally this mediator aids motility by alternately stimulating contraction of longitudinal smooth muscle and relaxation of circular smooth muscle (220) However production of PGE_2 is also linked to several diseases in the gut including colorectal cancer (221, 222). Research indicates that PGE_2 can enhance cellular proliferation, inhibit cell apoptosis, promote angiogenesis, and suppress tumor destruction mediated by the immune system (223).

PGE$_2$ production is also vital for blood flow, regulation of salt and water excretion, vascular resistance, and renin secretion in the kidney during periods of physiological stress (224). In the reproductive organs, this prostanoid contributes to vasodilation and smooth muscle stimulation.

PGE$_2$ promotes erection, ejaculation, and sperm transport in males while playing a role in embryo implantation and uterine contractions during the initiation of labor in females. (225, 226) Further, PGE$_2$ production has been implicated in both bone formation, as well as bone resorption, and research shows that this mediator can increase bone cancer growth (227, 228).

Prostaglandin E$_2$ and Leukocytes

Beyond its ability to mediate inflammatory responses through the potent actions it exerts on structural cells, PGE$_2$, has significant effects on the leukocytes that shape adaptive immune reactions, beginning with DCs and macrophages. In addition to being a major source of PGE$_2$ synthesis in the immune system, these APCs also show expression, to varying levels, of EP receptors in both humans and mice and can respond to PGE$_2$ released by bronchial epithelial cells (229, 230). While exceptions have been noted (231), most evidence indicates that early exposure of immature DCs to PGE$_2$ promotes cell maturation, and may be critical for the upregulation of certain costimulatory molecules present on these APCs (232, 233). Additionally PGE$_2$ has been shown to augment expression of chemokine receptors, such as CCR7, that promote migration of DCs to local lymph nodes (229, 234). This finding is supported by *in vivo* research showing that maturation and migration of Langerhans cells is impaired in mice deficient of the EP$_4$ receptor (235). PGE$_2$ may also determine how antigen is presented to naïve T cells by DCs. Data indicates that DCs cultured *in vitro* with exogenous PGE$_2$ have enhanced production of IL-10 and reduced production of IL-12p70 to favor either a T$_H$2 or a T$_{reg}$ cell response, possibly depending on cofactors present in the milieu. DCs matured in the presence of PGE$_2$ can either promote or suppress T cell proliferation depending on additional factors present during maturation (229-231, 233, 236). In macrophages, PGE$_2$ can inhibit phagocytosis (237). Additionally, this prostanoid reduces production of TNF-α and IL-6 by these cells resulting in part

through upregulation of IL-10 (238, 239). PGE_2 also suppresses MHC expression on macrophages (240)

Beyond shaping the reactions of T cells indirectly through its actions on DCs, PGE_2 can act directly on $CD4^+$ T cells to shape immune responses. Early experiments conducted *in vitro* demonstrated that PGE_2 downregulates T cell proliferation and T_H1 cytokine production. In these works, the addition of exogenous PGE_2 to either committed or naïve $CD4^+$ T cell populations resulted in inhibition of IL-2 and IFN-γ production. T_H2 cytokines, including IL-4 and IL-5, were unaffected or slightly elevated. These affects are thought to be mediated by elevation of intracellular cAMP and subsequent activation of protein kinase A (PKA). (241-245) Indeed, suppression of responder cell proliferation by PGE_2 is attenuated in the mixed lymphocyte response when cells are obtained from mice deficient of the EP_2 or EP_4 receptor (246).

It is now clear that the actions of PGE_2 on T cells are more complex. While inhibition of T_H1 proliferation has largely been assigned to elevated cAMP levels, the downstream events were not well understood. T cell activation is primarily influenced by T cell receptor (TCR) antigen stimulation and one downstream effect of this stimulation is activation of lymphocyte-specific protein tyrosine kinase (Lck). Recent work demonstrates that elevation of cAMP and PKA mobilization by PGE_2 signaling leads to the phosphorylation and activation of C-terminal Src kinase. This factor in turn phosphorylates the C-terminal tyrosine of Lck and inactivates it. Thus, PGE_2 suppresses T cell proliferation by antagonizing activation of Lck (247). Given this knowledge, Yao and colleagues have recently demonstrated that low concentrations of exogenous PGE_2 enhance T_H1 differentiation in the presence of strong TCR stimulation (248). Similar to PGE_2-mediated cell suppression, this stimulatory effect is dependent on EP_2/EP_4 signaling; however, instead of PKA activation, the phosphoinositide-3-kinase pathway is critical.

Accumulating evidence suggests that PGE_2 can also mediate T_H17 responses. *In vitro* data generated in murine cells shows that addition of PGE_2 suppresses T_H17 differentiation from naïve T cells. However, this prostanoid stimulates production of IL-23, a cytokine essential for T_H17 cell expansion, from DCs in an EP_4 and cAMP-dependent manner. Further, although IL-23, on its own, is able to only modestly stimulate T_H17 cell proliferation, PGE_2 significantly enhances this expansion through EP_2/EP_4 and upregulation of cAMP and PKA (248). In humans, IL-1β also enhances T_H17 expansion and work shows that PGE_2 enhances this process (249, 250). Using mice to model contact hypersensitivity and multiple sclerosis, diseases mediated by both T_H1 and T_H17 cells, Yao et al. show *in vivo* that loss a of PGE_2 signaling through the EP_4 receptor attenuates disease. Further, *ex vivo* cells obtained from sensitized animals show reduced proliferation and produce attenuated levels of IFN-γ and IL-17 when cocultured with an EP_4 antagonist (248).

PGE_2 additionally mediates activities of T_{reg} cells. *In vitro* data conducted with T cells from both mice and humans demonstrates that incubation with this prostanoid enhances the suppressive capacities of $CD4^+CD25^+$ T cells and confers regulatory T cell functions on $CD4^+CD25^-$ cells. This is accomplished through upregulation of *FOXP3* mediated by EP_2/EP_4 binding (251-253) Further, adaptive T_{reg} cells have been shown *in vitro* to produce PGE_2, although this production is not observed by naturally occurring T_{reg} cells (253).

Other cell populations involved in atopic immune responses can also be influenced by PGE_2. Actions of PGE_2 on B cells are complex. *In vitro* data generated using murine B cells suggests that while exogenous PGE_2 enhances production of IgE from uncommitted B cells by promoting class switching to the ε transcript, it also suppresses maturation and proliferation of these cells. These functions are mediated through binding of EP_2/EP_4 and upregulation of cAMP (254-256). Other works have argued that PGE_2 can suppress IgE formation (257). Mast cells, which bind IgE, can also

be influenced by this prostanoid. Both *in vitro* and *in vivo* data generated in mice supports a role for this prostanoid in mast cell recruitment and degranulation through an EP_3 dependent mechanism which becomes more pronounced with age (258-260). These findings have been corroborated using human mast cells (261).

Prostacyclin

Prostacyclin (PGI_2) is another prostanoid produced downstream of COX signaling. Discovery of this prostanoid occurred in 1976 when Vane et al. isolated an enzyme from the aortas of sheep and pigs that was found to inhibit platelet aggregation (262). PGI synthase (PGIS) is responsible for the conversion of prostacyclin from the intermediate PGH_2. This synthase is a hemoprotein originally purified from bovine aorta in the early 1980's and is a cytochrome p450 (263). PGIS colocalizes with both COX-1 and COX-2 in the nuclear envelope and endoplasmic reticulum (264). Following its synthesis, PGI_2 can be released into the extracellular milieu where it binds to the I-prostanoid (IP) receptor which, like the EP_2 and EP_4 receptors, preferentially couples with G_s and activates adenylyl cyclase to elevate intracellular cAMP levels. Accumulating evidence suggests that endogenously produced PGI_2 may also be capable of entering the nucleus and activating peroxisome proliferator activated receptors (265). PGI_2 is unstable under physiological conditions, giving it a very short half-life of less than 2 minutes *in vivo* before it is metabolized to its inactive form, 6-keto-$PGF_{1\alpha}$ (266).

PGI_2 is known most notably for its profound effects on the cardiovascular system. Indeed, prostacyclin is the most abundant prostanoid produced in vascular tissues with endothelial cells representing the most significant source of this mediator (267). Synthesis of PGI_2 is often coupled with COX-2 and it is believed that the cardiovascular risks associated with the use of coxibs results from inhibition of prostacyclin (268). In the vasculature, PGI_2 is a potent vasodilator and its release is

critical for preventing platelet aggregation, dispersing existing aggregates, relaxing and inhibiting proliferation of vascular smooth muscle cells, suppressing atherogenesis and controlling leukocyte adhesion (268-271).

Similar to PGE_2, the capacity of prostacyclin to relax smooth muscle cells and blood vessels is important in systems beyond the cardiovascular system. PGI_2 is produced at high levels by the kidneys and mice lacking PGIS develop kidney abnormalities (272). *In vivo* work shows this prostanoid contributes to renal blood flow and renin production and secretion (273). These attributes also contribute to inflammation. Mice lacking the IP receptor have reduced edema and vascular permeability in models of inflammation (274, 275) and this prostanoid is also implicated in nociceptive pain (274). Further, IP-deficient animals have attenuated arthritic scores compared to wildtype controls (276).

Prostacyclin can also serve as a protective mediator. An analog of this prostanoid, iloprost, is used in the treatment of pulmonary arterial hypertension where it has been shown to improve overall symptoms and confer a survival benefit (277). Similarly, PGI_2 appears to be beneficial in the prevention of idiopathic pulmonary fibrosis. Animals lacking the IP receptor are more susceptible to bleomycin-induced pulmonary fibrosis and treatment with iloprost is protective against this form of pulmonary fibrosis when administered to wildtype mice (278, 279).

Prostacyclin and Leukocytes

Like PGE_2, prostacyclin has profound effects on immune cells. DCs express the IP receptor and are capable of producing at least modest quantities of PGI_2 (280). *In vitro* stimulation of bone marrow derived DCs with various prostacyclin analogs suppresses maturation and upregulation of costimulatory molecules including CD86, CD40, and MHC II (281, 282). Additionally these analogs

inhibit the release of cytokines including IL-12, TNF-α, and IL-6 from DCs in a dose-dependent manner while enhancing the production of IL-10 (281), suggesting that PGI_2 has an anti-inflammatory effect on DC activity. These actions are controlled, in part, by IP-dependent protein kinase A (PKA) activation, given that the effects of these analogs were not observed in IP -/- mice and were attenuated when stimulation occurred in the presence of a PKA inhibitor (282). Further, analog stimulation suppresses the ability of DCs to promote T cell proliferation. BALB/c-derived OVA-specific TCR (DO11.10) T_H2 cells cocultured with analog-treated OVA-pulsed DCs produce attenuated T_H2 cytokines IL-4, IL-5 and IL-13 and elevated quantities of IFN-γ and IL-10 (281, 282).

Evidence suggests that naïve $CD4^+$ cells are not a source of prostacyclin (280). Further, while exceptions have been noted (280), expression of the IP receptor is not typically observed on these cells (283, 284), suggesting that PGI_2 does not directly mediate differentiation into effector T cell populations. Like naïve $CD4^+$ cells, committed T cells do not produce PGI_2 (283), however upregulation of the IP receptor is identified, to various degrees, by different T_H cell types. While reports are in agreement that activated T_H2 cells express high levels of this receptor (283, 284), there is disagreement in the field regarding IP expression on T_H1 cells. While some groups observe elevated levels of IP expression on this cellular population, others indicate that there is little to no expression of this receptor on T_H1 cells (283-285).

The impact of prostacyclin on T_H1 cells is not clearly defined. In one *in vitro* study, murine T_H1 cells restimulated in the presence of PGI_2 analog produced reduced levels of IFN-γ (285). However, when another group restimulated DO11.10 T_H1 cells, IFN-γ levels were unaffected by the addition of prostacyclin (284) and a third study found that analog enhanced IFN-γ levels (280). The responses of T_H1 cells in the presence of PGI_2 or its analogs may depend on the strength of costimulatory signals. IFN-γ production was reduced when CD28 engagement was absent (285);

however, iloprost significantly enhances production of IFN-γ from T_H1 cells in an anti-CD28 dose-dependent manner, even when IL-12 is absent (280). *In vivo* findings are equally ambiguous, and suggest that PGI_2-mediated responses on T_H1 cells may be organ-specific. In a model of respiratory syncytial virus (RSV) mice overexpressing PGIS in the respiratory epithelium have significantly reduced disease and IFN-γ protein expression in the lung while IP -/- animals have augmented lung IFN-γ protein (286). However, prostacyclin augments inflammation in a T_H1-biased contact hypersensitivity model (280).

The actions of prostacyclin on T_H2 cells are equally controversial. In one study, *in vitro* T_H2 cells cocultured with prostacyclin analog during restimulation produced attenuated levels of IL-4, IL-10, and IL-13 (285). However, a separate group has consistently observed that T_H2 cells produce comparable levels of IL-4 and IL-5 regardless of the presence of PGI_2 analog, instead measuring augmented IL-10 (284). It has also been demonstrated that unlike T_H1 cells, iloprost reduces the number of IL-4 producing cells regardless of the degree of CD28 engagement (280).

Little is known regarding the effect of prostacyclin on T_H17 and T_{reg} cells; however, experimental evidence suggests that both T_H17 cells and $CD4^+CD25^+Foxp3^+$ express the IP-receptor, suggesting that this mediator can indeed influence these cell types (283, 287) A recent report indicates that PGI_2 augments T_H17 responses in the lung. Using an OVA/alum model, Li and colleagues demonstrate that inflamed COX-2 deficient mice have reduced levels of T_H17 cells and T_H17 cytokines in their lungs and airways. T_H17 levels and IL-17a cytokine quantities could be restored when $CD4^+$ T cells were pretreated with a prostacyclin analog or when PGI_2 was administered by osmotic pump (287).

Recently, work was conducted to elucidate the actions of prostacyclin on AMs. This study found that while AMs express the IP receptor, PGI_2-mediated responses in these cells are weak

compared to the responses by peritoneal macrophages (PMs) to prostacyclin. *In* vitro analysis reveals that while prostacyclin analog has potent inhibitory effects on phagocytosis, cytokine release, and bacterial killing in PMs while only weakly controlling these responses in AMs (288).

Prostanoids in Lung Allergy

The relevance of prostanoids to asthma and lung inflammation is highlighted by the existence of aspirin-exacerbated respiratory disease (AERD) first described in 1922 by Widal, representing a severe form of asthma in which use of aspirin or other NSAIDs initiates an allergic response in asthmatic individuals. Symptoms of AERD include bronchospasms, profuse rhinorrhea, nasal congestion, sneezing, and itching. In severe instances, episodes of AERD can result in life-threatening anaphylactic reactions. The exact prevalence of AERD is unclear, given that many asthmatics avoid the use of NSAIDs or may not correlate worsening symptoms with NSAID ingestion; however review of studies administering aspirin challenges placed the prevalence of AERD at 20% in asthmatics. Patients with AERD tend to have elevated levels and activation of eosinophils and mast cells. (289)

The expression of COX enzymes in the airways of asthmatics is controversial. While some studies fail to see upregulation of either enzyme in these individuals, other reports have observed increased levels of one or both isoforms (290-292). Studies using genetically-engineered animals lacking these enzymes and the use of pharmaceutical agents which alter the functions of COX isoforms have enhanced our knowledge regarding the contribution of prostanoids to allergy in the lung **(Table 1.1)**. When lung allergy is induced, mice lacking either COX-1 or COX-2 have an augmented allergic response compared to wildtype (WT) animals, with elevated eosinophilia, IgE, and mucus production (182) suggesting that the ultimate effect of COX-dependent prostanoid production is to limit allergic responses in the lung. These finding are corroborated by studies

conducted using COX-inhibitors (293, 294). The degree to which each isotype promotes this protection and the range of allergic responses effected, however, is controversial. For instance, these works disagree on the ability of the individual COX isoforms to prevent AHR. Some data suggests that inhibition of COX-1 is more detrimental in the lung than COX-2, while other works find an equal contribution from each isoform. (182, 293, 294)

A loss of all prostanoid production by either COX enzyme may exacerbate allergy in the respiratory system; however, the contribution of individual prostanoids to this process is more complex. Research demonstrates that production of some of these prostanoids exacerbate disease manifestations. This implies that in addition to preventing pulmonary allergy, prostanoid production can also contribute to disease. PGD_2 is released in abundance by activated mast cells and is believed to promote the pro-inflammatory responses generated by these cells following allergen challenge in the lung (295). Results generated from overexpression of the PGD_2 synthase in the mouse lung are consistent with this assumption. These mice experience exacerbated allergy in response to antigen sensitization and challenge marked by elevated eosinophilia and T_H2 cytokine levels (296).

Actions of PGD_2 occur through binding to the D prostanoid 1 (DP_1) receptor and chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2, DP_2). Immunized mice lacking the DP_1 receptor display attenuated inflammation and airway responses following allergen challenge (297). CRTH2 is thought to be involved in leukocyte trafficking. Consistent with this, agonists for this receptor initiate recruitment of eosinophils (298, 299); however the use of CRTH2-deficient animals to elucidate the role of endogenous signaling has proven controversial. Dependent on the genetic background of the animals used, CRTH2-deficient mice display no differences in disease parameters or enhancement of allergy (298, 300)

TXA$_2$ binds to the T prostanoid (TP) receptor to exert its functions. This prostanoid is known most notably for its role in platelet aggregation. Data generated in our lab suggests that although loss of the TP -/- receptor does not alter pulmonary inflammation, a TP-agonist induces bronchoconstriction in naïve animals and AHR in the inflamed murine airway (301). The functions of PGF$_{2\alpha}$ are most well-studied in the female reproductive system where this prostanoid contributes to the birthing process (218). Data regarding the function of this prostanoid in lung allergy is scare however studies suggest that this mediator promotes airway constriction and plasma exudation (302, 303).

Prostaglandin E$_2$ and Lung Allergy

Elevated levels of PGE$_2$ are measured in the sputum and plasma of asthmatic individuals (304-306). Further, PGE$_2$ is produced by many cell types which contribute to disease in asthma including epithelial cells, ASM cells, AMs, and DCs, with an even broader range of cells expressing the receptors for this mediator (307-309). Evidence supporting both pro- and anti-inflammatory functions in asthmatic responses has been described for this prostaglandin.

PGE$_2$ is generally considered a potent bronchodilator. PGE$_2$ administration can limit AHR in asthmatic individuals and rodents (302, 310-314) and can also inhibit airway constriction in exercise and aspirin-induced asthma (315, 316). However, a subclass of individuals develop heightened bronchoconstriction in response to PGE$_2$ (302, 317) and administration is often associated with a coughing reflex (318) , limiting its usefulness as a clinical prophylactic. The ability of PGE$_2$ to serve as an airway relaxant has been assigned to selective EP$_2$ receptor binding, while the adverse effects are likely due to activation of cholinergic neurons by alternate EP receptors (319).

Animal models have also provided some evidence that PGE$_2$ may contribute to aspects of airway disease, however the exact nature of this mediation is unclear. In a model of OVA-induced

lung allergy, mice lacking the EP_3 receptor developed elevated eosinophilia and augmented T_H2 cytokine production, while OVA-specific IgE production was unaffected by signaling through this receptor (320). However in a pulmonary allergy model using house dust mite antigen, animals lacking the mPGES1 synthase and wildtype animals had similar levels of IgE, BALF cell infiltrate and eosinophilia while showing augmented airway remodeling (321). These results suggest that the effects of PGE_2 in the airway are complex and may differ depending on the model being studied.

Prostacyclin and Lung Allergy

In the lung PGI_2 is generally thought to be protective. For example, PGI_2 is thought to limit disease associated with respiratory syncytial virus (RSV). In a murine model of this condition, animals overexpressing PGIS in the lung were protected compared to wildtype animals, while mice lacking the IP-receptor had augmented illness (286). Further, this prostanoid appears to ameliorate bleomycin induced ideopathic pulmonary fibrosis. Animals lacking the IP receptor have augmented disease while administration of iloprost is protective (278, 279). Prostacyclin analogs are also used as an effective treatment for pulmonary arterial hypertension in humans (322).

Studies analyzing the contribution of PGI_2 to lung allergy suggest that this mediator attenuates all phases of disease. When either acute or chronic allergy are induced following sensitization and challenge with OVA antigen, mice lacking the IP-receptor have elevated cellularity in their BALF, predominantly reflecting augmented eosinophilia. Further, these receptor-deficient animals have enhanced IgE levels, elevated vascular permeability, and exacerbated T_H2 cytokines (323, 324). Additionally, when chronic lung allergy is induced, IP-deficient mice have significantly elevated TGF-β1 and hydroxyproline levels. Histological analysis additionally reveals augmented goblet cell hyperplasia and collagen deposition in these mice (324). Splenocytes from sensitized animals produced enhanced quantities of IL-4 and IFN-γ when restimulated with OVA (323).

Immunoglobulin levels in IP-deficient mice and their *ex vivo* splenocyte responses suggest that PGI signaling contributes to allergic sensitization. Idzko et al. employed a model in which antigen sensitization can be tracked. When tolerogenic pDCs are depleted from the lung, exposure of the airways to antigen causes sensitization and lung allergy following subsequent antigen challenge. This response relies on antigen presentation by mDCs (81). Adoptive transfer of OVA-pulsed mDCs into the airways of naïve pDC-depleted mice results in eosinophilia, goblet cell hyperplasia, and elevated BALF T_H2 cytokine levels. *In vitro* treatment of OVA-pulsed mDCs with iloprost prior to adoptive transfer significantly abolishes disease parameters in recipients, supporting the idea that PGI_2 mediates allergic sensitization in the airways. In addition, lymph node cells obtained from animals receiving iloprost treated OVA-pulsed mDCs produced attenuated levels of T_H2 cytokines when restimulated; however, IFN-γ and IL-10 cytokine levels were enhanced in these populations. (282)

Conversely, data indicates that PGI_2 can independently contribute to the effector phase of lung allergy. Administering iloprost to sensitized animals prior to each antigen challenge significantly reduces eosinophilia, AHR, and goblet cell hyperplasia. Further, restimulated lymph node cells from these animals produce attenuated T_H2 cytokine quantities (282).

Figure 1.1 Differentiation of Naïve T Cells
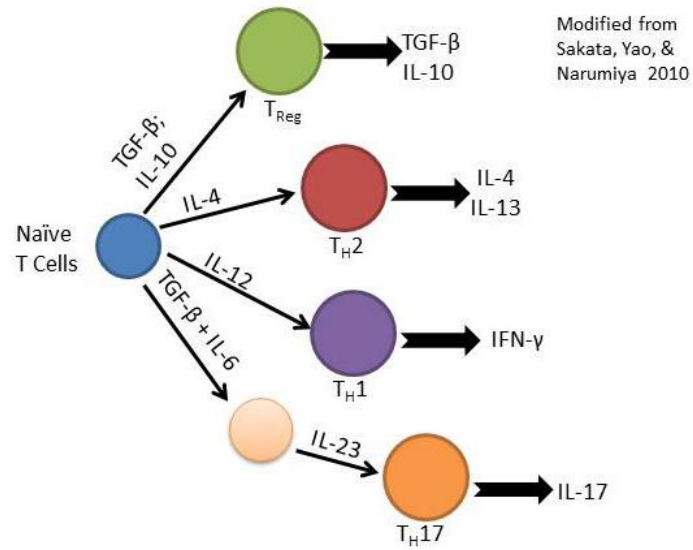
Figure 1.1 Differentiation of Naïve T cells The differentiation of naïve T cells into specific effector T cell subsets is controlled by the cytokines present during stimulation with antigen. TGF-β or IL-10 leads to the production of regulatory T (T_{Reg}) cells. Differentiation into the helper classes T_H1, T_H2, or T_H17 occurs in the presence of IL-12, IL-4, or TGF-β and IL-6 respectively. IL-23 stabilizes and expands the T_H17 population. These classes of T cells release unique cytokine profiles to exert their effects on immune responses.

Figure 1.2 Sensitization to Antigen

Figure 1.2 Sensitization to Antigen Particles entering the airway lumen can be sampled by dendritic cells (DCs) forming tight junctions with the epithelium. When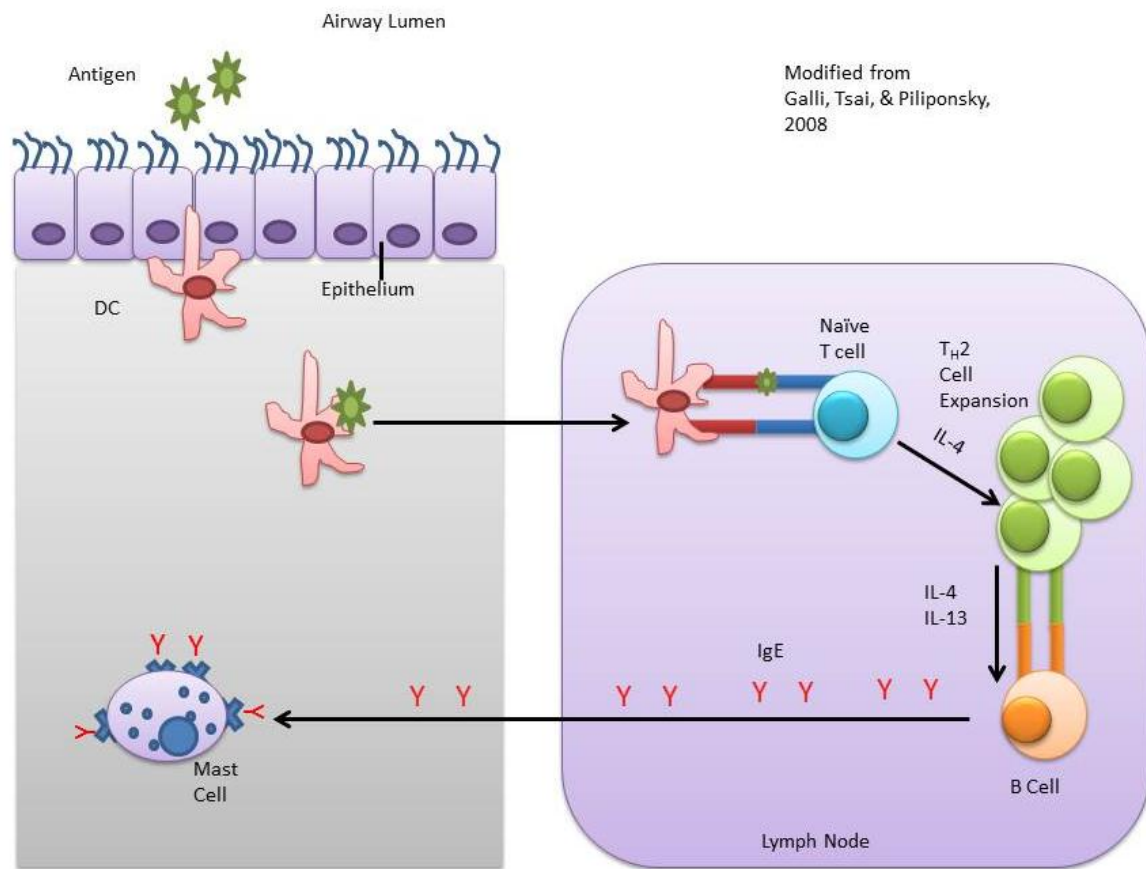 an antigen is taken up, DCs undergo a maturation process and travel to local lymph nodes where they can present antigen to naïve T cells. Activation occurs through interactions with the major histocompatibility complex (MHC) II, as well as other costimulatory receptors and, in the presence of IL-4, results in the acquisition of T_H2 characteristics. These antigen-specific T_H2 cells undergo expansion and produce large quantities of IL-4 and IL-13. Interaction of these T_H2 cells with costimulatory molecules on B cells initiates immunoglobulin class-switching resulting in the production of antigen-specific IgE antibody. IgE diffuses out of the lymph nodes and binds to the high-affinity receptor for IgE (FcεRI) on mast cells and basophils, sensitizing them to respond upon subsequent exposure to antigen.

Figure 1.3 Acute Allergic Response in the Lung

Increased Mucus Production

Goblet Cell

T_H2

Mast Cell

IL-4, IL-13
IL-9, IL-5

Eosinophil

Cell Recruitment

Increased vascular permeability

Bronchoconstriction

Vasodilation

ASM

Modified from Galli, Tsai, & Piliponsky, 2008
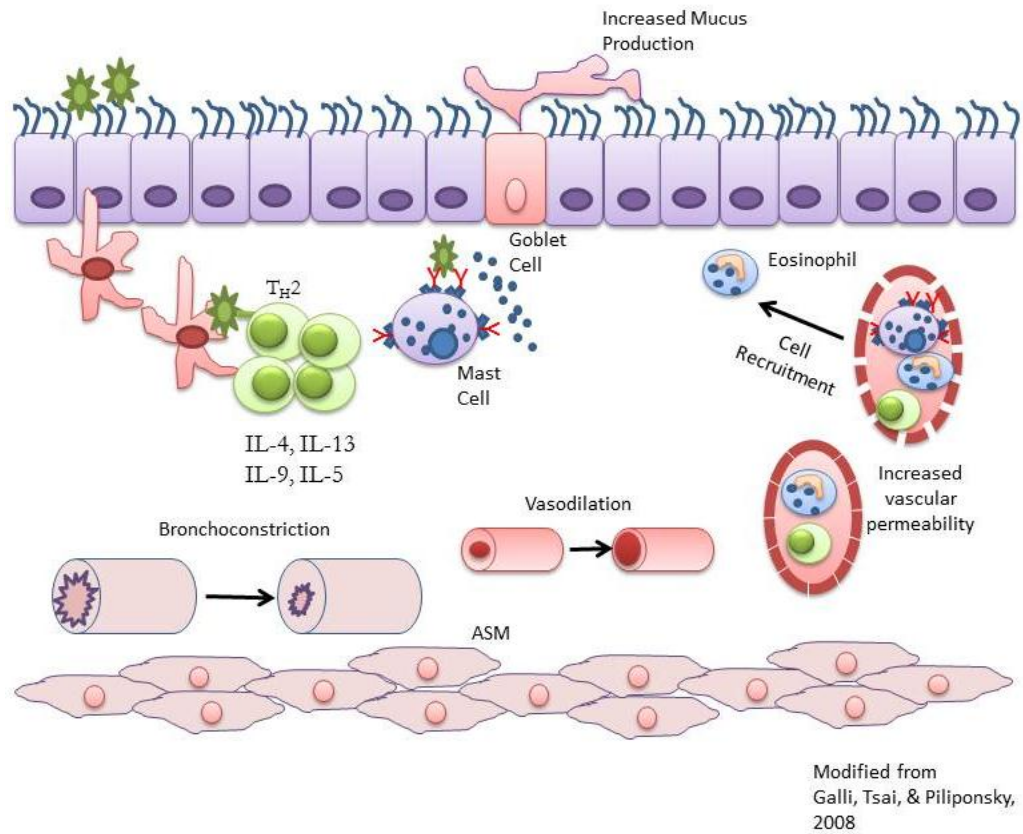
Figure 1.3 Acute Allergic Response in the Lung When an allergen gains entry to a previously sensitized host, it is taken up by DCs and presented to antigen-specific T_H2 cells circulating the mucosal system. Activated effector T cells release specific cytokines into the airways resulting in the symptoms associated with allergy in the lung including airway hyperresponsiveness (AHR), cell recruitment, vasodilation, elevated vascular permeability, and increased mucus. Antigen can also bind and aggravate FcεRI/IgE complexes on mast cells resulting in degranulation and the release of preformed mediators as well as de novo synthesized factors, which exacerbates allergic responses in the airway.

Figure 1.4 Prostanoid Biosynthesis Pathway
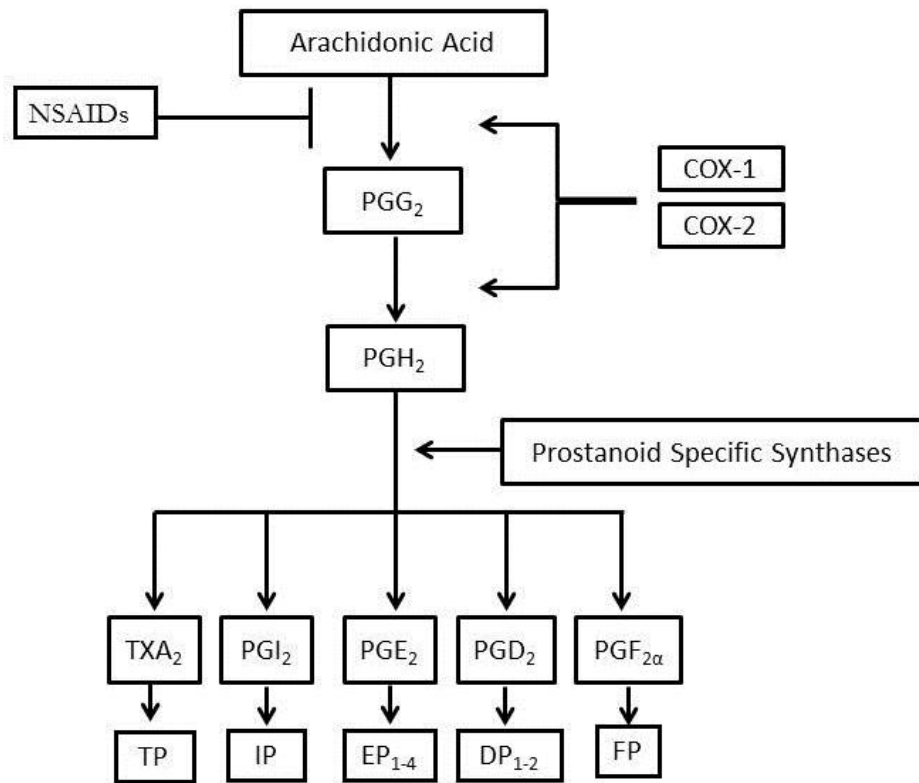
Figure 1.4 Prostanoid Biosynthesis Pathway Prostanoids are synthesized by the actions of cyclooxygenase (COX) enzymes on arachidonic acid (AA). AA is released from the plasma membrane by phospholipases and converted into unstable intermediate products, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by two isoforms of the COX enzyme, COX-1 and COX-2. The intermediate PGH_2 is converted to 5 bioactive prostanoids: thromboxane (TXA_2), prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) through synthesis by prostanoid-specific synthases. Prostanoids exert their autocrine and paracrine effects through binding to specific G-coupled protein receptors. Common analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), inhibit the actions of COX enzymes, thus preventing synthesis of prostanoids.

Table 1.1 *In Vivo* Contributions of Prostanoids to Pulmonary Allergy

Mouse	Treatment	Model	Eos	IgE	Th2 cyto	Mucus	AHR	Remod	Reference
COX-1 -/-		Acute OVA/alum	↑	↑	↑	↑	↑		Gavett 1999
COX-1 -/-		Acute OVA/alum	↑	≈	↑		≈		Church 2012
COX-2 -/-		Acute OVA/alum	↑	↑	≈	↑	≈		Gavett 1999
COX-2 -/-		Acute OVA/alum	↑	≈	↑		≈		Church 2012
WT	non-specific COX inhibitor	Acute OVA/alum		≈	↑	≈	↑		Peebles 2000
WT	COX-1 inhibitor	Acute OVA/alum	≈		↑	≈	↑		Peebles 2002
WT	coxib	Acute OVA/alum	≈		↑	≈	↑		Peebles 2002
WT	T_H2 DO11.10 + coxib	Adoptive Transfer	↑		↑	↑	↑		Jaffar 2002
DP -/-		Acute OVA	↓	≈	↓	↓	↓		Matsuoka 2000
CRTH2 -/-		Acute OVA/alum	↑						Chevalier 2005
CRTH2 -/-		Acute OVA/alum	≈						Shiriashi 2008
PGDS tg +		Acute OVA/alum	↑	≈	↑				Fujitani 2002
WT	TXA_2 Analog	Acute OVA/alum					↑		Allen 2006
TP -/-		Acute OVA/alum	≈	≈	≈	≈			Allen 2006
WT	$PGF_{2\alpha}$ Analog	naïve					↑		Arakawa 1993
EP_3 -/-		Acute OVA	↑	↑	↑				Kunikata 2003
mPGES1 -/-		Chronic der f	≈	≈		↑		↑	Lundequist 2010
mPGES1 -/-		Acute OVA/alum	↓	≈	↓		≈		Church 2012
IP -/-		Acute OVA/alum	↑	↑	↑				Takahashi 2002
IP -/-		Chronic OVA/alum	↑	↑	↑			↑	Nagao 2003
WT	T_H2 DO11.10 /coxib	Adoptive Transfer	↑		↑	↑	↑		Jaffar 2002
WT	T_H2 DO11.10/PGI_2 Analog	Adoptive Transfer	↓		↓		↓		Jaffar 2007
WT	PGI_2 Analog	Acute OVA/alum	↓		↓	↓	↓		Idzko 2007

Table 1.1 **In Vivo** *Contributions of Prostanoids to Pulmonary Allergy* Animals are a useful tool for delineating the *in vivo* contributions of a specific factor in a desired system through the use of genetic-engineering and pharmacological reagents. This table summarizes the major findings observed in the airways when aspects of the prostanoid pathway are manipulated. (Model identifies the manner in which an animal was sensitized.)

CHAPTER II

PGE$_2$ PRODUCED BY THE LUNG AUGMENTS THE EFFECTOR PHASE OF ALLERGIC INFLAMMATION[1]

Prostanoids are a family of bioactive lipid mediators produced in almost every cell type by the actions of prostaglandin-endoperoxide synthases (cyclooxygenase, COX) on arachidonic acid (AA). Synthesis is initialized when phospholipase A$_2$ releases AA from membrane phospholipids in response to a diverse range of stimuli. The AA is then catalyzed to prostaglandin H$_2$ (PGH$_2$) by one of two isoforms of the COX enzyme, COX-1 or COX-2 (218, 325) COX-1 is constitutively expressed by most cell types and is thought to be responsible for basal levels of prostanoid production while COX-2 expression is generally undetectable in most tissues under homeostatic conditions and is upregulated in response to inflammatory stimuli (218), although exceptions have been noted. For example, COX-1 expression increases dramatically in the lactating mammary gland (189) and COX -2 expression can easily be detected in the healthy lung of both mice and humans (186, 197). PGH$_2$ generated by either COX -1 or COX -2 is subsequently converted into a family of related molecules: prostaglandin D$_2$ (PGD$_2$), prostaglandin E$_2$ (PGE$_2$), prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$), prostacyclin (PGI$_2$), and thromboxane A$_2$ (TXA$_2$) by pathway specific synthases. These lipids mediate their actions through the selective binding to G-coupled protein receptors, each with a unique but overlapping pattern of expression (218, 325). PGE$_2$ binds with a high affinity to four receptors, E prostanoid (EP) 1-4, all of which are expressed in the lung (326, 327).

PGE$_2$ levels are elevated during most inflammatory responses; however, elevated synthesis of this lipid mediator may reflect an attempt by the organism to limit ongoing inflammation and protect the airways from collateral damage. Certainly, PGE$_2$ mediated pathways capable of limiting inflammation and restoring homeostasis in the lung have been identified. PGE$_2$ is a potent smooth muscle relaxant and through the EP$_2$ receptor can limit constriction of the airways (319). Indeed, administration of this mediator into the airways ameliorates airway hyperresponsiveness (AHR) caused by several bronchoconstrictive agents in humans and animals (302, 310-314) and attenuates aspirin-induced and exercise-induced bronchoconstriction (315, 316). PGE$_2$ can induce ion secretion and thus alter the composition of the airway surface liquid, facilitating mucociliary clearance (328). Furthermore, exogenous PGE$_2$ can limit T cell proliferation and T$_H$1 type cytokine release from LPS-stimulated macrophages through stimulation of the EP$_2$ and EP$_4$ receptors, respectively (246). In addition to its capacity to down-regulate pro-inflammatory cytokine release from immune cells, PGE$_2$ can also stimulate release of IL-10, a cytokine generally thought to be protective in the immune system (329).

Perhaps the strongest indication that PGE$_2$ might function to limit allergic inflammation in the lung comes from animal studies conducted using pharmacological and genetic approaches to limit prostaglandin synthesis in a model of ovalbumin (OVA) induced lung allergy. Mice of mixed genetic background and lacking either COX -1 or COX -2 were reported to develop far more severe disease than wildtype animals (182). Consistent with this, treatment of mice with indomethacin, a non-steroidal anti-inflammatory drug (NSAID) which suppresses the actions of both COX -1 and COX -2, or alternatively, with COX-specific NSAIDs induced elevated eosinophilia and IL-13 production in the lung (293, 294); although these approaches did not allow for the identification of the specific prostaglandin(s) responsible for limiting the allergic response. Another study, however, reported increased allergic inflammation in mice lacking EP$_3$ receptors (320), suggesting that loss of PGE$_2$ is at

least partly responsible for heightened inflammation observed in COX-deficient and NSAID treated animals.

