EFFECTS OF PROSTAGLANDIN D_2 AND THE DP_1 AND DP_2 RECEPTORS IN EOSINOPHIL RECRUITMENT INTO THE BROWN NORWAY RAT LUNGS

Wagdi Almishri

Department of Pathology
McGill University
Montreal, Canada

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ABSTRACT

The accumulation of eosinophils at sites of inflammation is one of the hallmarks of asthma. The aim of the present study was to investigate the involvement of PGD_2 and the DP_1 and DP_2 receptors in eosinophil recruitment in vivo. In this project, a group of Brown Norway rats were administered intratracheally with PGD_2, which activates both DP_1 and DP_2 receptors, as well as selective agonists of DP_1 (BW245C) and DP_2 (15R-methyl-PGD_2 and 13,14-dihydro-15-oxo-PGD_2) receptors. In addition, we have also tested the effect of 15-deoxy-$\Delta^{12,14}$-PGJ_2 which known to activate PPARγ and NF-κB, and DP_2 receptors. Negative control animals received saline alone while positive control animals received 5-oxo-ETE. In this work, we have shown for the first time that PGD_2 and selective DP_2 receptor agonists induce pulmonary eosinophilia in vivo in the following order of potency; (15R-methyl-PGD_2 > PGD_2 ≈ 15-deoxy-$\Delta^{12,14}$-PGJ_2 > dhk-PGD_2). This effect was time dependent with the maximal response being observed after 24 h. Interestingly; this response was somehow diminished when higher dose was tested (10μg). This effect is most likely to be mediated solely through the DP_2 receptor since this effect was not shared by the DP_1 specific agonist BW245C. These results are consistent with an important role for PGD_2 and the DP_2 receptor/$CRTH_2$ in allergic diseases such as asthma and suggest that this receptor may be an important therapeutic target for these conditions.

RESUME

L'accumulation d'éosinophiles aux sites d'inflammation est l'une des manifestations principales de l'asthme. L'objectif de la présente étude est d'étudier la participation de PGD_2 et des récepteurs DP_1 et DP_2 dans l'infiltration des éosinophiles in vivo. Dans ce projet, nous avons administré à des rats « Brown Norway », par voie intratrachéale, du PGD_2, qui est à la fois activateur des récepteurs DP_1 et DP_2, ainsi que les agonistes sélectifs du récepteur DP_1 (BW245C) et du récepteur DP_2 (15R-methyl-PGD_2 et 13,14-dihydro-15-oxo-PGD_2) . De plus, nous avons également examiné l'effet du 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) qui est connu pour activiver PPARγ NF-κB et le récepteur DP_2. Les animaux témoins négatifs ont reçu du salin, tandis que les témoins positifs ont reçu du 5-oxo-ETE. Dans ce travail, nous avons prouvé pour la première fois que le PGD_2 ainsi que les agonistes sélectifs du récepteur DP_2 induisent une hyperéosinophilies pulmonaire *in vivo* dans l'ordre suivant d'efficacité ;(15R-methyl-PGD_2 > PGD_2 ≈ 15-deoxy-$\Delta^{12,14}$-PGJ_2 > dhk-PGD_2). Cet effet est dépendant du temps, avec une réponse maximale observée après 24 h. De plus, cette réponse est diminuée lorsqu'une dose plus élevée a été testée (10μg). Cet effet est très probablement médié uniquement via le récepteur DP_2 puisque cet effet n'est pas observé avec l'agoniste spécifique de DP_1, le BW245C. Ces résultats suggèrent un rôle important pour PGD_2 et le récepteur DP_2/$CRTH_2$ dans les maladies allergiques telles que l'asthme et suggèrent que ce récepteur puisse être une cible thérapeutique importante pour ces conditions.

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ABBREVIATIONS

AA	**arachidonic acid**
AHR	**airway hyperresponsiveness**
ANOVA	**analysis of variance**
APC	**antigen presenting cells**
BAL	**bronchoalveolar lavage**
B cells	**B lymphocyte**
BN rat	**Brown Norway rat**
Ca^{2+}	**calcium**
COX	**cyclooxygenase**
CRTH2	**chemoattractant receptor-homologous molecule expressed on Th2 cells**
Cys-LTs	**cysteinyl leukotrienes**
DC	**dendritic cells**
dhk-PGD_2	**13,14-dihydro-15-ketoprostaglandin D_2**
15d-PGJ_2	**15-deoxy-$\Delta^{12,14}$-prostaglandin J_2**
ECP	**eosinophil cationic protein**
EDN	**eosinophil derived neurotoxin**
EPO	**eosinophil peroxidase**
EPX	**eosinophil protein X**
EPR	**early phase response**
E-selectin	**endothelial selectin**
GM-CSF	**granulocyte macrophage colony stimulating factor**
H-PGDS	**hematopoietic-type PGD synthase**

5-HETE	**5-hydroxy-6,8,11,14-eicosatetraenoic acid**
5-HPETE	**5-hydroperoxy-6,8,11,14-eicosatetraenoic acid**
ICAM-1	**intracellular adhesion molecule 1**
ICAM-3	**intracellular adhesion molecule**
ICAM-2	**intracellular adhesion molecule**
IFN-γ	**interferon gamma**
Ig	**immunoglobulin**
IL	**interleukin**
iNOS	**inducible nitric oxide synthase**
IV	**intravenous**
LAR	**late asthmatic response**
LTC_4	**leukotriene C_4**
LTD_4	**leukotriene D_4**
LTE_4	**leukotriene E_4**
L-PGDS	**lipocalin-type PGD synthase**
LTs	**leukotrienes**
5-LO	**5-lipoxygenase**
mAb	**monoclonal antibody**
MAP	**mitogen-activated protein**
MBP	**major basic protein**
MC	**mast cells**
M-CSF	**macrophage colony stimulating factor**
MDC	**macrophage-derived chemokine**
MHCII	**major histocompatibility complex class II**
NO	**nitric oxide**

OCT	**optimal cutting temperature**
OVA	**ovalbumin**
5-Oxo-ETE	**5-Oxo-6,8,11,14-eicosatetraenoic acid**
PAF	**platelet activating factor**
PGD_2	**prostaglandin D_2**
PGE_2	**prostaglandin E_2**
PGs	**prostaglandins**
PL A_2	**phospholipases A_2**
PPAR-γ	**peroxisome proliferator-activated receptor gamma**
P-selectin	**platelet-selectin**
RANTES	**regulated upon activation in normal T cells**
RXR	**retinoid X receptors**
15(R)-PGD_2	**15(R)-15-methyl PGD_2**
sECP	**serum eosinophil cationic proteins**
SMC	**smooth muscle cells**
TBS	**tris-buffered saline**
T cells	**T lymphocyte**
TGF-β_1	**transforming growth factor beta$_1$**
Th_1	**T helper type 1**
Th2	**T helper type 2**
TNF-α	**tumor necrosis factor - alpha**
TNF-γ	**tumor necrosis factor-gamma**
TXA_2	**thromboxanes A_2**
TXs	**thromboxanes**

VCAM-1	**vascular adhesion molecule-1**
VLA-1	**very late activation antigen-1**
VLA-4	**very late antigen (CD1149d/CD29)**
VLA-6	**very late antigen**

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction-Aim of study

Asthma is a chronic, allergic inflammatory airway disease characterized by a biphasic allergic reaction consisting of early and late phase responses. The late phase allergic reaction is accompanied by the infiltration of inflammatory cells into the lung, particularly eosinophils and $CD4^+$ Th2 cells [1]. Eosinophils are believed to be important effector cells in this disease since it has been clearly shown that there is a good correlation between their numbers in peripheral blood, bronchoalveolar lavage and bronchial mucosa and the severity of the disease [2]. Eosinophils and their mediators can contribute to asthma by different effects including damage to the lung epithelium [3], augmentation of the inflammatory response and the process of remodelling [4].

The identification of molecules that specifically regulate the recruitment of eosinophils and subsequently blocking the effect of these molecules offers new therapeutic strategies. The recruitment of eosinophils to sites of allergic inflammation is a complex process, occurring at a number of stages in the life cycle of the eosinophil where different mediators and adhesion receptors influence the accumulation process [5]. Among the mediators implicated in this process are the inflammatory cytokines (e.g., IL-3, IL-4, IL-5, IL-1β, GM-CSF and TNF-α), the chemokines (e.g., RANTES, monocyte chemoattractant protein-3, macrophage inflammatory protein-1α, and eotaxin), and lipid mediators [5]. Prostaglandin D_2 (PGD_2) is a major lipid mediator released mainly from mast cells and Th2 cells and elicits its biological actions through interaction with two PGD_2 receptors, the DP_1 receptor and the DP_2 receptor, also known as $CRTH_2$ (chemoattractant receptor-homologous molecule expressed on Th2 cells) [6]. PGD_2 levels in bronchoalveolar lavage (BAL) fluid increase dramatically following allergen challenge of asthmatic subjects and

hematopoietic-type PGD_2 synthase is abundant in cells that play an important role in asthma [7] [8]. However, the precise role of PGD_2 in allergic asthma remains unclear. An important area of research is now concerned with the effect of PGD_2 on eosinophil recruitment and a number of studies provide evidence for such role. For instance, Monneret et al demonstrated that PGD_2 is a potent eosinophil chemoattractant in vitro that acts via the novel DP_2 receptor rather than via the classic DP_1 receptor [9]. Another study by Matsuoka et al., showed that disruption of the DP_1 receptor gene attenuated allergen-induced airway eosinophilic inflammation, probably by reducing Th2 cytokine levels [10]. Gervais et al showed that PGD_2 triggers eosinophil degranulation and induces a rapid change in cell morphology through the DP_2 receptor/$CRTH_2$ [11].

The aim of this study was to test the hypothesis that administration of PGD_2 into the lungs results in pulmonary eosinophilia. If our hypothesis was correct, we then wanted to determine which of the two PGD_2 receptors mediated this response. To accomplish these aims, we investigated the effect on eosinophil recruitment of intratracheally administered PGD_2 which acts through both DP_1 and DP_2 receptors, as well as selective agonists of the DP_1 (BW245C) and the DP_2 (15R-methyl-PGD_2 and 13,14-dihydro-15-oxo-PGD_2) receptors. We also wanted to examine the effect of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) which could potentially have both inhibitory and stimulatory effects on eosinophils through its interaction with the DP_2 receptors and PPARγ/NF-κB, respectively [12].

The remainder of this chapter will provide background information pertinent to the current study. Asthma pathogenesis and the involvement of inflammatory cells in this disease will first be discussed, with particular emphasis placed on the role of eosinophils. In addition background information about arachidonic acid metabolites,

their receptors, and their involvement in inflammation in general and in asthma in particular will be presented.

1.2. Asthma

Asthma is a widespread disease in the industrialized world and its occurrence has almost doubled in the last 20 years [13]. Some cases of asthma can be fatal and approximately 5,000 people die each year due to this disease [14]. Although it has changed over time, the current definition of asthma covers most of the clinical and pathological aspects of this complex disease, which is "A chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals, the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment" [1]. Asthma also associated with characteristic airway remodelling which is accompanied by airway hyperresponsiveness to a variety of stimuli [15]. Even though asthma is multifactorial in cause, the most identifiable predisposing factor for the development of this disease is the genetic tendency for the development of an IgE-mediated response to distinct environmental allergens [16]. In general, the reaction to an allergen can be divided into two phases namely; the early phase response and late phase response [17].

1.2.1. The Allergic Reaction

The allergic reaction starts with a sensetization process that take place when environmental allergens are processed by antigen presenting cells (APC) that have the ability to present antigen to T and B cells. In the presence of an appropriate microenvironment differentiation of naive T cells into Th2 lymphocytes occurs. These Th2 cells interact with B cells initiating B cell production of IgE antibodies. This interaction is regulated by soluble and cell surface molecules (CD40L, CD154) provided by activated Th2 cells in the presence of Th2 cytokines including IL-4 and IL-13 [18, 19]. This results in the production of large amounts of IgE antibodies directed against specific environmental allergens. Mast cells in turn, have high affinity receptors on their surfaces that can bind to the Fc region of the IgE antibodies, so that after the initial exposure, mast cells will have large numbers of specific IgE attached to their surfaces [20].

On a second exposure to allergens, the allergens can cross-link the IgE antibody's Fab-site, on the mast cell, and induces lung mast cell activation resulting in the occurrence of the early phase response. The early response (EPR) is an acute bronchospastic event that occurs 15–30 minutes after exposure to allergen and resolves over a relatively short period of time [21]. Mast cells activation lead to a rapid release of their mediators (ie, histamine, cys-LTs, PGD_2, and tryptase) [20]. These mediators are largely appreciated to be the key for the development of airflow obstruction seen in early phase via the induction of bronchial smooth muscle constriction and bronchial wall oedema [20]. In most asthmatic subjects, the early asthmatic response is followed by a delayed bronchoconstrictor response termed the late asthmatic response (LAR) [22].

The LAR occurs between 6 to 9 h after allergen challenge and involves the recruitment and activation of eosinophils [23], $CD4^+$ T cells [24], basophils [25], neutrophils [26], and macrophages [27]. The release of preformed cytokines by mast cells is the likely initial trigger for the early recruitment of cells [1]. The interaction of these recruited cells with resident cells such as mast cells, macrophages, epithelial cells and endothelial cells generates a cascade of events that contributes to pathological changes seen in the airway [28, 29]. The role of some of these important cells in asthma pathogeneses will be highlighted later with special emphasis placed on the role of the eosinophils.

1.2.2. Airway Remodelling

One important chronic squeal of asthma is the structural alteration of lung tissue known as airway remodelling. Pathological changes seen in airways from asthmatic patients include: hyperplasia of mucus glands, mucus hypersecretion, smooth muscle hypertrophy, epithelial damage, subepithelial fibrosis, and inflammatory cell infiltrates [30] [17].These structural changes contribute to the severity and chronicity of asthma by amplifying airway narrowing through the increase in thickness of the bronchial wall [31].

Among all of the structural changes occurring, smooth muscle hypertrophy is considered the most important. In addition to contraction and relaxation, smooth muscle has other important roles in asthma including the secretion of immunomodulatory cytokines and chemokines, and expression of key surface receptors involved in cell adhesion and leukocyte activation [32]. Although the mechanisms underlying airway remodelling in asthma are still unclear, it is widely believed that it results from a complex interaction between different inflammatory

cells (i.e., mast cells, eosinophils, T cells, neutrophils and macrophages) and structural cells (i.e., fibroblasts, endothelial cells, smooth muscle cells) [33]. Eosinophils in particular may play an important role in the remodelling since they are a source of several mediators that could be implicated in this process including TGF-α, TGF-β_1, FGF-2, VEGF, TIMP-1, LTD_4 and IL-13 [34]. These structural alterations render airways more susceptible to various stimuli, a phenomenon known as airway hyperresponsiveness.

1.2.3. Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR), is a well-established feature of asthma in which exaggerated airway narrowing is easily elicited by a large number of stimuli such as histamine, methacholine or specific antigens [15]. Aairway hyperresponsi - veness is a result of a nonspecific increase in reactivity as well as sensitivity of the airways [35, 36]. Although the exact pathophysiology of AHR is not well defined, a variety of mechanisms may be involved in its development. For instance, airway epithelial damage is positively correlated with airway hyperreactivity [37]. The loss of airway epithelium may augment the response of the airways to various stimuli by increasing the level of exposure to these stimuli [17]. Structural changes in asthma may also contribute to AHR. Smooth muscle (SMC) hypertrophy is present in the lungs of asthmatics and may contribute to an exaggerated narrowing of the airways in response to a variety of environmental stimuli [17]. In addition, responsiveness to sympathetic input regulating relaxation of the airway via ß-adrenergic receptors on airway SMC has also been shown to be impaired in asthmatics [38]. Possibly due to the effect of IL-1 and TNF-α [39].

