The Role of the Canonical Beta-2 Adrenoceptor Gs Pathway in Development of the Asthma Phenotype in Murine Models

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A Dissertation Presentation to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirement for the Degree

Doctor of Philosophy

By

Gloria Serwaa Forkuo

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Abstract

Asthma is a chronic inflammatory disease of the airways characterized by variable degrees of inflammation, mucous metaplasia and airway hyperresponsiveness (AHR). Asthma causes over a quarter million deaths per year and affects 300 million people worldwide. Current treatment modalities including inhaled β_2-adrenoceptor (β_2AR) agonists, and inhaled corticosteroids, the main bronchodilating and anti-inflammatory agents respectively are associated with serious and adverse side effects. Therefore, there is the need to develop novel therapies for the management of asthma.

Using pharmacologic studies, we have previously shown in an allergen driven murine model of asthma that, chronic administration of certain "β-blockers" with inverse agonist properties such as nadolol, metoprolol, and ICI 118,551, but not the antagonists alprenolol, significantly attenuated three cardinal features of asthma: airway inflammation, mucous metaplasia and AHR. Also, in genetic studies, we established the requirement of the β_2AR in development of the asthma phenotype where ovalbumin sensitized and challenged β_2AR null mice, exhibited an attenuation of the asthma phenotype. These studies established the requirement of β_2AR signaling in the development of the asthma phenotype in murine models. In mild asthmatics, clinical studies have also shown that, chronic administration of nadolol dose-dependently increased the provocative concentration of methacholine causing a 20% fall in forced expiratory volume in 1 second (FEV_1). The β_2AR can signal in the presence of a ligand or spontaneously (constitutively active receptors) in the absence of a ligand. It can also

signal through at least two major pathways: the cAMP pathway and the β-arrestin pathway, which can lead to MAPK activation.

This project was designed to study the role of constitutive versus ligand activation of the β_2AR in development of the asthma phenotype in murine asthma models. We also investigated the pathways downstream of the β_2AR required for the development of the asthma phenotype. Using pharmacologic and genetic studies, we have shown that ligand activation of the β_2AR is required for the development of the asthma phenotype in murine asthma models. We also observed that, the Gs-cAMP pathway may demonstrate beneficial effects in a murine model of asthma.

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AC	Adenylyl cyclase
AHR	Airway hyperresponsiveness
AMP	Adenosine monophosphates
AP-1	Activator protein-1
BALF	Broncho-alveolar lavage fluid
β_2AR	β_2-adrenoceptor
β-AR	β-adrenoceptor
b-FGF	Basic fibroblast growth factor
β_2AR-KO	β_2-adrenoceptor null mice
cAMP	Adenosine 3′, 5′ cyclic monophosphate
CCL2	Chemokine ligand 2
CCL7	Chemokine ligand 17
CREB	Cyclic AMP response element binding protein
CHF	Congestive heart failure
COPD	Chronic obstructive pulmonary disease
EGF	Epidermal growth factor
Epi-KO	Epinephrine null
ERK1/2	Extracellular signal-regulated kinases 1/2
eIF2Bα	α-subunit of eukaryotic initiation factor 2B

EPAC	exchange proteins activated by cAMP
FDA	Food and Drug Administration
FEV_1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GRKs	G protein coupled receptor kinases
GPCR	G protein-coupled receptor
HDAC2	Histone deacetylase-2
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	Interleukins
I-CAM	Intercellular adhesion molecule 1
IGF-1	Insulin-like growth factor-1
i.p.	Intra-peritoneal
i.n.	Intra-nasal
IACUC	Institutional animal care and use committee
K	Airway reactivity
LABA	Long acting β_2AR agonists
MUC	Mucin glycoproteins
MAPK	Mitogen activated protein kinase

MAO	Monoamine oxidase
mMCP-1	Mouse monocyte chemotactic protein 1
mTARC	Mouse thymus and activation regulated chemokine
mRANTES	Mouse regulated on activation normal T cell expressed and secreted
NF-κB	Nuclear factor-κB
NF-AT	Nuclear factor of activated T-cells
NSF	N-ethylmaleimide-sensitive fusion protein
NHERF	Na+/H+-exchanger regulatory factor
NHE3	Na+/H+- exchanger type 3
Ova S/C	Ovalbumin sensitized and challenged
Ova	Ovalbumin
PDGF	Platelet-derived growth factor
PDE	Phosphodiesterase
PKA	Protein kinase A
PNMT	Phenylethanolamine N-methyltransferase
PCR	Polymerase chain reaction
PAF	Periodic acid fluorescent
PC_{100}	Methacholine concentration which doubles baseline resistance

PC_{20}	Provocative concentration causing a 20% fall in forced expiratory volume in 1 second
PKC	Protein kinase C
PDZ	PSD95, DLG, and ZO1
Rrs	Airway resistance
SMART	Salmeterol Multicenter Asthma Research Trial
STAT	Signal transduction-activated transcription factors
7TMR	7-transmembrane receptor
TIMP-1	Inhibitor of metalloproteinase-1
TGFβ	Transforming growth factor beta
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor-α
TLR	Toll-like receptors
TSLP	Thymic stromal lymphopoietin
Th2	T helper 2
VLA-4	Very late antigen-4
V-CAM	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VMAT	Vesicular monoamine transporter
WT	Wild-type

1 Introduction and statement of problem

Asthma is a chronic inflammatory disease of the conducting airways, leading to variable degrees of inflammation, structural changes of the airways and airway hyperresponsiveness (AHR) (Vignola, Kips et al. 2000, Pascual and Peters 2005, Kroegel 2009). Asthma causes over a quarter million deaths per year and affects 300 million people worldwide (2007). Most patients are well controlled by current treatment modalities including inhaled β_2-adrenoceptor (β_2AR) agonists, and inhaled corticosteroids (ICS), which are the main bronchodilating and anti-inflammatory agents respectively, currently in use. However, these drugs can have serious side effects, and in spite of intensive research and significant advances to enhance health care for asthmatic patients, asthma mortality rates in the United States have risen by 60% in the last 25 years (2007).

Several studies have reported negative outcomes, such as loss of asthma control and an increase in asthma related deaths with the chronic use of β_2AR agonists. The strongest evidence for negative outcomes has been mainly with the long-acting β_2AR agonists (LABA) (Salpeter, Buckley et al. 2006, Cates, Lasserson et al. 2009, Cates, Jaeschke et al. 2013). A recent example is the Salmeterol Multicenter Asthma Research Trial (SMART) which was prematurely stopped due to a small, but statistically significant increase in asthma-related deaths in the subjects receiving the LABA salmeterol, instead of as needed use of a short-acting β_2AR agonist (Nelson, Weiss et al. 2006).

The inhaled corticosteroids also have considerable side effects. These include pharyngitis, dysphonia, reflex cough and oropharyngeal candidiasis. Absorption of corticosteroids from the lungs into circulation can result in systemic side effects such as suppressed growth velocity and decreased leg length in children, suppressed immune response to infections, osteoporosis and bone fractures (Kroegel 2009, Ahmet, Kim et al. 2011, Chang 2012, Kelly, Sternberg et al. 2012). These findings reinforce the need to develop novel drugs and strategies for the management of asthma.

The paradigm shift that occurred with the use of 'β-blockers' (β-AR antagonists and inverse agonists) in congestive heart failure (CHF) is what inspired studies that began in our laboratory to test the effect of chronic administration of β-blockers in murine asthma models. In 1997, the Food and Drug Administration (FDA) approved carvedilol for CHF therapy, marking the first time a drug that was contraindicated in a disease became a drug of choice.

The impairment of cardiac output in CHF suggested logical treatment would be to use a β-adrenoceptor (β-AR) agonist, to enhance cardiac contractility; and contraindicate β-blockers which decrease cardiac contractility and heart rate due to their negative ionotropic properties. However, clinical trials with both β-agonists and certain β-blockers showed there are different clinical outcomes with acute versus chronic use of β-AR ligands. While β-AR agonist use acutely increase cardiac output as a result of increases in heart rate and contractility, their chronic use was associated with increased mortality (Weber, Andrews et al. 1982). On the other hand, clinical studies showed that, chronic

administration of β-blockers such as carvedilol and metoprolol resulted in significant reduction in mortality, and with time also produced increased cardiac contractility (Waagstein, Hjalmarson et al. 1975, Hall, Cigarroa et al. 1995, Bristow, Gilbert et al. 1996).

We hypothesized the paradigm shift that occurred in CHF could also be applicable in asthma. Similar to CHF, β-blockers are currently contraindicated for asthma therapy due to their potential acute bronchoconstricting properties in the airways. Current asthma guidelines recommend the use of β_2AR agonist for bronchodilation and symptomatic relief. However, as discussed above their chronic use is associated with loss of asthma control and an increase in asthma-related deaths.

Our laboratory then began studies to examine what happen with chronic β-blocker administration in murine asthma models. In these studies we observed that, in ovalbumin sensitized and challenged (Ova S/C) mice, chronic administration of β_2AR inverse agonists such as nadolol, metoprolol and ICI 118,551, but not the antagonists alprenolol, significantly attenuated three cardinal features of the asthma phenotype namely, airway inflammation, mucous metaplasia and AHR (Callaerts-Vegh, Evans et al. 2004, Nguyen, Omoluabi et al. 2008). Clinical studies have also shown that, chronic administration of nadolol dose-dependently increased PC_{20} (provocative concentration causing a 20% fall in forced expiratory volume in 1 second, FEV_1) in mild asthmatics (Hanania, Singh et al. 2008, Hanania, Mannava et al. 2010).

Additional studies from our laboratory established the requirement of β_2AR signaling in the development of the asthma phenotype in murine models. In allergen-driven murine models of asthma, Ova S/C β_2AR null mice (β_2AR-KO), exhibited an attenuation of the asthma phenotype (Nguyen, Lin et al. 2009). Taken together with our studies showing that certain β–blockers also attenuated the asthma phenotype, these studies established the requirement of β_2AR signaling in the development of the asthma phenotype in murine models. Due to the apparent requirement for inverse agonism as an essential property of beneficial ligands in murine models of asthma, we hypothesized that constitutive signaling was required for development of the asthma phenotype (Nguyen, Omoluabi et al. 2008, Nguyen, Lin et al. 2009).

Therefore, the purpose of my research was to understand the mechanisms of β_2AR stimulation (constitutive or spontaneous signaling versus ligand-induced β_2AR signaling) and the downstream signaling pathways in asthma pathogenesis and help determine better therapeutic options for asthma control.

2 Literature review

2.1 Asthma

Asthma is a complex syndrome which involves an interplay of genetic (atopy) and environmental factors such as exposure to viruses, exercise, drugs, allergens from cockroaches, animal dander and exposure to hazardous occupational chemicals (Prescott, Macaubas et al. 1998, Cookson 1999, Stein, Sherrill et al. 1999, Holgate 2008, Barnes 2008, Jacquet 2011, Holgate 2012, Venables and Chan-Yeung 1997).

These factors contribute to the inception and evolution of dominant features and clinical symptoms of asthma, which include variable airway inflammation, airflow obstruction and bronchial airway hyperresponsiveness (AHR). As inflammation becomes severe, the airway becomes susceptible to mucous cell metaplasia, smooth muscle proliferation, angiogenesis, fibrosis and nerve proliferation (Holgate 2012). The inflammatory cascade in asthma is a T helper 2 (Th2) driven adaptive immune response which increases immunoglobulin E (IgE) production with subsequent increase in recruitment of innate immune cells such as eosinophils, mast cells and basophils into the airways (Figure 1) (Holgate 2008, Barnes 2008, Jacquet 2011, Holgate 2012).

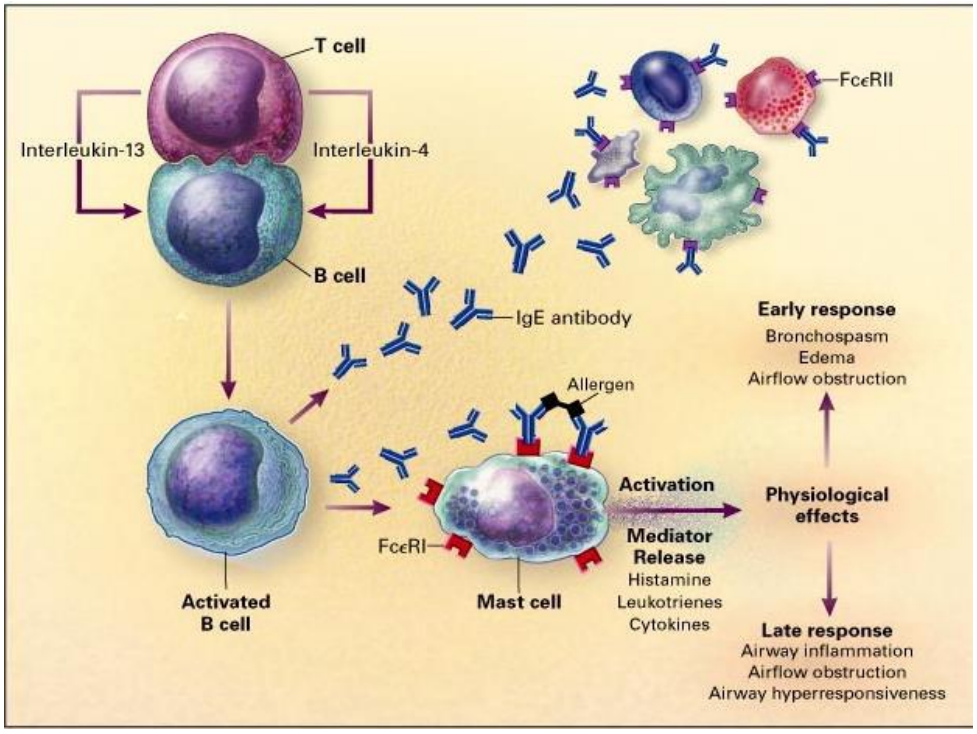

Figure 1: Schematic of interaction between T cells and B cells for IgE synthesis. After subsequent encounters with antigens, binding of the high-affinity IgE receptors produces the release of preformed and newly generated mediators. Once present in various tissues, mediators may produce various physiological effects depending on the target organ. Adapted from: (Busse and Lemanske 2001).

2.2 Major cell types in asthma

2.2.1 The role of the epithelium in asthma

Inhaled allergens interact with different receptors located on the surface of epithelial cells (Figure 2). These receptors include protease-activated receptors (PAR), toll-like receptors (TLR) and C-type lectin receptors. Activation of these receptors can lead to the release of pro-Th2 cytokines such as interleukins (IL) 33 and 25, thymic

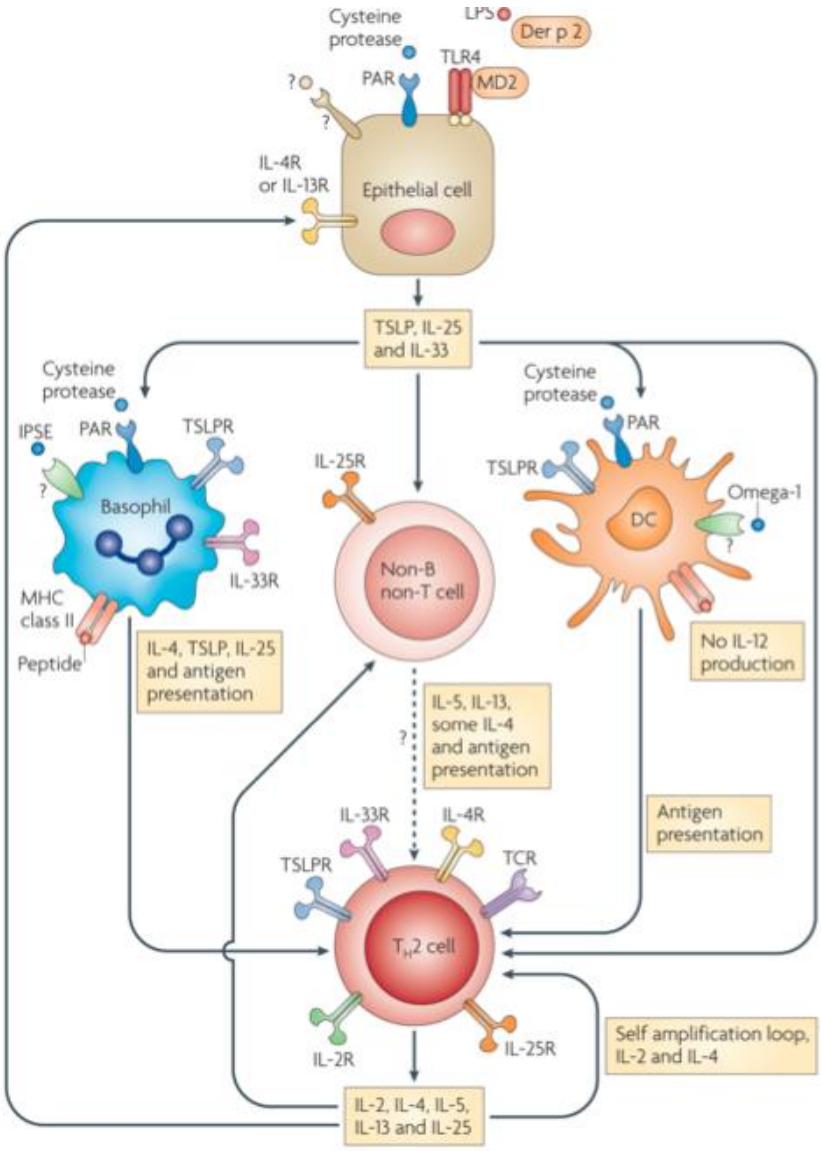

Figure 2: Schematic of the epithelium and its role in asthma. Cysteine proteases, lipopolysaccharide containing allergens and products of helminths can activate lung epithelial cells by acting on receptors located on the surface of the epithelium to produce thymic stromal lymphopoietin (TSLP), interleukin-25 (IL-25) and IL-33, which initiate T helper 2 type immune responses by acting on basophils, dendritic cells, B lymphocytes and non-T cells. Adapted from: (Paul and Zhu 2010).

stromal lymphopoietin (TSLP), and granulocyte-macrophage colony stimulating factor

(GM-CSF) (Figure 2). These cytokines enhance the release of IL-4 by innate immune cells to skew naïve T cell polarization to Th2 cell production. They also increase production of chemotactic factors for dendritic cell activation (Holgate 2008, Barnes 2008, Jacquet 2011, Holgate 2012).

The epithelium serves as an important barrier for the airways from harmful pathogens and stimuli from outside the environment. The airway epithelium consists of basal cells, ciliated cells and non-ciliated cells. Basal cells connect different cells of the epithelium to the basement membrane. They are attached to the basement membrane through hemidesmosomes (Evans, Van Winkle et al. 2001, Knight and Holgate 2003, Mary Mann-Jong Chang 2008). Ciliated cells on the other hand are present through out the entire conducting airways and they are mainly characterized by their columnar shape and their apical cilia (Mary Mann-Jong Chang 2008). Non-ciliated cells are mainly secretory cells with goblet cells being the major secretory cells in humans where as club cells are the major secretory cells in mice (Liu, Driskell et al. 2006, Mary Mann-Jong Chang 2008).

In asthma, there is increased epithelial detachment and denudation from the basement membrane. Defects in the epithelium also results in production of cytokines, growth factors and other pro-inflammatory mediators. Transforming growth factor beta (TGFβ), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF) and insulin-like growth factor-1 (IGF-1) are some of the

growth factors shown to be produced by the damaged epithelium, which contribute to airway remodeling. Goblet cell hyperplasia and metaplasia are major features of asthma. Goblet cell hyperplasia refers to the increase in the number of goblet cells in areas where they are usually present (Rogers 2003, Mary Mann-Jong Chang 2008). Goblet cell metaplasia on the other hand is the appearance of these goblet cells in areas where they are usually absent (Rogers 2003, Mary Mann-Jong Chang 2008). A typical example is the development of goblet cells from ciliated cells by the process of transdifferentiation (Tyner, Kim et al. 2006, Turner, Roger et al. 2011).

Also the mucus lining the airways protects the epithelium from external bacterial, cellular, and particulate debris. Mucus is made up of water and a complex mixture of proteins, lipid as well as mucin particles (Rogers 2002, Fahy and Dickey 2010). Mucin are high molecular weight proteins which are heavily glycosylated. The viscous nature of mucus is due to the presence of this heavily glycosylated high molecular weight protein (Kim, McCracken et al. 1997, Rogers 2002, Fahy and Dickey 2010). There are 13 mucin glycoproteins genes (MUC genes), which have been so far identified in humans. These are MUC1-4, MUC5AC, MUC5B, MUC6-9, MUC11-13. The predominant source of mucin glycoproteins is the goblet cells and mucous glands. It has been shown that, the main mucins present in mucus are MUC5AC and MUC5B (Thornton, Rousseau et al. 2008). Whereas MUC5AC is produced by goblet cells, MUC5B is secreted by submucosal glands (Thornton, Rousseau et al. 2008, Evans, Kim et al. 2009). In asthmatics, the predominant secreted mucin is MUC5AC, whereas studies have shown an

upregulation of Muc5ac in an antigen driven murine model of asthma (Evans and Koo 2009). Although MUC5B and Muc5b are both expressed in the airways, the levels of MUC5B are not increased in asthma (Evans, Kim et al. 2009). In asthmatic patients, there is increased number and size of mucous glands as well as increased goblet cells (Dunnill 1960, Dunnill, Massarella et al. 1969, Cho, Seo et al. 1996). Increased MUC5AC gene expression has been detected in asthmatics (Fahy 2001). Below in figure 3 are the difference in size of epithelium between a normal subject and an asthmatic. Compared to normal subjects, patients who died from mild-moderate asthma as well as severe asthma attacks had goblet cell hyperplasia and mucus plugs in peripheral airways (Aikawa, Shimura et al. 1992, Fahy 2002). The mucus plugs from asthmatic patients are rich in mucin (Sheehan, Richardson et al. 1995). Mucus plug can subsequently leads to airway obstruction and airflow resistance (Cohn 2006, Lai and Rogers 2010).

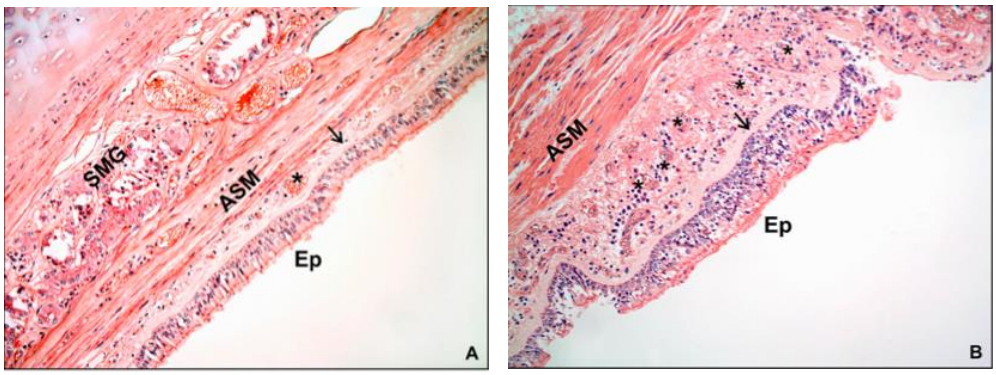

Figure 3: (A) Normal bronchial mucosa. The epithelium is intact and composed of ciliated columnar cells, and **(B) bronchial mucosa from a patient with fatal asthma.** There is epithelial damage and basement membrane thickening (arrow). The airway smooth muscle layer is thickened. Adopted from: (Mauad, Bel et al. 2007).

2.2.2 The role of the airway smooth muscles in asthma

Increase in size of airway smooth muscle is referred to as hypertrophy whereas the increase in number of airway smooth muscles is referred to as hyperplasia. These are predominant features, which are evident in both large and small airway walls of fatal and non-fatal cases of asthma (Figure 4) (Dunnill 1960, Cho, Seo et al. 1996).

One major difference between smooth muscle hypertrophy and hyperplasia is that, whereas the former occurs in the whole airway, the latter is found predominantly in the central airways. These mechanisms have been proposed to contribute to airway hyperresponsiveness.

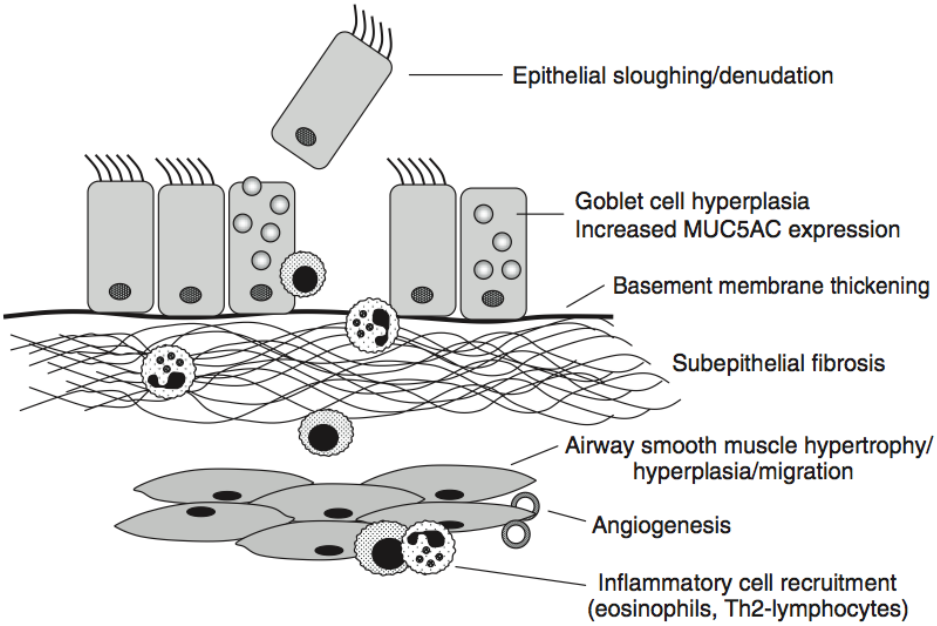

Figure 4: Airway remodeling in chronic asthma. These include goblet cell hyperplasia, subepithelial fibrosis, angiogenesis, smooth muscle hypertrophy and hyperplasia. Adopted from: (Tagaya and Tamaoki 2007).

2.2.3 The role of inflammatory cells in asthma

2.2.3.1 The role of Th2 cells in asthma

Polarization of naïve T cells to produce Th2 response requires a source of IL-4. IL-4 activates the transcription factors signal transduction-activated transcription factors 6 (STAT6) and GATA-3 (Paul and Zhu 2010). Innate immune cell types such as natural killer T cells, eosinophils, mast cells or basophils are the major sources of IL-4 for Th2 polarization (Ben-Sasson, Le Gros et al. 1990).

Although IL-4 plays a major role in Th2 cell polarization of naïve T cells, some studies have also reported that dendritic cells have the intrinsic ability to produce other cytokines such as IL-6, which contribute to Th2 cell polarization (Dodge, Carr et al. 2003, Feili-Hariri, Falkner et al. 2005). Apart from IL-4 and IL-6 signals, GM-CSF from dendritic cells is sufficient to induce a typical Th2 response (Lambrecht, De Veerman et al. 2000).

Selective expansion of Th2 cells secretes cytokines such as IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF. These cytokines modulate the pathogenesis of asthma as shown below in figure 5. These secreted cytokines have several functions which include survival of Th2 cells, B cell isotype switching to IgE synthesis, mast-cell differentiation and maturation, eosinophil maturation and survival and recruitment of basophils (Bowen, Kelly et al. 2008).

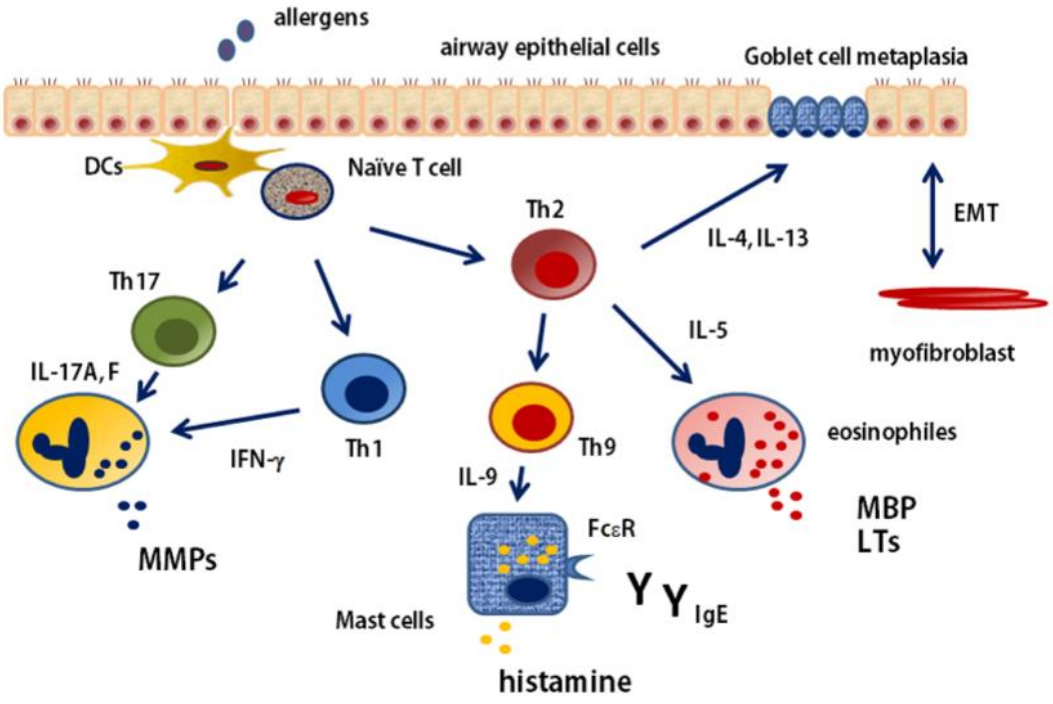

Figure 5: T cell immune response in the asthmatic airways. Naïve T cells receive allergen by dendritic cells. Th2 cells then produce cytokines: IL-4, IL-5 and IL-13. These cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth-muscle changes that contribute to the pathophysiology of asthma. Adapted from: (Kudo, Ishigatsubo et al. 2013).

The initial and sustained response of inhaled allergens is influenced by IgE levels. Clinical and epidemiologic studies have linked increased levels of IgE to the severity of asthma (Burrows, Martinez et al. 1989, Pastorello, Incorvaia et al. 1995).

Interaction of inhaled allergens with the epithelium and the dendritic cells which line the airways lead to initiation of IgE synthesis (Figure 6). Activated dendritic cells migrate to the lymph nodes and present antigens to T cells as shown in figure 9 (Noah and Becker 2000). Cytokines and co-stimulatory molecules influence the interaction

between T cells and presented allergens which influence isotype switching of B cells to production of a specific immunoglobulin.

The switching of B cells to IgE isotype is influenced by two major cytokines; IL-4 and IL-13 (Punnonen, Aversa et al. 1993, Pawankar, Okuda et al. 1997, Van der Pouw Kraan, Van der Zee et al. 1998, Harris, Chang et al. 1999, Kashiwada, Levy et al. 2010). The binding of co-stimulatory molecules such CD40 on B cells to its ligand on T cells influence isotype switching of B cells to IgE production (Tangye, Ferguson et al. 2002).

Once the levels of IgE is increased in the blood circulation, mast cells and peripheral blood basophils bind to IgE circulating in blood through the high IgE receptors (FcεRI) as shown in figure 5 (Boltz-Nitulescu, Melewicz et al. 1983, Baniyash and Eshhar 1984). The interaction of allergens and the high IgE receptors lead to cell activation and the release of preformed and newly generated mediators (Ishizaka, White et al. 1987, Stone, Prussin et al. 2010).

The early phase reaction is an IgE-mediated reaction, which occurs within minutes of allergen exposure. The IgE bound to FcεRI on mast cells and basophils is cross-linked by allergen, resulting in the release of preformed and newly synthesized mediators, which cause vasodilation, increased vascular permeability, bronchoconstriction and mucus secretion. The late phase reaction typically develops after 2–6 h and peaks 6–9 h after allergen exposure. It is characterized by airway narrowing and mucus hypersecretion. They reflect the local recruitment

and activation of Th2 cells, eosinophils, basophils and other leukocytes (Galli, Tsai et al. 2008).

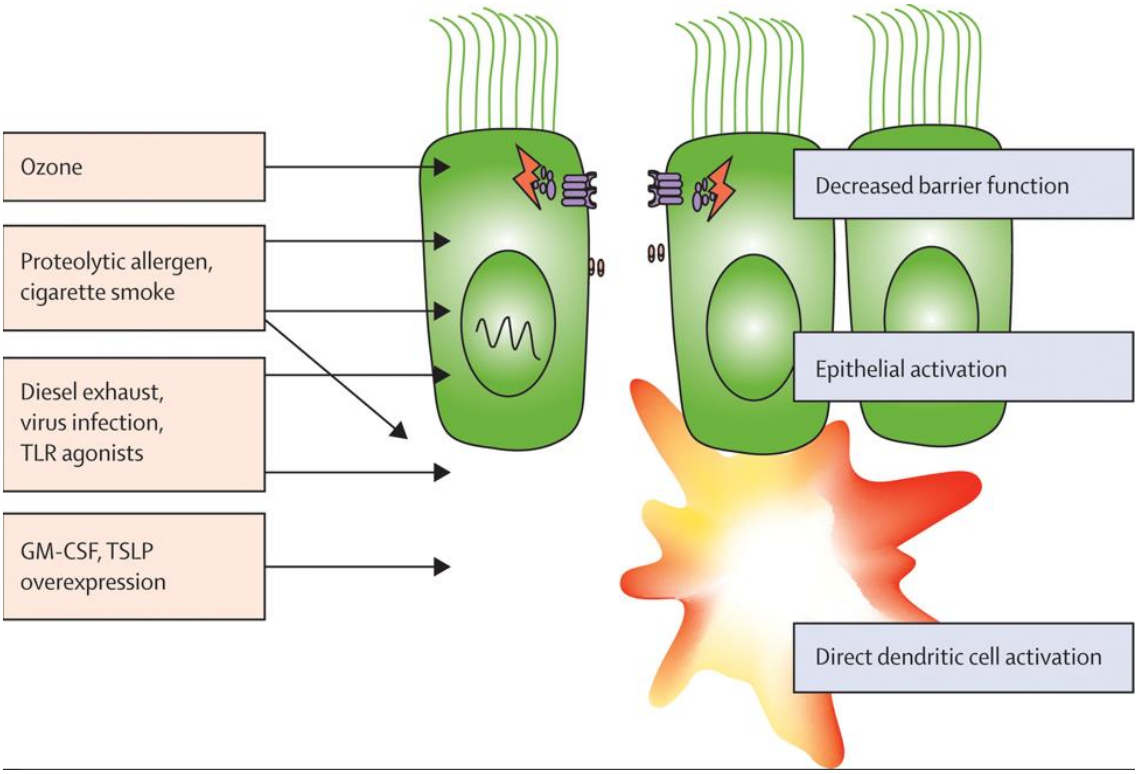

Figure 6: Schematic of mechanisms by which gene–environment interactions interfere with communication between epithelial and dendritic cells. Certain factors including those inside allergens interfere with epithelial permeability and barrier function, thereby exposing the dendritic cell network to the inhaled allergens. Other factors directly activate dendritic cells. Activated dendritic cells are better at antigen presentation. These risk factors lead to permanent changes in the epithelium (some of which are epigenetic, and long lived) and the dendritic cell system, so that allergens are better recognized. Adapted from: (Lambrecht and Hammad 2010).