PGE$_2$ production occurs through the metabolism of PGH$_2$ by the microsomal PGE$_2$ synthase-1 (mPGES1) (197). While initially two additional enzymes, cytosolic PGE$_2$ synthase (cPGES) and microsomal PGE$_2$ synthase-2 (mPGES2) were thought to be capable of this enzymatic conversion, studies using mutant mouse lines carrying mutations in the genes for these synthases, *mPges1*, *cPges/p23* or *mPges2* respectively, have failed to support this *in vivo* function for any product other than mPGES1 (199, 201, 204). mPGES1 is expressed in many tissues and cell types of both humans and animals, including in the lung and leukocytes (197, 330, 331). Similar to COX-2, the expression of mPGES1 increases dramatically in response to inflammatory mediators suggesting coupling with this enzyme; however, evidence demonstrates that mPGES1 can couple with both COX-1 and COX-2 to synthesize PGE$_2$ (197-199, 330). Furthermore, studies using mice lacking mPGES1 in models of pain nociception, rheumatoid arthritis, atherogenesis, and abdominal aortic aneurysm provide evidence that this synthase contributes to the pathogenesis of both acute and chronic inflammation (199, 200, 332, 333).

Here we elucidate the contribution of mPGES1-derived PGE$_2$ in the development of allergic lung disease, a mouse model of asthma. First, using congenic mouse lines lacking either COX-1 or COX-2, we confirm the role of this pathway in our model. We then evaluate the role of PGE$_2$ produced by mPGES1 expressed by resident airway cells and PGE$_2$ released from recruited inflammatory cells in this allergic response.

Materials and Methods

Experimental Animals

All animal colonies were maintained according to standard guidelines as defined by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill. Experiments were carried out on age and sex matched mice between 8-12 weeks of age. C57BL/6 (B6) (backcrossed >10 generations) COX-1 -/-, B6 x 129S6/SvEv (129) fililal generation (F)1 COX-2 -/-, and B6 (backcrossed >10 generations) mPGES1 -/- (187, 199, 334). B6 OVA-Specific TCR-Transgenic (OT-II) mice were purchased from The Jackson Laboratories (Bar Harbor, Ma). OT-II mice were crossed to mPGES1 -/- mice to generate OT-II/mPGES1 -/- animals.

OVA Sensitization and Challenge

All mice were sensitized systemically on days 0 and 14 with an i.p injection of 40μg OVA (Sigma-Aldrich) emulsified in aluminum hydroxide (alum) (Sigma-Aldrich). One week following the second injection, experimental mice were challenged for 5 consecutive days (days 21-25) with an aerosol instillation of 1% OVA in 0.9% sodium chloride (NaCl) for 1 hour each day. Control animals were challenged with 0.9% NaCl only. 24 hours after the final challenge, lung mechanics were measured and bronchoalveolar lavage fluid (BALF), serum, and whole lungs were collected for further analysis. For experiments examining allergic sensitization, animals were immunized as described and harvested on day 21.

Measurement of Cell Proliferation

Splenocytes were prepared by mechanical dispersion of spleen over a 70 μm cell strainer (BD Falcon). Red blood cells were lysed in lysis buffer (4.1g NH_2Cl, 0.5g $KHCO_3$, 100 μl 0.5M EDTA dissolved in 500 ml dH_2O) and splenocytes were washed twice in PBS. Cells were plated at a density of 2.5 X 10^6 cells/ml in RPMI 1640 media enriched with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and .29 μg/ml L-glutamine. OVA was added to splenocyte cultures at concentrations ranging from 0 μg /ml to 100 μg/ml. After incubation for 72 hours at 37°C, cell proliferation was assessed using WST-1 reagent (Roche) according to the manufacturer's instructions.

Measurement of Airway Mechanics in Intubated Mice

Mechanical ventilation and airway mechanic measurements were conducted as previously described (335). Briefly, mice were anesthetized with 70-90 mg/kg pentabarbitol sodium (American Pharmaceutical Partners, Los Angeles, CA), tracheostomized, and mechanically ventilated with a computer-controlled small-animal ventilator. Once ventilated, mice were paralyzed with 0.8 mg/kg pancaronium bromide. Forced Oscillatory Mechanics (FOM) was measured every 10 seconds for 3 minutes. These measurements allowed for the assessment of airway resistance in the central airways (Rn), as well as in tissue damping (G). Increasing doses (between 0 and 50mg/ml) of methacholine chloride (Mch) were administered to animals through a nebulizer to measure airway hyperresponsiveness (AHR). These data are presented as percent above baseline.

BALF Collection and Cell Counts

Following measurements of lung mechanics, mice were humanely euthanized. Lungs were lavaged with five 1ml aliquots of HBSS (Gibco). 100μl of BALF was reserved for cell count analysis and

total cell counts were measured by hemacytometer. Differential cell counts were collected using cytospin preparation stained with Hema 3 and/or Fast Green. All remaining BALF was centrifuged to remove cells, and stored at -80° c for immunoassay.

PGE$_2$, IgE and Cytokine Production

Levels of cytokines and PGE$_2$ were determined by immunoassay in BALF, lung tissue homogenate, and/or tissue culture supernatant. To determine cytokine production by stimulated splenocytes, cells were prepared as described above. Cells were cultured at a density of 1 X 10^7 cells/ml in the presence of 100 μg/ml OVA. After 72 hours, supernatants were collected and stored at -80°C prior to evaluation by ELISA. To determine lung cytokine levels, lungs were flash frozen in liquid nitrogen, weighed, and stored at -80°C. Lung tissue was pulverized and homogenized in buffer containing 150 mM NaCl, 15 mM Tris-HCl, 1mM CaCl$_2$, 1mM MgCl$_2$ supplemented with protease inhibitor (Roche). Values shown represent the total quantity of cytokine or mediator measured divided by tissue weight. Cytokines were determined by ELISA following manufacturer's protocols: IL-13 (R&D Systems), IFN-γ (R&D Systems), IL-4 (R&D) and IL-17a (eBiosciences). For quantification of PGE$_2$, lung tissue was pulverized then homogenized in 1XPBS/1mM EDTA and 10 μM indomethacin. Lipids were purified through octadecyl C$_{18}$ mini columns (Amersham Biosciences for mPGES1; Alltech Associates for COX-1 and COX-2), and prostanoid content was determined using an enzyme immunoassay kit (Assay Designs) according to the manufacturer's instructions. Blood was obtained by cardiac puncture, allowed to coagulate, and centrifuged to isolate serum. IgE levels were determined by immunoassay using 96 well EIA/RIA plates (Costar). Plates were coated with IgE capture antibody (Pharmingen; clone R35-72), blocked with 1% BSA/PBS and then incubated with IgE standard (Pharmingen) or serum followed by biotinylated rat anti-mouse IgE (Pharmingen; clone R35-118). Detection was carried out using streptavidin-horseradish peroxidase (HRP)

(Pharmingen) and hydrogen peroxide /2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Absorbance at 405nm was measured.

Bone Marrow Chimera Generation

Recipient mice were exposed to 5 grays irradiation from a Cesium g-irradiator at 0 and 3 hours. Femurs and tibias were collected from donor mice and flushed with cold PBS to isolate bone marrow. Bone marrow was introduced by tail vein injection into recipient mice immediately following the second round of radiation and after 8 weeks animals were sensitized and challenged with OVA.

Statistical Analysis

Statistical analysis was performed using Prism 4 (GraphPad Software). Comparisons of the mean were made by F test, Student's t-test or ANOVA followed by Tukey-Kramer's HSD *post hoc* test as necessary. Data are shown as mean ± SEM. Differences with *p<0.05* were considered statistically significant.

Results

Allergic Asthma in the COX-1 and COX-2 deficient mice

We first verified, using congenic mouse lines, the contribution of prostanoids to allergic lung disease induced by sensitization and challenge with OVA antigen. COX-1 -/- B6 congenic mice were generated by >10 crosses between mice carrying a null allele at this locus and commercially purchased B6 mice. COX-2 mice survive poorly on most inbred genetic backgrounds, in part due to a patent ductus arteriosus (190, 336), and thus most experiments assessing COX-2 function have utilized > F2 mice; mice expected to carry a random assortment of 129 and B6 derived alleles. To circumvent this problem, we generated two lines of COX-2 mutant mice, the first line on the co-isogenic 129 genetic background and the second congenic line on the B6 genetic background. 129 COX-2 +/- females were intercrossed with B6 +/- males to generate congenic F1 progeny. The COX-2 -/- and COX-2 +/+ littermates were used in the experiments presented here. Allergic airway disease was induced through sensitization by an i.p. injection of OVA emulsified in aluminum hydroxide and challenged with repeated aerosols of antigen. Inflammation and airway mechanics were assessed twenty four hours after the final antigen challenge.

As expected, our immunization protocol induced a robust cellular influx in B6 wildtype mice (COX-1 +/+), compared to saline treated animals **(Fig 2.1A)**. The F1 OVA-treated controls (COX-2 +/+) displayed a similar pattern of increased cellularity. Surprisingly, the loss of either COX-1 or COX-2 had comparable impacts on recruitment of cells into the airways: in each line, the total number of cells recovered by BALF was about twice that observed in the genetically matched controls. The cellular infiltrate present in the airways following antigen challenge was marked by heightened levels of eosinophils, typical of T_H2 type allergic responses **(Fig 2.1B)**. Characteristic of

this type of response, IL-13 levels were elevated in the BALF of wildtype mice with allergic lung disease compared to saline controls **(Fig 2.1C)**. Loss of either COX-1 or COX-2 led to increased levels of this cytokine in the airways. Again the magnitude of this increase, relative to the wildtype control, was surprisingly similar in the two lines. Elevated IgE levels were observed in both B6 COX-1 +/+ and B6/129 F1 COX-2 +/+ wildtype animals **(Fig 2.1D)**. However, unlike inflammatory disease in the lung, the loss of neither the COX-1 nor the COX-2 pathway significantly altered levels of this immunoglobulin isotype. AHR following challenge with methacholine (Mch), a potent airway constrictor, was quantified in intubated animals using a computer-controlled small-animal ventilator. This method allows for measurement of resistance in the central airway (Rn) and tissue damping (G). As reported previously for the B6 strain (337-339), we failed to observe AHR in response to Mch in mice with allergic lung disease. This was also true of the B6/129 F1 mice. Even in the COX-1 and COX-2 mice, which showed elevated levels of inflammation, no difference was observed in the response to methacholine in either parameter **(Fig 2.2)**.

Contribution of mPGES1 to PGE$_2$ production in the naïve and inflamed lung

The ability to catalyze the conversion of PGH$_2$ to PGE$_2$ has been assigned to three distinct enzymes, mPGES1, mPGES2 and cPGES (197, 340, 341). However, studies using mutant mouse lines have shown *in vivo* alteration of PGE$_2$ levels only in the mPGES1 mice (199, 342). We therefore first determined whether PGE$_2$ levels are elevated in this model of allergic lung disease and if so, whether this increase is dependent on the mPGES1 synthase. Allergic lung disease was induced in congenic mPGES1 -/- mice and their B6 controls (mPGES1 +/+), as described above. Lungs were harvested and the levels of PGE$_2$ were determined by enzyme immunoassay **(Fig 2.3)**. PGE$_2$ production could be detected in the lungs of healthy mice exposed to saline. This production was significantly reduced in the lungs obtained from mPGES1 -/- mice, indicating that mPGES1 is active

in the normal lung and contributes to the basal levels of PGE$_2$ in this organ. PGE$_2$ levels were

substantially potentiated in the lungs of wildtype mice with allergic lung disease. This increase was

entirely dependent on expression of the mPGES1 synthase, indicating that animals lacking this

synthase provide an appropriate model for determining whether loss of this COX1/2 downstream

pathway contributes to the increased disease observed in the COX-1 and the COX-2 deficient

animals.

Impact of PGE$_2$ on allergic lung inflammation

PGE$_2$ levels in the lung increase dramatically after allergic lung disease and this increase is

absent in both the COX-1 and the COX-2 deficient mice (**Fig 2.4**), supporting the hypothesis that loss

of this prostanoid could contribute to the increased disease observed in both of these mouse lines. To

test this hypothesis, mice lacking mPGES1, and their congenic controls, were sensitized and

challenged, as previously described. Twenty four hours after the final challenge, the impact of

mPGES1 synthase on inflammation of the airways, IgE production and airway mechanics was

assessed. Surprisingly, and contrary to our prediction based on analysis with the COX-1/COX-2

deficient mice, mice lacking mPGES1 had significantly less cellular infiltrate and associated

eosinophilia in comparison to OVA-challenged wildtype animals **(Fig 2.5A,B)**. A significant

decrease in IL-13 production was observed in the BALF of the mPGES1 -/- mice compared to

wildtype control animals **(Fig 2.5C)**. No difference in serum IgE levels was observed between

mPGES1-/- mice and their genetic controls **(Fig 2.5D)**. IL-4 production is critical for IgE isotype

switching (343), therefore we characterized IL-4 concentrations present in lung homogenate

following induction of allergy in this model **(Fig 2.5E)**. No difference was observed in the

production of this T$_H$2 cytokine between the inflamed lungs of wildtype and mPGES1 -/- mice. To

study this further, an additional experiment was carried out to examine proliferation and cytokine

production by splenocytes from immunized and challenged mice (**Fig 2.6**). The proliferative

response of the mPGES1 -/- cells to antigen did not differ significantly from that of control cultures

(**Fig 2.6A**), nor was a significant difference observed in the production of IFN-γ or IL-17a (**Fig

2.6C,D**). Similar to the BALF, a decrease in IL-13 levels was observed in these cultures (**Fig 2.6B**),

although in this case the decreased production by mPGES1 -/- cells did not achieve statistical

significance.

Changes in airway mechanics were determined, as previously described. As was the case for

the COX-1 and COX-2 deficient cohorts, inflammation and allergic airway disease induced using this

immunization protocol did not result in AHR in any parameter, either in the wildtype animals or in

the mPGES1 -/- line (**Fig 2.7**).

Effect of PGE$_2$ on lung inflammation in mice carrying a transgenic OVA- specific T cell receptor

The observation that IL-4 and IgE concentrations were unaffected by endogenous levels of

PGE$_2$ suggests that pro-inflammatory actions mediated by this prostanoid occur subsequent to the

sensitization phase of antigen. To explore this, we examined the induction of IgE and the response of

splenocytes to antigen in sensitized animals prior to challenge with aerosolized antigen (**Fig 2.8**). No

difference was observed in serum IgE between wildtype and mPGES1-deficient mice (**Fig 2.8A**).

Splenocytes isolated from wildtype and mPGES1 -/- animals a week after booster sensitization

demonstrate similar proliferative responses (**Fig 2.8B**) and no difference was observed in the

production of IL-13, IFN-γ, or IL-17a between groups (**Fig 2.8C-E**). Collectively, these results

indicate that the reduced inflammation observed in the mPGES1 -/- lungs is unlikely to reflect

alterations in the sensitization of mice to antigen, but rather the response of exposure of the lungs in

sensitized animals to antigen.

To explore this further, we determined whether loss of mPGES1 would alter the development of allergic lung disease in OT-II mice. These mice carry a transgenic T cell receptor specific to ovalbumin (344) and thus, exposure of the airways to this antigen results in inflammation in non-sensitized animals. In this case, however, the prominent cell type in the BALF is the neutrophil and thus this response is thought to model non-atopic allergic lung disease (345). Mice lacking mPGES1 were crossed to congenic B6 mice carrying the OT-II transgene to characterize the *in vivo* contribution of mPGES1 to the effector phase of allergic disease. As expected, mice lacking the transgene, OT-II (-), showed little inflammation in response to OVA challenge **(Fig 2.9A)**. In contrast, robust cell recruitment was observed in transgenic wildtype mice. Transgenic mPGES1 -/- animals showed a significant attenuation in cell infiltration compared to wildtype controls. This attenuation reflects significantly fewer granulocytes present in the BALF of the mPGES1-deficient animals **(Fig 2.9B)**. In addition, IL-13 levels were assessed. The levels of this cytokine in the BALF of wildtype animals was low, compared to that measured in OVA/alum sensitized animals, in agreement with the neutrophil-dominated response observed in this model. These levels were further reduced in animals lacking mPGES1, consistent with overall reduced levels of inflammation in the lungs of these mice (**Fig 2.9C**).

Individual contributions of lung and recruited inflammatory cells to PGE$_2$ mediated allergic responses

As shown above, mPGES1 contributes to the PGE$_2$ present in the healthy lung. To determine the relative contributions of mPGES1 produced by the lung and that produced by the recruited immune cells to this model of allergic lung disease, we studied the development of allergy in bone marrow chimeras. We first established that the contribution of mPGES1 to the allergic response was not altered in animals undergoing this experimental procedure. The difference in the OVA-induced

cellularity of the BALF between wildtype mice irradiated and reconstituted with wildtype marrow (WT→WT) compared to that observed in mPGES1 -/- animals irradiated and reconstituted with autologous marrow [knockout (KO→KO)] recapitulated the differences observed between these groups in previous experiments **(Fig 2.10 compared to Fig 2.5A)**.

We next asked whether PGE_2 produced by the immune cells recruited to the lung during allergic inflammation contributes to allergic airway disease. To address this, wildtype mice were irradiated and reconstituted with either wildtype (WT→WT) or mPGES1-/- (KO→WT) bone marrow. Reconstitution of mice with mPGES1-deficient bone marrow had only a modest impact on the inflammatory response; however, no statistically significant changes were seen in total BALF cellularity, eosinophil numbers, IL-13 production or serum total IgE **(Fig 2.11A-D)**.

Since the PGE_2 produced by recruited immune cells contributed little to the inflammation in the lung, we next addressed the possibility that the primary source of this pro-inflammatory PGE_2 was from the lung itself. We evaluated whether mPGES1 -/- animals reconstituted with wildtype bone marrow (WT→KO) would have attenuated allergy compared to animals in which all cells are capable of producing PGE_2 (WT→WT). Lung inflammation was attenuated in OVA sensitized and challenged WT→KO animals compared to animals in which both the lung and the bone marrow express mPGES1 (WT→WT). A significant decrease in BALF cellularity was observed, again primarily reflecting reduced recruitment of eosinophils **(Fig 2.12A, B)**. IL-13 production was also reduced in this group **(Fig 2.12C)**. These observations suggest that mPGES1 produced by cells in the lung, not recruited leukocytes, contributed to the development of allergic disease in response to ovalbumin. Consistent with the studies reported above, IgE levels were not significantly affected by loss of lung mPGES1 **(Fig 2.12D)**.

Figure 2.1 Effect of OVA Sensitization and Challenge on Inflammation in Congenic COX-1 and
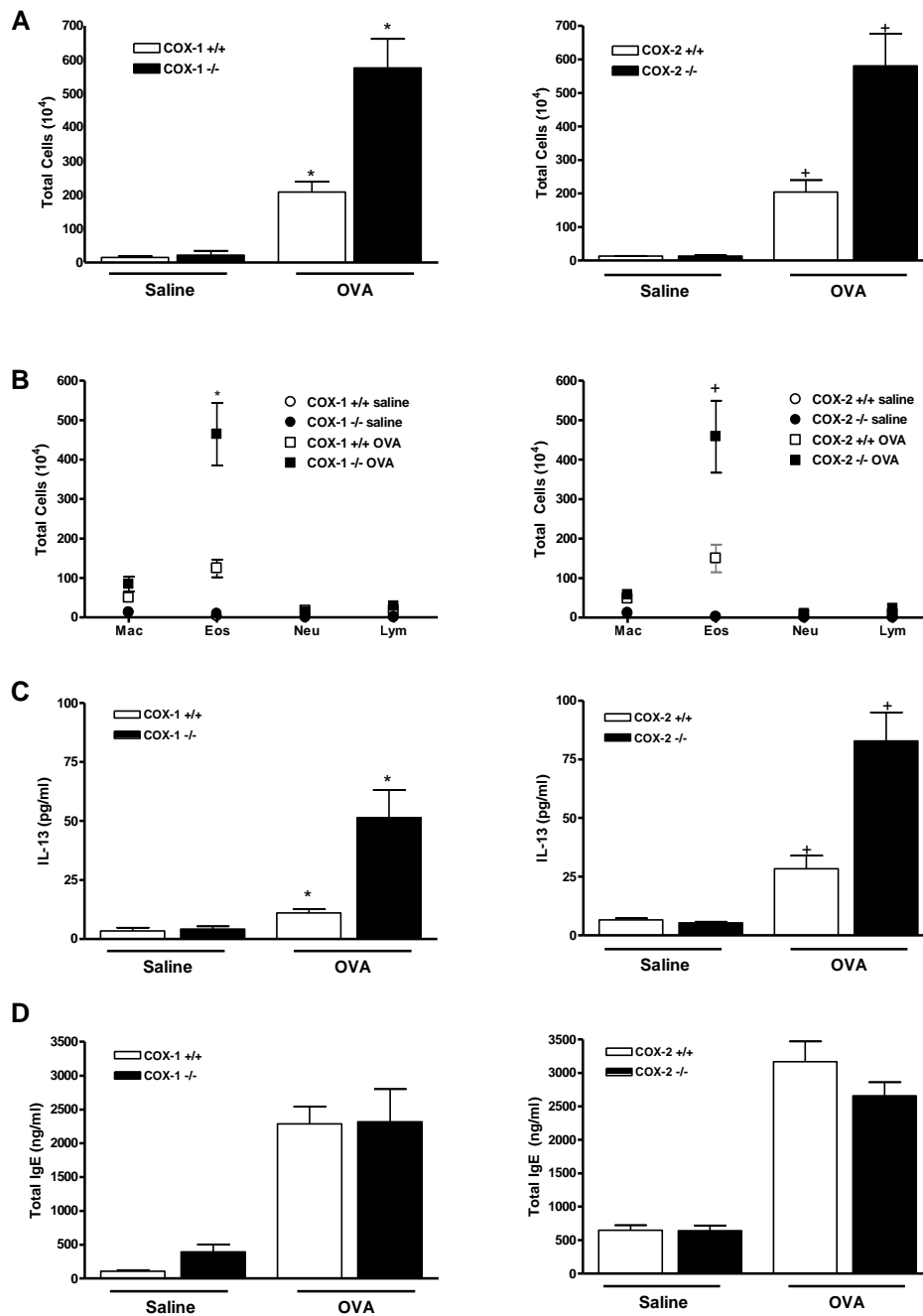
COX-2 -/- Mice

Figure 2.1 Effect of OVA Sensitization and Challenge on Inflammation in Congenic COX-1 and COX-2 -/- Mice Mice were sensitized i.p with OVA and alum and challenged with aerosolized antigen. Twenty-four hours after the final challenge serum IgE and lung inflammation were assessed. **A.** OVA exposed animals display an increase in the number of cells present in the BALF. Higher total cell counts are observed in the BALF collected from mice lacking either COX-1 or COX-2 compared to genetically matched controls (*,+ $p<0.001$). **B.** Cell differentials determined for the BALF showed that this increase is due primarily to an increase in the number of eosinophils. A significant increase in eosinophil numbers is observed in the wildtype OVA treated animals and this is further augmented in the COX-1 -/- and COX-2 -/- animals relative to the OVA treated controls (* $p<0.001$, + $p<0.05$). **C.** IL-13 levels in the BALF of the COX-1 -/- and COX-2 -/- mice are also significantly higher than levels measured in the OVA-treated wildtype controls (* $p<0.01$, + $p<0.001$). **D.** OVA sensitization and challenge leads to increased total serum IgE, however, IgE levels do not differ significantly between COX deficient and control animals. (For COX-1: +/+ saline n=5, -/- saline n=4, +/+ OVA n=8, -/- OVA n=9; for COX-2: +/+ saline n=5, -/- saline n=4, +/+ OVA n=11, -/- OVA n=12)

Figure 2.2 Measurements of Airway Mechanics in COX-1 -/- and COX-2 -/- Mice
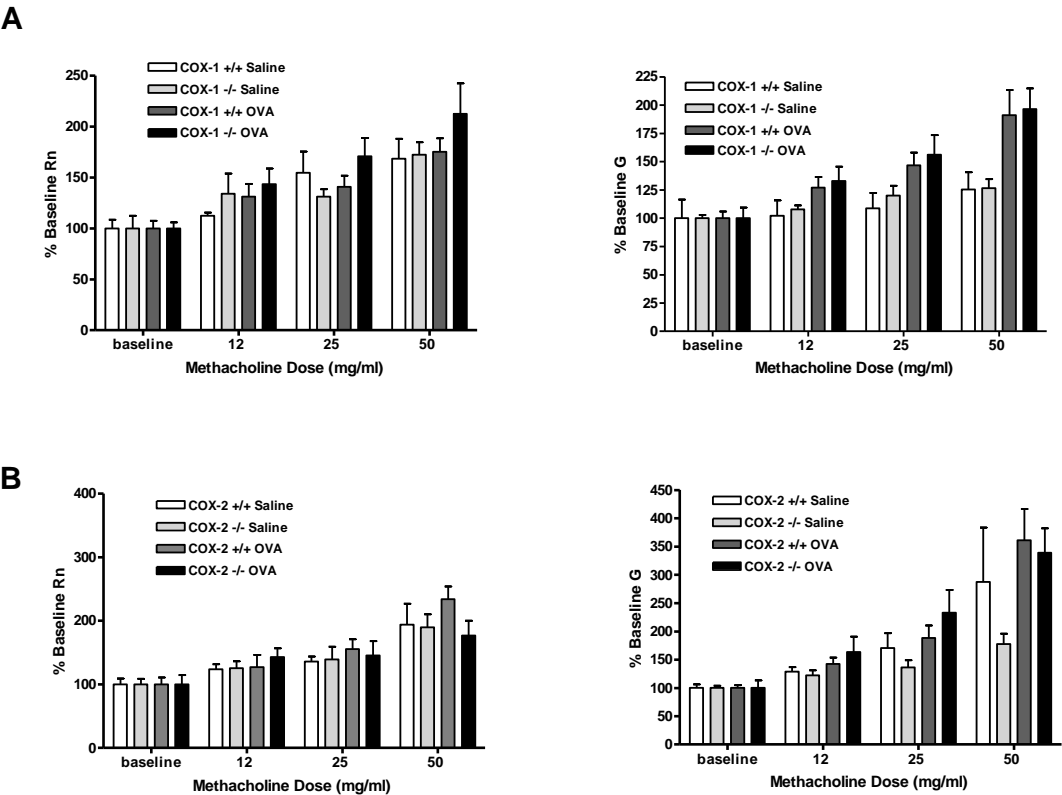
Figure 2.2 Measurements of Airway Mechanics in COX-1 -/- and COX-2 -/- Mice Airway

mechanics were assessed in COX-1 -/- mice and their congenic controls **(A)** and COX-2 -/- mice and

their congenic controls **(B).** Anesthetized and intubated mice, attached to a computer-controlled

small-animal ventilator, were exposed to increasing concentrations of aerosolized Mch and central

airway resistance (Rn) and tissue damping (G) were assessed. No significant differences are observed

between any groups at any concentration for either parameter. (COX-1 data representative of 2

independent experiments: +/+ saline n=7, -/- saline n=8, +/+ OVA n=15, -/- OVA n=13; for COX-2:

+/+ saline n=5, -/- saline n=4, +/+ OVA n=11, -/- OVA n=12)

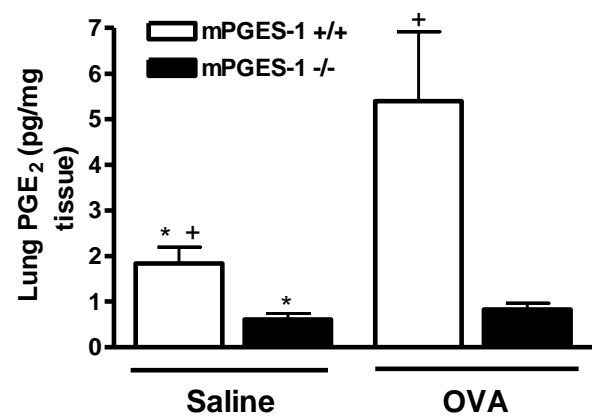**Figure 2.3 PGE₂ Production by the Naïve and Allergic mPGES1 -/- Lung**

Figure 2.3 PGE$_2$ Production by the Naïve and Allergic mPGES1 -/- Lung PGE$_2$ levels were measured in lung homogenate, prepared from naïve mice or after sensitization and challenge with OVA. In the naïve lung, concentrations of PGE$_2$ are significantly attenuated in mPGES1 -/- mice relative to wildtype mice (* $p<0.02$). PGE$_2$ levels increase significantly in lungs collected from mice sensitized and challenged with OVA (+ $p<0.05$). In contrast no significant increase in PGE$_2$ is observed in mPGES1 -/- mice. (mPGES1: +/+ saline n=5, -/- saline n=5, +/+ OVA n=7, -/- OVA n=11)

Figure 2.4 Production of PGE$_2$ in the Lungs of COX-1 and COX-2 Deficient Animals
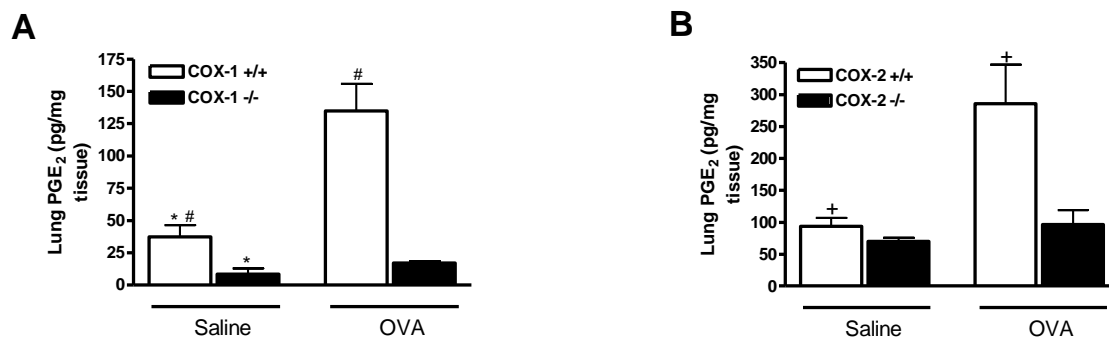
Figure 2.4 Production of PGE$_2$ in the Lungs of COX-1 and COX-2 Deficient Animals PGE$_2$ levels were assessed in lung homogenate obtained from naïve or OVA sensitized and challenged COX-1 -/- **(A)** or COX-2 -/- **(B)** mice and their congenic controls. **A.** The level of PGE$_2$ measured in the lungs of naive COX-1 -/- mice is reduced compared to congenic wildtype controls (*$p<0.05$). Following challenge with antigen in sensitized animals, wildtype mice have substantially elevated concentrations of lung PGE$_2$ (# $p<0.01$). No PGE$_2$ augmentation is observed in the COX-1 -/- inflamed lung. **B.** Naïve COX-2 -/- animals and their congenic controls have similar levels of PGE$_2$ measured in lung homogenate. Wildtype animals sensitized and challenged with OVA experience a significant enhancement of lung PGE$_2$ compared to saline treated controls (+ $p<0.05$). No elevation of PGE$_2$ is measured in the homogenate of inflamed lungs obtained from COX-2 -/- mice. (For COX-1: +/+ saline n=3, -/- saline n=3, +/+ OVA n=5, -/- OVA n=6; For COX-2: +/+ saline n=3, -/- saline n=3, +/+ OVA n=4, -/- OVA n= 6).

Figure 2.5 Inflammatory Response in mPGES1 -/- Mice Sensitized and Challenged with OVA
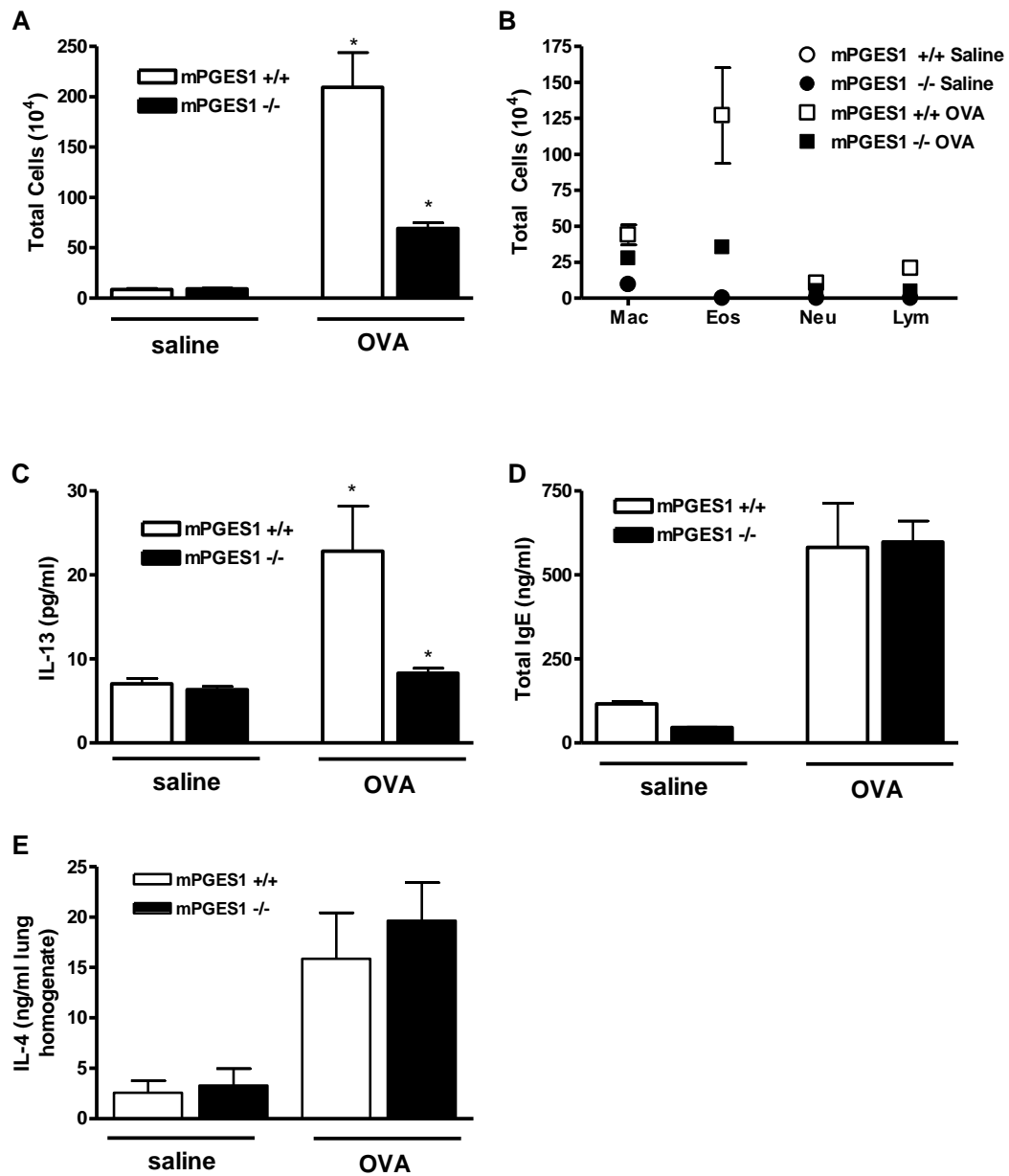
Figure 2.5 Inflammatory Response in mPGES1 -/- Mice Sensitized and Challenged with OVA **A.**
OVA sensitization and challenge leads to an increase in total cells present in the BALF of wildtype
mice. While increased numbers of cells are also observed in BALF collected from mPGES1 -/-
animals, the total cell count is significantly reduced compared to similarly treated wildtype control
animals (* $p < 0.001$). **B.** The decrease in the cellularity of the BALF of the mPGES1 -/- mice
correlates with a significant decrease in the number of eosinophils in the BALF of these animals
compared to the numbers present in the BALF from the control animals (* $p < 0.001$). **C.** IL-13 levels
in the BALF collected from OVA sensitized and challenged animals are significantly higher than
those measured in saline treated cohorts, both wildtype and mPGES1 -/- animals, however, higher
levels are observed in the samples collected from OVA treated wild type animals relative to levels in
BALF from mPGES1 -/- animals (* $p < 0.05$). **D.** OVA sensitization and challenge results in an
increase in total serum IgE of a similar magnitude in wildtype and mPGES1 -/- animals. **E.** IL-4
levels in whole lung homogenates do not differ significantly between samples prepared from
mPGES1-/- mice and controls. As expected both groups showed levels elevated in comparison to
samples prepared from saline treated cohorts. (For A-D: mPGES1 +/+ saline n=4, -/- saline n=3, +/+
OVA n=10, -/- OVA n=11; For E: mPGES1 +/+ saline n=2, -/- saline n=3, +/+ OVA n=5, -/- OVA
n=5)

Figure 2.6: Ex Vivo mPGES1 Splenocyte Responses in Antigen Challenged Mice
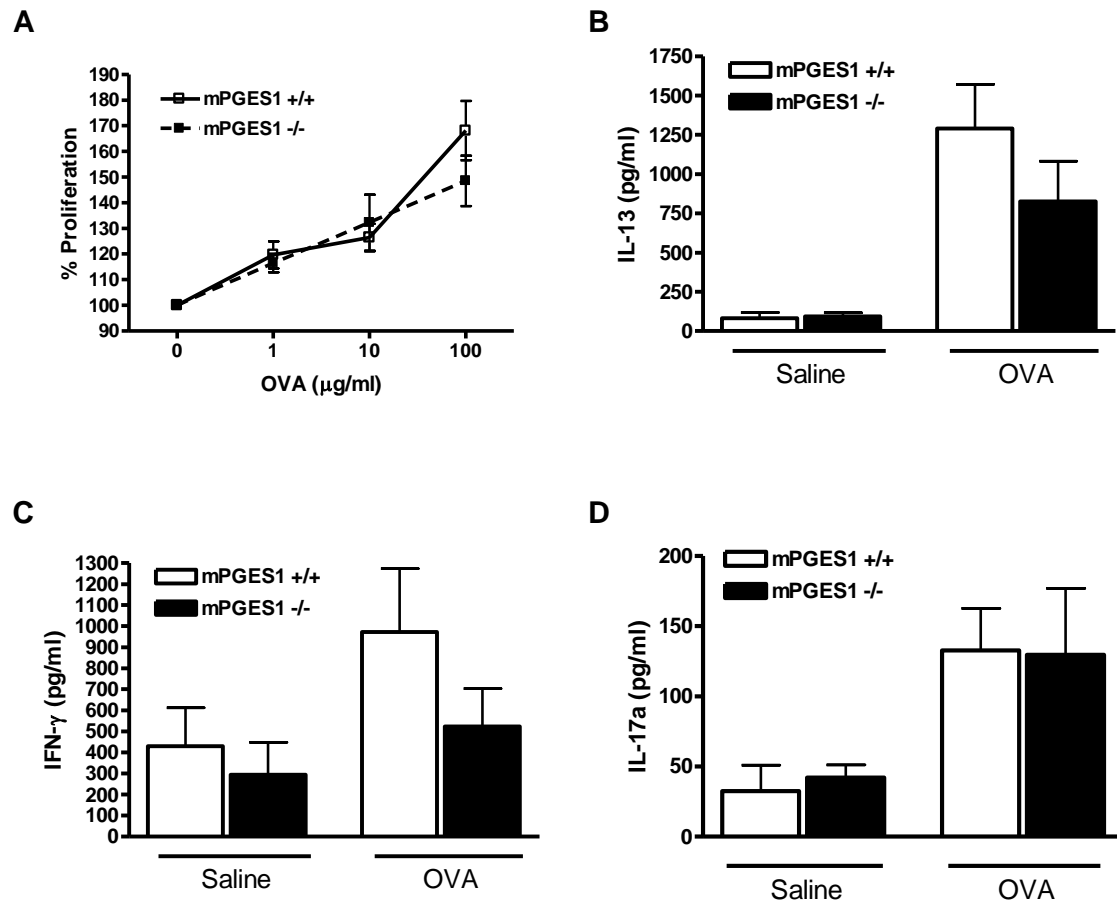
Figure 2.6: Ex Vivo mPGES1 Splenocyte Responses in Antigen Challenged Mice Wildtype and

mPGES1 -/- animals were sensitized and challenged with OVA antigen as described. 24 hours after

the final challenge, mice were euthanized and spleens were collected. **A.** Splenocytes were plated in

the presence of increasing concentrations of OVA. Proliferation was assessed using WST-1 reagent

after 72 hours. No significant difference in proliferation is observed between mPGES1-deficient

animals and wildtype controls. **B-D** Splenocytes were plated in media containing 100 μg/ml OVA.