Inflammation may also play an important role in AHR. Airway sensitivity was found to be related to sputum eosinophil numbers [40] and the concentration of eosinophil-derived major basic protein (MBP) in BAL fluid [37]. Additionally, the MBP has been shown to enhance the release of the neurotransmitter acetylcholine from parasympathetic nerve terminals by blocking activation of the M_2, inhibitory receptor on the nerve endings [41], resulting in enhanced narrowing of the airway wall for any given stimulus. It has also been shown that inflammation is associated with increased release of a number of sensory neuropeptides, including substance P and neurokinins, that probably enhances reflex constriction of airway smooth muscle [17]. Finally, an array of inflammatory mediators released by eosinophils and mast cells can directly constrict airways including PAF, histamine, leukotrienes, and eosinophil granule proteins [17].

1.2.4. The Role of Inflammatory Cells in Asthma

Asthma is a very difficult disease to study in view of its complexity and the large number of mediators and cellular components known to be involved in its pathogenesis. Samples of BAL fluid, sputum, and airway biopsy specimens and have revealed that inflammation of the airways exhibits distinctive patterns in asthma [42]. The key cells known to be involved in asthma are eosinophils, mast cells, antigen presenting cells, epithelial cells, fibroblasts, and bronchial SMC (fig.1) [16]. These cells either inflammatory or structural, orchestrate the process of asthma mainly by releasing a large number of inflammatory mediators, the important of which are histamine, PAF, PGs, cys-LTs, toxic oxygen radicals, and cytokines [43] [44]. In this section I will discuss the role of dendritic cells, mast Cells and Th2 cells in asthma. The role of eosinophils in asthma will be discussed separately in section 1.3.3. .

1.2.4.a Dendritic Cells

Dendritic cells (DC) are present in the upper airway, bronchial mucosa, lung interstitium, and pleura [45]. Their major role is to process environmental antigens and present them to T-cells [46]. This process is very important for naive T cells to proliferate and differentiate to become Th2-cells in response to allergen exposure [47]. The numbers of DC are notably higher in the airways of asthmatics compared with those of control subjects [48]. DC in the normal airway are immature, expressing low levels of MHC Class II and Fcε RI [49] and [50]. However, after antigen uptake DC turn into mature cells expressing higher levels of MHC Class II and Fcε RI [49] [50]. DC not only act as antigen presenting cells but also play an important role in the immune response. For instance, It has been shown that a subset of DC retains the ability to activate memory T-cells long after antigen inhalation and to play a role in the maintenance of chronic allergic airway inflammation [51].

1.2.4.b. Mast Cells

Mast cells arise in the bone marrow from hematopoietic pregenitor cells, enter the circulation as an immature cells, and travel to mucosal and submucosal sites in the airways where they undergo their terminal stages of differentiation/ maturation [16]. In asthma mast cells are believed to be vital during the early asthmatic response [52]. Increased mast cell number are present in the airways in asthma [37]. When an asthmatic subject encounters a second exposure to allergen, the allergen can cross-link the IgE antibody's Fab-site on the mast cell eliciting the release of both pre-formed (e.g. histamine, cytokines, and proteoglycans) [20] and newly synthesized (e.g. arachidonic acid metabolites including PGD_2 and LTC4) mediators [53]. Mast cell

mediators are largely responsible for the acute airway narrowing seen in the early phase of the asthmatic reaction. Mast cell mediators may contribute to airway narrowing directly by increasing muscle contraction [22], increasing the thickness of the airway by causing vasodilatation and microvascular leakage [54], and inducing mucus secretion [20]. Mast cells mediators also contribute to asthma by induceing leukocyte infiltration [20]. They could also contribute to airway remodelling by facilitating SMC hyperplasia through the mitogenic effects of their tryptases [55]. Furthermore, many studies related mast cell numbers in BAL fluid and airway biopsies to AHR [37]. There is wide agreement that these cells play a central role in the early asthmatic reaction, and they can also play a role in late phase responses [56].

1.2.4.c. Th2 Cells

High numbers of T lymphocytes are found in BAL fluid and bronchial biopsies from asthmatics [19, 57, 58]. Originally, T cells were subdivided into two broad subsets according to their surface cell markers and distinct functions: the $CD4^+$ (T helper) and the $CD8^+$ (T cytotoxic) cells. $CD4^+$ T lymphocytes can differentiate toward a T helper type 1 (Th_1) or Th2 phenotype each of which has its own cytokine profile [59]. The most important factor that determines the differentiation toward the Th2 phenotype in asthma is the presence of IL-4 [60]. Activated Th2 cells produce IL-4, IL-13, IL-5, IL-9, IL-6, and IL-10 [24]. In asthmatics, Th2 cells are not only increased in number but are also activated. Th2 cells from BAL, blood, and bronchial biopsies showed amplified expression of IL-2 receptor, class II histocompatibility antigens, and VLA-1 [61] [62]. Several lines of evidence support the involvement of Th2 cells in the pathogenesis of asthma. Firstly, the increased numbers of activated Th2 cells observed in asthmatics correlate with the numbers of activated eosinophils,

and the severity of the disease [63] [64]. Secondly, depletion of $CD4^+$ T cells in sensitized mice prior to local lung antigen challenge prevented the development of allergen-induced allergic airway responses [65]. Furthermore, AHR can be induced in naive mice by adoptive transfer of Th2 clones into their lungs [66]. Thirdly, studies have shown that the administration of agents that inhibit Th2 cytokine production and stimulate Th_1 pathways prevents the development of antigen-induced AHR and inflammation in murine models [66, 67]. The effect of Th2 cells in asthma are mediated by the release of cytokines including IL-4, IL-13, and IL-5 which results in stimulation of IgE production, AHR and eosinophil differentiation and activation [17]. In the following section, I will discuss in details the link between eosinophils as effector cells in asthma.

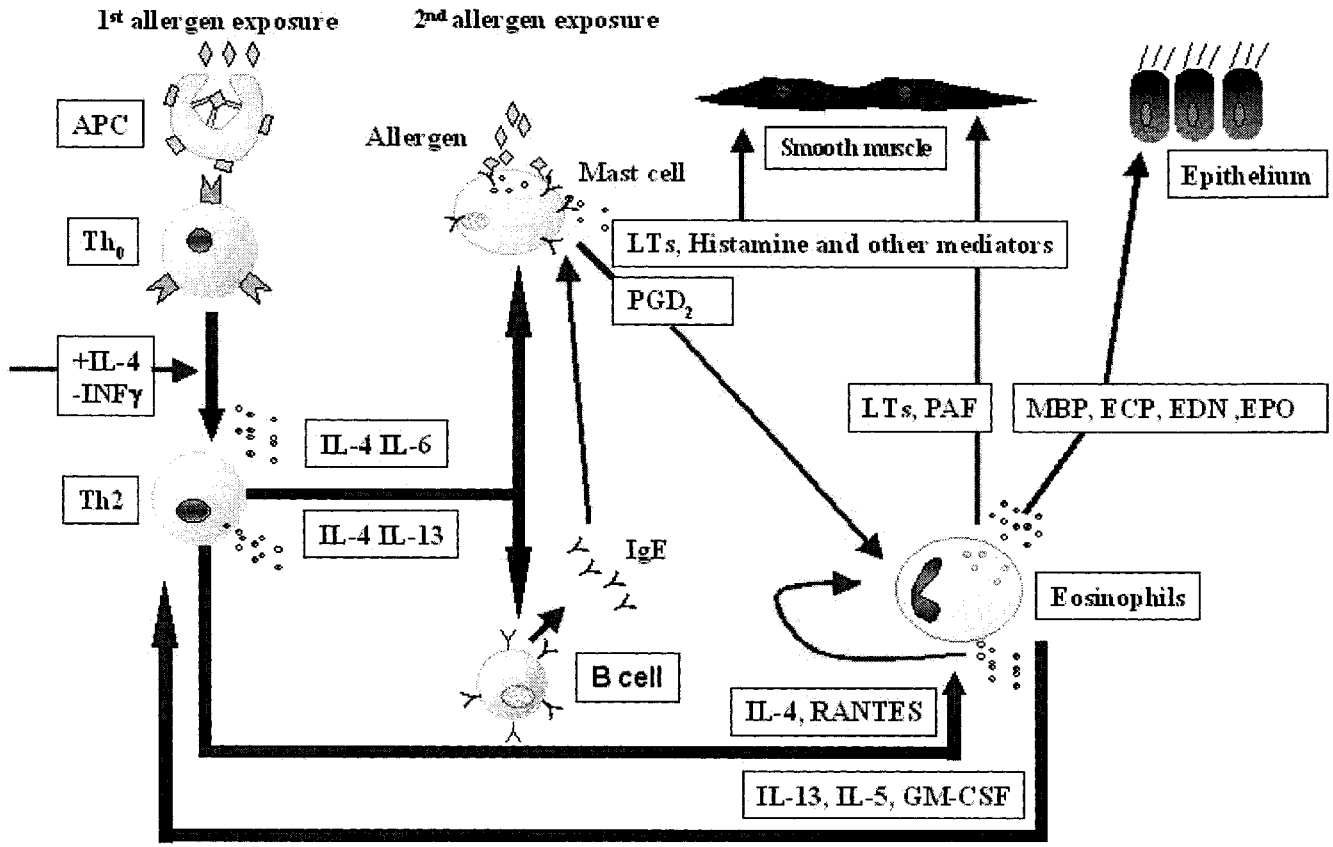

Fig.1. Inflammatory process in asthma. Several inflammatory and strctural cells interact in a complex manner and release multiple inflammatory mediators that act on various target cells in the airways to produce the characteristic pathophysiology of asthma.

1.3. Role of Eosinophils in Asthma

The eosinophil granulocyte is a white blood cell, the major role of which is to destroy invading parasites [68]. The eosinophil has a bilobed nucleus and a cytoplasm filled with characteristic granules, which have strong affinity for the negatively charged dye eosin [69]. In normal situations the eosinophils comprise only 1-5% of the leukocyte population in the circulation, and have a lifetime of about 3-4 days in blood, while in tissue their life span varies from a few days to several weeks, depending on their state of activation and the presence of survival factors such as IL-5, IL-3 and GM-CSF [70].

1.3.1. Eosinophil Cytoplasmic Structure

Distinct populations of granules are present within the eosinophil cytoplasm including primary granules, specific granules, and small granules [69]. Lipid bodies and a variety of vesicular structures are also present. The primary granules are spherical, membrane-bound structures that have a matrix without a core. MBP is diffused in the primary granules and during their maturation it forms a crystal core characteristic of the specific granules. Primary granules are abundant in eosinophil progenitors but rare in mature cells, where they constitute 5% of all granules [71].

The other set of cytoplasmic granular structures is the specific granule that contains a crystal core surrounded by homogeneous matrix. The crystal core consists mostly of MBP [72] in addition to other proteins such as IL-2, IL-4, IL-5,GM CSF [73-75], cathepsin and catalase [76] [77]. On the other hand, the matrix of the specific granules acts as a storage area for a large number of preformed eosinophil mediators, including eosinophil peroxidase (EPO), eosinophil protein X (EPX) and eosinophil cationic protein (ECP) [34]. In addition, the specific granule matrix also contains other important mediators such as IL-6 [78], TNF−α [79], RANTES [80] and proteoglycans

[81]. The third set of cytoplasmic granular structures is the lipid bodies which are considered to be a storage site for arachidonic acid [82] and of enzymes involved in lipid metabolism, such as cyclooxygenase and 5- and 15- lipoxygenase. Lipid bodies characteristically increase in size and number in activated eosinophils [83] [84].

1.3.2. Cell Receptors and Adhesion Molecules

Important elements of the eosinophil are the plasma membrane receptors, through which secretion is mediated or regulated. Among them are receptors for cytokines (e.g. IL-3, IL-5, GM-CSF, TNF-α, [85-88]), immunoglobulins (IgG [89] IgA [90] and IgE [91]) and lipid mediators (PGD_2, 5-oxo-ETE, LTD_4, PGE_2, and PAF [6]). In addition eosinophils express adhesion molecules on their surface including: β1 and β2 integrins [92, 93] , L-selectin [94], intracellular adhesion molecule (ICAM-1) [95, 96] and the counterligands for selectins E and P [97] [98]. Since eosinophil migration is the main focus of this thesis, the role of eosinophil adhesion molecules will be discussed in the following section.

Adhesion receptors are essential for the adherence of eosinophils to the blood vessel endothelium and their subsequent migration into the tissue. In general, eosinophil adhesion receptors can be divided into selectins and integrins [99]. The selectin family are responsible for the preliminary and reversible attachment of eosinophils to endothelial cells [100]. Selectins are characterized by a Ca^{2+}-dependent lectin domain which is able to bind to glycoproteins that contain the carbohydrate structure sialyl Lewis X [100].The selectin family consists of three proteins; E-selectin, P-selectin and L-selectin. L-selectin is expressed on most circulating leukocytes and is constitutively expressed on eosinophils [101]. It is rapidly shed from the cell surface, which may explain the reversible nature of

the binding. L-selectin interacts with MAdCAM-1, CD13 and with the other selectins [100].

Integrins are large heterodimeric proteins composed of $\propto$ and β-subunits [102]. They are usually expressed on cell surfaces and their ligands are preferentially expressed both on endothelial cells and in the extracellular matrix proteins [103]. Eosinophils express the β_1 integrins $\alpha_4\beta_1$ (CD49d/CD29), a heterodimer called very late antigen (VLA)-4, and $\alpha_6\beta_1$ (VLA-6) [104]. These *adhesion molecules* allow eosinophils to interact with the extracellular matrix proteins laminin, fibronectin and fibrinogen [100]. On the other hand, VLA-4 also binds to VCAM-1, which is expressed on endothelial cells and mediates selective eosinophil recruitment [105]. As for the β_2 integrins, eosinophils express $\alpha L\beta_2$ (CD11a/CD18), $\alpha M\beta_2$ (CD11b/CD18), $\alpha X\beta_2$ (CD11c/CD18) and $\alpha D\beta_2$. Their counter-structures are intercellular adhesion molecule (ICAM)-1, -2 and -3 as well as VCAM-1, fibrinogen and the complement fragment C3bi [106-110]. Integrins play a major role in allergic disease because of their involvement in adhesion and transendothelial migration of eosinophils and other leukocytes [111].

1.3.3. Eosinophils in Asthma

The presence of eosinophilic infiltration in the airways has been known for almost 100 years, having first been reported in 1908 in a patient who died of asthma [112]. Since then elevated numbers of eosinophils have consistently been found in bronchial biopsy specimens, BAL fluid, sputum [113] and peripheral blood of asthmatics [114]. Increased numbers of eosinophils have also been noted in bone marrow of atopic patients with asthma [115]. Moreover, progenitor CD 34^+ cells bear the IL-5 receptor with increased responsiveness to IL-5 suggesting they are primed for differentiation

toward eosinophils [116]. Higher levels of the eosinophil activation marker ECP have been reported in BAL fluid [117], sputum, nasal washes [118, 119] and serum [120, 121] from patients with asthma compared to healthy subjects. In addition, eosinophilia in response to allergen challenge is associated with elevated levels of eosinophil-derived cytokines in both the lung and peripheral blood [122].