2.2.3.2 The role of eosinophils in asthma

CD34+ precursor cells located in the bone marrow develop and mature to form eosinophils. These are responsive to the IL-5 cytokine. The IL-5 receptor α-chain is expressed on CD34+ precursor cells in the bone marrow upon allergen challenge (Sehmi, Wood et al. 1997). IL-5 produced from Th2 polarized cells may be the major source of this cytokine (Figure7).

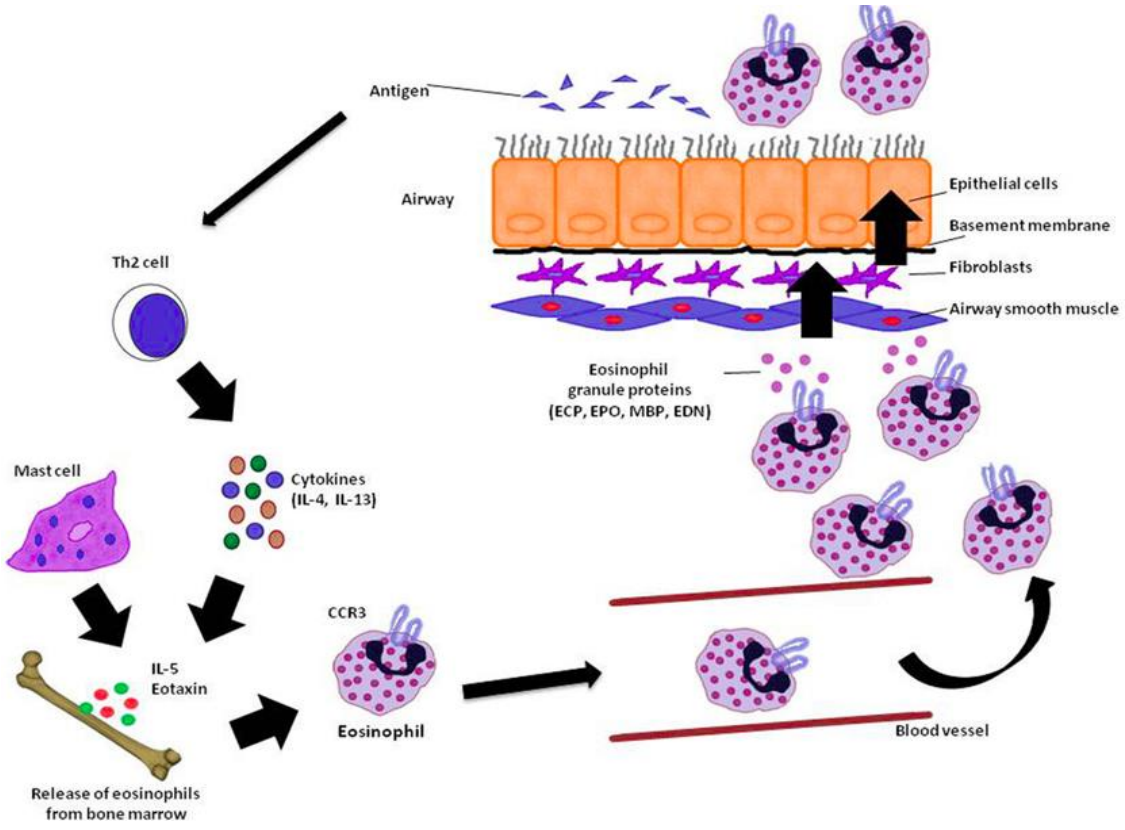

Figure 7: Eosinophil recruitment in asthma. Th2 cytokine producing T-cells as well as IL-5 and eotaxin stimulates the release of eosinophils from bone marrow. Elements like CC chemokine receptor (CCR3) also act on eosinophil recruitment to the lung. When eosinophils reach the airways they release granule proteins with cytotoxic, immunological, and remodeling-promoting properties in the lungs. Adapted from: (Possa, Leick et al. 2013)

During inflammation, eosinophils selectively infiltrate inflamed cells. Eosinophils in the circulating peripheral blood adhere to the endothelium of the blood vessels as shown in figure 8. Tethering of eosinophils to the endothelium causes the spreading and changing of shape of eosinophils. This is facilitated by proteins referred to as selectins. P and E-selectins are expressed on the endothelium where as L-selectin is expressed on leucocytes such as eosinophils (Sriramarao, Norton et al. 1996, Robinson, Frenette et al. 1999).

IL-4 activated endothelial cells also enhance binding of vascular cell adhesion molecule 1 (V-CAM) and intercellular adhesion molecule 1 (I-CAM) on activated endothelial cells to their ligand, very late antigen 4 (VLA-4) on eosinophils (Figure 8) (Sriramarao, Norton et al. 1996, Robinson, Frenette et al. 1999).

The activation of eosinophil is influenced by several cytokines such as IL-5, GM-CSF, and IL-3. Some studies have also shown that, eosinophil activation is influenced by the high affinity receptor for IgE, FcεRI (de Andres, Rakasz et al. 1997, Hasegawa, Pawankar et al. 1999, Kita, Kaneko et al. 1999).

Once eosinophils are activated via the high affinity receptor FcεRI, there is release of different proteins and toxins such as major basic protein, eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin and major basic

protein homolog which perpetuate inflammation (de Andres, Rakasz et al. 1997, Hasegawa, Pawankar et al. 1999, Kita, Kaneko et al. 1999).

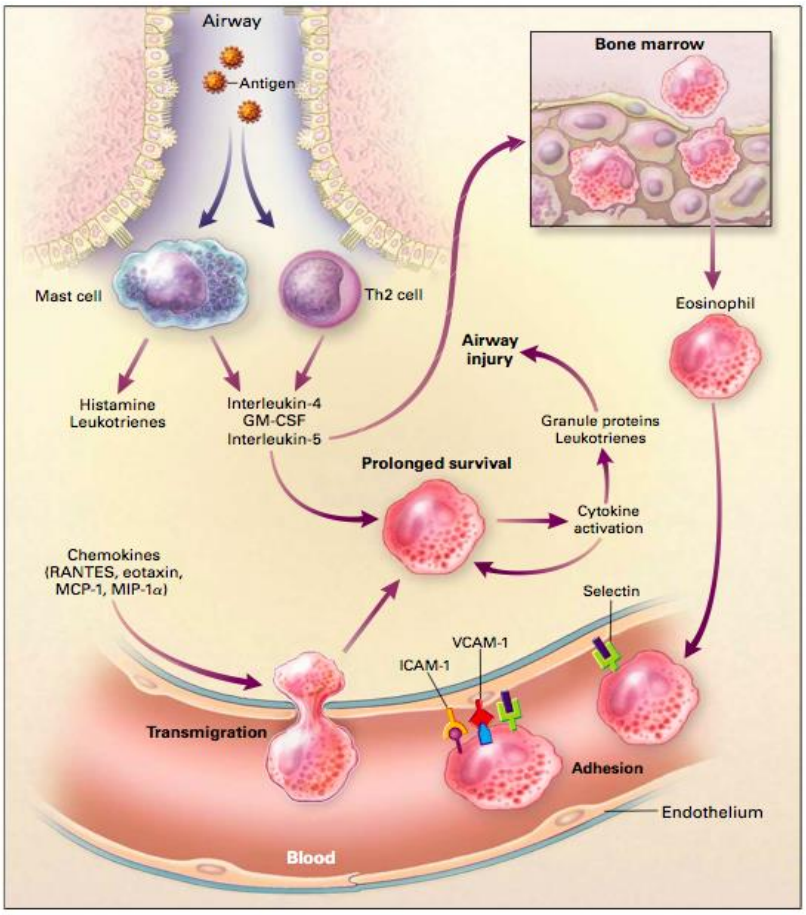

Figure 8: The role of eosinophils in allergic inflammation. Inhaled antigen activates mast cells and Th2 cells in the airway. They in turn induce the production of mediators of inflammation such as histamine, leukotrienes and cytokines including IL-4 and IL-5. IL-5 travels to the bone marrow and causes differentiation of eosinophils. Circulating eosinophils enter the area of allergic inflammation and begin migrating to the lung by rolling, through interactions with selectins and eventually adhering to endothelium through binding to adhesion proteins suchVCAM-1 and ICAM-1. Adapted from: (Busse and Lemanske 2001).

2.2.3.3 The role of mast cells in asthma

Monocuclear cells in bone marrow travel to mucosal and submucosal sites in the airway and mature into mast cells (Galli 1997). Cross linking of allergen to IgE on mast cells leads to activation of various cytosolic pathways which can release various pro-inflammatory mediators and cytokines. The release of histamine and leukotrienes for instance from mast cells as shown in figure 8 worsen airway obstruction by increasing constriction of airway smooth muscles (Conroy, Kennedy et al. 1990, Yamamura, Nabe et al. 1994).

Mast cells contain tryptase and chymase which have been shown to modulate responses of the airways to allergen (Clark, Abraham et al. 1995, Elrod, Moore et al. 1997). Major cytokines which regulate the Th2 response such as IL-3, IL-4, IL-5 and GM-CSF are also released upon crosslinking of IgE on mast cells to aeroallergens.

2.3 Other cell types in asthma

2.3.1 The role of dendritic cells in asthma

Pathogen or allergen disturbance of immune homeostasis of the airways results in recruitment of bone-marrow derived dendritic cells (GeurtsvanKessel, Willart et al. 2008, GeurtsvanKessel, Bergen et al. 2009).

Lung dendritic cells are located on the basolateral layer of the epithelium (Hammad and Lambrecht 2008). They are separated from allergens and pollutants by forming tight junctions with the epithelium (Sung, Fu et al. 2006, Kubo, Nagao et al. 2009). As a result of these tight junctions, interaction between dendritic cells and

allergens is by an active process (Hammad, Chieppa et al. 2009). This active process involves the ligation of allergens with TLR. This results in enhanced motility and antigen sampling behavior. Enhanced dendritic cell motility leads to translocation of dendritic cells to the mediastinal lymph node for antigen presentation to T lymphocytes as shown below in figure 9 (Vermaelen, Carro-Muino et al. 2001).

Antigen presentation by dendritic cells to naïve T cells results in T cell polarization to produce Treg, Th17, Th1 and Th2, which require a source of signal. This

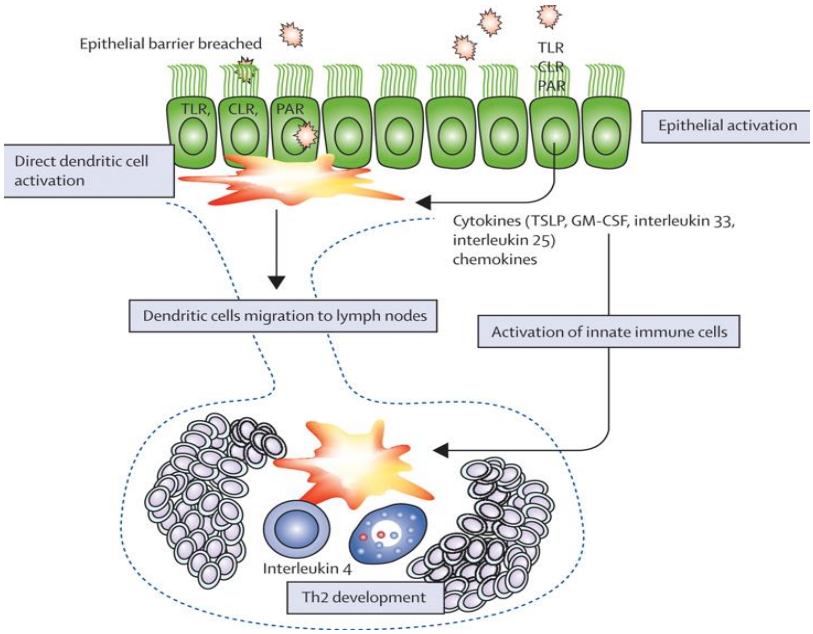

Figure 9: Schematic of dendritic cell function in allergic asthma. Dendritic cells just underneath the airway epithelial cells of the conducting airways sample inhaled antigens. Upon activation, they travel to the afferent lymphatic, the draining mediastinal nodes and present antigens to T cells. Adapted from: (Lambrecht and Hammad 2010).

process involves the interaction between major histocompatibility complexes class I and class II located on dendritic cells with T cell receptors. These sources of signal are shown below in figure 10. Therefore, Th2 polarization of naïve T cells requires a source of IL-4.

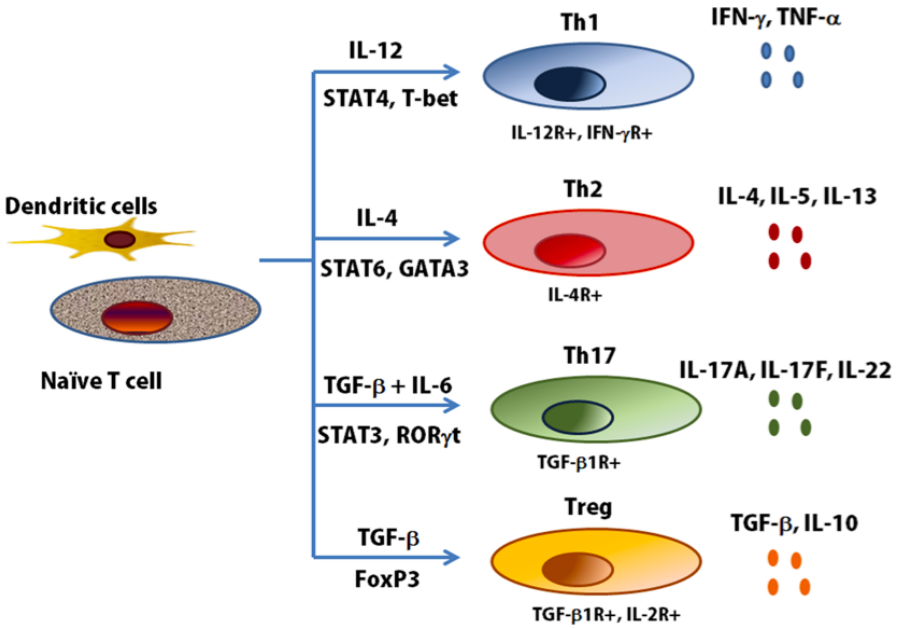

Figure 10: Schematic of T helper cell subsets and cytokine profiles. Th1, Th2, Treg and Th17 cells are a separate lineage of CD4+ T cells, distinct from other T cell subsets and every specific T helper cell produce its specific cytokines. Adapted from: (Kudo, Ishigatsubo et al. 2013).

2.3.2 The role of fibroblast in asthma

Submembrane thickening is also referred to as subepithelial fibrosis and it involves the thickening of the lamina reticularis, which is located just below the basement membrane (Overall, Wrana et al. 1989). The subepithelial myofibroblast releases

extracellular matrix proteins such as vitronectin, tenascin, proteoglycans, collagen I, collagen III, and fibronectin (Roche, Beasley et al. 1989). These molecules form a specialized network beneath the lamina reticularis which results in fibrosis (Brewster, Howarth et al. 1990).

TGFβ is a pro-fibrotic growth factor released by airway macrophages, epithelium, fibroblasts and eosinophils and it stimulates fibroblast to release extracellular matrix proteins. It also inhibits the synthesis of enzymes such as matrix metalloproteinase, which degrades extracellular matrix proteins. TGFβ also increases the synthesis of inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of matrix metalloproteinase (Overall, Wrana et al. 1989). These mechanisms can contribute to airway remodeling (Figure 11) and airway hyperresponsiveness.

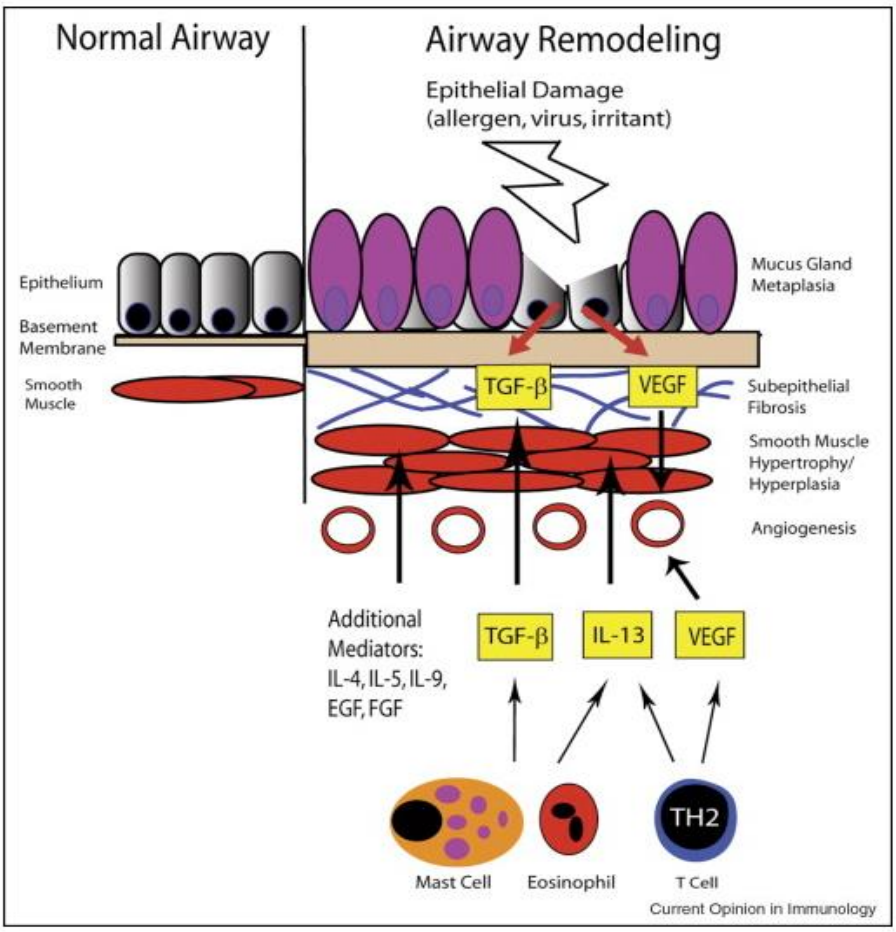

Figure 11: A diagram of normal and remodeled airway. Inflammatory and immune cells such as eosinophils, mast cells, Th2 cells as well as structural cells such as epithelium release cytokines, chemokines and growth factors, which contribute to mucous metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis, and angiogenesis. Adopted from: (Doherty and Broide 2007).

2.3.3 The role of blood vessels in asthma

In asthmatics there is neovascularization, which is increased number and size of blood vessels (Carroll, Cooke et al. 1997, Li and Wilson 1997). Two major growth factors have been shown to enhance angiogenesis and are increased in the broncho-

alveolar lavage (BALF) of asthmatic patients (Hoshino, Nakamura et al. 2001, Redington, Roche et al. 2001). These are vascular endothelial growth factor (VEGF) and FGF. Some studies have reported increased levels of VEGF and its receptor in asthmatic patients (Hoshino, Nakamura et al. 2001). These changes can contribute to airway hyperresponsiveness.

2.4 Transcriptional regulation in asthma

As mentioned earlier on, asthmatics present increased expression of inflammatory proteins such as cytokines, enzymes, receptors and adhesion molecules whose expression are regulated by transcription factors (Hunter and Karin 1992, Karin and Smeal 1992).

Transcription factors are proteins which bind to regulatory units on DNA to increase or decrease the rate of gene transcription hence increase or decrease the expression of proteins. Transcription factors bind to promotor regions of these genes in response to inflammatory stimuli and co-ordinate their expression (Barnes and Adcock 1998). Examples of these transcription factors are nuclear factor-κB (NF-κB), activator protein-1 (AP-1), nuclear factor of activated T-cells (NF-AT), cyclic AMP response element binding protein (CREB) and STAT (Shibasaki, Price et al. 1996). Cytokines themselves can serve as stimuli for the activation of transcription factors as shown in figure 12 below.

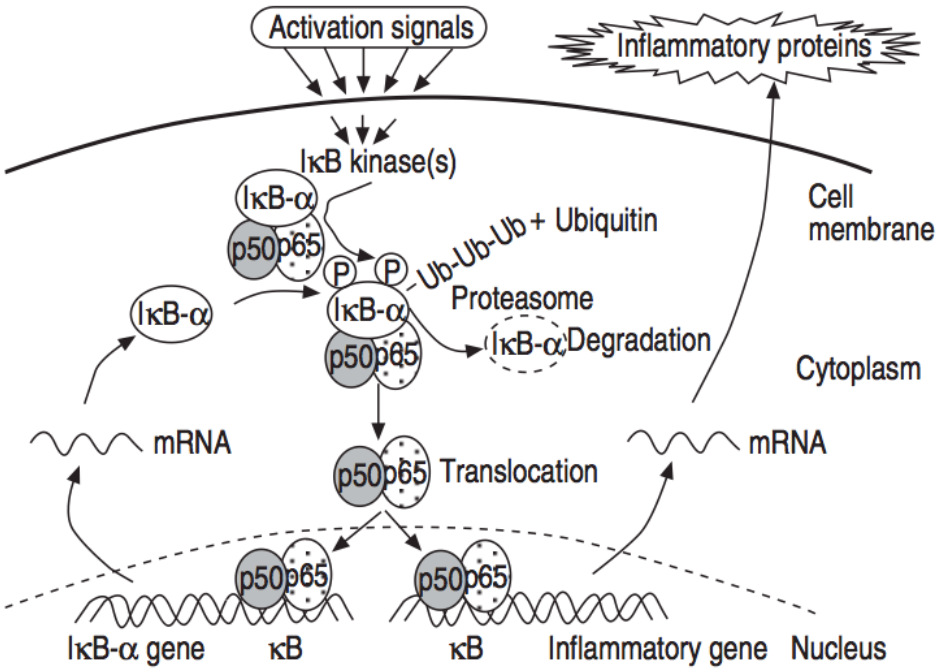

Figure 12: Inflammatory signals resulting in the coordinated expression of multiple inflammatory genes. These include cytokines, chemokine's, enzymes and adhesion molecules through their action on NFκ-B. Adopted from: (Barnes and Adcock 1998).

2.5 Asthma pharmacotherapy

Over the past 20 years, current medications have been modified to enhance disease therapy instead of developing new drugs for asthma management. Drugs that are currently used include:

- Inhaled β_2AR-agonists
- Inhaled corticosteroids (ICS)
- Theophyline
- Cromones
- Anticholinergics

2.5.1 β_2AR agonists

β_2AR expressed on smooth muscles from the trachea and throughout the bronchial tree of the airways are the major targets of inhaled β_2AR agonists (Carstairs, Nimmo et al. 1985, Hamid, Mak et al. 1991). β_2AR agonists can also affect other types of cells such as mast cells and epithelial cells (Butchers, Skidmore et al. 1980, Carstairs, Nimmo et al. 1985, Barnes 1995). β_2AR agonists act by increasing the intracellular messenger 3′, 5′ cyclic monophosphate (cAMP) with subsequent protein kinase A (PKA) activation, which is responsible for the control of smooth muscle tone (Johnson 1998).

β_2AR agonists are the most potent bronchodilatory drugs used for the management of asthma. There are subclasses of β_2AR agonist for asthma management. These include by duration of action:

- Short-acting β_2AR agonist (SABA)
- Long-acting β_2AR agonists (LABA)

- Ultra-long acting β_2AR agonists (Ultra-LABA).

Terbutaline and albuterol are examples of SABA available for asthma therapy. These drugs have a rapid onset of action hence are used as rescue medications. They however have a short duration of action (Linden, Rabe et al. 1996).

Salmeterol and formoterol on the other hand have a long duration of action of 12 hours hence can produce sustained bronchodilation. Although salmeterol has a slow onset of action, formoterol has a rapid onset of action hence in some cases can be used as rescue medications (Anderson 1993, Johnson, Butchers et al. 1993).

Ultra-LABAs currently under development for the management of asthma and chronic obstructive pulmonary disease (COPD) include indacaterol, carmoterol, milveterol and arformoterol (Cazzola, Matera et al. 2005, Cazzola, Segreti et al. 2010, Tashkin and Fabbri 2010). Although ultra-LABAs have not yet been approved for the management of asthma, indacaterol has been approved by the FDA and has recently been approved in Europe for the treatment of COPD (Cazzola, Bardaro et al. 2013).

β_2AR agonists used for the management of asthma can also be grouped as "rescue" medication for the acute relieve of symptoms of bronchospasm such as albuterol, terbutaline and formoterol as well as maintenance medications such as salmeterol and formoterol. Formoterol is used as both rescue and maintenance medication because of its rapid onset of action and long duration of action (Johnson 2001). They can

also be grouped into high efficacy β_2AR agonists such as formoterol and low efficacy β_2AR agonists such as albuterol, terbutaline and salmeterol (Johnson 2001).

Chronic administration of LABA is however associated with adverse effects, hence they are not used as monotherapy (Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006, 2007). LABAs are used in combination with inhaled corticosteroids for asthma therapy (Oppenheimer and Nelson 2008).

2.5.2 Glucocorticoids

Inhaled corticosteroids are the most effective anti-inflammatory agents in controlling asthma in all patients (Barnes 1995). Examples of currently used glucocorticoids include budesonide and fluticasone.

Several mechanisms have been proposed for their action in reducing inflammation. One mechanism by which corticosteroids switch off inflammatory response is by blocking the expression of inflammatory mediators through deacetylation of histones. This effect is mediated through histone deacetylase-2 (HDAC2). Once deacetylated, DNA is repackaged so that the promoter regions of inflammatory genes are unavailable for binding of transcription factors, which act to turn on inflammatory genes (Barnes, Adcock et al. 2005, Barnes 2006, Barnes 2006).

Some studies have shown they act on the airway epithelium to reduce the expression of nitric oxide, adhesion molecules, lipid mediators and cytokines (Mitchell,

Belvisi et al. 1994, Robbins, Barnes et al. 1994, van de Stolpe, Caldenhoven et al. 1993). Examples of these cytokines include IL-5, IL-8, RANTES and GM-CSF (Sousa, Poston et al. 1993, Kwon, Au et al. 1994, Kwon, Jose et al. 1995). In the epithelium, they also inhibit the expression of NF-κB and AP-1 (Manolitsas, Wang et al. 1995). These transcription factors have been shown to increase expression of cytokines and chemokines. They also inhibit the action of inflammatory cells such as T lymphocytes, macrophages, dendritic cells and eosinophils (Schleimer 1990).

In spite of their anti-inflammatory and immunomodulatory properties, deposition of inhaled corticosteroids in the airway can result in local side effects such as dysphonia (the inability to produce voice sounds using the vocal organs).

Although corticosteroids such as fluticasone propionate and budesonide undergo extensive first pass metabolism therefore allowing very little drug to enter the systemic circulation, their prolonged use can result in systemic side effects such as stunted growth in children, suppression of the immune system, bone fracture and osteoporosis in adults (Barnes 1995, Lipworth 1995). The side effects associated with the use of inhaled corticosteroids result in poor compliance of medication (Benbow and Naya 1994, Bosley, Parry et al. 1994).

2.5.3 Theophylline

Theophylline is a bronchodilator, which relaxes the airway smooth muscles. Recent studies have shown that, it may have anti-inflammatory and immunomodulatory

properties (Ward, McKenniff et al. 1993). Theophylline is a competitive nonselective phosphodiesterase (PDE) inhibitor (Essayan 2001). It raises the levels of cAMP and subsequently leads to PKA activation. Some studies have shown it inhibits TNF-α and leukotriene synthesis (Marques, Zheng et al. 1999, Peters-Golden, Canetti et al. 2005, Deree, Martins et al. 2008). See section 2.5.6 for more details on PDE inhibition and asthma. Theophylline can restore reduced HDAC2 activity as well as directly activate HDAC2 (Ito, Lim et al. 2002, Cosio, Mann et al. 2004). This reduces the expression of inflammatory genes. It reduces the late response to allergen (Pauwels, Van Renterghem et al. 1985, Ward, McKenniff et al. 1993). Theophylline can be administered orally as a slow-release medication hence it improves patient compliance compared to other inhaled medications such as corticosteroids (Kelloway, Wyatt et al. 1994).

The side effects associated with theophylline use include nausea, headache, seizures and cardiac arrhythmias. These side effects arise due to increased plasma concentration of the medication to toxic levels. Therefore patients on theophylline need to be monitored regularly. This can however be very expensive.

2.5.4 Cromones

Cromone medications are also referred to as mast cell stabilizers. These include nedocromil sodium and cromolyn sodium (Barnes, Holgate et al. 1995). Upon allergen exposure, mast cell destabilization and degranulation releases histamine, leukotrienes and other inflammatory mediators leading to bronchospasm. Cromone medications stabilize

mast cells by inhibiting IgE-regulated calcium channels (Heinke, Szucs et al. 1995). They have been shown to effectively reduce bronchospasm induced by allergens, exercise and sulphur dioxide. They also have steroid sparing properties (Svendsen and Jorgensen 1991).

However, they are very expensive and appear to be effective mainly in mild asthmatics. They also have a short duration of action hence they must be administered four times daily. This is inconvenient for patients and can reduce compliance to medication.

2.5.5 Anticholinergic agents

Anticholinergics act by binding to the muscarinic M3 receptors thereby blocking cholinergic vagal tone of the airway smooth muscles. These include ipratropium bromide, tiotropium bromide and oxitropium (Rebuck, Chapman et al. 1987, Gross 1988). Apart from their bronchodilatory properties, some studies have shown they reduce mucous hypersecretion (Tamaoki, Chiyotani et al. 1994). Although useful in COPD, they are however less effective bronchodilators compared to β_2AR agonists and also have a slow onset of action.

2.5.6 Phosphodiesterases 4 (PDE4) inhibitors as new therapeutics in development for the management of asthma

The activity of adenosine cAMP is tightly regulated by phosphodiesterases (PDEs). These enzymes catalyze the conversion of cAMP into adenosine

monophosphates (AMP) (Lugnier 2006). There are 11 distinct isoforms of PDEs and at least 44 distinct human PDEs. Among the 11 isoforms, those that are specific for cAMP are 4, 7 and 8 (Uzunov and Weiss 1972).

There are 4 distinct genes which encode the PDE4 enzyme. These genes are PDE4A, PDE4B, PDE4C and PDE4D. Depending on the number and length of the upstream conserved regions at the NH_2-terminal, PDE4 isoforms are long-, short- and super-short (Houslay and Adams 2003). PDE4 enzymes have anti-inflammatory and immunomodulatory properties. They are highly expressed in inflammatory cells such as mast cells, eosinophils, neutrophils, T cells and macrophages. They are also highly expressed in structural cells such as sensory nerves and epithelial cells (Torphy 1998). Although PDE4C is usually weakly expressed in cells PDE4A, PDE4B and PDE4D are highly expressed in inflammatory and immune cells.

Rolipram is a highly selective first generation PDE4 inhibitor. It has anti-inflammatory and immunomodulatory properties (Sanz, Cortijo et al. 2005). However, it has a narrow therapeutic window (the amount of drug that causes the therapeutic effect to the amount that causes toxicity) and it is associated with side effects such as diarrhea, nausea and vomiting (Burnouf, Auclair et al. 2000). This has necessitated the development of new second-generation PDE4 inhibitors such as roflumilast, which has a greater therapeutic index (Huang, Ducharme et al. 2001, Sturton and Fitzgerald 2002, Lagente, Martin-Chouly et al. 2005). Roflumilast for example has been shown to reduce

inflammation, fibrosis and goblet cell hyperplasia (Kumar, Herbert et al. 2003). In an antigen driven system of splenocytes from ovalbumin sensitized mice, rolipram reduced IL-5, a cytokine that increases eosinophilia (Foissier, Lonchampt et al. 1996).

Also, PDE3 and PDE7 are highly expressed in smooth muscles and T lymphocytes respectively. Therefore mixed PDE4/PDE3 as well as PDE4/PDE7 are under development to test if they have enhanced therapeutic benefits in patients with various inflammatory conditions.

2.6 β_2AR signaling

2.6.1 The canonical β_2AR cAMP/PKA pathway

G-protein coupled receptors (GPCRs), also referred to as 7-transmembrane receptors (7TMRs), are the largest, versatile and most ubiquitous of the plasma membrane receptors (Lefkowitz 2013). Rhodopsin is the prototypic GPCR whose crystal structure was obtained and it provided the first three-dimensional molecular model for GPCRs (Schertler, Villa et al. 1993, Krebs, Villa et al. 1998, Stenkamp, Teller et al. 2002).

The β_2AR is a member of the 7TMR, which is encoded by a gene on chromosome 5 (Kobilka, Dixon et al. 1987). When the β_2AR is activated by a ligand, it undergoes a conformational change resulting in trimeric $G\alpha\beta\gamma$-protein complex dissociating into a $G\alpha$ subunit and a $G\beta\gamma$ dimer as shown in figure 13 (Billington and Penn 2003). The $G\alpha$ subunit activates adenylyl cyclase (AC) whereas the $G\beta\gamma$ subunit transduces other

signals.

Adenylate cyclase is the enzyme, which catalyzes the conversion of intracellular ATP to cAMP and pyrophosphate (Beavo and Brunton 2002). Increased level of cAMP then leads to activation of the serine/threonine kinase, PKA and exchange proteins activated by cAMP (EPAC).

In the inactive state, PKA consist of two regulatory (R) and two catalytic (C) subunits as shown in figure 13 (Taylor, Kim et al. 2005). Four genes encode the regulatory subunit (RIα, RIβ, RIIα, and RIIβ) whereas three genes encode the catalytic subunit (Cα, Cβ, and Cγ) (Houslay 2006). Protein kinase A composed of the RI subunit are mainly located in the cytoplasm whereas those made up of the RII subunits are mainly associate with scaffold proteins (Tasken and Aandahl 2004, Smith, Langeberg et al. 2006).