Following a 72 hour incubation period, cell supernatants were collected and IL-13 **(B)**, IFN-γ **(C)**,

and IL-17a **(D)** cytokine levels were measured. OVA restimulation results in elevated cytokine levels

from the splenocytes of challenged animals. While a trend towards attenuation is observed in IL-13

levels measured from mPGES1 -/- mice, supernatant levels do not differ significantly between

challenged groups for any cytokine. (For A: mPGES1 +/+ n=4, mPGES1 -/- n=4; For B-D mPGES1:

+/+ saline n=4, -/- saline n=4, +/+ OVA n=9, -/- OVA n=7)

Figure 2.7 Evaluation of Airway Hyperresponsiveness in mPGES1 -/- Mice
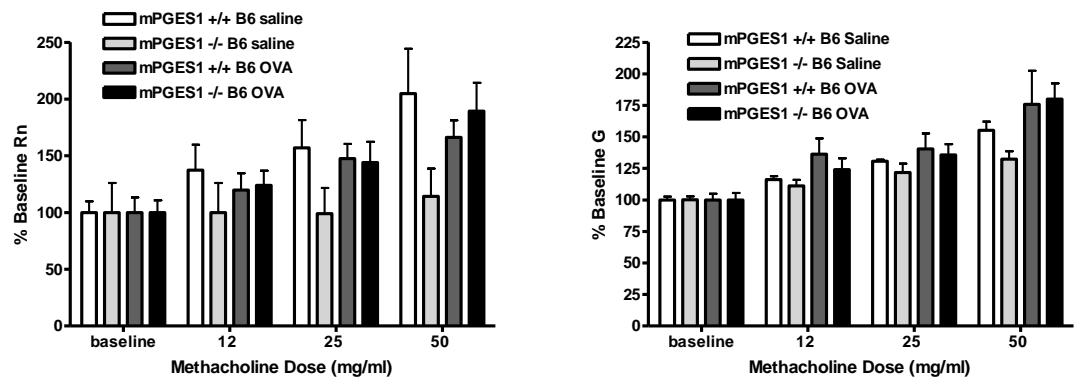
Figure 2.7 Evaluation of Airway Hyperresponsiveness in mPGES1 -/- Mice Increasing doses of aerosolized methacholine were administered to anesthetized and intubated mice attached to a computer-controlled small-animal ventilator to assess AHR in mPGES1 -/- mice. No significant differences are observed between any groups, at any concentration, for Rn or G (mPGES1: +/+ saline n=4, mPGES1 -/- saline n=4, mPGES1 +/+ OVA n=9, mPGES1 -/- OVA n=11)

Figure 2.8 Contribution of PGE$_2$ to Proliferation of Sensitized Splenocytes
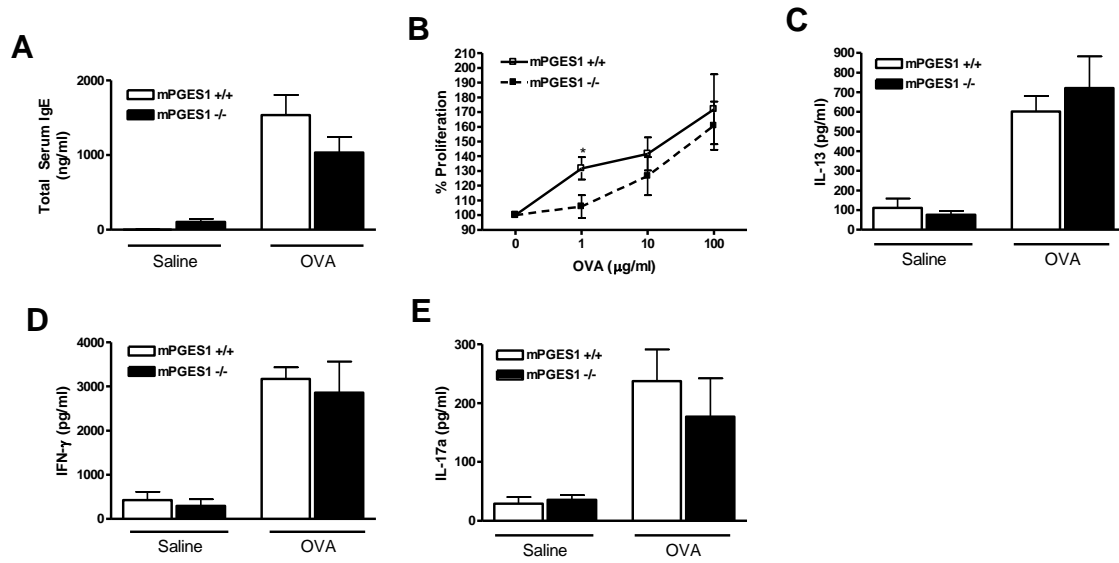
Figure 2.8 Contribution of PGE$_2$ to Proliferation of Sensitized Splenocytes Mice were sensitized on days 0 and 14 with OVA and one week later serum and splenocytes were collected. **A.** Sensitization with antigen results in elevated serum IgE in wildtype and mPGES1 -/- animals. This augmentation does not differ significantly between groups. **B.** Splenocytes were plated in media supplemented with increasing concentrations of OVA. Following 72 hours, cell proliferation was assessed by WST-1 reagent. Proliferation of splenocytes from mPGES1 -/- populations is significantly reduced at the 1 μg/ml OVA dose measured by t test analysis (*$p < 0.05$); however F test analysis reveals no overall differences in the proliferative response. **C-E.** Splenocytes from OVA and saline sensitized animals were plated with 100 μg/ml OVA. Following culture for 72 hours, cell supernatants were collected and IL-13 **(C)**, IFN-γ **(D)**, and IL-17a **(E)** levels were assessed.   Cytokine levels are elevated in both mPGES1 +/+ and mPGES1 -/- supernatants and these levels of augmentation do not differ significantly. (For A: mPGES1: +/+ saline n=4, -/- saline n=4, +/+ OVA n=6, -/- OVA n=7; For B mPGES1: +/+ OVA n=5, mPGES1 -/- n=7; For C and E mPGES1: +/+ saline n=6, -/- saline n=6, +/+ OVA n=5, -/- OVA n=7; For D mPGES1: +/+ saline n=4, -/- saline n=4, +/+ OVA n=4, -/- OVA n=4)

**Figure 2.9 OVA-induced Allergic Inflammation in mPGES1-/- Mice Carrying an OVA-specific**
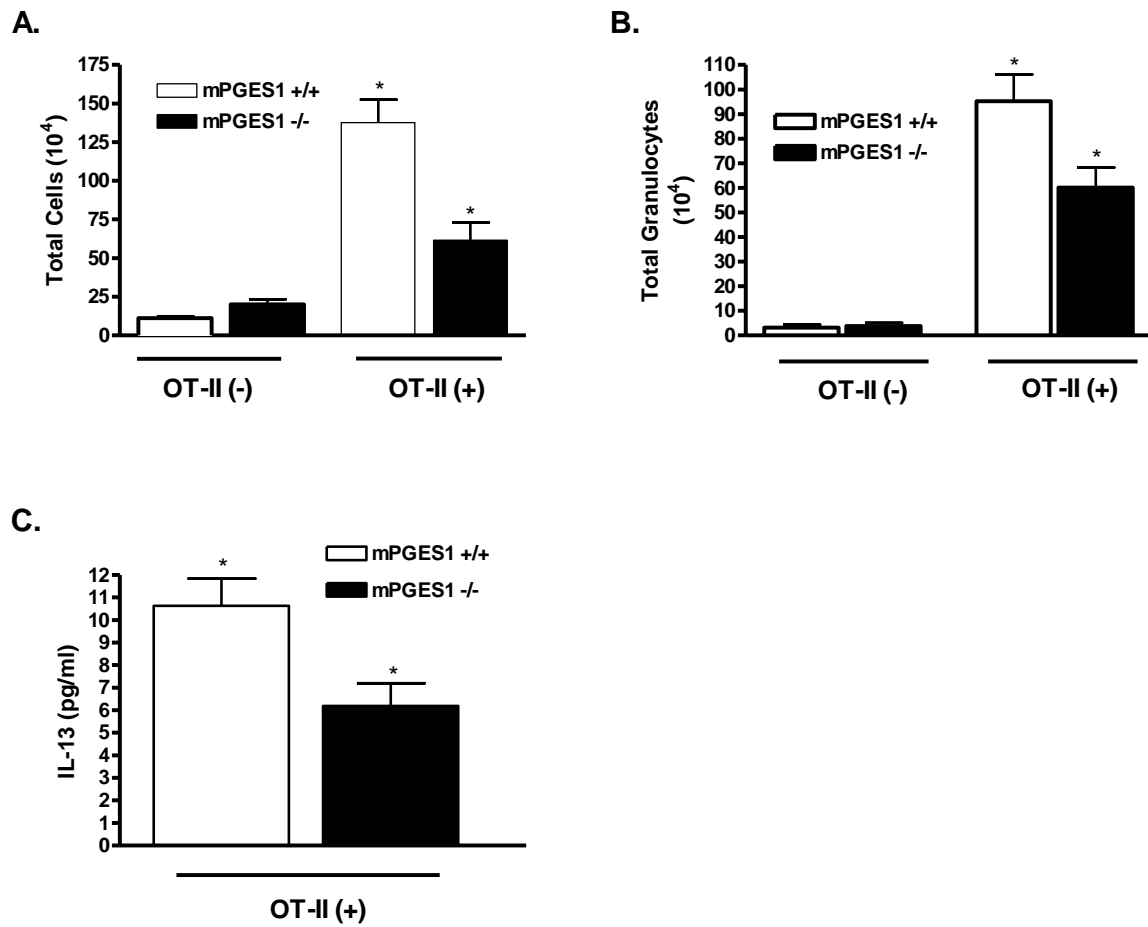
Transgene

Figure 2.9 OVA-induced Allergic Inflammation in mPGES1-/- Mice Carrying an OVA-specific

Transgene OT-II mice, mPGES1-/- mice, OT-II/mPGES1-/- mice and wild type animals were

challenged with aerosolized OVA and the development of lung inflammation was assessed. **A**.

BALF was collected 24 hours after the final challenge. Antigen challenge results in an increase in the

number of cells in the BALF of both OT-II expressing cohorts, however, the total number of cells is

significantly lower in the BALF from the OT-II/ mPGES1 -/- animals compared to OT-II/mPGES1

+/+ mice. (* $p < 0.001$) **B.** Differential cell analysis demonstrates that transgenic animals have

elevated granulocyte numbers measured in their BALF. Mice lacking mPGES1 had significantly

fewer granulocytes, compared to wildtype transgenic animals. (* $p < 0.05$) **C.** BALF IL-13 levels are

also significantly lower in samples collected from OT-II/mPGES1 -/- animals compared to OT-

II/mPGES1 +/+ animals expressing mPGES1 ($p < 0.02$). (For A: mPGES1 +/+ n=3, mPGES1 -/- n=4,

OT-II/ mPGES1 +/+ n=12, OT-II/mPGES1 -/- n=12; For B: mPGES1 +/+ n= 4, mPGES1 -/- n=5,

OT-II/mPGES1 +/+ n=6, OT-II/mPGES1 -/- n=9; For **C**, OT-II/ mPGES1 +/+ n=7, OT-II/mPGES1 -

/- n=7)

Figure 2.10 Development of Allergic Inflammation in mPGES1 Bone Marrow Chimeras
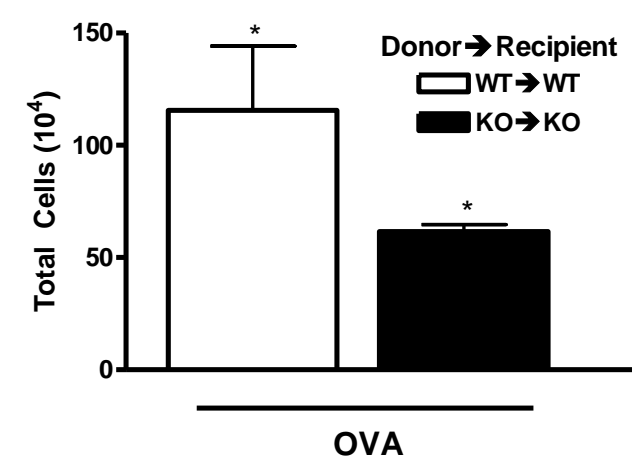
Figure 2.10 Development of Allergic Inflammation in mPGES1 Bone Marrow Chimeras Wildtype and mPGES1 -/- mice exposed to lethal doses of radiation, were reconstituted with bone marrow from autologous donors. Eight weeks after reconstitution, mice were sensitized and challenged with OVA. Wildtype mice reconstituted with wildtype bone marrow (WT→WT) have significantly elevated cell counts relative to mPGES1 -/- mice reconstituted with mPGES1 -/- bone marrow (KO→KO) (p=0.05). (For WT→WT n= 6; For KO→KO n=8)

Figure 2.11 Contribution of PGE$_2$ from Bone Marrow Derived Cell Populations to OVA-induced Lung Inflammation
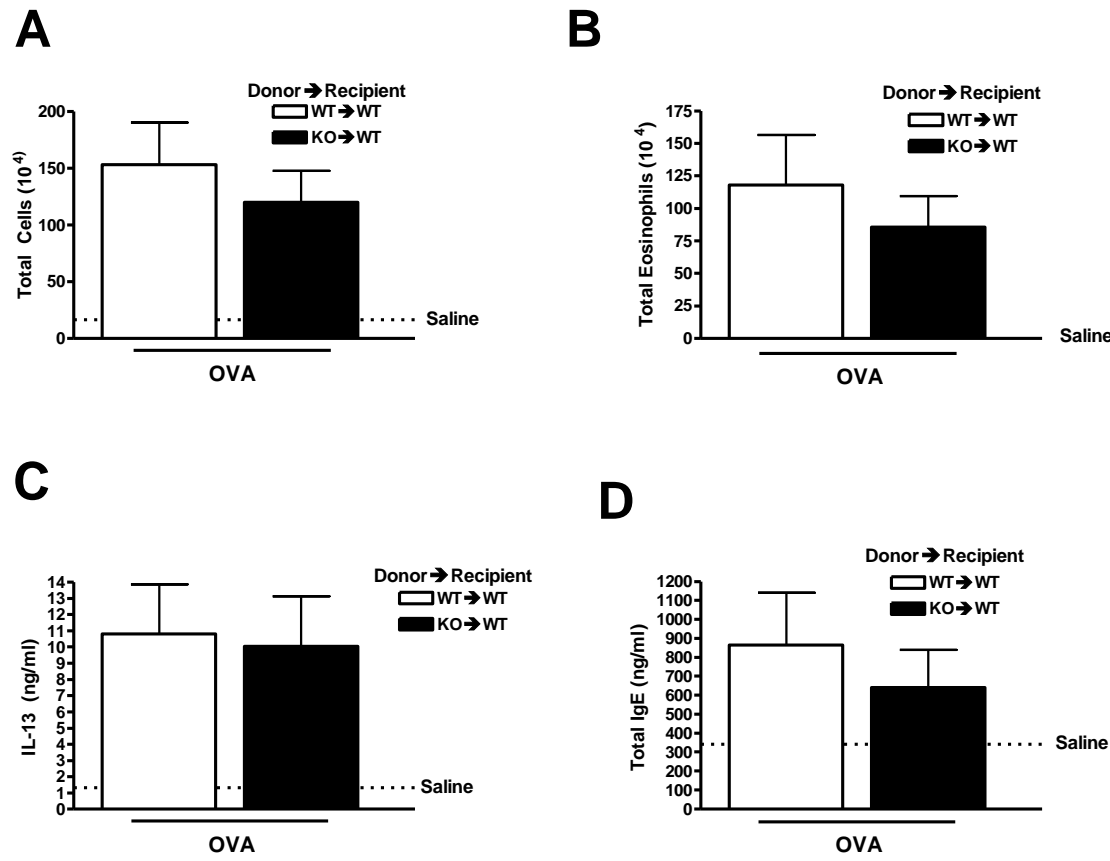
Figure 2.11 Contribution of PGE$_2$ from Bone Marrow Derived Cell Populations to OVA-induced

Lung Inflammation Lethally irradiated wildtype mice were reconstituted with either wildtype bone

marrow (WT→WT) or mPGES1 -/- bone marrow (KO→WT). **A.** As expected, an increase in the

cellularity of the BALF is observed in samples collected from the animals sensitized and challenged

with ovalbumin. No significant difference is measured in the total number of cells present in the

BALF of the two groups (WT→WT versus KO→WT). **B.** Morphological analysis of cell types

present in BALF revealed elevated levels of eosinophils in both OVA-treated groups and again the

numbers of these cells did not differ significantly between the animals that had received the wild type

versus the mPGES1 -/- marrow. **C.** No difference is observed in the level of IL-13 in the BALF of

the two OVA-treated groups. **D.** Total serum IgE concentrations are elevated to a similar degree in

groups sensitized and challenged with OVA. (For WT→WT: saline n=3, OVA n=8; KO→WT: saline

n=2, OVA n=8)

Figure 2.12 Contribution of PGE₂ Produced by Radiation Resistant Lung Populations to

Allergic Inflammation
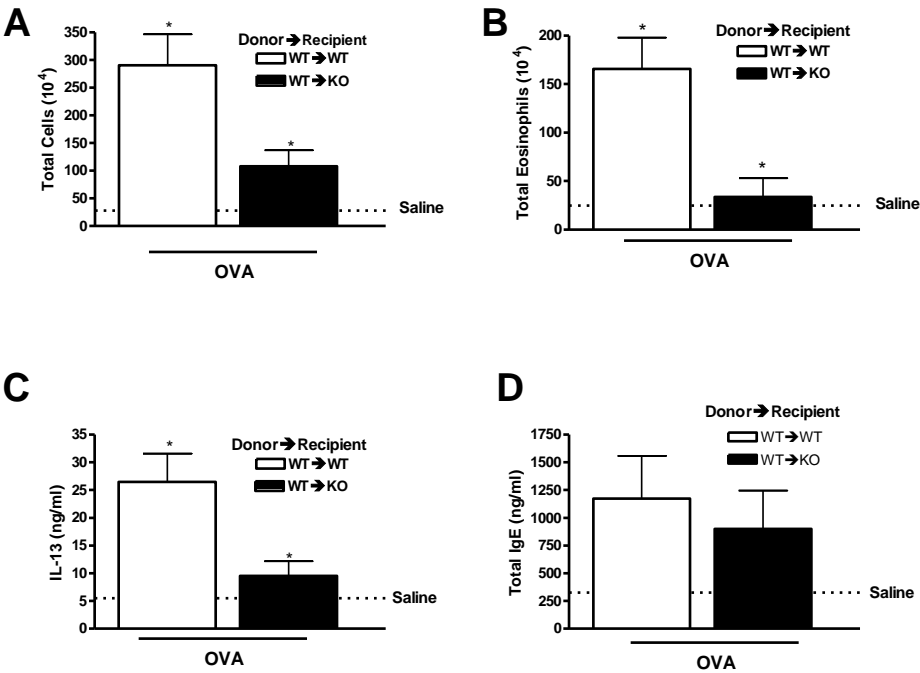
Figure 2.12 Contribution of PGE₂ Produced by Radiation Resistant Lung Populations to Allergic

Inflammation Wildtype mice or mPGES1 -/- mice exposed to lethal doses of radiation were

reconstituted with wildtype bone marrow, (WT→WT) and (WT→KO) respectively. BALF was

collected from OVA sensitized and challenged animals and total cell numbers **(A)** and cell

differentials **(B)** were determined. A decrease in both the total cell count and the number of

eosinophils in the BALF is observed in samples from mPGES1 -/- mice reconstituted with wildtype

marrow, compared to samples from similarly reconstituted and treated wildtype animals (WT→WT)

(* $p<0.01$). **C.** IL-13 levels are significantly higher in the BALF from OVA-sensitized and

challenged WT→WT mice compared to levels in BALF from similarly treated mPGES1-/- animals

that received wildtype bone marrow (WT→KO) (*$p<0.05$). **D.** Total serum IgE concentrations are

elevated to similar levels following OVA sensitization and challenge in both WT→WT and WT→KO

animals. (For WT→WT: saline n=4, OVA n=8; WT→KO: saline n=4, OVA n=7)

Discussion

Previous studies have shown that in the absence of COX-1 or COX-2, antigen exposure results in more severe allergic lung disease (182). Using mice lacking mPGES1 synthase, we show that attenuation of PGE_2 synthesis in the COX-1 and the COX-2 deficient mice does not account for the increase in disease observed in these mouse lines. In fact, in this model mPGES1 deficient mice showed reduced airway inflammation, indicating that PGE_2 enhances this aspect of allergic disease.

The impact of the genetic composition of mouse lines on the development of various aspects of allergic lung disease has been well established (346-349). The majority of the early studies assigning roles for COX-1 and COX-2 metabolites in inflammatory responses were carried out using mice of mixed genetic background, thus the representation of B6 and 129 genes in the COX deficient and control animals can be very different. We therefore first verified that the protection that COX-1 and COX-2 provided in this response could be observed when congenic animals were studied. Consistent with previous work, we report that both COX-1 and COX-2 dependent prostaglandins limit allergic inflammation in the lung. However, we show that both enzymes provided the mice with a similar level of protection. This differs from previous studies in which loss of COX-1 was reported to have a greater role than COX-2 both in production of PGE_2 in the naïve and inflamed lung as well as in limiting allergic inflammation (182). Since COX-1 and COX-2 have unique but overlapping patterns of expression and, depending on the cell type, can lead to the preferential production of a particular eicosanoid, this observation suggests that multiple prostaglandins or prostaglandins made by different cells types limit inflammation in this model.

We saw no development of AHR in either the COX-1 or the COX-2 deficient animals. This is not surprising given the genetic background of the mice. AHR is often absent in B6 mice (346, 347, 349). In previous studies, inflammation associated with loss of COX-1 but not COX-2 was

reported to result in increased sensitivity to methacholine (182). It is possible that this difference reflected differences in the segregation of 129 and B6 alleles in the two populations. This would be expected, as the closure of the ductus arteriosus in mice lacking EP_4 or COX-2 depends on the inheritance of a particular compliment of 129 and B6 alleles (190, 350). In contrast, no such selective pressure would skew inheritance of alleles in the COX-1 population.

Early work suggested that PGE_2 could play an important role in regulating the differentiation of mouse B lymphocytes to IgE secreting cells (351). However, this role was not supported by the report that IgE levels were actually higher in the COX-1 and COX-2 antigen treated animals (182). Our study did not observe this increase in the COX-1 and COX-2 deficient animals compared to their genetically matched controls and therefore does not support a role for PGE_2 in switching B cells to IgE production. No difference was noted in serum IgE levels between COX-1 -/-, COX-2 -/- or mPGES1 -/- and their control animals after induction of a T_H2 response. However, direct comparison of the IgE response of COX deficient animals reported here and those reported previously is difficult for a number of reasons. Not only do our studies utilize congenic mice, the cohort examined here were between 8 to 12 weeks of age while previous studies examined mice that ranged in age from 5 and 9 months. In addition, these studies evaluated IgE levels in the BALF, while we examined serum IgE.

In patients with allergic asthma, inhaled PGE_2 is reported to attenuate both the early and late phase response after exposure to antigen (302, 310-312). PGE_2 has also been shown to limit inflammation in animal models of asthma (313, 314, 352). Given this, it seemed likely that the heightened inflammation observed in the COX deficient mice reflected a loss of this protective prostanoid. Indeed, induction of allergic disease with ovalbumin dramatically increased PGE_2 levels in the lung and this augmentation was not observed when COX-1, COX-2, or mPGES1 was absent,

suggesting that both enzymes are capable of coupling with mPGES1 to promote prostanoid production during lung inflammation. However, unlike a genetic loss of COX enzymatic activity, a loss of mPGES1 did not result in heightened disease, in fact, quite the opposite; loss of this pathway attenuated the inflammatory response. Instead, our results are consistent with a model in which the primary protective COX dependent eicosanoid is prostacyclin, not PGE_2. Mice lacking the I-prostanoid (IP) receptor, specific for prostacyclin, were reported to have more severe allergic inflammation in the lung (323). Prostacyclin, but not PGE_2 was also shown to protect against the development of fibrosis in the bleomycin model of idiopathic pulmonary fibrosis (278), suggesting that, at least in the rodent lung, this might be the most important anti-inflammatory prostanoid.

We cannot rule out the possibility that the lack of a protective role for PGE_2 in this study is specific to this particular model and immunization protocol. A recent study examining the function of mPGES1 in a house dust mite antigen (Der f)-induced allergic model reported that PGE_2 limited vascular changes associated with chronic exposure to antigen while decreased PGE_2 had no significant impact on total recruitment of inflammatory cells to the lungs after Der f challenge (321). However, the vascular remodeling which this study showed was enhanced in the mPGES1 -/- mice is associated with chronic models of asthma and is not apparent in the acute model used in our study, preventing extension of this finding to this model of allergic lung disease. In contrast to our findings, decreased PGE_2 had no significant impact on recruitment of inflammatory cells to the lungs after Der f challenge. Again this difference might reflect different roles for PGE_2 in an acute allergic response, such as that induced by ovalbumin and adjuvant, versus a chronic model established by inhalation of a complex antigen with intrinsic ability to activate the innate immune response. Alternatively, it could reflect the fact that the mPGES1 -/- animals were compared to purchased wildtype B6 mice, whereas both the mPGES1 -/- and wildtype mice used in our studies were bred in the same facility, as studies have highlighted the importance of environmental factors, including the microbiome, in molding the

immune response (353-356) and it is possible that some phenotypes reflect such differences in addition to the genetic lesion under study.

Both our findings and the phenotype of mPGES1 -/- animals in the Der f allergic model do not support early reports of heightened inflammation in EP_3 -/- mice sensitized and challenged with ovalbumin (320). The reason for this discrepancy is not apparent, however, we have been unable to reproduce this finding using B6 congenic EP_3 -/- mice (unpublished data). Furthermore, previous work in our lab has indicated that PGE_2, through the EP_3 receptor, can promote inflammation by augmenting IgE mediated mast cell degranulation and in some circumstances PGE_2 alone is sufficient to mediate this response in rodents (258, 259).

Much of the support for the hypothesis that PGE_2 plays a protective role in the lung, limiting inflammation, comes from studies in which exposure of mice to antigen is accompanied by inhalation of PGE_2, its stable analog, or a PGE_2 receptor preferring antagonist and agonist (314, 352, 357). *In vitro* studies have reinforced this hypothesis, with studies such as those which have shown PGE_2 to be effective in limiting migration of eosinophils and increasing production of IL-10 by dendritic cells and naïve T cells (329, 357, 358). However, extrapolating findings from either or both of these types of studies to develop models which predict the contribution of PGE_2 to inflammatory responses *in vivo* has proven difficult. Some of this difficulty is related to the fact that very few of the pathways attributed to PGE_2 through pharmacological studies with inhaled PGE_2 or PGE_2 receptor preferring agonists/antagonists are supported by evaluation of mice lacking specific PGE_2 receptors or combination of receptors. In some cases, the discrepancies may reflect the effective dose and specificity of the reagents used. For example, early studies assigning anti-coagulatory properties to PGE_2 were later shown to reflect the ability of PGE_2 at concentrations used in these studies to activate the prostacyclin receptor (359). Thus it is possible that some of the protective actions of inhaled

PGE$_2$ and EP receptor agonist are incorrectly assigned to the PGE$_2$ pathway. Carrying out these experiments in mice lacking the IP receptor and or EP receptors should resolve many of these issues. In some cases, inconsistency between results obtained using the various approaches might simply reflect the fact that loss of a PGE$_2$ receptor may have far less consequence for the organism than stimulation of the same pathway, due to compensatory pathways active *in vivo*. For example, stimulation of naïve T cells with PGE$_2$ *in vitro* can inhibit production of a pro-inflammatory cytokines, such as IFN-λ (358), but *in vivo*, the absence of PGE$_2$ does not necessarily lead to altered expression of this cytokine following stimulation (321), emphasizing the point that many other inflammatory mediators, distinct from PGE$_2$, can activate the same downstream pathways to upregulate responses. Inhaled PGE$_2$ through the EP$_2$ receptor limits airway constriction to methacholine (319). However, in mice with inflamed airways, the dose response curve is not shifted to the left in mice lacking EP$_2$ (unpublished data), suggesting that in the inflamed airway other pathways available are capable of regulating airway tone.

Not only were we unable to assign a protective role to PGE$_2$, our studies indicate a novel role for PGE$_2$: in some allergic responses PGE$_2$ acts as a pro-inflammatory mediator, enhancing inflammation in the lung. To further define this pro-inflammatory action of PGE$_2$, we generated bone marrow chimeras, animals in which either the lung or the recruited immune cells were deficient in the enzyme. The results from studies with these animals indicated that PGE$_2$ produced by the lung, rather than from the recruited immune cells, contributed to the inflammatory response. Furthermore, PGE$_2$ does not alter the development of antigen specific T and B cell populations, but rather plays a role either in the expansion of these populations after challenge or in the recruitment of the cells to the lung. This interpretation was supported by study of mPGES1 deficient animals carrying an ovalbumin specific transgene. Loss of PGE$_2$ synthesis limited the development of inflammation when these animals were challenged with antigen, implicating PGE$_2$ in the effector phase of this

response in the lung. The lack of a role for PGE_2 in the sensitizing phase of the allergic response correlates well with the studies of these mice in the Der f allergic model (321). No difference was observed in the repertoire of T cells elicited by this antigen.

We cannot yet identify precise mechanisms by which PGE_2 contributes to the inflammatory response in the lung. As discussed above, PGE_2 can augment mast cell degranulation *in vitro* and *in vivo* and this action is mediated through the EP_3 receptor (258, 259), suggesting that perhaps PGE_2 augments inflammation by increasing the release of mediators from these cells. However, the immunization protocol used here is not mast cell dependent (158), making it unlikely that this effector cell contributes substantially to the inflammatory response. PGE_2 can also increase vascular permeability and thus increase vascular leakage and formation of inflammatory exudates (211). For example, instillation of PGE_2 was reported to increase migration of neutrophils into airways in response to complement exposure (360). This response was attributed to vascular changes as it was attenuated by treatment with a vasoconstrictor. PGE_2 has been reported to influence many aspects of epithelial cell physiology, including chemokine and cytokine profiles, release of mucins, ion transport and ciliary beat (361-365). For instance, PGE_2 can stimulate the release of IL-6 from many cell types (259, 361, 366, 367) and IL-6 can contribute to inflammation in some allergic models (367, 368). Additional experiments will be required to define precisely the circumstances and the mechanism by which inhibition of PGE_2 limits disease in this allergic lung.

In summary, our studies show that loss of mPGES1, the primary enzyme required for production of PGE_2 from COX-1 and COX-2 metabolites, is not required for T_H2 polarization following sensitization of mice to OVA. However, while PGE_2 has largely been considered protective, playing a role in limiting the inflammatory response during the effector phase to inhaled allergens, we show that under some circumstances, this is not the case. Acute inflammation in

response to ovalbumin is attenuated in mice with decreased levels of PGE_2, both in mice carrying an OVA specific transgene and in mice sensitized by exposure to antigen in the presence of adjuvant. These findings emphasize the complexity of the role for this prostanoid in immune responses and underscore the challenges of targeting PGE_2 and its receptors in the treatment of lung diseases.

CHAPTER III

PROSTACYCLIN ALTERS THE FUNCTIONS OF LEUKOCYTES TO ATTENUATE ALLERGIC LUNG INFLAMMATION

Prostacyclin (PGI_2) is one of 5 bioactive prostanoids produced from the enzymatic conversion of C20-unsaturated fatty acid. Synthesis is initiated by the actions of prostaglandin-endoperoxide synthases (cyclooxygenase, COX) on free arachidonic acid (AA) released from phospholipids by phospholipase A_2 (PLA_2) (325). COX-1 is responsible for the majority of basal level prostanoid production. Constitutive expression of this isoform is present in most cell types; however COX-1 expression can be upregulated and can contribute to inflammatory processes. In mice, COX-1 is the important isoform for mediating ear edema following treatment with AA (334). Conversely, expression of COX-2 is undetectable in most cells during homeostasis and is instead induced in response to a diverse range of stimuli including growth factors, LPS, and IL-1. Constitutive expression of COX-2 is detected, however, in multiple tissues of human and mice including the lung, brain, and testes (369). Both COX enzymes function by converting AA into the unstable intermediate products prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2). Pathway specific syntheses complete the conversion of prostanoids from these intermediates and prostaglandin I synthase (PGIS) is responsible for the final step in PGI_2 production (370). The autocrine and paracrine functions of PGI_2 are mediated through specific binding to the G_s-coupled IP receptor and upregulation of cAMP before this prostanoid is rapidly metabolized to its inactive byproduct 6-keto-$PGF_{1\alpha}$ (327).

PGI$_2$ is detected at high levels in the lung and production of this prostanoid is assigned to multiple cell types, most notably to endothelial cells (371). Consistent with this observation, PGI$_2$ contributes significantly to vasodilation and prevention of thrombosis (372). While PGI$_2$ synthesis promotes inflammation in some tissues (274, 276, 280), multiple lines of evidence suggest that this prostanoid is protective in the pulmonary system. Aerosolized iloprost, a PGI$_2$ analog, is a well-accepted form of therapy for the treatment of pulmonary arterial hypertension (322) and both pharmacological and genetic-based animal studies indicate that this prostanoid is protective in models of bleomycin-induced pulmonary fibrosis (278, 279). Further, prostacyclin attenuates disease parameters secondary to respiratory syncytial virus in mice (286)

Animal studies utilizing mice lacking either the COX-1 or COX-2 enzyme provide evidence that overall, prostanoid production in the respiratory system limits multiple disease parameters observed during allergic responses in the lung. Following sensitization and challenge with ovalbumin (OVA) antigen, both COX-deficient mutants have elevated eosinophilia and T$_H$2 cytokine levels in their airways (182) and these findings are corroborated by studies utilizing NSAIDs to pharmacologically inhibit prostanoid production (293, 294). Initially these prophylactic properties were ascribed to synthesis of PGE$_2$ by COX enzymes, given that this prostanoid is upregulated in the sputum of asthmatics (304-306) and its administration attenuates airway hyperresponsiveness (AHR) (302, 311). However, we have recently shown that loss of mPGES1 ameliorates OVA-induced lung inflammation (373), demonstrating that production of PGE$_2$ cannot account for the protection afforded by COX-dependent prostanoid synthesis during pulmonary allergy.

PGI$_2$ levels are elevated following allergic episodes in the lungs of both humans and animals and administration of this mediator inhibits bronchoconstriction (323, 374-378). Further, *in vitro* experiments suggest that PGI$_2$ analogs can alter the responses of both dendritic cells (DCs) and T cell populations (281, 282, 285). The most compelling evidence that PGI$_2$ limits allergic inflammation in the lung, however, comes from *in vivo* studies assessing the consequences of an IP deficiency in this

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system. Mice lacking the IP receptor (IP -/-) experience both exacerbated lung inflammation (323) and enhanced airway remodeling (324) following sensitization and challenge with antigen. Conversely, treatment with PGI_2 or its analogs is effective in attenuating pulmonary inflammation when administered either before antigen sensitization or before each antigen challenge (282, 283). Collectively these data imply that prostacyclin limits disease during both acute and chronic lung allergy and may potentiate COX-dependent protection in this system. However, to date, no study has been conducted which directly compares the development of pulmonary inflammation in IP -/- and COX-deficient cohorts.

Herein, we demonstrate that following sensitization and challenge with OVA, IP -/- mice have similar levels of pulmonary inflammation compared to COX-1-/- animals, although important exceptions are observed. We find that PGI_2 limits allergy during both the sensitization and effector phases, although its contribution appears to be more prolific following antigen challenge, which may reflect tissue specificity. Additionally, we present preliminary data that suggests administration of the prostacyclin analog, iloprost, may limit pulmonary inflammation in COX-1 -/- animals by suppressing inflammatory cytokine levels, although administration of analog was unable to significantly prevent augmented cellularity in the airways. This effect may extend beyond atopic allergy to other models of inflammation in the lung, including allergy induced in OT-II animals. Finally, we identify bone-marrow derived leukocytes as a significant target for PGI_2 mediation in this system. .

Materials and Methods

Experimental Animals

All animal colonies were maintained according to standard guidelines as defined by the NIH Guide for the Care and Use of Laboratory Animals were approved by the Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill. Experiments were carried out on age and sex matched mice. C57BL/6 (B6) (backcrossed >10 generations) COX-1 -/- and B6 (backcrossed >10 generations) IP -/- mice were generated as previously describe (268, 334) Wildtype (WT) B6 mice (backcrossed >10 generations) were used as controls. B6 OVA-Specific TCR-Transgenic (OT-II) mice were purchased from The Jackson Laboratories (Bar Harbor, Ma).

OVA Sensitization and Challenge

Mice were sensitized systemically with an i.p injection of 20μg OVA (Sigma-Aldrich) emulsified in aluminum hydroxide (alum) (Sigma-Aldrich) or saline on day 0. Two weeks later, animals were challenged for one hour with 1% aerosolized OVA on three consecutive days. In some experiments, animals received 0.2 μg iloprost (Cayman Chemicals) 30 minutes prior to antigen challenge by intratracheal (i.t.) instillation. 24 hours after the final antigen exposure, mice were euthanized and blood and BALF were collected for further analysis. For *ex vivo* experiments examining responses in sensitized animals, mice received an i.p. injection of 50 μg OVA emulsified in alum. Twelve days later, animals were euthanized and spleens and serum were collected for further analysis.

Measurement of Cell Proliferation

Splenocytes were prepared by mechanical dispersion of spleen over a 70 μm cell strainer (BD Falcon). Red blood cells were lysed in lysis buffer (4.1g NH_2Cl, 0.5g $KHCO_3$, 100 μl 0.5M EDTA

dissolved in 500 ml dH_2O) and splenocytes were washed twice in PBS. Cells were plated at a density of 2.5×10^6 cells/ml in RPMI 1640 media enriched with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and .29 µg/ml L-glutamine. OVA was added to splenocyte cultures at concentrations ranging from 0 µg /ml to 100 µg/ml. After incubation for 72 hours at 37°C, cell proliferation was assessed using WST-1 reagent (Roche) according to the manufacturer's instructions.

BALF Collection and Cell Counts

Following euthanasia, lungs were lavaged with five 1ml aliquots of HBSS (Gibco) and total cell counts were determined by hemacytometer. Cellular composition was evaluated morphologically using cytospin preparation stained with Hema 3 or Fast Green. All remaining BALF was centrifuged to remove cells and stored at -80° c for immunoassay.

6-keto-PGF$_{1\alpha}$, Cytokine, and Immunoglobulin Production

Levels of 6-keto-$PGF_{1\alpha}$ and cytokines present in BALF, lung homogenate and/or tissue culture supernatant were determined by immunoassay. To determine cytokine production by stimulated splenocytes, cells were prepared as described above. Cells were cultured at a density of 1×10^7 cells/ml in the presence of 100 µg/ml OVA. After 72 hours, supernatants were collected and stored at -80°C prior to evaluation by ELISA. Cytokines were determined by ELISA following manufacturer's protocols: IL-13 (R&D Systems), IFN-γ (R&D Systems) and IL-17a (eBiosciences). For quantification of 6-keto-$PGF_{1\alpha}$, lungs were flash frozen in liquid nitrogen, weighed, and stored at -80°C. The left lobe was pulverized then homogenized in 1XPBS/1mM EDTA and 10 µM indomethacin. Lipids were separated from tissue supernatant using octadecyl C_{18} mini columns (Alltech Associates), and prostanoid levels were determined using an enzyme immunoassay kit (Assay Designs) according to the manufacturer's instructions. Values shown represent the total

quantity of mediator measured divided by tissue weight. Blood was obtained by cardiac puncture, allowed to coagulate, and centrifuged to isolate serum. IgE levels were determined by immunoassay using 96 well EIA/RIA plates (Costar). Plates were coated with IgE capture antibody (Pharmingen; clone R35-72), blocked with 1% BSA/PBS and then incubated with IgE standard (Pharmingen) or serum followed by biotinylated rat anti-mouse IgE (Pharmingen; clone R35-118). Detection was carried out using streptavidin-horseradish peroxidase (HRP) (Pharmingen) and hydrogen peroxide /2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Absorbance at 405nm was measured.

Bone Marrow Chimera Generation

Recipient mice were exposed to 5 grays irradiation from a Cesium g-irradiator at 0 and 3 hours. Femurs and tibias were collected from donor mice and flushed with cold PBS to isolate bone marrow. Bone marrow was introduced by tail vein injection into recipient mice immediately following the second round of radiation and after 4.5 weeks animals were sensitized and challenged with OVA as described.

Statistical Analysis

Statistical analysis was performed using Prism 4 (GraphPad Software). Comparisons of the mean were made by F test, Student's t-test or ANOVA followed by Tukey-Kramer's HSD *post hoc* test as necessary. Data are shown as mean $\pm$ SEM. Differences with *$p<0.05$* were considered statistically significant.

Results

Allergic lung inflammation in IP -/- and COX-1 -/- animals

We have shown that sensitized congenic COX-deficient animals have heightened airway inflammation following challenge with OVA and that a loss of PGE$_2$ synthesis in this system cannot account for this augmented disease (373). Instead, a role for PGI$_2$ and its IP receptor in this process is supported by experimental evidence (323); however no reports exist that directly compare respiratory disease parameters arising in IP -/- and COX-deficient animals. To this end, we utilized our established allergy protocol to examine the development of lung inflammation in IP -/- and COX-1 -/- cohorts and their congenic wildtype (WT) controls in parallel. This COX isoform was selected for study because the knockouts (KOs) for both COX-1 and the IP-receptor are maintained on a B6 background. COX-2 -/- animals survive poorly on most inbred strains, due to a patent ductus arteriosus (190), and therefore experimental F1 progeny produced from intercrossing 129S6/SvEv and B6 heterozygotes are utilized. The genetic background of the COX-2 -/- animals makes direct comparisons to IP -/- animals challenging.