The occurrence of pulmonary eosinophila has been associated with the development of several aspects of asthma including airway hyperresponsiveness, epithelial damage, and airway remodelling [123, 124]. Eosinophils promote allergic inflammation, at least in part, via the release of a series of mediators that not only have proinflammatory effects but also act as growth factors, stimulants, and chemoattractants [125]. Among eosinophil mediators are GM-CSF, TGF-β_1, TNF-γ, IL -3, IL-4, IL-5, IL-6, IL-10, IL-11, IL -12, IL-13, IFN-γ, RANTES and eotaxin [126]. In addition to these mediators, eosinophils release granule proteins, including MBP, ECP and EDN [127]. The presence of eosinophils and their mediators can contribute to asthma by different effects including damage to the lung epithelium [3], augmentation of the inflammatory process and the process of remodelling [4].

Many studies provide support for a link between eosinophils and AHR in asthmatics. A study in IL-5-deficient transgenic mice showed that these animals failed to develop AHR and eosinophilia after allergen challenge [128]. Consistent with this, anti–IL-5 almost completely abrogated eosinophilia and airway hyperresponsiveness in an allergen challenged monkey model [129]. Another study by Justice *et al.*[130], in which airway, tissue and blood eosinophils were specifically depleted using a monoclonal antibody against CCR3 provides further support for this notion. In this study, the depletion of virtually all pulmonary eosinophils in OVA-treated mice led to a significant reduction of AHR following allergen challenge. On the other, a number

of recent studies[131] showed that eosinophils are not essential for airway hyperresponsiveness in asthma, the findings of these studies will be reviewed in the discussion section.

Despite the reported association between the presence of eosinophils and AHR, the precise mechanisms by which eosinophils influence AHR are not well defined. PAF-activated eosinophils cause contraction of human airway smooth muscle predominantly, but not solely, through the release of leukotrienes [132]. It has also been reported that supernatants from activated eosinophils causes hyperresponsiveness of airway smooth muscle in guinea pig trachea. This effect was attributed to the leukotrienes released from eosinophils [133]. Another study has demonstrated that eosinophil-drived MBP causes contraction of guinea pig airways through an epithelium-dependent mechanism [134]. Furthermore, intraepithelial administration of MBP augments the contraction of underlying canine tracheal smooth muscle elicited by acetylcholine [135]. Another possible explanation for the involvement of eosinophils in hyperresponssivness is its capacity to secrete IL-13 [136], which has potent spasmogenic properties [137].

Eosinophils can influence airway function by producing effects on airway remodelling. It has been shown that TGF-β_1 mRNA is overexpressed in severe asthmatics and that the main source of the mRNA is eosinophils [31]. Phipps *et al.* [138], using an allergen-induced skin model of asthmatic inflammation, showed that the release of transforming growth factor-β and IL-13 by eosinophils contributes to remodelling in allergic inflammation in human atopic skin. It also has been shown that eosinophils contribute to epithelial damage and loss particularly via their highly basic granule proteins – MBP, ECPs and eosinophil peroxidase [139]. In addition, many

other molecules secreted by eosinophils have been implicated in the remodelling processes including TGF-α, FGF-2, VEGF, TIMP-1, and LTD_4 [34].

In asthma there is a strong association between the two main effector cells eosinophils and Th2 cells. In both humans and rodent models, allergen-induced recruitment of eosinophils into the lung is correlated to $CD4^+$ T cells and their cytokines [126]. Studies showing expression of major histocompatibility complex class II (MHCII) and the T cell costimulatory molecules CD80 and CD86 by eosinophils from allergic lungs suggesting that these cells can directly communicate with helper T cells to regulate the immune response [140] [141]. This notion was confirmed in a study by Mackenzie et al. where they showed that eosinophils have a role in presenting allergen to stimulate $CD4^+$ T-helper cells [142]. In this study, eosinophils from the allergic lung rapidly internalized and processed Ag that was sampled from within the airway lumen. Ag-loaded eosinophils promoted the production of IL-4, IL-5, and IL-13 in cocultures with in vitro-polarized Th2 cells and induced IL-5 production from Ag-specific $CD4^+$ T cells isolated from allergic mice. Another recent study by Shi et al., further confirmed the above findings and demonstrated that antigen exposed airway eosinophils function both in vitro and in vivo as APCs to promote the secretion of Th2 cytokines [126]. These findings highlight the potential role of eosinophils to actively modulate immune responses by amplifying Th2 cell responses.

There are appealing data that attribute the affect of anti-asthmatic drugs at least in part to their effect on eosinophils. It has been shown that treatment of children with inhaled corticosteroids is associated with a significant decrease in the number of eosinophils and levels of serum eosinophil cationic proteins (sECP) [143] [144]. Corticosteroid treatment reduces the survival of eosinophils possibly by reducing the

levels of the eosinophil survival factors IL-5 and GM-CSF [145]. Recently, measurement of eosinophil proteins has been used to assess inflammatory status. A number of investigators have reported on the usefulness of measuring serum levels of ECP proteins in assessing airway inflammation and the efficacy of anti-inflammatory treatment in asthmatic children [146]. Eosinophil protein X is the only one of the four basic eosinophil granule proteins that can be accurately measured in urine and is most commonly used to investigate eosinophil activation and inflammation in children with asthma [147].

1.4. Lung Eosinophilia

In response to allergic stimuli in the lung, eosinophils are mobilized from the bone marrow and traffic to the airways. The migration of eosinophils into tissues is a multi-stage process involving interaction between eosinophils, endothelial cells and wide array of mediators [148]. This multi-stage process includes proliferation of eosinophils in the bone marrow, priming of the eosinophils, rolling along the endothelial cells, firm adhesion to the endothelium, trans-endothelial diapedesis and chemotaxis into the inflammatory site [149].

1.4.1. Proliferation and differentiation

Eosinophils are produced in the bone marrow from $CD34^{+}$/IL-5R pluripoten stem cells [150]. It also has been shown that the differentiation process can occurs at the tissue level in response to antigen challenge [150]. The commitment of multipotent cells to the eosinophil lineage is regulated principally by IL-3, IL-5 and GM-CSF [151], and possibly also eotaxin [152]. Of these cytokines, interleukin-5 (also known as eosinophil-differentiation factor) is the most specific for the eosinophil lineage and

is accountable for selective differentiation of eosinophils [149]. Interleukin-5 also stimulates the release of eosinophils from bone marrow into the peripheral circulation [153].The mature eosinophils enter the blood stream, where they remain for about 25 hours before migrating into the tissue although this appears to be shorter in pathological conditions [154].

1.4.2. Eosinophil Priming

Recruitment of cells into tissue including the airways wall is associated with their priming and activation [155]. Eosinophils from patients with asthma exhibit increased migratory responses [156, 157], adhesiveness [158, 159] and degranulation [160] compared with eosinophils from normal subjects. The priming process is dependent on cytokines such as IL-5 [161], GM-CSF and IL-3 [162, 163]. Chemokines such as RANTES [164] and eotaxin [165] also act on eosinophils to enhance markedly their recruitment . One mechanism underlying this change in responsiveness may be the induction of receptors on the cell surface [166].

1.4.3. Rolling

The initial reversible contact (rolling) of circulating eosinophils with the blood vessel wall prior to extravasation is mediated by selectins. The expression of E-and P selectins on the endothelial cells can be up-regulated by IL-1 and TNF-α, while L-selectin is constantly expressed on eosinophils [102, 167]. The reversible adhesion between the selectins and their ligands makes the eosinophils move slowly along the vascular endothelium. This permits interaction of integrins on eosinophils with their ligands on endothelial wall. During rolling the eosinophils become activated and the expression of integrins is upregulated, resulting in firm adhesion [102, 168]. The

molecules that are most important for firm adhesion of eosinophils to endothelial cells are CD11b/CD18 and VLA-4 on the eosinophils, and their ligands ICAM-1 and VCAM-1 on the endothelial cells. Expression of these adhesion molecules is stimulated by cytokines. For example, IL-1 and TNF-α induce ICAM-1 and VCAM-1 expression on endothelial cells. On the other hand IL-4 is a selective inducer of VCAM-1 while IFN-γ is a selective inducer of ICAM-1 [169].

1.4.4. Eosinophil Diapedesis and Chemotaxis

Eosinophils from the blood of normal individuals can adhere to IL-1- or TNF-α activated endothelial cells, but they are unable to migrate through this layer. In contrast, eosinophils from allergic or asthmatic individuals not only adhere to the endothelial cells, but also pass spontaneously through the cell layer. This capacity to transmigrate can be induced by pretreatment of eosinophils from normal individuals with IL-3, IL-5 or GM-CSF [170]. PAF and C5a are also involved in the transmigration of eosinophils across endothelial cells [171, 172]. Thus, priming of the eosinophils is a prerequisite for diapedesis. The expression of VCAM-1 and ICAM-1 on the endothelial cell is also necessary for eosinophil transmigration. After getting out of the blood vessels, eosinophils move into the targeted tissue in a process referred to as chemotaxis.

Chemotaxis is the movement of a cell in the direction of a chemoattractant gradient, and is characterized by adhesion/de-adhesion to extracellular matrix proteins. In response to a chemoattractant, the concentration of $Ca2^{+}$ in eosinophils rises and the cell undergoes polarization. A flat extension of the cytoplasm, called a lamellipod, is formed at the front of the moving cell, and a tail called an uropod is formed at the rear [158, 171]. The eosinophil moves by extending the lamellipod that

adheres to the substrate, and constricting the uropod while de-adhering from the substrate [158]. On the other hand, actin polymerisation and depolymerization supply the eosinophil with contractile forces. These forces are probably induced by phosphorylated myosin II interacting with actin filaments [173].

At the time this study was initiated, several factors were known to have chemotactic effect on eosinophils including PAF, complement factor C5a and 5-oxo-ETE [174]. All of these mediators, however, also act on neutrophils. On the other hand, IL-3, IL-5 and GM-CSF had been recognized as activators of eosinophil function, including migration [175]. Chemokines such as RANTES and eotaxin had also been found to be eosinophil chemoattractants [176]. More recently attention has been given to the role of PD2 as a chemotactic agent for eosinophils. The chemotactic effect of PGD_2 on eosinophils was first reported in dogs in a study by Emery et al. in 1988 [177]. This was followed recently in a study by Monneret et al. which showed that PGD_2 acting via the novel DP_2 receptor is a potent chemoattractant for human eosinophils in vitro [9]. The major goal of my thesis is determine whether PGD_2 can elicit eosinophil infiltration into the lungs and if so, to examine the nature of the receptors responsible for this effect.

1.5. Eicosanoids

Leukotrienes (LTs), and prostanoids belong to one family of lipid mediators known as eicosanoids [178]. These mediators play major roles in inflammation and have been implicated in the pathogenesis of many diseases, including asthma [179, 180]. Eicosanoids are formed mainly from arachidonic acid which is liberated from cell membrane phospholipids by phospholipases (PL) A_2 [181, 182]. Arachidonic acid is metabolized by two main pathways, namely the 5-lipoxygenase (5-LO) pathways,

which leads to the synthesis of leukotrienes and 5-oxo-ETE, and the cyclooxygenase pathway, which leads to the synthesis of PGs and thromboxanes [183].

1.5.1. 5-Lipoxygenase Pathways

5-LO converts AA into 5-HPETE [184], which is converted into a 5,6-epoxide, LTA_4, by the same enzyme [185]. LTA_4 is preferentially hydrolyzed to LTB_4 and LTC_4 by LTA_4 hydrolase and, LTC_4 synthase respectively. LTC_4 in turn is converted extracellularly to LTD_4 by transpeptidase and then to LTE_4 [186]. The effects of the cysteinyl leukotrienes (cys-LTs) are mediated via G protein-coupled receptors. In human lung distinct cys-LTs receptors appear to be present in bronchial smooth muscle and pulmonary veins [187]. The level of cys-LTs in BAL of asthmatics are elevated compared to non-asthmatics [188]. There is now substantial evidence that the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) play an important role in the pathophysiology of asthma [180]. Cys-LTs are potent in eliciting bronchoconstriction [189], increased endothelial membrane permeability leading to airway oedema, enhanced secretion of mucus [190], and may also increase bronchial hyperresponsiveness [191]. Additionally, cys-LTs may induce remodelling because they increase proliferation of airway smooth muscle [192] and airway epithelial cells [193]. LTC_4 in particular was shown to upregulate collagenase expression in human lung fibroblasts [194]. cys-LTs may also be involved indirectly in eosinophils recruitment because inhalation of LTE_4 [195] or LTD_4 [196] induces the recruitment of eosinophils in the airways. Interestingly, the cys-LTs are produced by cells believed to contribute to the pathogenesis of asthma including eosinophils, mast cells, basophils, Th2 and macrophages [197]. This ability of eosinophils to produce cys-LTs further substantiates their important role in asthma. In addition to cys-LT, the newly

identified class of AA metabolites the 5-oxo-ETE is formed from 5S-HETE by specific dehydrogenase through the 5-LO pathway [198]. This compound acts through a specific G-protein–coupled receptor and has been shown to be a potent stimulator of human eosinophil migration both in vitro and inviov[199] [200].

1.5.2. Cyclooxygenases pathway

Metabolism of arachidonic acid by two forms of cyclooxygenase leads to the synthesis of prostanoids which consist of the prostaglandins (PGs) and the thromboxanes (TXs) [201]. The first step in this pathway is the conversion of AA to PGH_2 by the cyclooxygenase enzymes [202]. PGH_2 can then be converted by cell-specific prostaglandin synthases into a series of PGs, including PGD_2 [203]. The two forms of cyclooxygenases involved in this pathway are COX-1 and COX-2. COX-1 is constitutive and is responsible for the basal release of prostanoids, whereas COX-2 is inducible by inflammatory stimuli such as proinflammatory cytokines [204]. Both cyclooxygenase isozymes are expressed in most tissues [205], including human airway epithelial cells which basally express COX-1, whereas COX-2 is induced by IL-1β and TNF-α [206, 207] and is enhanced by NO [207].

COX-1 in particular has been linked to a subgroup of asthmatics who have Aspirin-sensitive asthma. In this group of patients, aspirin and related drugs elicit asthma symptoms. It is thought this is due to inhibition of the formation of PGE_2 which has anti-inflammatory effects in asthma, and can suppress LTC_4 formation [208]. Clinical studies have demonstrated that selective inhibition of COX-2 does not induce airway narrowing in aspirin-sensitive asthmatics [208]. Indeed, it has been suggested that COX-2 may play a protective role in asthma, since COX-2 induction inhibits proliferation of human airway smooth muscle cells [208]. The major end

products of the cyclooxygenases pathway, the PGs, signal a wide variety of events through several G protein-coupled receptors [209]. These receptors have been named according to the natural prostanoid for which they have the greatest affinity. They include TP, IP, FP, and DP_1/DP_2 receptors, and four subtypes of PGE receptors, named EP_1, EP_2, EP_3 and EP_4, which all activate second messengers, such as cyclic adenosine monophosphate (cAMP) [210, 211]. In the following section I will focus on PGD_2. A sketch representing the major products of both 5-LO and cyclogenase pathway is presented in Fig 2.