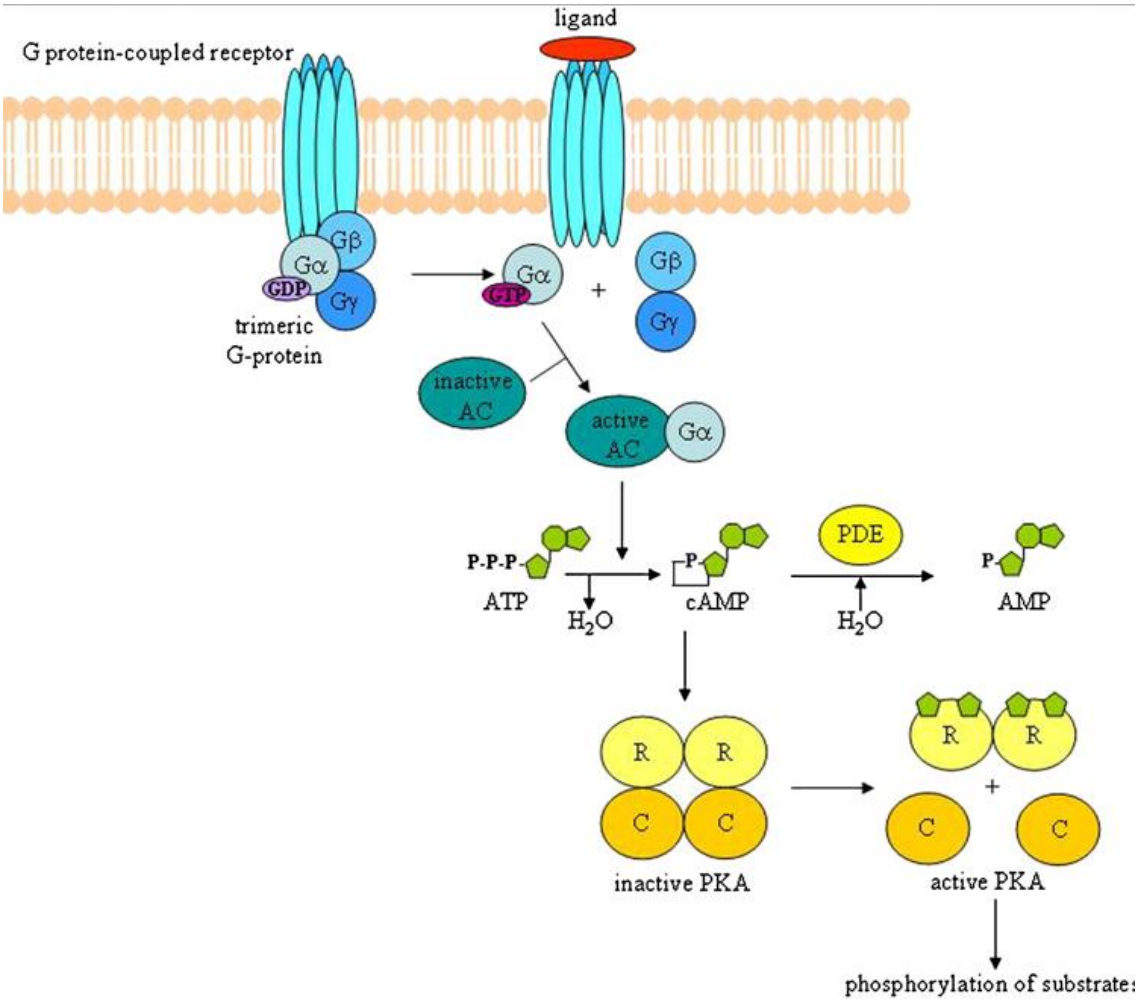

Figure 13: Schematic of the cAMP/PKA signaling pathway. Activation of a GPCR, leads to dissociation of the trimeric $G\alpha\beta\gamma$ protein to the active $G\alpha$ subunit. The $G\alpha$ subunit then activates adenylate cyclase which generates cAMP from ATP. cAMP then binds to the regulatory subunit of PKA holoenzyme and induces its dissociation. The catalytic subunit of PKA phosphorylates downstream substrates. cAMP is hydrolysed to AMP by phosphodiesterase enzymes. Adapted from: (Gerits, Kostenko et al. 2008).

As shown in figure 14 below, cAMP levels control muscle tone by activation of PKA, which in turn phosphorylates key regulatory proteins such as myosin light chain kinase and myosin light chain phosphatase. Activation of PKA also inhibits calcium ion release from intracellular stores, reduce calcium ion entry into the cells, and sequester intracellular calcium ions (Johnson 2001).

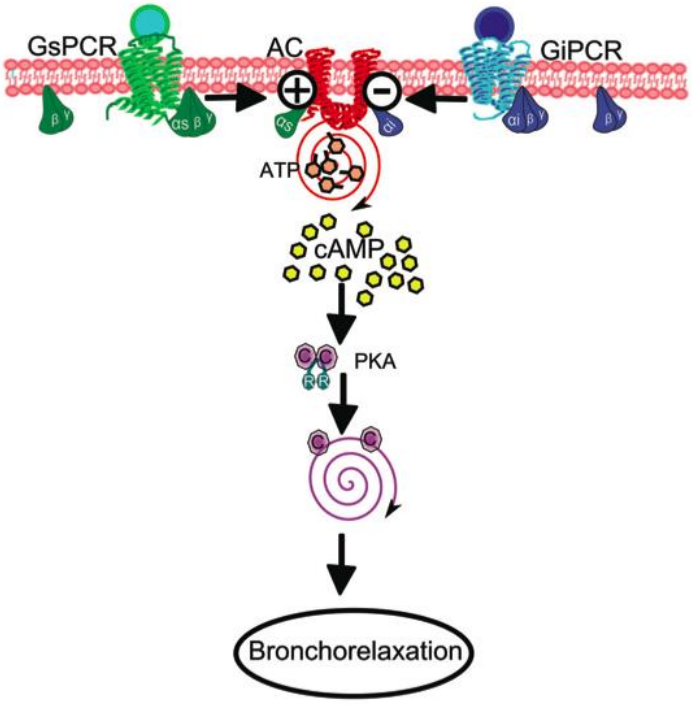

Figure 14: Schematic of the canonical cAMP signaling pathway. Following the binding of agonist, the β_2AR undergoes a conformational change promoting the dissociation of the Gα subunit from the Gβγ dimer. The Gαs-protein stimulates adenylate cyclase to catalyze the formation of cAMP from ATP. Binding of cAMP to PKA results in the release of the PKA catalytic subunits allowing them to phosphorylate a wide range of cellular targets, with the net result being bronchorelaxation. Adapted from: (Billington and Hall 2012).

2.6.2 Alternate β_2AR signaling pathways

Apart from the classic cAMP/PKA signaling of the β_2AR, emerging studies have shown that, the β_2AR can couple to diverse proteins as shown in figure 15 below and initiate other downstream signaling. Some of these signaling pathways are discussed below.

Recent findings suggest that, GPCRs undergo dimerization with important functional role. The β_2AR homodimerization is potentially enhanced by agonist and appears to involve the sixth transmembrane domain (Hebert, Moffett et al. 1996, Angers, Salahpour et al. 2000).

Also, AKAP79/150 promotes β_2AR phosphorylation after agonist stimulation and facilitates activation of MAPK pathways. They constitutively associate with the β_2AR, resulting in a complex that contains PKA, protein kinase C (PKC), and protein phosphatase 2B (Fraser, Cong et al. 2000).

The β_2AR has a consensus PDZ binding domain (DSLL) at the carboxyl terminus that has been shown to interact with the PDZ domain in the Na+/H+-exchanger regulatory factor (NHERF) in an agonist-dependent manner (Hall, Premont et al. 1998). NHERF is an ~44-kDa protein that binds to and attenuates the activity of the Na+/H+-exchanger type 3 (NHE3) (Weinman, Steplock et al. 1995). Moreover, the PDZ domain is an approximately 90 amino-acid domain that was initially identified in three structurally related proteins namely, PSD95, DLG, and ZO1 (Fanning and Anderson 1999).

Interacting with tyrosine kinases also regulates the β_2AR. Some studies have demonstrated the β_2AR is phosphorylated on tyrosine residues in an insulin-dependent manner (Hadcock, Port et al. 1992, Karoor, Baltensperger et al. 1995, Valiquette, Parent et al. 1995). The insulin promoted phosphorylation of the β_2AR in the carboxyl-terminal tail at Tyr350/354 and Tyr364 attenuates β_2AR activation of cAMP production in Chinese hamster ovary cells (Karoor, Baltensperger et al. 1995). Another study has shown that, insulin phosphorylation of the β_2AR on Tyr141 resulted in an enhanced ability to activate cAMP production in Chinese hamster fibroblasts (Valiquette, Parent et al. 1995).

N-ethylmaleimide-sensitive factor (NSF) has also been demonstrated to interact with the β_2AR. This is mediated by the C-terminal 3 amino acids of the β_2AR and has been shown to promote receptor internalization and recycling (Cong, Perry et al. 2001).

Finally, β_2AR has also been shown to interact with the α-subunit of eukaryotic initiation factor 2B (eIF2Bα), a nucleotide exchange factor that regulates translation (Klein, Ramirez et al. 1997).

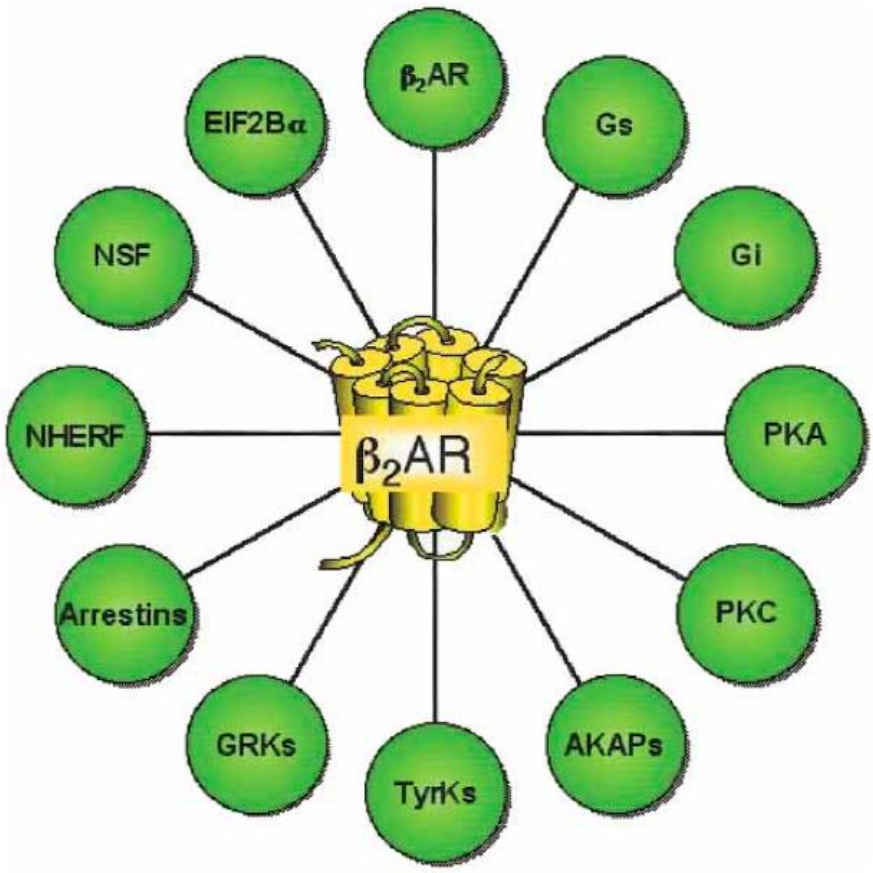

Figure 15: Schematic of known protein-protein interactions of the β2AR. Adapted from: (Benovic 2002)

2.6.2.1 β2AR Gs dependent ERK1/2 activation

The β2AR can activate as well as inhibit the ERK MAPK in a cAMP dependent fashion. The β2AR has been shown to activate ERK in a Gs-dependent manner in certain cell types such as HEK 293 cells and S49 lymphoma cells (Wan and Huang 1998, Schmitt and Stork 2000). This pathway is pertussis toxin-insensitive and involves Src,

Rap1 and B-Raf isoform, rather than c-Raf1. It is independent of Ras (Figure 16).

The Gs-dependent cAMP-mediated inhibition of ERK through the β_2AR has been extensively studied (Cook and McCormick 1993, Wu, Dent et al. 1993, Crespo, Cachero et al. 1995). This is mediated by the β_2AR Gs-dependent activation of PKA, which subsequently leads to phosphorylation and inhibition of c-Raf1 (Figure 16) (Cook and McCormick 1993, Wu, Dent et al. 1993).

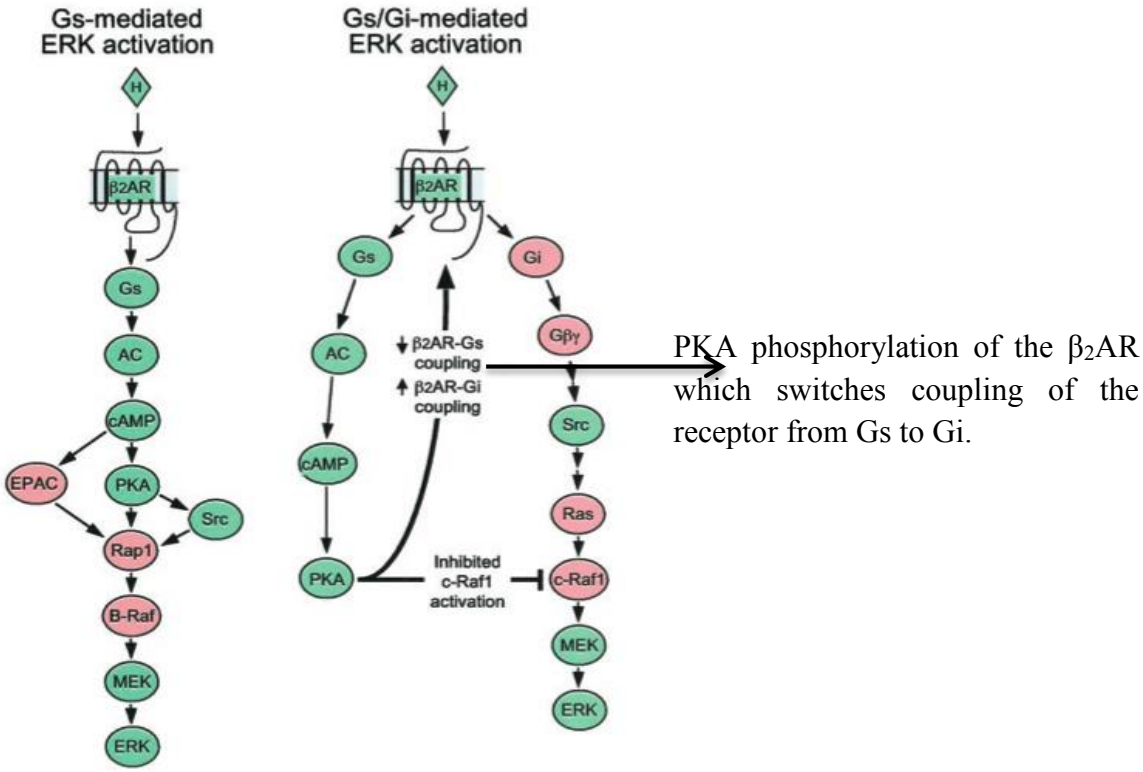

Figure 16: Schematic of Gs and Gi-mediated pathways for ERK activation by the β_2AR. Signaling intermediates colored green are shared by both pathways, whereas those in salmon are unique to one pathway. Adapted from: (Lefkowitz, Pierce et al. 2002)

2.6.2.2 β_2AR Gi signaling

There is substantial data, which shows that, the β_2AR can couple and transduce signal by binding to Gi proteins. Coupling of the receptor to Gi leads to ERK activation. This Gi mediated ERK activation is pertussis toxin-sensitive and it is transduced by Gβγ subunits, Src, Ras, and c-Raf1 as shown in figure 16 (Daaka, Luttrell et al. 1997, Luttrell, Daaka et al. 1999, Zou, Komuro et al. 1999). There is also data that suggest the PKA phosphorylation of the β_2AR, a mechanism which was previously shown to desensitize the receptor by reducing coupling to Gs proteins causes the receptor coupling to switch from Gs to Gi protein (Figure 16) (Abramson, Martin et al. 1988, Xiao, Ji et al. 1995).

2.6.2.3 β-arrestin signaling

β-arrestins function as signaling adaptors, where they bind the agonist-occupied form of the β_2AR. The β_2AR β-arrestin interaction leads to formation of complexes that cointernalize into endocytic vesicles. These complexes are also associated with sustained MAPK activity and function as signalosomes, which results in compartmentalization of 7TMR signaling. Signalosomes are signaling receptor complexes or scaffold associated with endosomes (Shenoy and Lefkowitz 2003, DeWire, Ahn et al. 2007). This results in the initiation of a second wave of signal transduction. β-arrestins bind Src, a tyrosine kinase involved in the Ras-dependent ERK1/2 activation (Luttrell, Ferguson et al. 1999). β-arrestins scaffolds upstream kinases such as ASK1, MEK1 and MKK4 for the activation of JNK3 MAPK (Figure 17) (McDonald, Chow et al. 2000). They also scaffold

the upstream kinases for ERK activation, which include Raf and MEK1 (Figure 17) (DeFea, Zalevsky et al. 2000, Luttrell, Roudabush et al. 2001). These discrete signaling modules localize the scaffolded kinases to specific areas in the cell. This helps to protect the active kinases from dephosporylation by phosphatases (Morrison and Davis 2003). By forming these scaffolding complexes, they act as signal transducers to direct signals from the cell membrane into the cell (Lefkowitz and Shenoy 2005). The scaffolding properties of β-arrestins leading to ERK1/2 activation and JNK3 activation are shown below in figure 17 (DeFea, Zalevsky et al. 2000, Luttrell, Roudabush et al. 2001).

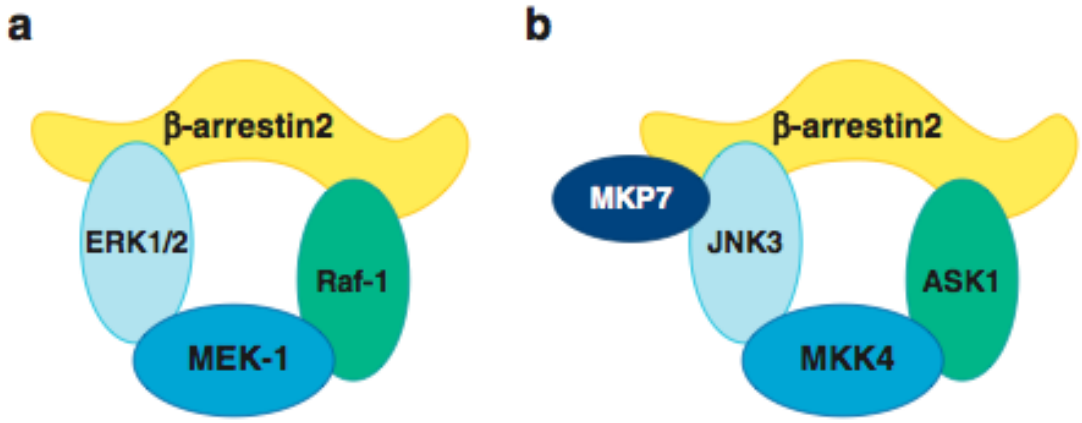

Figure 17: Model depicting β-arrestin as scaffold for ERK1/2 and JNK3 activation. Adapted from: (DeWire, Ahn et al. 2007)

2.6.2.4 Differences between G-protein-dependent and β-arrestin-dependent ERK1/2 activation

As previously described, activation of ERK1/2 by the β_2AR has two different components. This includes mechanisms involving the classical G-protein-stimulated production of the cAMP second-messenger-dependent kinase PKA, or through β-arrestins (Kobayashi, Narita et al. 2005, Shenoy, Drake et al. 2006, 2007). The G-protein-dependent ERK1/2 activation is rapid and transient and it is sensitive to inhibition by pertussis toxin or PKA inhibitors. The β-arrestin-dependent ERK1/2 activation on the other hand is slower in onset and more sustained in duration, and it is sensitive to depletion of β-arrestins but not inhibition by pertussis toxin or PKA inhibitors (Ebisuya, Kondoh et al. 2005, Shenoy, Drake et al. 2006). There are also G protein and β-arrestin independent mechanisms that can lead to ERK1/2 activation.

In most cells, ERK1/2 are mainly cytoplasmic. They can however move in between the cytoplasm and the nucleus. Activation of ERK1/2 dissociates them from MEK (their cytoplasmic anchoring proteins), and moves them from the cytoplasm into the nucleus. In the nucleus they phosphorylate several proteins such as transcription factors.

It is important to note that, activation of ERK1/2 does not always result in nuclear translocation and transcriptional regulation. Activation of ERK1/2 in a G-protein-

dependent fashion stimulates their translocation from the cytoplasm to the nucleus, whereas ERK1/2 activation in a β-arrestin dependent fashion restricts them to the cytoplasm (Lefkowitz and Shenoy 2005). The ERK1/2 activated by β-arrestin is mainly confined to the cytoplasm and hardly translocate into the nucleus (DeFea, Zalevsky et al. 2000, Luttrell, Roudabush et al. 2001, Tohgo, Choy et al. 2003, Ebisuya, Kondoh et al. 2005).

2.6.3 Compartmentalization of cAMP signaling

The cellular location and temporal organization of cAMP signaling can differ due to differences in cAMP micro-domains in different areas of the cell (Zaccolo and Pozzan 2002). Studies have shown how scaffold proteins and other molecules can finely tune cAMP micro-domains in cells and how the spatio-temporal differences in the levels of cAMP affect the specificity of signaling. Studies recording signals generated by cAMP using probes have observed startling disparities in signals generated in different areas of the cells such as the cytosol and the membrane (Wachten, Masada et al. 2010).

As shown in figure 18, a combination of different proteins, kinases and other molecules contribute to cAMP micro-domains. For example, different isoforms of PDEs, tethered PKA/AKAPs, phosphatases among others have been shown to compartmentalize cAMP signaling (Zaccolo and Pozzan 2002, Houslay, Baillie et al. 2007, Willoughby and Cooper 2007, Wachten, Masada et al. 2010).

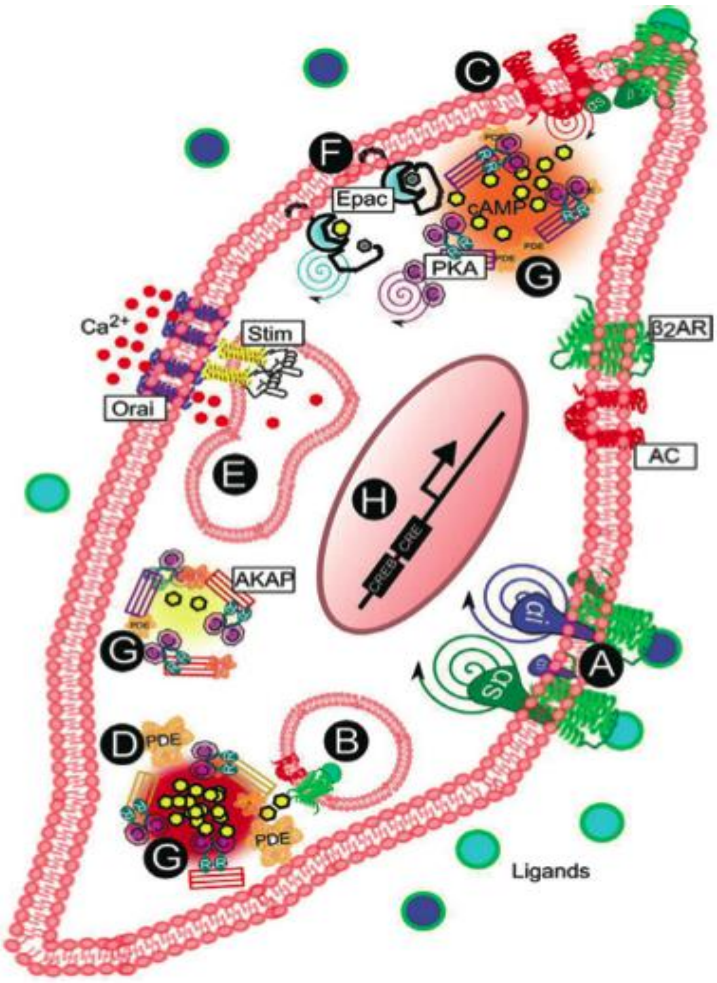

Figure 18: Schematic of the spatio-temporal control of cAMP. In G above, cAMP is localized to specific micro-domain of the cell. Adapted from: (Billington and Hall 2012).

2.6.4 β_2AR Desensitization

There are two type of β_2AR desensitization. These are homologous desensitization and heterologous desensitization.

2.6.4.1 Homologous desensitization

Homologous desensitization is mediated by phosphorylation of the receptor by G protein coupled receptor kinases (GRKs) and then subsequent binding to β-arrestin. Receptors in the agonist-occupied conformation are phosphorylated by GRKs.

There are seven known GRKs. GRK1 and GRK7 also referred to as rhodopsin kinase and cone opsin kinase respectively are highly expressed in the retina and they regulate the rhodopsin photoreceptor (Weiss et al., 1998) whereas GRK2-6 are ubiquitously expressed.

The GRK phosphorylation of the receptor requires membrane targeting of the GRK (Stoffel, Pitcher et al. 1997). The β-adrenergic receptor kinases GRK2 and GRK3 have C-terminal Gβγ-subunit-binding and pleckstrin-homology domains. The pleckstrin-homology domain binds free Gβγ subunits, which enables GRK2 and GRK3 translocation to the membrane. GRK phosphorylation alone is not sufficient for uncoupling receptors from G-protein, but requires β-arrestin binding.

There are four functional arrestin gene families (Freedman and Lefkowitz 1996, Ferguson 2001). Visual arrestin and cone arrestins are expressed in the retina whereas arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2) are ubiquitously expressed (Attramadal, Arriza et al. 1992, Lohse, Benovic et al. 1990). Binding of β-arrestins to the agonist occupied receptor results in complete receptor uncoupling from G proteins.

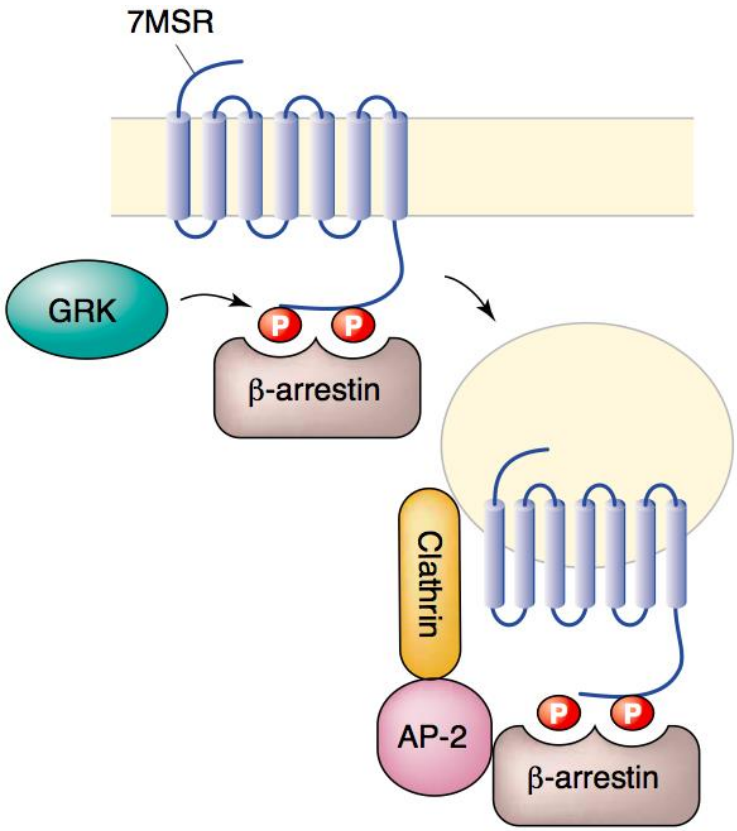

Figure 19: Schematic of β-arrestin-mediated clathrin /AP-2 dependent receptor endocytosis. Adapted from: (Lefkowitz and Whalen 2004)**.**

2.6.4.2 Heterologous desensitization

The process of heterologous desensitization begins within seconds after agonist exposure. This involves second-messenger-dependent PKA that phosphorylates serine threonine residues within the cytoplasmic loops and C-terminal tail domains of the β_2AR. Phosphorylation of these sites reduces the efficiency of receptor G-protein coupling (Benovic, Pike et al. 1985). However, agonist occupancy of the receptor is not required

for receptor desensitization (Lefkowitz 1993).

In heterologous desensitization, activation of the β_2AR can result in the inhibition of another GPCR. Therefore, activation of the β_2AR generates cAMP with PKA activation that causes inhibition of signaling by a second or heterologous GPCR. This type of desensitization is due to cross talk in signaling pathways that involves modifications of the activities of GPCRs, G proteins, or effectors. Therefore, the β_2AR for example can lead to inhibition of other Gs coupled receptors such as the histamine receptor (H_2 receptor) and prostaglandin E2 receptor (EP_2 receptor). The H_2 and EP_2 receptors are Gs coupled receptors (Freedman and Lefkowitz 1996, Daaka, Luttrell et al. 1997).

2.6.4.3 β-arrestins and receptor sequestration

The β_2AR internalization is a slow process that occurs within several minutes. GRK mediated phosphorylation of the receptor and subsequent β-arrestin binding promotes internalization of the receptor. β-arrestins contain two motifs that allow them to function as adapter proteins that link the β_2AR to components of the clathrin-dependent endocytic machinery as shown in figure 19 (Goodman, Krupnick et al. 1996). This interaction involves binding of a LIEF sequence of β-arrestins, with the N-terminal domain of the clathrin heavy chain (Krupnick, Goodman et al. 1997). Secondly, the RxR sequence on β-arrestin binds to the β2 adaptin subunit of the heterotetrameric AP-2 adaptor complex (Laporte, Oakley et al. 1999, Laporte, Oakley et al. 2000). The AP-2

complex then links the β_2AR to the clathrin endocytic machinery by binding to clathrin, dynamin and epidermal growth factor receptor substrate 15 (EPS-15). These molecules are involved in the initiation of clathrin-coated pit formation (Figure 19) (Kirchhausen 1999). Another molecule, the N-ethylmaleimide-sensitive fusion protein (NSF), an ATPase involved in intracellular transport has been shown to be important for receptor endocytosis (McDonald, Chow et al. 2000).

After endocytosis, the β_2AR receptor is either rapidly dephosphorylated by PP2A phosphatase in an acidified vesicle compartment and recycled back to the plasma membrane or targeted for degradation by lysosomes (Oakley, Laporte et al. 2000, Oakley, Laporte et al. 2001).

2.7 Receptor Theory

2.7.1 Classic receptor theory model

The canonical pathway for signaling by GPCRs is the G protein pathway. This is the classic model of receptor activation where receptors exist in an inactive state R. The inactive receptor R, can bind to a ligand to produce a binary complex. If the ligand is an agonist, the binary complex activates downstream effectors, whereas an antagonist does not activate downstream pathways (Figure 20) (Costa and Herz 1989).

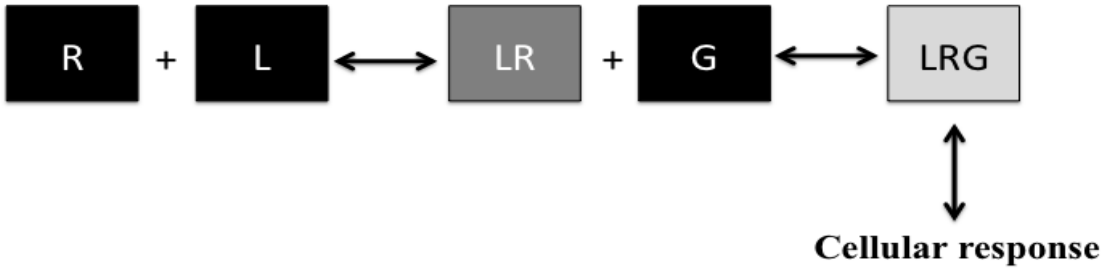

Figure 20: Classical model of GPCR activation. Receptor R when activated by a ligand L, forms a binary complex LR, that has high affinity for signaling molecules like G proteins. The LRG ternary complex can activate downstream signaling pathways eliciting cellular responses. Antagonist-bound receptor has low or no affinity for G and prevents downstream signaling. Adapted from: (De Lean, Stadel et al. 1980).

2.7.2 Two-state model of receptor activation

Constitutively active or spontaneously active receptors can signal in the absence of a ligand. Simultaneous with the discovery of constitutively active receptors was ligands for GPCRs, which are inverse agonists. Inverse agonists are ligands, which can turn off the activity of spontaneously active receptors. The classical model of receptor activation did not explain the existence of constitutively active receptors. It therefore became necessary to add another conformation usually termed the R* conformation (Barker, Westphal et al. 1994, Chidiac, Hebert et al. 1994, Bond, Leff et al. 1995, Leff 1995, Leff, Scaramellini et al. 1997, Stallaert, Dorn et al. 2012, van der Westhuizen, Breton et al. 2014).

The two-state model of receptor activation states that, receptors exist in equilibrium with each other in the R (inactive) and R* (active) conformations.

With this model, there exist three types of ligands:

- agonists
- antagonists
- inverse agonists.

An agonist A, has a relatively higher affinity for R* conformation and stabilizes the active conformation therefore shifting the equilibrium to produce more R*. An inverse agonist on the other hand, has a relatively higher affinity for the inactive conformation R, and stabilizes the inactive conformation therefore shifting the equilibrium to produce more R. An antagonist has equal affinity for both the R and R* conformation therefore does not shift the equilibrium in either direction, but will inhibit the effect of both agonists and inverse agonists (Figure 21).

The addition of a second receptor state has resulted in the theoretical possibility of an infinite number of receptor conformations. The addition of multiple states of the receptor require data from clinical or experimental studies.

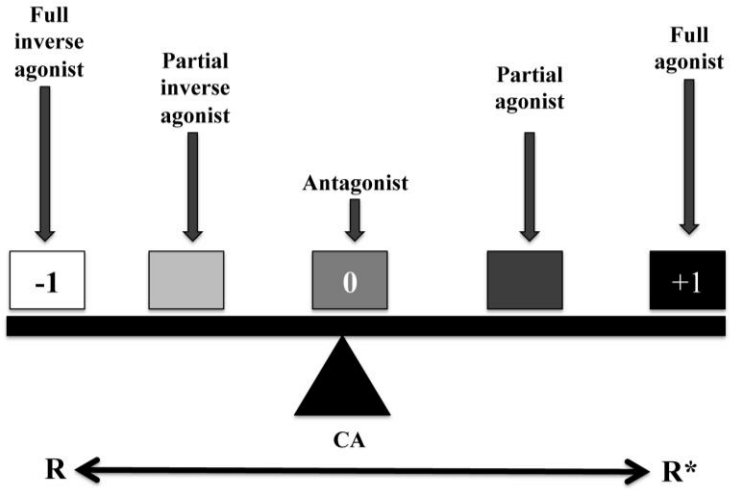

Figure 21: The two-state model of GPCR activation. Full agonists stabilize receptors in an active conformation (R*), whereas full inverse agonists stabilize the inactive receptor conformation (R). Neutral antagonists, simply referred to as antagonists, have no effect on the R/R* equilibrium, but blocks the effects of agonists and inverse agonists. Intrinsic activity is shown as -1, 0 and +1. Adapted from: (Walker, Penn et al. 2011)

2.7.3 Three-state model

Recent studies have shown that, GPCRs can activate more than one signaling pathway independent of each other (Barker, Westphal et al. 1994, Chidiac, Hebert et al. 1994, Bond, Leff et al. 1995, Leff 1995, Leff, Scaramellini et al. 1997, Stallaert, Dorn et al. 2012, van der Westhuizen, Breton et al. 2014).