Animals were sensitized systemically to antigen by an i.p. injection of 20 μg OVA emulsified in alum. Two weeks later, animals were challenged with saline or 1% aerosolized OVA for one hour on 3 consecutive days. Inflammation was assessed in these animals 24 hours after the final antigen challenge. As anticipated, sensitization and challenge with OVA induced a robust cellular infiltration in the airways of all animals, both WT controls and genetic KOs, compared to saline treated animals (**Fig 3.1A,B**). Cellularity was elevated approximately two-fold in the IP -/- animals, compared to IP +/+ controls (**Fig 3.1A**). While COX-1 -/- animals also experienced an enhanced cellular influx, compared to their WT controls (**Fig 3.1B**), the magnitude was not as substantial as that observed in IP -/- animals. IL-13 measurements, used as a marker for T$_H$2 cell cytokine release, were quantified in

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the BALF (**Fig 3.1C,D**). Levels of this cytokine were enhanced in the airways of all mice exposed to antigen. Loss of the IP-receptor **(Fig 3.1C)** or COX-1 **(Fig 3.1D)** induced similar increases in the levels of IL-13 measured above their respective WT controls. Finally, total IgE was quantified in these cohorts. Typical of atopic allergy, IgE was elevated in the serum of all rodents sensitized and challenged with OVA **(Fig 3.1 E,F)**. However, while IP -/- had IgE levels markedly higher than IP +/+ controls (**Fig 3.1E**), COX-1 -/- mice had IgE levels similar to WT mice **(Fig 3.1F)**.

Contribution of COX-1 to PGI_2 production in the inflamed lung

Our observations suggest that lost production of PGI_2 may account for the augmented lung inflammation observed in COX-1 -/- mice. Therefore, we next examined whether PGI_2 levels are altered in the inflamed COX-1 -/- murine lung. Prostacyclin is extremely labile at physiological pH and is rapidly metabolized to its inactive byproduct 6-keto-$PGF_{1\alpha}$ (327). We assessed concentrations of this metabolite, as a marker for PGI_2 production, present in the lung homogenate of naïve and allergic COX-1 -/- animals and their congenic controls **(Fig 3.2)**. 6-keto-$PGF_{1\alpha}$ is observed in the naïve lung of WT animals. Concentrations of this metabolite are reduced in lung tissue of naive COX-1 -/- animals, consistent with the idea that this isomer contributes to production of PGI_2 in the lung during homeostasis. Following challenge with antigen in sensitized animals, significantly more 6-keto-$PGF_{1\alpha}$ is measured in the lung homogenate of WT animals. In contrast, no increase in this metabolite is observed in the inflamed COX-1 -/- lung. This data confirms that production of PGI_2 is inhibited in the lungs of COX-1 deficient animals during atopic allergy, supporting the hypothesis that a loss of this prostanoid may account for the exacerbated lung inflammation observed in COX-1 -/- rodents.

Contribution of PGI$_2$ to **ex vivo** *immune responses*

Although the pulmonary inflammation observed in IP -/- mice is similar to the inflammation observed in COX-1 -/- animals, the phenotypes are not identical. We therefore sought to determine whether the heightened inflammation observed in the airways of COX-1 -/- mice can be reduced upon exogenous administration of the PGI$_2$ analog iloprost, used clinically in the treatment of pulmonary arterial hypertension (277). Experimental evidence suggests that local application of iloprost to the airways of immunized mice prior to each antigen challenge is sufficient to attenuate pulmonary inflammation in these animals (282). However, the elevated level of IgE measured in our IP -/- mice suggests that PGI$_2$ synthesis during allergic sensitization may be influential in its ability to limit inflammation in the lung.

To delineate the contribution of PGI$_2$ signaling to allergic sensitization, WT and IP -/-animals were sensitized i.p. with OVA/alum. IgE levels and *ex vivo* splenocyte responses were assessed 12 days later, prior to antigen challenge. We observed that antigen sensitization, alone, was sufficient to measure significant, albeit modest, elevations in the IgE levels of IP -/- mice compared to immunized WT controls **(Fig 3.3A)**. PGI$_2$ might limit antigen sensitization by suppressing the expansion of immune cells. To evaluate this possibility, splenocytes from immunized WT and IP -/- mice were isolated and incubated with increasing concentrations of OVA antigen. Proliferative responses were subsequently assessed using WST-1 reagent (**Fig 3.3B**). No difference was observed in the proliferative curves generated by cells isolated from WT and IP -/- animals. We next tested whether PGI$_2$ limits atopic allergic sensitization by altering T cell polarization. To do so, we measured IL-13 (**Fig 3.3C)**, IFN-γ (**Fig 3.3D**), and IL-17a (**Fig 3.3E)** levels present in splenocyte supernatants as markers for typical T$_H$2, T$_H$1, and T$_H$17 cytokine profiles, respectively. There were no significant differences observed in any of these cytokine levels between IP+/+ and IP -/- cell supernatants.

The effects of iloprost on lung inflammation in COX-1 -/- and IP -/- mice

While a modest elevation is observed in the IgE levels of IP -/- animals following antigen sensitization, our data suggests that the majority of protection afforded by this lipid mediator occurs during allergy elicitation in the lung. To this end, we examined the consequence of iloprost administration to pulmonary inflammation in the COX-1 -/- animals during the effector phase of allergy. COX-1 -/- mice and their WT controls were sensitized and challenged with antigen, as previously described. A subset of these COX-1 -/- animals received 0.2μg iloprost (Ilo) by i.t. administration 30 minutes prior to each antigen challenge (Ilo/COX-1 -/-) while all other animals were exposed to vehicle (Veh). As anticipated, BALF cellularity was enhanced in COX-1 -/- mice pretreated with vehicle (Veh/COX-1 -/-), compared to WT controls **(Fig 3.4A)**. This cellular infiltration, while modestly reduced, was not significantly attenuated in Ilo/COX-1 -/- mice. IL-13 levels were next assessed in the BALF. Veh/COX-1 -/- mice had significantly elevated cytokine levels, compared to WT animals; however, no significant elevation was observed in Ilo/COX-1 -/- mice **(Fig 3.4B).** As a final test, IgE was measured in the serum of experimental animals **(Fig 3.4C)**. Consistent with our previous observations, no significant difference was observed between WT and Veh/COX-1 -/- mice. Treatment with iloprost had no significant impact on the concentration of this immunoglobulin.

To confirm the involvement of the IP-receptor in our above generated data, an identical round of experiments was conducted utilizing IP -/- mice. Immunized animals were challenged following pretreatment with Veh (WT and Veh/IP -/-) or iloprost (Ilo/IP -/-) as previously described. The cellularity measured in the BALF of Veh/IP -/- animals was elevated compared to WT animals **(Fig 3.5A)**. Cellular levels quantified in Ilo/IP -/- mice did not significantly differ from Veh/IP -/-

controls. Additionally, BALF IL-13 levels and serum IgE were similarly augmented in Veh/IP-/- and Ilo/IP -/- mice when compared to WT mice (**Fig 3.5B,C**).

The effects of iloprost on OT-II mice

Our evidence suggests that iloprost significantly ameliorates aspects of lung inflammation which develop during atopic allergic responses, demonstrated by reduced levels of T_H2-specific cytokine IL-13. We examined whether this protection by iloprost could be extended to non-atopic models of lung inflammation, as well, utilizing OVA TCR-specific (OT-II) mice. These mice harbor a transgenic TCR specific for OVA-antigen and exposure of their airways to OVA induces lung inflammation without a prior necessity for antigen sensitization. The pattern of disease these animals develop is characterized by elevated neutrophilia and may reflect a T_H17-driven response (345). We challenged the airways of OT-II transgenic (OT-II tg +) mice and their WT (OT-II tg -) controls for 5 consecutive days with 1% OVA. Airway inflammation in these animals was assessed 24 hours later. A subset of these animals received 0.2μg i.t. iloprost 30 minutes prior to each challenge.

A robust cellular infiltration was observed in the airways of OT-II tg+ mice, compared to OT-II tg- animals. Administration of iloprost did not significantly alter the cellularity observed in OT-II tg+ mice (**Fig 3.6A**). As an additional test, IL-17a levels were quantified in the BALF of OT-II tg+ animals (**Fig 3.6B**). A modest reduction in the level of this cytokine was observed in iloprost treated animals; however this value failed to reach statistical significance (p=0.06).

Contribution of leukocytes to lung inflammation in IP -/- animals

Experimental evidence indicates that, in the inflamed lung, stromal cells are the predominant source of PGI_2; however, both leukocytes and structural cells express high levels of the IP receptor

(283). We therefore sought to determine which cell type is being altered by production of PGI_2 during allergy in the lung. To elucidate whether IP-signaling on leukocytes is responsible for the exacerbated pulmonary inflammation observed in IP -/- mice, we studied the development of allergy in bone marrow chimeras. Bone marrow, harvested from WT or IP -/- animals was transplanted into irradiated WT animals to generate mice with normal IP expression on structural cells of the lung while possessing either WT (WT→WT) or IP-deficient (knockout [KO]→WT) immune cells. Following reconstitution, these animals were sensitized and challenged as previously described.

While elevated cell infiltration was confirmed in the airways of both WT→WT and KO→WT animals immunized and challenged with OVA, this cellularity was significantly augmented in KO→WT mice (**Fig 3.7A**), reflecting a significantly enhanced eosinophil population (**Fig 3.7B**). Consistent with IP -/- mice, IL-13 cytokine levels were significantly increased in the BALF of OVA-treated KO→WT animals compared to OVA-treated WT→WT mice (**Fig 3.7C**). Additionally, KO→WT mice receiving antigen had substantially greater concentrations of serum IgE, compared to WT→WT controls (**Fig 3.7D**).

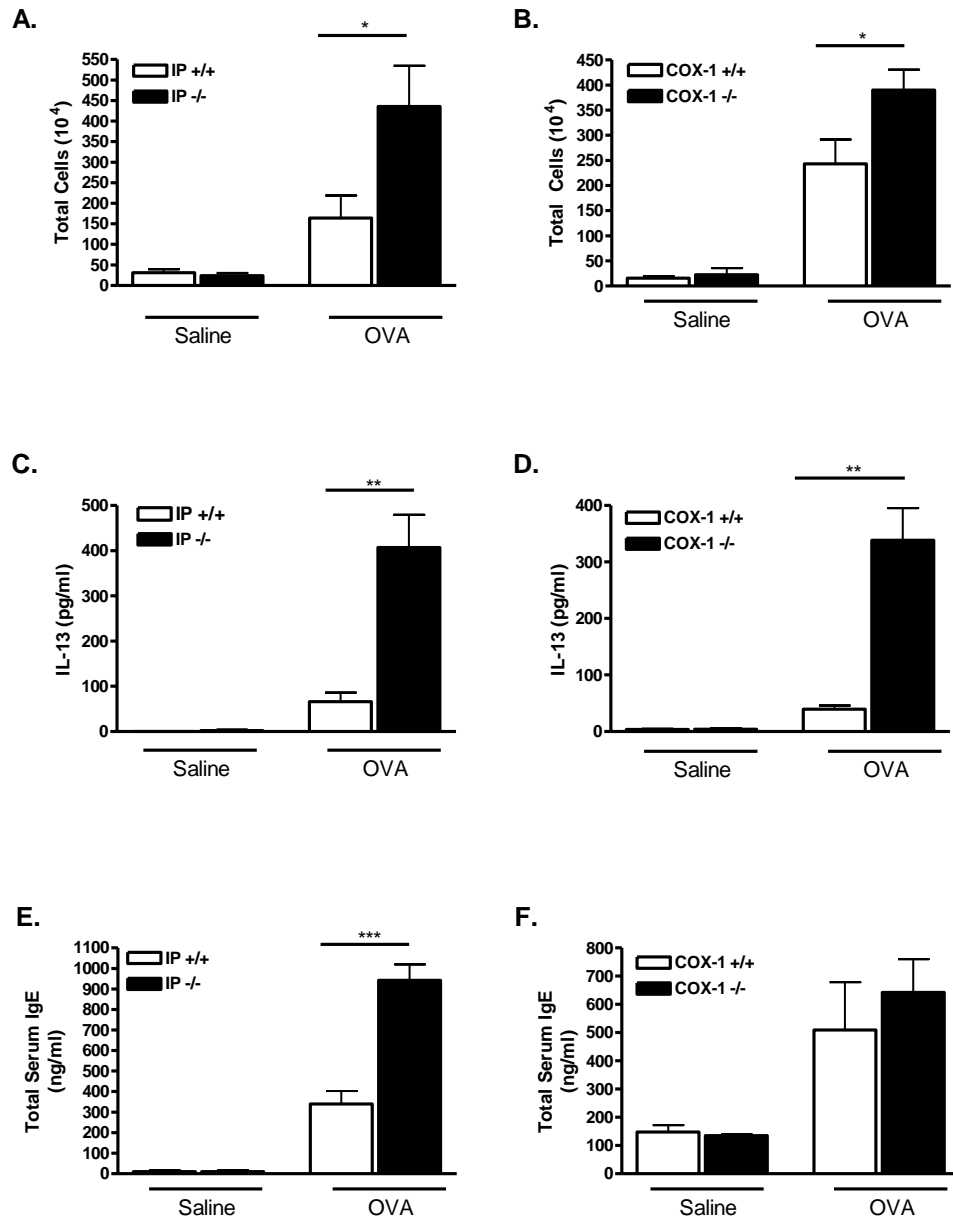**Figure 3.1 Allergic Lung Inflammation in IP -/- and COX-1 -/- Mice**

Figure 3.1 Allergic Lung Inflammation in IP -/- and COX-1 -/-mice IP -/- mice, COX-1 -/- mice and their WT controls were sensitized i.p. with saline or 20 μg OVA emulsified in alum. Two weeks following sensitization, animals were challenged for three consecutive days with saline or 1% aerosolized OVA. Twenty four hours after the final exposure, airway inflammation was assessed. **A,B.** Sensitization and challenge with antigen resulted in elevated cellularity in the BALF of IP -/- mice, COX-1 -/- mice, and their WT controls. This elevation is significantly augmented in both IP -/- mice compared to their inflamed WT controls **(A)** (*$p<0.01$) and COX-1 -/- animals compared to their OVA-treated WT controls **(B)** (*$p<0.05$). **C,D.** Induction of allergy augmented BALF IL-13 levels in all mice, compared to saline controls. IP -/- animals **(C)** and COX-1 -/- animals **(D)** both have cytokine measurements significantly higher than their WT controls (**$p<0.001$). **E,F.** Total serum IgE is elevated following allergy induction in all mice. IP -/- mice have increased IgE compared to WT controls **(E)** (***$p<0.0001$). In contrast, COX-1 -/- mice have IgE levels similar to their WT controls **(F)**. (For IP saline: IP +/+ n=3, IP -/- n=3; For IP OVA: IP +/+ n=7, IP -/- n=8; For COX-1 saline: COX-1 +/+ n=5, COX-1 -/- n=4; For COX-1 OVA: COX-1 +/+ n=7, COX-1 -/- n=8)

Figure 3.2 The Contribution of COX-1 to PGI$_2$ Production in the Inflamed Lung
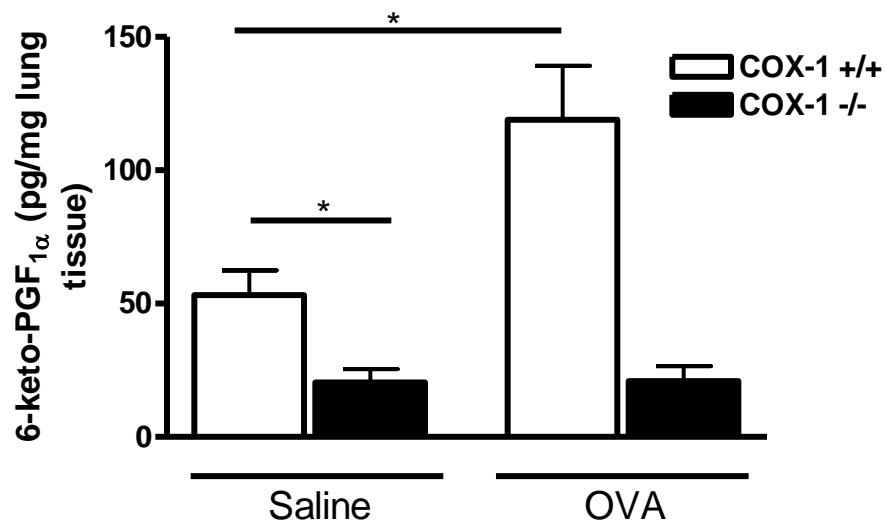
Figure 3.2 Contribution of COX-1 to PGI$_2$ Synthesis in the Inflamed Lung Levels of the PGI$_2$ metabolite, 6-keto-PGF$_{1\alpha}$, were assessed in the lung homogenate of naïve and inflamed COX-1 -/- animals and their WT controls. In the naïve lung, concentrations of 6-keto-PGF$_{1\alpha}$ are significantly attenuated in COX-1 -/- animals, compared to WT controls (*$p < 0.05$). 6-keto-PGF$_{1\alpha}$ levels increase significantly in lungs collected from WT mice sensitized and challenged with OVA (*$p < 0.05$). In contrast, no enhanced production of 6-keto-PGF$_{1\alpha}$ occurs in the inflamed COX-1 -/- lung. (For Saline: COX-1 +/+ n=5, COX-1 -/- n=4; For OVA COX-1 +/+ n=5; COX-1 -/- n=6)

Figure 3.3 Immune Responses in IP -/- Animals Following Antigen Sensitization
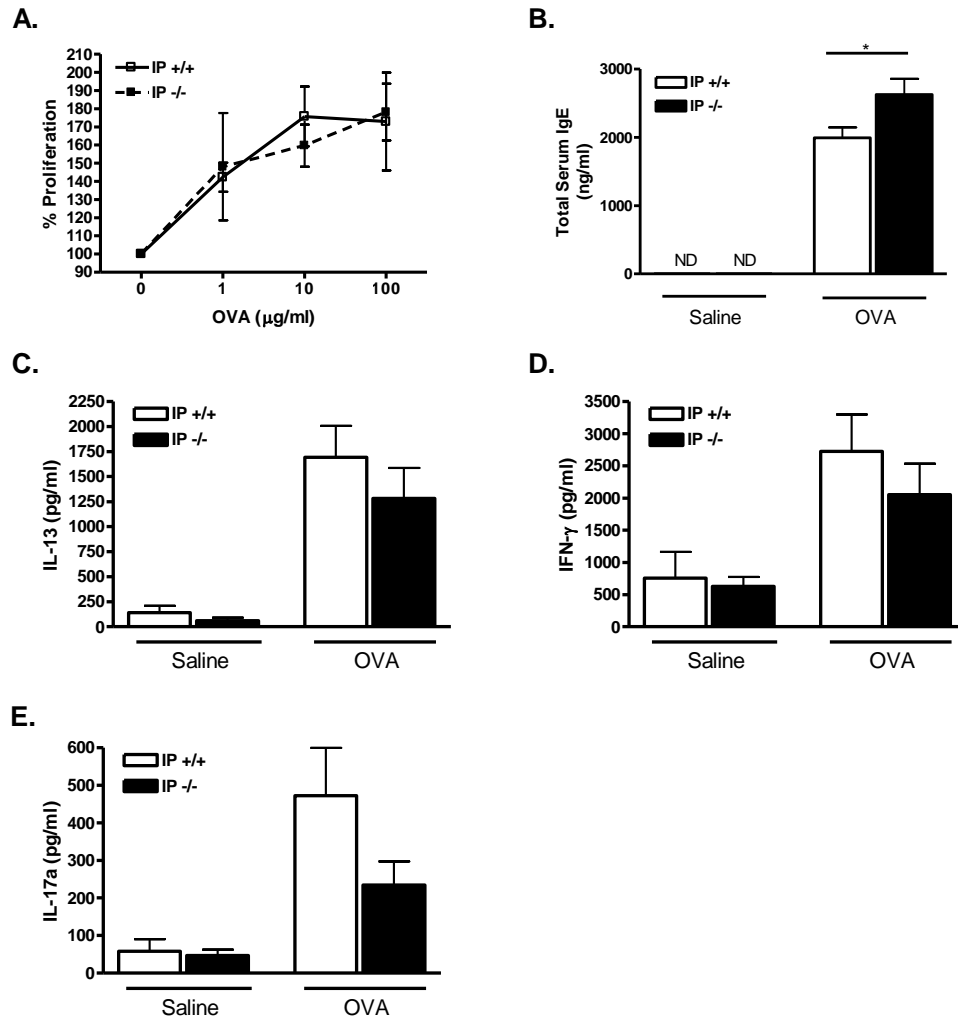
Figure 3.3 Immune Responses in IP -/- Animals Following Antigen Sensitization WT and IP -/-
mice were sensitized with 50 μg OVA in alum and 12 days later, mice were euthanized and blood and
spleens were collected for analysis. **A.** Total IgE levels were measured in the serum of antigen
sensitized animals. IP -/- mice have significantly higher serum IgE compared to WT controls (*
$p < 0.05$). **B.** Splenocytes isolated from WT and IP -/- sensitized animals with increasing
concentrations of OVA. Following a 72 hour incubation period, cell proliferation was assessed using
WST-1 reagent. No significant difference in splenocyte proliferation is observed between wildtype
and IP -/- cells. **C-E.** Splenocytes from naïve and OVA-sensitized animals were cultured with 100
μg/ml OVA for 72 hours. IL-13 **(C)**, IFN-γ **(D)**, and IL-17a **(E)** cytokine levels were quantified in
cell supernatant. No significant differences are observed in cytokine production between IP +/+ and
IP -/- cells. (For saline: IP +/+ n=4, IP -/- n=4; For OVA: IP +/+ n=5, IP -/- n=4)

Figure 3.4 The Effect of Iloprost on Lung Inflammation in COX-1 -/- Mice
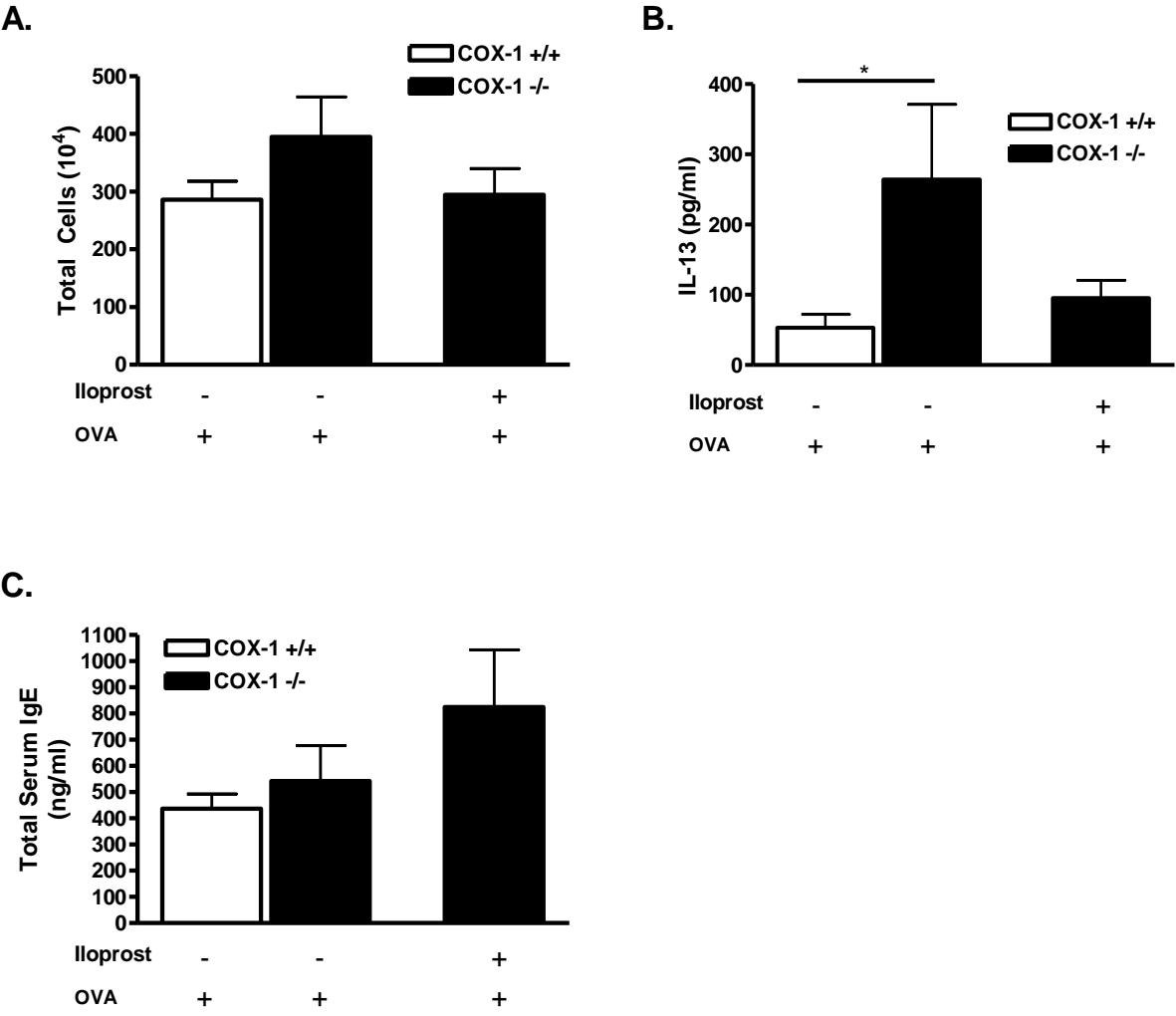
Figure 3.4 The Effect of Iloprost on Lung Inflammation in COX-1 -/- Mice COX-1 -/- animals and their wildtype congenic controls were sensitized and challenged with OVA antigen as previously described. 30 minutes prior to each challenge, mice received i.t. administration of 0.2μg iloprost (Ilo/COX-1 -/-) or vehicle (WT and Veh/COX-1-/-). 24 hours after the final challenge, animals were euthanized and BALF, serum, and lungs were collected. **A.** Veh/COX-1 -/- animals have elevated levels of cellularity in their BALF, compared to WT mice. Ilo/COX-1 -/- animals do not have significantly altered cellularity, compared to Veh/COX-1 -/- controls. **B.** IL-13 levels are significantly augmented in the BALF of allergic Veh/COX-1 -/- animals, compared to WT mice (*$p<0.05$). In contrast, Ilo/COX-1 -/- mice do not develop enhanced IL-13 levels, compared to WT controls **C.** WT and Veh/COX-1 -/- mice have comparable levels of total serum IgE. Ilo/COX-1-/- mice do not have a significant effect on IgE concentrations. (COX-1 +/+ n=8, Veh/COX-1 -/- n=4, Ilo/COX-1 -/- n=4)

Figure 3.5 The Effect of Iloprost on IP -/- Mice

A.

B.

C.
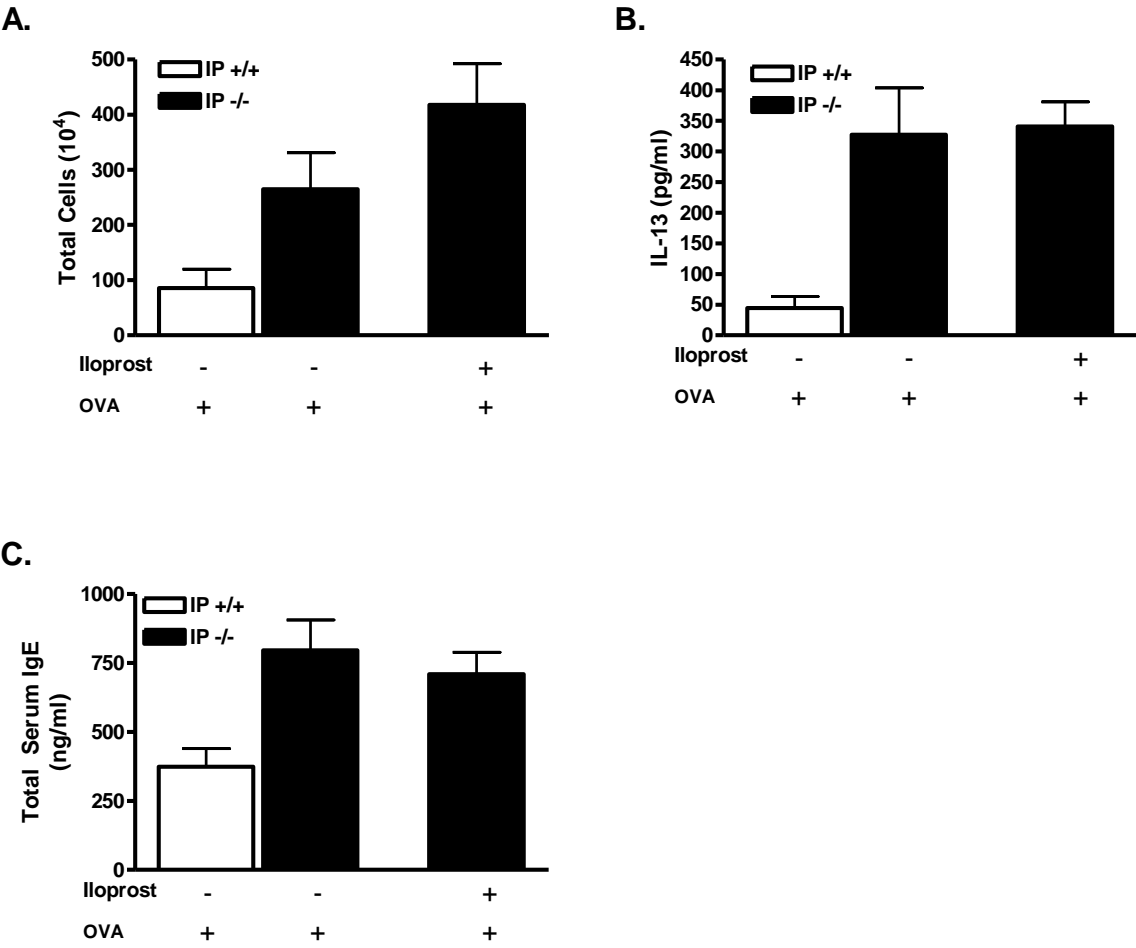
Figure 3.5 The Effect of Iloprost on IP -/- Mice Animals were sensitized and challenged with OVA antigen, as previously described. Prior to each antigen challenge, mice received an i.t. delivered dose of 0.2μg iloprost or vehicle (Ilo/IP -/- compared to WT and Veh/IP -/-). 24 hours after the final challenge, BALF, serum and lungs were collected from all animals. **A.** Cellularity in the BALF of Veh/IP-/- mice is augmented compared to WT animals. Ilo/IP -/- have no significant difference in the cell levels measured, compared to Veh/IP -/- controls. **B.** Il-13 cytokine levels measured in Veh/IP -/- mice are dramatically elevated, compared to WT controls. This augmentation is not affected in Ilo/IP-/- mice. **C.** Total serum IgE levels are enhanced in IP -/- mice compared to WT controls. No significant difference is observed between Veh/IP-/- mice and Ilo/IP -/- groups. (WT n=4, IP -/-/Veh n=5, IP -/-/Ilo n=5)

Figure 3.6 The Effect of Iloprost on OT-II Mice

A.

OT-II	-	-	+	+
Iloprost	-	+	-	+
OVA	+	+	+	+

B.
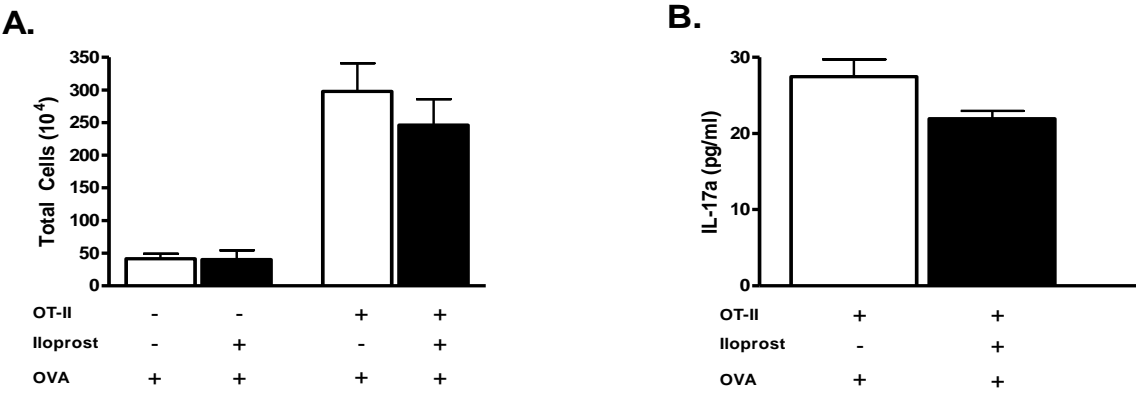
OT-II	+	+
Iloprost	-	+
OVA	+	+

Figure 3.6 The Effect of Iloprost on OT-II Mice Mice harboring a transgene specific for the OVA TCR (OT-II tg +) and their WT controls (OT-II tg -) were challenged for 5 consecutive days with 1% aerosolized OVA to induce inflammation. Half of these mice received 0.2μg i.t. iloprost 30 minutes prior to each challenge. 24 hours after the final challenge, BALF was collected for analysis. **A.** BALF cellularity is increased following OVA challenge in all OT-II tg + animals, compared to OT-II tg- controls. Administration of iloprost prior to challenge had no significant effect on BALF cell levels. **B** IL-17a cytokine levels were assessed in the BALF of OT-II tg + animals. Pretreatment with iloprost resulted in a modest, insignificant, reduction in IL-17a (p=0.06) (For OT-II tg-: Veh/WT n=3, Ilo/WT n=2, Veh/OT-II n=5, Ilo/OT-II n=5)

Figure 3.7 IP-signaling on Leukocytes
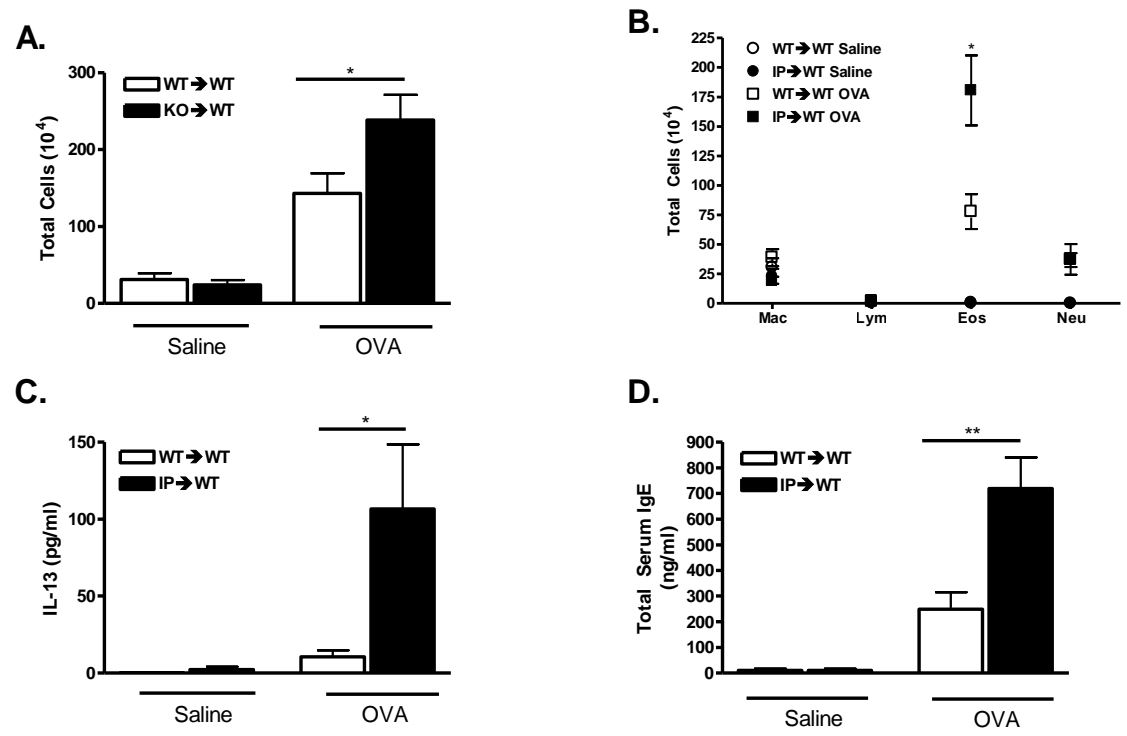
Figure 3.7 IP-signaling on Leukocytes Lethally irradiated WT mice were reconstituted with either WT (WT→WT) or IP-deficient (KO→WT) bone marrow. 4 ½ weeks following reconstitution, animals were sensitized and challenged with saline or OVA as described. **A.** All animals receiving OVA have elevated BALF cellularity; however cell levels are significantly augmented in KO→WT mice compared to allergic WT→WT controls (*$p<0.05$). **B.** The increased cellularity observed in the BALF of allergic KO→WT mice compared to WT→WT controls results from significantly enhanced eosinophilia (*$p<0.02$). **C.** IL-13 cytokine levels measured in the BALF following sensitization and challenge with OVA are significantly augmented in KO→WT mice compared to WT→WT mice (*$p=0.05$). **D.** Allergy-induction with OVA antigen results in significantly elevated concentrations of total serum IgE in KO→WT animals compared to WT→WT controls. (For saline: WT→WT n=3, KO→WT n=3; For OVA: WT→WT n=7, KO→WT n=9)

Discussion

Our previous work has demonstrated that COX-1 enzymatic activity limits the inflammation associated with atopic lung allergy; a loss of this isoform augments disease in mice, compared to congenic controls (373). This is consistent with data generated using animals of a mixed genetic background (182). While this phenomenon was initially thought to reflect a specific loss of PGE_2 synthesis in these animals, we have recently shown that PGE_2 enhances disease parameters in this model (373). Instead, utilizing IP -/- and COX-1 -/- cohorts, we have examined the contribution of PGI_2 to the heightened disease in COX-1 -/- animals. We observed that allergic IP -/- and COX-1 -/- cohorts develop similar levels of inflammation in the lung, although important differences between the two groups are observed. Further, we have demonstrated that although PGI_2 limits atopic disease during both the sensitization and effector phases, the contribution of this mediator is more prolific during allergy elicitation. We have additionally presented preliminary evidence suggesting that iloprost can attenuate effector phase atopic allergy in the airways of COX-1 -/- mice by suppressing IL-13 cytokine levels. Finally, we show that PGI_2 targets IP receptors on leukocytes to mediate its effects in the lung.

Using an identical protocol, we observed that a genetic loss of either COX-1 or IP increases parameters of lung inflammation following sensitization and challenge with OVA antigen. In this model, both COX-1 -/- and IP -/- animals have significantly elevated cellularity in their airways; however, the magnitude of this influx is more substantial in IP -/- animals, compared to COX-1 -/- mice. Two explanations might account for this difference. This observation most likely arises from the fact that, unlike IP -/- animals, COX-1 -/- mice lose the ability to produce all downstream prostanoids whose synthesis are dependent on this isoform, representing both pro- and anti-inflammatory mediators. IP -/- animals, however, lose PGI_2-signaling only. These animals retain the ability to produce and respond to alternate prostanoids. We have demonstrated that PGE_2 promotes

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inflammation in this system and therefore may be the source of the enhanced cellularity in IP -/- animals. Alternatively, although we show that production of the stable PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$, is drastically inhibited in the inflamed COX-1 -/- lung, we cannot rule out the possibility that low levels of this prostanoid are being produced by COX-2 in this system. This query can be addressed by examining the levels of 6-keto-$PGF_{1\alpha}$ in the inflamed lungs of COX-1 -/- animals pretreated with indomethacin. Unlike cellular infiltrate measurements, IL-13 cytokine levels in the airways of COX-1 -/- and IP -/- mice are comparable. This finding suggests that the attenuated IL-13 cytokine levels observed in COX-1 -/- may result specifically from aberrant PGI_2 synthesis.

Consistent with our previous findings, a loss of COX-1 enzymatic activity does not augment concentrations of serum IgE in response to OVA, compared to WT controls. In contrast, a specific loss of prostacyclin signaling significantly increases observed IgE levels. This is in agreement with other reports examining the actions of PGI_2 in the lung (323) and demonstrates that prostanoid production does in fact contribute to IgE concentrations. The lack of a measurable phenotype in the COX-1-/- animals suggests that a careful balance exists between prostanoids in this process and that the actions of pro- and anti-inflammatory prostanoids contribute equally to levels of IgE. Although no significant difference was measured in IgE levels resulting from a loss of mPGES1 (373), we have often observed a reduced trend in concentrations of this immunoglobulin in mPGES1 -/- mice following sensitization (data not shown). PGD_2, acting through the DP_1 receptor, is also described to enhance OVA-induced lung inflammation (297). Like mPGES1 -/- animals, DP_1-deficient mice have only modestly reduced levels of IgE that fail to reach statistical significance (297). Collectively this data suggests that although pro-inflammatory cytokines, including PGE_2 and PGD_2, independently contribute only minimally to IgE synthesis in the lung, synergy between these prostanoids can effectively antagonize the suppression PGI_2 exerts on IgE levels.

To more precisely delineate the mechanism underlying IP-dependent suppression of antigen sensitization, we conducted *ex vivo* experiments measuring the responses of splenocytes in the absence of IP signaling. Total splenocyte numbers quantified after mechanical dispersion and red blood cell lysis were comparable (data not shown), suggesting that endogenous PGI_2 does not prevent immune cell trafficking into this organ. During atopic antigen sensitization, antigen presentation results in antigen-specific T_H2 differentiation and proliferation of immune cells populations. No differences were observed in the proliferative curves or cytokine levels generated by IP +/+ and IP -/- splenocytes. These results imply that PGI_2 does not limit IgE in this model by suppressing cellular expansion or altering polarization.