1.5.3. PGD_2

Prostaglandin D_2 (PGD_2) is a prostanoid derived from arachidonic acid via the action of COX-1 and COX-2 and specific PGD synthases [211]. Prostaglandin (PG) D synthase catalyzes the isomerization of the 9,11-endoperoxide group of PGH_2, to produce PGD_2 with 9-hydroxy and 11-keto groups, in the presence of sulfhydryl compounds [212]. There are two PGD_2 synthases, namely lipocalin-type PGD synthase (L-PGDS) [213] and hematopoietic PGDS (H-PGDS) [214]. L-PGDS is expressed by meningeal cells, epithelial cells of the choroid plexus, and oligodendrocytes in the brain and by epithelial cells of the epididymis and Leydig cells in the testis [215]. In contrast, H-PGDS is responsible for the biosynthesis of PGD_2 by immune and inflammatory cells such as mast cells [8], antigen-presenting cells [216], and Th2 cells [217]. PGD_2 biosynthesis has been demonstrated in various tissues including brain, spleen, lung, bone marrow, stomach, and skin [218]. The major pathway for metabolism and degradation of PGD_2 is shown in Fig. 3.

PGD_2 exerts its action through two plasma membrane G-protein coupled receptors, the DP_1 which is coupled to $G_{\alpha s}$ protein [219], and the recently identified DP_2

receptor also known as chemoattractant receptor-homologous molecule expressed on Th2 cells ($CRTH_2$) which is coupled to $G_{\alpha i}$ protein [9, 220]. At higher concentrations PGD_2, can activate other prostanoid receptors, notably the TP receptor, resulting in airway constriction [221]. PGD_2 has various effects in mammals such as promotion of sleep, nerve cell function, hormone release, inhibition platelets aggregation and vasodilatation [222, 223].

1.5.3.a. PGD_2 in Asthma

There is evidence that PGD_2 may be an important mediator in allergic asthma. H-PGDS is abundant in mast cells [8], dendritic cells [216], and certain subpopulations of Th2 lymphocytes [217], all of which play critical roles in this asthma [1]. Furthermore, PGD_2 is rapidly released in large amounts from major cells following allergen challenge of asthmatic subjects [7].

A number of studies have been done to examine the role of PGD2 in asthma, and the results suggest that this compound may be an important proinfilmatory mediator in this disease. It has been known for some time that PGD_2 induces bronchoconstriction [224]. However, relatively high doses are required for this response which appears to be mediated by the interaction of PGD_2 with TP receptors as it is blocked by selective TP antagonists [225, 226].

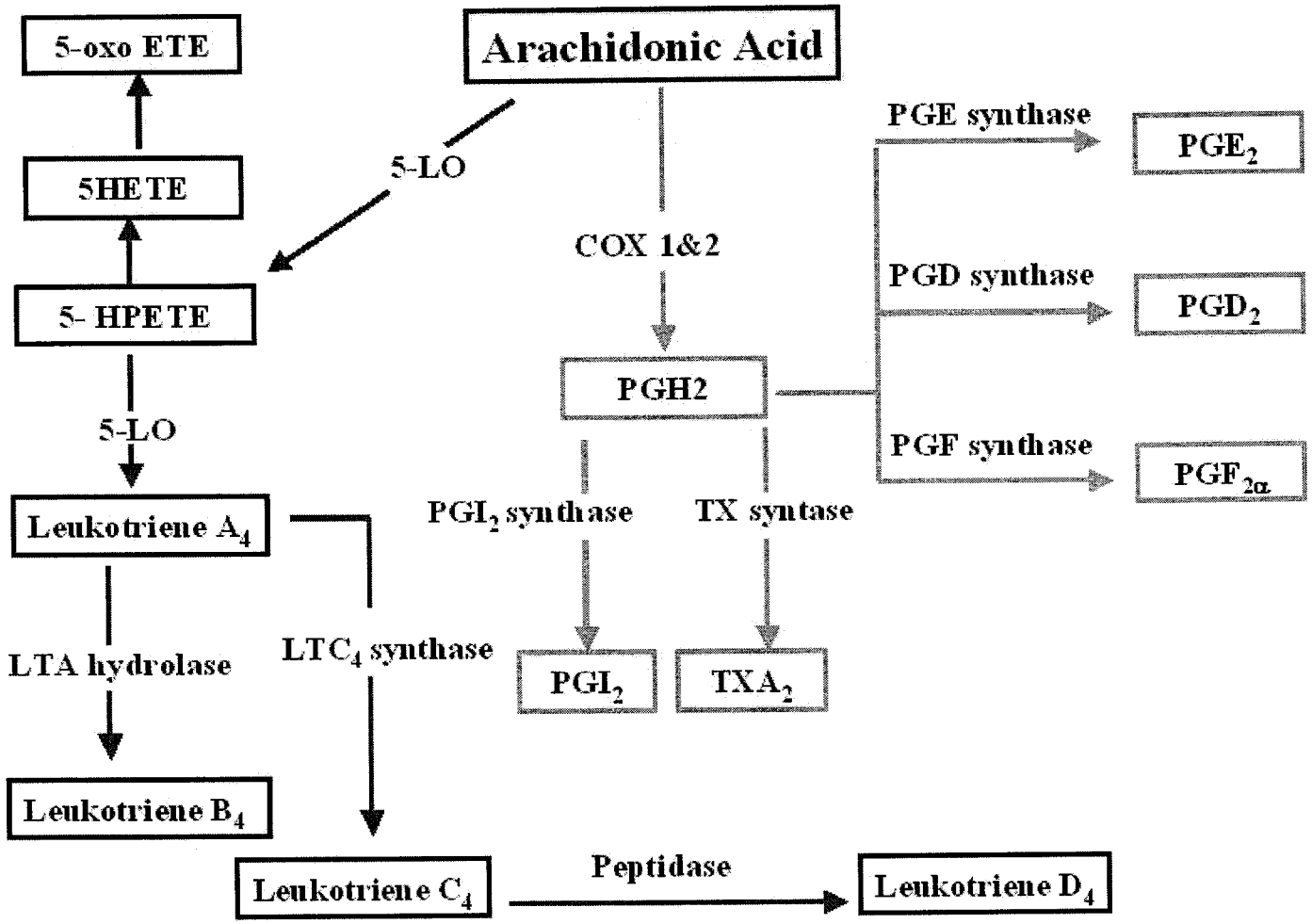

Figure 2. Generation of eicosanoids in the arachidonic acid cascade.Through the actions of the COX and 5-LO enzymes eicosanoids are formed .

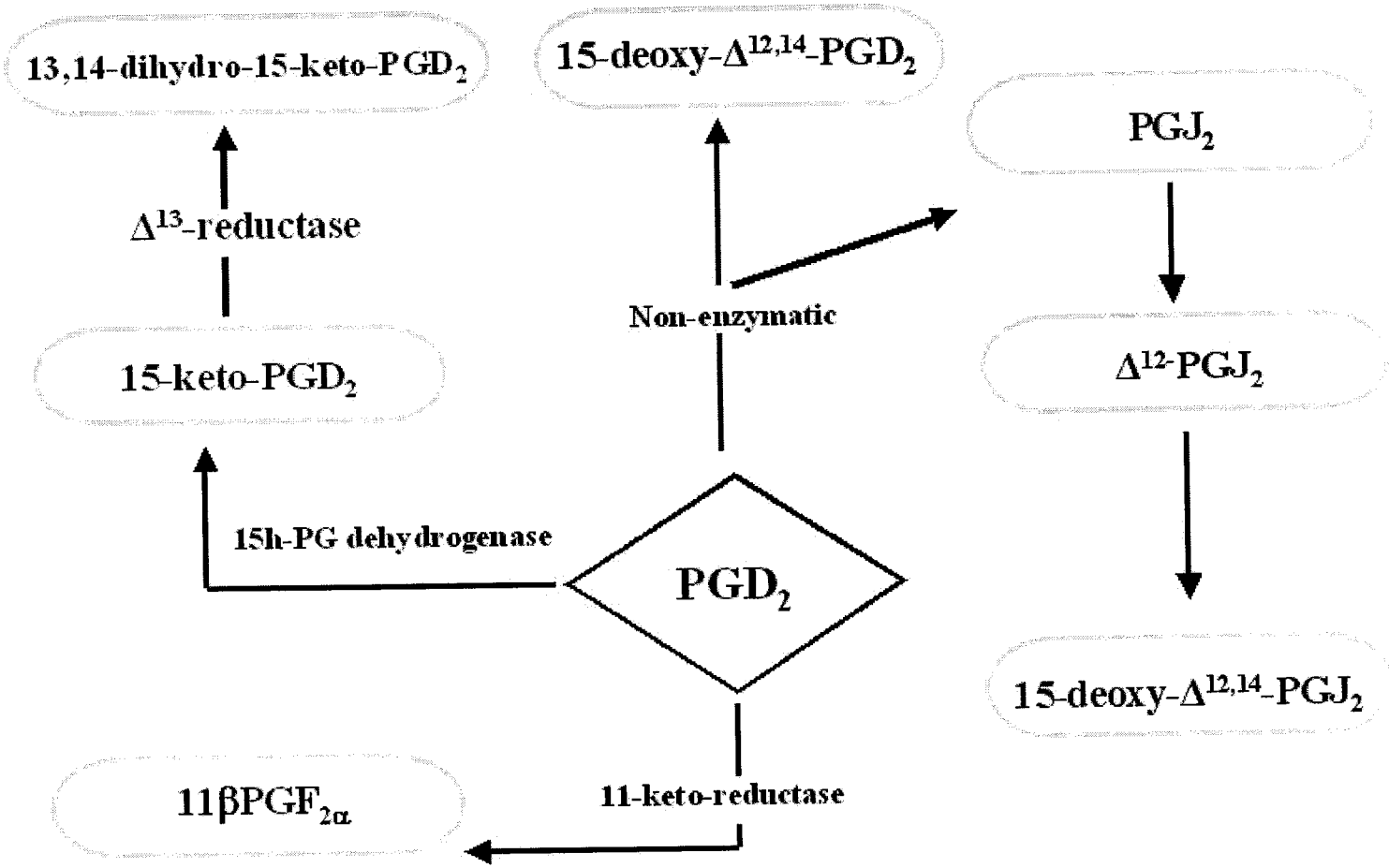

Figure. 3. The major pathway for metabolism and degradation of PGD_2.

IV administration of PGD_2 in guinea pigs also provokes airway wall thickening by inducing edema and enhancing bronchial smooth muscle constriction in response to histamine. This effect effect was also inhibited by TXA_2 antagonists [227]. The important role of PGD_2 in asthma was further supported by Fujitani et al. [228], who generated transgenic mice overexpressing the lipocalin-like PGD synthase in the lung, and subjected them to OVA-induced pulmonary allergic inflammation. They found increased IL-4 and IL-5 concentrations and enhanced eosinophilic numbers in BAL fluid in these mice.

Whereas the above studies suggest an important role of PGD_2 in the pathogenesis of asthma, there is a report suggesting that PGD_2 works to suppress mast cell activation. Chan et al.[229] found PGD_2 agonists exerted inhibition of histamine release form rat mast cells and this effect may be mediated by the DP_1 receptor since it was shared by the selective DP_1 agonist BW245C. Consistent with these findings, PGD_2 was demonstrated to have anti-inflammatory properties in rat pleurisy and colitis models possibly via PPARγ and/or the DP1 receptor [230] and [231].

As discussed above, eosinophilic infiltration is a characteristics feature of asthma. A handful studies have been conducted to investigate the possible role of PGD_2 on eosinophil infiltration. One of the earliest is a study by Emery et al. who showed that application of PGD_2 to the surface of dog trachea, using an endotracheal tube with two inflatable balloons, induced accumulation of eosinophils in the lumen [177] . Another group demonstrated that intravenous infusion of PGD_2 causes a rapid reduction in circulating eosinophils in dogs, presumably by promoting their adherence or migration through the endothelium [232].

Other animal models further highlighted a proinflamatory role for PGD_2. In the guinea pig, topical application of PGD_2 induced eosinophil infiltration into the conjunctiva. It seemed unlikely that this was mediated by the DP_1 receptor, since it was not shared by the DP_1 agonist BW245C [233]. An in vitro study by Monneret et al. recently showed that PGD_2 is a potent chemoattractant for human eosinophils. This effect was not mediated by the DP_1 receptor, as it was not shared by the selective DP_1 receptor agonist BW245C and was not prevented by the selective DP_1 receptor antagonist BWA868C [9]. Another study by Gervais et al., demonstrated the ability of PGD2 to induce a rapid change in human eosinophil morphology and increase eosinophil chemokinesis [11]. These results all support a role for inducing tissue eosinophilia.

Beside its effect on eosinophil migration, PGD_2 also induces a variety of other responses in eosinophils. Raible et al., first showed that PGD_2 increases cytosolic calcium in eosinophils and enhances the release of LTC_4 [234]. Then Monneret et al., showed that PGD_2 is a potent stimulator of actin polymerization and CD11b expression in these cells [9]. It was also demonstrated that PGD_2 induces eosinophil degranulation and that this effect was most likely to be mediated by the DP_2 receptor since it was reproduced by the selective DP_2 agonist dhk-PGD_2 but not by the selective DP_1 agonist BW245C [11].

1.5.4. DP_1 Receptor

PGD_2 elicits its biological actions mainly through interaction with the DP_1 and DP_2 receptors. The DP_1 is localized in the plasma membrane and is a member of the G-protein coupled receptor family. It coupled to $G_{\alpha s}$ and acts by activating adenylcyclase [219]. This receptor shares the highest sequence homology with the other prostanoid

receptors, TP, FP, EP_{1-4} and IP [220]. The DP_1 receptor has been cloned thus far from mouse [235] , human [221] and rat [236]. The identity of the amino acid sequence is 90% between the mouse and the rat receptors, 73% between the mouse and human receptors, and 70% between the rat and human receptors [237]. In mice, the DP_1 receptor is expressed moderately in the ileum and weakly in the lung, stomach, and uterus [235]. Consistently, only low levels of expression were detected in humans [221]. In relation to our current study, Monneret et al. were able to show for the first time that it is also present on human eosinophils [9]. In addition, immunoelectron microscopy using an antibody to mouse DP revealed that the DP receptor is highly expressed in ciliated and non-ciliated epithelial cells of the bronchioles and type II alveolar epithelial cells. Moderate expression was also observed in the type-I alveolar epithelial cells and inflammatory white blood cells [10]. More recently, Nantel et al., showed that the DP_1 receptor is present in the nasal mucosa and detected the DP_1 receptor mRNA in epithelial goblet cells, serous glands and in the vasculature[238].

Several selective DP_1 agonists and antagonists have been developed. Agonists include BW245C [239], and L-644,698 [240] whereas BWA868C [239] and S-5751 [241] are DP_1 antagonists. Although, the mouse DP_1 receptor showed different affinities for PGD_2, BWA868C and BW245C in the order of PGD_2 > BWA868C, BW245C [211], the cloned human DP_1 receptor showed almost equal ligand binding affinities for these compounds [221]. Through the activation of the DP_1 receptor, PGD_2 has been implicated in different physiological events including sleep induction, mucus production, cell survival, control of intraocular pressure and allergic responses [10, 11, 236, 242, 243].

1.5.4.a. Role of DP_1 receptor in inflammation

The DP_1 receptor have been thought to have inhibitory or relaxant effects on many cell types [211] and [220]. This is typically shown by the inhibitory effect of PGD_2 on platelet activation and its vasodilatory effects. With regard to leukocytes, signals from the DP_1 receptor have inhibitory effects on the migration of DP-transfected Jurkat cells, eosinophils, and dendritic cells [220], [9] and [230] . DP_1 signals also inhibit fibroblast migration [244] . Consistent with these findings, PGD_2 was demonstrated to have anti-inflammatory properties in colitis models possibly via the DP_1 receptor, since this anti-inflammatory effect was shared by the agonist BW245C [245]. Moreover, there is a report suggesting that DP_1 might be involved in suppression of mast cell activation. Chan et al. [229] found that the DP_1 agonist BW245C exerted strong inhibition of histamine release from rat mast cells.