To help explain this phenomenon, it became important to expand the two-state model of receptor activation to the three-state model. The three-state model postulates that, receptors can exist in three distinct conformations (Leff, Scaramellini et al. 1997, Scaramellini and Leff 1998). These are usually denoted the R (inactive conformation), R*

(an active conformation through pathway 1) and the R** (an active conformation through pathway 2) (Leff, Scaramellini et al. 1997, Scaramellini and Leff 1998).

3 Methods

3.1 Animals (mice)

Male and female six to eight weeks old wild-type (WT), SvJ/129 mice (Jackson Laboratory, BarHarbor, ME and Charles Rivers) and Epi-null mice (obtained from Steven Ebert, University of Central Florida) were housed under specific pathogen-free conditions. Mice used for the experiments each weighed approximately 20-25g. Animals were housed under standard conditions of humidity, temperature and a controlled 12 h light and dark cycle and had free access to food and water.

All animal experiments conformed to the rules and regulations of the University of Houston Institutional Animal Care and Use Committee (IACUC). We reported experiments involving animals in accordance with the ARRIVE guidelines (Kilkenny, Browne et al. 2010, McGrath, Drummond et al. 2010).

3.2 Genotyping

To ensure the mice used for the experiments had targeted disruption of the Phenylethanolamine N-methyltransferase (PNMT) gene and were null for the PNMT enzyme (Ebert, Rong et al. 2004), all Epi-null mice used for the experiment were genotyped. The PNMT enzyme converts norepinephrine to epinephrine as shown in figure 22 below.

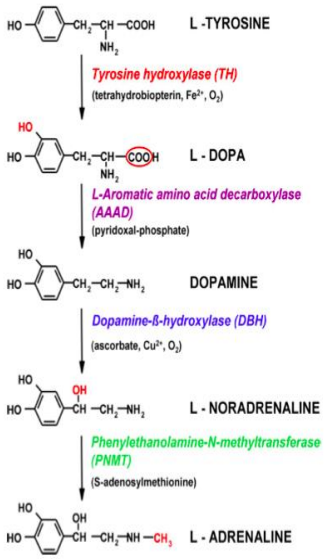

Figure 22: Pathway for catecholamine biosynthesis and its enzymatic steps. The step from the conversion of L-noradrenaline to L-adrenaline (epinephrine) is typical for the adrenal medullary cells. Adapted from: (Kvetnansky, Sabban et al. 2009)

Approximately 2-3 mm of the tails of mice were clipped and digested in 5 µL of proteinase K (Promega®) and 300 µL of tail digestion buffer. Tails were left overnight in an incubator set at 55-60^{O}C. 100 µL of protein precipitation solution was then added to the solution to precipitate the protein from the mixture of proteinase K and digestion buffer. Isolation of DNA proceeded by adding 300 µL of isopropanol to precipitate DNA, and was then washed with 70% ethanol and dissolved in 50 µL of sterile saline for polymerase chain reaction (PCR) analysis.

5` 3` Forward primer CAG GCG CCT CAT CCC TCA GCA GCC

5` 3` Reverse primer WT CTG GCC AGC GTC GGA GTC AGG GTC

5` 3` Reverse primer Epi-null GGT GTA CGG TCA GTA AAT TGG ACA CCG TCC TC

Approximately 80-100 ng of DNA combined with the above primers were used at temperatures and times listed in table 1 below using the PCR machine. The constituents of the PCR cocktail (25 µL), were GoTaq enzyme (Promega®), GoTaq green buffer (Promega®), $MgCl_2$ (Promega®), dNTP (Promega®), forward primer (IDT, Integrated DNA technologies), reverse primer (IDT, Integrated DNA technologies) Dep C treated water and 80-100 ng of DNA.

Steps in PCR	**Temperature**	**Time(min:sec)**
1	**94^0C**	**1:00**
2	**94^0C**	**0:30**
3	**60^0C**	**0:30**
4	**72^0C**	**1:00**
5	**72^0C**	**10:0**
6	**4^0C**	∞

Table 1: Details between each step for polymerase chain reaction (PCR).

The DNA bands were separated on a 1.5 % agarose gel mixed with ethidium bromide. The bands were visualized using fluochem with UV illumination. Using a 1 kb ladder, WT bands were located at 160 bp whereas knock-out bands were located at 100 bp.

3.3 Murine models of asthma

3.3.1 Ovalbumin sensitization and challenge model of asthma

Groups of randomized mice were sensitized once a week for three weeks with intra-peritoneal (i.p.) injections of 2 mg/ kg/ d of ovalbumin (Ova) (Sigma-Aldrich, St. Louis, MO) mixed with 2 mg of Alum (Imject Alum; Thermo Scientific, Pierce, Rockford, IL) on days 0, 7 and 14 (Figure 23). The mice were then challenged intra-nasally (i.n.) with 1 mg/ kg/ d Ova, for 5 days from days 41–45 (Figure 23). Control mice were sensitized with Ova and challenged with saline (Nguyen, Omoluabi et al. 2008, Thanawala, Forkuo et al. 2013).

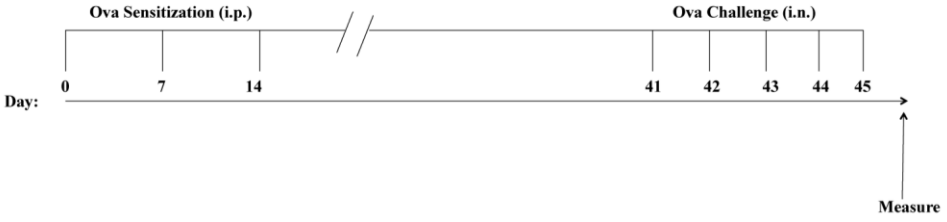

Figure 23: Ovalbumin sensitization and challenge (Ova S/C) protocol. Wild-type (WT) and Epi-null mice were sensitized intra-peritoneally (i.p.) with 2 mg/kg/day of ovalbumin (Ova) mixed with 2 mg of alum on days 0, 7, and 14. Following Ova sensitization, mice were challenged five times intra-nasally (i.n.) with 1mg/kg/day of Ova or vehicle from days 41-45.

3.3.2 IL-13 model of asthma

3.3.2.1 Anesthesia before IL-13 administration

Mice were anesthetized with 0.2 mL/10g i.p. Avertin® (2, 2, 2 tribromoethanol). Avertin stock solution was prepared by dissolving 25 g of Avertin in 15.5 ml of tert-amyl-alcohol (2-methyl 1-butanol) and stirring continuously in the dark for about 12 hours. Working solution was prepared by using 0.5 mL of stock solution and 39.5 mL of saline and filtered through 0.2 μ filters.

The mice became anesthetized (loss of righting reflex and no response to paw pinching) in approximately 5 minutes and remained anesthetized for about 15-20 minutes. Anesthetized mice were suspended on a plastic support for visualization of the trachea through the oropharynx. A light source was adjusted just below the vocal cords to provide the best view of the trachea. A metal laryngoscope was then used to raise the lower jaw of the mouse to keep the mouth open and displace the tongue to maximize oropharyngeal exposure, providing a clear view of the tracheal opening.

3.3.2.2 IL-13 administration

Recombinant IL-13 (Peprotech) was administered intra-tracheally to mice at a dose of 1.25 μg/25 g body weight using a Microsprayer® by Penn Century. The Microsprayer® was inserted into the tracheal opening and aerosolized IL-13 was released

into the trachea. Mice were euthanized 72 hours after IL-13 administration. Control mice were aerosolized to vehicle (Figure 24) (Wills-Karp, Luyimbazi et al. 1998).

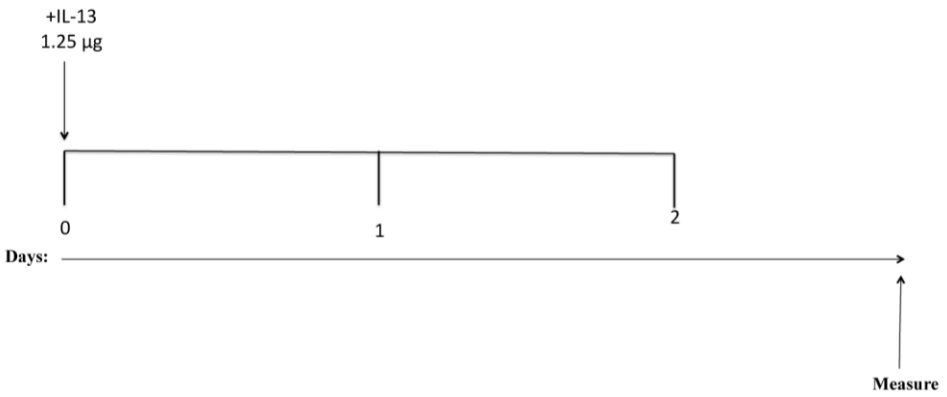

Figure 24: IL-13 treatment. Wild-type (WT) and Epi-null mice received vehicle or 1.25 µg IL-13 intra-tracheally. Intra-tracheal drug administration was done using the Microsprayer®. Mice were euthanized 72 hours after IL-13 administration.

3.4 Drug administration

3.4.1 Administration of β_2-adrenoceptor (β_2AR) agonists

Formoterol or salmeterol (Sigma-Aldrich, St. Louis, MO), both preferential long-acting β_2AR agonists were prepared in 99.95% saline and 0.05% DMSO (Merck KGaA, Darmstadt, Germany) or 0.05% ethanol (Sigma-Aldrich, St. Louis, MO) respectively. The solution was administered as twice-daily i.p. injections at a dose of 5 µg/kg (formoterol) or 1.5 µg/kg (salmeterol) in approximately100 µL per injection. Formoterol or salmeterol were administered to groups of ovalbumin sensitized and challenged (Ova

S/C) WT and Epi-null mice for a duration of 6, 9, 12 and 19 days. Drug treatment began 1, 4, and 7 and 14 days before the intranasal Ova challenge period and the treatment continued throughout the 5 days of Ova challenge. All final concentrations resulted in injections of approximately 100 μL per 20 g mice. Control mice received an equal volume of vehicle.

3.4.2 Administration of adenylate cyclase activator forskolin

Forskolin was administered via two routes; the oral route and inhalation of nebulized forskolin.

3.4.2.1 Oral forskolin

Forskolin at a dose of 4 mg/kg/day was triturated with powdered rodent diet chow (Lab Supply) and fed to mice *ad libitum* in cages using the Optimice J-feeders. Drug treatment began 14 days before the intranasal Ova challenge period and the treatment continued throughout the 5 days of Ova challenge. This resulted in a total of 19 days of drug treatment. Control mice received powdered rodent diet chow. Mice were monitored daily after drug administration, and also closely monitored to ensure 5 g food intake daily.

3.4.2.2 Nebulized Forskolin

Forskolin was suspended in 8 mL sterile saline to a final concentration of 0.0125 mg/mL and aerosolized to randomized groups of Ova S/C WT and Epi-null mice through a nebulizer (DeVILBISS/Palmo-Aide Compressor) driven by air for 30 minutes. Sterile

solutions of forskolin were prepared under the biological safety cabinet and filtered using a corning sterile syringe filter (0.2 μm) before aerosolized to the mice. Drug treatment began 14 days before the intranasal Ova challenge period and the treatment continued throughout the 5 days of Ova challenge. This resulted in a total of 19 days of drug treatment. Mice were monitored daily after drug administration. Control mice were aerosolized to an equal volume of vehicle.

3.4.3 Administration of phosphodiesterase 4 (PDE4) inhibitors

3.4.3.1 Roflumilast

Sterile solution of roflumilast (Santa Cruz) was prepared in 4% methylcellulose solution (Sigma-Aldrich, St. Louis, MO) and 2.5% polyethylene glycol (Sigma Aldrich, St. Louis, MO) under a biological safety cabinet. A fine suspension was obtained by stirring with a magnetic stirrer. Roflumilast was then administered at a dose of 5mg/ kg/ day (~150 μL) by oral gavage with 20G gavage needles to groups of Ova S/C WT and Epi-null mice once daily for 12 days. Control mice received an equal volume of vehicle. Mice were monitored daily after drug administration.

3.4.3.2 Rolipram

Sterile solution of rolipram (LC laboratories, Woburn MA) was suspended in 0.1% methylcellulose (Sigma-Aldrich, St. Louis, MO) solution and ground in a homogenizer to ensure a uniform suspension under the biological safety cabinet. A fine suspension was obtained by stirring with a magnetic stirrer. Rolipram was then

administered at a dose of 5mg/ kg/ day (~150µL) by oral gavage with 20G gavage needles to groups of Ova S/C WT and Epi-null mice once daily for 12 days. Control mice received an equal volume of vehicle. Mice were monitored daily after drug administration.

3.5 Total inflammatory cell count

At the end of the protocol, mice were euthanized with pentobarbitol (Sigma Aldrich, St. Louis, MO) at a dose of 100 mg/kg (i.p.). The lungs were dissected and tracheotamized with an 18G luer stub adapter. The left lung was clamped using a hemostat and the right lung perfused with sterile saline. Broncho-alveolar lavage fluid (BALF) was then pooled and aspirated into tubes. Total inflammatory cell count was obtained using a hemocytometer (Hausser Scientific, Horsham, PA) and light microscope as described previously (Nguyen, Omoluabi et al. 2008, Thanawala, Forkuo et al. 2013).

3.6 Differential cell count

After total inflammatory cell count, the remaining BALF was spun unto charged slides using Cytopsin®. Slides were stained with Wright Geimsa stain (Sigma-Aldrich, St. Louis, MO) and washed with double distilled water for 10 minutes. The slides were cover slipped with xylene based mounting media and the number of eosinophils were counted using light microscopy in five randomly chosen fields of view at 40X. The eosinophil was determined as a percentage of the total cells in each field and expressed as

eosinophil per mL of BALF (Nguyen, Omoluabi et al. 2008, Thanawala, Forkuo et al. 2013).

3.7 Chemokine measurements

Broncho-alveolar lavage fluid was collected from a different set of IL-13 treated mice. After total inflammatory cell count was completed, BALF was spun at 100 g for 5 minutes at 4^0C. The supernatant was collected into separate tubes, appropriately labeled and stored at -80^0C until chemokine measurements were evaluated. Chemokine measurements were performed by Pierce Searchlight (Woburn, MA) using multiplex enzyme-linked immunosorbent assay. The samples were provided to Pierce Searchlight in a blinded fashion.

3.8 Mucous metaplasia

After obtaining BALF from mice, the lungs were perfused with 4% cold formalin (Sigma-Aldrich, St. Louis, MO) through the tracheal cannula. After perfusion, the cannula was removed and the trachea was tied with a suture to avoid leakage of the formalin and to ensure the lungs are well fixed. The lungs were then isolated from the thoracic cavity and kept in formalin for 24 hours. The left lobe was then sectioned transversely into two halves after 24 hours as shown below in figure 25 A. These were placed in plastic cassettes, dehydrated and embedded in paraffin. The two halves

embedded in paraffin blocks were then sectioned afterwards (Piccotti, Dickey et al. 2012).

We then obtained 5 μm sections of each block unto charged slides using the microtome. The sections were dewaxed with histoclear and rehydrated in ethanol dilutions in the following order: 100%, 95%, 80%, 70% and phosphate-buffered saline. Following rehydration, the sections were oxidized in 1% periodic acid and incubated in fluorescent reagent for 20minutes at room temperature. The slides were then washed with distilled water, rinsed in acid alcohol and cover slipped with Canada balsam and methyl salicylate mounting medium to obtain periodic acid fluorescent (PAF) stained slides (Piccotti, Dickey et al. 2012).

The PAF-stained slides were examined under epifluorescence and images from random fields of the lung sections were acquired from the axial bronchi. The image J software was used to obtain mucin volume density by morphometrically determining the area of mucin glycoprotein in the epithelium per length of the basement membrane as shown below in figure 25 B (Evans, Williams et al. 2004, Kim, Kelemen et al. 2008, Piccotti, Dickey et al. 2012). Scale bars obtained during image acquisition were used to scale the images.

Mucin volume density= (MA)/LBM (4/3.142)

In this formular, MA= area of basement membrane

and LBM= length of basement membrane.

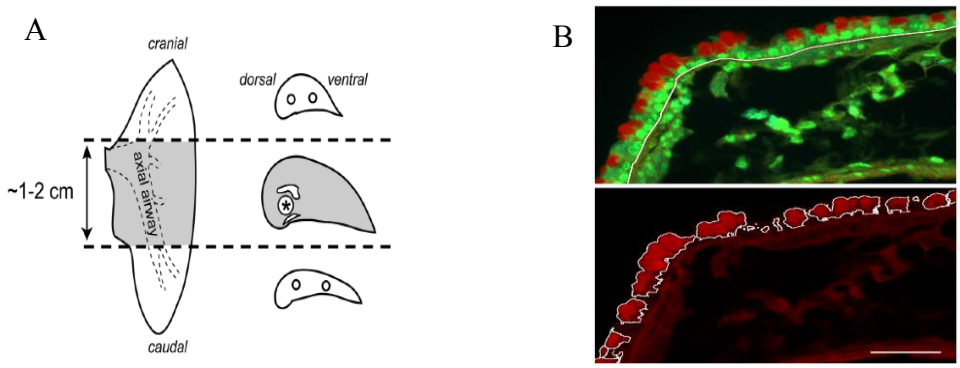

Figure 25: Schematic for lung sectioning. (A) The middle piece containing the main axial airway (gray) is sectioned into 2–3 mm thick segments. The segments are embedded in paraffin while keeping the airway positioned for sectioning onto charged microscope slides. Non-mucin producing regions are the white sections. (**B**) In two color images, the full length of the airway region to be analyzed is measured (white line along basal lamina). In red-only images, the area of red in PAF stained epithelium is identified and measured using Image J software. The area and length of staining are applied to the formula for volume density calculation shown above. Adapted from: (Piccotti, Dickey et al. 2012).

3.9 Airway hyperresponsiveness

At the end of the protocol on day 46, mice were anaesthetized with a cocktail of 240 mg/kg ketamine (Ketaject; Bioniche Teoranta Inverin Co., Galway, Ireland) and 48 mg/kg xylazine (AnaSed Akorn Inc., Lake Forest, IL) respectively for airway hyperresponsiveness. Airway hyperresponsiveness was measured using the forced oscillation method with the flexivent apparatus (SCIREQ, Montreal, PQ, Canada). The flexivent was calibrated with an 18G needle before the beginning of every experiment.

The trachea was exposed and mice were intubated with the calibrated 18G cannula. Mice were then connected to the Y-adaptor of the flexivent apparatus and ventilated with a tidal volume of 7.5 mL/kg, a respiratory rate of 150 breaths/min, and a positive end-expiratory pressure between 1.5 and 3 cmH_2O. Ventilator based assessment of airway resistance was obtained at baseline to saline and increasing doses of methacholine. Methacholine was nebulized at a dose of 3.125, 6.25, 12.5, 25 and 50 mg/mL directly into the ventilatory circuit using the flexivent nebulizer.

To prevent animal hypothermia, mice were maintained at a constant temperature of 37^0C by lying supine on heating pads. Anesthesia was maintained throughout the experiment by administration of 20% ketamine cocktail every 20 minutes until the end of the experiment.

Three peak airway resistance values were averaged at each dose as the airway resistance for each mouse. Airway resistance values were used to calculate airway sensitivity (PC_{100}, the provocative methacholine dose required to double baseline resistance) and airway reactivity (K, the slope) using non-linear regression by graph pad prism (Evans, Bond et al. 2003).

3.10 Statistical Analysis

Data are expressed as mean ±SEM. One-way analysis of variance (ANOVA) with Tukey-post hoc test was performed for statistical difference for multiple groups. For comparison of two groups, a two-tailed unpaired Student's t test was used. Statistical significance was defined as $p<0.05$ using GraphPad Prism 4 (Rosner B 2011). Power

analyses based on previous results were used to determine adequate group sizes and mice were randomly assigned into treatment or vehicle groups.

4 Determining whether constitutive or ligand-induced activation of the β_2AR was required in development of the asthma phenotype

*The data in the ovalbumin sensitization and challenge murine model of asthma has been published in the *Am. J. Respir. Cell Mol. Biol.* February 2013, Volume 48, Issue 2, pages 220-229.

4.1 Introduction

We have previously shown that, chronic administration of a subset of 'β-blockers' with inverse agonist properties such as nadolol, significantly attenuated cardinal features of the asthma phenotype including inflammation, mucous metaplasia, and airway hyperresponsiveness (AHR) in murine asthma models (Callaerts-Vegh, Evans et al. 2004, Lin, Peng et al. 2008, Nguyen, Omoluabi et al. 2008). In genetic studies, β_2-adrenoceptor (β_2AR) null mice (β_2AR-KO) also exhibited attenuated asthma phenotypes and phenocopied chronic administration of β_2AR inverse agonists (Nguyen, Lin et al. 2009). These studies identified β_2AR signaling as required for the pathogenesis of the asthma phenotype in murine models. However, we did not establish whether the β_2AR signaling allowing the development of the asthma phenotype was due to constitutive (spontaneous) signaling by empty receptors, or whether ligand-induced activation of the β_2AR was necessary.

We then tested the role of constitutive versus ligand-induced β_2AR signaling in an antigen-driven murine model of asthma. Due to the apparent requirement for inverse

agonism as an essential property of beneficial ligands in murine models of asthma, we hypothesized that constitutive signaling was required for development of the asthma phenotype (Callaerts-Vegh, Evans et al. 2004, Lin, Peng et al. 2008, Nguyen, Omoluabi et al. 2008). We adopted two approaches to test our hypothesis, the 'loss-of-function' approach where we depleted the endogenous ligand (primarily performed by my colleague, Vaidehi Jatin Thanawala), and the 'gain-of-function' approach where we replaced the β_2AR agonist (my experiment).

In loss-of-function studies, we depleted the endogenous hormone for the β_2AR, epinephrine. Whereas epinephrine activates all 9 adrenergic receptors, norepinephrine activates 8 of the adrenergic receptors with the exception of the β_2AR (Lands, Arnold et al. 1967). Pharmacological sympathectomy in wild-type (WT) mice was done using reserpine, which inhibits vesicular monoamine transporter (VMAT) required to load monoamines into vesicles and protect them from degradation by monoamine oxidase (MAO) and catecholamine O-methyltransferase (COMT) (Goodman LS 2011). In the previously described ovalbumin sensitization and challenge (Ova S/C) model of asthma, a loading dose of 5 mg/kg reserpine was administered to WT mice intra-peritoneally (i.p.) prior to intra-nasal (i.n.) ovalbumin (Ova) challenge. This was followed by a maintenance dose of 0.3 mg/kg for a duration of 5 days during Ova challenge. Reserpine treatment resulted in a significant reduction in circulating and adrenal levels of norepinephrine and epinephrine. The levels of both epinephrine and norepinephrine were below quantification in plasma, and the epinephrine content of the adrenal glands was reduced

to <3% of control mice (Table 2) (Thanawala, Forkuo et al. 2013). Reserpine and vehicle treated WT mice were used in an Ova S/C model of asthma. Reserpine treatment resulted in significant reductions in inflammatory cells, mucous metaplasia and AHR (Thanawala, Forkuo et al. 2013).

In a genetic model of epinephrine depletion, Cre recombinase produced targeted disruption of the gene coding for the enzyme that converts norepinephrine into epinephrine, phenylethanolamine N-methyltransferase (PNMT) (herein termed Epi-null mice) (Ebert, Rong et al. 2004). Mice with genetic deletion of the enzyme had no detectable epinephrine in plasma, and <2% of the epinephrine in adrenal glands as compared with control mice (Table 2) (Thanawala, Forkuo et al. 2013). In the Ova S/C model of asthma, Epi-null mice showed significant reductions in inflammatory cells, mucous metaplasia and AHR (Thanawala, Forkuo et al. 2013). Therefore, loss-of-function studies using pharmacologic and genetic sympathectomy attenuated three cardinal features of the asthma phenotype, namely reductions in inflammatory cells, mucous metaplasia and AHR (Thanawala, Forkuo et al. 2013).

In the next set of experiments, we used a gain-of-function pharmacologic approach to study the role of ligand-activated β_2AR receptors for the development of the asthma phenotype. We hypothesized that, treatment with the β_2AR agonist formoterol, a drug currently used in asthma therapy to Epi-null mice will paradoxically restore features of the asthma phenotype in murine asthma models.

	Plasma (pg/μl)		Adrenals (ng/mg of tissue)	
	Norepinephrine	**Epinephrine**	**Norepinephrine**	**Epinephrine**
WT	166.94 ± 18.06	135.88 ± 18.87	233.64 ± 71.45	244.13 ± 50.86
WT Ova S/C	96.64 ± 25.80	107.93 ± 27.98	185.85 ± 59.33	217 ± 43.26
Epi-KO	153.11 ± 7.52	BLQ	326.97 ± 54.06	12.36 ± 5.28
Epi-KO Ova S/C	86.91 ± 15.66	BLQ	374.12 ± 25.41	1.19 ± 0.37
WT + Reserpine	BLQ	BLQ	8.64 ± 1.55	6.48 ± 2.31
WT + Reserpine Ova S/C	BLQ	BLQ	1.12 ± 4.85	5.86 ± 2.91

Table 2: The levels of epinephrine and norepinephrine in wild-type (WT) and Epi-null mice in plasma and adrenals.

4.2 Approach

We treated Ova S/C (Figure 26) or IL-13 treated (Figure 27) Epi-null mice with twice-daily i.p. injections of the preferential β_2AR agonist formoterol at a dose of 5 μg/kg.

Formoterol was administered instead of epinephrine for several reasons. First, epinephrine has an extremely short half-life of approximately 2 minutes. Even when dissolved in 100 μM ascorbic acid to prevent oxidation, incubation in a water bath at 38^{O}C resulted in significant degradation after just 3 days. Second, formoterol has similar efficacy to epinephrine and a comparably rapid onset of action, but has a longer half-life of approximately 12 hours, allowing twice-daily administration (Anderson 1993). Lastly, formoterol is on the market for asthma therapy and has been associated with adverse effects with chronic administration, which made it an interesting drug to test (Mann, Chowdhury et al. 2003, Salpeter, Buckley et al. 2006, Wolfe, Laforce et al. 2006, Cates and Cates 2012).

To enhance the accuracy and validity of our data, we tested our hypothesis in two different murine models to of asthma (Ova S/C and the IL-13 murine model of asthma). In the Ova S/C and the IL-13 model of asthma, formoterol administration started 1, 4, 7 and 14 days prior to the first ovalbumin challenge or intra-nasal IL-13 treatments respectively, and continued till the end of the protocol. This resulted in a duration of 6, 9, 12 and 19 days in the Ova S/C model (Figure 26) and for a duration of 4, 7, 10 and 17 in

the IL-13 model (Figure 27). Formoterol was administered at different time points to study the short-term and long-term effects of β_2AR activation in murine models of asthma. We administered twice-daily i.p. injections of formoterol at a very low dose of 5 μg/kg. This dose was selected based on several studies using formoterol at a dose of 100 mg/kg/d, and a study using what was described as a "low dose" of 25 mg/kg/d of formoterol, but these were examining formoterol's effect as a drug (Harcourt, Schertzer et al. 2007, Pearen, Myers et al. 2008, Koopman, Gehrig et al. 2010, Leger, Koopman et al. 2011). In our study, we were attempting to "restore" β_2AR signaling, and not elicit a "drug" response.

Mice were phenotyped for inflammatory cell infiltration into the airways, chemokine production in broncho-alveolar lavage fluid (BALF), mucous metaplasia and AHR as previously described in methods.

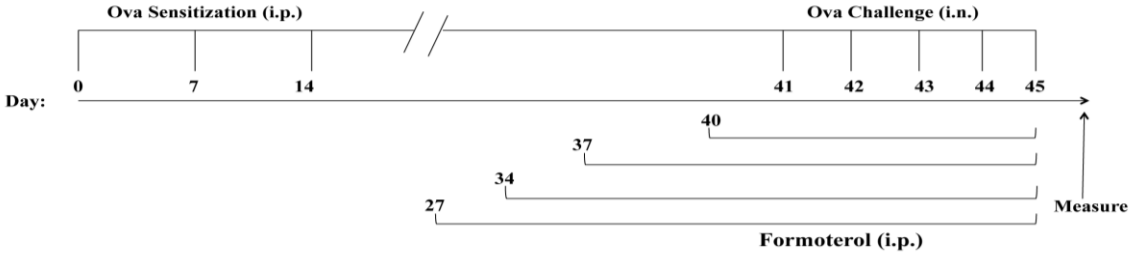

Figure 26: Ovalbumin sensitization and challenge (Ova S/C) treatment protocol. Wild-type (WT) and Epi-null mice were sensitized intra-peritoneally (i.p.) with 2 mg/kg/day of ovalbumin (Ova) mixed with 2mg of alum on days 0, 7, and 14. Following Ova sensitization, mice were challenged five times intra-nasally (i.n.) with 1mg/kg/day of Ova or vehicle from days 41-45. At different time points as shown above, groups of Ova

S/C Epi-null mice received twice daily i.p. injections of vehicle or formoterol at a dose of 5 µg/kg.

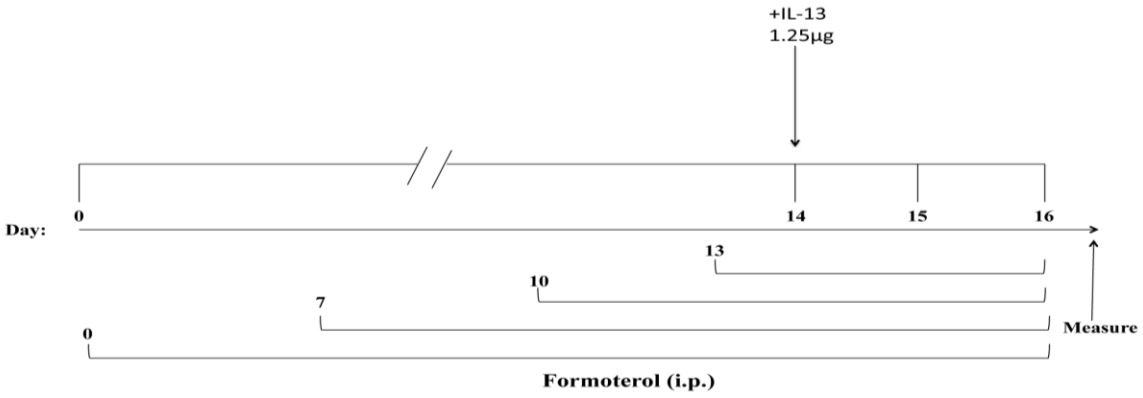

Figure 27: IL-13 treatment protocol: Wild-type (WT) and Epi-null mice received vehicle or 1.25 µg IL-13 intra-tracheally. At different time points as indicated above, groups of IL-13 treated Epi-null mice began to receive twice-daily i.p. injections of vehicle or formoterol at a dose of 5 µg/kg.

4.3 Results

4.3.1 Effect of formoterol administration in an ovalbumin sensitization and challenge murine model of asthma

4.3.1.1 Effect of formoterol administration on total inflammatory cells in the airways

In Ova S/C Epi-null mice, there was no significant increase in inflammatory cells in BALF compared to control Epi-null mice (Figure 28). In Ova S/C WT mice, there was significant increase in inflammatory cells compared to control WT mice ($p<0.05$) (Figure 28). Administration of formoterol to Ova S/C Epi-null mice for a duration of 6 days did not significantly increase total inflammatory cells in BALF compared to vehicle-treated

Ova S/C Epi-null mice (Figure 28). However, there was significant increase in total inflammatory cells in Ova S/C Epi-null mice with 9, 12 and 19 days of formoterol administration compared to vehicle-treated Ova S/C Epi-null mice ($p<0.05$) (Figure 28).

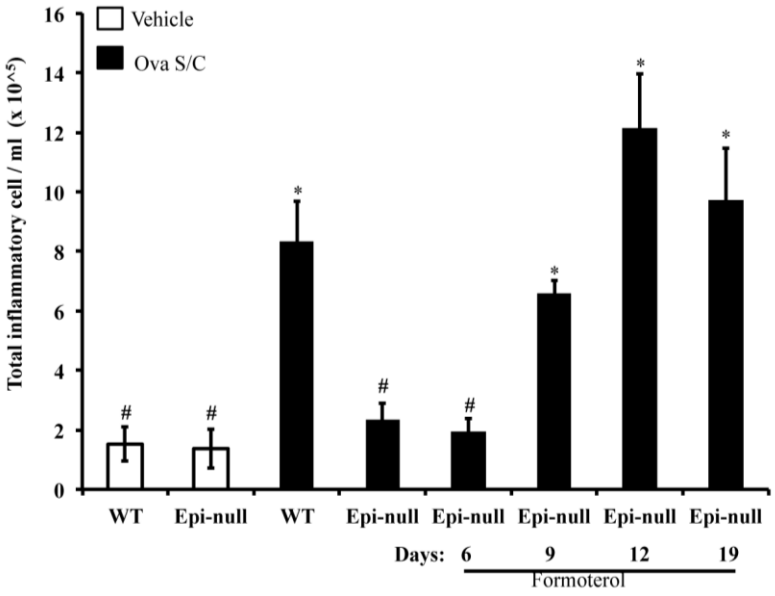

Figure 28 (Ova S/C model): Effect of formoterol administration on total inflammatory cells in BALF. Total inflammatory cell count from BALF of Ova S/C WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. #indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

4.3.1.2 Effect of formoterol administration on eosinophils in the airways

Eosinophil levels were not significantly increased in Ova S/C Epi-null mice but were significantly increased in Ova S/C WT compared to control mice ($p<0.05$) (Figure 29). Formoterol administration to Ova S/C Epi-null mice for 6 or 9 days did not significantly increase eosinophils compared to vehicle-treated Ova S/C Epi-null mice (Figure 29). However, compared to vehicle-treated Ova S/C Epi-null mice, we observed significant increases in eosinophil of Ova S/C Epi-null mice treated with formoterol for 12 and 19 days ($p<0.05$) (Figure 29).