Given that PGI_2 does not appear to abrogate T cell responses following antigen presentation, it is possible that IgE levels are altered in this model as a result of impaired immunoglobulin production by B cells. While IP is present on effector T_H2 cells, expression of this receptor is not observed on activated B cells (283), suggesting that PGI_2 acts indirectly on these cells to inhibit IgE synthesis. B cells require signals from activated T_H2 cells to induce immunoglobulin class switching to IgE. Among these, T_H2 cells physically interact with B cells through CD40L/CD40 and CD23/CD21 ligand-binding (379). Prostacyclin may potentially alter IgE levels by blunting expression of CD40L or CD23 on T_H2 cells, thus reducing their ability to stimulate activation of B cells. To our knowledge, expression of these ligands on T_H2 cells in response to PGI_2 has not been explored. Elucidating the levels of alternative immunoglobulins present in IP -/- animals subsequent to antigen sensitization may clarify this issue.

Additionally, release of IL-4 induces germline ε transcript expression and the presence of this cytokine is essential for isotype switching to occur (380). In agreement with our findings, Jaffar and colleagues observed in their studies that PGI_2 does not suppress T_H2 cytokine levels or cellular

proliferation following *in vitro* restimulation of OVA-specific DO11.10 (DO11.10) cells (283). Instead, this prostanoid augments the IL-10 levels measured (284). While levels of IL-10 cytokine were not assessed in our work, it stands to reason that a loss of this anti-inflammatory cytokine may indirectly enhance IgE levels by altering the IL-10/IL-4 balance and promoting ε transcript expression. Although we measured IL-4 concentrations in our supernatants, values in all splenocyte cultures were below the level of detection (data not shown). Finally, $CD4^+CD25^+$ T_{reg} cells produce IL-10 and have been shown to express IP receptors (283). A loss of IP-signaling may impede the suppressive capabilities of T_{reg} cells. Further work is necessary to elucidate which mechanism PGI_2 utilizes to inhibit IgE levels following antigen sensitization.

It is currently unclear why other groups have observed *in vitro* deviations in cellular proliferation and cytokine levels resulting from PGI_2 signaling; however variations in culturing protocols make direct comparisons challenging (281, 285, 323). For example, maturation of naïve cells *in vitro* may not accurately reflect the endogenous factors present during the *in vivo* stimulation of our splenocytes. Further, multiple studies measured cellular responses following addition of PGI_2 analog rather than examining the contribution of endogenous prostacyclin signaling through the IP receptor (281, 285). Indeed, one group reported that suppression of T cell responses by analogs was only partially inhibited in IP -/- cells, suggesting that addition of exogenous analog can stimulate multiple pathways (285). Differences in T cell responses may also reflect variations in costimulatory conditions. While PGI_2 inhibits T_H1 cytokine release when CD28 stimulation is absent; data shows that this prostanoid promotes T_H1 responses in a CD28 dose-dependent manner (280). In another report, the actions of IP-signaling on effector T cell cytokine release varied depending on whether cells were stimulated with antigen or anti-CD3 antibody (323).

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While a significant difference in IgE is measured in immunized IP -/- animals, this difference fails to achieve the magnitude of change observed following allergen challenge. This may suggest that the overwhelming contribution of PGI_2 in limiting IgE levels is specific to the respiratory tract. In support of this theory, additional groups administering antigen systemically have failed to observe a significant difference in IgE values until after challenge of the airways occurred (323, 324). Further, fewer DO11.10 cells were measured in the lungs of recipient rodents following adoptive transfer and subsequent antigen challenge, when cells were stimulated *in vitro* with PGI_2. In contrast, PGI_2- and Veh-treated DO11.10 cells were observed at similar levels in lymph nodes and in the spleen (283). Finally, recent work by Idzko and colleagues, employing a model in which tolerogenic plasmacytoid DCs are depleted from the airways, reports that the administration of iloprost during local sensitization to the airways is sufficient to inhibit the pulmonary inflammation arising in Veh-treated animals following antigen challenge (282).

To further delineate the contribution of aberrant PGI_2 production to the effector phase of lung inflammation in COX-1 -/- mice, we administered local doses of iloprost to COX-1 -/- animals prior to antigen challenge. While cellularity in the BALF of Ilo/COX-1 -/- mice was modestly reduced compared to Veh/COX-1 -/- controls, no statistically significant differences in airway cellularity and serum IgE levels were observed as a result of iloprost treatment. IL-13 concentrations were significantly augmented in the BALF of Veh/COX-1 -/- animals, compared to WT mice; however, no augmentation occurred in Ilo/COX-1 -/- mice. This result supports our observation in IP -/- and COX-1 -/- mice that elevated IL-13 levels result from a loss of PGI_2 synthesis. This effect was specifically occurring through the IP receptor; iloprost treatment had no consequence on IL-13 levels in IP -/- mice. Collectively, these results suggest that production of prostacyclin by COX enzymes suppresses cell recruitment, to a degree, but exerts most of its effects by suppressing cytokine release from immune cells. Research indicates that following antigen challenge, T cells are recruited to the

lung but require restimulation by DCs present in the lung for inflammation to occur (381) PGI$_2$ has been shown to suppress maturation of DCs, preventing their abilities to present antigen to T cells (281, 282). This could explain why cell levels are only modestly reduced, yet cytokine production is suppressed.

Although iloprost appears to suppress cytokine release in the lung following antigen challenge in a model of atopic allergy, we explored whether this effect could be extended to non-atopic models of lung inflammation. To this end, OT-II transgenic mice were administered iloprost prior to challenge with OVA. In agreement with our observations in COX-1 -/- mice, treatment with this analog did not significantly prevent the recruitment of cells to the airway, nor did it significantly suppress IL-17a levels. While a modest reduction was observed in measurements of this cytokine following iloprost treatment, these observations seem to suggest that the actions of PGI$_2$, in the airway, act predominantly on T$_H$2-driven responses.

While resident cells of the lung appear to be the predominant source of PGI$_2$ synthesis in the inflamed lung, both structural cells and leukocytes express elevated levels of the IP receptor (283). Given that prostanoids can act in both an autocrine and paracrine manner, it was of interest to elucidate which cell type PGI$_2$ manipulates to exert its inhibitory functions. To this end, we generated bone marrow chimeras with normal IP-signaling in the lung but lacking IP expression on leukocytes. Following induction of allergy, chimeras had significantly enhanced airway eosinophilia and IL-13 levels, as well as augmented IgE, compared to animals reconstituted with WT bone marrow. This data demonstrates that IP-signaling through recruited immune cells is important for the protection afforded by PGI$_2$ in the lung.

While further work is needed to clarify which class of leukocyte PGI$_2$ exerts its effects on, evidence supports a model in which multiple cell types can be controlled by this prostanoid. A recent

study reported that administrating iloprost prior to antigen challenge ameliorates lung inflammation and AHR, resulting from inhibition of DC maturation and migration (282). However, a separate group made the observation that EPO levels in the airways of naive IP +/+ and IP -/- animals transferred with WT OT-II T_H2 cells were similar following challenge. Additionally, naive mice transferred with PGI_2-treated DO11.10 T_H2 cells had reduced EPO levels compared to animals receiving Veh-treated cells (283), suggesting that PGI_2 acts on T_H2 cells during pulmonary inflammation. Collectively, these observations imply that the actions exerted by PGI_2 produced by stromal cells during atopic lung allergy are not limited to one cell type.

In conclusion, we have shown that PGI_2 production inhibits atopic inflammation in the murine lung following challenge in immunized recipients. While mediation by this prostanoid is active in both the sensitization and effector phases, in this model prostacyclin seems to play a more critical role following antigen challenge; possibly suggesting that protection from inflammation by this mediator is specific to the lung. We have also presented preliminary data suggesting that PGI_2 exerts its effects not by preventing recruitment to the lung, but by suppressing cytokine levels. Taken together, we propose a model in which COX-1 generation of PGI_2 in the lung limits inflammation by downregulating expression of costimulatory signals on leukocytes. Following local exposure to antigen, PGI_2 may suppress DC maturation thus reducing the ability of these APCs to migrate to local lymph nodes and activate naïve T cells. Suppression of DC maturation also prevents restimulation of effector T cells recruited to the airways and may additionally reduce costimulatory molecules on T_H2, attenuating their ability to release cytokines and stimulate B cells.

CHAPTER IV

AIRWAY IMMUNE TOLERANCE IN THE ABSENCE OF PGE$_2$ SIGNALING

Normal respiration exposes the mucosal surface of the airway to a continuous array of both pathogenic and innocuous antigens ubiquitous in the external environment. The ability to efficiently recognize and combat harmful material while avoiding unnecessary responses to non-pathogenic allergens, which would lead to chronic inflammation and barrier damage, is a central challenge the immune system faces at mucosal surfaces. Experimental evidence suggests that a tight regulation exists to promote immune tolerance to harmless environmental particles and this is controlled, in part, by the ability of epithelial cells to release mediators into the milieu, creating a protective microenvironment at the time of antigen exposure (68). A breakdown of this system precipitates chronic allergy, inflammation, and asthma (65).

Prostaglandin E$_2$ (PGE$_2$) is an important bioactive lipid mediator with pleiotropic functions, produced by most cells. The synthesis of this prostanoid is initialized when arachidonic acid (AA) is released from membrane phospholipids by phospholipase A$_2$. AA is converted into the intermediate products, prostaglandin G$_2$ (PGG$_2$) and prostaglandin H$_2$ (PGH$_2$) by cyclooxygenase (COX) enzymes existing in two isoforms encoded by unique genes, COX-1 and COX-2. Following conversion to PGH$_2$, production of PGE$_2$ is completed by a prostanoid specific synthase. Three distinct synthases have been described for synthesis of this mediator: microsomal PGE synthases (mPGES) -1 and -2, and cytosolic PGE synthase (cPGES) (197, 340, 341); however study of these synthases *in vivo,* using gene-specific knock out technology, only supports a role for mPGES-1 in mediating this conversion

(199, 201, 204). Following its synthesis and transport from the cell, the autocrine and paracrine actions of PGE_2 are initiated by ligand-binding to four distinct G-coupled E prostanoid (EP) receptors, EP_{1-4}, with overlapping functions. Ligand-binding to EP_1, a G_q-coupled receptor, results in elevation of intracellular calcium levels. Downstream actions following binding of EP_2 or EP_4, G_s-coupled receptors, occur through augmentation of cyclic AMP (cAMP). The EP_3 receptor is unique in that it exists in multiple isoforms which can couple with distinct G-protein receptors. Depending on the variant, EP_3 can couple with the G_i, G_s, and G_q-protein receptors to attenuate or increase cAMP levels or elevate intracellular calcium levels, respectively (382). Varying levels of expression have been observed in the murine lung for of all four EP receptors (327, 383).

The concept that prostanoids, including PGE_2, can influence the microenvironment at mucosal surfaces and promote antigen tolerance is supported by data generated in the gastrointestinal (GI) tract which, much like the airways, is a mucosal system with continuous exposure to innocuous particles in the form of food antigens and commensal bacteria. Chronic use of non-steroidal anti-inflammatory drugs (NSAIDs), analgesics that suppress COX-dependent synthesis of prostanoids, can augment disease in patients with inflammatory bowel syndrome (IBS) (384, 385). In fact, using a murine model in which all T cells recognize a single defined antigen, Newberry et al. demonstrated that prostanoid production by COX-2, constitutively expressed by the lamina propria, is crucial for induction of GI immune tolerance and this was attributed to production of PGE_2 by this enzyme (185, 386).

While evidence supports a role for PGE_2 in immune tolerance generated at the mucosal surface of the GI tract, little is known regarding its contribution to the establishment of antigen tolerance in the airways. However, similar to the gut mucosa, experimental evidence suggests that both COX isoforms are present during homeostasis in epithelial cells of the airway and constitutive

production of PGE_2 by these cells is noted in both humans and mice (329, 387). Further, constitutive expression of COX-2 and PGE_2 is reported in lung cancers where they are thought to prevent tumor destruction by promoting a microenvironment permissive for tumor development, in part by decreasing responses from host effector cells (388). In an *in vivo* model of murine lung cancer, neutralization of PGE_2 significantly reduced the presence of T_{reg} cells (252).

In vitro studies examining the actions of PGE_2 suggest several mechanisms this prostanoid might utilize to promote airway tolerance. Culture with this mediator is demonstrated to downregulate expression of MHC II (240, 389), reduce T and B cell proliferation (246, 390), and suppress the release of inflammatory cytokines such as IFN-γ and TNF-α while promoting the release of suppressive cytokines, such as IL-10 and TGF-β, from T cells, DCs, and macrophages (238, 241, 242, 246, 358, 391). Additionally, evidence suggests that PGE_2 can promote the differentiation and inhibitory functions of both naturally occurring and adaptive T regulatory (T_{reg}) cells (251, 253, 392).

In this chapter, we utilize mice deficient in key components of the PGE_2 pathway to evaluate the contribution of this prostanoid to immune tolerance in the airways in response to an innocuous antigen. We show that a loss of EP_2, EP_3, EP_4, or additionally a complete loss of PGE_2 does not prevent pulmonary immunosuppression to antigen in this model. Instead, we provide evidence that signaling through the prostacyclin-specific IP receptor may contribute to antigen tolerance in the lung.

Materials and Methods

Experimental Animals

All animal colonies were maintained according to standard guidelines as defined by the NIH Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee. Experiments were carried out on age and sex matched mice between 8-12 weeks of age. Mice lacking the EP_2, EP_3, EP_4, and IP receptors or mPGES1 were generated as previously described (268, 334, 350, 393, 394). EP_2, EP_3, and IP mice were backcrossed >10 generations onto the C57BL/6 (B6) background. mPGES1 mice were backcrossed >10 generation onto the BALB/c background. EP_4 -/- animals and their controls are maintained on a recombinant inbred strain consisting of B6, DBA/2, and 129/Ola.

OVA Tolerance, Sensitization and Challenge

To induce airway tolerance, a subset of experimental mice (OVA/OVA) were exposed to aerosolized 1% OVA (Sigma-Aldrich) for 10 minutes on 3 consecutive days. All other mice were exposed to aerosolized saline. One week later, OVA/OVA animals and inflamed controls (Sal/OVA) were sensitized with an i.p injection of 20μg OVA emulsified in aluminum hydroxide (alum) (Sigma-Aldrich). Two weeks following antigen sensitization, OVA/OVA and Sal/OVA mice were challenged for one hour on 3 consecutive days with 1% aerosolized OVA. Naïve controls (Sal/Sal) were challenged with saline. Airway inflammation was assessed 24 hours after the final challenge.

BALF Collection and Cell Counts

Following euthanasia, lungs were lavaged with five 1ml aliquots of HBSS (Gibco) and total cell counts were determined by hemacytometer. Cellular composition was evaluated morphologically

using cytospin preparation stained with Hema 3 or Fast Green. All remaining BALF was centrifuged to remove cells and stored at -80° c for immunoassay.

IgE Analysis

Blood was obtained by cardiac puncture, allowed to coagulate, and centrifuged to isolate serum. IgE levels were determined by immunoassay using 96 well EIA/RIA plates (Costar). Plates were coated with IgE capture antibody (Pharmingen; clone R35-72), blocked with 1% BSA/PBS and then incubated with IgE standard (Pharmingen) or serum followed by biotinylated rat anti-mouse IgE (Pharmingen; clone R35-118). Detection was carried out using streptavidin-horseradish peroxidase (HRP) (Pharmingen) and hydrogen peroxide /2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Absorbance at 405nm was measured.

Statistical Analysis

Statistical analysis was performed using Prism 4 (GraphPad Software). Comparisons of the mean were made by Student's t-test or ANOVA followed by Tukey-Kramer's HSD *post hoc* test as necessary. Data are shown as mean ± SEM. Differences with *p<0.05* were considered statistically significant.

Results

Inhalation Induced Airway Tolerance

Evidence suggests that exposure to antigen via the airway mucosa prior to systemic sensitization suppresses subsequent production of IgE, typical of atopic allergic reactions (60). However, to our knowledge no study has been conducted to assess the contribution of the PGE_2 pathway to tolerance in the airways. We first developed a method to effectively induce immunosuppression in response to our established protocol of sensitization and challenge with innocuous antigen. Antigen tolerant mice (OVA/OVA) received 1% aerosolized OVA exposure for 10 minutes daily on 3 consecutive days. One week later OVA/OVA animals and allergic controls (Sal/OVA) were i.p. immunized with 20μg OVA emulsified in adjuvant. Two weeks after systemic antigen sensitization, mice were exposed to 1% aerosolized OVA for 1 hour daily on 3 consecutive days. Naïve mice (Sal/Sal) received saline exposure. 24 hours after the final aerosol challenge, animals were euthanized and airway inflammation was assessed. As expected, Sal/OVA mice developed a robust cellular infiltration in their airways **(Fig 4.1A)** compared to Sal/Sal controls. In contrast, OVA/OVA animals had cellular levels comparable to naïve controls. Differential cell counts were established by histological analysis of cytospin preparation and granulocyte numbers were recorded. The elevated cell infiltrate observed in the airways of Sal/OVA mice reflected significantly increased granulocyte numbers **(Fig 4.1B)** compared to Sal/Sal and OVA/OVA animals. Finally, IgE levels, elevated in atopic allergic responses, were measured in the serum. Allergy induction increased IgE in Sal/OVA mice, compared to Sal/Sal controls **(Fig 4.1C)**. In contrast, OVA/OVA animals have no augmented production of IgE. These observations demonstrate that our protocol is appropriate for studying the contribution of the PGE_2 pathway to airway tolerance in mice.

Airway tolerance in EP-receptor deficient animals

The immunosuppressive effects of PGE_2 are overwhelmingly attributed to actions occurring through EP_2/EP_4 binding and elevation of intracellular cAMP. PGE_2 binding of these receptors is demonstrated to enhance IL-10 release and from DCs (230), reduce T cell proliferation (246, 247), and suppress cytokine release from macrophages (246). Further, EP_2 and EP_4 agonists upregulate expression of *Foxp3* and PGE_2-mediated *Foxp3* expression is attenuated in cells obtained from EP_2 -/- or EP_4 -/- mice (252). Therefore, we focused our work on receptors capable of altering cAMP levels.

We first examined airway tolerance in mice lacking the EP_2 receptor (EP_2 -/-) and their congenic controls (EP_2 +/+). Tolerance or sensitivity to OVA antigen was induced as described earlier. As expected, a robust cellular recruitment into the airways occurred in both EP_2 +/+ Sal/OVA and Sal/OVA EP_2 -/- controls **(Fig 4.2A)**. Pre-exposure to aerosolized antigen attenuated cellular reductions to comparable levels in both EP_2 +/+ and EP_2 -/- OVA/OVA mice. Elevated cell counts in Sal/OVA animals correspond to increased granulocyte numbers (**Fig 4.2B)**; however, this enhancement is not observed in either EP_2 +/+ or EP_2 -/- OVA/OVA cohorts. Serum IgE is similarly elevated in all Sal/OVA mice (**Fig 4.2C**). In contrast, OVA/OVA animals do not have augmented IgE levels, regardless of genotype.

We next analyzed lung inflammation in Sal/Sal, Sal/OVA, and OVA/OVA EP_4 -/- mice and their congenic controls. Our results following this round of experiments were similar to those observed in EP_2 animals. Sal/OVA EP_4 -/- mice experience similar levels of airway cellularity compared to EP_4 +/+ controls **(Fig 4.3A)**. All OVA/OVA mice have comparable cellular reductions. Differential cell analysis reveals elevated numbers of granulocytes in the BALF of both Sal/OVA groups, regardless of EP_4 expression **(Fig 4.3B)**. OVA/OVA animals have attenuated granulocyte content, compared to Sal/OVA mice, in both cohorts. This reduction does not vary significantly

between EP_4 +/+ and EP_4 -/- animals. Sensitization and challenge with OVA results in augmented concentrations of serum IgE in both EP_4 +/+ and EP_4 -/- Sal/OVA mice **(Fig 4.3C).** OVA/OVA animals have significantly less IgE. This reduction is comparable between EP_4 +/+ and EP_4 -/- mice.

Although our data generated with EP_2 and EP_4 receptor deficient mice suggests that PGE_2 elevation of intracellular cAMP does not contribute significantly to airway tolerance, alternative splicing of EP_3 allows this receptor to couple with the G_i and G_s proteins to reduce or elevate cAMP levels, respectively (206). Therefore, utilizing EP_3 -/- animals and their congenic controls, we evaluated whether a loss of this receptor would alter airway tolerance. Elevated cellularity is observed in the airways of all Sal/OVA mice following allergic sensitization and challenge **(Fig 4.4A)**. The reduced cell numbers measured in OVA/OVA animals does not differ significantly between EP_3 -/- mice and their EP_3 +/+ controls. Both EP_3 +/+ and EP_3 -/- Sal/OVA animals have augmented granulocyte numbers in their BALF **(Fig 4.4B)**. Similar reductions occur in all OVA/OVA mice. Measurements taken from the serum of wildtype Sal/OVA animals reveal elevated IgE levels **(Fig 4.4C)**. Surprisingly, levels of this immunoglobulin in EP_3 -/- mice are significantly diminished. Both OVA/OVA cohorts have reduced levels of IgE compared to their respective Sal/OVA controls. The percentage of IgE measured in wildtype OVA/OVA mice, compared to their Sal/OVA controls, is similar in degree to the percentage measured in OVA/OVA EP_3 -/-, compared to their Sal/OVA EP_3 -/- controls.

Airway tolerance in mPGES1-deficient mice

While an individual loss of the EP_2, EP_3, or EP_4 caused no apparent deviations on airway immune tolerance, this data could not rule out a role for the EP_1 receptor or multiple receptors with overlapping functions in this system. Therefore we next evaluated whether a complete loss of PGE_2 expression would alter immune tolerance to OVA antigen in the airways. While research has

suggested the existence of three PGE_2 synthases, *in vivo* data only supports a role for mPGES1 in this process (199, 228). Therefore, we utilized mPGES1 -/- mice and their congenic controls to study the overall contribution of endogenous PGE_2 to our model of airway immune tolerance.

Following harvest, OVA/OVA mice have reduced cellular levels in their BALF, compared to Sal/OVA controls **(Fig 4.5A)**. A similar trend is observed in mPGES1 -/- mice; however, this reduction fails to reach statistical significance. Differential cell analysis reveals lower granulocyte numbers in OVA/OVA mice **(Fig 4.5B)**. These reductions are similar in both mPGES1 +/+ and mPGES1 -/- OVA/OVA groups, compared to Sal/OVA controls. As expected, Sal/OVA mice have elevated levels of serum IgE **(Fig 4.5C)**. Loss of mPGES1 does not prevent or alter this enhancement. All OVA/OVA mice showed similar reductions in IgE concentrations, regardless of mPGES1 production.

OVA tolerance in IP receptor deficient mice

Prostacyclin, another prostanoid produced downstream of COX enzymatic activity on AA, exerts its effects through selective binding to the I prostanoid (IP) receptor and subsequent elevation of intracellular cAMP levels (327). Our research, presented earlier in this dissertation, supports a role for PGI_2 in attenuating atopic lung inflammation. Therefore, utilizing animals lacking the IP receptor (IP -/-) and their congenic controls, we evaluated whether this prostanoid contributes to airway immune tolerance. As we have previously shown, loss of the IP receptor leads to substantially elevated cellular recruitment in Sal/OVA mice, compared to wildtype controls **(Fig 4.6A)**. Both IP +/+ and IP -/- OVA/OVA mice have reduced airway cellularity, compared to their respective Sal/OVA controls. However, a comparison of the percentage of cells in OVA/OVA mice, in respect to their Sal/OVA controls, reveals that the attenuation observed in IP -/- mice is significantly less than in IP +/+ animals. Increased granulocyte numbers are observed in both groups of Sal/OVA animals,

compared to Sal/Sal controls, however the magnitude was much greater in IP -/- mice. In both

OVA/OVA groups, significantly fewer granulocytes are counted; however when the percent is

analyzed in terms of respective Sal/OVA controls, the decrease in granulocyte number is significantly

less in IP -/- mice **(Fig 4.6B)**. Sensitization and challenge with OVA results in elevated serum IgE

which is increased in IP -/- mice **(Fig 4.6C)**. OVA/OVA treatment significantly ameliorates IgE

concentrations in both IP +/+ and IP -/- groups. Analysis of the percentage of IgE in these animals

compared to their respective Sal/OVA controls reveals that the reduction occurs to a similar

magnitude in both IP +/+ and IP -/- animals.

Figure 4.1 Airway Tolerance Induction in Wildtype Mice

A.

B.

C.
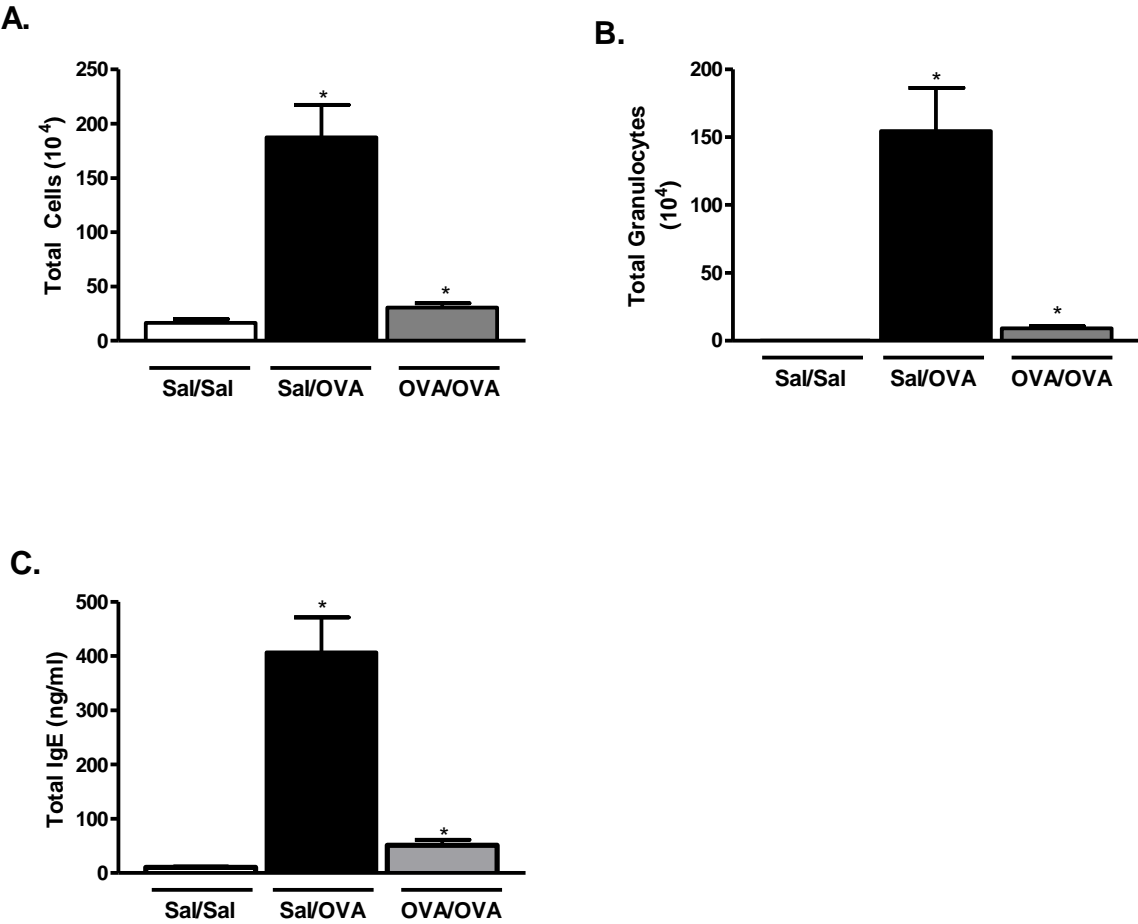
Figure 5.1 Airway Tolerance Induction in Mice Animals were exposed to 1% aerosolized OVA or saline for 10 minutes on days -9 through-7. On day 0, experimental mice were sensitized to antigen via an i.p. injection of 20ug OVA emulsified in alum. Mice were challenged two weeks later on 3 consecutive days with 1% OVA for an hour each day. 24 hours after the final challenge, mice were euthanized and inflammation was assessed. **A.** Increased cellular infiltrate is observed in the BALF of Sal/OVA animals, compared to Sal/Sal controls. The cellularity measured in OVA/OVA mice is significantly reduced compared to Sal/OVA mice (*p<0.001). **B.** Differential cell analysis reveals that cell recruitment in Sal/OVA mice results from elevation of granulocytes. OVA/OVA mice have significantly fewer granulocytes, compared to Sal/OVA mice (*p<0.001). **C.** Sal/OVA mice have heightened concentrations of IgE, compared to Sal/Sal controls. Total IgE levels are significantly less in OVA/OVA mice, compared to their allergic counterparts (*p<0.001). (Sal/Sal mice n=5; Sal/OVA n=10; OVA/OVA n=10)

Figure 4.2 Airway Tolerance in EP$_2$ -/- Mice
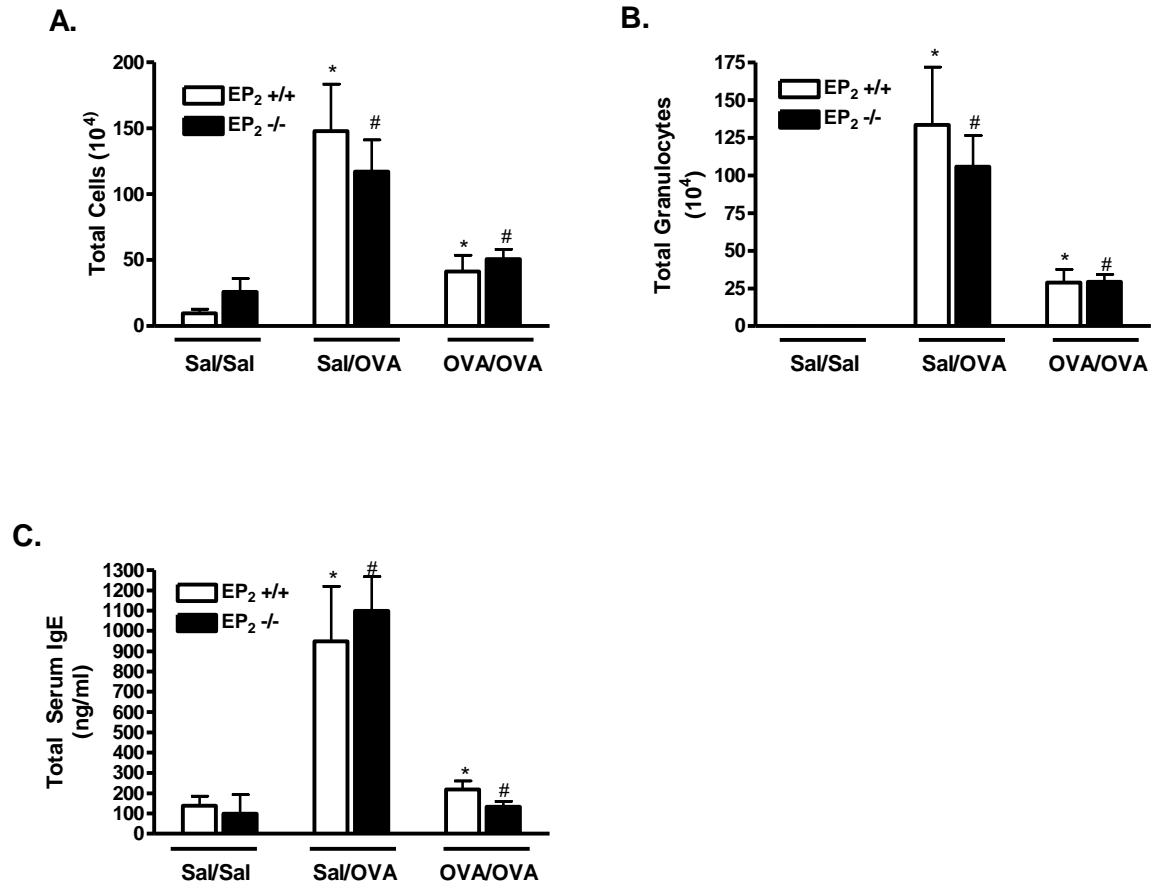
Figure 4.2 Airway Tolerance in EP$_2$ -/- Mice EP$_2$ -/- mice and their congenic controls were either

tolerized (OVA/OVA) or sensitized (Sal/OVA) to OVA antigen as previously described and

inflammation was subsequently assessed. **A.** Sensitization/challenge with antigen results in

comparable increases in cellularity in both Sal/OVA groups compared to saline controls. Similar

degrees of attenuation in cellularity are observed in EP$_2$ +/+ and EP$_2$ -/- OVA/OVA cohorts (*,#

$p<0.05$). **B.** All Sal/OVA animals have elevated BALF granulocyte numbers, independent of EP$_2$

receptor expression. Reduced granulocyte counts measured in OVA/OVA animals are unaffected by

the presence of absence of EP$_2$ (* $p<0.05$, # $p<0.01$). **C.** Total serum IgE concentrations were

assessed. Alterations in IgE measurements do not differ significantly as a result of EP$_2$ receptor

expression in either Sal/OVA or OVA/OVA groups (* $p<0.05$, # $p<0.001$). (For EP$_2$ +/+ mice:

Sal/Sal n=3, Sal/OVA n=7, OVA/OVA n=7; For EP$_2$ -/- mice: Sal/Sal n=2, Sal/OVA n=7,

OVA/OVA n=7)

Figure 4.3 Airway Tolerance in EP$_4$ -/- Mice

A.

B.

C.
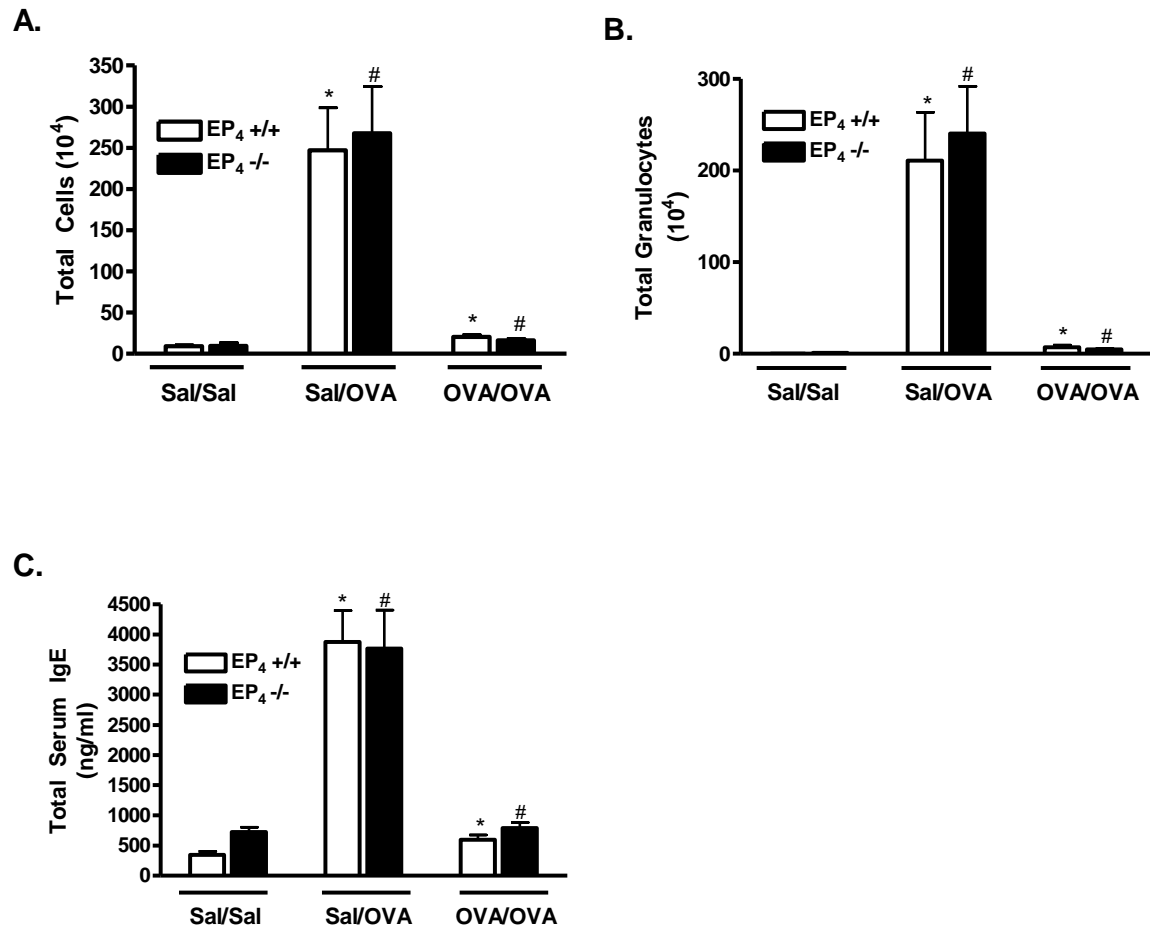
Figure 4.3 Airway Tolerance in EP$_4$ -/- Mice Lung inflammation was assessed in EP$_4$ +/+ and EP$_4$ -/- mice either tolerant (OVA/OVA) or sensitive (Sal/OVA) to antigen. **A.** All Sal/OVA mice, regardless of EP$_4$ expression, have significantly enhanced BALF cellularity compared to Sal/Sal controls. This cellularity is reduced to a similar degree in both OVA/OVA groups (* $p < 0.001$, # $p < 0.01$). **B.** Granulocyte levels are augmented in both Sal/OVA groups. OVA/OVA animals have reduced granulocytes in their airways. This reduction does not vary significantly between EP$_4$ +/+ and EP$_4$ -/- animals (* $p < 0.001$, # $p < 0.01$). **C.** Serum IgE is enhanced in both EP$_4$ +/+ and EP$_4$ -/- Sal/OVA groups. IgE levels are reduced to a similar degree in all OVA/OVA animals (* $p < 0.001$, # $p < 0.01$). (For EP$_4$ +/+ mice: Sal/Sal n=4, Sal/OVA n=10, OVA/OVA n=10; EP$_4$ -/- mice: Sal/Sal n=2, Sal/OVA n=9, OVA/OVA n=7)

Figure 4.4 Airway Tolerance in EP₃ -/- Mice
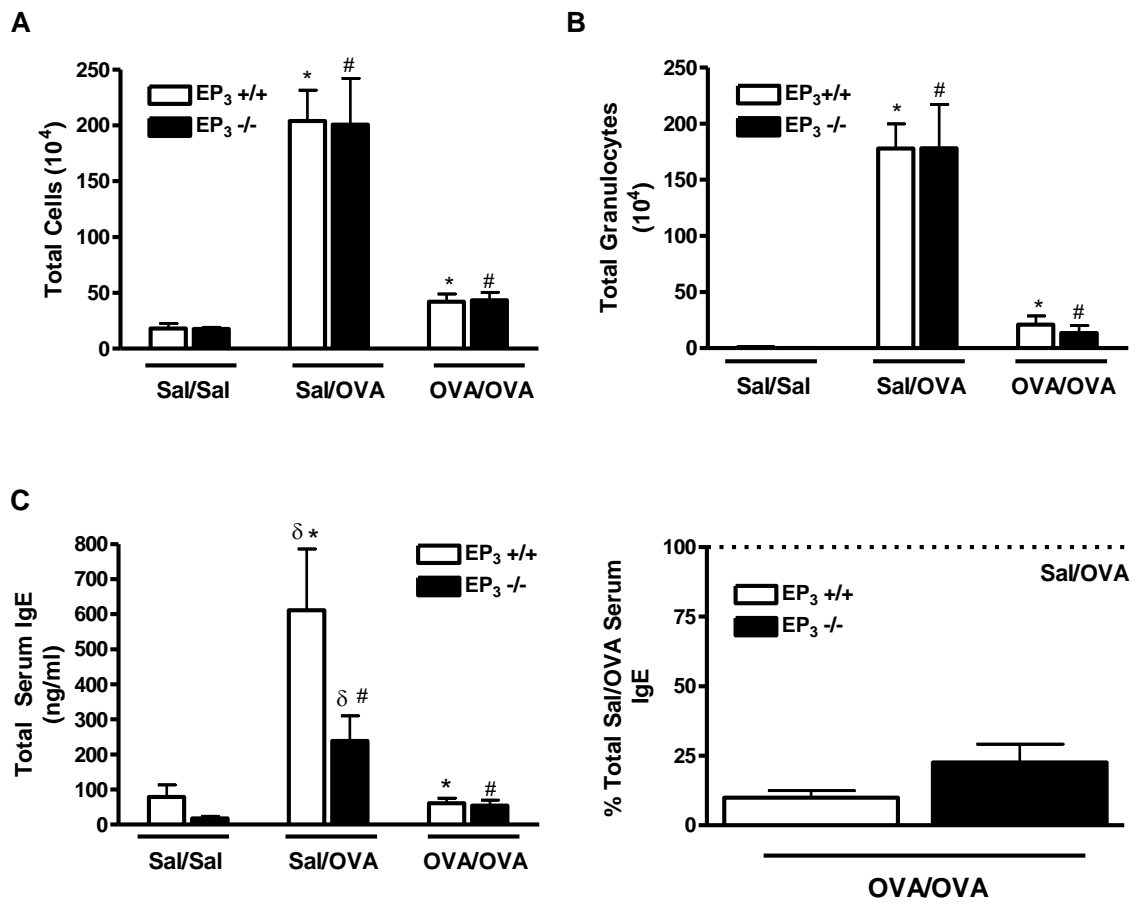
Figure 4.4 Airway Tolerance in EP$_3$ -/- Mice Tolerance or allergy were induced in the airways of