In contrast to these inhibitory effects, it was reported that signals from the DP_1 receptor prolong the survival of eosinophils and induce mucin secretion in a colonic adenocarcinoma cell line [11] and [246]. Moreover, in mice, DP_1 deficiency was associated with a significant reduction of allergic inflammation in an animal model of asthma. Matsuoka et al. [10] examined DP receptor-deficient mice in a model of ovalbumin (OVA)-induced allergic inflammation. The loss of the DP_1 receptor caused a significant reduction of Th2 cytokines, including IL-4, IL-5 and IL-13. Lymphocyte and eosinophil accumulation in BAL fluid was also significantly reduced in the knockout mice. In contrast to wild-type mice, few mucus-containing cells were detected in the airways of DP-deficient mice. Moreover, whereas OVA challenge significantly increased airway sensitivity to acetylcholine in wild-type mice, little

increase was detected in DP-deficient animals. Thus, PGD_2 appears to act on the DP_1 receptor to plays an important role in elicitation of certain key features of allergic asthma. This presumption was confirmed by other experiments. Firstly, Arimura et al. [241] administered the DP_1 antagonist S-5751 orally to guinea pigs subjected to the same model of OVA-induced allergic asthma, and found that it significantly reduced eosinophil infiltration into the lung. Secondly, although only moderate expression of DP_1 was detected in the lungs of non-immunized mice, OVA challenge to the airway markedly enhanced the expression of this receptor [10].

1.5.5. DP_2/CRHT2 Receptor

For some time, it was generally considered that most of the biological actions of PGD_2 are mediated through the classical DP receptor [211]. However, in 2001, two independent studies identified a second receptor for PGD_2. First Hirai et al. identified $CRTH_2$ as a novel PGD_2 receptor [220]. In the same year, Monneret et al., using a pharmacological approach identified a second PGD_2 receptor on eosinophils, and named it the DP_2 receptor to distinguish it from classical DP_1 receptor [9]. $CRTH_2$ and the DP_2 receptor are identical to one another.

The DP_2 receptor is a seven-transmembrane G protein–coupled receptor structurally related to members of the *N*-formyl peptide receptor (FPR) family [247]. DP_2 has no significant homology in amino acid sequence with the DP_1 receptor or any of the other known prostanoid receptors [211]. Interestingly, the highest amino acid sequence identity for the DP_2 receptor is found within members of the leukocyte chemoattractant receptor subfamily [248].The relationship among eicosanoid receptors in term of their amino acid sequences is shown in fig.4 . Apart from PGD_2 this receptor does not display high affinity binding to other eicsanoids, chemokines, or

chemoattractants [247]. Expression studies in mice [249], rats [250], and humans [251] revealed DP_2 receptor expression in many tissues including lung, thymus, liver, spleen and brain. This wide tissue distribution, may be due to its localization in leukocytes, as it has been shown to be largely expressed by Th2 cells [252]. This receptor is expressed in Th2 cells [247], basophils [202] and eosinophils [11]. Genetic studies shows that the overall identity between the human and rat DP_2 sequences is 75.6%, whereas it is 89.1% between the mouse and rat sequences [250]. It has also been shown that the mouse homologue is similar to human DP_2 with regard to ligand specificity, signalling pathways, and biological functions [253]. One major difference between the DP_2 and DP_1 receptors is the binding of DP_2 to $G_{\alpha i}$ as opposed to $G_{\alpha s}$ that used by DP_1 [250].

Several selective DP_2 receptor agonists have been identified including 15R-methyl PGD_2, 13, 14-dihydro-15-keto PGD_2 and 15-deoxy-$^{\Delta 12,14}$-PGD_2 [11]. At present, no DP_2-selective antagonist is available except for the anti-DP_2 monoclonal antibody BM7 [220]. Although, Ramatroban was first reported as a selective TP receptor antagonist, it was subsequently shown to also act as an antagonist for the DP_2 receptors [254].

As selective DP_2 ligands have only recently been identified, little is known about the physiological role of this receptor. However, activation of this receptor in leukocytes induces a variety of responses. For instance, in Th2 cells activation of the DP_2 receptor induces chemotaxis and/or chemokinesis and up-regulation of CD40 ligand [220]. In eosinophils activation of this receptor induces chemotaxis [11], eosinophil degranulation [11], up-regulation of CD11b expression [9], actin polymerization, L-selectin shedding, cell shape change, and mediator release [9] [11].

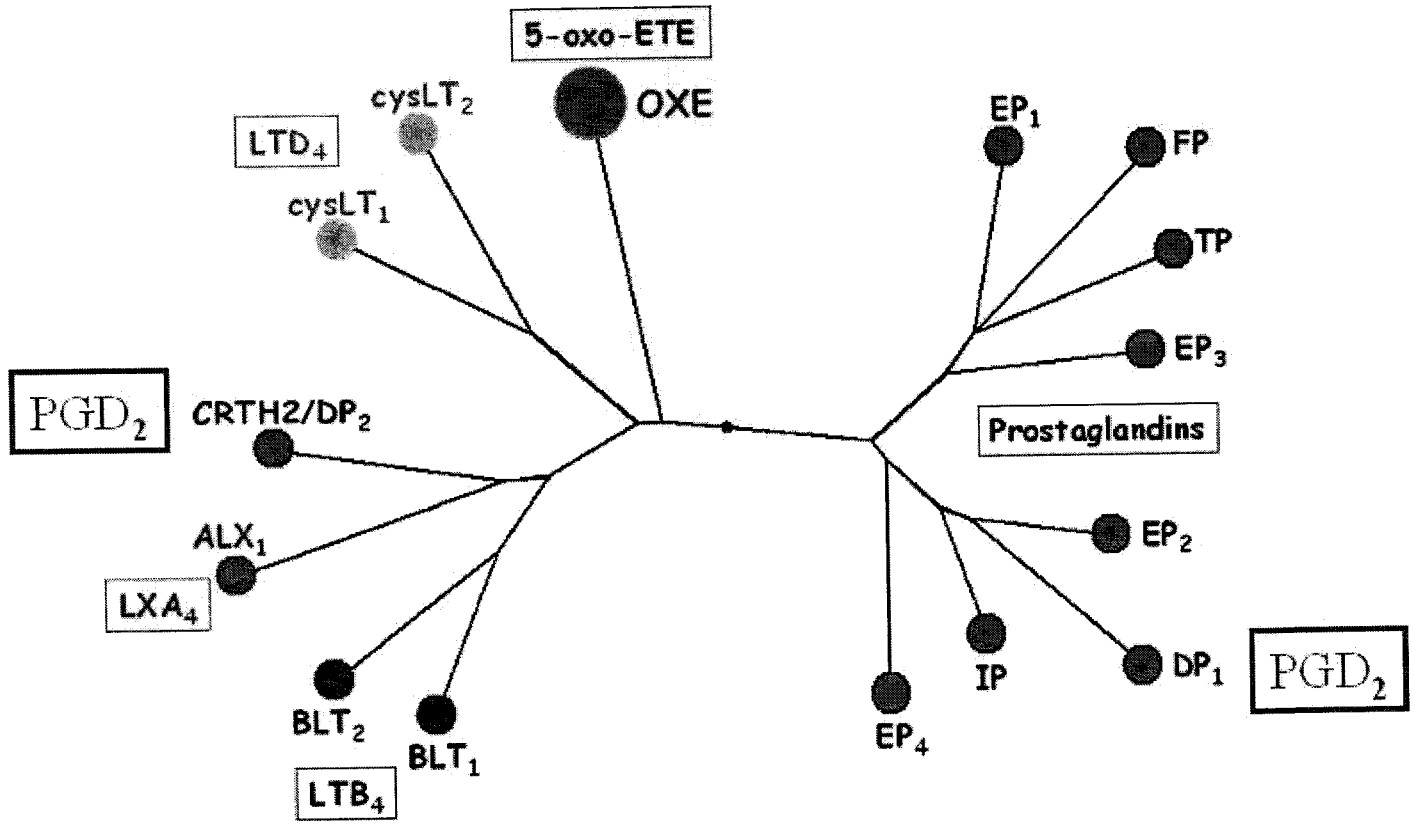

Figure 4. The relationship among eicosanoid receptors in terms of their amino acid sequence. Modified from Brink et al, Pharmacol Rev, 2004.

Similar effects were observed using transfected cell lines. It has been shown that DP_2-transfected Jurkat cells can migrate along a gradient of PGD_2, whereas DP_1 transfected Jurkat cells cannot [220]. It has been suggested that DP_1 and DP_2 co-operatively contribute to the development of allergic inflammation [220] [11].

1.5.6. 15d-$\Delta^{12,14}$-PGJ_2 And Its receptors

One of the compounds that were tested in the current study is 15d-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2). 15-d-PGJ_2 belongs to the J series of prostaglandins and is a degradation product of PGD_2 [203]. PGD_2 was shown to be dehydrated to PGJ_2 in aqueous solution [255] and is converted to 15deoxy-$\Delta^{12,14}$-PGD_2 (15d-PGD_2), Δ^{12}-PGJ_2, and 15-d-PGJ_2 in the presence of serum albumin [256] [257].

15d-PGJ_2 has been shown to have anti-inflammatory effects mediated by its stimulatory effect on peroxisome proliferator-activated receptorγ (PPARγ) [258]. However, more recent evidence has shown that there are also effects of 15d-PGJ_2 that are independent of PPARγ activation [259-261] . For instance, Vaidya and colleagues have shown that 15d-PGJ_2 can inhibit the production of oxygen free radicals by neutrophils in a PPARγ-independent manner [260]. Moreover, 15d-PGJ_2 induces apoptosis via a novel mechanism involving reactive oxygen species that is unrelated to activation of PPARγ in hepatic myofibroblasts [262]. Some of these effects may be mediated by inhibition of NF-κB. NF-κB is a transcription factor that exists in virtually all cell types in the cytoplasm in an inactive form because of its binding to IκB. The activation of NF-κB by various stimuli leads to phosphorylation of I-κB resulting in release of NF-κB from IκB and its subsequent translocation to the nucleus where it can upregulate transcription of specific proinflammatory genes [263]

15d-PGJ_2 has been shown to form covalent conjugates with IκB kinase and a subunit of NF-κB, as well as a number of other proteins, resulting in loss of their function. This is due to its reactive cyclopentenone structure, which readily reacts with protein thiol group (fig5). A recent study by Monneret et al. showed that 15d-PGJ_2 is DP_2 receptor agonist and can there by activate human eosinophils in vitro [264]. This proinflammatory effect is observed at low nanomolar concentrations in contrast to the μmolar concentrations required for its anti-inflammatory effects. One of the goals of the current study was to determine whether 15d-PGJ_2 can exert proinflammatory effects in vivo by inducing pulmonary eosinophilia.

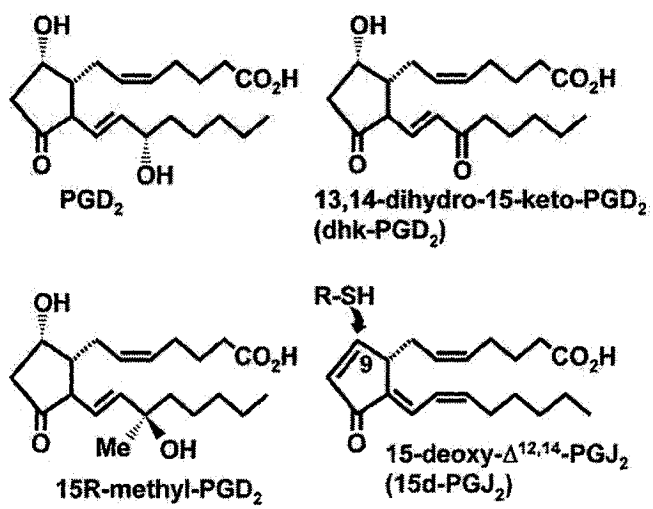

Figure 5. Structures of PGD_2 and selective DP_2 receptor agonists. Because of its reactive cyclopentenone structure 15d-PGJ_2 reacts with protein thiol groups to give conjugates at carbon-9.

1.5.6.a. PPARγ Receptor

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors [265]. PPARs promote the transcription of target genes by forming heterodimers with retinoid X receptors (RXR) and binding to specific motifs in the promoter regions of target genes termed PPAR-responsive elements (PPRE) [266]. Three receptor subtypes of PPARs, designated PPARα, PPARγ, and PPARδ have been identified [267]. Moreover, three mRNA isoforms of PPARγ have been detected in humans (termed $PPAR\gamma_1$, $PPAR\gamma_2$, and $PPAR\gamma_3$) [268] [269]. $PPAR\gamma_1$ and $PPAR\gamma_3$ mRNAs code for the same protein, while $PPAR\gamma_2$ codes for a different protein containing 28 additional amino acids at the N-terminus [270].

PPARγ has been cloned from a number of species, including mice [271], hamsters [272], pigs [273] rhesus monkeys [274],and humans [275]. Interestingly, the PPARγ protein shows a significant conservation across all the species from which it has been cloned [275] [276]. It is expressed in adipocytes, spleen, colon, bladder, smooth and skeletal muscle, bone marrow and intestine [266] [277]. High levels of expression of PPARγ were demonstrated in many cancer cell types [278] [279].

More recently, the receptor has been found in many immune cells, including neutrophils, macrophages, and B cells [280]. In mice, it has been shown that immature as well as mature spleen-derived DCs express PPARγ [281]. On the other hand, Sugiyama et al., found that human cultured mast cells (HCMC) express $PPAR\gamma_2$ weakly but increase its expression when activated with anti-IgE or with calcium ionophore plus phorbol ester [282]. In relation to our current study, Ueki et al demonstrated that human purified eosinophils express PPARγ [76]. PPARγ is also

expressed in human airway epithelial cells [283]. An interesting feature of PPARγ expression is its sensitivity to the microenvironment. It has been shown that slight alterations in culture conditions can significantly effect whether PPARγ is expressed or not [266]. Several studies have identified a number of agents that regulate PPARγ expression including TNFα [284], GM-CSF, M-CSF, and IL-4 [285].

A number of potential PPARγ endogenous ligands have been identified including linolenic acid, arachidonic acid [286], 13-hydroxyoctadecadienoic acid (13-HODE) [287] and 15d-PGJ_2 [288], all of which bind to PPARγ at micromolar concentrations. PPARγ is also activated by a series of synthetic compounds such as thiazolidinediones [289], L-tyrosine-based compounds [290], and some nonsteroidal anti-inflammatory agents [291].

1.5.6.b. 15d-$\Delta^{12,14}$-PGJ_2 and PPARγ in Inflammation

A number of reports have proposed an anti-inflammatory role for 15d-PGJ_2 PPARγ. For example, 15d-PGJ_2 inhibits the production of iNOS, TNF-α and IL-1β by mouse and human macrophages by a mechanism involving the inhibition of mitogen-activated protein (MAP) kinases, NF-κB or IκB kinase [261, 292]. Several studies provide further evidence for a potential role for 15d-PGJ_2 as an anti-inflammatory agent. It was shown that 15d-PGJ_2 could inhibit the expression of genes coding for IL-1β, TNF-α, cyclooxygenase-2, and NO synthase-2 in Monocytes/macrophages [293, 294]. It has also been shown that 15d-PGJ_2 inhibit the release of the proinflammatory cytokines IL-8 in IL-4 from airway epithelial cells [283]. Furthermore, the PPARγ agonists 15d-PGJ_2, and troglitazone, attenuated the production of GM-CSF by anti-IgE-stimulated HCMC [282].