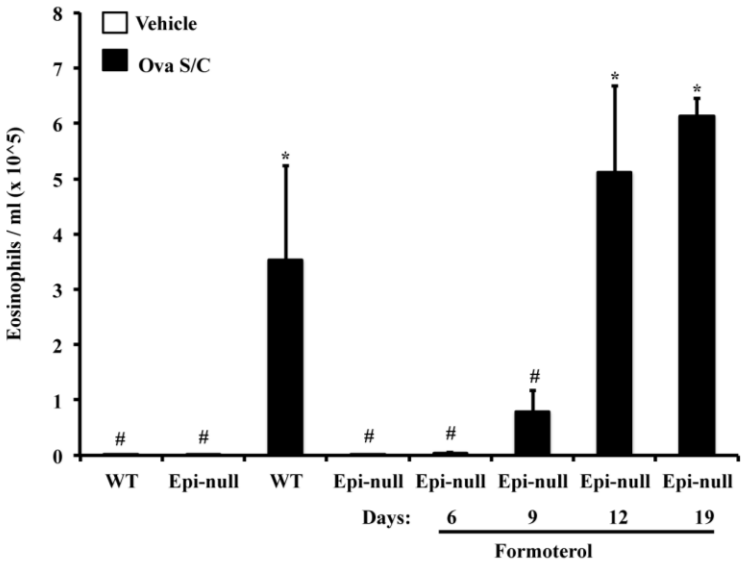

Figure 29 (Ova S/C model): Effect of formoterol administration on eosinophilia in BALF. Eosinophil count from BALF of Ova S/C WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. #indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

4.3.1.3 Effect of formoterol administration on mucous production in the airways

In Ova S/C Epi-null mice but not Ova S/C WT mice, there was no significant increase in mucous production in the airways compared to control mice (Figures 30 and 31). Formoterol treatment of Ova S/C Epi-null mice for 6, 9, 12 and 19 days significantly increased mucous production compared to vehicle-treated Ova S/C Epi-null mice ($p<0.05$) (Figures 30 and 31). Moreover, 19 days of formoterol treatment in Ova S/C Epi-null mice significantly increased mucous production compared to vehicle-treated Ova S/C WT mice ($p<0.05$) (Figures 30 and 31).

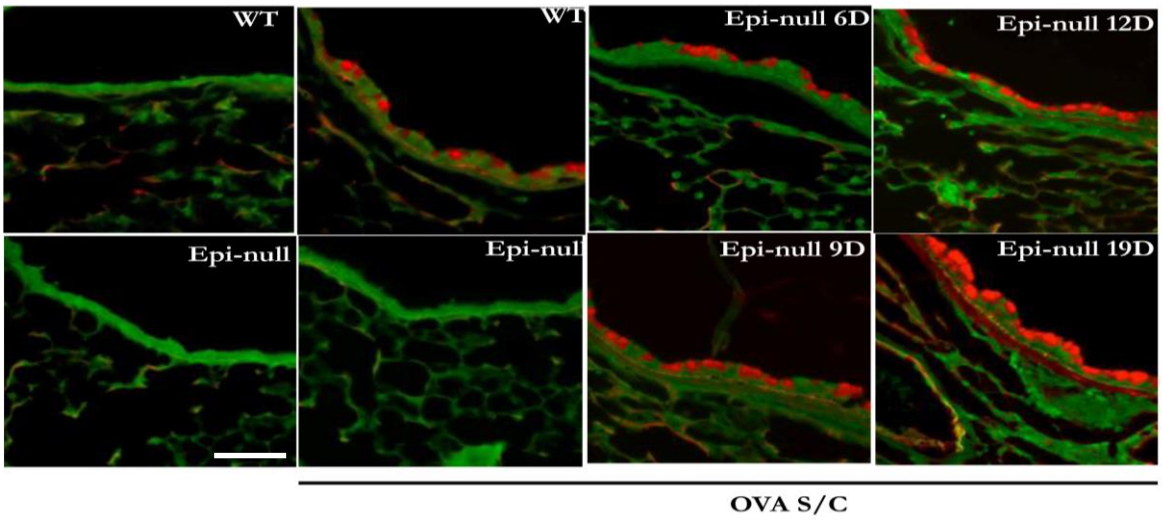

Figure 30 (Ova S/C model): Representative images of the effect of formoterol administration on mucin content. Mucin (red) content in the airway epithelia (green) of Ova S/C WT and Epi-null mice with or without formoterol administration. Mucin content in the airway epithelia was measured after periodic acid fluorescent (PAF) staining. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 μg/kg. Scale bar (white), 50 μm.

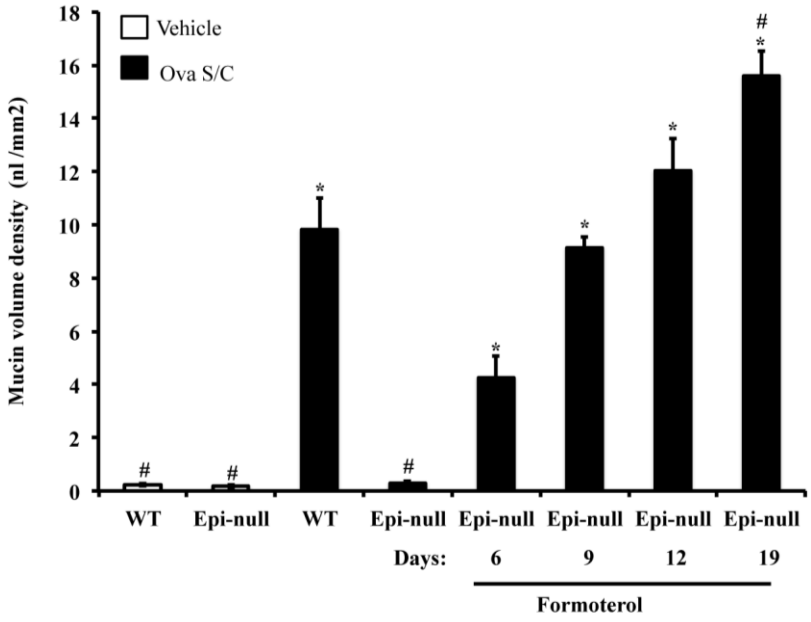

Figure 31 (Ova S/C model): Quantification of the effect of formoterol administration on mucin content in the airways. Morphometric quantification of the mucin volume density assessed from Ova S/C WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 μg/kg. Data represent the mean (±SEM) from five to six mice in each group. # indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

4.3.1.4 Effect of formoterol administration on peak airway resistance, airway sensitivity (PC_{100}) and airway reactivity (K)

We also studied the effect of formoterol administration on three parameters of airway hyperresponsiveness; peak airway resistance, airway sensitivity and airway reactivity to nebulized methacholine.

In Ova S/C Epi-null mice, there was no significant increase in peak airway resistance, airway reactivity, and airway sensitivity (as reflected by an increased PC_{100} value) compared to control mice (Figures 32-34). Methacholine at a dose of 50 mg/mL did not significantly increase peak airway resistance in Ova S/C Epi-null mice compared to control mice (Figure 32). In Ova S/C WT mice, there was significant increase in peak airway resistance, airway reactivity, and airway sensitivity (as reflected by a decreased PC_{100} value) compared to control mice (Figures 32-34). In Ova S/C Epi-null mice, 9, 12 and 19 days of formoterol administration significantly increased airway sensitivity and airway reactivity compared to vehicle-treated Ova S/C Epi-null mice ($p<0.05$) (Figures 33 and 34). Six days of formoterol treatment significantly increased airway sensitivity and airway reactivity compared to vehicle-treated Ova S/C Epi-null mice and it was also significantly different from Ova S/C WT mice ($p<0.05$) (Figures 33 and 34).

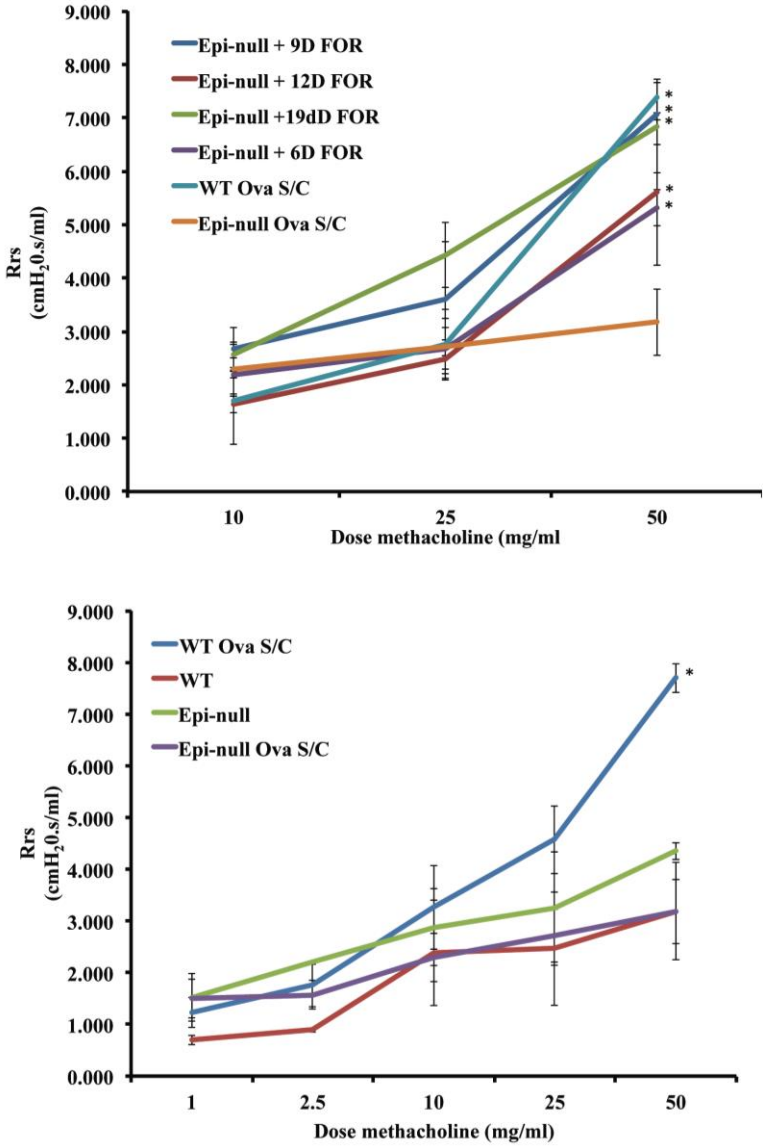

Figure 32 (Ova S/C model): Effect of Formoterol administration on airway resistance (Rrs). Airway resistance to increasing doses of nebulized methacholine (0–50 mg/mL) was measured using forced oscillation technique in Ova S/C WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from three to five mice in each group. # indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

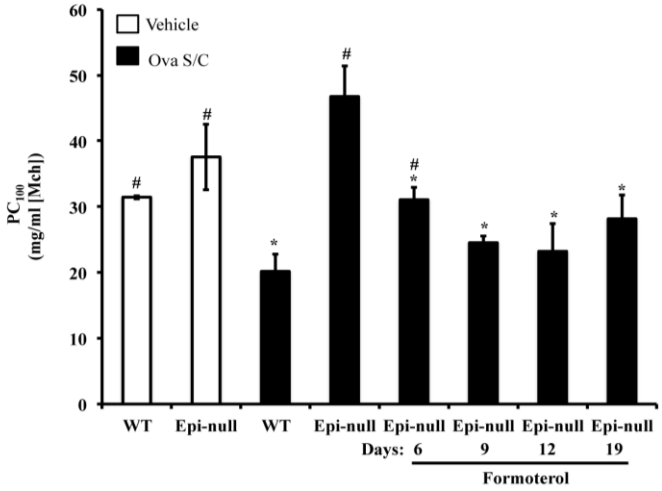

Figure 33 (Ova S/C model): Effect of formoterol administration on airway sensitivity (PC_{100}). Airway sensitivity values in Ova S/C WT and Epi-null mice with or without formoterol administration. A lower value for PC_{100} (the provocative methacholine dose required to double baseline resistance) represents increased airway responsiveness. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from four to five mice in each group. # indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

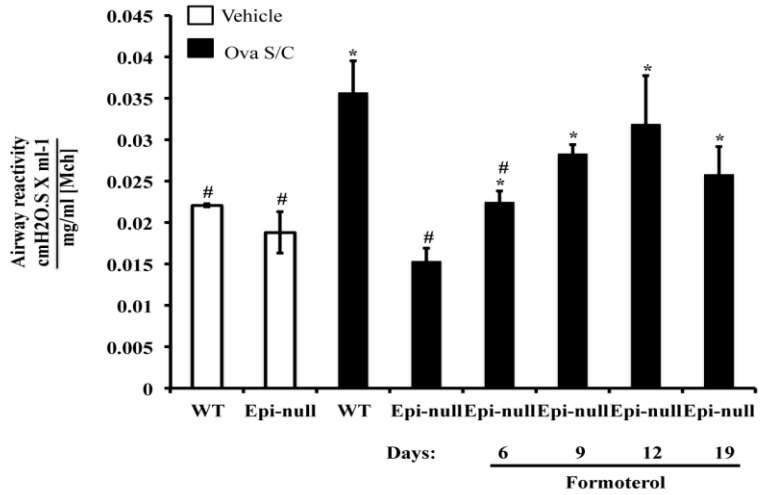

Figure 34 (Ova S/C model): Effect of formoterol administration on airway reactivity (K). Airway reactivity in Ova S/C WT and Epi-null mice with or without formoterol administration. A higher value for reactivity represents increased airway responsiveness. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from four to five mice in each group. # indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

4.3.2 Effect of formoterol administration on total inflammatory cells in an IL-13 murine model of asthma

4.3.2.1 Effect of formoterol administration on total inflammatory cells in the airways

Intra-tracheal administration of IL-13 to Epi-null mice but not WT mice resulted in no significant increase in total inflammatory cells compared to control mice (Figure 35). In IL-13 treated Epi-null mice, 4 and 7 days of formoterol treatment did not significantly increase total inflammatory cells compared to vehicle-treated Epi-null mice. However, administration of formoterol for 10 and 17 days to IL-13-treated Epi-null mice significantly increased total inflammatory cells compared to vehicle-treated Epi-null mice ($p<0.05$) (Figure 35).

4.3.3 Effect of formoterol administration on chemokine production in an IL-13 murine model of asthma

4.3.3.1 Effect of formoterol administration on mGRO/KC production in the airways

The mGRO/KC chemokine increases recruitment of neutrophils into the airways (Moser, Schumacher et al. 1991, Schumacher, Clark-Lewis et al. 1992). There was no significant increase in mGRO/KC in both IL-13 treated Epi-null and WT mice compared to control mice. In IL-13 treated Epi-null mice, administration of formoterol for 4, 7, 10 and 17 days did not show any significant increase in mGRO/KC compared to IL-13 treated WT and Epi-null mice (Figure 36).

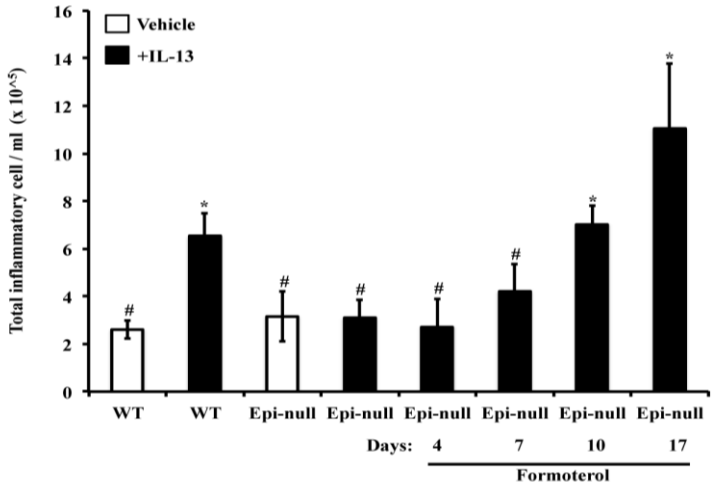

Figure 35 (IL-13 model): Effect of formoterol administration on total inflammatory cell in BALF. Total inflammatory cell count from BALF of IL-13 treated WT and Epi-null mice with or without formoterol administration in IL-13 treated Epi-null mice. The numbers indicate the days of formoterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. # indicates $p<0.05$ significance as compared to IL-13 treated WT mice; * indicates $p<0.05$ significance as compared to IL-13 treated Epi-null mice.

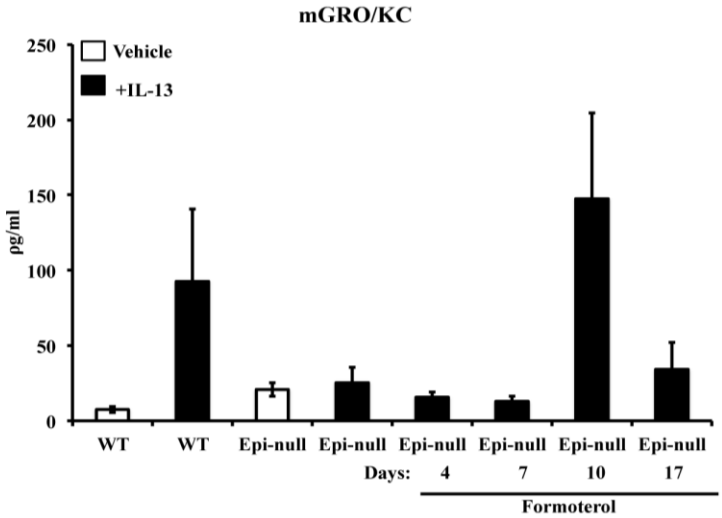

Figure 36 (IL-13 model): Effect of formoterol administration on mGRO/KC in BALF. The levels of mGRO/KC from BALF of IL-13 treated WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in IL-13 treated Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. # Indicates $p<0.05$ significance as compared to IL-13 treated WT mice; * indicates $p<0.05$ significance as compared to IL-13 treated Epi-null mice.

4.3.3.2 Effect of formoterol administration on mTARCand and mMCP-1 production in the airways

Mouse thymus and activation regulated chemokine (mTARC) and mouse monocyte chemotactic protein 1 (mMCP-1) are chemotactic factors, which increases recruitment of various inflammatory cells into the airways (Butcher and Picker 1996, Baggiolini 1998). Recruitment of T cells, monocytes and dendritic cells is regulated by mMCP-1 (Alam, York et al. 1996) whereas mTARC is mainly involved in T cell recruitment (Alam, York et al. 1996, Imai, Nagira et al. 1999, Kawasaki, Takizawa et al. 2001). There was no significant increase in mTARC and mMCP-1 in IL-13 treated Epi-null and WT mice compared to control mice (Figures 37 A and B). In IL-13 treated Epi-null mice 4, 7 and 17 days of formoterol administration did not significantly increase mTARC and mMCP-1 compared to vehicle-treated Epi-null mice (Figures 37 A and B). However, there was a significant increase in mTARC and mMCP-1 with 10 days of formoterol administration in IL-13 treated Epi-null mice compared to vehicle-treated Epi-null mice ($p<0.05$) (Figures 37 A and B).

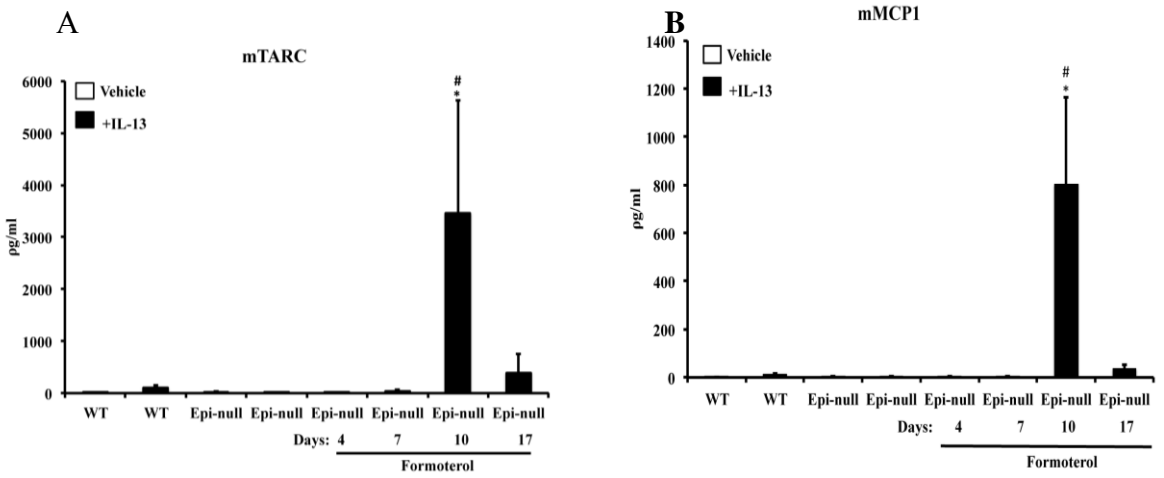

Figure 37 (IL-13 model): Effect of formoterol administration on (A) mTARC and (B) mMCP1 in BALF. The levels of mTARC and mMCP-1 from BALF of IL-13 treated WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in IL-13 treated Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 μg/kg. Data represent the mean (±SEM) from five to six mice in each group. # Indicates $p<0.05$ significance as compared to IL-13 treated WT mice; * indicates $p<0.05$ significance as compared to IL-13 treated Epi-null mice.

4.3.3.3 Effect of formoterol administration on meotaxin and mRANTES production in the airways

Mouse regulated activation normal T cell expressed and secreted (mRANTES) and meotaxin play major role in eosinophilia associated with models of allergic airway diseases (Butcher and Picker 1996, Baggiolini 1998). The chemokine meotaxin is chemotactic for eosinophil where as mRANTES is chemotactic for basophils, T cells and eosinophil (Butcher and Picker 1996, Baggiolini 1998). There was no significant increase

in meotaxin and mRANTES in IL-13 treated Epi-null and WT mice compared to control mice (Figures 38 A and B). In IL-13 treated Epi-null mice 4, 7 and 17 days of formoterol administration did not significantly increase meotaxin and mRANTES compared to vehicle-treated Epi-null mice (Figures 38 A and B). However, there was a significant increase in meotaxin and mRANTES with 10 days of formoterol administration in IL-13 treated Epi-null mice compared to IL-13 treated WT and Epi-null mice ($p<0.05$) (Figures 38 A and B).

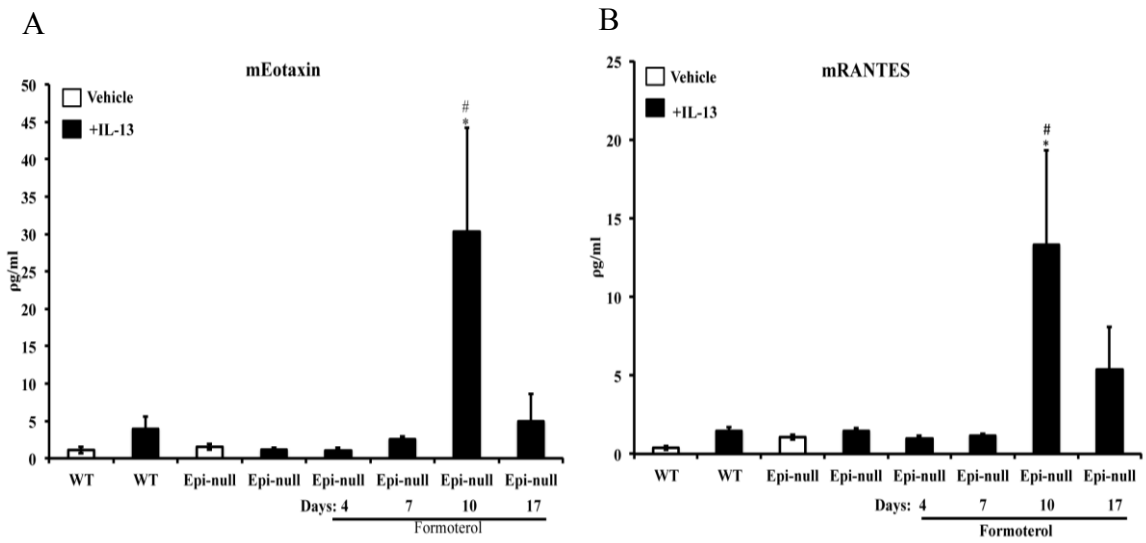

Figure 38 (IL-13 model): Effect of formoterol administration on (A) meotaxin and (B) mRANTES in BALF. The levels of meotaxin and mRANTES from BALF of IL-13 treated WT and Epi-null mice with or without formoterol administration. The numbers indicate the days of formoterol administration in IL-13 treated Epi-null mice. Twice-daily i.p. injections of formoterol was administered at a dose of 5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. # indicates $p<0.05$ significance as compared to IL-13 treated WT mice; * indicates $p<0.05$ significance as compared to IL-13 treated Epi-null mice.

4.4 Discussion

In the Ova S/C model of asthma 9, 12 and 19 days of formoterol administration restored total inflammatory cells in BALF of Epi-null mice (Figure 28). We also observed significant increase in eosinophils in the airways of Epi-null mice with 12 and 19 days of formoterol treatment (Figure 29). Administration of formoterol for 6, 9, 12 and 19 days time dependently restored mucous production in the airways (Figures 30 and 31). Airway hyperresponsiveness assessed as airway sensitivity (PC_{100}) and airway reactivity (K) was also significantly increased with formoterol treatment (Figures 33-34).

In the IL-13 model of asthma, 10 and 17 days of formoterol administration significantly increased total inflammatory cells (Figure 35). Formoterol administration produced significant increases in the levels of four chemokines; mRANTES, meotaxin, mMCP-1 and mTARC in IL-13 treated Epi-null mice (Figures 37 and 38). These chemokines increase recruitment of inflammatory cells such as T cells, dendritic cells, basophils and eosinophils into the airways (Butcher and Picker 1996, Baggiolini 1998). There was no significant increase in mGRO/KC chemokine with formoterol administration (Figure 36). This chemokine is chemotactic for neutrophils (Moser, Schumacher et al. 1991, Schumacher, Clark-Lewis et al. 1992).

Xie Chen and colleagues tested the anti-inflammatory effects of oral formoterol in an Ova S/C murine model of asthma. In this study it was observed that, administration of formoterol for 7 days caused a dose-dependent and almost complete inhibition of total

inflammatory cells at 2 mg/kg (Xie, Chen et al. 2003). They also studied the changes in lung resistance and dynamic lung compliance, to intra-gastric gavage of formoterol and observed that, formoterol protected lung against increase in lung resistance and reduction of lung compliance induced by bronchial challenge to ovalbumin. However, these parameters were measured following 7 days of formoterol treatment and this shorter duration may explain why formoterol protected against inflammation, increase in lung resistance and reduced lung compliance induced by bronchial challenge of Ova (Xie, Chen et al. 2003).

Another group studied the effect of formoterol on airway goblet cell hyperplasia and protein Muc5ac expression in a murine model of asthma. In this study it was observed that, administration of 1 mg/kg formoterol i.p., three days per week for a total of 6 weeks reduced airway goblet cell hyperplasia and protein Muc5ac expression in an Ova S/C model of asthma (Tan, Zhang et al. 2011). The differences in observation between our study and those conducted by Tan Zhang and colleagues could be due to several reasons. First, there was a major difference in protocols for drug administration used for the two studies. Whereas we administered formoterol on a consistent basis (each consecutive day for different treatment periods), Tan Zhang and colleagues administered formoterol three days per week for a total of 6 weeks. Secondly, there were differences in strain of mice and dose of formoterol administered.

In the IL-13 model of asthma, we observed significant increase in the levels of mRANTES, meotaxin, mMCP-1 and mTARC with 10 days of formoterol administration. In murine models of asthma and humans with asthma, studies have shown significant increase in RANTES, eotaxin, MCP-1 and TARC (Hu, Chen et al. 2012, Hatchwell, Girkin et al. 2014, Chihara, Yasuba et al. 1997, Alam, York et al. 1996, Nakamura, Weiss et al. 1999, Lilly, Woodruff et al. 1999, Sallusto, Lenig et al. 1998, Imai, Nagira et al. 1999). The IL-13 model of asthma used in our study is an eosinophilic model and that explains why we did not observe any significant increase in mGRO/KC, a chemokine chemotactic for neutrophils in any of the groups administered formoterol.

The RANTES chemokine is chemotactic for inflammatory cells such as eosinophils, memory T cells, and monocytes and it plays an important role in various immune and allergic disorders. Chihara and colleagues in 1997 observed that, the plasma RANTES level was significantly elevated in patients with acute asthma attacks compared to control groups (Chihara, Yasuba et al. 1997). Similar observations were made in studies conducted by Alam York and colleagues in 1996, where they observed that; levels of RANTES in BALF were significantly higher in asthmatic patients than in control subjects (Alam, York et al. 1996). Furthermore, a direct correlation of increased eotaxin levels has been observed in asthmatics with compromised lung function (Nakamura, Weiss et al. 1999). Also patients with acute asthma have displayed significantly higher plasma eotaxin levels than normal subjects (Lilly, Woodruff et al. 1999). Alam York and colleagues have also shown elevated levels of MCP-1in BALF of asthmatic patients than

in control subjects (Alam, York et al. 1996). Lastly, TARC has been identified to induce chemotaxis of Th2 type $CD4^+$ human T lymphocytes (Sallusto, Lenig et al. 1998, Imai, Nagira et al. 1999). In a murine model of asthma, TARC was involved in the development of AHR and eosinophilia through the recruitment of Th2 type $CD4^+$ T lymphocytes (Kawasaki, Takizawa et al. 2001)

The data in our murine model of asthma also supports clinical observations where the chronic use of formoterol can be associated with serious and life-threatening asthma related events as well as asthma mortality (Mann, Chowdhury et al. 2003, Salpeter, Buckley et al. 2006, Wolfe, Laforce et al. 2006, Cates and Cates 2012).

In conclusion, we have shown that, ligand activation of the β_2AR receptor is required for the development of the asthma phenotype in murine models of asthma. This is yet another paradoxical finding since epinephrine was introduced at the beginning of the 20^{th} century for asthma management, and was considered the first widely accepted treatment for asthma (Rau 2000). These data further support the hypothesis that, certain "β-blockers" may have a role in chronic asthma management.

5 The role of the β_2AR Gs-cAMP pathway in development of the asthma phenotype in a murine model of asthma

5.1 Introduction

We have previously identified the requirement of ligand activation of the β_2-adrenoceptor (β_2AR) in development of the asthma phenotype in murine models (Thanawala, Forkuo et al. 2013). The β_2AR is a seven transmembrane receptor (7TMR) known to signal through at least two major pathways. These include the canonical Gs pathway, which leads to activation of adenylate cyclase, cAMP accumulation and protein kinase A (PKA) activation, and the β-arrestin pathway, which can lead to activation of MAPKs (Shenoy, Drake et al. 2006, Wisler, DeWire et al. 2007, Drake, Violin et al. 2008, Walker, Penn et al. 2011). Therefore, we started investigating the pathways downstream of the receptor required for the development of the asthma phenotype.

Tran and colleagues have measured the intrinsic efficacy (this describes the activation function of a ligand per receptor) of adenylate cyclase of β_2AR agonists in membrane preparations. In this study, the efficacies of salmeterol and formoterol relative to epinephrine were 13% and 63% respectively as shown in table 3 below (Tran, Friedman et al. 2004). Although salmeterol and formoterol have different intrinsic efficacies at the Gs pathway, both have been given black box warning due to studies reporting increased morbidity and mortality with their chronic administration (Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006, 2007, Cates, Jaeschke et al. 2013). This suggests that, the

Gs pathway may not be the major pathway involved in the negative effect of β_2AR agonists.

Rajagopal and colleagues in 2011 screened a number of β_2AR ligands in an attempt to quantify the bias ligands at the β_2AR (Rajagopal, Ahn et al. 2011). A biased ligand at the β_2AR is one, which exhibits different capacities to activate either the Gs or the β-arrestin pathway relative to a reference ligand (usually the endogenous β_2AR ligand, epinephrine) (Walker, Fong et al. 2003, Rajagopal, Rajagopal et al. 2010). In this study, it was observed that relative to epinephrine, formoterol and salmeterol were β-arrestin-biased agonists of the β_2AR (Rajagopal, Ahn et al. 2011) although others have reported salmeterol poorly recruits β-arrestin upon activation of the β_2AR (Moore, Millman et al. 2007). Furthermore, ERK1/2 a downstream kinase mainly activated by β-arrestin, can also be stimulated in a β-arrestin-independent fashion (Wisler, DeWire et al. 2007, van der Westhuizen, Breton et al. 2014). Another study reported that in contrast to its low efficacy at cAMP, salmeterol was 80% as effective as epinephrine at ERK1/2 activation (Friedman, Babu et al. 2002, Rajagopal, Ahn et al. 2011, Walker, Penn et al. 2011). It has also been reported that, salmeterol is 19-fold better at activating ERK1/2 than cAMP relative to isoproterenol (van der Westhuizen, Breton et al. 2014).

In genetic studies, Walker and colleagues have shown β-arrestin-2 knockout mice show attenuation of cardinal features of the asthma phenotype in an allergen driven model of asthma (Walker, Fong et al. 2003).

We therefore began investigating the β_2AR-Gs pathway in development of the asthma phenotype in murine models. First, we studied if two drugs with significant differences in activation of the Gs pathway, but comparable at activating ERK1/2, would result in different asthma phenotypes.

Epinephrine	**100%**
Fenoterol	**67%**
Formoterol	**63%**
Terbutaline	**33%**
Salbutamol	**25%**
Salmeterol	**13%**
Alprenolol	**-14%**
Carvedilol/Propranolol	**-37%/-38%**
ICI-118,551	**-76%**
Nadolol	**-82%**

Table 3: Intrinsic efficacies of β-AR ligands. Adopted from: (Bond, Leff et al. 1995, Nagaraja, Iyer et al. 1999, Tran, Friedman et al. 2004)

5.2 Approach

Salmeterol was administered to Ova S/C Epi-null for a duration of 6, 9, 12 and 19 days using the protocol previously described for formoterol and the results were compared to those obtained for formoterol (see pages 68-75) (Figure 39). We also wanted to study the short-term and long-term effects of β_2AR activation in murine models of asthma.

Similar to formoterol, salmeterol has a long half-life of approximately 12 hours allowing the convenience of twice-daily administration (Johnson, Butchers et al. 1993). It

is also highly stable to heat, oxygen and does not degrade easily (Johnson, Butchers et al. 1993). We administered twice-daily i.p. injections of salmeterol at a dose of 1.5 μg/kg. As with formoterol, this dose was again selected to "restore" β_2AR signaling and not elicit any drug response (Maris, van der Sluijs et al. 2004, Singam, Jena et al. 2006, Riesenfeld, Sullivan et al. 2010, Qian, Wu et al. 2011).

Mice were phenotyped for inflammatory cell infiltration into the airways and mucous metaplasia.

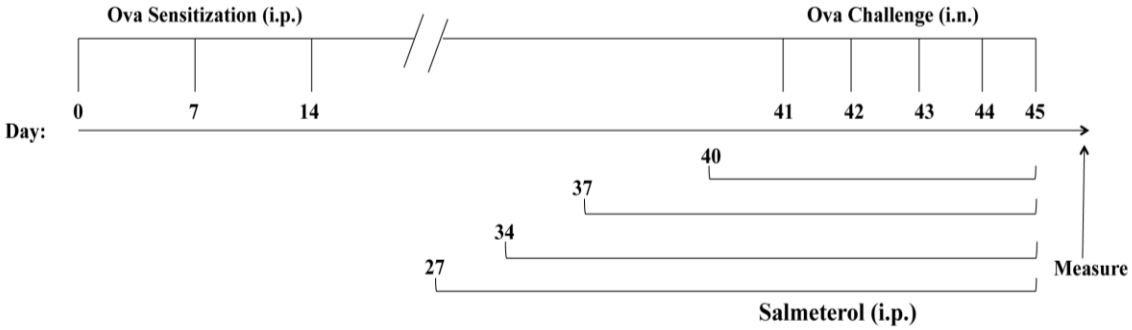

Figure 39: Ovalbumin sensitization and challenge (Ova S/C) treatment protocol: Wild type (WT) and Epi-null mice were sensitized intra-peritoneally (i.p.) with 2mg/kg/day of ovalbumin (Ova) mixed with 2mg of alum on days 0, 7, and 14. Following Ova sensitization, mice were challenged five times intra-nasally (i.n.) with 1mg/kg/day of Ova or vehicle from days 41-45. At different time points as shown above, groups of Ova sensitized and challenged Epi-null mice received twice-daily i.p. injections of vehicle or salmeterol at a dose of 1.5 μg/kg.