EP$_3$-/- mice and their congenic controls, as previously described. **A.** All Sal/OVA animals, regardless

of EP$_3$ expression, have similar elevations in BALF cellularity compared to Sal/Sal controls. Cellular

reductions observed in OVA/OVA mice are unaffected by a loss of the EP$_3$ receptor (* $p<0.001$, #

$p<0.01$). **B.** Sal/OVA animals have enhanced granulocyte numbers and these levels are significantly

attenuated in OVA/OVA mice. The observed reduction is similar in EP$_3$ +/+ and EP$_3$ -/- cohorts (*

$p<0.001$, # $p<0.01$). **C.** Total serum IgE is increased in Sal/OVA animals, however this increase is

significantly less in EP$_3$ -/- animals (δ $p<0.05$). Both OVA/OVA groups have significantly attenuated

IgE levels compared to their Sal/OVA controls (*,# $p<0.05$). The percent of IgE measured in

OVA/OVA groups, compared to respective Sal/OVA controls, does not differ significantly between

EP$_3$ +/+ and EP$_3$ -/- animals. (For EP$_3$ +/+ mice: Sal/Sal n=4, Sal/OVA n=10, OVA/OVA n=10; EP$_3$

-/- mice: Sal/Sal n=5, Sal/Ova n=10, OVA/OVA n=10)

Figure 4.5 Airway Tolerance in mPGES1 -/- Mice

Figure 4.5 Airway Tolerance in mPGES1 -/- Mice Following treatment with aerosolized OVA or saline, mPGES1 -/- mice and their congenic controls were sensitized and challenged with OVA. Airway inflammation was assessed following challenge. **A.** Both mPGES1 +/+ and mPGES1 -/- Sal/OVA animals have similar increases in BALF cellularity. Reduced cellularity is observed in OVA/OVA mice, compared to Sal/OVA controls, however this difference only reaches statistical significance in mPGES1 +/+ animals (* $p < 0.01$). **B.** Granulocyte numbers are enhanced to a similar degree in all Sal/OVA animals. Comparable reductions of granulocyte numbers occur in both mPGES1 +/+ and mPGES1 -/- mice, although these reductions do not reach statistical significance. **C.** All Sal/OVA animals have elevated serum IgE. OVA/OVA animals have similar reductions in IgE, regardless of mPGES1 production (*,# $p < 0.01$). (For mPGES1 +/+ mice: Sal/Sal n=3, Sal/OVA n=6, OVA/OVA n=7; mPGES1 -/- mice: Sal/Sal n=5, Sal/OVA n=6, OVA/OVA n=8)
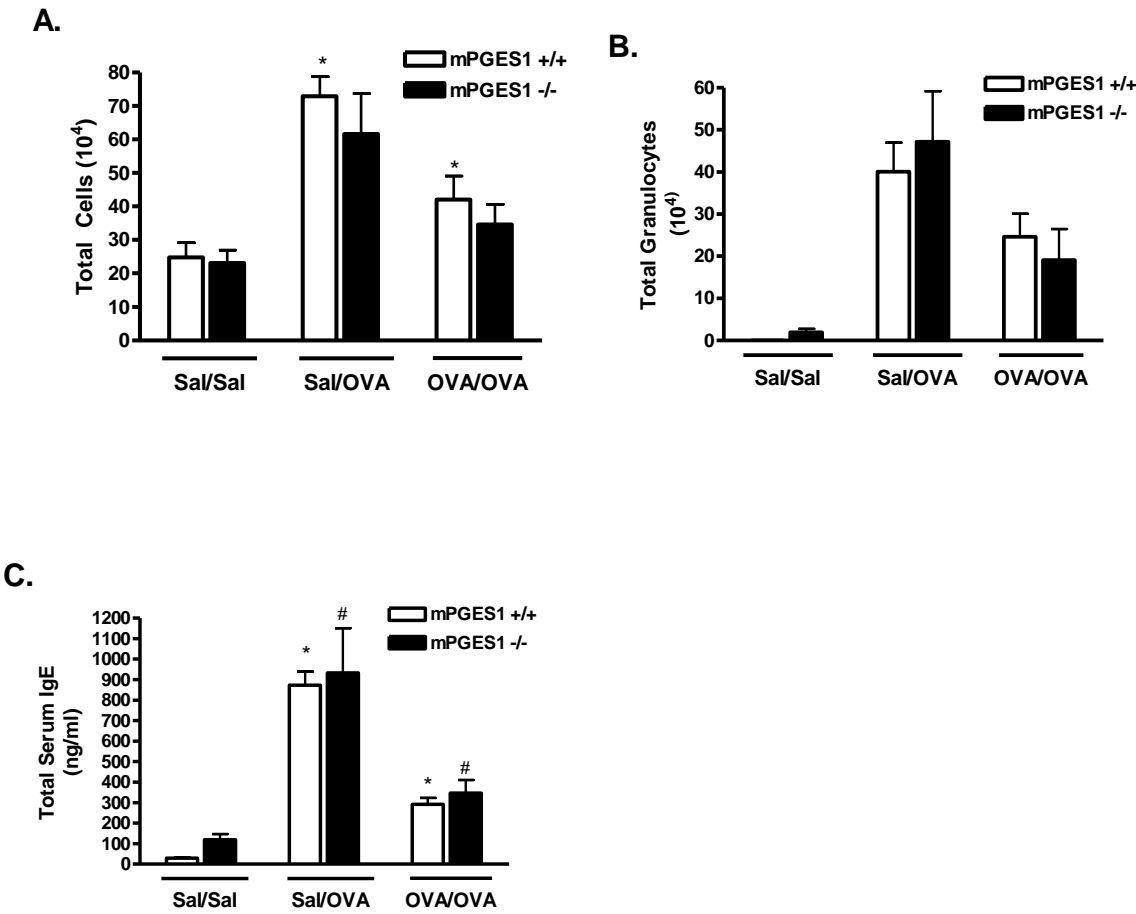
Figure 4.6 Airway Tolerance in IP -/- Mice
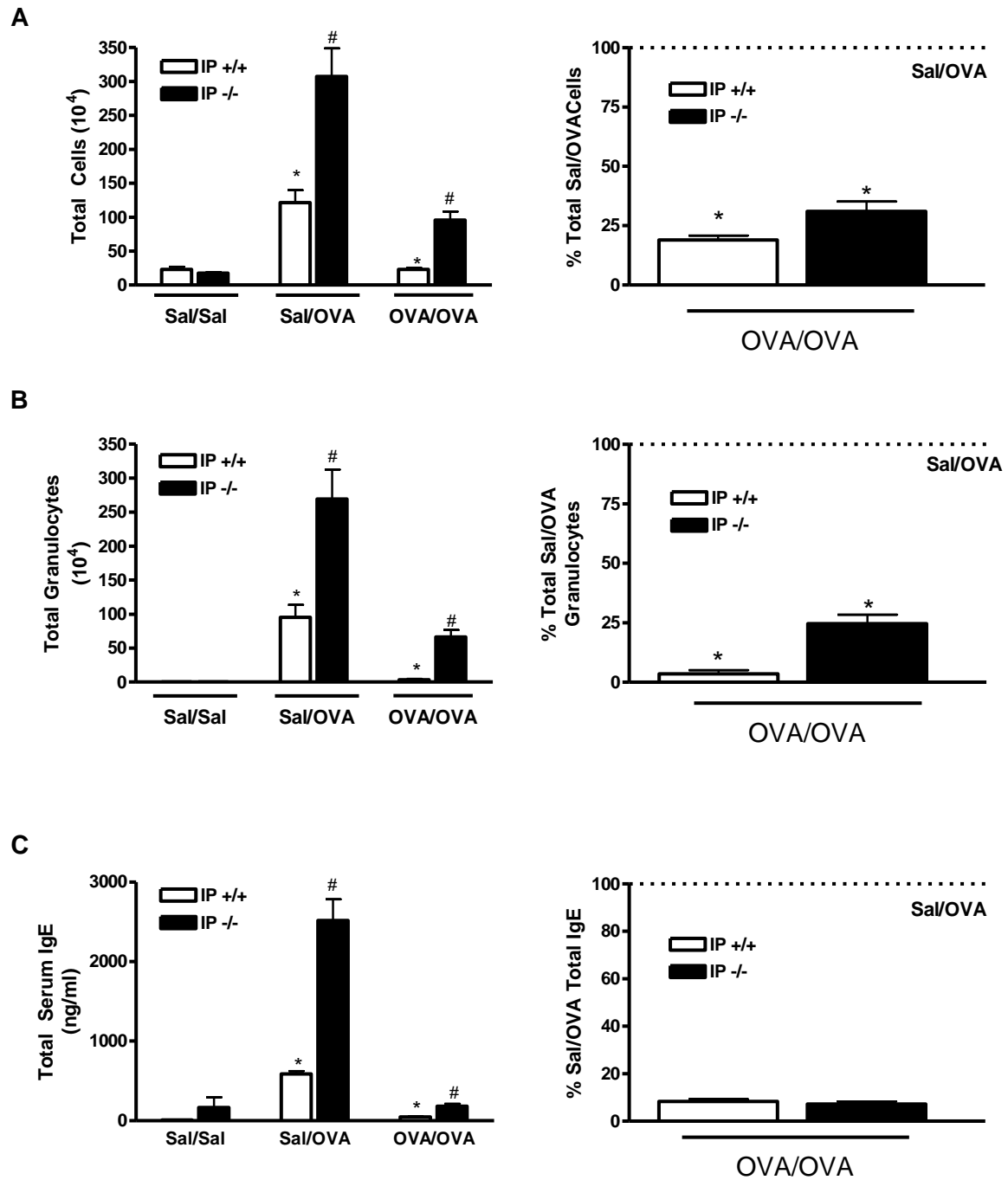
Figure 4.6 OVA Tolerance in IP -/- Mice Lung inflammation in Sal/Sal, Sal/OVA, and OVA/OVA animals was studied using IP -/- mice and their congenic controls. **A.** Increased cellularity is observed in the BALF of all Sal/OVA animals compared to Sal/Sal controls. This enhancement is dramatically greater in IP -/- mice. OVA/OVA animals have significantly attenuated cell counts in both IP +/+ and IP -/- OVA/OVA groups (*,# $p<0.001$). The percent of cells in OVA/OVA mice, compared to respective Sal/OVA controls, is significantly greater in IP -/- mice (* $p<0.02$). **B.** Increased cellularity following OVA treatment corresponds to elevated granulocyte numbers in both IP +/+ and IP -/- animals, however the increase is substantially higher in IP -/- mice. Inducing tolerance results in decreased granulocyte numbers in both OVA/OVA groups, compared to their Sal/OVA controls (*,# $p<0.001$). The percent of granulocytes counted in OVA/OVA animals, compared to respective Sal/OVA controls, is greater in IP -/- animals (* $p<0.0001$). **C.** While more substantial in IP -/- mice, all Sal/OVA mice have augmented IgE compared to Sal/Sal controls. Significantly reduced IgE concentrations are measured in all OVA/OVA mice (*,# $p<0.001$). The percent of IgE measured in OVA/OVA groups, compared to respective Sal/OVA controls, is not significantly different between IP +/+ and IP -/- cohorts. (For IP +/+ mice: Sal/Sal n=5, Sal/OVA n=11, OVA/OVA n=9; IP -/- mice: Sal/Sal n=5, Sal/OVA n=11, OVA/OVA n=9)

Discussion

While both COX-2 and PGE$_2$ have been implicated in mucosal immune tolerance established in the GI tract in response to innocuous antigens (386), the contribution of the PGE$_2$ pathway to this process in the airways has not been elucidated. To evaluate the role of PGE$_2$ in this system, we utilized a protocol in which exposure to antigen via the airways prior to sensitization and challenge attenuates subsequent allergic lung inflammation. In agreement with previous data (60), we observed that when a novel antigen is encountered by the airway mucosa prior to systemic sensitization, secondary challenge results in a dramatic attenuation of total serum IgE and BALF granulocyte cellularity.

We subjected mice deficient in individual EP receptors to our model of airway tolerance in order to characterize the *in vivo* contribution of PGE$_2$ signaling to our model of mucosal immunosuppression in the respiratory tract. We found that a genetic loss of the EP$_2$, EP$_3$, or EP$_4$ receptor was insufficient to alter the suppressed inflammation observed when wildtype animals are pre-exposed to antigen. An inability to signal through these receptors did not prevent reduction in serum IgE levels or BALF granulocyte counts.

Surprisingly, we found that a loss of the EP$_3$ receptor results in significantly reduced IgE levels in Sal/OVA animals, compared to EP$_3$ +/+ controls, suggesting that a pathway downstream of EP$_3$-signaling enhances IgE during allergy in the lung. In Chapter 3, we demonstrated that a loss of the IP receptor significantly augments IgE levels in the serum of animals sensitized and challenged with antigen. However, no alterations in this immunoglobulin are observed in the inflamed airways of mice lacking either isoform of the COX enzyme (373). Collectively, this suggests that actions of one or more pro-inflammatory prostanoid can effectively balance the PGI$_2$-mediated suppression of

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IgE during lung allergy. Our data supports a role for PGE_2-dependent activation of EP_3 in enhancing IgE levels.

Alternative splicing produces distinct EP_3 isoforms that can activate unique downstream pathways. In a model of cutaneous edema, activation of the EP_3 receptor, and upregulation of intracellular calcium, promotes mast cell degranulation, indirectly enhancing inflammation (211, 258, 259). EP_3 signaling may elevate IgE levels in our system by enhancing the release of factors such as chymase, from mast cells, which have been shown to increase IgE production (395). EP_3 coupling with G_s or G_i proteins can elevate or reduce intracellular cAMP levels, respectively. Increased levels of this second messenger have been shown to promote IgE isotype switching while suppressing proliferation of B cells (396). Therefore it is conceivable that elevated levels of this immunoglobulin may result from EP_3 coupling with G_s protein to enhance IgE isotype switching or alternatively, EP_3 coupling with G_i to reduce cAMP and promote proliferation of B cells. Additionally, recent evidence by Singh and colleagues suggests that EP_3-dependent signaling promotes the development of DCs both *in vitro* and *in vivo* and animals lacking this receptor have reduced DC numbers in their bone marrow and spleen (397). It is possible that our animals lacking EP_3 have fewer APCs available to activate the adaptive immune system in response to antigen, indirectly resulting in lower quantities of IgE. Unlike EP_3 -/- mice, IgE levels measured in mPGES1 -/- animals do not differ significantly from wildtype controls. However we do tend to see a slight attenuation in levels of this immunoglobulin in mPGES1 -/- animals (data not shown). This suggests that one or more of the alternative EP receptors antagonizes EP_3 in its capacity to stimulate IgE.

Our observation that EP_3-signaling promotes IgE production is in contrast to work by Kunikata et al. who observed that animals lacking this receptor have elevated inflammation following sensitization and challenge with OVA and no apparent difference in IgE levels (320). It is unclear

why the results of Kunikata and colleagues differ from our own; however we have been unable to repeat the findings of this group using our own congenic EP_3 -/- animals (unpublished observations). Additionally, we cannot rule out the possibility that differences in protocol account for these discrepancies, given that the method of allergy induction employed by Kunikata and colleagues utilizes a mechanism distinct from our own (158).

All three of the receptors studied in this work are capable of coupling with G_s protein to elevate levels of cAMP. It is therefore conceivable that the contribution of these receptors to airway immune tolerance is redundant and that multiple receptors can perform an identical task. In this scenario, a genetic loss of a single receptor may not be sufficient to observe an altered phenotype. Additionally, an elevation of intracellular calcium, following activation of an EP_1-dependent pathway, could potentially prevent T_H2 lung inflammation in this model of airway immune tolerance. In a model of contact hypersensitivity, a T_H1 cell-type response, animals lacking the EP_1 receptor had attenuated inflammation. In this study, cells isolated from the lymph nodes of EP_1 -/- mice produced significantly fewer T_H1 cells when stimulated with antigen (398). Therefore, activation of an EP_1-dependent pathway could potentially promote mucosal tolerance in the respiratory tract by suppressing T_H2 cell differentiation in favor of T_H1 cell polarization.

To determine whether the lack of phenotype in our mice was due to pathway redundancy or a dependency on EP_1 signaling, we evaluated airway tolerance in mice deficient of the mPGES1 synthase, the only synthase with a documented contribution to PGE_2 synthesis *in vivo* (199). We observed that mPGES1 -/- animals exposed to aerosolized antigen prior to immunization experienced immunoglobulin and granulocyte levels that were comparable to their wildtype counterparts following allergen challenge. While the attenuation in cellularity failed to reach statistical significance in mPGES1 -/- animals, the magnitude of change was similar to that observed in

wildtype controls, suggesting that tolerance was unaffected in these animals. However, mice on this genetic background, BALB/c, often have poor granulocyte recruitment into the BALF (399). We cannot rule out the possibility that a significant difference may have been observed in the BALF of mPGES1 -/- mice on a more permissible genetic background. This can be clarified by studying the establishment of tolerance in C57BL/6 mice lacking mPGES1.

We and others have demonstrated that prostacyclin-mediated signaling suppresses inflammatory responses in models of lung allergy (278, 284, 323), therefore we evaluated the role of this prostanoid in airway tolerance. In agreement with our previous data, we found that aspects of immunosuppression in our model were altered in mice lacking the IP receptor. Specifically, IP -/- OVA/OVA mice did not experience the same magnitude of granulocyte cell attenuation observed in wildtype OVA/OVA animals. Surprisingly, we found that suppression of IgE occurred independently of prostacyclin signaling, implying that prostacyclin contributes to airway tolerance subsequent to initial antigen presentation or alternatively, that antigen sensitization must occur locally, in agreement with our previous data.

Our findings support a model in which PGI_2 released from stromal cells of the lung suppresses the actions of leukocytes including DCs and T cells. This mediator may promote airway immune tolerance by inhibiting chemokine receptors on DCs that allow them to home to lymph nodes. In support of this theory, Idzko et al. demonstrate that administration of iloprost, a stable prostacyclin analog, to mice not only inhibits the maturation of lung DC's but also prevents the migration of these APC's to local lymph nodes (282). While our data fails to show a role for prostacyclin in the suppression of initial APC functions in this model, primed T cells require restimulation by antigen-loaded DCs to initiate secondary allergic responses (381). Secondary

immune responses are effectively suppressed when iloprost is given to immunized mice prior to challenge resulting from suppressed maturation of DCs (282).

Expression of prostacyclin may also suppress T cells recruited to the lung which escaped earlier tolerance mechanisms. Our previous findings suggest that iloprost can attenuate IL-13 levels in the BALF. Conversely, IP expression is upregulated on Foxp3$^+$ T$_{regs}$ (283) and treatment with prostacyclin enhances IL-10 levels released from CD4$^+$ cells. Elevated prostacyclin expression may therefore attract T$_{regs}$ to the lung and enhance the release of anti-inflammatory cytokines that prevent the initiation of inflammatory responses mounted against innocuous antigens.

In summary, our work provides evidence that the PGE$_2$ pathway does not contribute to immune tolerance of innocuous antigens in the airway. Instead, prostacyclin may promote immune suppression to innocuous antigens by selectively recruiting regulatory T cells to the mucosa while preventing the activation of effector T cells that would enhance deleterious immune responses.

CHAPTER V

CONCLUDING REMARKS

Atopic allergy is a major risk factor for asthma: a chronic disease of the respiratory tract characterized by episodes of reversible airway obstruction and airway hyperresponsiveness. Worldwide, 300 million individuals are thought to suffer from asthma, making this affliction a major public health concern. The etiology of asthma is complex; both environmental and genetic factors contribute to this disease emphasizing the necessity for research delineating underlying mechanisms. Atopic asthma develops when the immune system mistakenly interprets inhaled innocuous environmental antigens as harmful and mounts T_H2-driven responses against them. The severity of an attack is controlled, in part, by the cytokines and mediators released in the airways in response to that antigen.

Prostanoids are lipid mediators produced by almost all cell types of the body. These mediators are not stored but instead are de novo synthesized from arachidonic acid (AA) in response to a variety of stimuli. Free AA, liberated from the phospholipid membrane by the actions of phospholipases is presented to cyclooxygenase (COX) enzymes which convert it into unstable intermediate products. Synthesis into the five bioactive prostanoids is completed by prostanoid specific synthases. These mediators exert their autocrine and paracrine functions through binding of specific G-coupled receptors that activate a diverse array of downstream pathways. Prostanoids are implicated in a broad range of processes from tissue regulation to inflammation and tumorogenesis.

(400) In this dissertation, we explored the overall contribution of prostanoids, and the specific

contributions of PGE_2 and PGI_2, to allergy and mucosal tolerance in the lung.

Our work in Chapter 2 demonstrates that while overall, COX-dependent prostanoid

production limits allergic airway inflammation in the lung, a specific loss of PGE_2 attenuates disease

parameters in mice, suggesting that this prostanoid promotes inflammation in this system. We

provide evidence that structural cells of the lung, likely epithelial cells or airway smooth muscle cells,

are responsible for the synthesis of this pro-inflammatory prostanoid. However, more work is

necessary to elucidate which cell type, specifically, is the source of this PGE_2. The use of conditional

knockout animals may help to clarify this issue. Mice homozygous for a loxP-flanked *mPges1* gene

can be crossed to animals expressing cre recombinase under promoters specific for smooth muscle

cells or epithelial cells. *Tgln* cre mice are used to study the loss of PGE_2 production by smooth

muscle cells while mice expressing cre under the human *SP-C* promoter are widely used to study

pulmonary epithelial cells (401, 402).

The work conducted in Chapter 2 also does not identify which PGE_2 receptor is being

activated during allergy in the pulmonary system to promote inflammation. However, allergy was

induced in animals lacking receptors EP_2, EP_3, or EP_4 in Chapter 4 of this dissertation, to establish

controls for studying airway tolerance. No differences in total cellularity or granulocyte numbers

were observed in any of these knockout animals, compared to their WT controls, suggesting that an

individual loss of these receptors cannot account for the phenotype we observed in mPGES1 -/- mice

in Chapter 2. Several possibilities exist that may explain this finding. Chapter 4 did not study allergy

in animals lacking the EP_1 receptor so it is possible that a loss of this receptor may account for the

attenuated inflammation observed when PGE_2 production is absent. Along with EP_3, recent evidence

suggests that EP_1-signaling can promote the development of DCs both *in vitro* and *in vivo*. Indeed,

animals lacking this receptor have reduced levels of this APC in their bone marrow and spleen (397). It is therefore conceivable that signaling through the EP_1 receptor promotes lung inflammation by potentiating the development of DCs and enhancing the presentation of antigen to the adaptive immune system. However, given that PGE_2 appears to exert its pro-inflammatory actions mainly during the effector phase of allergy in the airways, subsequent to antigen presentation, this possibility seems unlikely. The contribution of EP_1 to this process can be addressed by analyzing OVA-induced lung inflammation in mice lacking this receptor.

More likely, our data may imply that the ability of PGE_2 to enhance inflammation during allergy in the respiratory tract is controlled, redundantly, by multiple receptors and therefore a loss of only one receptor is not sufficient to reproduce the attenuated inflammation observed in mPGES1 -/- animals. In Chapter 2, we hypothesize that PGE_2 promotes inflammation by increasing vasodilation and vasculature permeability. At least some research indicates that these functions can be controlled dually by the EP_2 and the EP_4 receptors and elevation of intracellular cAMP (403). These receptors may work in concert to promote inflammation in our model of lung inflammation. This idea is substantiated by data generated in a model of arthritis, showing that arthritic scores in mice are only attenuated when both the EP_2 and the EP_4 signaling pathways are inhibited (276). Given that EP_4 mice do not survey on most common inbred backgrounds, this possibility may be addressed by treating EP_2 -/- animals with an EP_4 antagonist during antigen challenge. Additionally, we observed in Chapter 4 that EP_3 -/- animals have reduced IgE levels, compared to wildtype controls, suggesting that this receptor may also contribute to the pro-inflammatory actions of PGE_2 in the lung. Like, EP_2 and EP_4, EP_3 can alter intracellular cAMP levels, suggesting that all three of these receptors may act redundantly. To identify whether EP_2, EP_3, and EP_4 all contribute to allergic lung inflammation, EP_2 and EP_4 antagonists can be given to EP_3 animals.

Unlike PGE_2, we show in Chapter 3 that PGI_2 signaling through the IP receptor limits allergic inflammation in the lung during both the sensitization and effector phase. Following sensitization with antigen, IP -/- mice have significantly elevated serum IgE levels. Our *ex vivo* experiments with splenocytes provide evidence that PGI_2 does not suppress IgE by altering immune cell proliferation or polarization; however, given that DCs represent only a small fraction of the cells present in splenocyte populations (397), this system may not accurately model antigen presentation and subsequent sensitization. Analysis of cellular responses by cells obtained from draining lymph nodes or alternatively, coculture of isolated DC and naïve T cell populations may be more informative. Indeed, evidence in rats suggests that, following stimulation with antigen, IgE-producing plasma cells are concentrated at the highest levels in local draining lymph nodes (404). Additionally, PGI_2 may act downstream of T_H2 cell differentiation and expansion. This prostanoid may limit interactions between T_H2 cells and B cells or alternatively, skew immunoglobulin production. To test for these possibilities, costimulatory molecules on these immune cells which influence activation of plasma cells, such as CD23 or CD40 can be measured. Finally, analysis of alternate immunoglobulin levels present in the serum of sensitized IP -/- mice may help determine how IgE levels are being limited by PGI_2.

While IP -/- animals have significantly increased levels of serum IgE following sensitization, compared to WT controls, the magnitude of this elevation is less than that observed following antigen challenge. Given that our model relies on systemic sensitization, this finding may imply that the anti-inflammatory actions of PGI_2, in this system, are specific to the lung. In support of this hypothesis, Idzko and colleagues have demonstrated that pulmonary inflammation can be suppressed when sensitization occurs in the airways using mDCs treated with iloprost (282). Further work is needed to explore this possibility. Data by Eisenbarth et al. has shown that inhaled OVA can induce atopic antigen sensitization when combined with low levels of LPS (74). Utilizing this model to study

pulmonary allergy in IP-/- animals may provide information on tissue-specificity for PGI_2-mediated airway protection. Additionally, PGI_2 may contribute mainly to the effector phase of an allergic response in the lung. This can be examined by generating OT-II mice lacking the IP receptor. While aerosolizing OVA to these animals does not provoke an atopic response, naïve T cells can be isolated from these animals and cultured to favor T_H2 cell polarization. These cells can be transferred into naïve IP -/- animals, allowing us to specifically study the contribution of IP-signaling to the effector phase of atopic allergy.

Utilizing bone marrow chimeras, we demonstrate in Chapter 2 that PGI_2 binds to receptors on leukocytes to limit inflammation during pulmonary allergy. Several classes of immune cells upregulate IP expression in response to airway antigen challenge, including DCs and T_H2 cells (103). Our work is unable to define which class of immune cell is being altered by IP-signaling in this system. Employing the cre/lox system will again allow us to more precisely define the contributions of specific immune cell populations to PGI_2-mediated protection in the lung. Mating animals homozygous for a loxP-flanked *Ptgir* gene to mice expressing cre recombinase downstream of the *Cd11c* promoter (405) or alternatively the *Cd4* promoter will delineate the importance of IP-signaling on DCs and T cells respectively. IP -/- animals have augmented airway remodeling in a chronic allergy model (324). Given that IP is upregulated on fibroblasts (103), it may be interesting to determine if IP-signaling on structural cells becomes important in limiting disease during chronic allergy. Subjecting bone marrow chimeras expressing IP only on radioresistant cells to a chronic allergy protocol may provide more information on this question.

We have provided preliminary evidence that while iloprost treatment causes only a slight reduction in BALF cellularity in COX-1 -/- animals, this drug has a more profound ability to limit IL-13 levels in these mice. Repeating these experiments with a larger cohort may give a more precise

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understanding of the effect this PGI_2 analog has on lung inflammation in these animals. Given that IP-signaling on leukocytes appears to be important for the protection afforded by PGI_2 in the airways, we propose that this prostanoid prevents the reactivation of memory T cells recruited to the lung following antigen challenge. This may be explored further by isolating DCs from the inflamed lung and measuring their maturation state in terms of cell surface receptor levels along with their ability to stimulate T cells. Additionally, crossing the leukocyte cell-specific cre recombinase animals outlined earlier to COX-1 -/- animals with a homozygous loxP-flanked *Ptgir* may further define the contribution of DC's and T helper cells to the reductions in IL-13 levels measured following iloprost treatment.

A novel cell type which produces high levels of IL-13 has recently been described by several groups (406-409). These are non T/non B cells which respond to IL-25 and IL-33, both of which are thought to contribute to allergic responses in the lung following OVA sensitization and challenge (410, 411). It stands to reason that this novel cell type is recruited to the airways during episodes of pulmonary allergy, although research has not yet confirmed this. Given that treatment with iloprost reduces the levels of IL-13 measured in COX-1 -/- animals, it is possible that PGI_2 alters the recruitment or function of these newly characterized cells. Further work is needed to explore this possibility. These cells lack conventional lineage markers but express c-Kit, as well as the receptors for IL-25 and IL-33 (406). Analysis of cells with this spectrum of surface markers in iloprost-treated COX-1 -/- animals, and their vehicle treated controls, may provide further information on the effects of PGI_2 on this class of cells.

Given that iloprost treatment appears to attenuate certain parameters of inflammation in COX-1 -/- animals, it will be interesting to repeat these experiments in animals lacking COX-2 to elucidate whether iloprost can suppress the pulmonary allergy that develops in these mice and if so, if

the effects of this PGI_2 analog are identical to those observed in COX-1 -/- animals. Additionally, it will be important to identify whether iloprost can ameliorate allergy induced in the lungs of WT animals or if this drug is only capable of limiting disease in animals lacking COX production.

REFERENCES

1.   Passalacqua, G., and G. Ciprandi. 2008. Allergy and the lung. *Clin Exp Immunol* 153 Suppl 1:12-16.

2.   Larche, M., C. A. Akdis, and R. Valenta. 2006. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 6:761-771.

3.   Kim, H. Y., R. H. DeKruyff, and D. T. Umetsu. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol* 11:577-584.

4.   Caramori, G., D. Groneberg, K. Ito, P. Casolari, I. M. Adcock, and A. Papi. 2008. New drugs targeting Th2 lymphocytes in asthma. *J Occup Med Toxicol* 3 Suppl 1:S6.

5.   Braman, S. S. 2006. The global burden of asthma. *Chest* 130:4S-12S.

6.   Bloom, B., R. A. Cohen, and G. Freeman. Summary health statistics for U.S. children: National Health Interview Survey, 2009. *Vital Health Stat 10*:1-82.

7.   Sullivan, P. W., V. H. Ghushchyan, J. F. Slejko, V. Belozeroff, D. R. Globe, and S. L. Lin. The burden of adult asthma in the United States: evidence from the Medical Expenditure Panel Survey. *J Allergy Clin Immunol* 127:363-369 e361-363.

8.   Akinbami, L. J., J. E. Moorman, and X. Liu. Asthma prevalence, health care use, and mortality: United States, 2005-2009. *Natl Health Stat Report*:1-14.

9.   Morgan, W. J., D. A. Stern, D. L. Sherrill, S. Guerra, C. J. Holberg, T. W. Guilbert, L. M. Taussig, A. L. Wright, and F. D. Martinez. 2005. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. *Am J Respir Crit Care Med* 172:1253-1258.

10.   Strachan, D. P. 1989. Hay fever, hygiene, and household size. *BMJ* 299:1259-1260.

11.   Braun-Fahrlander, C., M. Gassner, L. Grize, U. Neu, F. H. Sennhauser, H. S. Varonier, J. C. Vuille, and B. Wuthrich. 1999. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* 29:28-34.

12.   Kilpelainen, M., E. O. Terho, H. Helenius, and M. Koskenvuo. 2000. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* 30:201-208.

13.   Von Ehrenstein, O. S., E. Von Mutius, S. Illi, L. Baumann, O. Bohm, and R. von Kries. 2000. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 30:187-193.

14.   Strachan, D. P. 1997. Allergy and family size: a riddle worth solving. *Clin Exp Allergy* 27:235-236.

15.   Celedon, J. C., R. J. Wright, A. A. Litonjua, D. Sredl, L. Ryan, S. T. Weiss, and D. R. Gold. 2003. Day care attendance in early life, maternal history of asthma, and asthma at the age of 6 years. *Am J Respir Crit Care Med* 167:1239-1243.

16.   Ahmad-Nejad, P., S. Mrabet-Dahbi, K. Breuer, M. Klotz, T. Werfel, U. Herz, K. Heeg, M. Neumaier, and H. Renz. 2004. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. *J Allergy Clin Immunol* 113:565-567.

17.   Fageras Bottcher, M., M. Hmani-Aifa, A. Lindstrom, M. C. Jenmalm, X. M. Mai, L. Nilsson, H. A. Zdolsek, B. Bjorksten, P. Soderkvist, and O. Vaarala. 2004. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. *J Allergy Clin Immunol* 114:561-567.

18.   Kim, Y. S., K. S. Kwon, D. K. Kim, I. W. Choi, and H. K. Lee. 2004. Inhibition of murine allergic airway disease by Bordetella pertussis. *Immunology* 112:624-630.

19.   Bakir, M., F. Tukenmez, N. N. Bahceciler, I. B. Barlan, and M. M. Basaran. 2000. Heat-killed Mycobacterium bovis-bacillus Calmette Guerin-suppressed total serum IgE response in ovalbumin-sensitized newborn mice. *J Asthma* 37:329-334.

20.   Gerhold, K., K. Bluemchen, A. Franke, P. Stock, and E. Hamelmann. 2003. Exposure to endotoxin and allergen in early life and its effect on allergen sensitization in mice. *J Allergy Clin Immunol* 112:389-396.

21.   Blumer, N., U. Herz, M. Wegmann, and H. Renz. 2005. Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma. *Clin Exp Allergy* 35:397-402.

22.   Weitzman, M., S. Gortmaker, and A. Sobol. 1990. Racial, social, and environmental risks for childhood asthma. *Am J Dis Child* 144:1189-1194.

23.   Gold, D. R., A. Rotnitzky, A. I. Damokosh, J. H. Ware, F. E. Speizer, B. G. Ferris, Jr., and D. W. Dockery. 1993. Race and gender differences in respiratory illness prevalence and their relationship to environmental exposures in children 7 to 14 years of age. *Am Rev Respir Dis* 148:10-18.

24.   Priftis, K. N., E. C. Mantzouranis, and M. B. Anthracopoulos. 2009. Asthma symptoms and airway narrowing in children growing up in an urban versus rural environment. *J Asthma* 46:244-251.

25.   Hill, T. D., L. M. Graham, and V. Divgi. Racial disparities in pediatric asthma: a review of the literature. *Curr Allergy Asthma Rep* 11:85-90.

26. O'Neill, M. S., M. Jerrett, I. Kawachi, J. I. Levy, A. J. Cohen, N. Gouveia, P. Wilkinson, T. Fletcher, L. Cifuentes, and J. Schwartz. 2003. Health, wealth, and air pollution: advancing theory and methods. *Environ Health Perspect* 111:1861-1870.

27. Romieu, I., R. Garcia-Esteban, J. Sunyer, C. Rios, M. Alcaraz-Zubeldia, S. R. Velasco, and F. Holguin. 2008. The effect of supplementation with omega-3 polyunsaturated fatty acids on markers of oxidative stress in elderly exposed to PM(2.5). *Environ Health Perspect* 116:1237-1242.

28. Castillejos, M., D. R. Gold, A. I. Damokosh, P. Serrano, G. Allen, W. F. McDonnell, D. Dockery, S. Ruiz Velasco, M. Hernandez, and C. Hayes. 1995. Acute effects of ozone on the pulmonary function of exercising schoolchildren from Mexico City. *Am J Respir Crit Care Med* 152:1501-1507.

29. Gauderman, W. J., H. Vora, R. McConnell, K. Berhane, F. Gilliland, D. Thomas, F. Lurmann, E. Avol, N. Kunzli, M. Jerrett, and J. Peters. 2007. Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. *Lancet* 369:571-577.

30. Diaz-Sanchez, D., A. R. Dotson, H. Takenaka, and A. Saxon. 1994. Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. *J Clin Invest* 94:1417-1425.

31. Peters, J. M., E. Avol, W. J. Gauderman, W. S. Linn, W. Navidi, S. J. London, H. Margolis, E. Rappaport, H. Vora, H. Gong, Jr., and D. C. Thomas. 1999. A study of twelve Southern California communities with differing levels and types of air pollution. II. Effects on pulmonary function. *Am J Respir Crit Care Med* 159:768-775.

32. Kinney, P. L., G. D. Thurston, and M. Raizenne. 1996. The effects of ambient ozone on lung function in children: a reanalysis of six summer camp studies. *Environ Health Perspect* 104:170-174.

33. Strachan, D. P., and D. G. Cook. 1998. Health effects of passive smoking. 6. Parental smoking and childhood asthma: longitudinal and case-control studies. *Thorax* 53:204-212.

34. Strachan, D. P., and D. G. Cook. 1997. Health effects of passive smoking. 1. Parental smoking and lower respiratory illness in infancy and early childhood. *Thorax* 52:905-914.

35. Li, S., S. Batterman, E. Wasilevich, H. Elasaad, R. Wahl, and B. Mukherjee. Asthma exacerbation and proximity of residence to major roads: a population-based matched case-control study among the pediatric Medicaid population in Detroit, Michigan. *Environ Health* 10:34.

36. McConnell, R., K. Berhane, L. Yao, M. Jerrett, F. Lurmann, F. Gilliland, N. Kunzli, J. Gauderman, E. Avol, D. Thomas, and J. Peters. 2006. Traffic, susceptibility, and childhood asthma. *Environ Health Perspect* 114:766-772.

37.   Gehring, U., A. H. Wijga, M. Brauer, P. Fischer, J. C. de Jongste, M. Kerkhof, M. Oldenwening, H. A. Smit, and B. Brunekreef. Traffic-related air pollution and the development of asthma and allergies during the first 8 years of life. *Am J Respir Crit Care Med* 181:596-603.

38.   Whitekus, M. J., N. Li, M. Zhang, M. Wang, M. A. Horwitz, S. K. Nelson, L. D. Horwitz, N. Brechun, D. Diaz-Sanchez, and A. E. Nel. 2002. Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J Immunol* 168:2560-2567.

39.   Ohta, K., N. Yamashita, M. Tajima, T. Miyasaka, J. Nakano, M. Nakajima, A. Ishii, T. Horiuchi, K. Mano, and T. Miyamoto. 1999. Diesel exhaust particulate induces airway hyperresponsiveness in a murine model: essential role of GM-CSF. *J Allergy Clin Immunol* 104:1024-1030.

40.   Kim, C. S., N. E. Alexis, A. G. Rappold, H. Kehrl, M. J. Hazucha, J. C. Lay, M. T. Schmitt, M. Case, R. B. Devlin, D. B. Peden, and D. Diaz-Sanchez. Lung function and inflammatory responses in healthy young adults exposed to 0.06 ppm ozone for 6.6 hours. *Am J Respir Crit Care Med* 183:1215-1221.

41.   Alexis, N. E., J. C. Lay, M. Hazucha, B. Harris, M. L. Hernandez, P. A. Bromberg, H. Kehrl, D. Diaz-Sanchez, C. Kim, R. B. Devlin, and D. B. Peden. Low-level ozone exposure induces airways inflammation and modifies cell surface phenotypes in healthy humans. *Inhal Toxicol* 22:593-600.

42.   Diaz-Sanchez, D., A. Tsien, J. Fleming, and A. Saxon. 1997. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J Immunol* 158:2406-2413.

43.   Mastrangelo, G., E. Clonfero, S. Pavanello, U. Fedeli, E. Fadda, A. Turato, S. Piccinni, R. Montagnani, and G. Marcer. 2003. Exposure to diesel exhaust enhances total IgE in non-atopic dockers. *Int Arch Occup Environ Health* 76:63-68.

44.   Devouassoux, G., A. Saxon, D. D. Metcalfe, C. Prussin, M. G. Colomb, C. Brambilla, and D. Diaz-Sanchez. 2002. Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J Allergy Clin Immunol* 109:847-853.

45.   Diaz-Sanchez, D., M. P. Garcia, M. Wang, M. Jyrala, and A. Saxon. 1999. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol* 104:1183-1188.

46.   Takenaka, H., K. Zhang, D. Diaz-Sanchez, A. Tsien, and A. Saxon. 1995. Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: direct effects on B-cell IgE production. *J Allergy Clin Immunol* 95:103-115.