15d-PGJ_2 also has been implicated in cell apoptosis in both inflammatory and tumour cells. It has been shown that 15d-PGJ_2 can induce the apoptosis of mouse T and B cells [295, 296]. It has also been shown that 15d-PGJ_2 can induce eosinophil apoptosis via PPARγ-independent inhibition of NF-κB activation [297]. Additionally, Chinett et al. demonstrated that ligand activation of PPARγ results in apoptosis induction in macrophages and that PPARγ inhibits the transcriptional activity of the NF-κB p65/RelA subunit. This findings suggest that PPARγ induces macrophage apoptosis by negatively interfering with the anti-apoptotic NF-κB signaling pathway [298]. It has been also shown that 15d-PGJ_2 induces endothelial cell apoptosis via a PPARγ dependent pathway [299]. Furthermore, the PPARγ ligand Troglitazone has been shown to inhibit vascular smooth muscle cell proliferation [300]. Moreover, there are reports of the PPARγ-mediated inhibition of tumor-cell growth both *in vitro* and *in vivo* by 15d-PGJ_2 in a variety of tissues, including breast, prostate, colon, lung, bladder and oesophagus [301, 302]. Other PPARγ agonists also inhibit carcinogenesis [303].

On the other hand, not all studies support an anti-inflammatory role for 15d-PGJ_2 and the PPARγ receptor. A study by Thieringer et al. have failed to observe an inhibitory effect of 15d-PGJ_2 on induced expression of TNF-α and IL-6 in freshly prepared human macrophages [304]. Additionally, there is some evidence that 15d-PGJ_2 can promote inflammation. 15d-PGJ_2 has been reported to induce expression cyclooxygenase2 in epithelial cells [305]. It also can stimulate the production of proinflammatory mediators, such as IL-8 and activate MAP kinases, in some systems [306-308]. Interestingly, it has been found that 15d-PGJ_2 is a potent activator of eosinophils by its interaction with the DP_2 receptor [264]. As the above-mentioned

data indicates both anti- and- proinflamatory roles for this compound, a caution needs to be exercised before reaching a general conclusion in this regard.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Eicosanoids and reagents

5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) was synthesized chemically in the laboratory of Joshua Rokach, Florida Institute of Technology. All other eicosanoids as well as BW245C were purchased from Cayman Chemical Co, Ann Arbor, MI. Monoclonal antibody for eosinophil-derived MBP was provided by Dr R. Moqbel (University of Alberta, Edmonton, Canada). Rabbit antimouse immunoglobulin (Ig), and Mouse APAAP, were purchased from Dykocytomation.

2.2 Animals and in vivo procedures

The animals used in this study were 8 to 10 weeks old male Brown Norway rats (175-215 gm; RijHsd substrain), which were purchased from Harlan, Indianapolis, IN. All experimental procedures were approved by an institutional animal care committee and carried out in accordance with McGill University's policy on Handling and Treatment of Laboratory animals and the Canadian Council on Animals Care guidelines. The rats were housed in groups of three with food and water provided *ad libitum*. They were allowed to become acclimatized to laboratory conditions for 4 to 6 days prior to experimentation. Prior to treatment with eicosanoids, the animals were anaesthetized by intraperitonial injection of xylazine (7 mg/kg IP) and pentobarbital (50 mg/kg IP). Different amounts of test compounds in 100-µl vehicle (saline containing 0.5% ethanol) were administered to the lung by intratracheal instillation while control animals received vehicle alone.

Briefly, the rat was positioned against an angled restraining stand. An elastic ring was used to hold the mouth open and to facilitate the instillation procedure. A directed light source was used to provide Illumination. A 6 cm long PE-90 polyethylene tube

was inserted trans-orally into the tracheal lumen, then inserted into the mouth and placed between the vocal cords and into the lumen of the trachea. We used two simple techniques to determine if the intubation device is in the respiratory tract, rather than the gastrointestinal tract. Firstly, we sensed the movement of the polyethylene tube as it moved over the cartilage rings of the trachea. Secondly, after inserting the polyethylene tube we placed a mirror in front of its free end and verified that the animal was breathing by observing the condensation of moisture on the shiny face of the mirror. After confirming the correct positioning, the tube was then connected to a 1 ml syringe containing the test compound followed by 0.6 ml of air to aid in the distribution of the injected compound throughout the lung.

2.3. Preparation of tissue sections

At various times after administration of eicosanoids, animals were sacrificed by intraperitonial injection of an overdose of pentobarbital (150 mg/kg IP). Following induction of anaesthesia, the abdominal cavity was opened and rats were bled via the abdominal aorta to drain as much blood as possible. The chest cavity then was carefully opened, and the trachea was exposed, and freed from surrounding fascia without cutting the trachea. Using a fine tipped scissors, a tiny "V" shaped cut in the top of the trachea was made. A 6 cm long PE-240 polyethylene tube was carefully inserted into the V shaped cut, and fixed in place using a surgical knot. The whole lung with the polyethylene tube attached to it was taken out and washed briefly in phosphate-buffered saline (PBS). The lung was then placed over a clean gauze moisture with PBS and a 5 ml syringe filled with 100% OCT was attached to the free end of the polyethylene tube. Then the lung was filled slowly with OCT (3.5ml) and the polyethylene tube was slowly pulled out and the trachea was quickly clamped with

a small artery forceps just below the tiny cut to keep the OCT in the lung. A section of the left lobe of the lungs around the hilum was removed and placed in an aluminium foil basket filled with OCT embedding medium. The section was then snap frozen in isopentane precooled in liquid nitrogen and stored at –80 °C. Sections (6 μm) were cut in a cryostat, air dried for 1 h, fixed in acetone/methanol (60:40) for 7 minutes and further air dried for 30 min. The slides were then wrapped in aluminium foil and stored at –20 °C.

2.4. Immunocytochemistry

To identify eosinophils in the lung sections, the slides were allowed to defrost and treated with a monoclonal antibody to MBP using the alkaline phosphatase-anti-alkaline phosphatase technique as previously described by Frew and Kay [309]. Briefly, tissue sections were incubated with Universal blocking solution for 15 min. Excess blocker was drained, and the slides were incubated overnight at 4 °C with primary antibodies diluted in antibody diluting buffer (1:100) in a humidified chamber. The primary antibodies used here were mouse anti-human monoclonal antibody to MBP. Slides then were washed twice with Tris-buffered saline (TBS) for 5 min, followed by treatment with diluted rabbit antimouse immunoglobulin (1:60) as a secondary antibody at room temperature for 30 min. They were then washed with TBS for 5 min, and incubated with mouse APAAP diluted in antibody diluting buffer (1:60) for 30 min at room temperature. Again, slides were washed with TBS, and the reaction was developed and visualized with fast red dissolved in alkaline phosphatase substrate. The sections were counterstained with Gill II hematoxyline for 2 min, washed with water, placed in lithium carbonate and then a thin layer of Crystal Mount was placed over the tissue and the slides were left to dry in the oven at 37 °C overnight. The next day slides were mounted

under glass coverslip using Permount mounting medium. When dried the slides were coded and read in a blind fashion by two independent observers at a magnification of 200x. Eosinophils were observed in clusters around both large and small airways. For each slide, positive cells were counted in at least four non-overlapping eosinophil-containing areas (1 mm^2) around the airways using a squared eyepiece graticule. For each slide, the numbers of cells in the four regions with the highest numbers of cells were averaged.

2.5. Data analysis

Mean values of the cell counts determined for each slide by the two independent observers were used for all further analyses. All values are expressed as means ± SE of the numbers of immunoreactive cells per mm^2. The statistical significance of differences among groups were assessed using either one-way or two-way ANOVA as appropriate, with the Student-Newman-Keuls test as a multiple comparison method. Differences with a P value of less than 0.05 were considered to be statistically significant.

CHAPTER 3

EXPERIMENTAL RESULTS

3. RESULTS

3.1. PGD_2 elicits pulmonary infiltration of eosinophils

Monneret et al [9], demonstrated that Prostaglandin D_2 is a potent chemoattractant for human eosinophils in vitro. To test this effect in vivo, Brown Norway rats were treated with either saline vehicle or PGD_2 (5 μg), which were administered by tracheal instillation. Twenty-four hours later the rats were sacrificed, the lungs removed, and sections were taken for immunostaining for MBP using a mouse anti-human antibody that cross-reacts with rat MBP. PGD_2 induced a dramatic increase in the number of eosinophils in the lung (Fig. 6B) compared to vehicle-treated controls (Fig. 6A). The MBP positive cells were found principally around the airways. However, there were also some eosinophils around blood vessels and within the parenchyma (Fig. 7). No staining was observed in controls in which the antibody was omitted (data not shown). The response to PGD_2 was highly reproducible ($P < 0.001$), and was only slightly less than that to the potent eosinophil chemoattractant 5-oxo-ETE, which we used as a positive control data shown (Fig. 8).

3.2. Pulmonary eosinophilia is induced by DP_2 but not by DP_1 receptor agonists

As PGD_2 activates both DP_1 and DP_2 receptors, we wished to determine which of these receptors is responsible for the effect of this prostaglandin on eosinophil infiltration. To address this issue we compared its effects to those of BW245C which is a potent DP_1 receptor agonist with a potency similar to PGD_2 and has no activities on the DP_2 receptors [9]. We also examined the effect of the selective DP_2 receptor

agonists dhk-PGD_2 and 15R-methyl-PGD_2. The effect of 15d-PGJ_2 were also investigated because it is a potent DP_2 receptor agonist and we wanted to determine whether its DP_2-mediated effects would predominate over its reported anti-inflammatory effects as discussed in section 1.5.6.b. Figure 8. shows the effect of PGD_2 and other eicosanoids (5μg) on the number of eosinophils in the lung 24h after treatment. Treatment with PGD_2 results in an increase in eosinophil numbers of over 3-fold after 24h ($P< 0.001$) compared to vehicle. The response to PGD_2 was also slightly less than that of the potent eosinophil chemoattractant 5-oxo-ETE. Unlike PGD_2, BW245C had no effect on the numbers of eosinophils present in the lung. In contrast, all of the selective DP_2 receptor agonists tested induced eosinophil infiltration. 15R-Methyl-PGD_2 was found to be a potent stimulator of eosinophil migration in vitro [310]. This also was the case in the current study where this compound was found to be more potent than PGD_2 in inducing tissue eosinophilia (Fig. 6C; $P < 0.001$). Despite the reported studies that suggest an anti-inflammatory role for 15d-PGJ_2, it has been demonstrated that this compound is a potent activator of human eosinophils [264]. We showed here that 15d-PGJ_2 is a potent stimulator of eosinophil migration in vivo (Fig.6 1D; $P < 0.001$). Meanwhile dhk-PGD_2 induced eosinophil infiltration but was less potent than other DP_2 tested agonists ($P < 0.05$).

3.3. Time course for the effects of DP_2 receptor agonists on eosinophil infiltration

To investigate the time courses for the effects of DP_2 receptor agonists on eosinophil infiltration, sections were taken from lungs immediately following vehicle injection and 4, 12, 24, and 48 h after intratracheal instillation of PGD_2, 15R-methyl-PGD_2, and 15d-PGJ_2 (Fig. 9). As it has been previously shown in our lab that lung eosinophil numbers were constant during this time period following instillation of

vehicle alone [200], we did not evaluate responses to vehicle at all of the time points tested. However, the numbers of eosinophils in lungs immediately following vehicle injection (4.0 ± 0.7 eosinophils/mm^2) were the same as the numbers 24 h after injection of vehicle (4.1 ± 0.6 eosinophils/mm^2). Although none of the substances tested altered eosinophil numbers by 4 h, PGD_2 ($P < 0.05$) and the two selective DP_2 receptor agonists ($P < 0.005$) significantly increased their numbers by 12 h. The maximal response to 15R-methyl-PGD_2 was observed after 24 h. Although PGD_2 and 15d-PGJ_2 elicited near maximal responses by this time, the responses to these agonists continued to rise up to 48h.

3.4. Dose-response relationship for PGD_2, 15d-PGJ_2, and 15R-methyl-PGD_2

To further investigate the effects of PGD_2 and DP_2 receptor agonists on eosinophil recruitment into the rat lung their dose-response relationships were investigated and compared (Fig10). Rats were insufflated intratracheally with various amounts of PGD_2, 15d-PGJ_2, and 15R-methyl-PGD_2. After 24h, the lungs were removed and the numbers of eosinophils counted in lung sections after immunostaining for MBP. Of the three compounds tested, 15R-methyl-PGD_2 was the most potent ($P < 0.005$) with an ED_{50} of about 0.6 μg. PGD_2, which had an ED_{50} of about 1.5 μg induced a smaller response at all doses tested. 15d-PGJ_2 appeared to have potency similar to that of PGD_2, although the responses to lower doses of this compounds were somewhat variable. Interestingly, diminished responses to all three compounds were observed at the highest dose tested (10μg). At this dose, none of the PGD_2 analogs had a significant effect on eosinophil numbers in the lungs.

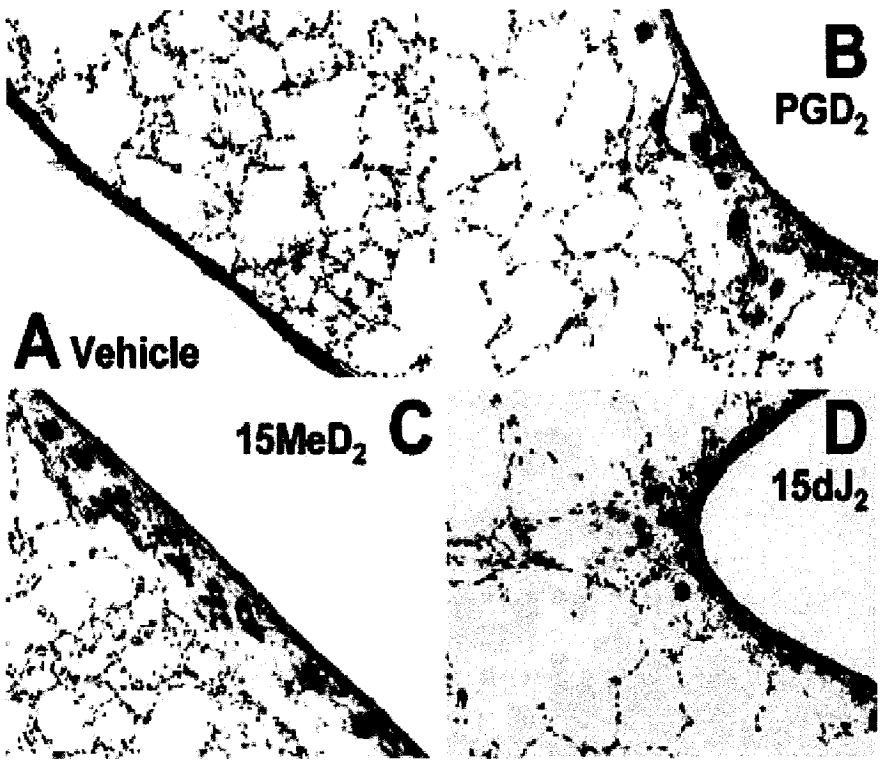

Fig. 6. Representative slides of the effects of PGD_2 and selective DP_2 receptor agonists on lung eosinophil numbers. Brown Norway rats were treated by intratracheal administration of either vehicle (A), PGD_2 (5 μg; B), 15R-methyl-PGD_2 (5 μg; C), or 15d-PGJ_2 (5 μg, D). Tissue sections were removed after 24 h and treated with an antibody to MBP, which stains eosinophils red.