5.3 Results

5.3.1 Effect of salmeterol administration on total inflammatory cells in the airways

In Ova S/C Epi-null mice, administration of salmeterol for 6, 9, 12 and 19 days significantly increased total inflammatory cell infiltration in BALF compared to vehicle-treated Ova S/C Epi-null mice ($p<0.05$) (Figure 40).

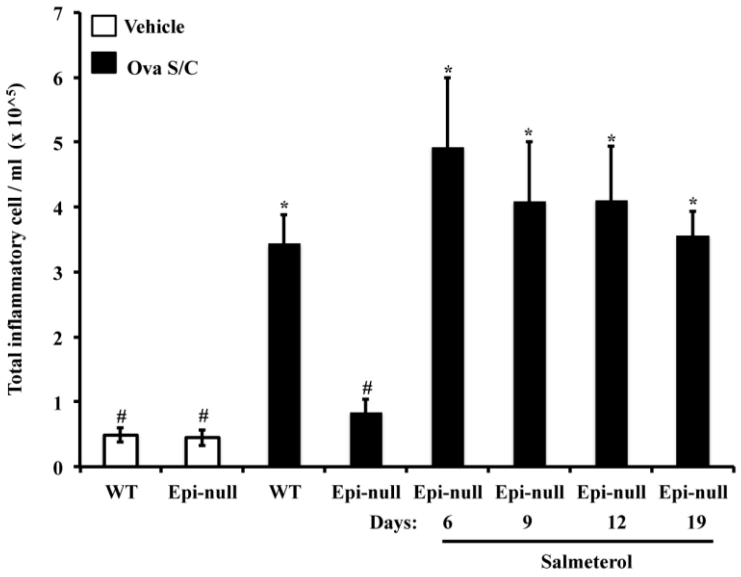

Figure 40: Effect of salmeterol administration on total inflammatory cell in BALF. Total inflammatory cell count from BALFof Ova S/C WT and Epi-null mice with or without salmeterol administration. The numbers indicate the days of salmeterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of salmeterol was administered at a dose of 1.5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. # Indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

5.3.2 Effect of salmeterol administration on eosinophils in the airways

Administration of salmeterol in Ova S/C Epi-null mice for 6, 9, 12 and 19 days significantly increased eosinophils in BALF compared to Ova S/C Epi-null mice ($p<0.05$) (Figures 41 and 42).

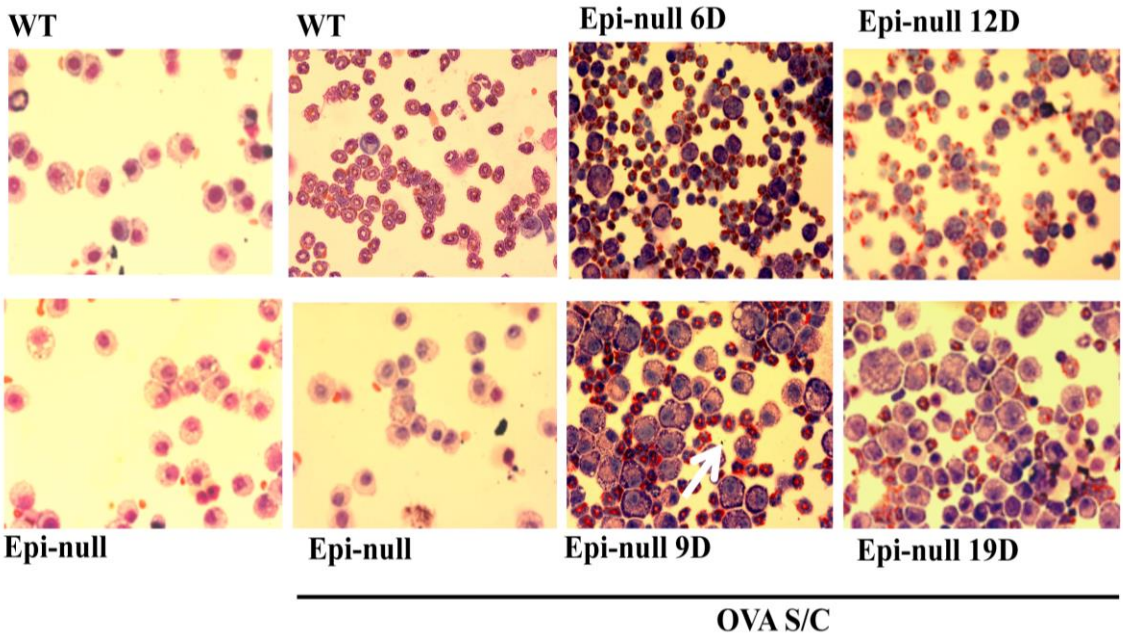

Figure 41: Representative images of the effect of salmeterol administration on eosinophil in BALF. Eosinophil in BALF of Ova S/C WT and Epi-null mice with or without salmeterol administration. Eosinophil (cells with pink granules as indicated by white arrow) content in BAL fluid were obtained after staining cells with Wreight Geimsa stain for 6, 9, 12, and 19 days of salmeterol administration. The numbers indicate the days of salmeterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of salmeterol was administered at a dose of 1.5 µg/kg.

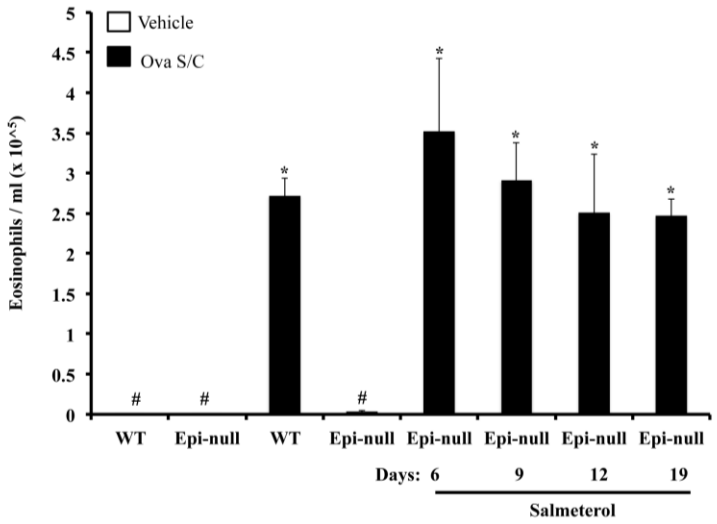

Figure 42: Quantification of the effect of salmeterol administration on eosinophilia in BALF. Eosinophil count from BALF of Ova S/C WT and Epi-null mice with or without salmeterol administration. The numbers indicate the days of salmeterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of salmeterol was administered at a dose of 1.5 μg/kg. Data represent the mean (±SEM) from five to six mice in each group. # Indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

5.3.3 Effect of salmeterol administration on mucous production in the airways

Administration of salmeterol to Ova S/C Epi-null mice for 6, 9, 12 and 19 days significantly increased mucous production in the airways compared to vehicle-treated Ova S/C Epi-null mice ($p<0.05$) (Figures 43 and 44).

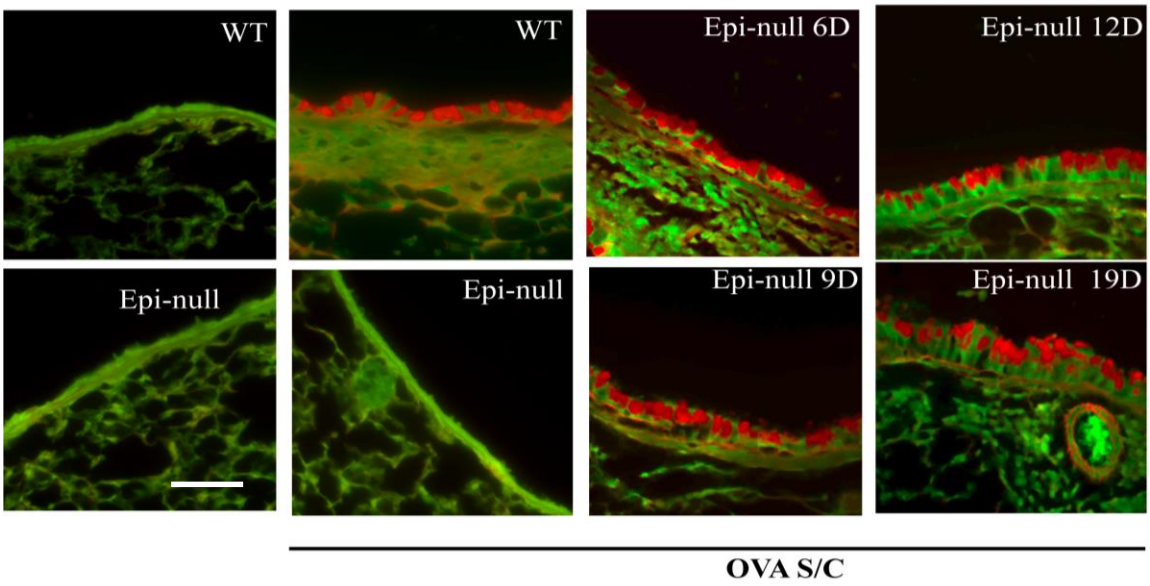

Figure 43: Representative images of the effect of salmeterol administration on mucin content in the airways. Mucin (red) content in the airway epithelia (green) for Ova S/C WT and Epi-null mice with or without salmeterol administration. Mucin content in the airway epithelia was measured after periodic acid fluorescent (PAF) staining for 6, 9, 12, and 19 days of salmeterol administration. The numbers indicate the days of salmeterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of salmeterol was administered at a dose of 1.5 µg/kg. Scale bar (white), 50µm.

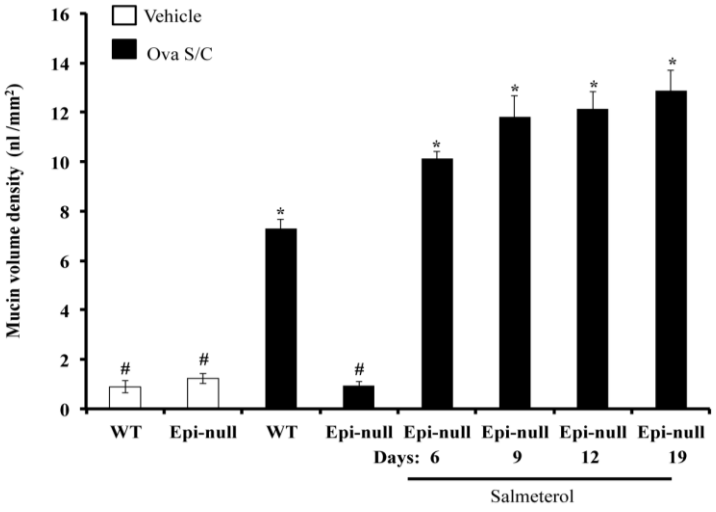

Figure 44: Quantification of the effect of salmeterol administration on mucin content in the airways. Morphometric quantification of the mucin volume density assessed from Ova S/C WT and Epi-null mice with or without salmeterol administration. The numbers indicate the days of salmeterol administration in Ova S/C Epi-null mice. Twice-daily i.p. injections of salmeterol was administered at a dose of 1.5 µg/kg. Data represent the mean (±SEM) from five to six mice in each group. # indicates $p<0.05$ significance as compared to Ova S/C WT mice; * indicates $p<0.05$ significance as compared to Ova S/C Epi-null mice.

5.4 Discussion

In this study, salmeterol restored cardinal features of the asthma phenotype similar to what we had observed with formoterol. Salmeterol increased inflammatory cells in BALF and mucous metaplasia in the airways.

However, there were some temporal differences in features of the asthma phenotype. Salmeterol significantly increased inflammation and mucous metaplasia within 6 days of drug administration, whereas at least 12 days of formoterol treatment were required to restore the same parameters. Even though formoterol has a rapid onset of action compared to salmeterol, we observed that salmeterol restored inflammation and mucous metaplasia quicker in the murine asthma model (Anderson 1993) (Figure 46). This temporal difference could be due to differences to the binding characteristics of formoterol and salmeterol to the receptor as shown in figures 45 and 46. Salmeterol is more lipophilic compared to formoterol and it has a higher affinity for the β_2AR (Anderson 1993). Salmeterol appears to pseudo-competitively bind to the receptor and does not easily dissociate whereas formoterol competitively associates with the receptor. *In-vitro* studies have not been able to measure the dissociation constant of salmeterol from the receptor (Anderson 1993, Johnson, Butchers et al. 1993). The long lipophilic chain and the saligenin head of salmeterol has also been suggested to dock at an exo-site of the receptor and hold the drug at the active site of the receptor as shown in figure 46 (Johnson, Butchers et al. 1993).

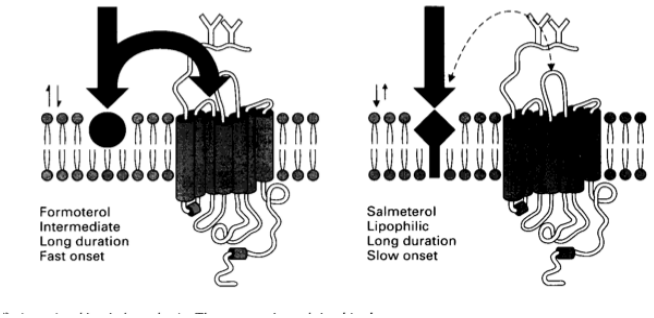

Figure 45: Diagrammatic representation of the diffusion microkinetic hypothesis. Adapted from: (Anderson 1993)

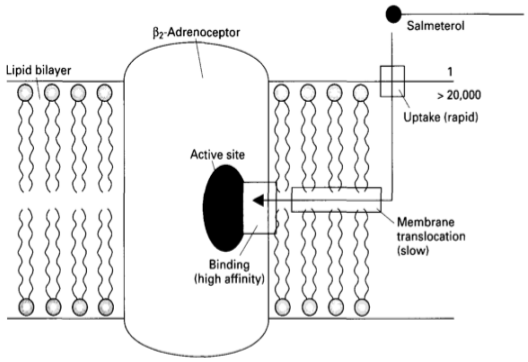

Figure 46: Proposed interaction of salmeterol with the cell membrane. Adapted from:(Johnson, Butchers et al. 1993)

Our results are in agreement with those conducted by Riesenfeldet al. in 2010, where 20 minutes nebulization of 3 mg/mL salmeterol for a duration of 9 days significantly increased total inflammatory cells, eosinophils and mucous production in the airways of mice. They also observed a significant increase in elastance, an index of airway hyperresponsivess with 9 days administration of nebulized salmeterol (Riesenfeld, Sullivan et al. 2010).

Our data are also consistent with clinical observations where use of either salmeterol or formoterol, are associated with adverse outcomes. In July 2005, an FDA

panel convened to decide how to deal with the negative outcomes. The conclusion of the panel was to recommend the labels on these drugs include strong warning of serious and life threatening events (black box warning).

Other studies have reported an opposite effect for salmeterol (Hu, Chen et al. 2012, Hatchwell, Girkin et al. 2014). In one study, salmeterol administration significantly reduced total inflammatory cells, eosinophils, mucous production and airway hyperresponsiveness. There was also inhibition of the NF-κB transcription factor, decreased activation of the MAPK ERK, and induced apoptosis of dendritic cells, an antigen presenting cell which plays a key role in inflammation (Hu, Chen et al. 2012). We speculate the reason for this discrepancy is the differences in duration of drug treatments. In this study, salmeterol was administered for a duration of 3 days, which may not be long enough to exercabate the asthma phenotype (Hu, Chen et al. 2012). Also, in a house dust mite model of allergic airway disease, 5 days of salmeterol treatment at a dose of 0.4 mg/kg decreased total inflammatory cells, eosinophil, chemokines and rhinovirus induced airway hyperresponsivness (Hatchwell, Girkin et al. 2014). The higher dose, shorter duration of drug treatment, differences in model of asthma and differences in mouse strain are possible explanations for disparities in results obtained.

One would question if 13% compared to the 63% activation by formoterol activation of adenylate cyclase by salmeterol relative to epinephrine is of any clinical

relevance as both drugs may cross the necessary threshold for adenylate cyclase activation for downstream Gs-cAMP signaling to occur.

In conclusion, we observed that, two drugs with completely different intrinsic efficacies at the canonical β_2AR-Gs-cAMP pathway restored key features of the asthma phenotype in a murine asthma model. The findings suggest the canonical Gs pathway may not be responsible for development of the asthma phenotype in Epi-null mice.

6 Evaluating the effect of selective amplification of the β_2AR Gs-cAMP pathway in the development of the asthma phenotype

6 .1 Introduction

The ideal ligand to pharmacologically test the role of the β_2AR-Gs pathway in asthma pathogenesis would be one biased for Gs pathway activation. However, unlike carvedilol and propranolol, which activate ERK1/2 pathway in a β-arrestin dependent and independent manner respectively, but are inverse agonists at Gs-cAMP, no drug which activates Gs-cAMP but inhibit ERK1/2 has been identified yet (Wisler, DeWire et al. 2007, Rajagopal, Rajagopal et al. 2010).

Therefore, we decided to use an alternative strategy and amplify the Gs pathway downstream to the β_2AR using phosphodiesterase 4 (PDE4) inhibitors. Phosphodiesterases including PDE4, break down cAMP and cGMP and play a key role in inflammatory and immune responses (Sanz, Cortijo et al. 2005). Phosphodiesterase 4 are the major isoenzymes which are highly expressed in immune cells, airway epithelial cells and smooth muscle cells (Torphy 1998).

6.2 Approach

We used the PDE4 inhibitors, roflumilast or rolipram in wild type (WT) and Epi-null mice co-treated with formoterol or salmeterol (Figure 47). We tested two chemically diverse PDE4 inhibitors to ensure the effect seen on inflammation, mucous production

and airway hyperresponsiveness (AHR) with the drugs was a result of PDE4 inhibition, and not a unique or ~~an~~ off-target drug effect of one of the compounds (Figure 48). Roflumilast or rolipram (5 mg/kg) via oral gavage was co-administered with intra-peritoneal (i.p.) twice-daily injections of either formoterol (5 µg/kg) or salmeterol (1.5 µg/kg) to ovalbumin sensitized and challenged (Ova S/C) WT and Epi-null mice for 12 days (Figure 49). In our previous studies, at least 12 days of formoterol or salmeterol treatment in Ova S/C Epi-null mice restored cardinal features of the asthma phenotype. Mice were phenotyped for inflammatory cells in the broncho-alveolar lavage fluid (BALF), airway epithelial mucous content and AHR.

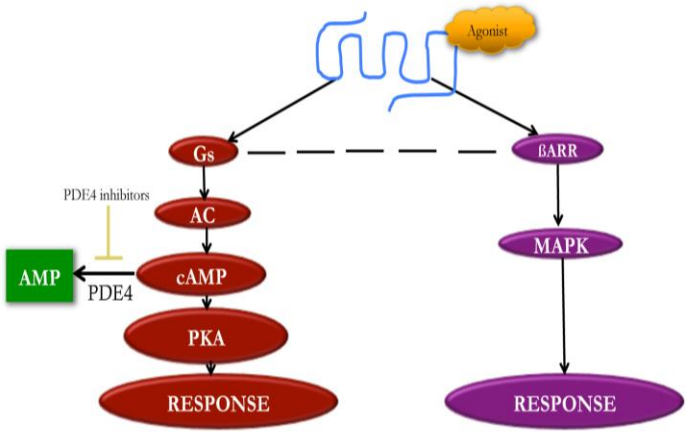

Figure 47: Schematic of amplification of the Gs-cAMP pathway with phosphodiesterase 4 (PDE4) inhibitors.

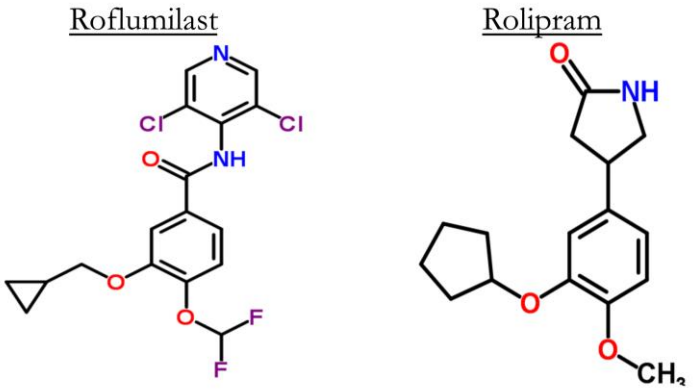

Figure 48: The structure of roflumilast and rolipram

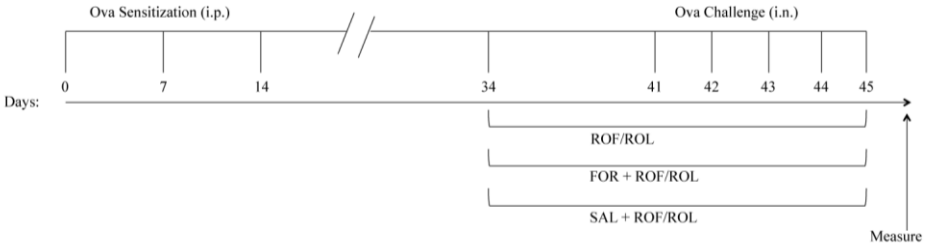

Figure 49: Ovalbumin sensitization and challenge (Ova S/C) treatment protocol Groups of Ova S/C WT and Epi-null mice were treated via oral gavage with roflumilast (5 mg/kg) or rolipram (5 mg /kg) alone. In other groups of mice, roflumilast (5 mg/kg) or rolipram (5 mg/kg) were co-administered with twice daily i.p. injections of either formoterol (5 µg/kg), or salmeterol (1.5 µg/kg). Duration of all drug treatment was 12 days.

6.3 Results

6.3.1 Effect of roflumilast or rolipram on total inflammatory cells in the airways

Administration of roflumilast or rolipram to Ova S/C Epi-null mice did not significantly increase inflammation compared to vehicle-treated Ova S/C Epi-null mice (Figures 50 A and 51 A). The increase in inflammation in Ova S/C WT mice was not significantly attenuated with roflumilast or rolipram treatment compared to vehicle-treated Ova S/C WT (Figures 50 B and 51 B).

6.3.2 Effect of formoterol or salmeterol on total inflammatory cells in the airways

As previously shown, administration of formoterol or salmeterol to Ova S/C Epi-null mice produced a significant increase in inflammatory cells in BALF, compared to vehicle-treated Ova S/C Epi-null mice ($p<0.05$) (Figures 50 A and 51 A). Treatment of Ova S/C WT mice with either formoterol or salmeterol did not demonstrate any change in total cells in BALF compared to vehicle-treated Ova S/C WT mice (Figures 50 B and 51 B).

6.3.3 Effect of co-administration of roflumilast or rolipram with either formoterol or salmeterol on total inflammatory cells in the airways

The increase in inflammation produced by formoterol and salmeterol treatment in Ova S/C Epi-null mice was significantly reduced when either drug were co-administered with roflumilast or rolipram ($p<0.05$) (Figures 50 A and 51 A). In Ova S/C WT mice, co-administration of formoterol and roflumilast but not formoterol and rolipram significantly

reduced inflammatory cell in BALF compared to formoterol treated Ova S/C WT mice ($p<0.05$) (Figures 50 B and 51 B).

On the other hand, co-administration of salmeterol with either roflumilast or rolipram in Ova S/C WT mice did not significantly reduce total inflammatory cells compared to Ova S/C WT mice treated with salmeterol (Figures 50 B and 51 B).

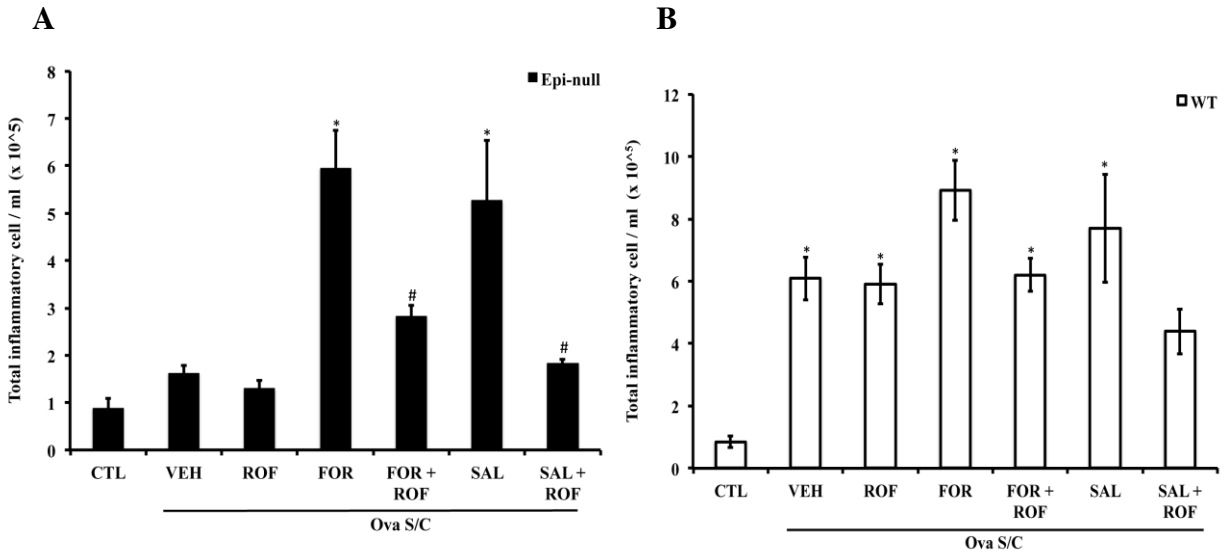

Figure 50: Effect of roflumilast administration, with or without formoterol or salmeterol co-administration on total inflammatory cells in BALF. Total inflammatory cells in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C Epi-null and WT mice were treated with different drug combinations. In these groups of mice, roflumilast (5 mg/ kg) was administered once daily via oral gavage, or co-administered with twice-daily i.p. injections with either formoterol (5 µg/kg) or salmeterol (1.5 µg/ kg) for a duration of 12 days. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ significance as compared to respective CTL, # indicates $p<0.05$ indicates significance as compared to respective treatment in the absence of ROF.

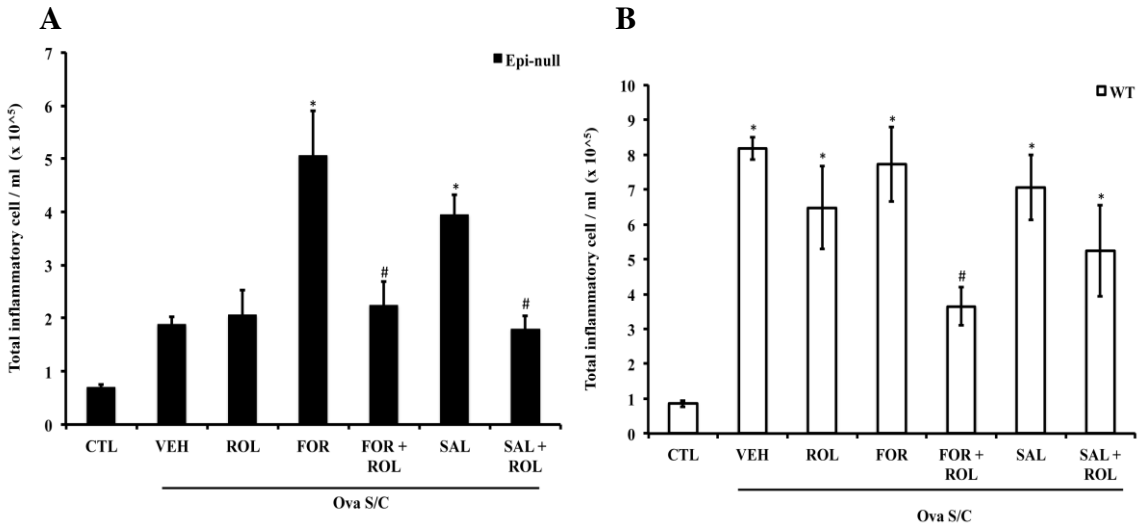

Figure 51: Effect of rolipram administration, with or without formoterol or salmeterol co-administration on total inflammatory cells in BALF. Total inflammatory cells in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C Epi-null and WT mice were treated with different drug combinations. In these groups of mice, rolipram (5 mg/kg) was administered once daily via oral gavage, or co-administered with twice daily i.p. injections with either formoterol (5 µg/kg) or salmeterol (1.5 µg/kg) for a duration of 12 days. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ significance as compared to respective CTL, # indicates $p<0.05$ indicates significance as compared to respective treatment in the absence of ROL.

6.3.4 Effect of roflumilast or rolipram on eosinophils in the airways

Administration of roflumilast or rolipram to Ova S/C Epi-null mice did not significantly increase airway eosinophils compared to vehicle-treated Ova S/C Epi-null mice (Figures 52 A and 53 A). The eosinophil levels in Ova S/C WT mice treated with

rolipram but not roflumilast was significantly different from vehicle-treated Ova S/C WT mice ($p<0.05$) (Figures 52 B and 53 B).

6.3.5 Effect of formoterol or salmeterol on eosinophils in the airways

Administration of formoterol or salmeterol significantly increased BALF eosinophils in Ova S/C Epi-null mice ($p<0.05$)) (Figures 52 A and 53 A). Administration of formoterol or salmeterol in Ova S/C WT mice showed no change in eosinophils compared to vehicle-treated Ova S/C WT mice (Figures 52 B and 53 B).

6.3.6 Effect of co-administration of roflumilast and rolipram with either formoterol or salmeterol on eosinophils in the airways

The increase in eosinophils observed in Ova S/C Epi-null mice treated with formoterol and salmeterol was significantly reduced when both drugs were co-administered with roflumilast or rolipram ($p<0.05$)) (Figures 52 A and 53 A). Co-administration of either roflumilast or rolipram with formoterol or salmeterol in Ova S/C WT mice significantly reduced eosinophils compared to formoterol and salmeterol treated Ova S/C WT mice ($p<0.05$)) (Figures 52 B and 53 B).

6.3.7 Effect of roflumilast and rolipram on mucous production in the airways

Treatment of Ova S/C Epi-null mice with roflumilast and rolipram did not significantly increase mucus in the airways compared to vehicle-treated Ova S/C Epi-null mice (Figures 54, 56 A, 57 and 59 A). In Ova S/C WT mice, roflumilast or rolipram

treatment did not significantly reduce mucus compared to vehicle-treated Ova S/C WT mice (Figures 55, 56 B, 58, 59 B).

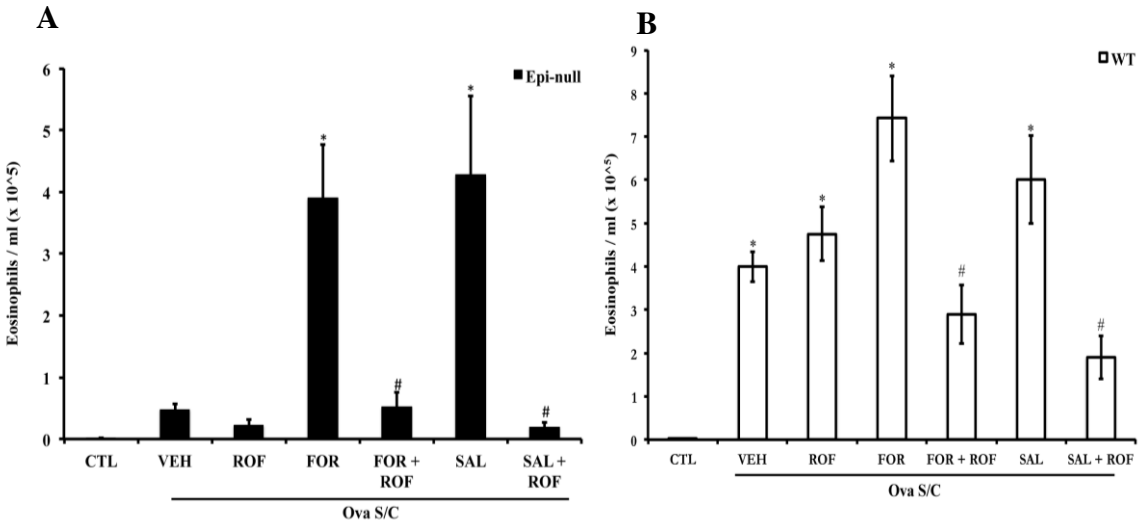

Figure 52: Effect of roflumilast administration, with or without formoterol or salmeterol co-administration on airway eosinophilia in BALF. Eosinophils in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C WT and Epi-null mice. Groups of Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice were treated with different drug combinations. In these groups of mice, roflumilast (5 mg/ kg) was administered once daily via oral gavage or co-administered with twice-daily i.p. injections with either formoterol (5 μg/kg) or salmeterol (1.5 μg/kg) for a duration of 12 days. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ indicates significance as compared to respective CTL, # indicates $p<0.05$ significance as compared to respective treatment in the absence of ROF.

6.3.8 Effect of formoterol and salmeterol on mucous production in the airways

In Ova S/C Epi-null mice, formoterol and salmeterol treatment increased mucous production in the airways of Ova S/C Epi-null ($p<0.05$) (Figures 54, 56 A, 57 and 59 A). Mucous production in Ova S/C WT mice treated with either formoterol or salmeterol was not different from vehicle-treated Ova S/C WT mice (Figures 55, 56 B, 58, 59 B).