47.   Tsien, A., D. Diaz-Sanchez, J. Ma, and A. Saxon. 1997. The organic component of diesel exhaust particles and phenanthrene, a major polyaromatic hydrocarbon constituent, enhances IgE production by IgE-secreting EBV-transformed human B cells in vitro. *Toxicol Appl Pharmacol* 142:256-263.

48.   Diaz-Sanchez, D., A. Tsien, A. Casillas, A. R. Dotson, and A. Saxon. 1996. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. *J Allergy Clin Immunol* 98:114-123.

49.   Marano, F., S. Boland, V. Bonvallot, A. Baulig, and A. Baeza-Squiban. 2002. Human airway epithelial cells in culture for studying the molecular mechanisms of the inflammatory response triggered by diesel exhaust particles. *Cell Biol Toxicol* 18:315-320.

50.   Hiura, T. S., M. P. Kaszubowski, N. Li, and A. E. Nel. 1999. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J Immunol* 163:5582-5591.

51.   Griffiths, H. R. 2000. Antioxidants and protein oxidation. *Free Radic Res* 33 Suppl:S47-58.

52.   Lim, H. B., T. Ichinose, Y. Miyabara, H. Takano, Y. Kumagai, N. Shimojyo, J. L. Devalia, and M. Sagai. 1998. Involvement of superoxide and nitric oxide on airway inflammation and hyperresponsiveness induced by diesel exhaust particles in mice. *Free Radic Biol Med* 25:635-644.

53.   Thomsen, S. F., S. van der Sluis, K. O. Kyvik, A. Skytthe, and V. Backer. Estimates of asthma heritability in a large twin sample. *Clin Exp Allergy* 40:1054-1061.

54.   Yilmaz-Demirdag, Y., B. Prather, and S. L. Bahna. Does heredity determine the allergy manifestation or the sensitisation to a specific allergen? *Allergol Immunopathol (Madr)* 38:56-59.

55.   Van Eerdewegh, P., R. D. Little, J. Dupuis, R. G. Del Mastro, K. Falls, J. Simon, D. Torrey, S. Pandit, J. McKenny, K. Braunschweiger, A. Walsh, Z. Liu, B. Hayward, C. Folz, S. P. Manning, A. Bawa, L. Saracino, M. Thackston, Y. Benchekroun, N. Capparell, M. Wang, R. Adair, Y. Feng, J. Dubois, M. G. FitzGerald, H. Huang, R. Gibson, K. M. Allen, A. Pedan, M. R. Danzig, S. P. Umland, R. W. Egan, F. M. Cuss, S. Rorke, J. B. Clough, J. W. Holloway, S. T. Holgate, and T. P. Keith. 2002. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 418:426-430.

56.   Vercelli, D. 2008. Discovering susceptibility genes for asthma and allergy. *Nat Rev Immunol* 8:169-182.

57.   Moffatt, M. F., M. Kabesch, L. Liang, A. L. Dixon, D. Strachan, S. Heath, M. Depner, A. von Berg, A. Bufe, E. Rietschel, A. Heinzmann, B. Simma, T. Frischer, S. A. Willis-Owen, K. C. Wong, T. Illig, C. Vogelberg, S. K. Weiland, E. von Mutius, G. R. Abecasis, M. Farrall, I. G. Gut, G. M. Lathrop, and W. O. Cookson. 2007. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* 448:470-473.

58.   2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447:661-678.

59.   Holgate, S. T. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 242:205-219.

60.   Holt, P. G., J. E. Batty, and K. J. Turner. 1981. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 42:409-417.

61.   Hurst, S. D., B. W. Seymour, T. Muchamuel, V. P. Kurup, and R. L. Coffman. 2001. Modulation of inhaled antigen-induced IgE tolerance by ongoing Th2 responses in the lung. *J Immunol* 166:4922-4930.

62.   Van Hove, C. L., T. Maes, G. F. Joos, and K. G. Tournoy. 2007. Prolonged inhaled allergen exposure can induce persistent tolerance. *Am J Respir Cell Mol Biol* 36:573-584.

63.   Fahy, J. V., and B. F. Dickey. Airway mucus function and dysfunction. *N Engl J Med* 363:2233-2247.

64.   Proud, D., and R. Leigh. Epithelial cells and airway diseases. *Immunol Rev* 242:186-204.

65.   Macaubas, C., R. H. DeKruyff, and D. T. Umetsu. 2003. Respiratory tolerance in the protection against asthma. *Curr Drug Targets Inflamm Allergy* 2:175-186.

66.   Wan, H., H. L. Winton, C. Soeller, E. R. Tovey, D. C. Gruenert, P. J. Thompson, G. A. Stewart, G. W. Taylor, D. R. Garrod, M. B. Cannell, and C. Robinson. 1999. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 104:123-133.

67.   Wan, H., H. L. Winton, C. Soeller, G. W. Taylor, D. C. Gruenert, P. J. Thompson, M. B. Cannell, G. A. Stewart, D. R. Garrod, and C. Robinson. 2001. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. *Clin Exp Allergy* 31:279-294.

68.   Mayer, A. K., H. Bartz, F. Fey, L. M. Schmidt, and A. H. Dalpke. 2008. Airway epithelial cells modify immune responses by inducing an anti-inflammatory microenvironment. *Eur J Immunol* 38:1689-1699.

69.   Rate, A., J. W. Upham, A. Bosco, K. L. McKenna, and P. G. Holt. 2009. Airway epithelial cells regulate the functional phenotype of locally differentiating dendritic cells: implications for the pathogenesis of infectious and allergic airway disease. *J Immunol* 182:72-83.

70.   Bleck, B., D. B. Tse, M. A. Curotto de Lafaille, F. Zhang, and J. Reibman. 2008. Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation and polarization via thymic stromal lymphopoietin. *J Clin Immunol* 28:147-156.

71.   Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, and B. N. Lambrecht. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 15:410-416.

72.   Schon-Hegrad, M. A., J. Oliver, P. G. McMenamin, and P. G. Holt. 1991. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J Exp Med* 173:1345-1356.

73.   Jahnsen, F. L., D. H. Strickland, J. A. Thomas, I. T. Tobagus, S. Napoli, G. R. Zosky, D. J. Turner, P. D. Sly, P. A. Stumbles, and P. G. Holt. 2006. Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus. *J Immunol* 177:5861-5867.

74.   Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 196:1645-1651.

75.   Stumbles, P. A., J. A. Thomas, C. L. Pimm, P. T. Lee, T. J. Venaille, S. Proksch, and P. G. Holt. 1998. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. *J Exp Med* 188:2019-2031.

76.   Vermaelen, K. Y., I. Carro-Muino, B. N. Lambrecht, and R. A. Pauwels. 2001. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 193:51-60.

77.   Akbari, O., R. H. DeKruyff, and D. T. Umetsu. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2:725-731.

78.   Akbari, O., G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff, and D. T. Umetsu. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 8:1024-1032.

79.   Tsitoura, D. C., R. H. DeKruyff, J. R. Lamb, and D. T. Umetsu. 1999. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4+ T cells. *J Immunol* 163:2592-2600.

80.   Ostroukhova, M., C. Seguin-Devaux, T. B. Oriss, B. Dixon-McCarthy, L. Yang, B. T. Ameredes, T. E. Corcoran, and A. Ray. 2004. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* 114:28-38.

81.   de Heer, H. J., H. Hammad, T. Soullie, D. Hijdra, N. Vos, M. A. Willart, H. C. Hoogsteden, and B. N. Lambrecht. 2004. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 200:89-98.

82.	Holt, P. G., D. H. Strickland, M. E. Wikstrom, and F. L. Jahnsen. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* 8:142-152.

83.	MacLean, J. A., W. Xia, C. E. Pinto, L. Zhao, H. W. Liu, and R. L. Kradin. 1996. Sequestration of inhaled particulate antigens by lung phagocytes. A mechanism for the effective inhibition of pulmonary cell-mediated immunity. *Am J Pathol* 148:657-666.

84.	Thepen, T., N. Van Rooijen, and G. Kraal. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med* 170:499-509.

85.	Jakubzick, C., F. Tacke, J. Llodra, N. van Rooijen, and G. J. Randolph. 2006. Modulation of dendritic cell trafficking to and from the airways. *J Immunol* 176:3578-3584.

86.	Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen. 1993. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med* 177:397-407.

87.	Murai, M., O. Turovskaya, G. Kim, R. Madan, C. L. Karp, H. Cheroutre, and M. Kronenberg. 2009. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* 10:1178-1184.

88.	Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389-400.

89.	Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.

90.	Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4:337-342.

91.	Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

92.	Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.

93.	Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.

94.	Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory T

cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 172:5986-5993.

95.   Kearley, J., J. E. Barker, D. S. Robinson, and C. M. Lloyd. 2005. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 202:1539-1547.

96.   Kearley, J., D. S. Robinson, and C. M. Lloyd. 2008. CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J Allergy Clin Immunol* 122:617-624 e616.

97.   Lewkowich, I. P., N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Sproles, J. S. Shah, J. Kohl, Y. Belkaid, and M. Wills-Karp. 2005. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 202:1549-1561.

98.   Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287-296.

99.   Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10:1969-1980.

100.   Rubtsov, Y. P., J. P. Rasmussen, E. Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W. R. Henderson, Jr., W. Muller, and A. Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28:546-558.

101.   Oh, J. W., C. M. Seroogy, E. H. Meyer, O. Akbari, G. Berry, C. G. Fathman, R. H. Dekruyff, and D. T. Umetsu. 2002. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol* 110:460-468.

102.   Grunig, G., D. B. Corry, M. W. Leach, B. W. Seymour, V. P. Kurup, and D. M. Rennick. 1997. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J Exp Med* 185:1089-1099.

103.   Jaffar, Z., T. Sivakuru, and K. Roberts. 2004. CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J Immunol* 172:3842-3849.

104.   Barnes, M. J., and F. Powrie. 2009. Regulatory T cells reinforce intestinal homeostasis. *Immunity* 31:401-411.

105.   Till, S. J., J. N. Francis, K. Nouri-Aria, and S. R. Durham. 2004. Mechanisms of immunotherapy. *J Allergy Clin Immunol* 113:1025-1034; quiz 1035.

106. Pajno, G. B., G. Barberio, F. De Luca, L. Morabito, and S. Parmiani. 2001. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. *Clin Exp Allergy* 31:1392-1397.

107. Moller, C., S. Dreborg, H. A. Ferdousi, S. Halken, A. Host, L. Jacobsen, A. Koivikko, D. Y. Koller, B. Niggemann, L. A. Norberg, R. Urbanek, E. Valovirta, and U. Wahn. 2002. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* 109:251-256.

108. Nouri-Aria, K. T., P. A. Wachholz, J. N. Francis, M. R. Jacobson, S. M. Walker, L. K. Wilcock, S. Q. Staple, R. C. Aalberse, S. J. Till, and S. R. Durham. 2004. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 172:3252-3259.

109. O'Hehir, R. E., L. M. Gardner, M. P. de Leon, B. J. Hales, M. Biondo, J. A. Douglass, J. M. Rolland, and A. Sandrini. 2009. House dust mite sublingual immunotherapy: the role for transforming growth factor-beta and functional regulatory T cells. *Am J Respir Crit Care Med* 180:936-947.

110. Vissers, J. L., B. C. van Esch, G. A. Hofman, M. L. Kapsenberg, F. R. Weller, and A. J. van Oosterhout. 2004. Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model. *J Allergy Clin Immunol* 113:1204-1210.

111. Jacobsen, L. 2001. Preventive aspects of immunotherapy: prevention for children at risk of developing asthma. *Ann Allergy Asthma Immunol* 87:43-46.

112. Pajno, G. B. 2007. Sublingual immunotherapy: the optimism and the issues. *J Allergy Clin Immunol* 119:796-801.

113. Larche, M., and D. C. Wraith. 2005. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med* 11:S69-76.

114. Seder, R. A., W. E. Paul, M. M. Davis, and B. Fazekas de St Groth. 1992. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. *J Exp Med* 176:1091-1098.

115. Cote-Sierra, J., G. Foucras, L. Guo, L. Chiodetti, H. A. Young, J. Hu-Li, J. Zhu, and W. E. Paul. 2004. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci U S A* 101:3880-3885.

116. Zhu, J., L. Guo, C. J. Watson, J. Hu-Li, and W. E. Paul. 2001. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* 166:7276-7281.

117. Zhu, J., J. Cote-Sierra, L. Guo, and W. E. Paul. 2003. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19:739-748.

118. Rochman, Y., R. Spolski, and W. J. Leonard. 2009. New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol* 9:480-490.

119. Freeman, G. J., V. A. Boussiotis, A. Anumanthan, G. M. Bernstein, X. Y. Ke, P. D. Rennert, G. S. Gray, J. G. Gribben, and L. M. Nadler. 1995. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* 2:523-532.

120. Geha, R. S., H. H. Jabara, and S. R. Brodeur. 2003. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* 3:721-732.

121. Emson, C. L., S. E. Bell, A. Jones, W. Wisden, and A. N. McKenzie. 1998. Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. *J Exp Med* 188:399-404.

122. Huh, J. C., D. H. Strickland, F. L. Jahnsen, D. J. Turner, J. A. Thomas, S. Napoli, I. Tobagus, P. A. Stumbles, P. D. Sly, and P. G. Holt. 2003. Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma. *J Exp Med* 198:19-30.

123. Galli, S. J., J. Kalesnikoff, M. A. Grimbaldeston, A. M. Piliponsky, C. M. Williams, and M. Tsai. 2005. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 23:749-786.

124. Cohn, L., J. S. Tepper, and K. Bottomly. 1998. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol* 161:3813-3816.

125. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 186:1737-1747.

126. Hershey, G. K., M. F. Friedrich, L. A. Esswein, M. L. Thomas, and T. A. Chatila. 1997. The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. *N Engl J Med* 337:1720-1725.

127. Rosa-Rosa, L., N. Zimmermann, J. A. Bernstein, M. E. Rothenberg, and G. K. Khurana Hershey. 1999. The R576 IL-4 receptor alpha allele correlates with asthma severity. *J Allergy Clin Immunol* 104:1008-1014.

128. McKenzie, G. J., P. G. Fallon, C. L. Emson, R. K. Grencis, and A. N. McKenzie. 1999. Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *J Exp Med* 189:1565-1572.

129. Coyle, A. J., G. Le Gros, C. Bertrand, S. Tsuyuki, C. H. Heusser, M. Kopf, and G. P. Anderson. 1995. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* 13:54-59.

130. Moser, R., J. Fehr, and P. L. Bruijnzeel. 1992. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J Immunol* 149:1432-1438.

131. Walter, D. M., J. J. McIntire, G. Berry, A. N. McKenzie, D. D. Donaldson, R. H. DeKruyff, and D. T. Umetsu. 2001. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 167:4668-4675.

132. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261-2263.

133. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258-2261.

134. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 103:779-788.

135. Bochner, B. S., D. A. Klunk, S. A. Sterbinsky, R. L. Coffman, and R. P. Schleimer. 1995. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol* 154:799-803.

136. Zhu, Z., B. Ma, T. Zheng, R. J. Homer, C. G. Lee, I. F. Charo, P. Noble, and J. A. Elias. 2002. IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 168:2953-2962.

137. Warren, D. J., and M. A. Moore. 1988. Synergism among interleukin 1, interleukin 3, and interleukin 5 in the production of eosinophils from primitive hemopoietic stem cells. *J Immunol* 140:94-99.

138. Stern, M., L. Meagher, J. Savill, and C. Haslett. 1992. Apoptosis in human eosinophils. Programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. *J Immunol* 148:3543-3549.

139. Yamaguchi, Y., T. Suda, S. Ohta, K. Tominaga, Y. Miura, and T. Kasahara. 1991. Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood* 78:2542-2547.

140. Simon, H. U., S. Yousefi, C. Schranz, A. Schapowal, C. Bachert, and K. Blaser. 1997. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *J Immunol* 158:3902-3908.

141. Collins, P. D., S. Marleau, D. A. Griffiths-Johnson, P. J. Jose, and T. J. Williams. 1995. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 182:1169-1174.

142.   Akutsu, I., T. Kojima, A. Kariyone, T. Fukuda, S. Makino, and K. Takatsu. 1995. Antibody against interleukin-5 prevents antigen-induced eosinophil infiltration and bronchial hyperreactivity in the guinea pig airways. *Immunol Lett* 45:109-116.

143.   Nakajima, H., I. Iwamoto, S. Tomoe, R. Matsumura, H. Tomioka, K. Takatsu, and S. Yoshida. 1992. CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 146:374-377.

144.   Van Oosterhout, A. J., A. R. Ladenius, H. F. Savelkoul, I. Van Ark, K. C. Delsman, and F. P. Nijkamp. 1993. Effect of anti-IL-5 and IL-5 on airway hyperreactivity and eosinophils in guinea pigs. *Am Rev Respir Dis* 147:548-552.

145.   Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 183:195-201.

146.   Tominaga, A., S. Takaki, N. Koyama, S. Katoh, R. Matsumoto, M. Migita, Y. Hitoshi, Y. Hosoya, S. Yamauchi, Y. Kanai, and et al. 1991. Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production. *J Exp Med* 173:429-437.

147.   Dent, L. A., M. Strath, A. L. Mellor, and C. J. Sanderson. 1990. Eosinophilia in transgenic mice expressing interleukin 5. *J Exp Med* 172:1425-1431.

148.   Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, T. T. Hansel, S. T. Holgate, P. J. Sterk, and P. J. Barnes. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356:2144-2148.

149.   Uyttenhove, C., R. J. Simpson, and J. Van Snick. 1988. Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. *Proc Natl Acad Sci U S A* 85:6934-6938.

150.   Hultner, L., and J. Moeller. 1990. Mast cell growth-enhancing activity (MEA) stimulates interleukin 6 production in a mouse bone marrow-derived mast cell line and a malignant subline. *Exp Hematol* 18:873-877.

151.   Vink, A., G. Warnier, F. Brombacher, and J. C. Renauld. 1999. Interleukin 9-induced in vivo expansion of the B-1 lymphocyte population. *J Exp Med* 189:1413-1423.

152.   Nicolaides, N. C., K. J. Holroyd, S. L. Ewart, S. M. Eleff, M. B. Kiser, C. R. Dragwa, C. D. Sullivan, L. Grasso, L. Y. Zhang, C. J. Messler, T. Zhou, S. R. Kleeberger, K. H. Buetow, and R. C. Levitt. 1997. Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci U S A* 94:13175-13180.

153.	Godfraind, C., J. Louahed, H. Faulkner, A. Vink, G. Warnier, R. Grencis, and J. C. Renauld. 1998. Intraepithelial infiltration by mast cells with both connective tissue-type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice. *J Immunol* 160:3989-3996.

154.	Temann, U. A., P. Ray, and R. A. Flavell. 2002. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J Clin Invest* 109:29-39.

155.	Temann, U. A., G. P. Geba, J. A. Rankin, and R. A. Flavell. 1998. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med* 188:1307-1320.

156.	Townsend, J. M., G. P. Fallon, J. D. Matthews, P. Smith, E. H. Jolin, and N. A. McKenzie. 2000. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity* 13:573-583.

157.	McMillan, S. J., B. Bishop, M. J. Townsend, A. N. McKenzie, and C. M. Lloyd. 2002. The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity. *J Exp Med* 195:51-57.

158.	Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp Med* 192:455-462.

159.	Reber, L., C. A. Da Silva, and N. Frossard. 2006. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. *Eur J Pharmacol* 533:327-340.

160.	Dvorak, A. M. 2005. Ultrastructural studies of human basophils and mast cells. *J Histochem Cytochem* 53:1043-1070.

161.	Lalloo, U. G., P. J. Barnes, and K. F. Chung. 1996. Pathophysiology and clinical presentations of cough. *J Allergy Clin Immunol* 98:S91-96; discussion S96-97.

162.	Galli, S. J., M. Tsai, and A. M. Piliponsky. 2008. The development of allergic inflammation. *Nature* 454:445-454.

163.	Gutierrez-Ramos, J. C., C. Lloyd, and J. A. Gonzalo. 1999. Eotaxin: from an eosinophilic chemokine to a major regulator of allergic reactions. *Immunol Today* 20:500-504.

164.	Gonzalo, J. A., C. M. Lloyd, L. Kremer, E. Finger, A. C. Martinez, M. H. Siegelman, M. Cybulsky, and J. C. Gutierrez-Ramos. 1996. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 98:2332-2345.

165.	Humbles, A. A., D. M. Conroy, S. Marleau, S. M. Rankin, R. T. Palframan, A. E. Proudfoot, T. N. Wells, D. Li, P. K. Jeffery, D. A. Griffiths-Johnson, T. J. Williams, and P. J. Jose. 1997. Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *J Exp Med* 186:601-612.

166. Trivedi, S. G., and C. M. Lloyd. 2007. Eosinophils in the pathogenesis of allergic airways disease. *Cell Mol Life Sci* 64:1269-1289.

167. Justice, J. P., M. T. Borchers, J. R. Crosby, E. M. Hines, H. H. Shen, S. I. Ochkur, M. P. McGarry, N. A. Lee, and J. J. Lee. 2003. Ablation of eosinophils leads to a reduction of allergen-induced pulmonary pathology. *Am J Physiol Lung Cell Mol Physiol* 284:L169-178.

168. Lee, J. J., D. Dimina, M. P. Macias, S. I. Ochkur, M. P. McGarry, K. R. O'Neill, C. Protheroe, R. Pero, T. Nguyen, S. A. Cormier, E. Lenkiewicz, D. Colbert, L. Rinaldi, S. J. Ackerman, C. G. Irvin, and N. A. Lee. 2004. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305:1773-1776.

169. Humbles, A. A., C. M. Lloyd, S. J. McMillan, D. S. Friend, G. Xanthou, E. E. McKenna, S. Ghiran, N. P. Gerard, C. Yu, S. H. Orkin, and C. Gerard. 2004. A critical role for eosinophils in allergic airways remodeling. *Science* 305:1776-1779.

170. Brottman, G. M., W. E. Regelmann, A. Slungaard, and O. D. Wangensteen. 1996. Effect of eosinophil peroxidase on airway epithelial permeability in the guinea pig. *Pediatr Pulmonol* 21:159-166.

171. Frigas, E., D. A. Loegering, and G. J. Gleich. 1980. Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab Invest* 42:35-43.

172. MacPherson, J. C., S. A. Comhair, S. C. Erzurum, D. F. Klein, M. F. Lipscomb, M. S. Kavuru, M. K. Samoszuk, and S. L. Hazen. 2001. Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. *J Immunol* 166:5763-5772.

173. Ayars, G. H., L. C. Altman, M. M. McManus, J. M. Agosti, C. Baker, D. L. Luchtel, D. A. Loegering, and G. J. Gleich. 1989. Injurious effect of the eosinophil peroxide-hydrogen peroxide-halide system and major basic protein on human nasal epithelium in vitro. *Am Rev Respir Dis* 140:125-131.

174. Agosti, J. M., L. C. Altman, G. H. Ayars, D. A. Loegering, G. J. Gleich, and S. J. Klebanoff. 1987. The injurious effect of eosinophil peroxidase, hydrogen peroxide, and halides on pneumocytes in vitro. *J Allergy Clin Immunol* 79:496-504.

175. Cho, J. Y., M. Miller, K. J. Baek, J. W. Han, J. Nayar, S. Y. Lee, K. McElwain, S. McElwain, S. Friedman, and D. H. Broide. 2004. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest* 113:551-560.

176. Jacobsen, E. A., S. I. Ochkur, N. A. Lee, and J. J. Lee. 2007. Eosinophils and asthma. *Curr Allergy Asthma Rep* 7:18-26.

177. Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M. S. Ludwig, N. Barnes, D. Robinson, and A. B. Kay. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins

in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 112:1029-1036.

178.   Leslie, C. C. 2004. Regulation of the specific release of arachidonic acid by cytosolic phospholipase A2. *Prostaglandins Leukot Essent Fatty Acids* 70:373-376.

179.   Yu, Y., J. Fan, X. S. Chen, D. Wang, A. J. Klein-Szanto, R. L. Campbell, G. A. FitzGerald, and C. D. Funk. 2006. Genetic model of selective COX2 inhibition reveals novel heterodimer signaling. *Nat Med* 12:699-704.

180.   Tanabe, T., and N. Tohnai. 2002. Cyclooxygenase isozymes and their gene structures and expression. *Prostaglandins Other Lipid Mediat* 68-69:95-114.

181.   Deininger, M. H., and H. J. Schluesener. 1999. Cyclooxygenases-1 and -2 are differentially localized to microglia and endothelium in rat EAE and glioma. *J Neuroimmunol* 95:202-208.

182.   Gavett, S. H., S. L. Madison, P. C. Chulada, P. E. Scarborough, W. Qu, J. E. Boyle, H. F. Tiano, C. A. Lee, R. Langenbach, V. L. Roggli, and D. C. Zeldin. 1999. Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest* 104:721-732.

183.   Harris, R. C., J. A. McKanna, Y. Akai, H. R. Jacobson, R. N. Dubois, and M. D. Breyer. 1994. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Invest* 94:2504-2510.

184.   Kaufmann, W. E., P. F. Worley, J. Pegg, M. Bremer, and P. Isakson. 1996. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. *Proc Natl Acad Sci U S A* 93:2317-2321.

185.   Newberry, R. D., J. S. McDonough, W. F. Stenson, and R. G. Lorenz. 2001. Spontaneous and continuous cyclooxygenase-2-dependent prostaglandin E2 production by stromal cells in the murine small intestine lamina propria: directing the tone of the intestinal immune response. *J Immunol* 166:4465-4472.

186.   Asano, K., C. M. Lilly, and J. M. Drazen. 1996. Prostaglandin G/H synthase-2 is the constitutive and dominant isoform in cultured human lung epithelial cells. *Am J Physiol* 271:L126-131.

187.   Morham, S. G., R. Langenbach, C. D. Loftin, H. F. Tiano, N. Vouloumanos, J. C. Jennette, J. F. Mahler, K. D. Kluckman, A. Ledford, C. A. Lee, and O. Smithies. 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 83:473-482.

188.   Bogar, L. J., L. L. Bartula, H. P. Parkman, and S. I. Myers. 1999. Enhanced bradykinin-stimulated prostaglandin release in the acutely inflamed guinea pig gallbladder is due to new synthesis of cyclooxygenase 1 and prostacyclin synthase. *J Surg Res* 84:71-76.

189. Chandrasekharan, S., N. A. Foley, L. Jania, P. Clark, L. P. Audoly, and B. H. Koller. 2005. Coupling of COX-1 to mPGES1 for prostaglandin E2 biosynthesis in the murine mammary gland. *J Lipid Res* 46:2636-2648.

190. Loftin, C. D., D. B. Trivedi, H. F. Tiano, J. A. Clark, C. A. Lee, J. A. Epstein, S. G. Morham, M. D. Breyer, M. Nguyen, B. M. Hawkins, J. L. Goulet, O. Smithies, B. H. Koller, and R. Langenbach. 2001. Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. *Proc Natl Acad Sci U S A* 98:1059-1064.

191. Shitashige, M., I. Morita, and S. Murota. 1998. Different substrate utilization between prostaglandin endoperoxide H synthase-1 and -2 in NIH3T3 fibroblasts. *Biochim Biophys Acta* 1389:57-66.

192. McAdam, B. F., I. A. Mardini, A. Habib, A. Burke, J. A. Lawson, S. Kapoor, and G. A. FitzGerald. 2000. Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid and isoeicosanoid production in inflammation. *J Clin Invest* 105:1473-1482.

193. Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 231:232-235.

194. Rao, P., and E. E. Knaus. 2008. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *J Pharm Pharm Sci* 11:81s-110s.

195. Ogino, N., T. Miyamoto, S. Yamamoto, and O. Hayaishi. 1977. Prostaglandin endoperoxide E isomerase from bovine vesicular gland microsomes, a glutathione-requiring enzyme. *J Biol Chem* 252:890-895.

196. Moonen, P., M. Buytenhek, and D. H. Nugteren. 1982. Purification of PGH-PGE isomerase from sheep vesicular glands. *Methods Enzymol* 86:84-91.

197. Jakobsson, P. J., S. Thoren, R. Morgenstern, and B. Samuelsson. 1999. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A* 96:7220-7225.

198. Murakami, M., H. Naraba, T. Tanioka, N. Semmyo, Y. Nakatani, F. Kojima, T. Ikeda, M. Fueki, A. Ueno, S. Oh, and I. Kudo. 2000. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem* 275:32783-32792.

199. Trebino, C. E., J. L. Stock, C. P. Gibbons, B. M. Naiman, T. S. Wachtmann, J. P. Umland, K. Pandher, J. M. Lapointe, S. Saha, M. L. Roach, D. Carter, N. A. Thomas, B. A. Durtschi, J. D. McNeish, J. E. Hambor, P. J. Jakobsson, T. J. Carty, J. R. Perez, and L. P. Audoly. 2003. Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. *Proc Natl Acad Sci U S A* 100:9044-9049.

200.   Kamei, D., K. Yamakawa, Y. Takegoshi, M. Mikami-Nakanishi, Y. Nakatani, S. Oh-Ishi, H. Yasui, Y. Azuma, N. Hirasawa, K. Ohuchi, H. Kawaguchi, Y. Ishikawa, T. Ishii, S. Uematsu, S. Akira, M. Murakami, and I. Kudo. 2004. Reduced pain hypersensitivity and inflammation in mice lacking microsomal prostaglandin e synthase-1. *J Biol Chem* 279:33684-33695.

201.   Lovgren, A. K., M. Kovarova, and B. H. Koller. 2007. cPGES/p23 is required for glucocorticoid receptor function and embryonic growth but not prostaglandin E2 synthesis. *Mol Cell Biol* 27:4416-4430.

202.   Watanabe, K., K. Kurihara, Y. Tokunaga, and O. Hayaishi. 1997. Two types of microsomal prostaglandin E synthase: glutathione-dependent and -independent prostaglandin E synthases. *Biochem Biophys Res Commun* 235:148-152.

203.   Murakami, M., K. Nakashima, D. Kamei, S. Masuda, Y. Ishikawa, T. Ishii, Y. Ohmiya, K. Watanabe, and I. Kudo. 2003. Cellular prostaglandin E2 production by membrane-bound prostaglandin E synthase-2 via both cyclooxygenases-1 and -2. *J Biol Chem* 278:37937-37947.

204.   Jania, L. A., S. Chandrasekharan, M. G. Backlund, N. A. Foley, J. Snouwaert, I. M. Wang, P. Clark, L. P. Audoly, and B. H. Koller. 2009. Microsomal prostaglandin E synthase-2 is not essential for in vivo prostaglandin E2 biosynthesis. *Prostaglandins Other Lipid Mediat* 88:73-81.

205.   Reid, G., P. Wielinga, N. Zelcer, I. van der Heijden, A. Kuil, M. de Haas, J. Wijnholds, and P. Borst. 2003. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci U S A* 100:9244-9249.

206.   Namba, T., Y. Sugimoto, M. Negishi, A. Irie, F. Ushikubi, A. Kakizuka, S. Ito, A. Ichikawa, and S. Narumiya. 1993. Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature* 365:166-170.

207.   Tai, H. H., H. Cho, M. Tong, and Y. Ding. 2006. NAD+-linked 15-hydroxyprostaglandin dehydrogenase: structure and biological functions. *Curr Pharm Des* 12:955-962.

208.   Blotman, F., J. Chaintreuil, P. Poubelle, O. Flandre, A. Crastes de Paulet, and L. Simon. 1980. PGE2, PGF2 alpha, and TXB2 biosynthesis by human rheumatoid synovia. *Adv Prostaglandin Thromboxane Res* 8:1705-1708.

209.   Sturge, R. A., D. B. Yates, D. Gordon, M. Franco, W. Paul, A. Bray, and J. Morley. 1978. Prostaglandin production in arthritis. *Ann Rheum Dis* 37:315-320.

210.   Juhlin, L., and G. Michaelsson. 1969. Cutaneous vascular reactions to prostaglandins in healthy subjects and in patients with urticaria and atopic dermatitis. *Acta Derm Venereol* 49:251-261.

211. Goulet, J. L., A. J. Pace, M. L. Key, R. S. Byrum, M. Nguyen, S. L. Tilley, S. G. Morham, R. Langenbach, J. L. Stock, J. D. McNeish, O. Smithies, T. M. Coffman, and B. H. Koller. 2004. E-prostanoid-3 receptors mediate the proinflammatory actions of prostaglandin E2 in acute cutaneous inflammation. *J Immunol* 173:1321-1326.

212. Stock, J. L., K. Shinjo, J. Burkhardt, M. Roach, K. Taniguchi, T. Ishikawa, H. S. Kim, P. J. Flannery, T. M. Coffman, J. D. McNeish, and L. P. Audoly. 2001. The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure. *J Clin Invest* 107:325-331.

213. Coceani, F., and E. S. Akarsu. 1998. Prostaglandin E2 in the pathogenesis of fever. An update. *Ann N Y Acad Sci* 856:76-82.

214. Ding, M., Y. Kinoshita, K. Kishi, H. Nakata, S. Hassan, C. Kawanami, Y. Sugimoto, M. Katsuyama, M. Negishi, S. Narumiya, A. Ichikawa, and T. Chiba. 1997. Distribution of prostaglandin E receptors in the rat gastrointestinal tract. *Prostaglandins* 53:199-216.

215. Tani, S., M. Okuda, R. Morishige, and T. Tanaka. 1997. Gastric mucin secretion from cultured rat epithelial cells. *Biol Pharm Bull* 20:482-485.

216. Hirokawa, M., O. Furukawa, P. H. Guth, E. Engel, and J. D. Kaunitz. 2004. Low-dose PGE2 mimics the duodenal secretory response to luminal acid in mice. *Am J Physiol Gastrointest Liver Physiol* 286:G891-898.

217. Horton, E. W. 1979. Prostaglandins and smooth muscle. *Br Med Bull* 35:295-300.

218. Miller, S. B. 2006. Prostaglandins in health and disease: an overview. *Semin Arthritis Rheum* 36:37-49.

219. Nishio, H., S. Terashima, M. Nakashima, E. Aihara, and K. Takeuchi. 2007. Involvement of prostaglandin E receptor EP3 subtype and prostacyclin IP receptor in decreased acid response in damaged stomach. *J Physiol Pharmacol* 58:407-421.

220. Okada, Y., A. Hara, H. Ma, C. Y. Xiao, O. Takahata, Y. Kohgo, S. Narumiya, and F. Ushikubi. 2000. Characterization of prostanoid receptors mediating contraction of the gastric fundus and ileum: studies using mice deficient in prostanoid receptors. *Br J Pharmacol* 131:745-755.

221. Nakanishi, M., D. C. Montrose, P. Clark, P. R. Nambiar, G. S. Belinsky, K. P. Claffey, D. Xu, and D. W. Rosenberg. 2008. Genetic deletion of mPGES-1 suppresses intestinal tumorigenesis. *Cancer Res* 68:3251-3259.

222. Pugh, S., and G. A. Thomas. 1994. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E2. *Gut* 35:675-678.

223. Harris, S. G., J. Padilla, L. Koumas, D. Ray, and R. P. Phipps. 2002. Prostaglandins as modulators of immunity. *Trends Immunol* 23:144-150.

224.   Breyer, M. D., and R. M. Breyer. 2000. Prostaglandin E receptors and the kidney. *Am J Physiol Renal Physiol* 279:F12-23.

225.   Challis, J. R., F. H. Bloomfield, A. D. Bocking, V. Casciani, H. Chisaka, K. Connor, X. Dong, P. Gluckman, J. E. Harding, J. Johnstone, W. Li, S. Lye, K. Okamura, and M. Premyslova. 2005. Fetal signals and parturition. *J Obstet Gynaecol Res* 31:492-499.

226.   Bach, D., and H. Walker. 1982. How important are prostaglandins in the urology of man? *Urol Int* 37:160-171.

227.   Blackwell, K. A., L. G. Raisz, and C. C. Pilbeam. Prostaglandins in bone: bad cop, good cop? *Trends Endocrinol Metab* 21:294-301.

228.   Isono, M., T. Suzuki, K. Hosono, I. Hayashi, H. Sakagami, S. Uematsu, S. Akira, Y. A. Declerck, H. Okamoto, and M. Majima. Microsomal prostaglandin E synthase-1 enhances bone cancer growth and bone cancer-related pain behaviors in mice. *Life Sci*.

229.   Scandella, E., Y. Men, S. Gillessen, R. Forster, and M. Groettrup. 2002. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* 100:1354-1361.

230.   Harizi, H., C. Grosset, and N. Gualde. 2003. Prostaglandin E2 modulates dendritic cell function via EP2 and EP4 receptor subtypes. *J Leukoc Biol* 73:756-763.

231.   Harizi, H., M. Juzan, V. Pitard, J. F. Moreau, and N. Gualde. 2002. Cyclooxygenase-2-issued prostaglandin e(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J Immunol* 168:2255-2263.

232.   Krause, P., M. Bruckner, C. Uermosi, E. Singer, M. Groettrup, and D. F. Legler. 2009. Prostaglandin E(2) enhances T-cell proliferation by inducing the costimulatory molecules OX40L, CD70, and 4-1BBL on dendritic cells. *Blood* 113:2451-2460.

233.   Jonuleit, H., U. Kuhn, G. Muller, K. Steinbrink, L. Paragnik, E. Schmitt, J. Knop, and A. H. Enk. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 27:3135-3142.

234.   Legler, D. F., P. Krause, E. Scandella, E. Singer, and M. Groettrup. 2006. Prostaglandin E2 is generally required for human dendritic cell migration and exerts its effect via EP2 and EP4 receptors. *J Immunol* 176:966-973.

235.   Kabashima, K., D. Sakata, M. Nagamachi, Y. Miyachi, K. Inaba, and S. Narumiya. 2003. Prostaglandin E2-EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. *Nat Med* 9:744-749.

236. Kalinski, P., P. L. Vieira, J. H. Schuitemaker, E. C. de Jong, and M. L. Kapsenberg. 2001. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood* 97:3466-3469.

237. Aronoff, D. M., C. Canetti, and M. Peters-Golden. 2004. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J Immunol* 173:559-565.

238. Strassmann, G., V. Patil-Koota, F. Finkelman, M. Fong, and T. Kambayashi. 1994. Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med* 180:2365-2370.

239. Shinomiya, S., H. Naraba, A. Ueno, I. Utsunomiya, T. Maruyama, S. Ohuchida, F. Ushikubi, K. Yuki, S. Narumiya, Y. Sugimoto, A. Ichikawa, and S. Oh-ishi. 2001. Regulation of TNFalpha and interleukin-10 production by prostaglandins I(2) and E(2): studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. *Biochem Pharmacol* 61:1153-1160.

240. Snyder, D. S., D. I. Beller, and E. R. Unanue. 1982. Prostaglandins modulate macrophage Ia expression. *Nature* 299:163-165.

241. Betz, M., and B. S. Fox. 1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol* 146:108-113.

242. Katamura, K., N. Shintaku, Y. Yamauchi, T. Fukui, Y. Ohshima, M. Mayumi, and K. Furusho. 1995. Prostaglandin E2 at priming of naive CD4+ T cells inhibits acquisition of ability to produce IFN-gamma and IL-2, but not IL-4 and IL-5. *J Immunol* 155:4604-4612.

243. Abe, N., K. Katamura, N. Shintaku, T. Fukui, T. Kiyomasu, J. Iio, H. Ueno, G. Tai, M. Mayumi, and K. Furusho. 1997. Prostaglandin E2 and IL-4 provide naive CD4+ T cells with distinct inhibitory signals for the priming of IFN-gamma production. *Cell Immunol* 181:86-92.

244. Goodwin, J. S., and J. Ceuppens. 1983. Regulation of the immune response by prostaglandins. *J Clin Immunol* 3:295-315.

245. Goodwin, J. S., A. D. Bankhurst, and R. P. Messner. 1977. Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. *J Exp Med* 146:1719-1734.

246. Nataraj, C., D. W. Thomas, S. L. Tilley, M. T. Nguyen, R. Mannon, B. H. Koller, and T. M. Coffman. 2001. Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest* 108:1229-1235.

247. Chemnitz, J. M., J. Driesen, S. Classen, J. L. Riley, S. Debey, M. Beyer, A. Popov, T. Zander, and J. L. Schultze. 2006. Prostaglandin E2 impairs CD4+ T cell activation by inhibition of lck: implications in Hodgkin's lymphoma. *Cancer Res* 66:1114-1122.

248.   Yao, C., D. Sakata, Y. Esaki, Y. Li, T. Matsuoka, K. Kuroiwa, Y. Sugimoto, and S. Narumiya. 2009. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med* 15:633-640.

249.   Boniface, K., K. S. Bak-Jensen, Y. Li, W. M. Blumenschein, M. J. McGeachy, T. K. McClanahan, B. S. McKenzie, R. A. Kastelein, D. J. Cua, and R. de Waal Malefyt. 2009. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med* 206:535-548.

250.   Chizzolini, C., R. Chicheportiche, M. Alvarez, C. de Rham, P. Roux-Lombard, S. Ferrari-Lacraz, and J. M. Dayer. 2008. Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. *Blood* 112:3696-3703.

251.   Baratelli, F., Y. Lin, L. Zhu, S. C. Yang, N. Heuze-Vourc'h, G. Zeng, K. Reckamp, M. Dohadwala, S. Sharma, and S. M. Dubinett. 2005. Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. *J Immunol* 175:1483-1490.