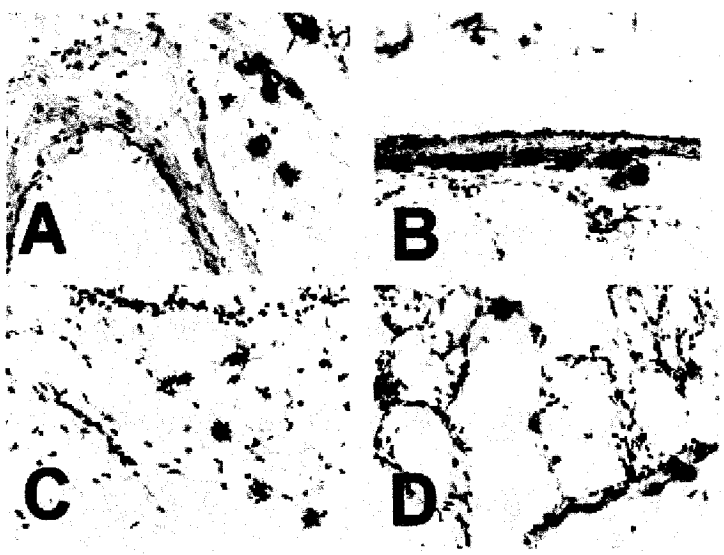

Figure 7. Representative slides of the presence of eosinophils around the blood vessels A and B and within the parenchyma C and D. These slides were taken from animals treated with PGD_2 (5 μg) and tissue sections were removed after 24 h.

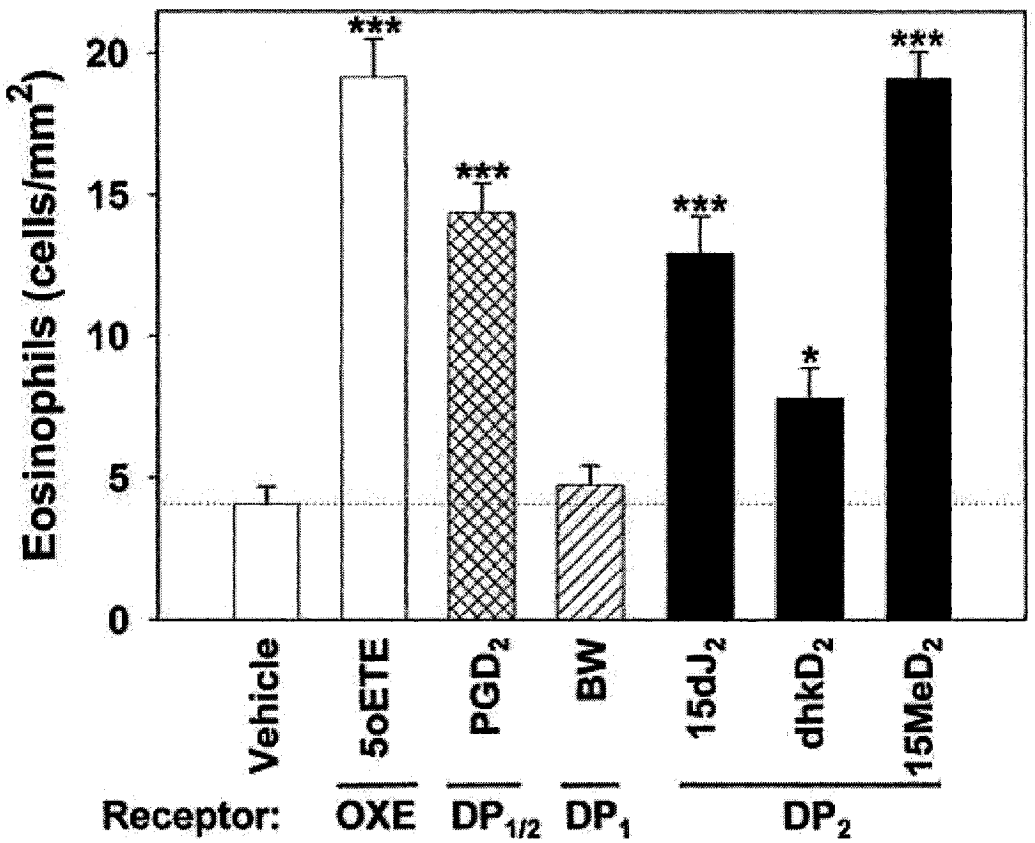

Figure 8. Effects of PGD_2 and selective PGD_2 receptor agonists on lung eosinophil numbers.Brown Norway rats were treated with either vehicle or 5 μg of 5-oxo-ETE (5-oETE), PGD_2, BW245C (BW), 15d-PGJ_2 ($15dJ_2$), dhk-PGD_2 ($dhkD_2$), or 15R-methyl-PGD_2 ($15MeD_2$). Lung sections were taken 24 h later and stained for MBP as shown in Fig. 1. ***, $P < 0.001$; *, $P < 0.05$ when compared to the vehicle-treated control. Except for vehicle (n = 9) and PGD_2 (n = 10), there were 5 rats in each group.

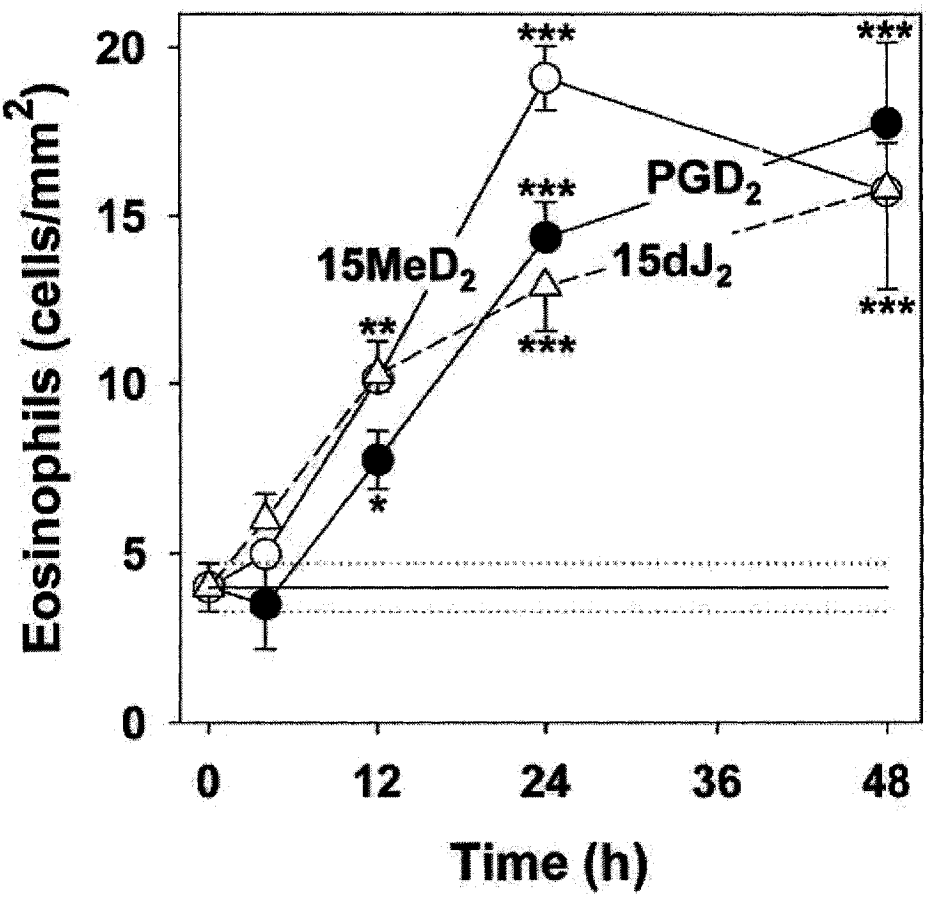

Figure 9. Time courses for the effects of PGD_2 and selective DP_2 receptor agonists on lung eosinophils. Brown Norway rats were treated with 5 μg PGD_2 (□), 15d-PGJ_2 (Δ; $15dJ_2$), or 15Rmethyl-PGD_2 (○; $15MeD_2$). Lung sections were taken either immediately following treatment with vehicle or after different periods of time. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ when compared to combined data from controls treated with vehicle for 0 and 24 h. n = 4 except for vehicle (n = 3), PGD_2 (4 h, n = 5; 24 h, n = 10), 15R-methyl-PGD_2 (24 h, n = 5), and 15d-PGJ_2 (24 h, n = 5).

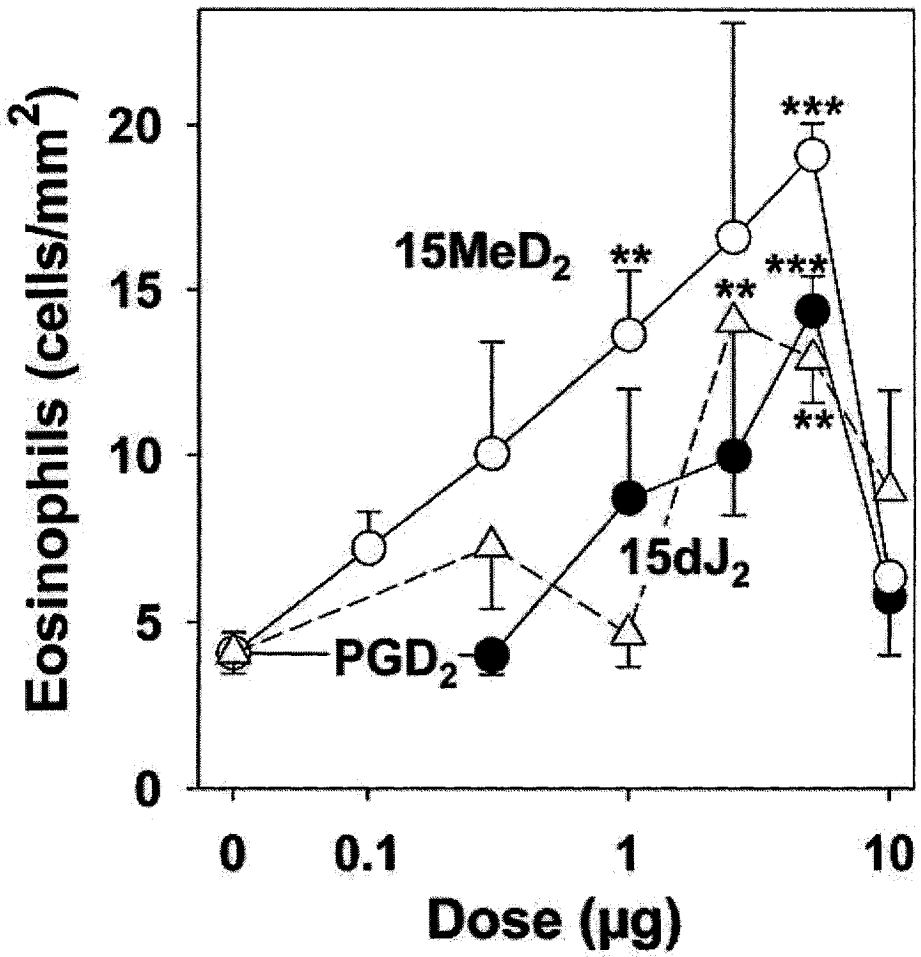

Figure 10. Dose-response relationship for the effects of PGD_2 and related compounds on lung eosinophils. Brown Norway rats were treated with vehicle or different doses of PGD_2 (•), 15d-PGJ_2 (Δ; $15dJ_2$), or 15R-methyl-PGD_2 (○; $15MeD_2$). Lung sections were taken after 24 h later and immunostained for MBP. ***, $P < 0.001$; **, $P < 0.01$ when compared to vehicle treated controls. n = 5 except vehicle (n = 9), PGD_2 (2.5 µg, n = 3; 5 µg, n = 10; 10 µg, n = 3), 15R-methyl-PGD_2 (2.5 µg, n = 2; 10 µg, n = 3), and 15d-PGJ_2 (2.5 µg, n = 3; 10 µg, n = 4). All values are means ± SE except when n was equal to 2 in which case the value is the means ± range.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. DISCUSSION

The central role of eosinophils in the pathogenesis of asthma is long known [126]. Activated eosinophils play an important role in asthma through their capacity to release toxic proteins, oxygen radicals and lipid mediators as well as an array of proinflammatory cytokines [311]. However, in 2000, Leckie and his group concluded that eosinophils do not have a significant role in the development of asthma in humans after a humanized monoclonal antibody against IL-5, effectively depleted eosinophils from blood and sputum in mild atopic subjects with asthma, but had no effect on airway hyperresponsiveness [312]. Nevertheless, these results may be viewed as the inability of anti-IL-5 to abolish bronchial mucosal eosinophils to a degree sufficient to reduce the symptoms of asthma. A similar study by Flood-Page et al., using the same monoclonal antibody against IL-5 on human subjects with mild asthma, showed that this antibody only reduced airway tissue and bone marrow eosinophils by 50% [313]. Interestingly, very recently Lee and his group created a transgenic line of mice that are specifically devoid of eosinophils, and demonstrated that these cells were required for pulmonary mucus accumulation and airway hyperresponsiveness associated with asthma [314]. Interestingly, Humbles et al., used the same transgenic mice model and found that total ablation of the eosinophil has no effect on AHR when compared to wild-type mice. Meanwhile, they found that eosinophil ablation was protective from peribronchiolar collagen deposition and increases in airway smooth muscle [131]. Although this study used the same animal model that Lee et al. used, they obtained a different result, which further complicates our understanding of the relationship between eosinophils and AHR.

There are currently two types of drugs used to treat asthma. The first consists of bronchodilators that are aimed primarily at providing symptomatic improvement through the use of bronchodilators. These include both long- and short- acting inhaled β_2-agonists, which are the most effective [315], followed by anticholinergics [316]. The second type of drug aims at suppression of airway inflammation and reduction of bronchial hyperresponsiveness [30]. Presently, the most effective anti-inflammatory drugs used in the treatment of asthma are glucocorticoids, which act primarily by inhibiting the late-phase reaction [317]. Leukotriene-modifying drugs are also used to reduce airway inflammation and have been shown to improve symptoms especially when used in combination with inhaled steroids [318].

However, there is reason for concern about currently used asthma therapy. There are concerns that long-term use of both long-acting and short-acting inhaled beta-agonists may cause a loss of asthma control in some patients [319]. On the other hand, steroids are rather non-specific in their actions, and their use also raises concerns over side effects. Furthermore, a minority of patients with asthma fail to respond to glucocorticoid therapy [117]. In addition, corticosteroid treatment has shown limited, if any, benefit in reducing airway remodelling [320]. Moreover, lung function in asthmatics declines more rapidly compared with that of normal individuals, indicating disease progression despite the use of currently available asthma therapy [321]. These concerns over the current asthma therapy have stimulated a search for alternative drug targets that are primarily involved in the inflammatory process in asthma. These new therapeutic approaches in asthma include, specific immunotherapy and cytokine, chemokine or adhesion molecule agonists/antagonists as well as monoclonal antibodies [30]. Other strategies include enhancing the effect of cytokines

that promote the antiallergic pathway (e.g. IFN-γ and IL-12) which favor the Th_1 response over the Th2 response [322, 323].