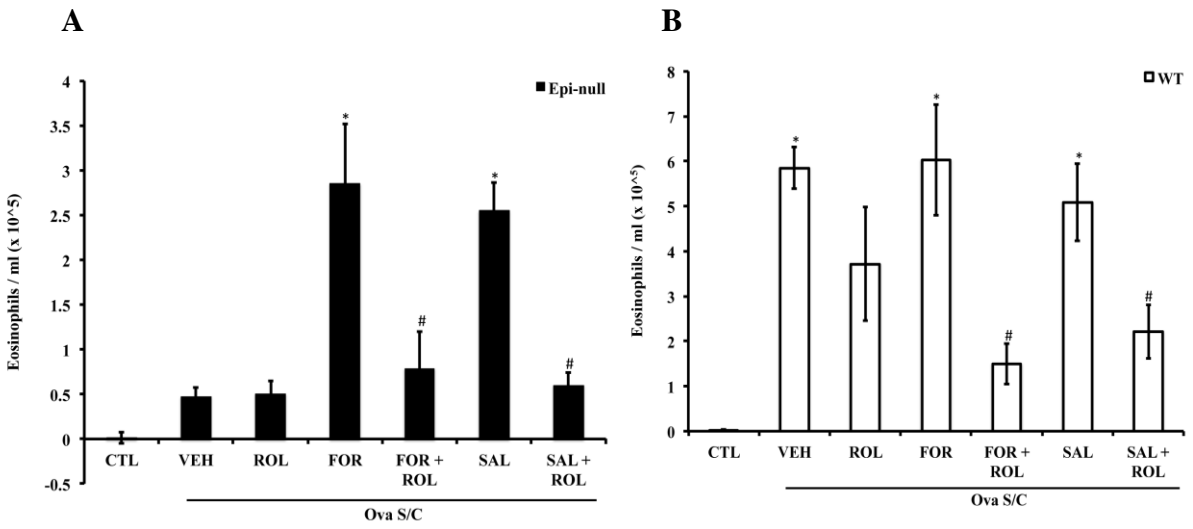

Figure 53: Effect of rolipram administration, with or without formoterol or salmeterol co-administration on airway eosinophilia in BALF. Eosinophils in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C WT and Epi-null mice. Groups of Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice were treated with different drug combinations. In these groups of mice rolipram (5 mg/kg) was administered once daily via oral gavage or co-administered with twice-daily i.p. injections with either formoterol (5 µg/kg) or salmeterol (1.5 µg/kg) for a duration of 12 days. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ indicates significance as compared to respective CTL, # indicates $p<0.05$ significance as compared to respective treatment in the absence of ROL.

6.3.9 Effect of co-administration of roflumilast and rolipram with either formoterol or salmeterol on mucous production in the airways

The increase in mucous production observed with both formoterol and salmeterol in Ova S/C Epi-null mice was significantly attenuated when co-administered with roflumilast or rolipram ($p<0.05$) (Figures 54, 56 A, 57 and 59 A). With the exception of Ova S/C WT mice treated with formoterol and rolipram, Ova S/C WT mice treated with the other drug combinations displayed significant reduction in mucous production ($p<0.05$) (Figures 55, 56 B, 58, 59 B).

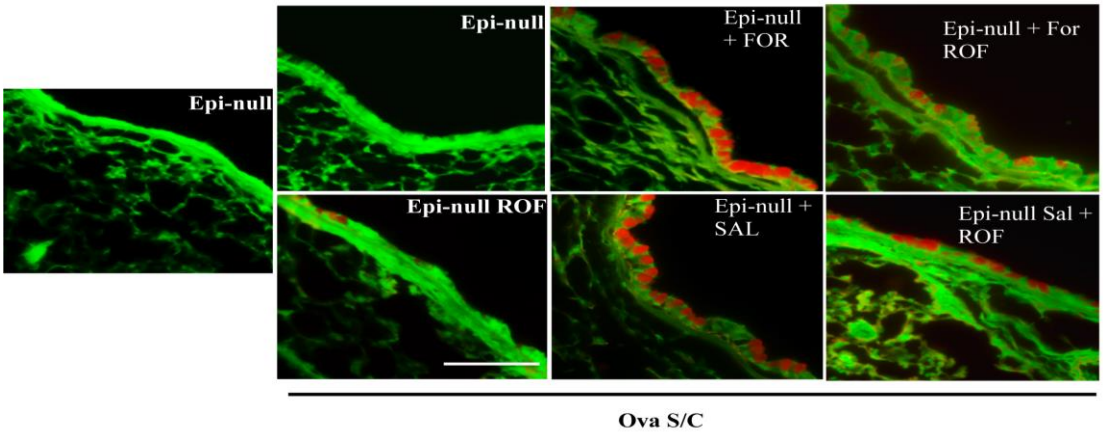

Figure 54: Representative images of the effect of roflumilast administration, with or without formoterol or salmeterol on mucous metaplasia. Representative microscopic images of mucin (red) content in the airway epithelia (green) of saline challenged (CTL) and vehicle (VEH) treated Ova S/C Epi-null mice. Groups of Ova S/C Epi-null mice were treated with different drug combinations. In these groups of mice roflumilast (5 mg/ kg) was administered via oral gavage daily or co-administered with twice-daily i.p. injections with either formoterol (5µg/kg) or salmeterol (1.5µg/kg) for a duration of 12 days. Images were acquired after periodic acid fluorescent (PAF) staining using the confocal microscope at 40X magnification. Scale bar (white), 50 µm.

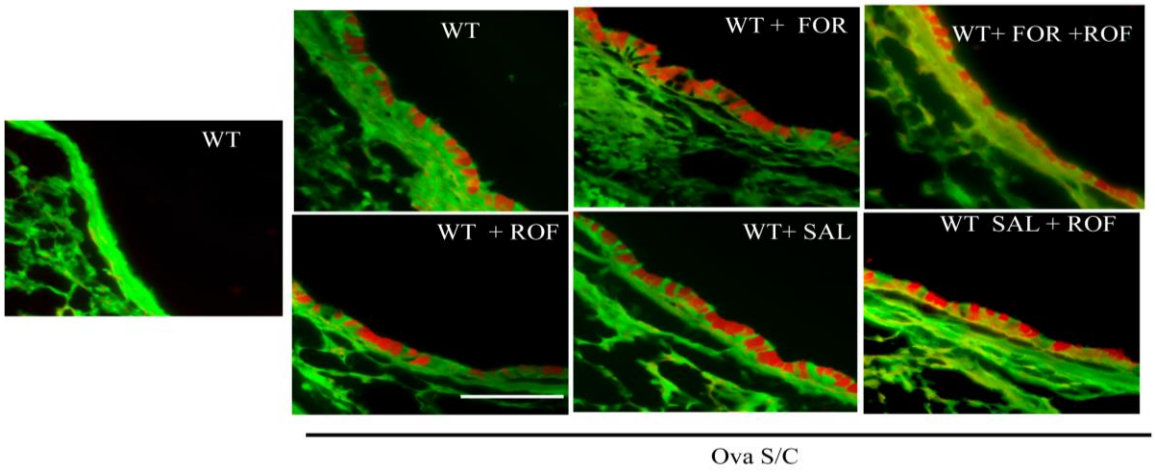

Figure 55: Representative images of the effect of roflumilast administration, with or without formoterol or salmeterol on mucous metaplasia. Representative microscopic images of mucin (red) content in the airway epithelia (green) of saline challenged (CTL) and vehicle (VEH) treated Ova S/C WT mice. Groups of Ova S/C WT mice were treated with different drug combinations. In these groups of mice roflumilast (5 mg/kg) was administered via oral gavage daily or co-administered with twice daily i.p. injections with either formoterol (5 µg/kg) or salmeterol (1.5 µg/kg) for a duration of 12 days. Images were acquired after periodic acid fluorescent (PAF) staining using the confocal microscope at 40X magnification. Scale bar (white), 50 µm

6.3.10 Effect of formoterol on airway resistance, airway reactivity (K) and airway sensitivity (PC_{100})

Airway responsiveness was not significantly increased in Ova S/C Epi-null mice compared to control groups (Figures 61-63). There was significant increase in airway sensitivity (reflected as a lower PC_{100} value) and reactivity in formoterol treated Ova S/C Epi-null mice compared to vehicle treated Ova S/C Epi-null mice ($p<0.05$) (Figures 61 A and 62 A). Additionally, 25 and 50 mg/mL of methacholine significantly increased

airway resistance values in formoterol treated Ova S/C Epi-null mice compared to vehicle treated Ova S/C Epi-null mice ($p<0.05$) (Figure 60 A).

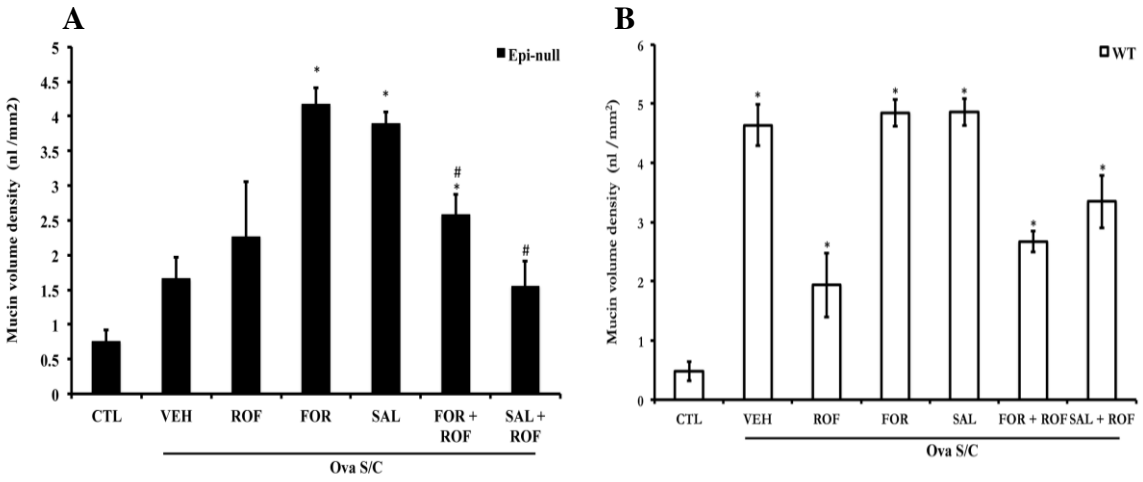

Figure 56: Quantification of the effect of roflumilast administration, with or without formoterol or salmeterol on mucous metaplasia. Morphometric quantification of the mucin volume density assessed from saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups of mice, roflumilast (5 mg/kg) was administered alone via oral gavage or co-administered once daily with twice-daily i.p. injections with either formoterol (5 µg/ kg) or salmeterol (1.5 µg/kg) for a duration of 12 days Data represent the mean (±SEM) from n=5-13 in each group. * indicates $p<0.05$ significance as compared to respective CTL, # indicates $p<0.05$ significance as compared to respective treatment in the absence of ROF.

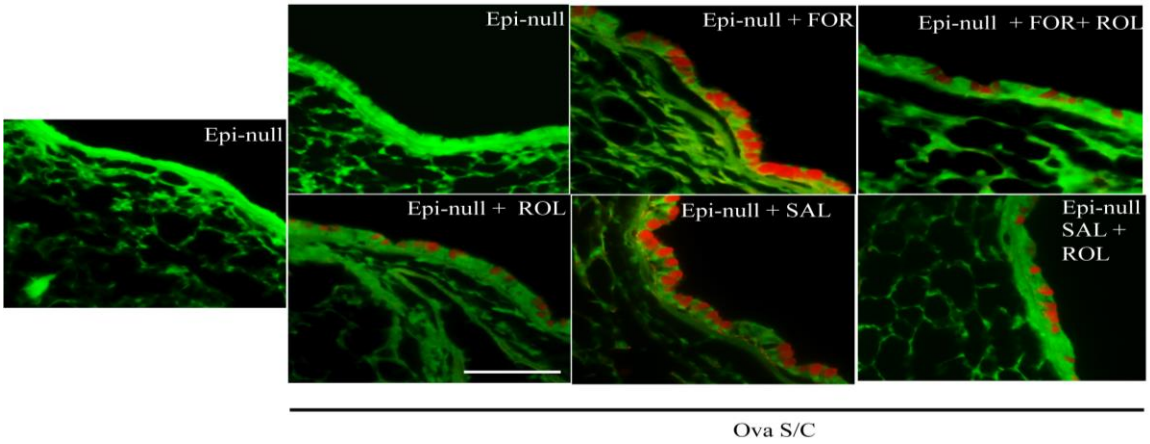

Figure 57: Representative images of the effect of rolipram administration, with or without formoterol or salmeterol on mucous metaplasia. Representative microscopic images of mucin (red) content in the airway epithelia (green) of saline challenged (CTL) and vehicle (VEH) treated Ova S/C Epi-null mice. Groups of Ova S/C Epi-null mice were treated with different drug combinations. In these groups of mice, rolipram (5 mg/ kg) was administered via oral gavage daily or co-administered with twice daily i.p. injections with either formoterol (5 µg/kg) or salmeterol (1.5 µg/kg) for a duration of 12 days. Images were acquired after periodic acid fluorescent (PAF) staining using the confocal microscope at 40X magnification. Scale bar (white), 50 µm.

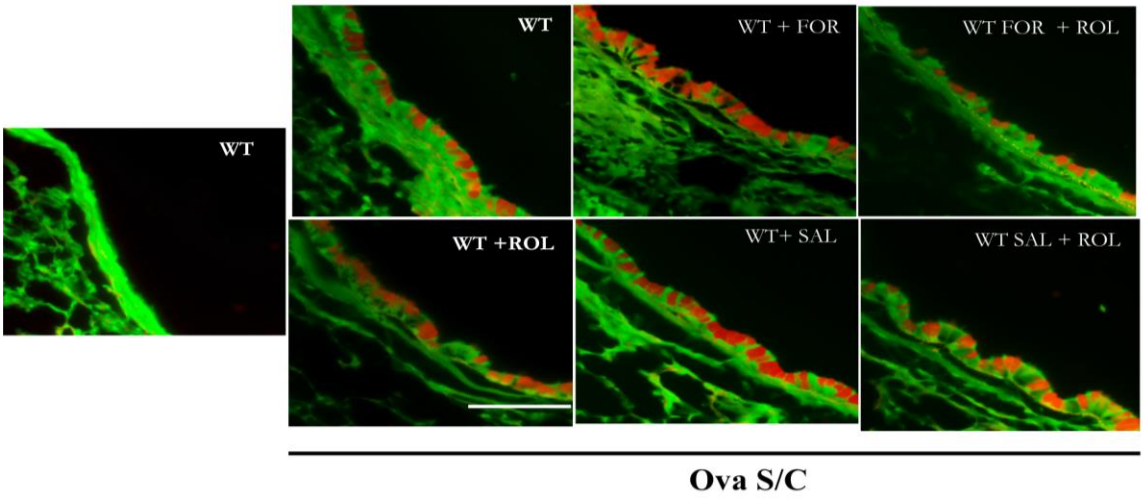

Figure 58: Representative images of the effect of rolipram administration, with or without formoterol or salmeterol on mucous metaplasia. Representative microscopic images of mucin (red) content in the airway epithelia (green) of saline challenged (CTL) and vehicle (VEH) treated Ova S/C WT mice. Groups of Ova S/C WT mice were treated with different drug combinations. In these groups of mice, rolipram (5 mg/ kg) was administered via oral gavage daily or co-administered with twice daily i.p. injections with either formoterol (5 µg/kg) or salmeterol (1.5 µg/kg) for a duration of 12 days. Images were acquired after periodic acid fluorescent (PAF) staining using the confocal microscope at 40X magnification. Scale bar (white), 50 µm.

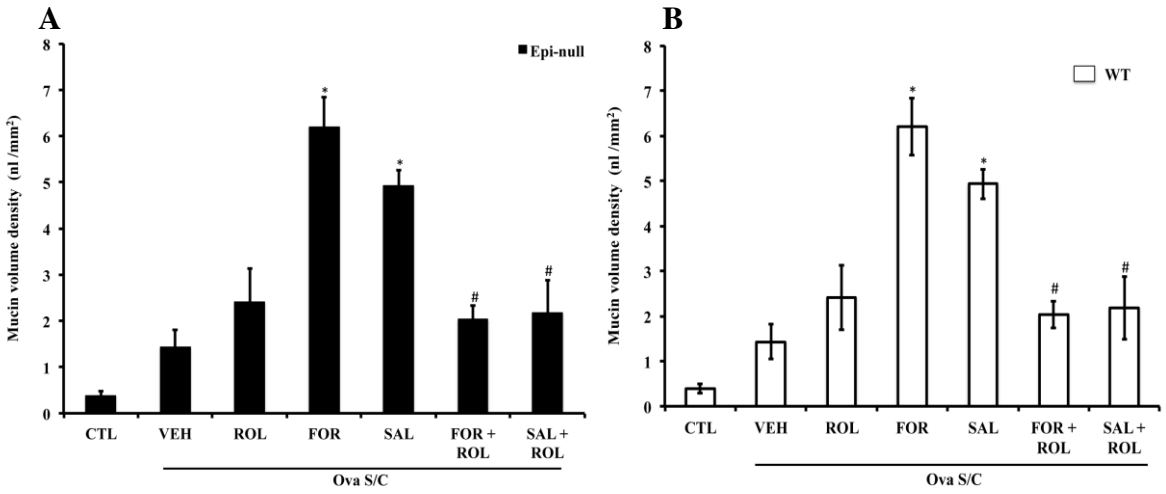

Figure 59: Quantification of the effect of rolipram administration, with or without formoterol or salmeterol on mucous metaplasia. Morphometric quantification of the mucin volume density assessed from saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups of mice, rolipram (5 mg/kg) was administered alone via oral gavage or co-administered once daily with twice-daily i.p. injections with either formoterol (5 μg/kg) or salmeterol (1.5 μg/kg) for a duration of 12 days. Data represent the mean (±SEM) from n=5-13 in each group. * indicates $p<0.05$ significance as compared to respective CTL, # indicates $p<0.05$ significance as compared to respective treatment in the absence of ROL.

6.3.11 Effect of roflumilast on airway resistance, airway reactivity and airway sensitivity

Sroflumilast were not significantly different from vehicle-treated Ova S/C Epi-null mice (Figures 61 A and 62 A).

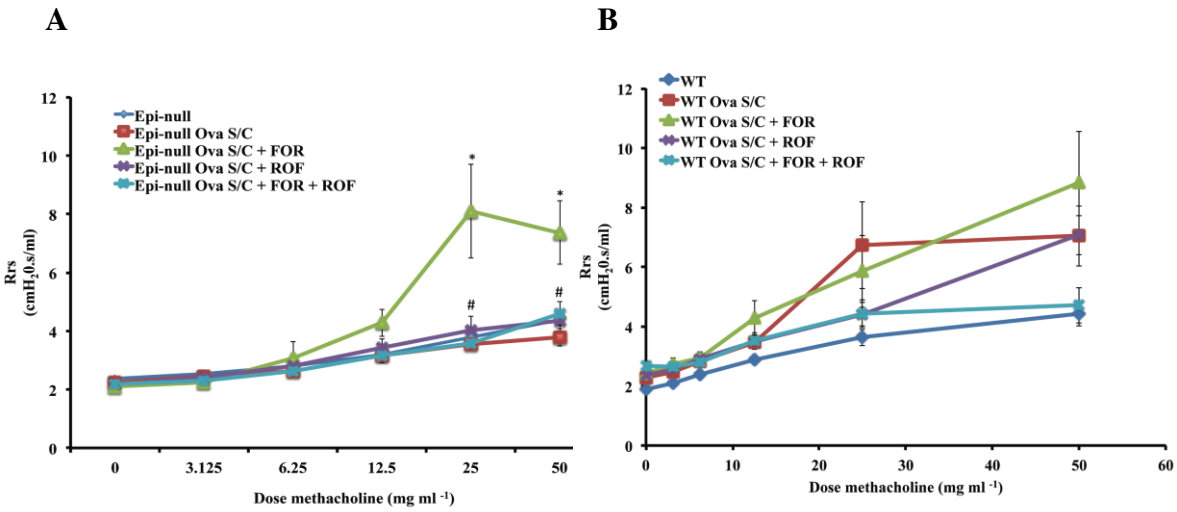

Figure 60: Effect of roflumilast administration, with or without formoterol on airway resistance (Rrs). Airway resistance of saline challenged and vehicle treated Ova S/C (A) Epi-null and (B) WT mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups of mice, roflumilast (5 mg/ kg) was administered once daily via oral gavage, or co-administered with twice-daily i.p. injections of formoterol (5 µg/kg) for a duration of 12 days. Airway resistance to increasing doses of nebulized methacholine (0–50 mg/mL) were measured using forced oscillation technique. Airway resistance was determined by averaging the three highest resistance responses produced for each mouse by each methacholine dose. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ significance as compared to respective control, # indicates $p<0.05$ indicates significance as compared to respective treatment in the absence of ROF.

6.3.12 Effect of roflumilast plus formoterol on airway resistance, airway reactivity and airway sensitivity

Co-administration of roflumilast and formoterol in Ova S/C Epi-null mice significantly reduced airway reactivity ($p<0.05$) but had no effect on airway sensitivity compared to vehicle-treated Ova S/C Epi-null mice (Figures 61 A and 62 A). There was also significant reduction in airway resistance to 25 and 50 mg/mL of methacholine with co-administration of roflumilast and formoterol in Ova S/C Epi-null mice compared to formoterol treated Ova S/C Epi-null mice ($p<0.05$) (Figure 60 A).

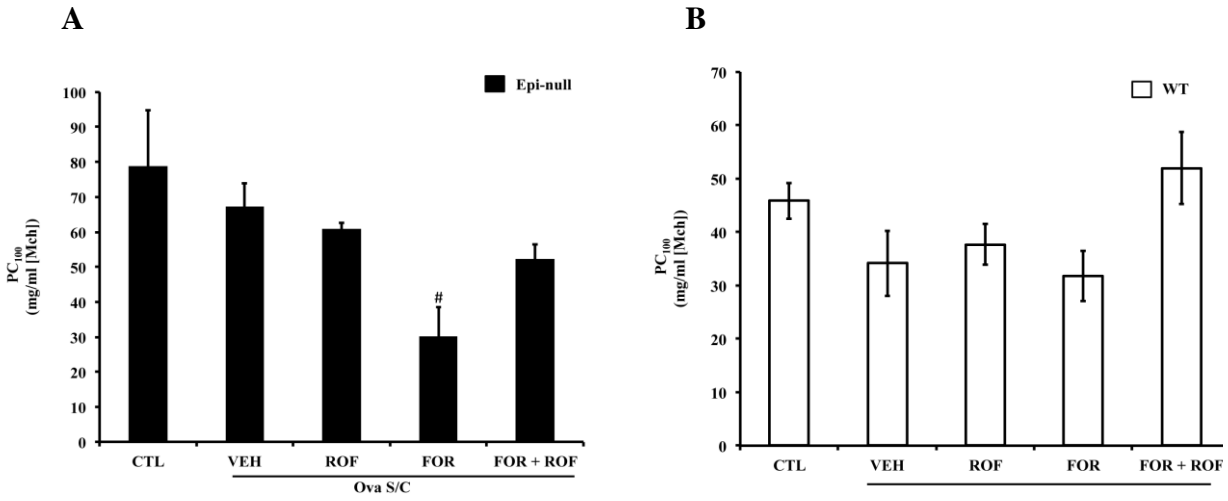

Figure 61: Effect of roflumilast administration, with or without formoterol on airway sensitivity (PC_{100}). Airway sensitivity of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars)mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups of mice, roflumilast (5 mg/kg) was administered once daily via oral gavage, or co-administered with twice daily i.p. injections of formoterol (5 µg/kg) for a duration of 12 days. A lower value for PC_{100} represents increased airway responsiveness. Data represent the mean (±SEM) from n=4-9 mice in each group. * indicates $p<0.05$ significance as compared to respective control, # indicates $p<0.05$ indicates significance as compared to respective treatment in the absence of ROF.

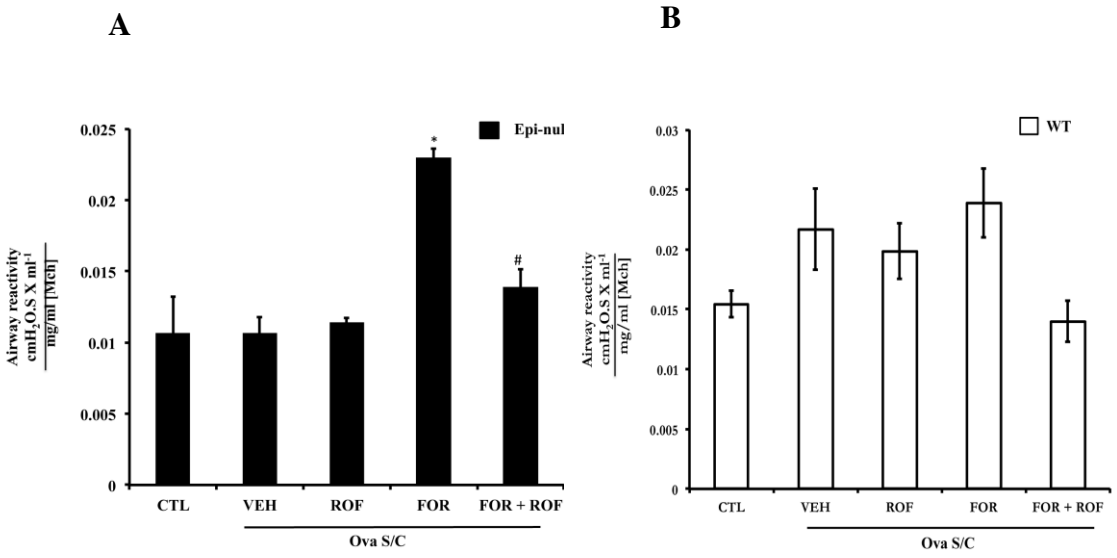

Figure 62: Effect of roflumilast administration, with or without formoterol on airway reactivity (K). Airway reactivity of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups of mice, roflumilast (5 mg/kg) was administered once daily via oral gavage, or co-administered with twice daily i.p. injections of formoterol (5 µg/ kg) for a duration of 12 days. A higher value for reactivity represents increased airway responsiveness. Data represent the mean (±SEM) from n=4-9 mice in each group. * indicates $p<0.05$ significance as compared to respective CTL, # indicates $p<0.05$ indicates significance as compared to respective treatment in the absence of ROF.

6.4 Discussion

Concurrent administration of roflumilast or rolipram with either β-arrestin biased ligands, formoterol or salmeterol (Rajagopal, Ahn et al. 2011) to Ova S/C Epi-null mice significantly decreased inflammatory cell infiltration in BALF (Figures 50 A-53 A), and mucous hypersecretion (Figures 54, 56 A, 57 and 59 A) in the airways. Administration of roflumilast and formoterol to Ova S/C Epi-null mice significantly reduced airway reactivity (Figure 62 A), an effect that was not seen in Ova S/C WT mice (Figure 62 B).

The effect seen on inflammation, mucous metaplasia and airway hyperresponsiveness with co-administration of the long-acting β_2AR agonists salmeterol and formoterol with the PDE4 inhibitors roflumilast and rolipram in Ova S/C Epi-null mice may explain recent findings that addition of PDE4 inhibitors to β_2AR agonists, both approved therapeutics for chronic obstructive pulmonary disease (COPD), may provide superior therapeutic benefits and greater efficacy (Fabbri, Calverley et al. 2009, Tannheimer, Sorensen et al. 2012, Tannheimer, Wright et al. 2012).

Additional studies have shown roflumilast in combination with either salmeterol or formoterol, can lead to increased anti-inflammatory activity with regards to inflammatory cytokine production from LPS-stimulated human peripheral blood mononuclear cells. In this study, the effect of salmeterol in combination with roflumilast was shown to be PKA dependent (Tannheimer, Sorensen et al. 2012). Another study investigating the effect of co-treatment of roflumilast and indacaterol (ultra long-acting

β_2AR agonist), on fibroblast function observed therapeutic impact on lung fibroblast pro-inflammatory and pro-fibrotic mediator release. These inflammatory mediators contribute to small airway remodeling and airway obstruction in COPD (Tannheimer, Wright et al. 2012). Also in COPD patients, roflumilast improved post-bronchodilator forced expiratory volume in 1 second (FEV_1) in patients treated with salmeterol (Fabbri, Calverley et al. 2009).

These observations explain why there is recent development of novel bifunctional molecules, which have potent β_2AR agonist and PDE4 inhibitor activity. These include small molecule GS-5759 (Tannheimer, Sorensen et al. 2014), hybrids consisting of pharmacophores of salmeterol and roflumilast (Liu, Huang et al. 2013) and a novel class of dual pharmacology compound targeting both β_2AR and PDE4 (Luini, Lewis et al. 1986, Shan, Huang et al. 2012).

In WT mice, the protective effects of PDE4 inhibition were less clear. In Ova S/C WT mice treated with roflumilast or rolipram alone, there was no significant reduction in inflammatory cell infiltration in BALF (Figures 50 B and 51 B) or mucous production (Figures 55, 56 B, 58, 59 B) in the airways. Also, co-treatment with either formoterol and roflumilast or salmeterol and rolipram did not significantly reduce total inflammatory cells in the airways of Ova S/C WT mice (Figures 50 B and 51 B). In contrast, other studies have shown roflumilast and rolipram inhibit key features of inflammation in WT

mice (Kumar, Herbert et al. 2003, Martorana, Beume et al. 2005, Herbert, Hettiaratchi et al. 2008, Cortijo, Iranzo et al. 2009).

Other studies have reported that in a mouse model of mild chronic asthma, roflumilast significantly reduced pro-inflammatory cytokines and growth factors that play critical roles in the pathogenesis of chronic asthma (Herbert, Hettiaratchi et al. 2008). Martorana and colleagues in 2005 studied acute lung inflammation and chronic lung changes induced by cigarette smoke in WT mice and observed that, roflumilast partially decreased lung inflammation and fully prevented parenchymal destruction (Martorana, Beume et al. 2005). Another study using WT mice showed that, roflumilast prevented the development of bleomycin-induced lung injury, lung fibrosis and vascular remodeling (Cortijo, Iranzo et al. 2009). Lastly, a study has reported in a chronic model of asthma that, roflumilast significantly attenuated airway inflammation and airway remodeling. Although roflumilast showed a modest decrease in airway responsiveness, it did not reach statistical significance, which is what we observed in our study (Kumar, Herbert et al. 2003).

The PDE4 inhibitors are approved for the management of COPD but not asthma. Some studies have reported PDE4 inhibitors have a greater suppressive effect on Th2 than on Th1 response, suggesting they may be useful in asthma, (Essayan, Huang et al. 1994, Van Wauwe, Aerts et al. 1995, Essayan, Huang et al. 1997) whereas others have reported they preferably inhibit Th1 responses more effectively than Th2 responses

(Ross, Williams et al. 1997, Navikas, Matusevicius et al. 1998, Dinter, Tse et al. 2000).

Our data suggest that PDE4 inhibition produces beneficial effects on inflammation, mucous metaplasia and AHR only when the β_2AR is activated by a β-arrestin-biased ligand, relative to the endogenous ligand epinephrine in WT mice. The reason for this discrepancy of a significant effect of roflumilast and rolipram being observed only when epinephrine is replaced with formoterol or salmeterol is unclear. One possible explanation for this observation is the model depicted in figure 63 below where selective Gs amplification is beneficial in Epi-null mice stimulated with a β-arrestin-biased ligand but not in WT mice as epinephrine which activates both pathways with 'equal' bias and the higher Gs activation could generate enough cAMP to be protective without the need to inhibit its breakdown.

The PDE4 enzyme activity is increased in asthma and other inflammatory conditions. A study investigated the changes in PDE4 activity of the peripheral blood leukocytes in patients with bronchial asthma and observed that leukocyte PDE4 activity was significantly elevated during asthmat attacks and in active asthmatics, irrespective of the drug treatment (Mue, Ise et al. 1976). Also, in mononuclear leucocytes of patients suffering from allergic rhinitis and atopic dermatitis, Grewe and colleagues have also shown that, PDE4 activity was significantly increased (Grewe, Chan et al. 1982). Similar observations were made by Sawai and colleagues where 100 patients with atopic dermatitis, presented with significant increases in PDE4 activity in peripheral blood

mononuclear cells (Sawai, Ikai et al. 1998). A study conducted in an Ova S/C murine model of asthma observed significant induction of PDE4 activity (Sun, Deng et al. 2006). This increase in activity directly correlated with increased cytokine production such as eotaxin, TNF-α and IL-4 as well as increased total inflammatory cells in the airways. They also observed mucous metaplasia and airway hyperresponsivenss, measured as indices of airway resistance and dynamic airway compliance (Sun, Deng et al. 2006).

The suppressive effects of PDE4 inhibitors on inflammation may be accompanied by the elevations in cAMP levels. Borger and colleagues studied the effect of cAMP elevating agents such as dibutyryl-cAMP and IBMX on activated T lymphocytes and observed reduced expression of IL-3 and GM-CSF which play key roles in the development of asthma (Borger, Kauffman et al. 1996). Basophil-enriched leukocytes obtained from 27 atopic asthmatic patients when challenged with mite antigen resulted in increased release of IL-4 and IL-13 due to cross-linking of cell surface antigen with IgE. However, when the cells were treated with various cAMP elevating agents such as forskolin, dibutyryl-cAMP, theophylline and the PDE4 selective inhibitor rolipram, IL-4 and IL-13 cytokine release was significantly suppressed (Shimizu, Shichijo et al. 1998). Pharmacologic studies carried out to investigate the regulation of IL-13 expression by PDE4 inhibitors in T cell clones obtained from ragweed allergic asthmatic subject have shown that both IL-13 gene expression and protein secretion into culture supernatants were down regulated by the PDE4 inhibitor rolipram (Essayan, Kagey-Sobotka et al. 1997). They also observed significant effect on the proliferative responses of these cells

with rolipram treatment (Essayan, Kagey-Sobotka et al. 1997). The inductions of two major cytokines, IL-4 and IL-13, which play major roles in the inflammatory response were significantly suppressed in a mite allergen activated basophil (Shimizu, Shichijo et al. 1998).

Some studies have also shown that, direct stimulation of the PKA system, a kinase downstream of cAMP, downregulates IL-4 at mRNA and protein expression levels. This downregulation was a result of transcriptional and posttranscriptional processes, which depends on the degree of T lymphocyte activation (Borger, Kauffman et al. 1996).

Also, recent observations have shown that, the PKA and β-arrestin pathways could converge on the same effectors. A typical example is the MAPK ERK1/2, whose activation is conventionally downstream β-arrestin signaling. The ERK1/2 kinase could also be activated through the Gs-cAMP pathway in a cAMP/PKA/Rap-1/B-Raf/MEK dependent pathway. In spite of the crosstalk that exist between these two pathways, studies have shown spatiotemporal differences, and the kinetics and intensity of the downstream effector activation result in distinct biochemical, physiological and functional consequences.

In conclusion we have shown that, amplification of the Gs-cAMP signaling pathway demonstrated beneficial effects in the presence of a β-arrestin biased ligand in murine models of asthma. Although further studies are needed to fully comprehend the role of the various β_2AR signaling pathways, our results suggest the ideal β_2AR drug for asthma management may be a Gs biased ligand that activates the canonical Gs pathway while inhibiting β-arrestin signaling.