252.   Sharma, S., S. C. Yang, L. Zhu, K. Reckamp, B. Gardner, F. Baratelli, M. Huang, R. K. Batra, and S. M. Dubinett. 2005. Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. *Cancer Res* 65:5211-5220.

253.   Mahic, M., S. Yaqub, C. C. Johansson, K. Tasken, and E. M. Aandahl. 2006. FOXP3+CD4+CD25+ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. *J Immunol* 177:246-254.

254.   Fedyk, E. R., and R. P. Phipps. 1996. Prostaglandin E2 receptors of the EP2 and EP4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE-secreting cells. *Proc Natl Acad Sci U S A* 93:10978-10983.

255.   Roper, R. L., D. H. Conrad, D. M. Brown, G. L. Warner, and R. P. Phipps. 1990. Prostaglandin E2 promotes IL-4-induced IgE and IgG1 synthesis. *J Immunol* 145:2644-2651.

256.   Roper, R. L., D. M. Brown, and R. P. Phipps. 1995. Prostaglandin E2 promotes B lymphocyte Ig isotype switching to IgE. *J Immunol* 154:162-170.

257.   Pene, J., F. Rousset, F. Briere, I. Chretien, J. Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K. Arai, J. Banchereau, and et al. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. *Proc Natl Acad Sci U S A* 85:6880-6884.

258.   Nguyen, M., A. J. Pace, and B. H. Koller. 2005. Age-induced reprogramming of mast cell degranulation. *J Immunol* 175:5701-5707.

259.   Nguyen, M., M. Solle, L. P. Audoly, S. L. Tilley, J. L. Stock, J. D. McNeish, T. M. Coffman, D. Dombrowicz, and B. H. Koller. 2002. Receptors and signaling mechanisms required for

prostaglandin E2-mediated regulation of mast cell degranulation and IL-6 production. *J Immunol* 169:4586-4593.

260.   Weller, C. L., S. J. Collington, A. Hartnell, D. M. Conroy, T. Kaise, J. E. Barker, M. S. Wilson, G. W. Taylor, P. J. Jose, and T. J. Williams. 2007. Chemotactic action of prostaglandin E2 on mouse mast cells acting via the PGE2 receptor 3. *Proc Natl Acad Sci U S A* 104:11712-11717.

261.   Wang, X. S., and H. Y. Lau. 2006. Prostaglandin E potentiates the immunologically stimulated histamine release from human peripheral blood-derived mast cells through EP1/EP3 receptors. *Allergy* 61:503-506.

262.   Moncada, S., R. Gryglewski, S. Bunting, and J. R. Vane. 1976. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* 263:663-665.

263.   DeWitt, D. L., and W. L. Smith. 1983. Purification of prostacyclin synthase from bovine aorta by immunoaffinity chromatography. Evidence that the enzyme is a hemoprotein. *J Biol Chem* 258:3285-3293.

264.   Liou, J. Y., S. K. Shyue, M. J. Tsai, C. L. Chung, K. Y. Chu, and K. K. Wu. 2000. Colocalization of prostacyclin synthase with prostaglandin H synthase-1 (PGHS-1) but not phorbol ester-induced PGHS-2 in cultured endothelial cells. *J Biol Chem* 275:15314-15320.

265.   Lim, H., and S. K. Dey. 2002. A novel pathway of prostacyclin signaling-hanging out with nuclear receptors. *Endocrinology* 143:3207-3210.

266.   Stitham, J., C. Midgett, K. A. Martin, and J. Hwa. Prostacyclin: an inflammatory paradox. *Front Pharmacol* 2:24.

267.   MacIntyre, D. E., J. D. Pearson, and J. L. Gordon. 1978. Localisation and stimulation of prostacyclin production in vascular cells. *Nature* 271:549-551.

268.   Cheng, Y., S. C. Austin, B. Rocca, B. H. Koller, T. M. Coffman, T. Grosser, J. A. Lawson, and G. A. FitzGerald. 2002. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* 296:539-541.

269.   Egan, K. M., J. A. Lawson, S. Fries, B. Koller, D. J. Rader, E. M. Smyth, and G. A. Fitzgerald. 2004. COX-2-derived prostacyclin confers atheroprotection on female mice. *Science* 306:1954-1957.

270.   Kobayashi, T., Y. Tahara, M. Matsumoto, M. Iguchi, H. Sano, T. Murayama, H. Arai, H. Oida, T. Yurugi-Kobayashi, J. K. Yamashita, H. Katagiri, M. Majima, M. Yokode, T. Kita, and S. Narumiya. 2004. Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *J Clin Invest* 114:784-794.

271.   Szczeklik, A., R. J. Gryglewski, R. Nizankowski, J. Musial, R. Pieton, and J. Mruk. 1978. Circulatory and anti-platelet effects of intravenous prostacyclin in healthy men. *Pharmacol Res Commun* 10:545-556.

272.   Yokoyama, C., T. Yabuki, M. Shimonishi, M. Wada, T. Hatae, S. Ohkawara, J. Takeda, T. Kinoshita, M. Okabe, and T. Tanabe. 2002. Prostacyclin-deficient mice develop ischemic renal disorders, including nephrosclerosis and renal infarction. *Circulation* 106:2397-2403.

273.   Fujino, T., N. Nakagawa, K. Yuhki, A. Hara, T. Yamada, K. Takayama, S. Kuriyama, Y. Hosoki, O. Takahata, T. Taniguchi, J. Fukuzawa, N. Hasebe, K. Kikuchi, S. Narumiya, and F. Ushikubi. 2004. Decreased susceptibility to renovascular hypertension in mice lacking the prostaglandin I2 receptor IP. *J Clin Invest* 114:805-812.

274.   Murata, T., F. Ushikubi, T. Matsuoka, M. Hirata, A. Yamasaki, Y. Sugimoto, A. Ichikawa, Y. Aze, T. Tanaka, N. Yoshida, A. Ueno, S. Oh-ishi, and S. Narumiya. 1997. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 388:678-682.

275.   Ueno, A., H. Naraba, Y. Ikeda, F. Ushikubi, T. Murata, S. Narumiya, and S. Oh-ishi. 2000. Intrinsic prostacyclin contributes to exudation induced by bradykinin or carrageenin: a study on the paw edema induced in IP-receptor-deficient mice. *Life Sci* 66:PL155-160.

276.   Honda, T., E. Segi-Nishida, Y. Miyachi, and S. Narumiya. 2006. Prostacyclin-IP signaling and prostaglandin E2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis. *J Exp Med* 203:325-335.

277.   Barst, R. J., J. S. Gibbs, H. A. Ghofrani, M. M. Hoeper, V. V. McLaughlin, L. J. Rubin, O. Sitbon, V. F. Tapson, and N. Galie. 2009. Updated evidence-based treatment algorithm in pulmonary arterial hypertension. *J Am Coll Cardiol* 54:S78-84.

278.   Lovgren, A. K., L. A. Jania, J. M. Hartney, K. K. Parsons, L. P. Audoly, G. A. Fitzgerald, S. L. Tilley, and B. H. Koller. 2006. COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 291:L144-156.

279.   Zhu, Y., Y. Liu, W. Zhou, R. Xiang, L. Jiang, K. Huang, Y. Xiao, Z. Guo, and J. Gao. A prostacyclin analogue, iloprost, protects from bleomycin-induced pulmonary fibrosis in mice. *Respir Res* 11:34.

280.   Nakajima, S., T. Honda, D. Sakata, G. Egawa, H. Tanizaki, A. Otsuka, C. S. Moniaga, T. Watanabe, Y. Miyachi, S. Narumiya, and K. Kabashima. Prostaglandin I2-IP signaling promotes Th1 differentiation in a mouse model of contact hypersensitivity. *J Immunol* 184:5595-5603.

281.   Zhou, W., K. Hashimoto, K. Goleniewska, J. F. O'Neal, S. Ji, T. S. Blackwell, G. A. Fitzgerald, K. M. Egan, M. W. Geraci, and R. S. Peebles, Jr. 2007. Prostaglandin I2 analogs inhibit proinflammatory cytokine production and T cell stimulatory function of dendritic cells. *J Immunol* 178:702-710.

282. Idzko, M., H. Hammad, M. van Nimwegen, M. Kool, N. Vos, H. C. Hoogsteden, and B. N. Lambrecht. 2007. Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function. *J Clin Invest* 117:464-472.

283. Jaffar, Z., M. E. Ferrini, M. C. Buford, G. A. Fitzgerald, and K. Roberts. 2007. Prostaglandin I2-IP signaling blocks allergic pulmonary inflammation by preventing recruitment of CD4+ Th2 cells into the airways in a mouse model of asthma. *J Immunol* 179:6193-6203.

284. Jaffar, Z., K. S. Wan, and K. Roberts. 2002. A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *J Immunol* 169:5997-6004.

285. Zhou, W., T. S. Blackwell, K. Goleniewska, J. F. O'Neal, G. A. Fitzgerald, M. Lucitt, R. M. Breyer, and R. S. Peebles, Jr. 2007. Prostaglandin I2 analogs inhibit Th1 and Th2 effector cytokine production by CD4 T cells. *J Leukoc Biol* 81:809-817.

286. Hashimoto, K., B. S. Graham, M. W. Geraci, G. A. FitzGerald, K. Egan, W. Zhou, K. Goleniewska, J. F. O'Neal, J. D. Morrow, R. K. Durbin, P. F. Wright, R. D. Collins, T. Suzutani, and R. S. Peebles, Jr. 2004. Signaling through the prostaglandin I2 receptor IP protects against respiratory syncytial virus-induced illness. *J Virol* 78:10303-10309.

287. Li, H., J. A. Bradbury, R. T. Dackor, M. L. Edin, J. P. Graves, L. M. DeGraff, P. M. Wang, C. D. Bortner, S. Maruoka, F. B. Lih, D. N. Cook, K. B. Tomer, A. M. Jetten, and D. C. Zeldin. Cyclooxygenase-2 regulates Th17 cell differentiation during allergic lung inflammation. *Am J Respir Crit Care Med* 184:37-49.

288. Aronoff, D. M., C. M. Peres, C. H. Serezani, M. N. Ballinger, J. K. Carstens, N. Coleman, B. B. Moore, R. S. Peebles, L. H. Faccioli, and M. Peters-Golden. 2007. Synthetic prostacyclin analogs differentially regulate macrophage function via distinct analog-receptor binding specificities. *J Immunol* 178:1628-1634.

289. Farooque, S. P., and T. H. Lee. 2009. Aspirin-sensitive respiratory disease. *Annu Rev Physiol* 71:465-487.

290. Demoly, P., D. Jaffuel, N. Lequeux, B. Weksler, C. Creminon, F. B. Michel, P. Godard, and J. Bousquet. 1997. Prostaglandin H synthase 1 and 2 immunoreactivities in the bronchial mucosa of asthmatics. *Am J Respir Crit Care Med* 155:670-675.

291. Sousa, A., R. Pfister, P. E. Christie, S. J. Lane, S. M. Nasser, M. Schmitz-Schumann, and T. H. Lee. 1997. Enhanced expression of cyclo-oxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma. *Thorax* 52:940-945.

292. Taha, R., R. Olivenstein, T. Utsumi, P. Ernst, P. J. Barnes, I. W. Rodger, and A. Giaid. 2000. Prostaglandin H synthase 2 expression in airway cells from patients with asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 161:636-640.

293. Peebles, R. S., Jr., R. Dworski, R. D. Collins, K. Jarzecka, D. B. Mitchell, B. S. Graham, and J. R. Sheller. 2000. Cyclooxygenase inhibition increases interleukin 5 and interleukin 13

production and airway hyperresponsiveness in allergic mice. *Am J Respir Crit Care Med* 162:676-681.

294.   Peebles, R. S., Jr., K. Hashimoto, J. D. Morrow, R. Dworski, R. D. Collins, Y. Hashimoto, J. W. Christman, K. H. Kang, K. Jarzecka, J. Furlong, D. B. Mitchell, M. Talati, B. S. Graham, and J. R. Sheller. 2002. Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 165:1154-1160.

295.   Ricciotti, E., and G. A. FitzGerald. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 31:986-1000.

296.   Fujitani, Y., Y. Kanaoka, K. Aritake, N. Uodome, K. Okazaki-Hatake, and Y. Urade. 2002. Pronounced eosinophilic lung inflammation and Th2 cytokine release in human lipocalin-type prostaglandin D synthase transgenic mice. *J Immunol* 168:443-449.

297.   Matsuoka, T., M. Hirata, H. Tanaka, Y. Takahashi, T. Murata, K. Kabashima, Y. Sugimoto, T. Kobayashi, F. Ushikubi, Y. Aze, N. Eguchi, Y. Urade, N. Yoshida, K. Kimura, A. Mizoguchi, Y. Honda, H. Nagai, and S. Narumiya. 2000. Prostaglandin D2 as a mediator of allergic asthma. *Science* 287:2013-2017.

298.   Shiraishi, Y., K. Asano, K. Niimi, K. Fukunaga, M. Wakaki, J. Kagyo, T. Takihara, S. Ueda, T. Nakajima, T. Oguma, Y. Suzuki, T. Shiomi, K. Sayama, S. Kagawa, E. Ikeda, H. Hirai, K. Nagata, M. Nakamura, T. Miyasho, and A. Ishizaka. 2008. Cyclooxygenase-2/prostaglandin D2/CRTH2 pathway mediates double-stranded RNA-induced enhancement of allergic airway inflammation. *J Immunol* 180:541-549.

299.   Shiraishi, Y., K. Asano, T. Nakajima, T. Oguma, Y. Suzuki, T. Shiomi, K. Sayama, K. Niimi, M. Wakaki, J. Kagyo, E. Ikeda, H. Hirai, K. Yamaguchi, and A. Ishizaka. 2005. Prostaglandin D2-induced eosinophilic airway inflammation is mediated by CRTH2 receptor. *J Pharmacol Exp Ther* 312:954-960.

300.   Chevalier, E., J. Stock, T. Fisher, M. Dupont, M. Fric, H. Fargeau, M. Leport, S. Soler, S. Fabien, M. P. Pruniaux, M. Fink, C. P. Bertrand, J. McNeish, and B. Li. 2005. Cutting edge: chemoattractant receptor-homologous molecule expressed on Th2 cells plays a restricting role on IL-5 production and eosinophil recruitment. *J Immunol* 175:2056-2060.

301.   Allen, I. C., J. M. Hartney, T. M. Coffman, R. B. Penn, J. Wess, and B. H. Koller. 2006. Thromboxane A2 induces airway constriction through an M3 muscarinic acetylcholine receptor-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 290:L526-533.

302.   Smith, A. P., M. F. Cuthbert, and L. S. Dunlop. 1975. Effects of inhaled prostaglandins E1, E2, and F2alpha on the airway resistance of healthy and asthmatic man. *Clin Sci Mol Med* 48:421-430.

303. Arakawa, H., J. Lotvall, I. Kawikova, C. G. Lofdahl, and B. E. Skoogh. 1993. Leukotriene D4- and prostaglandin F2 alpha-induced airflow obstruction and airway plasma exudation in guinea-pig: role of thromboxane and its receptor. *Br J Pharmacol* 110:127-132.

304. Aggarwal, S., Y. P. Moodley, P. J. Thompson, and N. L. Misso. Prostaglandin E2 and cysteinyl leukotriene concentrations in sputum: association with asthma severity and eosinophilic inflammation. *Clin Exp Allergy* 40:85-93.

305. Nemoto, T., H. Aoki, A. Ike, K. Yamada, and T. Kondo. 1976. Serum prostaglandin levels in asthmatic patients. *J Allergy Clin Immunol* 57:89-94.

306. Profita, M., A. Sala, A. Bonanno, L. Riccobono, L. Siena, M. R. Melis, R. Di Giorgi, F. Mirabella, M. Gjomarkaj, G. Bonsignore, and A. M. Vignola. 2003. Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J Allergy Clin Immunol* 112:709-716.

307. Long, J. A., M. Fogel-Petrovic, D. A. Knight, P. J. Thompson, and J. W. Upham. 2004. Higher prostaglandin e2 production by dendritic cells from subjects with asthma compared with normal subjects. *Am J Respir Crit Care Med* 170:485-491.

308. Hempel, S. L., M. M. Monick, and G. W. Hunninghake. 1994. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J Clin Invest* 93:391-396.

309. Widdicombe, J. H., I. F. Ueki, D. Emery, D. Margolskee, J. Yergey, and J. A. Nadel. 1989. Release of cyclooxygenase products from primary cultures of tracheal epithelia of dog and human. *Am J Physiol* 257:L361-365.

310. Gauvreau, G. M., R. M. Watson, and P. M. O'Byrne. 1999. Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation. *Am J Respir Crit Care Med* 159:31-36.

311. Manning, P. J., C. G. Lane, and P. M. O'Byrne. 1989. The effect of oral prostaglandin E1 on airway responsiveness in asthmatic subjects. *Pulm Pharmacol* 2:121-124.

312. Pavord, I. D., C. S. Wong, J. Williams, and A. E. Tattersfield. 1993. Effect of inhaled prostaglandin E2 on allergen-induced asthma. *Am Rev Respir Dis* 148:87-90.

313. Selg, E., M. Andersson, L. Lastbom, A. Ryrfeldt, and S. E. Dahlen. 2009. Two different mechanisms for modulation of bronchoconstriction in guinea-pigs by cyclooxygenase metabolites. *Prostaglandins Other Lipid Mediat* 88:101-110.

314. Tanaka, H., S. Kanako, and S. Abe. 2005. Prostaglandin E2 receptor selective agonists E-prostanoid 2 and E-prostanoid 4 may have therapeutic effects on ovalbumin-induced bronchoconstriction. *Chest* 128:3717-3723.

315. Melillo, E., K. L. Woolley, P. J. Manning, R. M. Watson, and P. M. O'Byrne. 1994. Effect of inhaled PGE2 on exercise-induced bronchoconstriction in asthmatic subjects. *Am J Respir Crit Care Med* 149:1138-1141.

316. Sestini, P., L. Armetti, G. Gambaro, M. G. Pieroni, R. M. Refini, A. Sala, A. Vaghi, G. C. Folco, S. Bianco, and M. Robuschi. 1996. Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma. *Am J Respir Crit Care Med* 153:572-575.

317. Mathe, A. A., and P. Hedqvist. 1975. Effect of prostaglandins F2 alpha and E2 on airway conductance in healthy subjects and asthmatic patients. *Am Rev Respir Dis* 111:313-320.

318. Maher, S. A., M. A. Birrell, and M. G. Belvisi. 2009. Prostaglandin E2 mediates cough via the EP3 receptor: implications for future disease therapy. *Am J Respir Crit Care Med* 180:923-928.

319. Tilley, S. L., J. M. Hartney, C. J. Erikson, C. Jania, M. Nguyen, J. Stock, J. McNeisch, C. Valancius, R. A. Panettieri, Jr., R. B. Penn, and B. H. Koller. 2003. Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. *Am J Physiol Lung Cell Mol Physiol* 284:L599-606.

320. Kunikata, T., H. Yamane, E. Segi, T. Matsuoka, Y. Sugimoto, S. Tanaka, H. Tanaka, H. Nagai, A. Ichikawa, and S. Narumiya. 2005. Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3. *Nat Immunol* 6:524-531.

321. Lundequist, A., S. N. Nallamshetty, W. Xing, C. Feng, T. M. Laidlaw, S. Uematsu, S. Akira, and J. A. Boyce. Prostaglandin E(2) exerts homeostatic regulation of pulmonary vascular remodeling in allergic airway inflammation. *J Immunol* 184:433-441.

322. Hoeper, M. M., M. Schwarze, S. Ehlerding, A. Adler-Schuermeyer, E. Spiekerkoetter, J. Niedermeyer, M. Hamm, and H. Fabel. 2000. Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. *N Engl J Med* 342:1866-1870.

323. Takahashi, Y., S. Tokuoka, T. Masuda, Y. Hirano, M. Nagao, H. Tanaka, N. Inagaki, S. Narumiya, and H. Nagai. 2002. Augmentation of allergic inflammation in prostanoid IP receptor deficient mice. *Br J Pharmacol* 137:315-322.

324. Nagao, K., H. Tanaka, M. Komai, T. Masuda, S. Narumiya, and H. Nagai. 2003. Role of prostaglandin I2 in airway remodeling induced by repeated allergen challenge in mice. *Am J Respir Cell Mol Biol* 29:314-320.

325. Harizi, H., J. B. Corcuff, and N. Gualde. 2008. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends Mol Med* 14:461-469.

326. Kiriyama, M., F. Ushikubi, T. Kobayashi, M. Hirata, Y. Sugimoto, and S. Narumiya. 1997. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* 122:217-224.

327. Ushikubi, F., M. Hirata, and S. Narumiya. 1995. Molecular biology of prostanoid receptors; an overview. *J Lipid Mediat Cell Signal* 12:343-359.

328. Palmer, M. L., S. Y. Lee, P. J. Maniak, D. Carlson, S. C. Fahrenkrug, and S. M. O'Grady. 2006. Protease-activated receptor regulation of Cl- secretion in Calu-3 cells requires prostaglandin release and CFTR activation. *Am J Physiol Cell Physiol* 290:C1189-1198.

329. Schmidt, L. M., M. G. Belvisi, K. A. Bode, J. Bauer, C. Schmidt, M. T. Suchy, D. Tsikas, J. Scheuerer, F. Lasitschka, H. J. Grone, and A. H. Dalpke. Bronchial epithelial cell-derived prostaglandin E2 dampens the reactivity of dendritic cells. *J Immunol* 186:2095-2105.

330. Mancini, J. A., K. Blood, J. Guay, R. Gordon, D. Claveau, C. C. Chan, and D. Riendeau. 2001. Cloning, expression, and up-regulation of inducible rat prostaglandin e synthase during lipopolysaccharide-induced pyresis and adjuvant-induced arthritis. *J Biol Chem* 276:4469-4475.

331. Radi, Z. A., and R. Ostroski. 2007. Pulmonary and cardiorenal cyclooxygenase-1 (COX-1), -2 (COX-2), and microsomal prostaglandin E synthase-1 (mPGES-1) and -2 (mPGES-2) expression in a hypertension model. *Mediators Inflamm* 2007:85091.

332. Wang, M., E. Lee, W. Song, E. Ricciotti, D. J. Rader, J. A. Lawson, E. Pure, and G. A. FitzGerald. 2008. Microsomal prostaglandin E synthase-1 deletion suppresses oxidative stress and angiotensin II-induced abdominal aortic aneurysm formation. *Circulation* 117:1302-1309.

333. Wang, M., A. M. Zukas, Y. Hui, E. Ricciotti, E. Pure, and G. A. FitzGerald. 2006. Deletion of microsomal prostaglandin E synthase-1 augments prostacyclin and retards atherogenesis. *Proc Natl Acad Sci U S A* 103:14507-14512.

334. Langenbach, R., S. G. Morham, H. F. Tiano, C. D. Loftin, B. I. Ghanayem, P. C. Chulada, J. F. Mahler, C. A. Lee, E. H. Goulding, K. D. Kluckman, H. S. Kim, and O. Smithies. 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* 83:483-492.

335. Allen, I. C., A. J. Pace, L. A. Jania, J. G. Ledford, A. M. Latour, J. N. Snouwaert, V. Bernier, R. Stocco, A. G. Therien, and B. H. Koller. 2006. Expression and function of NPSR1/GPRA in the lung before and after induction of asthma-like disease. *Am J Physiol Lung Cell Mol Physiol* 291:L1005-1017.

336. Dinchuk, J. E., B. D. Car, R. J. Focht, J. J. Johnston, B. D. Jaffee, M. B. Covington, N. R. Contel, V. M. Eng, R. J. Collins, P. M. Czerniak, and et al. 1995. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* 378:406-409.

337. Ewart, S. L., D. Kuperman, E. Schadt, C. Tankersley, A. Grupe, D. M. Shubitowski, G. Peltz, and M. Wills-Karp. 2000. Quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice. *Am J Respir Cell Mol Biol* 23:537-545.

338. Fukunaga, J., M. Abe, A. Murai, Y. Akitake, M. Hosokawa, and M. Takahashi. 2007. Comparative study to elucidate the mechanism underlying the difference in airway hyperresponsiveness between two mouse strains. *Int Immunopharmacol* 7:1852-1861.

339. Shinagawa, K., and M. Kojima. 2003. Mouse model of airway remodeling: strain differences. *Am J Respir Crit Care Med* 168:959-967.

340. Tanioka, T., Y. Nakatani, N. Semmyo, M. Murakami, and I. Kudo. 2000. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. *J Biol Chem* 275:32775-32782.

341. Tanikawa, N., Y. Ohmiya, H. Ohkubo, K. Hashimoto, K. Kangawa, M. Kojima, S. Ito, and K. Watanabe. 2002. Identification and characterization of a novel type of membrane-associated prostaglandin E synthase. *Biochem Biophys Res Commun* 291:884-889.

342. Boulet, L., M. Ouellet, K. P. Bateman, D. Ethier, M. D. Percival, D. Riendeau, J. A. Mancini, and N. Methot. 2004. Deletion of microsomal prostaglandin E2 (PGE2) synthase-1 reduces inducible and basal PGE2 production and alters the gastric prostanoid profile. *J Biol Chem* 279:23229-23237.

343. Del Prete, G., E. Maggi, P. Parronchi, I. Chretien, A. Tiri, D. Macchia, M. Ricci, J. Banchereau, J. De Vries, and S. Romagnani. 1988. IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *J Immunol* 140:4193-4198.

344. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76:34-40.

345. Nakae, S., H. Suto, G. J. Berry, and S. J. Galli. 2007. Mast cell-derived TNF can promote Th17 cell-dependent neutrophil recruitment in ovalbumin-challenged OTII mice. *Blood* 109:3640-3648.

346. Schulz, H., C. Johner, G. Eder, A. Ziesenis, P. Reitmeier, J. Heyder, and R. Balling. 2002. Respiratory mechanics in mice: strain and sex specific differences. *Acta Physiol Scand* 174:367-375.

347. Tankersley, C. G., R. Rabold, and W. Mitzner. 1999. Differential lung mechanics are genetically determined in inbred murine strains. *J Appl Physiol* 86:1764-1769.

348. Zhu, W., and M. I. Gilmour. 2009. Comparison of allergic lung disease in three mouse strains after systemic or mucosal sensitization with ovalbumin antigen. *Immunogenetics* 61:199-207.

349. Van Hove, C. L., T. Maes, D. D. Cataldo, M. M. Gueders, E. Palmans, G. F. Joos, and K. G. Tournoy. 2009. Comparison of acute inflammatory and chronic structural asthma-like responses between C57BL/6 and BALB/c mice. *Int Arch Allergy Immunol* 149:195-207.

350. Nguyen, M., T. Camenisch, J. N. Snouwaert, E. Hicks, T. M. Coffman, P. A. Anderson, N. N. Malouf, and B. H. Koller. 1997. The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. *Nature* 390:78-81.

351. Fedyk, E. R., S. G. Harris, J. Padilla, and R. P. Phipps. 1997. Prostaglandin receptors of the EP2 and EP4 subtypes regulate B lymphocyte activation and differentiation to IgE-secreting cells. *Adv Exp Med Biol* 433:153-157.

352. Martin, J. G., M. Suzuki, K. Maghni, R. Pantano, D. Ramos-Barbon, D. Ihaku, F. Nantel, D. Denis, Q. Hamid, and W. S. Powell. 2002. The immunomodulatory actions of prostaglandin E2 on allergic airway responses in the rat. *J Immunol* 169:3963-3969.

353. Ivanov, II, K. Atarashi, N. Manel, E. L. Brodie, T. Shima, U. Karaoz, D. Wei, K. C. Goldfarb, C. A. Santee, S. V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D. R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485-498.

354. Wu, H. J., Ivanov, II, J. Darce, K. Hattori, T. Shima, Y. Umesaki, D. R. Littman, C. Benoist, and D. Mathis. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 32:815-827.

355. Wen, L., R. E. Ley, P. Y. Volchkov, P. B. Stranges, L. Avanesyan, A. C. Stonebraker, C. Hu, F. S. Wong, G. L. Szot, J. A. Bluestone, J. I. Gordon, and A. V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109-1113.

356. Mazmanian, S. K., J. L. Round, and D. L. Kasper. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453:620-625.

357. Sturm, E. M., P. Schratl, R. Schuligoi, V. Konya, G. J. Sturm, I. T. Lippe, B. A. Peskar, and A. Heinemann. 2008. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol* 181:7273-7283.

358. Demeure, C. E., L. P. Yang, C. Desjardins, P. Raynauld, and G. Delespesse. 1997. Prostaglandin E2 primes naive T cells for the production of anti-inflammatory cytokines. *Eur J Immunol* 27:3526-3531.

359. Fabre, J. E., M. Nguyen, K. Athirakul, K. Coggins, J. D. McNeish, S. Austin, L. K. Parise, G. A. FitzGerald, T. M. Coffman, and B. H. Koller. 2001. Activation of the murine EP3 receptor for PGE2 inhibits cAMP production and promotes platelet aggregation. *J Clin Invest* 107:603-610.

360. Downey, G. P., R. S. Gumbay, D. E. Doherty, J. F. LaBrecque, J. E. Henson, P. M. Henson, and G. S. Worthen. 1988. Enhancement of pulmonary inflammation by PGE2: evidence for a vasodilator effect. *J Appl Physiol* 64:728-741.

361. Li, T., J. Qi, and E. A. Cowley. Activation of the EP prostanoid receptor induces prostaglandin E and pro-inflammatory cytokine production in human airway epithelial cells. *Pulm Pharmacol Ther* 24:42-48.

362. Song, K. S., Y. H. Choi, J. M. Kim, H. Lee, T. J. Lee, and J. H. Yoon. 2009. Suppression of prostaglandin E2-induced MUC5AC overproduction by RGS4 in the airway. *Am J Physiol Lung Cell Mol Physiol* 296:L684-692.

363. Kim, Y. D., E. J. Kwon, D. W. Park, S. Y. Song, S. K. Yoon, and S. H. Baek. 2002. Interleukin-1beta induces MUC2 and MUC5AC synthesis through cyclooxygenase-2 in NCI-H292 cells. *Mol Pharmacol* 62:1112-1118.

364. Clayton, A., E. Holland, L. Pang, and A. Knox. 2005. Interleukin-1beta differentially regulates beta2 adrenoreceptor and prostaglandin E2-mediated cAMP accumulation and chloride efflux from Calu-3 bronchial epithelial cells. Role of receptor changes, adenylyl cyclase, cyclo-oxygenase 2, and protein kinase A. *J Biol Chem* 280:23451-23463.

365. Wanner, A., M. Sielczak, J. F. Mella, and W. M. Abraham. 1986. Ciliary responsiveness in allergic and nonallergic airways. *J Appl Physiol* 60:1967-1971.

366. Tavakoli, S., M. J. Cowan, T. Benfield, C. Logun, and J. H. Shelhamer. 2001. Prostaglandin E(2)-induced interleukin-6 release by a human airway epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* 280:L127-133.

367. Raychaudhuri, N., R. S. Douglas, and T. J. Smith. PGE2 induces IL-6 in orbital fibroblasts through EP2 receptors and increased gene promoter activity: implications to thyroid-associated ophthalmopathy. *PLoS One* 5:e15296.

368. Neveu, W. A., J. B. Allard, O. Dienz, M. J. Wargo, G. Ciliberto, L. A. Whittaker, and M. Rincon. 2009. IL-6 is required for airway mucus production induced by inhaled fungal allergens. *J Immunol* 183:1732-1738.

369. Smith, W. L., R. M. Garavito, and D. L. DeWitt. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* 271:33157-33160.

370. Wu, K. K., and J. Y. Liou. 2005. Cellular and molecular biology of prostacyclin synthase. *Biochem Biophys Res Commun* 338:45-52.

371. Oida, H., T. Namba, Y. Sugimoto, F. Ushikubi, H. Ohishi, A. Ichikawa, and S. Narumiya. 1995. In situ hybridization studies of prostacyclin receptor mRNA expression in various mouse organs. *Br J Pharmacol* 116:2828-2837.

372. Kawabe, J., F. Ushikubi, and N. Hasebe. Prostacyclin in vascular diseases. - Recent insights and future perspectives. *Circ J* 74:836-843.

373. Church, R. J., L. A. Jania, and B. H. Koller. Prostaglandin E2 Produced by the Lung Augments the Effector Phase of Allergic Inflammation. *J Immunol*.

374. Walmrath, D., U. Schneider, B. Kreusler, F. Grimminger, M. Ennis, and W. Seeger. 1991. Intravascular anti-IgE challenge in perfused lungs: mediator release and vascular pressor response. *J Appl Physiol* 71:2499-2506.

375. Schulman, E. S., N. F. Adkinson, Jr., and H. H. Newball. 1982. Cyclooxygenase metabolites in human lung anaphylaxis: airway vs. parenchyma. *J Appl Physiol* 53:589-595.

376. Schulman, E. S., H. H. Newball, L. M. Demers, F. A. Fitzpatrick, and N. F. Adkinson, Jr. 1981. Anaphylactic release of thromboxane A2, prostaglandin D2, and prostacyclin from human lung parenchyma. *Am Rev Respir Dis* 124:402-406.

377. Hardy, C. C., P. Bradding, C. Robinson, and S. T. Holgate. 1988. Bronchoconstrictor and antibronchoconstrictor properties of inhaled prostacyclin in asthma. *J Appl Physiol* 64:1567-1574.

378. Bianco, S., M. Robuschi, R. Ceserani, and C. Gandolfi. 1980. Effects of prostacyclin on aspecifically and specifically induced bronchoconstriction in asthmatic patients. *Eur J Respir Dis Suppl* 106:81-87.

379. Bonnefoy, J. Y., J. F. Gauchat, P. Life, P. Graber, J. P. Aubry, and S. Lecoanet-Henchoz. 1995. Regulation of IgE synthesis by CD23/CD21 interaction. *Int Arch Allergy Immunol* 107:40-42.

380. Lebman, D. A., and R. L. Coffman. 1988. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J Exp Med* 168:853-862.

381. van Rijt, L. S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H. C. Hoogsteden, and B. N. Lambrecht. 2005. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 201:981-991.

382. Breyer, R. M., C. K. Bagdassarian, S. A. Myers, and M. D. Breyer. 2001. Prostanoid receptors: subtypes and signaling. *Annu Rev Pharmacol Toxicol* 41:661-690.

383. Sugimoto, Y., S. Narumiya, and A. Ichikawa. 2000. Distribution and function of prostanoid receptors: studies from knockout mice. *Prog Lipid Res* 39:289-314.

384. Bjarnason, I., J. Hayllar, A. J. MacPherson, and A. S. Russell. 1993. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* 104:1832-1847.

385. Aabakken, L., and M. Osnes. 1989. Non-steroidal anti-inflammatory drug-induced disease in the distal ileum and large bowel. *Scand J Gastroenterol Suppl* 163:48-55.

386. Newberry, R. D., W. F. Stenson, and R. G. Lorenz. 1999. Cyclooxygenase-2-dependent arachidonic acid metabolites are essential modulators of the intestinal immune response to dietary antigen. *Nat Med* 5:900-906.

387. Holtzman, M. J. 1992. Arachidonic acid metabolism in airway epithelial cells. *Annu Rev Physiol* 54:303-329.

388. Huang, M., M. Stolina, S. Sharma, J. T. Mao, L. Zhu, P. W. Miller, J. Wollman, H. Herschman, and S. M. Dubinett. 1998. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. *Cancer Res* 58:1208-1216.

389. Simkin, N. J., D. F. Jelinek, and P. E. Lipsky. 1987. Inhibition of human B cell responsiveness by prostaglandin E2. *J Immunol* 138:1074-1081.

390. Murn, J., O. Alibert, N. Wu, S. Tendil, and X. Gidrol. 2008. Prostaglandin E2 regulates B cell proliferation through a candidate tumor suppressor, Ptger4. *J Exp Med* 205:3091-3103.

391. Harizi, H., and G. Norbert. 2004. Inhibition of IL-6, TNF-alpha, and cyclooxygenase-2 protein expression by prostaglandin E2-induced IL-10 in bone marrow-derived dendritic cells. *Cell Immunol* 228:99-109.

392. Bryn, T., S. Yaqub, M. Mahic, K. Henjum, E. M. Aandahl, and K. Tasken. 2008. LPS-activated monocytes suppress T-cell immune responses and induce FOXP3+ T cells through a COX-2-PGE2-dependent mechanism. *Int Immunol* 20:235-245.

393. Tilley, S. L., L. P. Audoly, E. H. Hicks, H. S. Kim, P. J. Flannery, T. M. Coffman, and B. H. Koller. 1999. Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor. *J Clin Invest* 103:1539-1545.

394. Fleming, E. F., K. Athirakul, M. I. Oliverio, M. Key, J. Goulet, B. H. Koller, and T. M. Coffman. 1998. Urinary concentrating function in mice lacking EP3 receptors for prostaglandin E2. *Am J Physiol* 275:F955-961.

395. Yoshikawa, T., T. Imada, H. Nakakubo, N. Nakamura, and K. Naito. 2001. Rat mast cell protease-I enhances immunoglobulin E production by mouse B cells stimulated with interleukin-4. *Immunology* 104:333-340.

396. Roper, R. L., and R. P. Phipps. 1992. Prostaglandin E2 and cAMP inhibit B lymphocyte activation and simultaneously promote IgE and IgG1 synthesis. *J Immunol* 149:2984-2991.

397. Singh, P., J. Hoggatt, P. Hu, J. M. Speth, S. Fukuda, R. M. Breyer, and L. M. Pelus. Blockade of prostaglandin E2 signaling through EP1 and EP3 receptors attenuates Flt3L-dependent dendritic cell development from hematopoietic progenitor cells. *Blood* 119:1671-1682.

398. Nagamachi, M., D. Sakata, K. Kabashima, T. Furuyashiki, T. Murata, E. Segi-Nishida, K. Soontrapa, T. Matsuoka, Y. Miyachi, and S. Narumiya. 2007. Facilitation of Th1-mediated immune response by prostaglandin E receptor EP1. *J Exp Med* 204:2865-2874.

399. Kodama, M., K. Asano, T. Oguma, S. Kagawa, K. Tomomatsu, M. Wakaki, T. Takihara, S. Ueda, N. Ohmori, H. Ogura, J. Miyata, K. Tanaka, N. Kamiishi, K. Fukunaga, K. Sayama, E. Ikeda, T. Miyasho, and A. Ishizaka. Strain-specific phenotypes of airway inflammation and bronchial hyperresponsiveness induced by epicutaneous allergen sensitization in BALB/c and C57BL/6 mice. *Int Arch Allergy Immunol* 152 Suppl 1:67-74.

400. Legler, D. F., M. Bruckner, E. Uetz-von Allmen, and P. Krause. Prostaglandin E2 at new glance: novel insights in functional diversity offer therapeutic chances. *Int J Biochem Cell Biol* 42:198-201.

401. Wert, S. E., S. W. Glasser, T. R. Korfhagen, and J. A. Whitsett. 1993. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev Biol* 156:426-443.

402. Cyphert, J. M., I. C. Allen, R. J. Church, A. M. Latour, J. N. Snouwaert, T. M. Coffman, and B. H. Koller. Allergic inflammation induces a persistent mechanistic switch in thromboxane-mediated airway constriction in the mouse. *Am J Physiol Lung Cell Mol Physiol*.

403. Kabashima, K., M. Nagamachi, T. Honda, C. Nishigori, Y. Miyachi, Y. Tokura, and S. Narumiya. 2007. Prostaglandin E2 is required for ultraviolet B-induced skin inflammation via EP2 and EP4 receptors. *Lab Invest* 87:49-55.

404. McMenamin, C., B. Girn, and P. G. Holt. 1992. The distribution of IgE plasma cells in lymphoid and non-lymphoid tissues of high-IgE responder rats: differential localization of antigen-specific and 'bystander' components of the IgE response to inhaled antigen. *Immunology* 77:592-596.

405. Caton, M. L., M. R. Smith-Raska, and B. Reizis. 2007. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med* 204:1653-1664.

406. Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, H. E. Jolin, and A. N. McKenzie. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.

407. Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.

408. Price, A. E., H. E. Liang, B. M. Sullivan, R. L. Reinhardt, C. J. Eisley, D. J. Erle, and R. M. Locksley. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* 107:11489-11494.

409. Saenz, S. A., M. C. Siracusa, J. G. Perrigoue, S. P. Spencer, J. F. Urban, Jr., J. E. Tocker, A. L. Budelsky, M. A. Kleinschek, R. A. Kastelein, T. Kambayashi, A. Bhandoola, and D. Artis. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464:1362-1366.

410. Tamachi, T., Y. Maezawa, K. Ikeda, S. Kagami, M. Hatano, Y. Seto, A. Suto, K. Suzuki, N. Watanabe, Y. Saito, T. Tokuhisa, I. Iwamoto, and H. Nakajima. 2006. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. *J Allergy Clin Immunol* 118:606-614.

411. Oboki, K., T. Ohno, N. Kajiwara, K. Arae, H. Morita, A. Ishii, A. Nambu, T. Abe, H. Kiyonari, K. Matsumoto, K. Sudo, K. Okumura, H. Saito, and S. Nakae. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A* 107:18581-18586.