Although eosinophils have long been considered to be a major target in the therapeutic strategy for bronchial asthma there is not yet any available therapy that is selectively directed at these cells [312]. Eosinophils can be targeted by different strategies at different levels of the eosinophil life cycle (fig11). It is theoretically possible that therapeutic interventions could be directed at inhibiting eosinophilopoiesis. However, abolishing eosinophils completely might constitute a risk of infection to the host [324]. Another strategy is to prevent eosinophil recruitment. Eosinophil recruitment into sites of inflammation is a complex process involving interaction between eosinophils, endothelial cells and a wide array of mediators [148]. Theoretically, targeting the adhesion molecules can prevent eosinophil recruitment. However, this approach is potentially dangerous because all leukocytes express adhesion molecules and none is selectively expressed by eosinophils [324]. Another appealing strategy is to prevent eosinophil entry into the tissue by targeting the chemotactic factors.

At the time of the start of this study, several factors had been suggested as being chemotactic for eosinophils including PAF, complement factor C5a and LTB_4 [174]. All of these mediators, however, act on both eosinophils and neutrophils. A number of mediators belonging to the chemokine family, were shown to be effective eosinophil chemoattractants including RANTES, eotaxin 1, 2 and 3 and monocyte chemoattractant protein 2, 3 and 4 [325]. On the other hand, IL-3, IL-5 and GM-CSF had been recognized as activators of eosinophil function, including migration [175]. IL-2 had also been claimed to induce eosinophil migration [100]. Lipid mediators also appear to be important mediators of eosinophil migration.

PGD_2 is released in large amounts during asthmatic attacks in humans [7].The important role of PGD_2 in asthma was further confirmed by the finding that antigen sensitized transgenic mice over expressing the lipocalin-like PGD synthase in the lung displayed increased IL-4 and IL-5 levels and enhanced eosinophil numbers in BAL fluid following antigen challenge [228]. One possible role for PGD_2 is its involvement as a chemotactic agent for eosinophils in this disease. The importance of PGD_2 as an eosinophil chemoattractant is supported by a study by Emery et al. in dogs [177], and by the finding that PGD_2 is a potent chemotactic factor for human eosinophils [9]. The main goal of the current study was to determine whether PGD_2 could induce pulmonary eosinophilia in vivo.

This study demonstrates for the first time that intratracheal administration of PGD_2 induces pulmonary eosinophilia. This response was almost as intense as that induced by the potent eosinophil chemoattractant 5-oxo-ETE [199], which acts through the recently cloned OXE receptor [326, 327] and elicits a robust eosinophilic response in Brown Norway rat lungs following intratracheal administration [200]. The present results are consistent with an *in vivo* study in dogs demonstrating that superfusion of tracheal segments with PGD_2 induced the accumulation of eosinophils in the superfusion fluid [177].

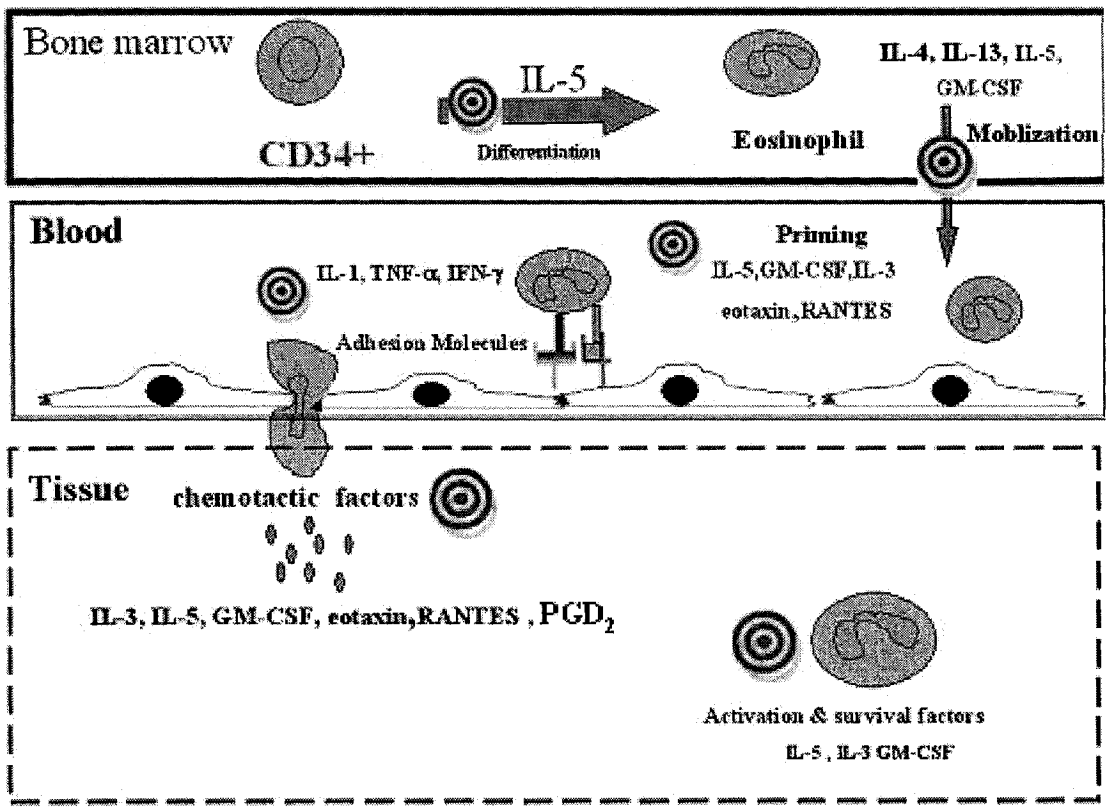

Figure 11. Different levels at which eosinophils can be targeted to reduce their numbers in the body. Theoretically, different strategies can be used to prevent tissue eosinophilia including, targeting the eosinophilopoiesis process, targeting factors that help mobilizing eosinophils from bone marrow to the blood, targeting factors that prime eosinophils for migration, targeting the adhesion molecules or the factors that up regulate them, targeting the chemotactic factors that attract eosinophils to the tissue, and targeting the factors that activate and prolong the survival of eosinophils in the tissue

The response to PGD_2 is clearly mediated by DP_2 rather than DP_1 receptors, as it was not shared by BW245C, which is a potent and highly selective DP_1 receptor agonist [328]. In contrast, all of the selective DP_2 agonists tested induced pulmonary eosinophilia with relative potencies similar to those predicted by their *in vitro* effects on human eosinophils. The most potent of these is 15R-methyl-PGD_2, which is the most potent DP_2 receptor agonist reported to date [310]. Interestingly, this compound has the unnatural R configuration at C_{15}, and is much more potent *in vitro* than the corresponding analog with the S configuration at C_{15}, which is common to all naturally occurring prostaglandins. The present study demonstrates that 15R-methyl-PGD_2 is active not only in vitro, but also in vivo, suggesting that it may be a very useful tool for examining DP_2 receptor-mediated responses in animal models.

15d-PGJ_2 was approximately equipotent with PGD_2 in inducing pulmonary eosinophilia, consistent with its potent stimulatory effects on human eosinophils in vitro [264]. This is quite interesting in view of the abundant reports in the literature on the anti-inflammatory effects of this compound [12]. 15d-PGJ_2 is formed by the albumin-catalyzed degradation of PGD_2 [257]. Although this clearly occurs in vitro, the presence of this substance in vivo in amounts compatible with its anti-inflammatory effects remains controversial [12, 231, 329]. The anti-inflammatory effects of 15d-PGJ_2 appear to be mediated by activation of PPARγ or by covalent binding to components of the NF-κB system or other cellular proteins [330]. The reactivity of 15d-PGJ_2 is due to its cyclopentenone ring structure, which can form adducts with protein thiol groups as shown in Fig. 5. Although it can clearly induce a variety of responses by this mechanism, including suppression of the expression of cyclooxygenase-2, inducible nitric oxide synthase, and cytokines, the concentrations required are considerably higher than those needed for DP_2 receptor-mediated

responses [264]. There are also a number of *in vivo* studies demonstrating anti-inflammatory effects of this prostaglandin in various animal models, including carrageenan-induced pleurisy, in which case it was reported to inhibit the infiltration of both neutrophils [331] and mononuclear cells [231] in the early and late phases, respectively, of this response. The present study clearly demonstrates that 15d-PGJ_2 can also induce proinflammatory effects *in vivo* through activation of the DP_2 receptor, suggesting that caution should be used in the development of such compounds as therapeutic agents [231].

The third DP_2 agonist we tested was dhk-PGD_2, which was the first selective agonist reported for this receptor [9, 220]. Although this compound is often used in studies on the DP_2 receptor, it is less potent than PGD_2 *in vitro*, and the present study would suggest that this is also true *in vivo*, as it was considerably less active than the other was PGD_2 derivatives tested. It would therefore seem preferable to use 15R-methyl-PGD_2 as a selective DP_2 agonist in studies on this receptor.

The absence of a response to the selective DP_1 receptor agonist BW245C is interesting in view of the inhibitory effects of both deletion of the gene for the DP_1 receptor [10] and the selective DP_1 antagonist S-5751 [241] on pulmonary eosinophilia in animal models of asthma. The lack of *in vitro* chemotactic activity for human eosinophils of this compound [9] suggests that activation of the DP_1 receptor affects lungs eosinophil numbers by an indirect mechanism, possibly through effects on cytokine production, as $DP_1^{-/-}$ mice display reduced Th2 cytokine levels following antigen challenge compared to wild type mice [10]. Activation of DP_1 receptors might also augment the responses to inflammatory mediators by increasing blood flow and vascular permeability in the affected tissue [332], consistent with the expression of these receptors on endothelial cells [238]. Alternatively, stimulation of DP_1 receptors

on eosinophils could increase their survival in the lung, as shown for BW245C (although not for PGD_2 itself) *in vitro* [11].

On the other hand, there is evidence that the DP_1 receptor might play an inhibitory role in allergic responses. PGD_2, when co-administered intratracheally with FITC-labelled OVA, inhibited the migration of dendritic cells from the lungs to draining lymph nodes. This effect was shared by BW245C but not by dhk-PGD_2, indicating the involvement of DP_1 receptors [333]. Furthermore, the selective DP_1 receptor antagonist BWA868C augments the activation of eosinophils by PGD_2 [9], suggesting that this receptor might serve to attenuate DP_2 receptor-meditated responses in these cells.

The response to PGD_2 and selective DP_2 receptor agonists was delayed, as it was not observed after 4 h and was only about half maximal by 12 h. This is similar to the time course for 5-oxo-ETE-induced pulmonary eosinophilia in Brown Norway rats [200].The delay in eosinophil infiltration suggests that this response may be dependent on mobilization of these cells from the bone marrow. The DP_2 receptor agonist Δ^{12}-PGJ_2 has been shown to induce the release of eosinophils from the bone marrow in the guinea pig isolated perfused hind limb preparation [334]. Moreover, intravenous injection of dhk-PGD_2 into Brown Norway rats has been reported to result in increased numbers of circulating eosinophils [250]. Thus, it is possible that PGD_2 and the DP_2 agonists used in the present study first acted on the bone marrow to induce the release of eosinophils, and then promoted their accumulation in the lung through their chemoattractant properties. Alternatively, we cannot rule out the possibility that DP_2 receptor agonists act indirectly, by stimulating the release of an eosinophil chemoattractant from another cell-type.

All three compounds tested in the dose response study elicited diminished responses at the highest dose tested. Although in the case of PGD_2 this could potentially be explained by an inhibitory effect of the DP_1 receptor, this would not pertain to the selective DP_2 receptor agonists. PGD_2 is known to have some agonist activity at TP receptors for thromboxane A_2, resulting in bronchoconstrictor responses to higher doses [335]. It is possible that 15R-methyl-PGD_2 and 15d-PGJ_2 could also activate these receptors, and thereby elicit bronchoconstrictor responses. This could possibly have limited the distribution of agonist throughout the airways, resulting in a reduced eosinophil response.

Our results differ from a previous report which demonstrated that inhalation of aerosolized PGD_2 by antigen-sensitized mice does not result in elevated eosinophil numbers in BAL fluid between 24 and 48 h after treatment [336]. In contrast, exposure of these mice to low-dose antigen 24 h after administration of PGD_2 induced a strong eosinophilic response that could be blocked by an antibody to macrophage-derived chemokine (MDC). Furthermore, PGD_2 was shown to increase the expression of MDC by airway epithelial cells [336]. The nature of the receptor responsible for these effects is not known, although the DP_1 receptor would appear to be the most likely candidate. Expression of the DP_2 receptor is fairly limited, being restricted principally to eosinophils, Th2 cells, and basophils [220] and [238]whereas the DP_1 receptor is expressed in a larger number of cells, including airway epithelial cells [238]. There are several possible explanations for the differences between the two studies. It is possible that the rat may respond more strongly than the mouse to PGD_2. Alternatively, the dose of PGD_2 used in the mouse may have been too high to observe a direct effect of PGD_2 on eosinophil infiltration. Consistent with this, we observed

diminished responses to PGD_2 at the highest dose tested (cf. Fig. 10). Finally, eosinophil numbers were evaluated by a more selective method (immunocyto-Chemistry) in lung tissue in the present study.

4.2. Claims to Original Research

1. **This thesis presents the first published data concerning the direct effect of PGD_2 on eosinophil infiltration into the lung. This raises the possibility that PGD_2 and/or its receptors may be an important target for future asthma therapy.**

2. **The in vivo effects of PGD_2 were shown to be mediated by DP_2 receptor.**

3. **This is the first study to show that the selective DP_2 agonist 15R-methyl PGD_2 is active in vivo, and is more potent than PGD_2 in inducing pulmonary eosinophilia.**

4. **In spite of its reported anti-inflamatory properties, we have shown that 15d-PGJ_2 induces infiltration of eosinophils into the lung.**

4.3. Conclusion and Future Considerations

As discussed earlier, eosinophils are thought to play a central role in the pathogenesis of asthma. Therapeutic strategies that targeting eosinophils in bronchial asthma could be potentially valuable. There is appealing evidence that PGD_2 is an important mediator in asthma, because of its rapid release from mast cells and its chemotactic effect on eosinophils. The results presented herein show for the first time that PGD_2 and selective DP_2 receptor agonists induce pulmonary eosinophilia *in vivo* in a time- and dose-dependent manner. On the other hand, the selective DP_1 receptor agonist BW245C is inactive. 15R-methyl-PGD_2 is the most potent among these compounds, and should serve as an excellent selective DP_2 receptor agonist for *in vivo* studies. 15d-PGJ_2 is also a potent inducer of eosinophil infiltration in vivo, which raises some doubt about its potential usefulness as an anti-inflammatory agent. These results are consistent with an important role for PGD_2 and the DP_2 receptor/$CRTH_2$ in allergic diseases such as asthma.

To conclude, although the findings presented here suggest that PGD_2 may be a potent eosinophil-chemoattractant, further evidence is clearly required to demonstrate that it has a pathophisiological role in the recruitment of this cell type in asthma. A tool that might be used to further explore this issue is blocking the formation of PGD_2 or antagonizing its effect to demonstrate that it has direct effect in blocking or reducing eosinophil recruitment in asthma models. Another interesting way to study this is to create animal models that lack both DP_1 and DP_2 receptors and investigate the effect on eosinophil infiltration and other asthmatic responses following antigen challenge.

CHAPTER 5

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