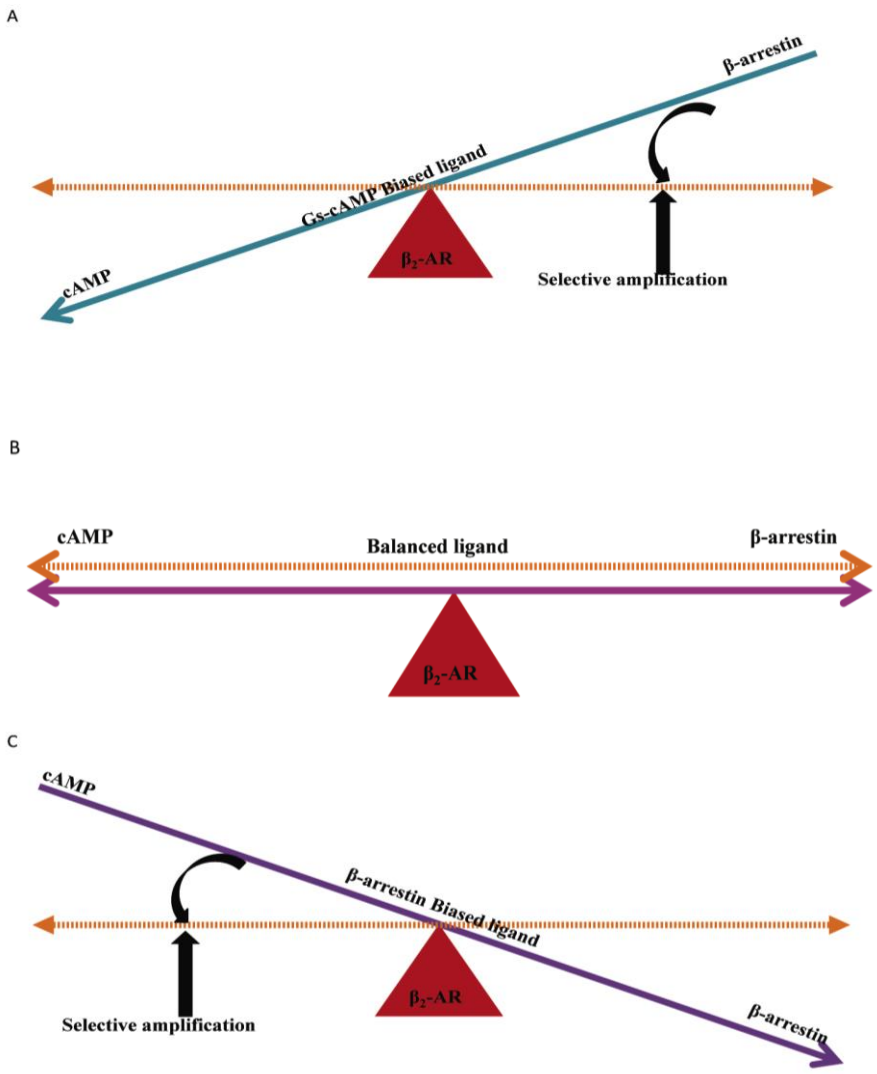

Figure 63: Schematic representation of our proposed theory to explain the effect of selective amplification of the β2AR signaling in the presence of (A) Gs-cAMP biased ligand, the (B) reference ligand (endogenous ligand epinephrine) and (C) β-arrestin biased ligand. We hypothesize that, amplification of either pathways in the presence of a biased ligand disrupts the bias with a huge delta in cAMP accumulation (A) or (C) β-arrestin recruitment relative to the reference ligand hence the proposed beneficial or detrimental effects that will be seen respectively. However, these effects are not seen with (B) the reference ligand where there is an already existent balance in cAMP and β-arrestin levels. Aqua represents baseline cAMP accumulation and β-arrestin recruitment of a Gs-cAMP biased ligand relative to epinephrine. Orange represents baseline cAMP accumulation and β-arrestin recruitment in the presence of the reference ligand epinephrine. Purple represent baseline cAMP accumulation and βarrestin recruitment in the presence of a βarrestin biased ligand relative to epinephrine.

7 The effect of a more global amplification of the Gs-cAMP pathway in a murine model of asthma

7.1 Introduction

The PDE4 isozyme plays a critical role in inflammatory and immunomodulatory cells. It is predominantly found in mast cells, eosinophils, neutrophils, T cells, macrophages and structural cells such as sensory nerves and epithelial cells (Torphy 1998).

β-arrestins form a complex with PDE4 enzymes, thereby providing a means of delivering an enzyme degrading cAMP to the site of active cAMP synthesis in the plasma membrane in an agonist-dependent fashion (Perry, Baillie et al. 2002). PDE4 isoforms from all four subfamilies can interact with β-arrestin1 and 2 (Perry, Baillie et al. 2002, Conti, Richter et al. 2003, Houslay and Adams 2003). Challenge of cells with a β_2AR agonist recruits PDE4–β-arrestin complex to the β_2AR. Therefore, PDE4 enzymes are targeted and concentrated to specific intracellular sites in the cell (Perry, Baillie et al. 2002, Conti, Richter et al. 2003, Houslay and Adams 2003).

Therefore, roflumilast and rolipram may affect PDE4 enzyme activity in specific cell types and in specific micro-domains in cells, therefore leading to compartmentalization of cAMP signaling.

Molecular cloning techniques have identified nine mammalian genes that encode

membrane-bound adenylate cyclase (AC) (Sunahara, Dessauer et al. 1996, Hanoune, Pouille et al. 1997, Smit and Iyengar 1998, Patel, Du et al. 2001), and one gene encoding a soluble isoform (Buck, Sinclair et al. 1999). Most isoforms of adenylate cyclase are widely expressed and the broad distribution of AC isoforms suggests that any given cell contains multiple isoforms (Sunahara and Taussig 2002). The diterpene forskolin (from *Coleus forskohlii*) activates all known isoforms of mammalian membrane-bound ACs (Premont, Matsuoka et al. 1996) with the exception of AC9 (Iourgenko, Kliot et al. 1997). Forskolin-dependent activation of AC2, AC4, AC5, AC6, and AC7 is synergistic with Gαs-mediated coactivation, whereas activation by forskolin and Gαs is additive for isoforms AC1, AC3, and AC8 (Sunahara, Dessauer et al. 1996).

We studied the effect of increasing cAMP using forskolin, the direct activator of adenylate cyclase on features of the asthma phenotype namely, airway inflammation and mucous metaplasia in a murine asthma model (Figure 64).

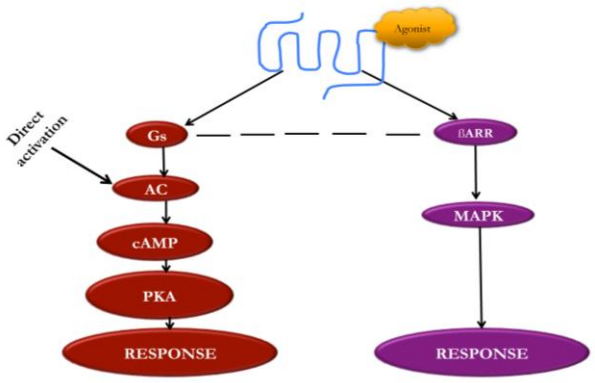

Figure 64: Schematic of amplification of the cAMP pathway with forskolin.

7.2 Approach

Forskolin was administered to Ova S/C WT and Epi-null mice by two routes; oral (forskolin mixed with mouse chow) and inhaled forskolin (nebulization of forskolin). Forskolin was administered for a duration of 19 days since 19 days of formoterol administration completely restored all three features of the asthma phenotype and showed increased mucous production in previous studies (Figure 65). Groups of mice received other drug combinations; oral forskolin and formoterol or oral forskolin and roflumilast. Forskolin was administered at a dose of 4mg/kg/day. This dose of forskolin was used to avoid toxic effects (Virgona, Yokotani et al. 2012, Yokotani, Chiba et al. 2012, Virgona, Taki et al. 2013, Yokotani, Chiba et al. 2013).

Mice were phenotyped for inflammatory cell infiltration into the airways and mucous metaplasia.

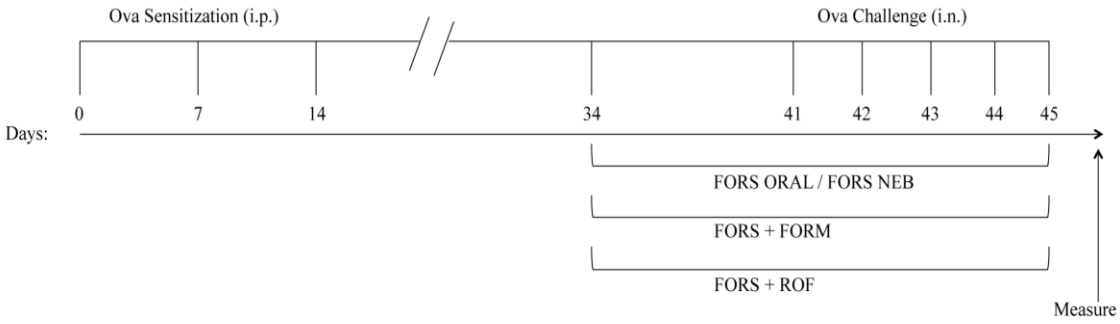

Figure 65: Ovalbumin sensitization and challenge (Ova S/C) treatment prototcol. Groups of Ova S/C WT and Epi-null mice were treated with oral or nebulized forskolin (4 mg/kg/day) alone. In other groups of mice, oral forskolin was co-administered with twice-daily i.p. injections of either formoterol (5 μg/kg) or roflumilast (5 mg/kg). Duration of all drug treatment was 19 days.

7.3 Results

7.3.1 Effect of oral or nebulized forskolin on total inflammatory cells in the airways

In Ova S/C Epi-null mice, oral or nebulized forskolin significantly increased total inflammatory cells in BALF compared to vehicle-treated Ova S/C Epi-null mice (Figure 66 A). Oral or nebulized forskolin in Ova S/C WT mice did not show significant increase in total inflammatory cells in BALF compared to vehicle-treated Ova S/C WT mice (Figure 66 B).

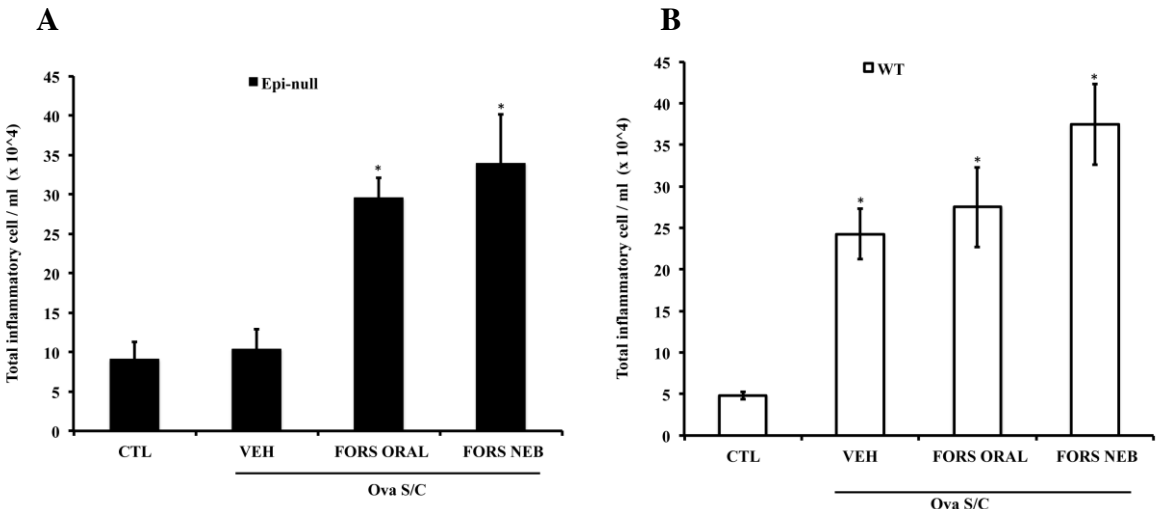

Figure 66: Effect of oral and nebulized forskolin on total inflammatory cells in BALF. Total inflammatory cells in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with either oral or nebulized forskolin at a dose of 4 mg/kg/day for duration of 19 days. Vehicle-treated mice received respective vehicle for forskolin. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ significance as compared to respective CTL.

7.3.2 Effect of Oral or nebulized forskolin on eosinophils in the airways

In Ova S/C Epi-null mice, oral or nebulized forskolin significantly increased eosinophils in BALF compared to vehicle-treated Ova S/C Epi-null mice (Figure 67 A). Oral or nebulized forskolin in Ova S/C WT mice did not show significant increase in eosinophils in BALF compared to vehicle-treated Ova S/C WT mice (Figure 67 B).

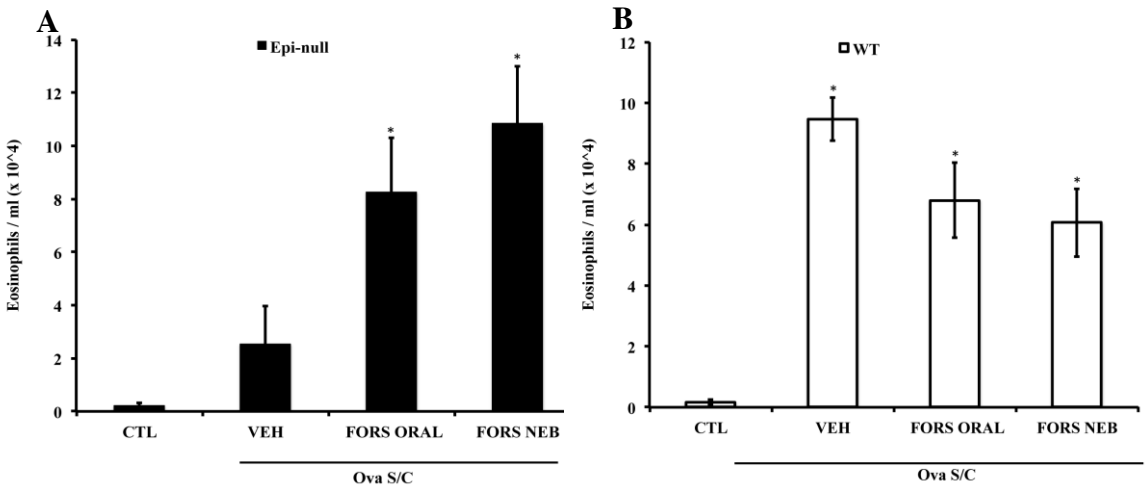

Figure 67: Effect of oral and nebulized forskolin on airway eosinophilia in BALF. Eosinophils in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with either oral or nebulized forskolin at a dose of 4 mg/kg/day for duration of 19 days. Vehicle-treated mice received respective vehicle for forskolin. Differential counting of cells were done with Wright Geimsa staining. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ indicates significance as compared to respective CTL.

7.3.3 Effect of oral or nebulized forskolin on mucus production in the airways

In Ova S/C Epi-null mice, oral or nebulized forskolin significantly increased mucous production compared to vehicle-treated Ova S/C Epi-null mice (Figure 68 and 69 A). Oral and nebulized forskolin in Ova S/C WT mice did not show significant increase in mucous production compared to vehicle-treated Ova S/C WT mice (Figure 68 and 69 B).

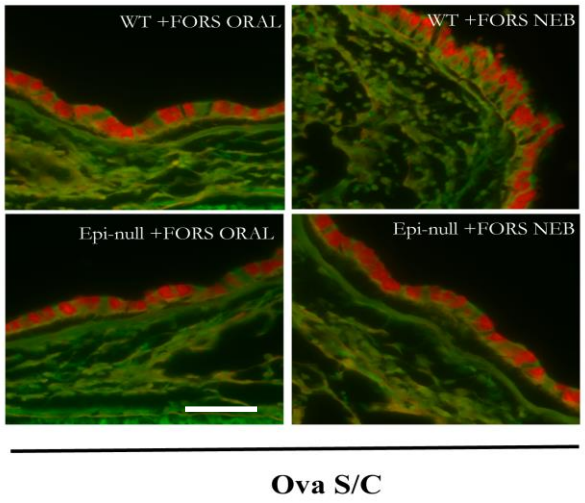

Figure 68: Representative images of the effect of oral and nebulized forskolin on mucous metaplasia. Representative microscopic images of mucin (red) content in the airway epithelia (green) of Ova S/C WT and Epi-null mice. Groups of Ova S/C WT and Epi-null mice were treated with either oral or nebulized forskolin at a dose of 4 mg/kg/day for duration of 19 days. Vehicle-treated mice received respective vehicle for forskolin. Images were acquired after periodic acid fluorescent (PAF) staining using the confocal microscope at 40X magnification. Scale bar (white), 50 µm.

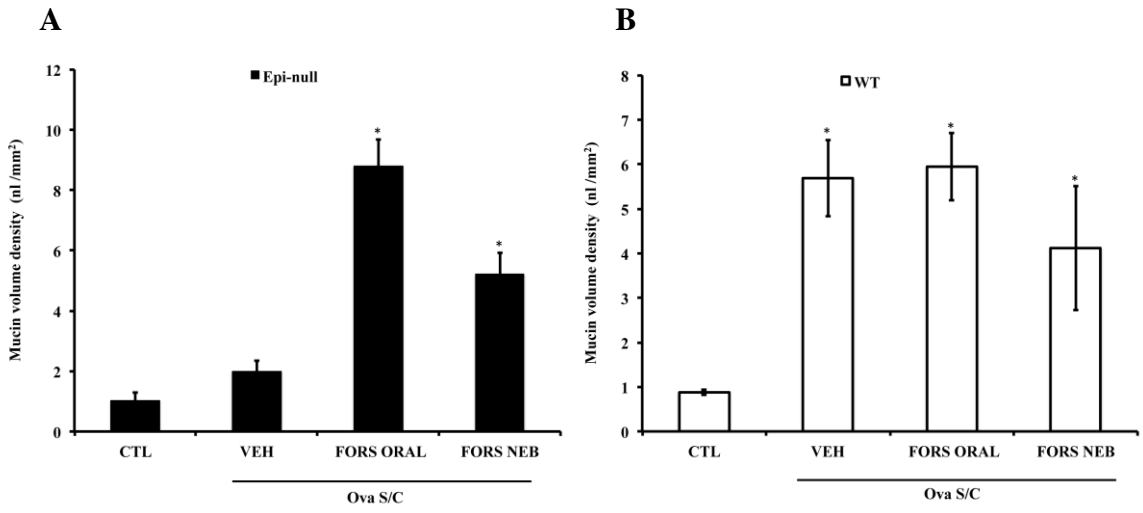

Figure 69: Quantification of the effect of oral and nebulized forskolin on mucous metaplasia. Morphometric quantification of the mucin volume density assessed from saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with either oral or nebulized forskolin at a dose of 4 mg/kg/day for duration of 19 days. Vehicle-treated mice received respective vehicle for forskolin. Morphometric quantification of the images was done using Image J software. Data represent the mean (±SEM) from n=5-13 in each group. * indicates $p<0.05$ significance as compared to respective CTL.

7.3.4 Effect of co-administration of oral forskolin and formoterol on total inflammatory cells in the airways

Total inflammatory cells in Ova S/C Epi-null mice co-treated with forskolin and formoterol was not significantly different from Ova S/C Epi-null mice treated with formoterol or forskolin alone (Figure 70 A). Co-administration of oral forskolin and formoterol in Ova S/C WT mice significantly reduced total inflammatory cells in BALF compared to Ova S/C WT mice treated with formoterol or forskolin alone (Figure 70 B).

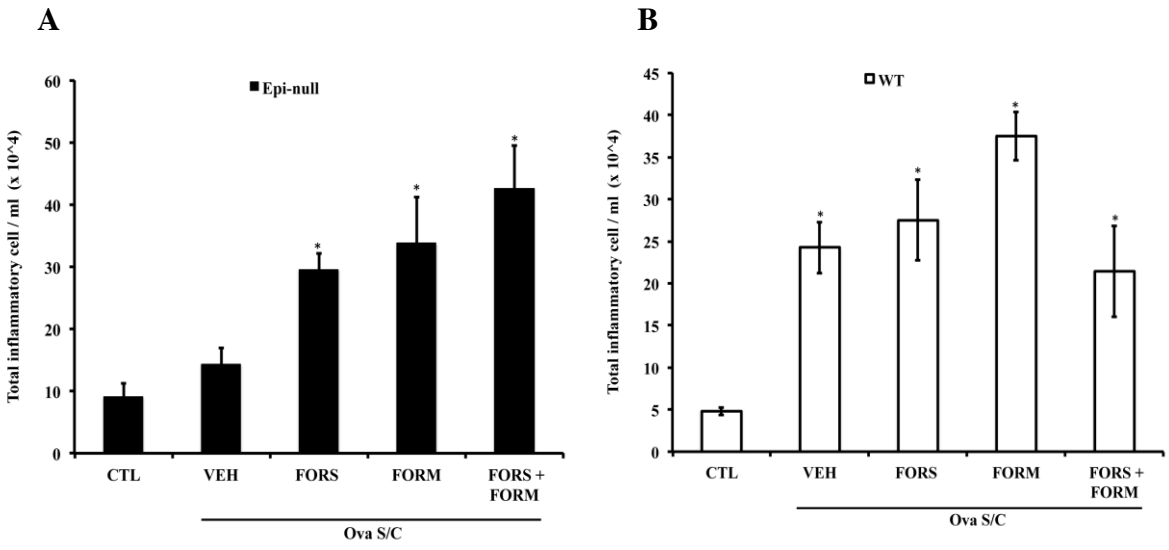

Figure 70: Effect of oral forskolin administration, with or without formoterol on total inflammatory cells in BALF Total inflammatory cells in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars)mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups, mice were fed with oral forskolin (4 mg/kg/day) or co-administered with twice-daily i.p. injections with formoterol (5 µg/kg) for a duration of 19 days. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ significance as compared to respective CTL.

7.3.5 Effect of co-administration of oral forskolin and formoterol on eosinophils in the airways

Eosinophils in Ova S/C Epi-null mice co-treated with forskolin and formoterol was not significantly different from Ova S/C Epi-null mice treated with formoterol or forskolin alone (Figure 71 A). Co-administration of oral forskolin and formoterol in Ova S/C WT mice significantly reduced eosinophils in BALF compared to Ova S/C WT mice treated with formoterol or forskolin alone (Figure 71 B).

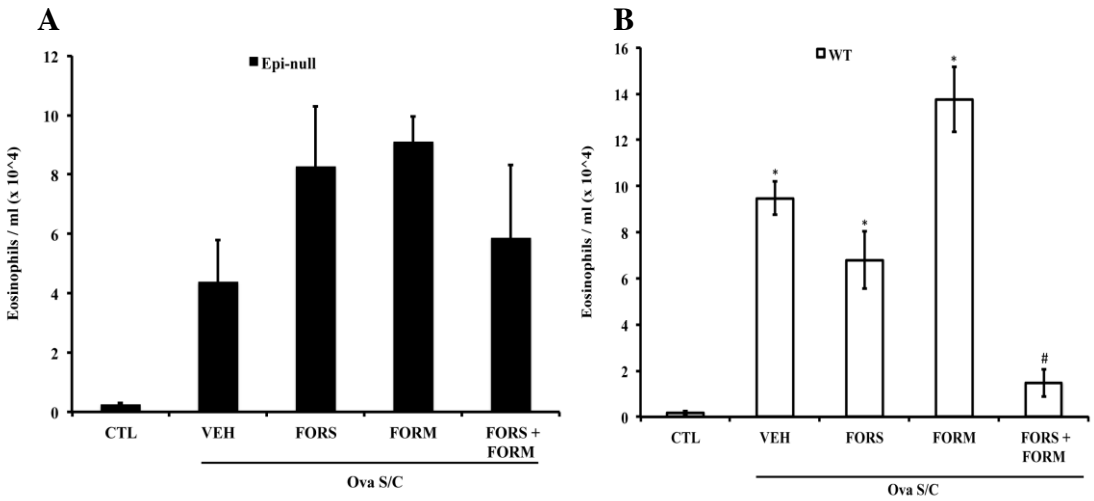

Figure 71: Effect of oral forskolin administration, with or without formoterol on airway eosinophilia in BALF. Eosinophils in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C WT and Epi-null mice. Groups of Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice were treated with different drug combinations. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups, mice were fed with oral forskolin (4 mg/kg/day) or co-administered with twice-daily i.p. injections with formoterol (5 µg/kg) for a duration of 19 days. Differential counting of cells were done with Wright Geimsa staining. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ indicates significance as compared to respective CTL

7.3.6 Effect of co-administration of forskolin and roflumilast on total inflammatory cells in the airways

In Ova S/C Epi-null mice, co-administration of forskolin and roflumilast significantly increased total inflammatory cells in BALF compared to forskolin treated Ova S/C Epi-null mice (Figure 72 A). Co-administration of forskolin and roflumilast in Ova S/C WT mice did not show significant increase in total inflammatory cells in BALF compared to forskolin treated Ova S/C WT mice (Figure 72 B).

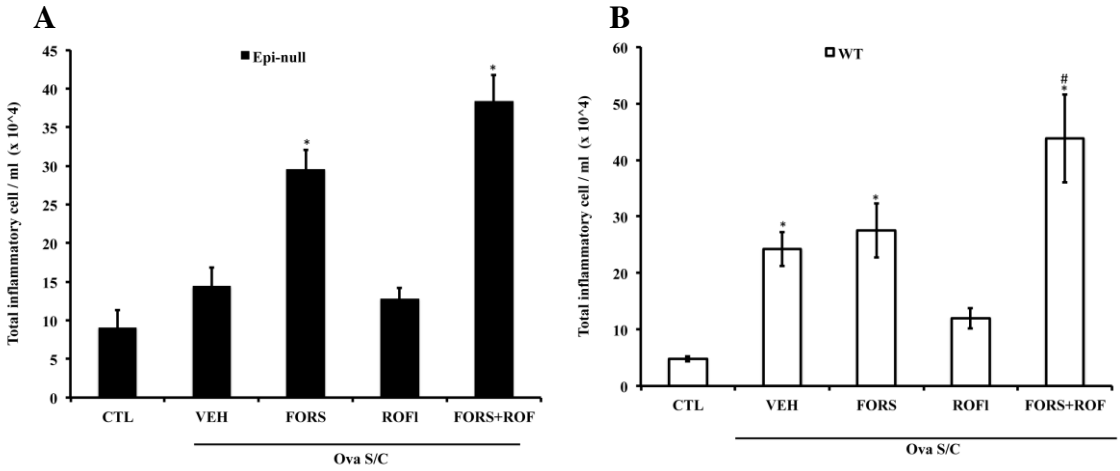

Figure 72: Effect of oral forskolin administration, with or without roflumilast on total inflammatory cells in BALF. Total inflammatory cells in BALF of saline challenged (CTL) and vehicle (VEH) treated Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups, mice were fed with oral forskolin (4 mg/kg/day) or co-administered with twice-daily i.p. injections with formoterol (5 µg/kg) for a duration of 19 days. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ significance as compared to respective CTL.

7.3.7 Effect of co-administration of oral forskolin and roflumilast on eosinophils in the airways

In Ova S/C Epi-null mice, co-administration of forskolin and roflumilast significantly increased eosinophils in BALF compared to forskolin treated Ova S/C Epi-null mice (Figure 73 A). Co-administration of forskolin and roflumilast in Ova S/C WT mice did not show significant increase in eosinophils in BALF compared to forskolin treated Ova S/C WT mice (Figure 73 B).

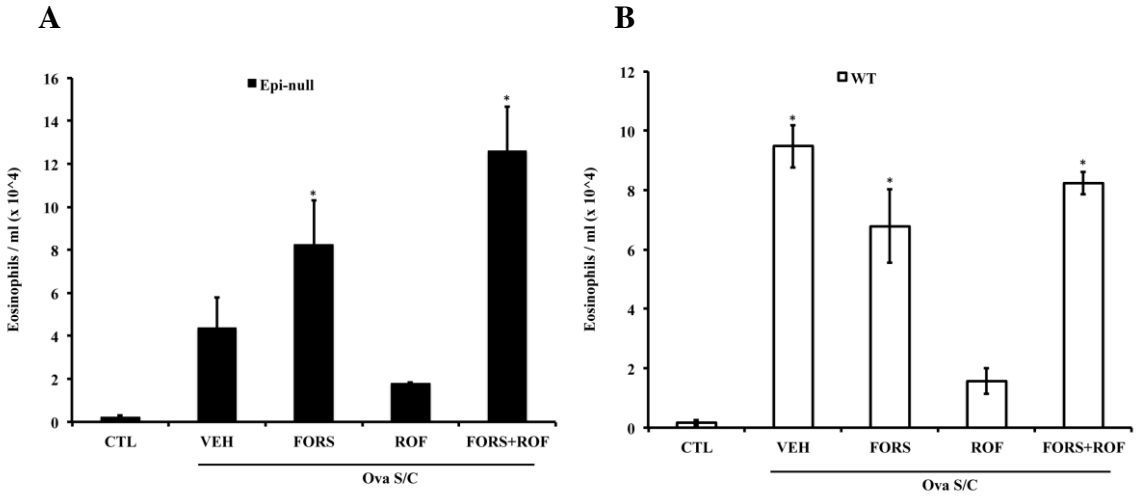

Figure 73: Effect of oral forskolin administration, with or without roflumilast on airway eosinophilia in BALF. Eosinophils in BAL fluid of saline challenged (CTL) and vehicle (VEH) treated Ova S/C WT and Epi-null mice. Groups of Ova S/C (A) Epi-null (black bars) and (B) WT (white bars) mice were treated with different drug combinations. Groups of Ova S/C WT and Epi-null mice were treated with different drug combinations. In these groups, mice were fed with oral forskolin (4 mg/kg/day) or co-administered with twice-daily i.p. injections with formoterol (5 µg/kg) for a duration of 19 days. Differential counting of cells were done with Wright Geimsa staining. Data represent the mean (±SEM) from n=5-9 mice in each group. * indicates $p<0.05$ indicates significance as compared to respective CTL.

7.4 Discussion

Marone and colleagues have shown that, forskolin inhibited the IgE-mediated release of histamine and leukotriene from basophils and mast cells (Marone, Columbo et al. 1987). Another study demonstrated forskolin inhibited antigen-induced histamine release from human basophil. In a second series of experiments, they evaluated the effect of forskolin on antigen-induced histamine release from chopped human lung passively sensitized with serum from an allergic patient. In this study, forskolin dose dependently inhibited the release of histamine from lung mast cells (Marone, Columbo et al. 1986). Lastly, forskolin also reduced the expression of the adhesion molecule ICAM-1 and the release of GM-CSF evoked by IL-1β in airway smooth muscle cells. IL-1β induced IL-8 release was also repressed by forskolin (Kaur, Holden et al. 2008). These effects could be due to cell-type-specific amplification of cAMP signaling as previously demonstrated with PDE4 inhibitors such as roflumilast and rolipram.

However, in our studies we observed that, direct activation of adenylate cyclase by administration of forskolin for a duration of 19 days in Ova S/C Epi-null mice inflammatory cell infiltration into the airways and mucous metaplasia. Our data suggest that, amplification of cAMP in other cell types and in other domains in the same cell type may contribute to inflammation and mucous production in Ova S/C Epi-null mice.

The effects of chronic forskolin treatment in Ova S/C Epi-null mice may not be mediated through signaling by the β_2AR. Other pro-inflammatory Gs coupled receptors such as EP2 and EP4 receptors could mediate the effects of chronic forskolin treatment in Ova S/C Epi-null mice (Kalinski 2012,Legler, Krause et al. 2006, Lambrecht and Hammad 2010). These are receptors for prostaglandin E2 (PGE2) (Kalinski 2012). Prostaglandin E2 has been shown to be a mandatory factor for the development of a migratory phenotype of human monocyte-derived as well as for peripheral blood myeloid dendritic cells (Legler, Krause et al. 2006). Dendritic cells play an important role in presenting antigens during the inflammatory cascade (Lambrecht and Hammad 2010). Another study has shown that, PGE2 directly promotes differentiation and pro-inflammatory functions of human and murine IL-17 producing T helper (Th17) cells through their action on EP2 and EP4 receptor mediated signaling and cyclic AMP pathways (Boniface, Bak-Jensen et al. 2009). Although Th2 cells are believed to be critical contributors of asthma, recent studies have shown that, the IL-17 T-helper lymphocyte-associated cytokine, potentially mediates critical cascades in the pathogenesis of asthma (Silverpil and Linden 2012). Currently, several drugs that target IL-17 signaling are being tested in clinical trials for the management of asthma (Miossec and Kolls 2012, Silverpil and Linden 2012, Morishima, Ano et al. 2013). Other studies have implicated PGE2 action on EP2 and EP4 receptors in the pathogenesis of other inflammatory conditions (Inoue, Takamori et al. 2002, McCoy, Wicks et al. 2002, Akaogi, Yamada et al. 2004, Honda, Segi-Nishida et al. 2006).

In conclusion we observed that, a more general increase in cAMP increased inflammatory cells and mucous production and suggests compartmentalized and cell-type-specific amplification of the β_2AR-Gs-cAMP signaling pathway is needed to attenuate these parameters.

8 Summary and conclusion

A. In Ova S/C Epi-null mice, there was attenuation of three main features of the asthma phenotype; inflammatory cell infiltration into the airways, mucous metaplasia and airway hyperresponsiveness (AHR). However, administration of formoterol, a preferential β_2AR agonist to Ova S/C Epi-null mice significantly increased inflammatory cells in the airways, mucous metaplasia and AHR. The β_2AR can signal in the presence of a ligand or spontaneously (constitutively active receptors) in the absence of a ligand. This suggests that, *constitutive activity of the β_2AR is not sufficient and that ligand activation is required for the development of the asthma phenotype in the Ova S/C model of asthma*.

B. Administration of salmeterol, another preferential β_2AR agonist to Ova S/C Epi-null mice, significantly increased inflammation and mucous production in the airways. This supports our previous conclusion that, *ligand activation of the β_2AR is required for the development of the asthma phenotype in an Ova S/C model of asthma*.

C. The intrinsic efficacies of salmeterol and formoterol relative to epinephrine at cAMP are 13% and 63% respectively. However, both drugs increased inflammatory cell infiltration into the airways and mucous production in Ova S/C Epi-null mice. The β_2AR can signal through at least two pathways: the canonical Gs-cAMP pathway and the β-arretsin pathway, which can activate MAPKs. Our data suggests that, *β_2AR-Gs-cAMP intrinsic efficacies do not correlate with the development of the asthma phenotype in Ova S/C Epi-null mice hence; it is unlikely to be the major pathway involved in the pathogenesis of the asthma phenotype*.

D. In Ova S/C Epi-null mice, amplification of cAMP with chronic administration of either PDE4 inhibitors roflumilast or rolipram, did not restore cardinal features of the asthma phenotype. However, concurrent administration of either roflumilast or rolipram with formoterol or salmeterol significantly reduced inflammation and mucous metaplasia in Ova S/C Epi-null mice. Also, concurrent administration of roflumilast with formoterol significantly reduced airway resistance and airway reactivity in Ova S/C Epi-null mice. In unpublished data, both carvedilol and propranolol, ligands which activate ERK1/2 but inhibit cAMP activation restored cardinal features of the asthma phenotype. These results suggest that, *β_2AR Gs pathway may be protective and the ideal β_2AR drug for asthma management may*

be a Gs biased ligand that activates the canonical Gs pathway while inhibiting ERK1/2 activation.

E. In Ova S/C Epi-null mice, accumulation of cAMP by direct adenylate cyclase activation with oral or nebulized forskolin, increased total inflammatory cells in the airways and mucous production. This suggests *a more general increase in cAMP increased inflammatory cells and mucous production, hence suggests compartmentalized and cell-type-specific amplification of the β_2AR-Gs-cAMP signaling pathway is needed to attenuate these parameters*.

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(2007). "Trends in asthma morbidity and mortality." American Lung Association. American Lung Association epidemiology & statistics unit research and program services.

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